Resistance to *Bean pod mottle virus* in Transgenic Soybean Lines Expressing the Capsid Polyprotein

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ABSTRACT

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Transgenic fertile soybean plants were generated from somatic embryos of soybean (*Glycine max*) cv. Jack transformed via particle bombardment with the capsid polyprotein (pCP) gene of *Bean pod mottle virus* (BPMV). The plant transformation vector (pHIG/BPMV-pCP) utilized in these experiments contained the BPMV-pCP coding sequence, an intron-containing GUS gene, and the hygromycin phosphotransferase gene. Southern blot hybridization analysis showed that 19 transgenic soybean plants selected for resistance to hygromycin contained the genes for GUS and BPMV-pCP. The progeny of five of these transgenic soybean plants (plants 137, 139, 157, 183, and 186) were characterized in detail. An additional transgenic plant (plant 200) contained the intron-GUS and hygromycin resistance genes, but lacked the BPMV-pCP gene and was used as a negative control. Southern blot hybridization analysis of the five transgenic plants showed the presence of three copies of the T-

DNA in a similar banding pattern suggesting that they were derived from a single transformation event. Western and northern blot analyses showed that the expression levels of BPMV-pCP and pCP transcript were high in these five pCP plants. Infectivity assays with detached leaves demonstrated that all five pCP plants exhibited resistance to virus infection because they accumulated lower levels of BPMV compared with plant 200 and nontransformed controls. Unlike the T₂ progeny of line 183-1 that segregated with respect to the pCP gene and, consequently, to BPMV resistance, the T₂ progeny of the homozygous line 183-2 showed little or no symptoms in response to rub-inoculation with virions of a severe strain of BPMV. Although BPMV accumulation was evident in leaves on which viruliferous beetles were allowed a 72-h inoculation access period, the upper noninoculated leaves of the T₂ progeny of line 183-2 plants were symptomless and accumulated little or no virus. Because the progeny of this homozygous transgenic line exhibited systemic resistance, they could potentially be useful in generating commercial cultivars resistant to BPMV.

Additional keywords: coat protein, pathogen-derived resistance.

Bean pod mottle virus (BPMV) is a member of the genus *Comovirus* in the family *Comoviridae* (15). BPMV has a bipartite positive-strand RNA genome consisting of RNA-1 and RNA-2, which are separately encapsidated in isometric particles 28 nm in diameter. Both genomic RNAs are polyadenylated and have a small basic protein, viral genome-linked protein (VPg), covalently linked to their 5' termini. Like other comoviruses, the BPMV genome is expressed via the synthesis and subsequent cleavage of large polyprotein precursors (19). The complete nucleotide sequences of the two genomic RNAs of BPMV strain KY-G7 have been reported (7,21). BPMV RNA-1 codes for five mature proteins required for replication (from 5' to 3', a protease cofactor [32K], a putative helicase [58K], a VPg, a protease [24K], and a putative RNA-dependent RNA polymerase [87K]), whereas RNA-2 codes for a putative cell-to-cell movement protein and the two coat proteins (CPs) (7,21).

BPMV is widespread in the major soybean-growing areas in many of the southern and southeastern states (13,16,34,39). Over the past 2 to 3 years, it has become an increasingly serious production problem in the North Central and Northern Great Plains states (J. Hill, *personal communication*). BPMV is efficiently transmitted in nature, within and between soybean fields, by several species of chrysomelid beetles (12,16,32). The deleterious effects of BPMV infection are not limited to reductions in seed

yield, but extend to seed quality because BPMV has been reported to predispose soybeans to *Phomopsis* spp. seed infection (40), a major cause of poor seed quality in soybean (38). Furthermore, BPMV interacts synergistically with *Soybean mosaic virus*, resulting in more severe yield losses than caused by either virus alone (1,5,33).

Disease management through genetic resistance is not possible at present because no soybean cultivars with resistance to BPMV are commercially available. The concept of pathogen-derived resistance (PDR), first proposed in 1985 (36), has been successfully utilized over the past 15 years to confer resistance against viruses in many crop plants. PDR involves the expression of viral genes in a host plant and the subsequent disruption of the essential pathogenic processes of challenge virus, thus conferring resistance. PDR has been attained by expressing various forms of the viral CP, replicase, protease, and movement protein genes. PDR-mediated protection phenotypes range from a delay in symptom development, reduced symptoms, and virion accumulation to apparent immunity (3,4,10,20,26). The diversity in PDR phenotypes suggests that there are multiple mechanisms underlying the attained protection. The first example of PDR reported was in transgenic tobacco that accumulated CP of *Tobacco mosaic virus*, and the significant resistance achieved was termed CP-mediated resistance (4). Protection against viruses with two CPs (like comoviruses) could be achieved by expressing the capsid polyprotein (pCP; referred to in earlier studies as the precursor to the two CPs), or by expressing both the individual CP genes (8,28,29).

An earlier report showed that it is possible to obtain resistance to BPMV by expressing the pCP in transgenic soybean plants (8). However, the transgene utilized in that study was not stable,

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resulting in its loss in advanced generations and a subsequent loss of resistance (S. A. Ghabrial, *unpublished data*). The PDR against BPMV, reported in the present study, was generated by transformation of soybean with the BPMV-pCP gene via particle bombardment of somatic embryo cultures. Resistance to BPMV was demonstrated in the T₂ progeny of transgenic lines in plants inoculated mechanically and with viruliferous bean leaf beetles.

MATERIALS AND METHODS

Construction of the binary plasmid pHIG/BPMV-pCP. The 1.9-kbp *EcoRI/ClaI* fragment with the BPMV-pCP gene was removed from pMON1900 (8), blunt-ended with the T₄ DNA polymerase, and inserted into pBSFMA, a Bluescript-based plasmid containing a duplicated *Figwort mosaic virus* (FMV) 35S promoter (22) and rubisco small subunit gene (*rbcS*) terminator. The FMV 35S²-BPMV-pCP-*rbcS* 3' construct was ligated into the binary vector pHIG (44) to generate the recombinant plasmid pHIG/BPMV-pCP (Fig. 1).

Tissue culture and transformation. Somatic embryogenic cultures were initiated by plating immature (5 to 7 mm) zygotic embryogenic cotyledons (cv. Jack) on initiation media (Murashige and Skoog [MS]-based medium [27] containing [per liter] 40 mg of 2,4-D, 20 g of sucrose, and 2 g of phytagel, pH 7.0), and maintained in a culture room (23:1 h light/dark at 23°C) as detailed by Trick et al. (42). Embryos were transferred to proliferation media (MS-based medium containing, per liter, 20 mg of 2,4-D, 20 g of sucrose, and 2 g of phytagel, pH 5.8) for embryogenic proliferation of somatic embryos, with transfers made to fresh media every 21 to 24 days. Green embryos from raspberry-shaped clumps were selected for transformation by a dissecting microscope (Zeiss SV6; Carl Zeiss, Oberkochen, Germany). Selected material was placed in the center of a moist filter paper in sterile petri plates for 2 h (approximately 100 to 150 mg of somatic embryos per plate) and dried in a laminar flow hood for 15 min prior to bombardment. Transformation was carried out via particle bombardment with a gene gun (Dupont PDS1000; Bio-Rad Laboratories, Hercules, CA) by gold/DNA microprojectile preparations. Modifications to the standard protocol (42) included targeting each culture with a single shot (for nine shots, 25 µg of DNA was used to coat 7.5 mg of 0.6 µm gold). Cultures were bombarded by 1,550-lb/in² rupture disks (Bio-Rad) coupled with a focusing device (41) at 11.0 cm from rupture disk to target tissue.

Immediately after bombardment, embryogenic cultures were placed on proliferation media without any selective agent for 7 days. Selection of transformed material was done by transferring embryo cultures to proliferation media augmented with 15 mg/liter of hygromycin, pH 5.8, (Sigma Chemical, St. Louis) for 14 days,

then transferred to proliferation media with 30 mg/liter of hygromycin for 5 to 6 months, including transfer to fresh media every 4 weeks. During these transfers, some of the putative transgenic embryos were fractured. Such broken embryos could have given rise to one or more clones derived from a single transformation event. Surviving dark green clumps of embryogenic cultures were transferred onto maturation media (MS-based medium containing per liter, 60 g of maltose, 2 g of phytagel, and 0.5 g of activated charcoal, pH 5.8) for 21 days. Final maturation was obtained by transferring individual embryos onto maturation media until embryos had grown to 1.0 cm in length with visible cotyledons. Groups of six to eight embryos were desiccated in plastic petri plates sealed with Parafilm for 3 to 7 days. Embryos were removed as they became creamy white in color and were transferred to germination media (MS-based medium containing 30 g per liter of sucrose and 2 g of phytagel, pH 5.8). Most embryos began to form shoots and roots in 14 to 21 days, with some embryos requiring up to 8 weeks. These plantlets were transferred to Phytatrays (Sigma Chemical) containing half-strength B5 medium (11) for 14 days before being planted into artificial soil (ProMix BX, Premier Horticulture Inc., Red Hill, PA) and placed in humidity domes for acclimatization and subsequent transfer to the greenhouse.

Southern blot hybridization analysis. Genomic DNA was isolated from young leaves of soybean by homogenizing 100 mg of leaf tissue in a 1.5-ml microfuge tube containing 500 µl of extraction buffer (100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.5 M NaCl, 0.5% [wt/vol] sodium dodecyl sulfate [SDS], and 0.5% [vol/vol] β-mercaptoethanol). The homogenate was extracted with 500 µl of a phenol/chloroform/isoamyl alcohol mixture (25:24:1) and centrifuged at 12,000 × g for 5 min. The aqueous phase was collected, mixed with 1 µl of 10 mg/ml of RNase A, and incubated at room temperature for 20 min. The samples were re-extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), followed by two re-extractions with chloroform/isoamyl alcohol (24:1). DNA was precipitated by adding 2.5 volumes of ethanol and spooled out with a sterile glass Pasteur pipette. The spooled DNA was washed with 70% ethanol, dried, and resuspended in 100 µl of sterile water.

Five micrograms of DNA from each plant was digested overnight with *HindIII* or *EcoRI*, fractionated in 0.8% agarose gels, and blotted onto Zetaprobe membrane (Bio-Rad). Hybridization was done overnight at 42°C in a hybridization solution containing 50% formamide, 0.12 M Na₂HPO₄, 0.25 M NaCl, and 1 mM EDTA with either a 2.1-kb *HindIII/EcoRI* GUS-specific fragment or a 0.35-kb *HindIII* BPMV-pCP gene-specific fragment random prime labeled with [α -³²P] dCTP (Prime-It II Random Primer Labeling Kit; Stratagene, La Jolla, CA) as a probe. The membrane was washed three times at room temperature in 0.1× SSC (1×

Fig. 1. Diagrammatic representation of the binary plasmid pHIG/*Bean pod mottle virus* (BPMV) capsid polyprotein (pCP). The coding sequence of BPMV-pCP was inserted between the *Figwort mosaic virus* (FMV) 35S promoter and the rubisco small subunit gene (*rbcS*) terminator. P35S, *Peanut chlorotic streak virus* 35S promoter with duplicated enhancer domain; HPT, hygromycin phosphotransferase gene; C35S, *Cauliflower mosaic virus* 35S promoter. The bars, A and B, represent the 2.1-kb *HindIII/EcoRI* fragment (used as the GUS gene probe) and the 0.36-kb *HindIII* fragment (the BPMV-pCP gene-specific probe), respectively. The recombinant binary vector contains two *EcoRI* sites and four *HindIII* sites. The horizontal line at bottom (1.0 kb) indicates the size scale for the vector.

SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% SDS and exposed in a phosphorImager cassette (Molecular Dynamics, Sunnyvale, CA). The intensity of hybridized DNA bands was estimated using the ImageQuant software program (Molecular Dynamics).

Northern blot analysis. Total RNA was isolated from 100 mg of young leaf tissue using the Trizol reagent as suggested by the supplier (Gibco-BRL, Rockville, MD). Ten micrograms of total RNA was fractionated in 1.0% formaldehyde-agarose gels and transferred onto a Zetaprobe membrane. Conditions for hybridization and washing of membranes were as described for Southern blot hybridization analysis.

Immunoblot analysis. Leaf samples from transgenic plants were homogenized in 10 mM sodium phosphate buffer, pH 6.8, containing 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at $12,000 \times g$ for 10 min, and the supernatant was collected. The protein concentration was estimated with a protein assay (Bio-Rad). For immunoblot analysis, 15 μ g of protein was analyzed by SDS-polyacrylamide gel electrophoresis in 12% polyacrylamide gels according to the method of Laemmli (18). Proteins were electroblotted onto a nitrocellulose membrane in 25 mM Tris, 192 mM glycine, and 20% methanol (pH 8.2). The membrane was blocked for 2 h in 5% dry milk in Tris-buffered saline containing 0.05% Tween 20 and incubated with an antiserum to BPMV (14) in the same solution. Antigen-antibody complexes were visualized with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G and an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Buckinghamshire, England).

GUS staining. Plant tissue (leaf disks or flowers) was placed in vials containing the GUS assay buffer (50 mM sodium phosphate, 500 mM potassium ferricyanide, 500 mM potassium ferrocyanide, 10 mM EDTA, 1 mM X-gluc [5-bromo-4-chloro-3-indolyl- β -D-glucuronide], and 0.05% Triton X-100, pH 7.0) and incubated overnight at 37°C with gentle agitation (17). The assay buffer was decanted, and the seedlings were bleached in 95% EtOH to remove the chlorophyll.

Accumulation of BPMV-pCP in transgenic soybeans and evaluation of resistance to BPMV infection. To quantify the

accumulation of BPMV-pCP, four leaf disks were collected from the leaves of BPMV-pCP transgenic soybean plants and homogenized in 500 μ l of phosphate-buffered saline (PBS)-polyvinylpyrrolidone (PVP)-Tween buffer (pH 7.4). Serial 10-fold dilutions were made, and the BPMV-pCP levels were estimated by a double-antibody sandwich, enzyme-linked immunosorbent assay (ELISA) as described by Ghabrial and Schultz (14). Leaf extract dilutions, which produce ELISA absorbance values that fall within the linear phase of the standard sigmoid curve generated with purified virus concentration versus absorbency readings, were used.

The resistance of the primary transformants was evaluated with a detached leaf assay. Briefly, young fully expanded trifoliolate leaves from the BPMV-pCP soybeans were collected. Two of the Carborundum-dusted leaflets from each trifoliolate leaf were rub-inoculated with 10 μ g/ml (100 μ l per leaflet) of purified BPMV virions resuspended in 0.05 M potassium phosphate buffer, pH 7.0 (strain KY-G7, a relatively mild strain, was used). The third leaflet was mock inoculated with 0.05 M potassium phosphate buffer, pH 7.0. The inoculated trifoliolate leaves were placed in magenta boxes (Sigma Chemical) containing sterile cotton moistened with 0.02% of Peter's professional 20-20-20 fertilizer (Scotts Sierra Horticultural Products Co., Marysville, OH). The leaves were incubated at 26°C in a growth room for 7 days. Four leaf disks were sampled from each leaflet, homogenized in 500 μ l of PBS-PVP-Tween 20, and tested by ELISA as described previously.

Seedlings, at V2 stage (9), representing the T_2 progeny of transgenic soybean lines were assessed for virus resistance by rub-inoculation with purified virions (10 μ g/ml) or with viruliferous bean leaf beetles that were allowed to feed on the test transgenic seedlings for 72 h. A severe BPMV strain, Hopkins, was used in these inoculation experiments.

RESULTS AND DISCUSSION

Integration of the BPMV-pCP gene in soybean. In the first transformation experiment, 14 embryos that were green, and thus resistant to hygromycin after a 5 to 7 month selection, were removed for maturation and germination. Twelve of these embryos

Fig. 2. Southern blot hybridization analysis of total genomic DNA from putative transgenic soybean plants generated from two transformation experiments. Total DNA from primary transformants (T_0 generation) was digested with *Hind*III and probed with the GUS-intron gene probe. **A**, Lanes 137 to 200 (first experiment) and **B**, lanes 1 to 17 (second experiment) represent the putative transgenic plants; and lane WT, DNA from nontransformed wild-type 'Jack' soybean. The positions of the size markers in the 1-kb DNA ladder (Life Technologies, Rockville, MD) are indicated to the right.

developed shoots and roots and 10 of these were taken to the greenhouse, whereas the other two died in the humidity dome. Likewise, 22 hygromycin-resistant transgenic plants were also recovered in a second transformation experiment. Total genomic DNA was isolated from all 32 putative transgenic plants generated in the two experiments. Southern blot hybridization analysis of *Hind*III-digested DNA with the GUS gene probe indicated that the GUS gene was present in 6 of 10 plants from experiment 1 (Fig. 2A) and in 14 of 22 plants from experiment 2 (Fig. 2B). The hybridization profiles depicted in Figure 2A and B suggest that the transgenic plants recovered in experiments 1 and 2 represent two and eight independent integration events, respectively. The finding that some of the hygromycin-resistant plants lacked the transgenes indicates that selection in the presence of hygromycin was not very stringent.

Fig. 3. Southern blot hybridization analysis of DNA from primary transformants and progeny. DNA was digested with *Hind*III and hybridized with the 0.36-kb *Hind*III fragment of the *Bean pod mottle virus* (BPMV) capsid polyprotein (pCP) gene as a probe. **A**, The Southern blot shown in Figure 2A was reprobed with the BPMV-pCP gene probe after the GUS gene probe was stripped, lanes WT, nontransformed wild-type 'Jack' soybean; 137, 139, 157, 183, 186, and 200 are the primary transformants. **B**, T₁ generation: lanes 139-1 and 200-1 were the lone progeny lines of 139 and 200, respectively, and 183-1 and 183-2 were the two progeny lines obtained from 183. **C**, T₂ generation: lanes 183-1-1, 183-1-2, 200-1-1, 200-1-2, 183-2-1, 183-2-2, and 183-2-3 are representative T₂ progeny of lines 183-1, 200-1, and 183-2.

Fig. 4. Southern blot hybridization analysis of DNA from T₂ progeny of lines 183-1 and 183-2. Total DNA from the T₂ progeny of **A**, line 183-1 (plants 2 to 16, and **B**, line 183-2 (plants 1 to 21, panel B) was digested with *Hind*III and probed with the *Bean pod mottle virus* capsid polyprotein (pCP) gene probe. Note that the progeny of line 183-1, but not that of line 183-2, segregated for the pCP transgene.

The results of the Southern blot hybridization analysis shown in Figure 2A revealed the presence of three copies of the GUS gene in each of five transgenic plants (137, 139, 157, 183, and 186), whereas there was only one copy detected in plant 200. When the

Fig. 5. Southern blot hybridization analysis of DNA from T₂ progeny of line 183-1. **A**, Total DNA from the T₂ progeny of line 183-1 (plants 2 to 12) was digested with *Hind*III and probed with the GUS-intron gene probe, or **B**, digested with *Eco*RI and probed with the *Bean pod mottle virus* capsid polyprotein (pCP) gene probe. Note that the T₂ progeny of line 183-1 is segregating for the pCP gene and that the transgenic progeny maintained the three copies of T-DNA intact.

Fig. 6. Northern blot hybridization analysis of T₀ and T₁ transgenic soybean plants. Blots of total RNA from **A**, leaves of the primary transformants 137 to 200, different tissues from **B**, the T₀ plant 183, and from **C**, leaves of T₁ progeny of 139 lines (139-1), 183 (183-1 and 183-2), and 200 (200-1) were hybridized with the 0.36-kb *Bean pod mottle virus* (BPMV) capsid polyprotein (pCP) gene-specific fragment or with a 28S rRNA gene-specific probe. Lane WT, nontransformed wild-type 'Jack' soybean.

GUS gene probe was stripped from the *Hind*III-digested DNA blot and the blot was reprobed with the BPMV-pCP gene probe, only five of the six GUS-positive transgenic plants from experiment 1 contained the BPMV-pCP gene (Fig. 3A). The five transgenic plants containing three copies of T-DNA were probably from the same integration event, whereas plant 200 likely had an incomplete copy of T-DNA (i.e., lacking the BPMV-pCP gene) and was from a different transformation event. All 14 GUS-positive transgenic plants recovered in experiment 2 contained the pCP gene when the GUS gene probe was stripped and the blot (Fig. 2B) was reprobed with the pCP gene probe (data not shown). Further analysis of the transgenic plants was confined to advanced generations (i.e., T₁ and T₂ progeny) of plants recovered in experiment 1.

Southern blot hybridization analysis showed that the T₁ progeny of plant 139 (line 139-1) and plant 183 (lines 183-1 and 183-2) inherited the BPMV-pCP gene (Fig. 3B). Likewise, the T₂ progeny of lines 183-1 and 183-2 inherited the BPMV-pCP gene (Fig. 3C). Furthermore, Southern blot hybridization analysis of 21 T₂ progeny of line 183-2 showed that all the T₂ progeny inherited the pCP gene (Fig. 4B). The T₂ progeny of line 183-1, on the other hand, segregated for the transgene (Fig. 4A). Of a total of 19 T₂ progeny tested, 12 were positive for the transgenes. Although the ratio of positive to negative plants was lower than the expected 3:1 ratio, it could be explained based on the small size of the population examined. Altogether, the results clearly support the conclusion that line 183-2 is homozygous, whereas line 183-1 is hemizygous. Further analysis of the T₂ progeny of line 183-1 revealed that the three copies of T-DNA are linked because they were inherited intact even in the T₂ generation (Fig. 5A). Moreover, Southern blot hybridization analysis of *Eco*RI-digested genomic DNA with the BPMV-pCP gene probe suggest that two of the T-DNA copies were inserted side by side in the genomic DNA in a tail-to-tail arrangement. The approximate 9.0-kb *Eco*RI fragment shown in Figure 5B could be accounted for by the tail-to-tail orientation of two of the T-DNA copies, whereas the 4.3-kb *Eco*RI fragment is predicted to originate from the third copy, which is probably closely located (Fig. 1 for estimating the size of the resultant *Eco*RI fragments).

Fig. 7. Immunoblot analysis of T₀ transgenic soybean plants. Total soluble proteins isolated from leaves of the primary transformants (137 to 200) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, and the separated proteins were transferred to a nitrocellulose membrane and probed with an antiserum to *Bean pod mottle virus* (BPMV). Lane CP, purified BPMV virions containing the large (41 kDa) and small (22 kDa) coat protein subunits; MW, Kaleidoscope prestained standards (BioRad Laboratories, Hercules, CA); WT, protein from nontransformed wild-type 'Jack' soybean; and 137 to 200, protein from the indicated primary transformants. The position of the 63-kDa capsid polyprotein (pCP) is indicated by an arrow. The high molecular weight signal observed for pCP transgenic plants (lanes 137, 139, 157, 183, and 186) is probably due to aggregation of pCP.

The instability of the BPMV-pCP transgene reported by Di et al. (8) could be explained if some of the T₀ transgenic lines derived from *Agrobacterium*-mediated transformation of cotyledonary nodes were chimeric. Problems associated with transgenic chimeras from transformation of meristematic tissues have been previously described (6,25,45). In addition, *Agrobacterium*-mediated transformation of soybean is not routine due to the recalcitrance of soybean to infection by *Agrobacterium tumefaciens* (42,44). Particle bombardment of soybean shoot apical meristems (2,24) or embryogenic suspension cultures (23,30,31) has been successfully used to circumvent the problems posed by *Agrobacterium*-mediated transformation. However, transgenic soybean plants derived by particle bombardment of shoot apical meristems also can be chimeric (6). Somatic embryogenic cultures offer advantages over other methods for soybean transformation. Secondary somatic embryos appear to be derived from single cells or from a few epidermal cells of primary embryos, thereby reducing the chances of chimerism. However, there is a possibility of obtaining multiple transgenic lines derived from the same initial transformation event. Recent developments have increased the efficiency of the embryogenic system and decreased the time needed for initiation of somatic embryos and regeneration (35,37,43). Hence, in the present study, somatic embryos were used as the explants to generate transgenic soybean lines.

TABLE 1. Double antibody sandwich, enzyme-linked immunosorbent assay (ELISA) of detached leaves from transgenic T₀ soybean plants previously inoculated with *Bean pod mottle virus* (BPMV)^a

Sample	ELISA absorbance values (A _{405nm})	
	Mock-inoculated	BPMV-inoculated ^b
T-137	0.321	0.484
T-139	0.329	0.411
T-157	0.275	0.397
T-183	0.329	0.370
T-186	0.237	0.386
T-200	0.011	0.756
'Jack'	0.010	0.807
Purified BPMV		
75 ng/ml	0.226	...
150 ng/ml	0.422	...

^a Values are averages of two replicates of each of two leaflets per treatment.

^b Carborundum-dusted leaflets were rub-inoculated with purified BPMV virions (10 µg/ml; 100 µl per leaflet). Two leaflets of each trifoliolate leaf were virus-inoculated and the third leaflet was mock-inoculated with inoculation buffer (0.05 M potassium phosphate buffer, pH 7.0).

TABLE 2. Reactions of T₂ transgenic plants to inoculation with *Bean pod mottle virus* (BPMV) as evaluated by symptoms development and double antibody sandwich, enzyme-linked immunosorbent assay (ELISA)^a

Progeny (+/- pCP gene) ^b	Symptoms ^c	ELISA values A _{405nm}	Southern blot analysis ^d
183-1-1 (-)	...	1.277	Lane 183-1-1
183-1-2 (+)	Figure 8C	0.884	Lane 183-1-2
183-2-1 (+)	...	0.164	Lane 183-2-1
183-2-2 (+)	Figure 8D	0.145	Lane 183-2-2
183-2-3 (+)	...	0.156	Lane 183-2-3
200-1-1 (+)	Figure 8B	1.325	Lane 200-1-1
200-1-2 (+)	...	1.891	Lane 200-1-2
Jack-1 (-)	Figure 8A	1.174	Lane WT
Jack-2 (-)	...	1.291	...

^a ELISA values for noninoculated transgenic plants were in the range of 0.1 to 0.3. ELISA values for noninoculated controls ('Jack' and T₂ progeny of line 200-1, nontransformed and vector-transformed controls, respectively) were in the range of 0.010 to 0.015.

^b (-) or (+) indicates plants lacking or containing the capsid polyprotein (pCP) gene, respectively, as determined by Southern blot hybridization analysis.

^c The symptoms developed in response to BPMV inoculation are shown in Figure 8 as indicated.

^d The pertinent lanes from the Southern blot shown in Figure 3C are indicated.

Partial sterility in the transgenic soybeans. Primary transformants 137, 139, 157, 183, 186, and 200 exhibited varying degrees of fertility, but in all cases, the majority of flowers aborted or failed to set seed. Most of the remaining flowers produced pseudo-pods that were approximately 1 cm in length and without any seed. However, we were able to recover two, three, and one seed from plants 139, 183, and 200, respectively. Upon germination, progeny of plant 183 (183-1, 183-2) and of plants 139 and 200 (139-1 and 200-1, respectively) were recovered. The progeny of plants 139, 183, and 200 set normal pods. These sterility problems may be related to the long period in tissue culture (6 to 8 months), but are apparently evident only in the initial generation. No long-term phenotypic changes or heritable fertility decreases have been noted in subsequent generations.

Expression of BPMV-pCP in the transgenic plants. Using the GUS gene probe, northern blot hybridization analysis of total RNA isolated from T_0 plants 137, 139, 157, 183, 186, and 200

revealed the presence of the GUS transcript in all plants (data not shown). GUS activity was high in these plants, revealed by the intense blue of the stained leaves (data not shown). Northern blot hybridization analysis with the BPMV-pCP gene probe (Fig. 6A) showed that the BPMV-pCP transcript was present at high levels in all plants except T-200, which was consistent with the Southern blot hybridization data. Moreover, the transcript levels of the BPMV-pCP gene in these plants were similar, most likely because they were all derived from the same integration event. Further analyses with total RNA isolated from pod, leaf, stem, and root from plant 183 revealed that the BPMV-pCP transcript levels were highest in leaves compared with roots, stems, and pods, respectively (Fig. 6B). This pattern of differential expression was evident in tissues from plants 157 and 186 (data not shown). Northern blot analysis of T_1 progeny of lines 139-1, 183-1, 183-2, and 200-1 revealed that the BPMV-pCP transcript levels were also high, therefore, comparable to those noted in the T_0 generation (Fig. 6C).

Fig. 8. Symptom development on transgenic and control soybean plants inoculated with *Bean pod mottle virus* (BPMV). **A to D**, Symptoms observed on the nontransformed wild-type 'Jack' soybean and the T_2 progeny of lines 200-1 (plant 200-1-1), 183-1 (plant 183-1-2), and 183-2 (plant 183-2-1) are shown, respectively. The plants were mechanically inoculated with a severe strain (Hopkins) of BPMV and were monitored for symptom development for 3 weeks postinoculation. Chlorotic/necrotic symptoms appeared on the inoculated leaves of the susceptible control plants 4 to 5 days after inoculation, and systemic symptoms of severe mottling, leaf distortion, and blistering were observed 1 to 2 weeks. Progeny of line 183-2 showed little or no symptoms throughout the duration of the experiment, including the flowering and pod development stages. The youngest fully expanded leaves 5 weeks postinoculation were photographed.

Western blot analysis of total soluble proteins from leaves of T₀ transgenic plants 137, 139, 157, 183, and 186 showed a high level of expression of the 63-kDa pCP (Fig. 7, arrow). As expected, the 63-kDa pCP was not detected in transgenic plant 200. It has previously been shown that the pCP, produced in transgenic plants transformed with the pCP gene, neither undergoes proteolytic processing to the large (L) and small (S) capsid protein subunits (41 and 22 kDa in size, respectively) nor assemble into viruslike particles (28). The virion L and S capsid proteins are shown in Figure 6, lane CP. The expression levels of the 63-kDa pCP were high in both young and mature leaves, and were estimated by ELISA to be as high as 0.02% of the total soluble protein in leaves from T₀ transgenic plants 137, 139, 157, 183, and 186.

Resistance to BPMV in transgenic plants. Primary transformants 137, 139, 157, 183, 186, and 200 were tested for resistance to the KY-G7 strain of BPMV by the detached leaf assay. When the detached leaves were tested by ELISA 7 days after inoculation, all transgenic leaves, with the exception of T-200, showed some resistance to BPMV infection, demonstrated by the lower levels of BPMV accumulation compared with the nontransformed control 'Jack' (Table 1). Because the T-200 plant lacks the BPMV-pCP gene, its ELISA value was similar to the nontransgenic wild-type cv. Jack (Table 1). Virus movement from the BPMV-inoculated leaflets (two of the three leaflets of the trifoliolate detached leaf) to the mock-inoculated third leaflet was negligible as clearly shown by ELISA of the nontransformed control or vector-transformed plants (Table 1). Thus, the higher ELISA values for the mock-inoculated transgenic plants compared with the nontransgenic ones are due to the expression of the pCP gene.

The reactions of the progeny of lines 183-1 and 183-2 to rub-inoculation with a purified preparation of the severe strain Hopkins of BPMV are shown in Table 2 and Figure 8. The progeny of line 183-1 developed symptoms, but plant 183-1-2 was moderately resistant (based on level of BPMV accumulation) compared with the progeny of line 200-1 and nontransformed controls (Table 2). The susceptible plant 183-1-1, which did not inherit the pCP gene (Fig. 3C, lane 183-1-1), accumulated BPMV to a level comparable to that of the nontransgenic lines (Table 2). As stated earlier, line 183-1 is hemizygous and its progeny are predicted to segregate with respect to the transgene. Thus far, all progeny of homozygous line 183-2 analyzed to date were resistant, because they showed little or no symptoms throughout the duration of the experiment, including flowering and pod development stages.

TABLE 3. Double antibody sandwich, enzyme-linked immunosorbent assay (ELISA) of T₂ transgenic plants on which viruliferous bean leaf beetles were allowed to feed for 72 h^a

Plant number (+/- pCP gene) ^b	ELISA values (A _{405nm}) ^c	
	Upper leaves	Lower leaves with visible feeding sites
183-1-10 (-)	>2.00	1.838
183-1-20 (+)	0.119	0.208
183-1-30 (+)	0.149	0.236
183-1-40 (+)	0.155	0.964
183-2-10 (+)	0.121	1.374
183-2-11 (+)	0.435	0.603
183-2-12 (+)	0.467	1.036
183-2-13 (+)	0.664	1.403
183-2-14 (+)	0.215	1.027
'Jack' (-)	>2.0	1.911
200-1-3 (-)	1.978	1.140

^a ELISA values for noninoculated transgenic plants were in the range of 0.1 to 0.3. ELISA values for noninoculated controls ('Jack' and 200-1-3, nontransformed and vector-transformed controls, respectively) were in the range of 0.010 to 0.015.

^b (-) or (+) indicates plants lacking or containing the capsid polyprotein (pCP) gene, respectively, as determined by Southern blot hybridization analysis.

^c Values are averages for two replicates. Results obtained with leaf extract dilutions of 1:10,000 (wt/vol) are shown.

Likewise, there was little or no accumulation of BPMV detected by ELISA (Table 2). A total of six 183-2 T₂ progeny plant, including the three plants presented in Table 2, were tested by rub-inoculation with a purified virion preparation, and all plants were resistant, with a mean ELISA value of 0.150 (data not shown). The higher resistance of the T₂ progeny of line 183-2 compared with that of 183-1 is probably due to the fact that it is homozygous for the transgene. The limited number of T₂ progeny tested in these experiments was due to the fact that only 22 and 25 seeds, respectively, were recovered from lines 183-1 and 183-2.

In experiments with viruliferous bean leaf beetles, ELISA testing of leaves with visible feeding activities indicated that 9 of 11 plants tested became infected (Table 3). The two progeny plants that did not become infected were derived from line 183-1 (183-1-20 and 183-1-30); their ELISA values for the inoculated leaves were higher than those of the mock-inoculated nontransgenic controls because they express the pCP gene. Inoculated leaves of transgenic plant 183-1-40, on the other hand, did become infected but, interestingly, this plant did not become systemically infected. The response of this plant to virus infection suggests that it is homozygous for the pCP gene (Table 3). The response of plant 183-1-10, which did not inherit the pCP gene, to virus infection is comparable to that of nontransformed or vector-transformed plants (Table 3). Thus, the response of the segregating progeny of line 183-1 to virus infection is consistent with the conclusion that it is hemizygous. The upper noninoculated leaves of progeny of the homozygous line 183-2 showed reduced or no symptoms and accumulated little or no BPMV, determined by ELISA (Table 3, compare ELISA values with those of mock-inoculated transgenic plants). The difference in virus accumulation in the T₂ progeny of line 183-2 between rub-inoculation of virions (Table 2) compared with beetle inoculation (Table 3) may reflect different responses to the inoculation methods.

In summary, some of the transgenic soybean lines generated in this study exhibited resistance to systemic infection by BPMV. Progeny of the homozygous transgenic line 183-2 could provide valuable material for incorporation into commercial varieties. Because of the current outbreak of BPMV in the major soybean-growing regions and the absence of natural resistance, there is a need to develop commercial cultivars with BPMV resistance.

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Erratum

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In the manuscript entitled "Resistance to *Bean pod mottle virus* in Transgenic Soybean Lines Expressing the Capsid Polyprotein" by M. S. S. Reddy, S. A. Ghabrial, C. T. Redmond, R. D. Dinkins, and G. B. Collins (*Phytopathology* 91:831-838), the first column of Table 2 incorrectly reports that progeny 200-1-1 and 200-1-2 contain the capsid polyprotein gene. Progeny 200-1-1 and 200-1-2 do not contain the capsid polyprotein gene.