RNA-1 Dependent Seed Transmissibility of Cucumber Mosaic Virus in *Phaseolus vulgaris*

R. O. Hampton and R. I. B. Francki

U.S. Department of Agriculture, Agricultural Research Service, Department of Botany and Plant Pathology, Oregon State University, Corvallis 97331, and Department of Plant Pathology, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, South Australia, 5064.

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ABSTRACT

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Cucumber mosaic viral RNAs-1, -2, and -3 were isolated by successive gel electrophoresis and recombined between cucumber mosaic virus (CMV) strains that were (CMV-Pg) or were not (CMV-Le) seed transmissible in *Phaseolus vulgaris* 'Top Crop.' Twenty local-lesion isolates were derived for each of six pseudorecombinants, and selected isolates of each were assayed for seed transmissibility in cv. Top Crop. An average of 603 seedlings arising from Top Crop plants inoculated with respective pseudorecombinants were assayed for presence of seedborne CMV by means of double antibody sandwich enzyme-linked immunosorbent assay. Seedling symptoms of seedborne pseudorecombinants varied with seasonal differences in the greenhouse environment; in numerous cases infected seedlings were symptomless. Pseudorecombinants containing RNA-1 from CMV-Pg were seed-transmitted at a frequency of 64/2,032. Pseudorecombinants containing RNA-1 from CMV-Le were seed-transmitted at a frequency of 4/2,386. The latter seed transmissions were evenly divided among the three pseudorecombinants: LLP, 1/714; LPL, 2/1,091; and LPP, 1/581. Seed transmission rates of parental strains were, CMV-Pg, 22/60, and Le, 0/207. Thus, CMV RNA-1, co-governing viral replication and affecting virus movement, decisively influenced seed transmissibility of recombined CMV-Pg and CMV-Le genomic RNAs.

Cucumber mosaic cucumovirus (CMV) contains three single-stranded positive-sense genomic RNAs, RNA-1, -2, and -3, with molecular weights of about 1.35×10^6 (3.4 kb), 1.16×10^6 (3.0 kb), and 0.85×10^6 (2.2 kb), respectively (11,16). Subgenomic CMV RNA-4, with molecular weight of 0.35×10^6, is the mRNA for the coat protein (same sequence not expressed in RNA-3b) (5,19) and is encapsidated with RNA-3 (4).

In prior CMV RNA pseudorecombination studies, defined pathological traits have been ascribed to individual RNAs or joint functions of RNAs. The ability of CMV to infect cowpea systemically was associated with RNA-2 (3,7,12), as was systemic necrosis in *Nicotiana* × *edwardsonii*, leaf distortion in several host plant species, and the ability to systemically infect maize (17) and systemically infect *Pisum* pea and *Phaseolus* bean (3). Serological specificity was associated with RNA-3 (7,12,14), as was aphid transmissibility (14) and local lesion type on *Gomphrena globosa* and *Vicia faba* (17). The following traits were jointly determined by CMV RNA-2 and -3: local lesion type on cowpea (12) and on *Datura stramonium* and *Solanum melongena* (17), yellow mosaic symptoms induced in several CMV host plant species (17), and systemic infection of cowpea (12) and of *Lactuca saligna* (3). Both CMV RNA-1 and -2 are now known to code for viral replication (15). The RNA-1 of CMV-NL was found by Lakshman and Gonsalves (10) to be associated with the induction of necrotic local lesions (cotyledons), to restrict systemic viral movement in squash, and to completely localize infection of tobacco. Pseudorecombinants of raspberry ringspot nepovirus strains E and L (bipartite genome) were tested for seed transmissibility in *Stellaria media* in 1977 (6). In this pioneering research, viral seed transmissibility was found to be associated with RRV-E RNA-1. The purpose of the present study was to determine which genomic CMV RNA(s) was (were) associated with CMV seed transmissibility in *Phaseolus vulgaris*. CMV-Pg RNA-1 was closely associated with viral seed transmissibility.

MATERIALS AND METHODS

Parental CMV strains. CMV-Pg, selected for its high rate of seed transmissibility, originated as a seedborne isolate in *P. vulgaris* germ plasm accession PI 271998 (2). Other CMV isolates seedborne in *P. vulgaris* were CMV-F, kindly provided by J. C. Devergne (13) and CMV-Uh, kindly provided by J. Horvath (9). CMV-Le, a legume-strain type isolate from Japan (21) that is not seed transmissible in *P. vulgaris*, was kindly provided by T. Inouye. Neither CMV-Pg nor CMV-Le contained detectable satellite RNA.

Preparation of CMV and RNAs. CMV isolates were maintained in Top Crop bean, transferred to *Nicotiana clevelandii* for propagation, and purified by slight modifications of the method of Peden and Symons (16); extracts were constantly maintained at 4 C. Infected *N. clevelandii* leaves were prechilled and mechanically blended in the presence of 0.5 M sodium citrate buffer (1:2, w/v), pH 6.5, containing 0.5% thioglycolic acid and 5 mM EDTA. The extract was centrifuged 15 min at 10,000 *g*, and the resulting supernatant liquid was emulsified with an equal volume of chloroform. The aqueous phase was collected, stirred with 10% (w/v) polyethylene glycol (M_r, 8,000), and precipitated virus was centrifuged 15 min at 15,000 *g*. Resulting pellets were suspended by stirring in 5 mM sodium borate buffer, pH 9.0, containing 0.5 mM EDTA and 2% Triton X-100. The suspension was clarified by centrifugation 10 min at 10,000 *g* and subjected to two cycles of high- and low-speed centrifugation of 144,000 *g* for 60 min and 5,000 *g* for 10 min. If not used for RNA extraction immediately, the preparation was thoroughly mixed 1:1 with glycerol and stored at −28 C until ready for use.

RNA was extracted from purified CMV preparations by a modification of the method of Mossop and Francki (14). In essence, the virus suspension was emulsified 1:1:1 (v/v/v) with 90% phenol (10% sterile distilled water containing 0.1% 8-hydroxyquinoline) and with NE buffer (500 mM sodium acetate and 10 mM EDTA, pH 7.6) containing 1% sodium dodecyl sulfate (SDS), immediately before use. The emulsion was gently stirred (rotary shaker) for 60 min at 25 C, chilled, and partitioned by swinging-bucket centrifugation for 10 min, 10,000 *g*. The aqueous portion was re-extracted with an equal volume of fresh phenol, and the phenol portion was back-extracted with NE buffer. Aqueous portions were combined, thoroughly mixed with three volumes of re-distilled absolute ethanol, and precipitated for 4–18 h at −70 C. The precipitate was centrifuged 10 min at 10,000 *g* and washed three times with cold ethanol by vortexing and centrifugation before use.

RNA precipitates were resuspended in TE buffer (10 mM Tris-HCl buffer, pH 8.3, containing 10 mM EDTA), and yields were estimated spectrophotometrically. Genomic RNAs were separated by successive tube-gel electrophoresis (Fig. 1) in 3.36% polyacrylamide (19:1 ratio of acrylamide to bis acrylamide) gels (PAG) containing 7.0 M urea and in 1.5% agarose (9:1, w/w, low-gelling-temperature agarose [SeaPrep, FMC Corp., Rockland, ME] to standard agarose [Sigma Type II, Sigma Chemical Co., St. Louis, MO]). TBE buffer (0.1 M Tris, 90 mM boric acid and 20 mM EDTA) was used to prepare gels and as electrophoresis running buffer. Tube gels (12 × 140 mm) were loaded with 100 μg of RNA and run for 16 h, at 200 V and 36 mA. After electrophoresis, gels were gently extruded from tubes into toluidine blue (0.05% w/v) for 6–10 min. Lightly stained RNA bands were excised with sterile blades, guarding against RNA mixtures, and either stored at −28 C, or fitted directly onto the tops of freshly prepared agarose tube gels of the same diameter (tops of gels excised to provide a flat loading surface). RNAs were electrophoresed from PAG sections directly into agarose gels, typically for 4 h. Agarose gels were gently extruded into toluidine blue, lightly stained, destained, and examined for any evidence of mixed components. RNA migration distances were recorded, and respective RNA bands were again recovered by excision. Matching gel sections were homogenized (forcefully extruded-intruded through 18-gauge needle) in NE buffer containing 1% SDS, the RNA was phenol-extracted, as described for whole CMV, and was precipitated with cold ethanol.

Production of pseudorecombinants. Precipitates of each of the six separated RNAs (i.e., Le RNAs 1, 2, 3 and Pg RNAs 1, 2, 3) were resuspended in NE buffer, quantitated spectrophotometrically, and proportionately combined into two-component (experimental controls) and three-component RNA recombinants (Fig. 1). Eight 6-μl aliquots of each recombinant were applied as inoculum to separate *Chenopodium quinoa* leaves. Preliminary inoculations of *C. quinoa* plants with CMV RNA indicated that bentonite was neither necessary nor helpful as an RNA protectant and that development of CMV-RNA-induced local lesions was enhanced by treatment of plants with 24 h of darkness before inoculation and continuous light after inoculation.

Two-component recombinants of parental RNAs were applied to leaves of *C. quinoa*, as controls against undetected RNA mixtures (i.e., any infections by double-component combinations indicated incomplete RNA separation). Successful separations and recombinations of RNAs were assessed by production of local lesions by inoculation with three-component heterologs and production of few or no local lesions by inoculations with two-component homologs (i.e., <5% of the number produced by three-component heterologs).

In trials yielding infectious pseudorecombinants, with two-component negative controls inducing few or no *C. quinoa* local lesions, 10 or more pseudorecombinant-induced lesions were individually subtransferred to leaves of Early Globe beet (*Beta vulgaris*) plants (found in preliminary tests to produce local lesions when inoculated with homogenates of single CMV-induced *C. quinoa* lesions). Twenty well isolated beet lesions, in turn, were each excised, homogenized, and inoculated onto leaves of a *Nicotiana clevelandii* plant (Fig. 1). Each inoculated *N. clevelandii* plant was tested for CMV infection and assigned a separate pseudorecombinant isolate number.

Seed transmissibility of pseudorecombinants in Top Crop was assayed by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (1), using antiserum produced against CMV-Pg specifically for this purpose. Antibodies generated by four intradermal and intramuscular injections each of 0.5–1.5 mg of purified virus (alternately unfixed and glutaraldehyde-fixed preparations), over three months, were equally reactive to CMV-Pg, Le, and the pseudorecombinants. Top Crop seedlings were assayed individually, to optimize virus detection sensitivity.

RESULTS

Seed transmissibility of pseudorecombinants from Pg and Le

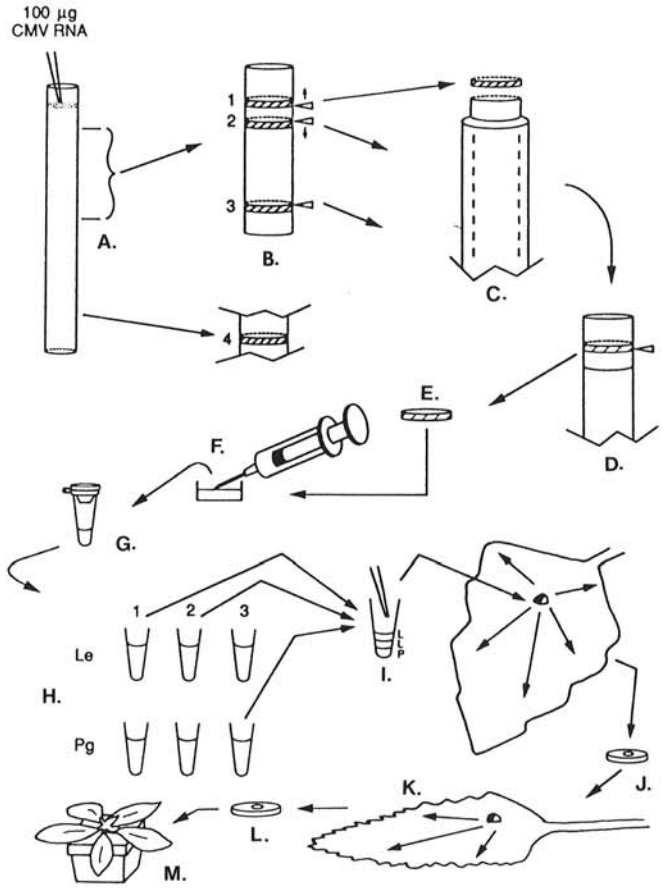

Fig. 1. Procedure by which pseudorecombinants of CMV-Pg and CMV-Le RNAs-1, -2, and -3 were prepared and established as local lesion isolates (see also Materials and Methods). **A,** denaturing 3.36% polyacrylamide tube gel (PAG) loaded with 100 μl of total genomic viral RNA. **B,** first-cycle electrophoretic separation of RNAs, with a thin section of gel. **C,** gel section containing RNA-1 being fitted to the top of a slightly extruded, trimmed agarose tube gel; RNA-2 and -3 processed the same. **D,** resulting band of RNA-1 after agarose (1.5%) electrophoresis, thicker relative to PAG bands, with discriminative thin section of gel; RNA-2 and -3 processed the same. **E–F,** combined agarose gel sections of RNA-1 homogenized by extrusion-intrusion in NE buffer (500 mM sodium acetate and 10 mM EDTA, pH 7.6) through syringe equipped with 18-gauge needle; RNA-2 and -3 processed the same. **G,** respective RNAs phenol-extracted from gel and precipitated in cold ethanol, −70 C. **H,** respective precipitates washed three times in cold phenol and resuspended in NE buffer. **I,** production of LLP pseudorecombinant and application of 6-μl droplet to leaf of *Chenopodium quinoa*; other five pseudorecombinants prepared accordingly. **J,** 4-mm leaf disk containing a single pseudorecombinant-induced *C. quinoa* local lesion. **K,** leaf disk homogenized in droplet of NE buffer in small mortar and applied to leaf of Early Globe beet (*Beta vulgaris*). **L,** 6-mm leaf disk containing a single pseudorecombinant-induced beet local lesion. **M,** leaf disk homogenized and applied to plant of *Nicotiana clevelandii* to provide inoculum supply of the six respective pseudorecombinants.

CMV RNAs is presented in Table 1, with five seed generations from Top Crop bean plants inoculated with one of three or four isolates of each pseudorecombinant. Seed transmissibility of pseudorecombinants was principally associated with the RNA-1 of parent Pg. Despite lower seed transmissibility of all pseudorecombinants relative to CMV-Pg, pseudorecombinants containing the RNA-1 of seed-transmissible Pg were nonetheless seed-transmitted at an average frequency of 3.1% (64/2,032) or 18 times the rate of pseudorecombinants containing the RNA-1 of parent Le (4/2,386 = 0.17%). There was no indication of a secondary seed transmission-supportive role for either CMV-Pg RNA-2 or RNA-3 (i.e., the presence of these RNAs did not influence seed transmission of pseudorecombinants).

The trace seed transmission frequencies of pseudorecombinants LPL and LPP could suggest possible contamination by Pg RNA-1 (i.e., separation of RNA-2 from Pg RNA-1 is difficult, and failure to do so could generate traces of an artifact, PPL). However, the similar low frequency seed transmissibility of putative pseudorecombinant LLP is not amenable to the same logic, because no such artifact was possible when isolated Le RNA-1 and -2 were recombined to produce this pseudorecombinant. It is also possible that CMV-Le is innately capable of trace levels of seed transmission in *P. vulgaris*.

Detection of seed-transmitted pseudorecombinants in Top Crop bean seedlings was dependent on ELISA, because of variable virus-induced symptoms. Variations in symptomatology were related to seasonal fluctuations in glasshouse environment more than to seed generations from pseudorecombinant-infected mother plants. Of 64 pseudorecombinant-infected seedlings (Table 1), only 36 (56%) showed unequivocal mosaic symptoms at the first trifoliate leaf stage. Of the 22 control seedlings containing seedborne parent, CMV-Pg (Table 1), 18 (82%) developed mosaic symptoms. Seedlings arising from pseudorecombinant-infected mother plants and producing ELISA absorbance (A_{405}) values barely exceeding the detection threshold (T = average, healthy Top Crop extract A_{405} value per plate, plus 5 × standard deviation of A_{405} values for two or more replicate wells) were subsequently bioassayed by mechanical inoculations to *N. clevelandii* plants (i.e., plant tissue was sampled, homogenized, and applied as inoculum to Carborundum-dusted leaves). Seedlings producing questionable ELISA results and negative bioassays were counted as "non-detections, non-seed transmissions." ELISA was more sensitive in detecting CMV, however, than assays on *N. clevelandii* seedlings, readily detecting purified CMV-Pg concentration of 20–50 pg of virus per milliliter. Parallel assays of the same virus preparations on *N. clevelandii* plants yielded plant infection rates of 30–40%.

DISCUSSION

Rao and Francki (18) and Edwards et al (3) indicated that no pathological effect could be attributed to CMV RNA-1. However, Lakshman and Gonsalves (10) found that CMV RNA-1 influenced localized infection in squash and tobacco and reviewed

TABLE 1. Seed transmission frequency (STF) of cucumber mosaic virus (CMV) genomic pseudorecombinants between strains Pg (seed transmissible) and Le (non-seed-transmissible), in cv. Top Crop bean (*Phaseolus vulgaris*) seeds, as determined by double antibody immunosorbent assay (DAS-ELISA)

		STF per seed generation (SG) number[a]					
Pseudorecombinant[b]	Isolate number[c]	SG-1	SG-2	SG-3	SG-4	SG-5	Total
LLP	1	0/200					
	2		0/58				
	27				0/25		
	22					1/431	1/714
LPL	2		0/61				
	4			0/80	1/52	0/429	
	5				1/421		
	9	0/48					2/1,091
LPP	8	0/21					
	21					1/410	
	22				0/150		1/581
Subtotal, pseudorecombinants with Le RNA-1							4/2,386
PPL	1	6/105					
	3					3/267	
	5		0/81				
	6			7/38	7/71		23/562
PLP	3	8/40					
	9				0/338	8/469	
	10		0/25				18/872
PLL	3	1/12	2/19	4/25	12/53		
	7				0/113		
	8	0/25	2/24			2/227	23/598
Subtotal, pseudorecombinants with Pg RNA-1							64/2,032
Parent strains (controls)							
LLL		0/100	0/107				0/207
PPP		19/40	3/20				22/60

[a] Seed transmitted pseudorecombinants were detected by DAS-ELISA of individual Top Crop seedlings arising from mother plants inoculated with designated single-lesion-derived recombinant isolates. Seedlings were assayed 5–10 days after emergence by a standardized ELISA capable of detecting 20–50 pg of purified CMV per milliliter. Numerator = number of seedlings containing ELISA-detectable pseudorecombinant; denominator = total number of seedlings assayed.

[b] Pseudorecombinants designated as the parental, Le or Pg, source of genomic RNAs 1, 2, and 3, respectively; e.g., LLP = RNAs 1 and 2 from parent CMV-Le and RNA 3 from parent CMV-Pg, etc.

[c] Twenty isolates of each pseudorecombinant were derived by means of successive local lesion transfers from *Chenopodium quinoa* (LL), to beet cv. Early Globe (LL), to *Nicotiana clevelandii* (systemic). Isolates were stored in desiccated tissue of *N. clevelandii*, at −28 C. Three or four isolates of each pseudorecombinant were randomly selected from the 20-isolate resource and tested for seed transmissibility.

reported RNA-1 effects for other multicomponent viruses. Except for the association between RNA-1 of raspberry ringspot virus and seed transmission in *Stellaria media* (6), seed transmission had not been previously attributed to a genomic viral RNA.

The mechanism(s) by which CMV RNA-1 functions in viral seed transmissibility was beyond the scope of this study. It seems appropriate, however, to attempt an explanation based on two reported roles of CMV RNA-1: co-participation with RNA-2 in viral replication (15) and movement of CMV from localized infections (10). Viral replication could limit CMV transmission through seeds more conceivably after rather than before viral entry into the embryo. Conversely, if RNA-1 were to function by promoting or limiting cell-to-cell movement, this function should be expressed by means of viral entry or nonentry into the embryo. Our experience with several seedborne virus-host systems indicates that the frequency of viral presence in developing embryos may significantly exceed the frequency of viral transmission from the seeds into germinating seedlings. This distinction would seem to favor an RNA-1 co-replicative role after viral entry into the embryo, in facilitating viral transmission through seeds. A replicative role (rather than a viral-movement role) in CMV-Pg RNA-1 seed transmissibility seems to be further strengthened by evidence of Suzuki et al (20) that CMV RNA-3 (both 3a and coat protein) is involved in CMV transport (i.e., CMV-Pg RNA-3 in our study was not associated with seed transmissibility).

Lower seed transmissibility of pseudorecombinants relative to CMV-Pg suggests possible incomplete complementarity among recombined RNAs of Pg and Le. A similar suggestion of "reduced" pathogenicity or viral function was suggested by the performance of some recombinations reported by Rao and Francki (18) and Edwards et al (3). On the other hand, the failure of pseudorecombinant isolates PPL-5 and PLL-7 to seed-transmit, though their symptomatology in *C. quinoa*, Early Globe beet, and *N. clevelandii* was normal, suggests possible RNA damage, such as CMV-Pg RNA-1 deletions during its isolation or recombinations.

Hayes and Buck (8) and Suzuki et al (20) recently prepared and assembled infectious transcripts of cloned cDNAs of CMV RNA-1, -2, and -3, providing alternative procedures by which pseudorecombinants can now be produced for the mapping of phenotypic characteristics of multicomponent viruses.

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