

Resistance

A Cowpea Line Has Distinct Genes for Resistance to Tobacco Ringspot Virus and Cowpea Mosaic Virus

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ABSTRACT

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A previous survey of cowpea (*Vigna unguiculata*) lines identified one, Arlington (USDA plant introduction number 293453), that was both resistant to the comovirus cowpea mosaic virus strain SB (CPMV) and a source of protoplasts that restrict CPMV replication. We report that the budblight strain of tobacco ringspot virus (TobRV), which infects several cowpea lines including Blackeye 5, failed to induce symptoms in Arlington cowpea seedlings or to be detected at 10-12 days after inoculation by enzyme-linked immunosorbent assay. F1 progeny of Blackeye 5 × Arlington all resisted infection by TobRV. Among F2 progeny, resistance

to TobRV and to CPMV were inherited as single dominant characters, but they segregated distinctly. TobRV, as well as CPMV, interfered with the increase of the comovirus cowpea severe mosaic virus in Arlington cowpeas. Previous results showed that Arlington cowpeas possess an inhibitor of CPMV-SB proteinase. No similar activity was detected against a TobRV proteinase. Thus, the resistances of Arlington cowpeas to TobRV and to CPMV, although similar in several respects, are controlled by separate genetic loci and probably operate under distinct mechanisms.

Additional keywords: concurrent protection, operational immunity, polyprotein processing, translation inhibitor.

Beier et al (2,3) identified the cowpea (*Vigna unguiculata*) line Arlington as the only one among more than 1,000 lines tested that was a source both of seedlings that are "operationally immune" to cowpea mosaic virus strain SB (CPMV) and of protoplasts that are resistant to the same virus. "Operational immunity" refers to a situation in which no virus increase was detected in plants exposed even to concentrated inocula. It implies no conclusion about whether virus replication had or had not actually occurred to some low, undetected level or any specific mechanism for the observed immunity.

Kiefer et al (16) obtained evidence suggesting that the immunity trait is the result of restriction of CPMV accumulation in

inoculated cells and is genetically controlled by a dominant allele (16). The results of Sanderson et al (22) and Ponz et al (20) implicated a plant-encoded inhibitor of CPMV-controlled processing of the CPMV-encoded polyproteins, and possibly an inhibitor of the translation of CPMV RNAs, as mediators of the immunity of Arlington seedlings against CPMV. A surprising interference phenomenon also was observed in this system (4,12). CPMV, although unable to replicate and accumulate to detected levels in Arlington cowpea seedlings, interfered with the accumulation of, and expression of symptoms by, cowpea severe mosaic virus strain DG (CPSMV).

With the advent of technologies for generating transgenic plants,

the search for potentially useful resistance genes need not be restricted to the cultivar of interest or even to plants with which it may be crossed genetically. Tobacco ringspot virus (TobRV), the type member of the nepovirus group, has a very broad host range and the ability to infect and seriously damage many crop plants, including cowpeas (24,25). Although TobRV is not known to be an important disease agent of cowpeas, it is able to infect many lines of this plant, such as Blackeye 5, and to kill seedlings in a few days (14). We report here the resistance of Arlington cowpeas to TobRV and the ability of TobRV to induce the protection of Arlington cowpeas against CPSMV.

MATERIALS AND METHODS

Viruses, hosts, and inoculation procedures. The source of the inoculum of budblight strain TobRV was leaves of Harosoy soybean (*Glycine max*) seedlings germinated from infected seeds. Satellite RNA of TobRV has not been detected in such inocula (14). CPMV and CPSMV were from a collection maintained in this laboratory. The Blackeye 5 and Arlington lines of cowpeas are USDA plant introduction numbers 293458 and 293453, respectively.

For inoculations, 2-g samples of frozen Harosoy soybean tissue were ground in a mortar, and the extract was diluted to 10 ml with 0.05 M potassium phosphate buffer, pH 7. Purified CPMV and CPSMV were diluted in the same buffer. TobRV concentration was determined by enzyme-linked immunosorbent assay (ELISA); CPMV and CPSMV concentrations were determined spectrophotometrically. We inoculated each of the two corundum-dusted unifoliate leaves of a set of 7-day-old cowpea seedlings with the same two 35- μ l aliquots, either virus inoculum plus buffer or two virus inocula (14). Mixing of the 70 μ l of liquid and inoculation of both leaves was accomplished with a gloved finger or glass paddle.

Assessment of infection. At 7–11 days after inoculation symptoms were recorded, and cowpea leaf extracts were analyzed for virus by immunodiffusion or ELISA. A weighed portion (80–120 mg) of leaf tissue was frozen on dry ice in a 13 \times 100 mm culture tube and ground with a glass rod. Addition of 0.2 ml of 50 mM sodium phosphate buffer, pH 7, and further homogenization produced a thick slurry. Ten μ l aliquots of slurry and of polyclonal rabbit antiserum to the appropriate virus were pipetted into 2-mm-diameter wells 8 mm apart in 2.5-mm-thick 1% agarose gel in the same phosphate buffer. Precipitin lines were noted 1 and 2 days later.

For direct ELISA eight 5-mm-diameter leaf disks were homogenized in 2 ml of extraction solution as described (21). Detection was with immunoglobulin G linked to horseradish peroxidase, the substrate was o-phenylenediamine, and the absorbance was measured at 450 nm.

Inhibition of translation and polyprotein processing. As described by Ponz et al (20), extracts of Arlington cowpea seedlings were chromatographed on 6% agarose beads (Sephacrose CL-6B) to yield four major "peaks," with "peak IV" containing activities inhibitory to translation of CPMV RNAs and to the processing of CPMV polyproteins. In the assay for inhibition of the translation of TobRV RNAs, aliquots of the chromatographic peak fractions were submitted to rabbit reticulocyte lysate (Promega Biotec), programmed with TobRV RNAs 1 and 2, and incubated with [³⁵S]methionine. Polyacrylamide gel electrophoresis and fluorography revealed the extent and pattern of proteins synthesized (20). Polyprotein processing was assessed with a modification of this reaction. Processing occurred in the reaction mixture, apparently catalyzed by proteinase(s) that are part of the polyprotein translated from RNA 1 (13,15). Based on the observation that most of the translation of TobRV RNAs, but little of the polyprotein processing, occurred in the first 40 min of incubation, fractionated cowpea leaf extract was tested for its ability to inhibit polyprotein processing by adding it to the translation reaction mixtures at 40 min. Conditions of in vitro translation, and subsequent polyprotein processing and analysis, were as described for virion RNAs of cherry leafroll virus and CPMV (19,20).

RESULTS

Immunity of Arlington cowpeas against TobRV. As previously observed (14), seedlings of cowpea cultivar Blackeye 5 developed large necrotic local lesions on the inoculated unifoliate leaves and necrosis of the hypocotyl region a few days after inoculation with TobRV. The plants usually collapsed and died before they could expand their trifoliate leaves. In contrast, we detected no symptoms on Arlington cowpea seedlings so inoculated. Extracts of buffer-rubbed and TobRV-inoculated Arlington cowpeas gave comparable background ELISA values, whereas extracts from TobRV-inoculated Blackeye 5 cowpea seedlings, in 30 μ l of buffer per mg of tissue, generated ELISA values greater than 2. Thus, Arlington cowpea seedlings may be defined as being operationally immune to TobRV under the specified conditions of inoculation, just as they are operationally immune to CPMV (2).

Arlington cowpeas were crossed to Blackeye 5 cowpeas. The F1 progeny and their F2 progeny were inoculated with TobRV. None of the 22 F1 progeny tested developed symptoms when inoculated with TobRV, and the extracts of the three of these that were tested proved also to be negative by ELISA for TobRV. F2 progeny seedlings were inoculated with either TobRV or CPMV. Some of the seedlings that proved to be immune to the virus first inoculated subsequently were inoculated with the other virus. The results, in Table 1, show that immunity to TobRV, as well that to CPMV (16), was inherited as a single dominant trait. The immunity to TobRV and the immunity to CPMV are under the control of different genetic loci, as indicated by the second and fourth lines of entries in Table 1.

The distinctness of the traits of immunity against the two viruses was confirmed by inoculating, with TobRV, other cowpea progeny lines that had been selected for immunity to CPMV. Two lines were F3 derivatives from Arlington \times Blackeye 5 (16), and seven lines were derived from a seven-generation backcross begun with Blackeye 5 and a homozygous immune F3 derivative of Arlington and Blackeye 5 (20). Seedlings from these CPMV-immune lines all efficiently supported the increase of TobRV as judged by development of symptoms.

Tests of interference with polyprotein processing and with translation of TobRV RNAs. To test for the possibility that an inhibitor of TobRV polyprotein processing is the mediator of immunity to TobRV, we assessed the ability of the four peak fractions from the chromatography of extracts from Arlington seedlings to interfere with the in vitro processing of TobRV polyproteins synthesized under the direction of a mixture of TobRV RNAs 1 and 2. Although the proteolytic processing of polyproteins reported for TobRV (13,15) was confirmed, no inhibitory activity was detected for the added fractions (data not shown).

TABLE 1. Segregation of resistance to TobRV and CPMV in F2 progeny of crosses of Arlington and Blackeye 5 cowpeas

Experiment	Virus	Number		Chi-square, 3:1 ratio ^a
		Immune	Susceptible	
1 ^b	TobRV	142	59	2.03
1 ^c	CPMV	49	13	0.54
2 ^d	CPMV	141	52	0.39
2 ^e	TobRV	50	22	1.19

^a The observed chi-square values are smaller than the $P=0.05$ value of 3.84 (1).

^b Seedlings that developed symptoms were scored as susceptible. ELISA values were less than three times background for the nine symptomless seedlings that were tested.

^c Sixty-two of the 142 seedlings that had been reported as immune in line 1 were inoculated with CPMV. Ten days later, seedlings that exhibited an increase in CPMV, as assessed by immunodiffusion of extracts, were scored as susceptible.

^d Susceptibility assessed by immunodiffusion and appearance of symptoms.

^e Seventy-two of the 141 uninfected cowpeas reported in the third line were inoculated with TobRV. Scored as susceptible were those seedlings that died and those that, at 10 days after inoculation, gave extracts with ELISA signals more than three times background.

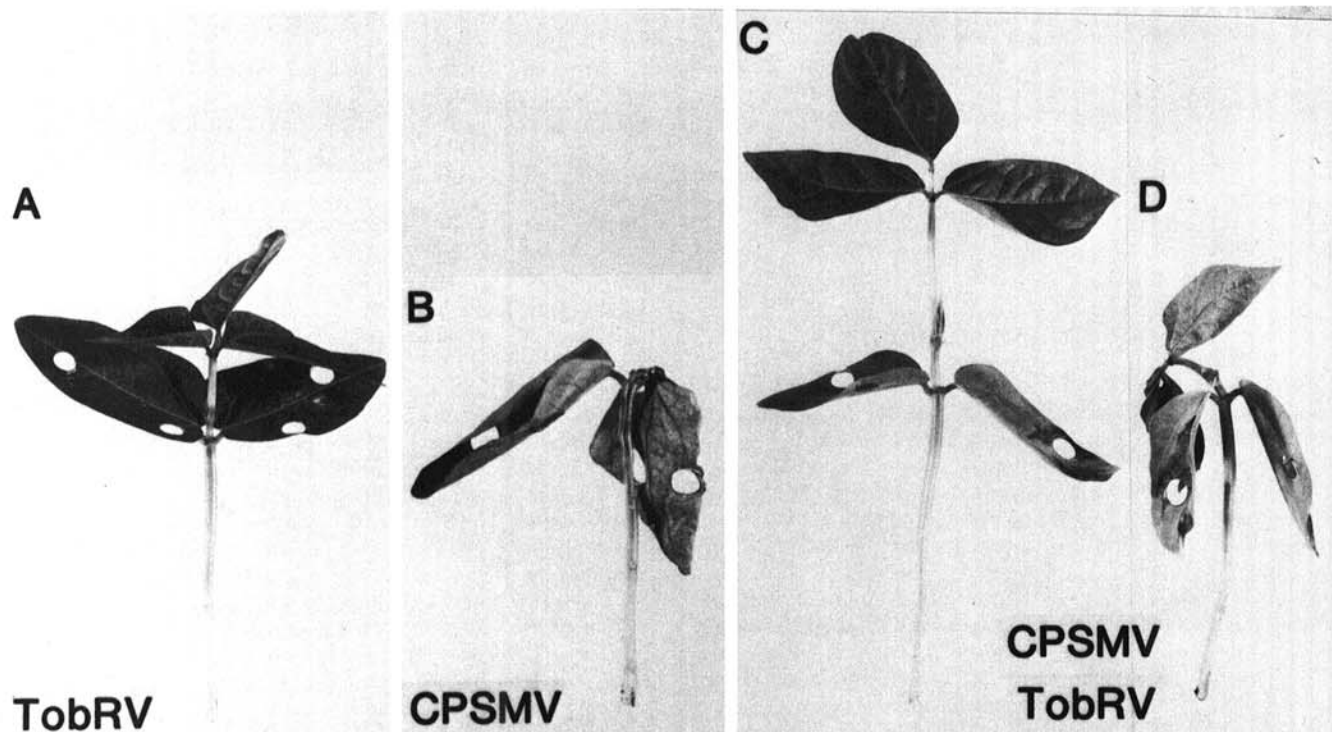


Fig. 1. Protection of Arlington cowpea seedlings against CPSMV by simultaneous inoculation of TobRV. Seedlings were inoculated 1 wk after planting and were photographed 10 days later. Inocula contained CPSMV at a final concentration 2.5 µg/ml (B-D) and TobRV at 2 µg/ml (A and C) or 0.2 µg/ml (D). The holes in the inoculated unifoliate leaves are sites from which samples were taken for ELISA analysis. Samples from the trifoliate leaves were taken later. Abrasion-induced leaf damage is visible on the unifoliate leaves in A.

TABLE 2. Protection between TobRV and CPSMV in co-inoculated Arlington cowpea seedlings^a

Entry	Inoculum	ELISA absorbance values			
		TobRV antiserum		CPSMV antiserum	
		unifoliate leaves	trifoliate leaves	unifoliate leaves	trifoliate leaves
1	None	0.026 ±.001	0.033 ±.001	0.042 ±.004	0.055 ±.003
2	TobRV	0.034 ±.009	0.036 ±.004	0.030 ±.002	0.033 ±.002
3	CPSMV	0.016 ±.013	0.030 ±.003	0.85 ±.28	1.07 ±.31
4	CPSMV + TobRV	0.025 ±.019	0.039 ±.004	0.74 ±.25	0.057 ±.010
5	CPSMV + TobRV	0.034 ±.010	0.036 ±.009	1.06 ±.26	1.60 ±.03
6	CPSMV + TobRV	0.034 ±.004	0.035 ±.002	1.12 ±.28	1.44 ±.40

^aInocula contained CPSMV at a final concentration of 2.5 µg/ml and TobRV, as dilutions of sap, at 2 µg/ml (entries 2 and 4), 0.4 µg/ml and 0.2 µg/ml (entries 5 and 6, respectively). Pooled leaf disk samples were collected from four plants for ELISA, at 6 days after inoculation from the inoculated unifoliate leaves and at 10 days from trifoliate leaves. Extracts were diluted with 250 µl of buffer per mg of leaf disk. ELISA absorbance readings were expressed as the average and range of results derived from two experiments (entries 1, 3, 5, and 6) and as the average and standard deviation from three experiments (entries 2 and 4), one of which corresponds to Figure 1 (A-D and entries 2, 3, 4 and 6, respectively).

Ponz et al (20) found that extracts of Arlington seedlings contain inhibitors of the translation of CPMV RNAs, in addition to inhibitor(s) of polyprotein processing. At least one of these translation inhibitors has the characteristics of virus specificity and inheritance in cowpea crosses that are expected of an agent capable of mediating resistance to CPMV. The chromatographic peak IV fraction that was active as an inhibitor of the translation of CPMV RNAs also inhibited translation of TobRV RNAs.

Protection against CPSMV induced by TobRV. Because

CPMV is able to interfere with CPSMV increase without itself accumulating to a detected level (4,12), we tested for a similar ability of TobRV to interfere with CPSMV infections of Arlington cowpeas. CPSMV induces large necrotic local lesions, necrosis of stems and veins, and often the death of inoculated Arlington cowpea seedlings. Results presented in Figure 1 show that TobRV protected Arlington seedlings, co-inoculated with CPSMV and TobRV, from the symptoms that were induced by CPSMV alone. This was demonstrated by reduced number and size of the local lesions on the inoculated leaves, and by the lack of stem necrosis and symptoms on the uninoculated trifoliate leaves (Fig. 1C).

The protective effect of TobRV was dose dependent (Fig. 1, compare C and D). The degree of protection against symptom development was correlated with reduced accumulation of CPSMV capsid antigen (Table 2). Unifoliate leaves inoculated with both viruses supported a slightly less extensive accumulation of CPSMV than those inoculated with CPSMV alone (Table 2, entries 3 and 4), and no CPSMV accumulation was detected in the uninoculated trifoliate leaves (entries 1 and 4). The same pattern of interference was observed in two additional experiments, one assessed by observation of symptoms, the other by symptoms and ELISA. In contrast, TobRV and CPSMV did not interfere with each other in a TobRV-susceptible host, Blackeye 5 cowpea seedlings, as assessed by induced symptoms and ELISA of extracts from inoculated and uninoculated leaves (data not shown).

We measured the effects of delaying the inoculation of CPSMV, after inoculation of TobRV. Groups of four 1-wk-old Arlington cowpea seedlings were inoculated with TobRV and CPSMV separately or together, and CPSMV also was inoculated after TobRV. The inocula and the total number of lesions on inoculated leaves at 5 days after inoculation were: TobRV alone, none; CPSMV alone, >> 200; concurrently inoculated TobRV and CPSMV, 53. Results from treatments with the delayed inoculation of CPSMV were 11 lesions at a 5-min delay, 7 lesions at 15 min, 37 lesions at 90 min, and 55 lesions at 255 min. Eleven days after inoculation all seedlings of some groups, those inoculated with CPSMV alone or with CPSMV at 90 or 255 min after inoculation with TobRV, were dead. The other seedlings had developed trifoliate leaves and were continuing to grow.

DISCUSSION

Operational immunity against TobRV. The Arlington cowpea was received in this laboratory (2) more than 10 yr ago as a donation from the USDA Plant Introduction collection at Experiment, Georgia. The origin of this cowpea line is listed as the U.S.A., and we do not know its original source or history. We report here that Arlington cowpea seedlings are operationally immune not only to the comovirus CPMV (2) but also to the nepovirus TobRV, the immunity against each virus being controlled by a separate dominant allele. Any or a combination of several possible mechanisms may underlie the operational immunity to TobRV.

Several examples of restrictions of nepoviruses are known. Plants infected with nepoviruses frequently exhibit a recovery phase in which a growing part of the plant develops nearly free of virus and virus-induced symptoms (17). In contrast to the recovery phenomenon, Arlington cowpea seedlings inoculated with TobRV show no initial increase of the virus. Another protection phenomenon is that induced by satellite RNA (23). The action of satellite RNA also does not seem to be connected to the observed operational immunity to TobRV by Arlington cowpeas, because no satellite RNA could be detected in the TobRV inoculum (14) and the immunity is controlled by host genetic factors.

Operational immunity against TobRV is controlled by a dominant allele, strongly suggesting that the immunity is due to the action of some Arlington-derived interfering biochemical factor, rather than the absence of a factor necessary for TobRV replication. Contrary to results with CPMV (20,22), tests for a factor from Arlington cowpeas that is active against TobRV polypeptide processing were negative, which neither supports nor eliminates involvement of such an inhibitor in immunity against TobRV. The inhibitory activity that was detected against the translation of TobRV RNAs is almost certainly the result of a mixture of substances (20). An inhibitor of translation that would be a candidate mediator of the restriction of TobRV in the Arlington cowpea should be co-inherited with immunity in cowpea crosses. We do not have at present the progeny of genetic crosses of Arlington and Blackeye 5 cowpeas that are required to test for co-inheritance.

A partial localization of TobRV was demonstrated by hypersensitive resistance of certain cowpea lines (8). It is possible that the operational immunity of Arlington cowpeas against TobRV is due to extreme localization of the infection. Such restrictions are exemplified by what has been termed "subliminal infections" (6,7), which appear to be the result of limiting the infection to the inoculated cell (27). This type of operational immunity often can be overcome by co-infection with another virus that can supply mobilizing factor(s), as demonstrated in several systems (5,10,28). Our attempts at mobilizing TobRV by the action of CPSMV (tested here) and several nepoviruses (data not presented) were unsuccessful.

Protection against CPSMV. Co-inoculation of CPSMV and either TobRV or CPMV caused Arlington cowpea seedlings to be protected partially against CPSMV. CPMV did not interfere with CPSMV replication in a CPMV-susceptible cowpea (4), nor did TobRV in results reported here. This implies that TobRV itself, and not the sap of Harosoy soybean, was responsible for the observed interference of TobRV with CPSMV in inoculated Arlington cowpeas and that the interference was with CPSMV replication per se and not simply an inactivation of CPSMV particles in the inoculum.

Protection by TobRV was most effective in the uninoculated, trifoliate leaves (Table 2, entry 4). Classical virus cross-protection also is most effective in the leaves to which the challenging virus otherwise would spread efficiently, in the absence of a systemically-infecting, protecting virus (9,11). However, cross-protection requires extensive replication of the protecting virus, which TobRV does not achieve in inoculated Arlington cowpea seedlings. Hence, protection against CPSMV by TobRV seems not to correspond to well-documented examples of cross protection.

The interference of CPMV with CPSMV in Arlington cowpea

seedlings (4,12) was termed "concurrent protection" by Ponz and Bruening (18) because only when both viruses were present in the same inoculum was the maximum protective effect achieved. Efficient protection would be expected to appear only after co-inoculation because the protecting virus, CPMV, appears to be incapable of increasing in the inoculated cell and therefore must act entirely through those CPMV virion RNA molecules that actually enter the cytoplasm during the inoculation process. Presumably, only rarely would the same cell be inoculated in two successive inoculations. It was of interest to determine whether TobRV protects Arlington cowpea seedlings against CPSMV in a concurrent manner. The most effective protection was obtained not by co-inoculation but with a slight delay (15 min) between the inoculations of TobRV and CPSMV. In contrast, even 5 to 10 min between inoculations of CPMV and CPSMV greatly reduced the protection of Arlington cowpeas against CPSMV (4).

Thus, the TobRV effect on CPSMV replication in Arlington cowpeas resembles the CPMV effect but does not exactly correspond to the previously defined concurrent protection with regard to the timing of the inoculations. In this regard, Sterk and de Jager (26) have reported a second cowpea line that is operationally immune to CPMV under control of a dominant allele and in which CPMV interferes with CPSMV. However, only simultaneous inoculations of the two viruses were tested, so whether concurrent protection describes this system remains to be seen.

This is the first report, to our knowledge, of a locus for operational immunity to TobRV that prevents any detected increase of the inoculated TobRV or appearance of symptoms. If resistance to TobRV in the Arlington cowpea is controlled by a single gene, it may be a candidate for transfer to agronomically important plants that are at economically significant risk due to TobRV infections. The system also exhibits a second phenomenon, protection against CPSMV by TobRV, that may have potential in virus control strategies.

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