

Survey of Susceptibility to Cowpea Mosaic Virus Among Protoplasts and Intact Plants from *Vigna sinensis* Lines

H. Beier, D. J. Siler, M. L. Russell, and G. Bruening

Postdoctoral Research Associate, Graduate Student, Staff Research Associate, and Professor, respectively, Department of Biochemistry and Biophysics, University of California, Davis, CA 95616.

Author H. Beier was the recipient of a fellowship from the Deutsche Forschungsgemeinschaft. Her present address is Biologisches Institut der Universität, 7 Stuttgart, West Germany.

The present address of D. J. Siler is Louis Berger International, East Orange, New Jersey. She was supported by NIH Training Grant No. GM 00119.

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ABSTRACT

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One thousand thirty-one lines of *Vigna sinensis* (cowpea) were surveyed for response to mechanical inoculation of the SB isolate of the yellow subgroup of cowpea mosaic virus on the primary leaves of seedlings. Sixty-five lines (6.3% of the total) were classified as operationally immune because no symptoms were observed and no virus was recovered at 7 to

12 days after the inoculation with purified virus at a concentration which was one hundred times that which would uniformly infect susceptible lines. Protoplasts were recovered from primary leaves of 55 of the immune lines. Protoplasts from 54 of the immune lines proved to be susceptible to the SB isolate.

A particular plant may not detectably support the replication of a particular virus after mechanical inoculation, or the virus may replicate with effects on the plant which vary in severity according to the virus-host combination and the conditions under which the plant was inoculated and maintained. Some lines of the same plant species can exhibit immunity even though others exhibit various degrees of susceptibility. This has been of practical value in situations in which it has been possible to introduce immunity or resistance into susceptible cultivars by performing genetic crosses with the immune or resistant lines. However, very little is known about the biochemical bases of immunity or resistance. We began investigating the phenomenon of immunity because it represents a valued property when introduced into a plant line and probably represents, at the molecular level, the most extreme (and therefore the most easily investigated) contrast to full susceptibility.

The SB isolate of the yellow subgroup of cowpea mosaic virus (CPMV-SB) is considered the type member of the comovirus group (5) and is a two-component virus system (7). We found that it was not capable of increasing in cowpea line 'Black'. The observation of this example of immunity to CPMV-SB inoculated on the primary leaves of seedlings stimulated us to survey other cowpea lines to detect genetically similar immune and susceptible plants that could be compared biochemically. The results of the

survey are summarized here. As a first step in elucidating possible mechanisms of immunity and in classifying the immune lines, protoplasts from the immune (to mechanical inoculation on the primary leaves of seedlings) lines were inoculated with CPMV-SB. Susceptible protoplasts were recovered from all but one of the immune lines.

MATERIALS AND METHODS

Viruses and seed.—The SB isolate (1) of cowpea mosaic virus was from the collection maintained in this laboratory. Isolate CPMV-DG was obtained from N. G. Vakili, Federal Experiment Station, Mayaguez, PR 00708. It is so designated because of the dull-green symptoms it induces on some cowpeas. The CPMV-DG used in these experiments was recovered after serial local lesion transfer in Pinto bean. In immunodiffusion tests CPMV-DG reacted with antiserum to CPMV-SB but appeared to be more closely related to Arkansas CPMV (11) than to SB, which confirmed the observations of Vakili (*personal communication*). Isolate CPMV-DG has the pattern of centrifugal (4), and electrophoretic (8) ribonucleoprotein components and of capsid proteins (13) that are characteristic of CPMV except that the top component is either absent or possibly present only in very small amounts and the electrophoretic mobilities of DG components are less than those of SB at neutral pH (Beier et al., *unpublished*). Virus was isolated as described (4) and virion concentrations were estimated

TABLE 1. Responses of 1,031 *Vigna sinensis* lines to mechanical inoculation with CPMV-SB (the SB isolate of the yellow subgroup of cowpea mosaic virus)

Response	Plant Introduction or other designation
Immune ^c (6.3%)	142779, 145198, 147563, 152(196, 197) ^a , 271256, 291094, 292893, 293(453, 465-467, 470, 491, 514, 537, 543, 545, 581, 582), 304164, 339(609, 610, 709), 352(765 ^b , 770, 854, 880, 905, 915, 941, 972, 997), 353(007, 049, 050, 270 ^b , 272, 383), 354(450, 451, 466 ^b , 469, 489 ^b , 495, 523, 524 ^b , 555, 556, 629, 667, 687, 760, 762, 833, 838, 857 ^b , 860, 877 ^b , 881 ^b), 367(919 ^b , 925), 382124 ^b Black ^d , La. Purchase ^e .
Hypersensitive ^f (5.6%)	194(204-207, 210-213), 205141, 293(460, 461, 469, 472, 486-490, 506, 507, 542, 558, 559, 565, 569), 325775, 326161, 337506, 339573, 349(674, 675), 352(879, 881, 893, 894, 919, 926, 935, 946), 353(057, 088, 089, 094, 106), 354(484, 499, 509, 531, 532, 635, 783, 787, 799-801, 805, 854, 859).
Resistant ^g (6.0%)	180497, 195417, 223720, 255(773, 776), 279845, 291(096, 140), 292(894, 909), 293(452, 471, 475, 476, 512, 523, 580, 589), 307554, 339(623, 639), 352(772, 904, 918, 971, 991), 353(004, 028, 040, 047, 063, 064, 066, 068, 076, 081, 086, 110, 111, 115, 121, 137, 147, 152, 260, 325, 332, 340), 354(448, 474, 507, 512, 542, 603, 624, 680, 718, 721, 771), 367921, T-(2940, 2941) ^d .
Sensitive ^h (82.0%)	115(674, 679, 681, 683, 729, 737, 739), 121433, 123267, 124(608, 609), 141355, 145732, 146(113, 618), 147(071, 076, 077, 561, 562), 148(674, 678, 681), 151(562, 563), 152(194, 195, 199), 154134, 162(699, 924, 925), 163(142, 448), 164(337, 641), 165(486, 493, 941), 166146, 167284, 170(844, 849, 857, 861, 863, 865, 868, 871, 872), 171(651, 891), 173(164, 165, 827), 174411, 175(327, 332, 959, 962, 963), 176796, 177(101, 578, 579), 178963, 179(124-126, 553-555), 180(013, 014, 355, 494), 181833, 182(316, 317), 183(089, 250, 251, 363, 482), 184(952, 953), 185647, 186(360, 386, 452, 454, 458-460, 465-467, 471), 188(701, 702, 704), 189(099, 230, 374, 375, 378, 380, 416), 190191, 194(202, 203, 208, 209, 214, 215), 196301, 197(056, 057), 200867, 201(024, 498), 202802, 205(139, 140, 240, 241), 208(771, 845), 209971, 211(109-111, 641, 642, 754-756), 212(635, 928, 930), 214(069, 352, 354), 215659, 218(122, 123), 220(210, 847-850), 221(534, 729-732), 222756, 223(023, 420, 548), 225(921, 922), 226106, 227(396, 397, 827, 829-831), 229(551, 734, 796), 244571, 246(131, 132), 250(238, 416, 587, 759), 251(223, 233), 253428, 255(755, 760, 765, 766, 771, 774, 781-785, 788, 789, 791, 792, 795, 811, 812, 815), 256(341, 342), 257463, 262179, 269(664-667), 270(066-068), 271(257-259, 263, 264), 274006, 277(784, 786), 286439, 291(095, 139, 385), 292(870, 871, 888-893, 896-908, 910-914), 293(446-451, 454-459, 462-464, 468, 473, 474, 477, 479-483, 492-502, 504, 505, 509, 513, 515-522, 524-532, 534-536, 538, 540, 541, 544, 546-557, 560-564, 566-568, 570-577, 579, 583, 585-588, 590), 297(561, 562), 298051, 300173, 302(458, 547), 304150, 307(556-561), 311(119, 120), 312(202, 203, 206-208, 210, 211), 315750, 318(820, 821), 319383, 321640, 323325, 325(773, 776), 326156, 331141, 337044, 339(564-566, 576, 580, 584, 587, 588, 590-593, 595-599, 603, 604, 611-613, 616, 619, 620, 638), 347639, 352(769, 773, 774, 826, 827, 829, 830, 832-838, 848, 851-853, 855, 857, 859-866, 870, 872, 873, 882-884, 887, 889, 892, 895, 897-903, 908, 909, 911, 912, 914, 916, 921, 922, 931, 933, 934, 939, 942, 945, 947-950, 953-956, 959-962, 964-966, 969, 970, 973, 974, 978, 980, 984-989, 994-996, 998, 999), 353(000, 008, 010, 011, 014, 015, 021, 022, 025, 027, 030, 031, 039, 041, 045, 046, 051, 055, 056, 058, 059, 067, 070, 072-074, 087, 092, 102, 103, 105, 107-109, 112, 117, 119, 122, 128, 130, 131, 135, 136, 139-141, 143-146, 149, 155, 157, 161, 164, 166, 167, 171, 172, 179, 181, 183, 211, 216, 217, 225-227, 232, 239, 243, 251, 258, 259, 265, 266, 271, 274, 275, 277, 279, 281-283, 289-291, 300, 307, 309-311, 318, 327, 329, 331, 341, 349, 350, 352, 355, 363-365, 377-382), 354(443-445, 447, 457, 463, 465, 468, 473, 475, 476, 481, 488, 490, 493, 494, 498, 500-504, 506, 508, 513-516, 536, 537, 539, 543, 544, 559, 564, 566-569, 571, 579-582, 584, 592, 596, 599, 607, 630, 631, 636, 640, 642, 648, 649, 659, 660, 662-664, 666, 668, 671-673, 675, 677-679, 682-684, 686, 691, 693, 694, 699, 700, 702, 704, 706-708, 711, 713, 717, 719, 724, 725, 727-729, 732, 734-736, 740, 742-745, 748, 759, 761, 763-768, 774, 777-779, 782, 786, 791, 793-797, 802, 807-809, 813, 815-818, 820-823, 825-832, 835, 837, 840-845, 847, 848, 850-853, 861, 863-865, 867-870, 878), 358(304, 716), 364685, 367(860-863, 865, 866, 868, 869, 920, 923, 927), 376(864, 865), 382(087, 088, 103, 107-111, 113-123, 125, 127-138), 390(420, 421, 831, 832), Crimson ^e , Dixielee ^e , Floricream ^e , G-21 ^d , Miss. Silver ^e , Mz-3769 ^d , PR-V-71-(10-R1, 10-R3, 10-R58, 12-8), Purplehull Pinkeye ^e , Topset ^e , T-(2881, 2882, 2884, 2890, 2891, 2949) ^d .

^aAll Plant Introduction numbers listed have six digits; some are abbreviated [e.g., 152(196,197) = 152196, 152197, etc.]; all are in numerical order within each group.

^bImmune lines from which, later, protoplasts were not isolated because of poor germination or heterogeneous appearance of the seeds (see Table 2).

^cImmune by the criteria of no symptoms 7-12 days after inoculation with 250 µg/ml CPMV-SB and no virus detectable in inoculated or secondary tissue by transfer to Blackeye 5 cowpeas or by immunodiffusion. Sixty-five lines are in this category.

^dLines not obtained from the Plant Introduction collection of the United States Department of Agriculture.

^eFrom a Plant Introduction collection, but no P.I. number is available.

^fPinpoint, necrotic local lesions on the primary leaves and no symptoms on the secondary leaves; no virus was detected in the secondary tissue. Fifty-eight lines are in this category.

^gChlorotic and/or necrotic local lesions on inoculated primary leaves and no symptoms on, nor detectable virus from, secondary leaves. Sixty-two lines are in this category.

^hChlorotic and/or necrotic local lesions on inoculated primary leaves; mosaic on, and/or stunting of secondary leaves or no symptoms on secondary leaves. Virus was detected in the secondary tissue. Eight hundred forty-six lines are in this category.

photometrically using an absorbivity of $8 \text{ cm}^2 \text{ mg}^{-1}$ at 260 nm (8). Cultivars and lines (referred to here collectively as 'lines') of cowpeas [*Vigna sinensis* (Toner) Savi] were of several sources: Black from R. J. Shepherd and Blackeye 5 from C. Tucker, both of the University of California at Davis; line G-21 from B. B. Brantly of the Georgia Experiment Station at Experiment, Georgia; four hybrid or sib-crossed lines, designated in the form PR-V-71-00-R00, from N. G. Vakili; and all other *V. sinensis* lines from G. Sowell, Jr., U.S. Department of Agriculture (USDA) Plant Introduction Station at Experiment, Georgia. Eight lines designated in the form T-0000, which are of the subspecies *sesquipedalis* ("yard-long bean") were from N. G. Vakili.

Inoculation of seedlings.—Primary leaves of seedlings were inoculated, using 45- μm (320-mesh) corundum (Fisher Scientific, Carborundum) and cotton-tipped swabs, with 2.5 $\mu\text{g}/\text{ml}$ of purified CPMV-SB in 0.05 *M* potassium phosphate, pH 7.0, 6 to 8 days after sowing in a glasshouse equipped with evaporative cooling. Plant lines were assigned to the categories of "sensitive," "resistant," and "hypersensitive," according to the criteria listed in footnotes h, g, and f, respectively, of Table 1. The first category differs from the second in that infection was systemic for lines classified as sensitive. Resistant and hypersensitive lines were distinguished on the basis of the formation of pinpoint necrotic local lesions on the inoculated leaves of the latter. The lines which tentatively were considered "immune", because no local lesions or other symptoms were apparent, were retested in a controlled environment chamber with day and night temperatures of 27 and 23 C and corresponding relative humidities of 72 and 69% (18 hr of daily fluorescent and incandescent illumination, $\sim 21,500$ lux at the leaf height). Seedlings (at least 10 per trial for those lines for which sufficient viable seed was available) were inoculated with 250 $\mu\text{g}/\text{ml}$ CPMV-SB and were considered to be operationally immune if no symptoms appeared on any plants of the line and, in the interval 7 to 12 days after inoculation, no virus could be detected in expressed sap of either the inoculated primary leaves or the secondary leaves by immunodiffusion or by inoculation to Blackeye 5 cowpeas. Sap from the inoculated Blackeye 5 cowpeas also was analyzed by immunodiffusion in most cases.

Isolation and inoculation of protoplasts.—Protoplasts of primary leaves (~ 0.5 g of tissue) were isolated (usually 8-9 days after sowing) essentially as described (2) except that the step of immersion of the leaf in 70% ethanol was omitted. Because suitable polyornithine (2) was no longer available, protamine sulfate was substituted as a potentiator of infection by CPMV. The inoculum was purified CPMV-SB at 10 $\mu\text{g}/\text{ml}$ in 0.45 *M* mannitol, 0.01 *M* potassium citrate, pH 5.6, 75 $\mu\text{g}/\text{ml}$ protamine sulfate. It was incubated at 25 C for 5 min before exposing protoplasts to it. Eight ml of protoplast suspension ($\sim 10^8/\text{ml}$) were centrifuged at 100 *g*, and the protoplasts were suspended in 2.5 ml of the virus solution. The suspension was incubated for 15 min; the protoplasts were washed and the incubation was continued at 28 C as described (2) except that chloramphenicol was omitted from the incubation medium. The protoplasts were washed, disrupted, and assayed for virus, also as described (2).

RESULTS AND DISCUSSION

Responses of seedlings to inoculation with cowpea mosaic virus.—Sixty-five out of the 1,031 varieties tested were operationally immune (Table 1), by our criteria, to CPMV-SB when inoculated on the primary leaves at one hundred times a concentration of virions which uniformly infected susceptible cowpeas. For each line the immunity has been confirmed in at least two separate experiments. Most (82%) of the lines proved to be sensitive. Wells and Deba (12) found that six out of 116 Plant Introduction lines and 16 out of 342 "indigenous pure-lines" were immune or highly resistant to a CPMV of Nigeria which had been inoculated as sap from infected plants. Robertson (10) reported that 14 out of 62 USDA Plant Introduction lines, when inoculated with either of two Nigerian CPMV's in crude sap, failed to develop symptoms or to develop a level of virus detectable by transfer to susceptible cowpeas. In the summary below, comparisons are based on the assumption that identically spelled names for USDA Plant Introduction lines refer to the same cultivar. The following were found to be highly resistant or immune to Nigerian CPMV's by Wells and Deba (12) and by Robertson (10) and, in this work, to be immune to CPMV-SB: 293453 (Arlington), 293467 (Brabham K892), 293514 (Groit), 293582 (Victor K798), and La. Purchase. The following were immune to Nigerian CPMV's, according to Robertson (10), and to CPMV-SB according to our results: 293466 (Brabham), 293491 (Columbia), and 293581 (Victor). Number 293522 (Jackson Alabama) was found to be highly resistant to a Nigerian CPMV (12) but sensitive to CPMV-SB. Five lines were found to be immune to the two Nigerian CPMV's (10) but sensitive to CPMV-SB: 293457 (Blackeye), 293468 (Brabham Victor), 293473 (Buff), 293567 (Six Weeks Alabama) and 293568 (Six Weeks Georgia). Bliss and Robertson (3) reported that line Dixielee did not show *systemic* symptoms when inoculated with the Nigerian CPMV's, and "no virus could later be recovered from previously inoculated plants." Dixielee was found to be sensitive to CPMV-SB. Vakili (*personal communication*) found that most individuals of 503 Plant Introduction accessions available to him were susceptible to the Puerto Rican CPMV, giving dull-green symptoms under field conditions. (The lines designated in the form PR-V-71-00-R00 were derived from individuals showing resistance to the CPMV giving dull-green symptoms.) All the lines listed in Table 2 have proved (in our unpublished tests) to be susceptible to CPMV-DG, though 367925 and La. Purchase seemed to be more resistant than the others. Vakili (*personal communication*) observed that resistance to a Puerto Rican CPMV which induces yellow symptoms is controlled by genes which are distinct from those for resistance to the virus inducing dull-green symptoms.

Inoculation of protoplasts from immune lines.—Seed from 10 of the 65 lines listed as being immune in Table 1 had poor germination or appeared heterogeneous. We did not attempt to isolate protoplasts from these. The susceptible cultivar Blackeye 5 was the source of protoplasts in the previously developed procedure (2) for protoplast isolation. That procedure proved to be successful in recovering protoplasts from primary leaves

TABLE 2. Responses of protoplasts, isolated from 55 immune lines of *Vigna sinensis*, to inoculation with CPMV-SB^a

Cultivar or P.I. Number	Recovered infectivity (lesions/leaf)	Cultivar or P.I. Number	Recovered infectivity (lesions/leaf)	Cultivar or P.I. Number	Recovered infectivity (lesions/leaf)
Blackeye 5 ^b	840	293545	380	353383	810
		293581	640	354450	750
142779	450	293582	570	354451	505
145198	370	304164	640	354469	410
147563	540	339609	225	354495	522
152196	680	339610	264	354523	830
152197	660	339709	210	354555	590
271256	586	352770	620	354556	650
291094	632	352854	324	354629	486
292895	720	352880	410	354667	760
293453	12	352905	290	354687	730
293465	820	352915	360	354760	550
293466	270	352941	744	354762	596
293467	580	352972	574	354833	346
293470	430	352997	580	354838	406
293491	790	353007	480	354860	374
293514	570	353049	530	367925	582
293537	534	353050	390	La. Purchase ^c	492
293543	445	353272	700	Black ^d	236

^aInoculation was in 0.45 M mannitol, 0.01 M potassium citrate, 75 µg/ml protamine sulfate, pH 5.6, using protoplasts at ~ 3 × 10⁶/ml and virus at ~ 10 µg/ml. Protoplasts were harvested 24 hr after inoculation and were assayed for protoplast-associated virus by local lesion assay.

^bSeed obtained locally; it is listed as P.I. 293458 and serves as a control since it is a systemic host for the virus.

^cFrom a Plant Introduction collection of the United States Department of Agriculture; no P.I. number available.

^dSeed obtained locally.

of the remaining 55 immune lines with no modification except that from certain lines the primary leaves were taken on day 7 or day 10, rather than at 8-9 days after sowing, in order to achieve >80% viability of the isolated protoplasts.

Under the conditions used (2) CPMV-SB accumulates in Blackeye 5 protoplasts in 25 hr to a level which is one-third to one-half the level attained in 40 hr. In a preliminary experiment CPMV-SB infectivity in inoculated Blackeye 5 and Black cowpea protoplasts was comparable after 40 to 50 hr of incubation. However, at 25 hr the infectivity in the Blackeye 5 protoplasts was about three times that in Black protoplasts. That is, there was a significant and reproducible lag in CPMV-SB increase in Black protoplasts, as compared to Blackeye 5 protoplasts. Therefore, the protoplasts from the immune lines were incubated for 25 hr after inoculation with the intention of possibly identifying other sources of protoplasts in which CPMV-SB replication is delayed. The results, in Table 2, show that protoplasts from 54 of the 55 lines which are immune as seedlings nevertheless were a source of susceptible protoplasts. However, in only a few cases was the amount of infectivity at 25 hr comparable to the level found in inoculated Blackeye 5 protoplasts. Protoplasts of 339609 tended to die after inoculation with CPMV-SB but not after mock inoculation, so a delayed replication may not be the explanation for the low yield of virus, at 25 hr, from these particular protoplasts.

Protoplasts from variety 293453 (Arlington) appeared to be immune or highly resistant to CPMV-SB. The infectivity at 25 hr after inoculation, corresponding to 12 lesions per leaf inoculated with the protoplast extract, can

be attributed to residual inoculum. A similar amount of infectivity remains associated with Blackeye 5 protoplasts which have been inoculated with CPMV-SB and incubated in the presence of cycloheximide to inhibit the synthesis of capsid (6, 9) and other proteins. Protoplasts from line Arlington supported the replication of CPMV-DG (data not presented). Other experiments concerned with the interaction of CPMV-SB and Arlington protoplasts will be reported later.

If none of the 10 lines which apparently were immune as seedlings (but which were not tested as protoplasts) can yield immune protoplasts, then immunity or high resistance at the protoplast level to CPMV-SB has proved to be very rare; probably only one in 1,031 would yield such protoplasts. At least 54 lines are immune as seedlings but susceptible as protoplasts. The implication is that for these lines the immunity of the seedling requires some entity external to the cytoplasm or interaction or communication between different cells. Alternatively, the process of releasing the protoplasts may have in some way changed the cytoplasm so that it becomes susceptible. Protoplasts have an intact plasma membrane. However, the plasma membrane cannot be eliminated as a potential site for blocking CPMV-SB infection because protamine sulfate may induce virion entry by a pathway other than those operating in the intact seedling.

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