The Influence of Bromegrass Mosaic Virus on the Replication of Tobacco Mosaic Virus in Hordeum vulgare

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ABSTRACT

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Inoculation of the first leaf of barley (*Hordeum vulgare* 'Black Hulless') with tobacco mosaic virus (TMV) and bromegrass mosaic virus (BMV) resulted in systemic infection of the host by both viruses whereas in plants inoculated with TMV only, the virus remained in the inoculated leaf. The yield of TMV from systemic infection of leaves 2-4 was 5-10 times that in the inoculated leaf. Systemic infection by TMV was dependent upon high temperature (30 C), but a temperature-shift experiment indicated that replication of TMV in systemically infected leaves occurred at 25 C. No evidence was obtained for encapsidation of TMV-RNA in BMV protein. No systemic infection of Black Hulless barley was obtained by inoculation with alfalfa mosaic, cowpea chlorotic mottle, southern bean mosaic, tobacco necrosis, or turnip yellow mosaic viruses in the presence or absence of BMV.

Additional key words: TMV infection of cereals.

Previous studies on the replication of tobacco mosaic virus (TMV) in barley *(Hordeum vulgare)* (1, 3) have shown that the virus remains largely restricted to the inoculated leaf (leaf 1) of seedlings inoculated with TMV only, but that systemic infection readily occurs when they are inoculated simultaneously with TMV and barley stripe mosaic virus (BSMV).

In mixed infections, TMV accumulates in younger noninoculated leaves, both viruses occur together in infected cells, and heterologous encapsidation (genomic masking) of TMV-RNA in BSMV-protein occurs (2). Recent experiments have demonstrated that systemic infection and enhanced accumulation of TMV also occur in barley simultaneously inoculated with brome mosaic virus (BMV). Both BSMV and BMV can be considered as "helper viruses" in their association with TMV in barley. The purpose of this report is to characterize the BMV/TMV infection system and to contrast certain of its features with those of the BSMV/TMV infection.

MATERIALS AND METHODS

Viruses.—Brome mosaic virus (BMV), originally derived from the type culture (AC 66, American Type Culture Collection) was used throughout. The TMV strain was the same as used previously (3); two local isolates, one from systemically infected *Lycopersicon esculentum* and one from systemically infected *Chenopodium amaranticolor*, were used in some experiments. Inocula (0.2-0.5 mg/ml) of these viruses for most experiments were from stocks previously purified by differential- and density-gradient centrifugation or occasionally from crude sap frozen until use. Alfalfa mosaic virus (AMV), cowpea chlorotic mottle virus (CCMV), southern bean mosaic virus (SBMV), tobacco necrosis virus (TNV), and turnip yellow mosaic virus (TYMV) were from local stocks maintained in appropriate hosts.

Inoculations.—Black Hulless barley seedlings, 7 days from seeding and containing only the first leaf (leaf 1) with a length of 6-7 cm, were selected for inoculation. Leaves which developed after inoculation are referred to as leaves 2, 3, and 4. The first leaf was inoculated with one of the viruses singly or in a mixture with BMV and then incubated in a controlled environment (31 C, 16 hr light, 16,140 lux at soil level) for the duration of the experiment. In temperature-shift experiments, inoculated seedlings were moved from the starting temperature (31 C or 25 C) to the final temperature (25 C or 31 C) after a prescribed interval. The proportion of inoculated plants infected with BMV or TMV was determined at 8-10 days postinoculation by infectivity tests of extracts from leaf 3 on *C. amaranticolor* or *Nicotiana tabacum* 'Xanthi nc' (TMV) or by microprecipitin serological assays (BMV). Systemic infection of barley by AMV and SBMV was assessed by inoculation of extracts to primary leaves of *Phaseolus vulgaris* 'Pinto'; the presence of CCMV, TNV, and TYMV was assessed by inoculation of extracts on *Vigna unguiculata* 'Early Ramshorn', *N. tabacum* 'Xanthi nc', and *Brassica pekinensis* Rupr. 'Wong Bok', respectively.

Quantitation of viruses.—The proportion of doubly inoculated plants infected with both BMV and TMV ranged from 70-95% in most experiments. Ten leaves from each leaf position (leaves 1-4) from BMV-inoculated and BMV + TMV-inoculated plants showing BMV

symptoms were harvested at 5, 7, 11, 14, 18, and 21 days postinoculation and used as the sources of virus for quantitation; once a plant had been sampled, it was discarded. The sample of 10 leaves was homogenized in 0.02 M Tris-citric acid, pH 6.5 (1:3, w/v), the juice was extracted through cheesecloth, and clarified by heating at 40 C for 1 hr followed by low-speed centrifugation (6,000 *g*, 20 min). Supernatant fluid (0.2 ml) was floated on linear sucrose gradient columns (10-40% ribonuclease-free sucrose, 0.02 M Tris-citric acid, pH 6.5) and centrifuged (SW 41 rotor, 40,000 rpm, 5 C, 1 hr). Adequate separation of BMV (86S) and TMV (187S) in these gradients allowed direct estimation of the virus content of each zone by ultraviolet densitometry (ISCO UA-5 analyzer, ISCO Model 690 density gradient fractionator) using previously determined standard curves relating area under the peak and virus concentration (1). Yields of virus were expressed in milligrams of virus per gram fresh weight (fr. wt.) of tissue.

Analysis for genomic masking.—Two approaches to detecting genomic masking were used. Artificial and natural mixtures of the two viruses were fractionated on sucrose gradient columns and samples collected at the position of genomically masked TMV-RNA (150S) (6) were tested for TMV infectivity on Xanthi nc tobacco. In the second method, the two types of virus mixture were fractionated into TMV-rich and BMV-rich populations by selective polyethylene glycol (PEG) fractionation and further purified by sucrose gradient centrifugation. The virus preparations were dissociated to RNA and protein by incubating virus at 50 C for 15 min with an equal volume of dissociation buffer (0.04 M Tris-HCl, pH 9.0, 0.002 M EDTA, 2% sodium dodecyl sulfate, 2.0 M urea, 0.1 M 2-mercaptoethanol, and 2.0 mg bentonite/ml). The protein:RNA mixture from each population was electrophoresed on polyacrylamide gels (2), stained with Coomassie Blue, and assessed for the presence of the genomically masked RNA by visual scanning.

Electron microscopy.—Small pieces of leaf tissue from doubly infected leaves were fixed in 5% glutaraldehyde buffered with 0.1 M potassium phosphate, pH 7.2, for 1.5 hr. They were then rinsed twice for 15 min in 0.1 M potassium phosphate, pH 7.2, and then postfixed in 1% osmium tetroxide in Palade's buffer for 1 hr, dehydrated, and embedded in Epon. Thin-sections were cut with a diamond knife, stained with lead citrate, and examined with a Philips EM 300 transmission electron microscope operated at 60 KV.

RESULTS

Inoculation of the first leaf of Black Hulless barley seedlings with TMV only resulted in infection of that leaf, but no evidence was obtained for systemic spread of TMV by indexing of extracts of noninoculated leaves on *C. amaranticolor*. When seedlings were co-inoculated with TMV and BMV, most (95%) of the plants became systemically infected with BMV; 80-90% of these BMV-infected plants were infected with TMV as determined either by microprecipitin or by infectivity tests. Barley inoculated with both BMV and TMV usually showed a chlorotic mottle in noninoculated leaves in addition to the general mosaic pattern of similar leaves of plants inoculated with BMV alone.

Virus yields in single and double infections.—The yield of BMV in tissue from four leaf positions sampled over an interval of 5-21 days after inoculation is illustrated in Fig. 1. Considerably higher yields (3-9 mg/g fr. wt.) were obtained from the inoculated leaf (leaf 1) and leaf 2 than from leaves 3 and 4 (1-2 mg/g fr. wt.) in both single and double virus infections. In doubly infected plants, the yield of BMV in leaf 2 continued to rise (4-9 mg/g fr. wt.) during the course of the experiment, whereas the yield of BMV in comparable leaves of singly infected plants remained at approximately 4.5 mg/g fr. wt. during the same interval.

In barley inoculated with TMV alone, the virus could be detected only in extracts from the inoculated leaf; the yield of TMV ranged from 0.1-0.5 mg/g fr. wt. over the 5-21 day postinoculation period. Similar yields of TMV were obtained from the inoculated leaves of barley infected with both BMV and TMV, but in those cases, TMV also was recovered from leaves 2, 3, and 4. The yield of TMV from these leaves (0.5-2.5 mg/g) was generally 2-5 times that of the inoculated leaf in later stages of infection and by 21 days postinoculation, the yield of TMV was 5-6 mg/g fr. wt. in leaf 4.

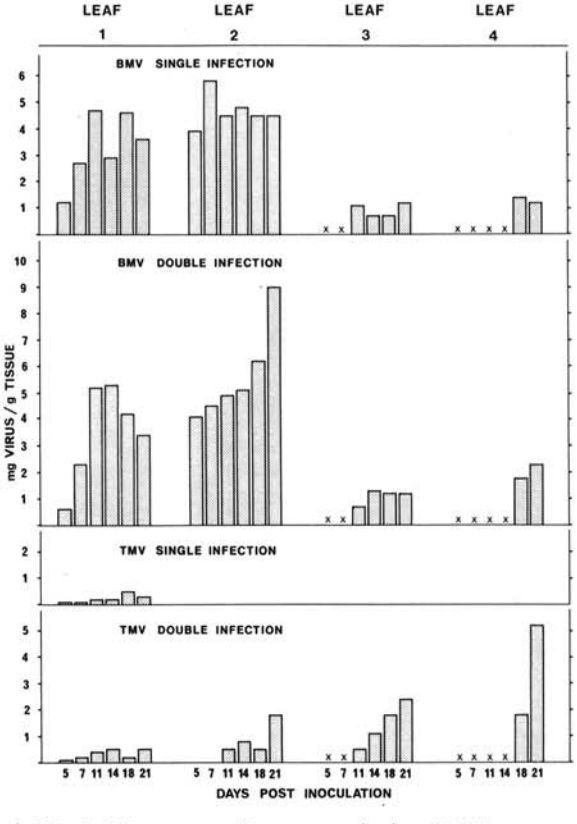

Fig. 1. The amount of brome mosaic virus (BMV) or tomato mosaic virus (TMV) (milligrams per gram fresh weight) recovered from barley leaves infected with BMV, TMV, or both viruses. Results are the average of two experiments. The symbol "X" indicates that the leaf had not developed to sampling stage.

The yield of TMV expressed as a proportion of the total virus obtained from doubly infected leaves is shown in Table 1. Less than 20% of the total virus obtained from leaves 1 and 2 during the 5- to 21-day infection period was TMV, whereas it constituted 40-70% of the total virus produced in leaves 3 and 4.

There was no difference in the capacity of three different isolates of TMV to invade barley systemically in the presence of BMV. The type strain, an isolate from tomato and another that systemically infected *C. amaranticolor* all behaved similarly in doubly infected barley.

Electron microscopy of doubly infected plants.—Both viruses could be detected readily within the same cell (Fig. 2), but the proportion of doubly infected cells was not determined. Although some sections of cells showed BMV virions only, none was found which showed TMV only.

Analysis for genomic masking.—The results of experiments (outlined below) that were designed to detect infectious TMV-RNA encapsidated in BMV coat protein were uniformly negative.

Sucrose gradient centrifugation.—Natural and artificial mixtures of TMV and BMV were centrifuged through sucrose gradients and fractions between the BMV and TMV zones were tested for TMV infectivity on Xanthi nc tobacco. These fractions would have contained TMV-RNA masked in BMV coat protein if such virions occurred because such genomically masked RNA, obtained in in vitro reassembly experiments with TMV-RNA and BMV protein (6) sedimented at about 150S. No infectivity corresponding to TMV could be detected in this region of the gradient from either mixture in three experiments, although such fractions did contain BMV virions as determined by infectivity assays on barley seedlings.

Polyacrylamide gel electrophoresis.—Real and artificial mixtures were separated into a BMV-rich fraction by treatment with 4% PEG-6000 and NaCl (0.1 M) and the precipitated TMV was separated by low-speed centrifugation; the BMV population then was centrifuged through a 10-40% sucrose gradient in the SW 27 rotor and the BMV zone was collected and then concentrated by ultracentrifugation. When BMV from both real and artificial mixtures in two experiments was dissociated in virus dissociation buffer and then electrophoresed in 2.5% polyacrylamide gels, BMV-RNA, but not TMV-RNA, could be detected by Coomassie Blue staining. When TMV prepared from real and artificial mixtures in a similar fashion was dissociated and electrophoresed, both TMV and BMV-RNA could be detected in each population of TMV, indicating contamination of the TMV-RNA by RNA derived from BMV virions. Such virions were readily detectable in the TMV population of both mixtures by electron microscopy and by analytical gradient centrifugation. Appropriate controls of TMV and BMV yielded only the homologous RNA upon electrophoresis in gels.

TABLE 1. Yield of tobacco mosaic virus (TMV) as a proportion of the total amount of brome mosaic virus (BMV) and TMV in doubly infected barley grown at 31 C

Days post-inoculation	Leaf 1		Leaf 2		Leaf 3		Leaf 4	
	Total virus (mg/g fr. wt.)	TMV (%)	Total virus (mg/g fr. wt.)	TMV (%)	Total virus (mg/g fr. wt.)	TMV (%)	Total virus (mg/g fr. wt.)	TMV (%)
5	0.7	14.3	4.1	0.0	. . .[a]		. . .	
7	2.5	8.3	4.5	0.0	. . .		. . .	
11	5.6	7.2	5.4	9.3	1.2	41.7	. . .	
14	5.8	8.6	5.9	13.6	2.4	42.1	. . .	
18	4.4	4.5	6.7	7.5	3.0	60.0	3.6	50.0
21	3.9	12.8	10.8	16.7	3.6	66.7	7.5	69.3

[a]Leaf had not developed to sampling stage.

TABLE 2. Yield of brome mosaic virus (BMV) and tomato mosaic virus (TMV) in doubly inoculated barley incubated at different temperatures

Temperature (C)	Days post-inoculation	Virus yield (mg/g fr. wt.)							
		Leaf 1		Leaf 2		Leaf 3		Leaf 4	
		BMV	TMV	BMV	TMV	BMV	TMV	BMV	TMV
31	5	0.7	0.1	1.9	0.0	. . .[a]		. . .	
	8	1.2	0.1	2.4	0.0	0.5	0.0	. . .	
	12	1.9	ND[b]	3.7	0.0	1.1	0.6	. . .	
	15	ND	ND	3.1	0.0	1.3	2.8	1.4	1.5
	19	ND	ND	3.4	0.1	1.2	3.4	1.3	3.4
25	5	0.3	0.1	0.7	0.0				
	8	0.2	0.0	2.0	0.0	2.0	0.0		
	12	1.0	0.0	1.6	0.0	2.7	0.0		
	15	ND	ND	4.0	0.0	2.2	0.0	1.7	0.0
	19	ND	ND	3.6	0.0	1.2	0.0	1.4	0.0

[a]Leaf had not developed to sampling stage.
[b]Not determined.

Temperature-shift experiment.—The previous experiments utilized plants maintained at 31 C after inoculation until the completion of the experiments; under these conditions, TMV could be detected readily and quantitated in the noninoculated leaves which developed after inoculation. The results of several experiments demonstrated that a relatively high and constant temperature (30-31 C) is required for this phenomenon; no TMV could be detected by gradient centrifugation in noninoculated leaves of doubly infected plants if they were maintained at a constant temperature (20 C or 25 C) after inoculation. The results of an experiment which compared virus yields at 25 C and 31 C are shown in Table 2. However, in a temperature-shift experiment, some TMV could be detected in noninoculated leaves (leaves 3 and 4) of plants that were incubated at 25 C for 1-3 days and then moved to 31 C until the end of the experiment (Table 3). If, however, the plants were kept at 25 C for 4-6 days before being moved to 31 C, no TMV was detected. Plants incubated at 31 C for 1 day and then moved to 25 C did not become systemically infected with TMV, but the virus was detected in plants kept at 31 C for 2-6 days before being moved to 25 C. In both cases, the amount of TMV detected was about 10-15% of that detected in plants maintained at a constant temperature of 31 C; however, only about 20% of the plants were systemically infected with TMV compared to 86% for those maintained at a constant temperature of 31 C.

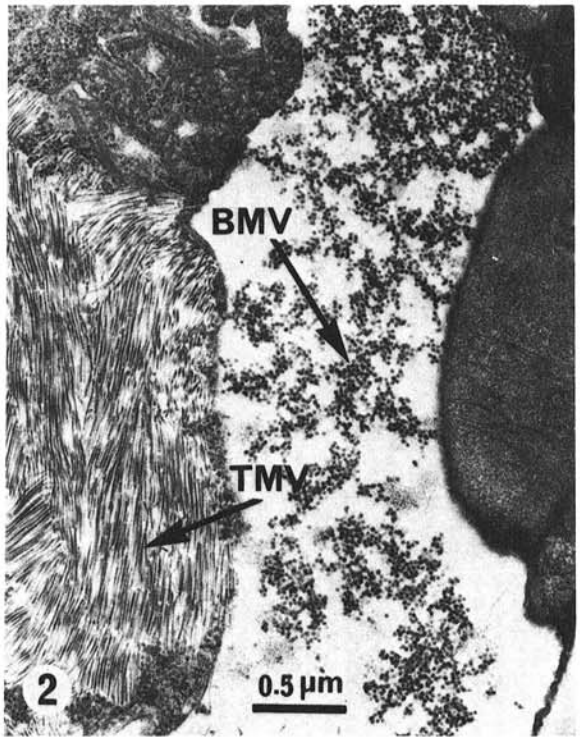

Fig. 2. Electron micrograph of a parenchyma cell of leaf 3 of barley infected with brome mosaic virus (BMV) and tomato mosaic virus (TMV).

Mixed inoculation with BMV and other viruses.—No evidence of systemic infection was obtained in barley inoculated with AMV, CCMV, SBMV, and TNV in single inoculations or in mixed inoculation with BMV in a greenhouse or in growth chambers at 30 C. All inoculations of appropriate indicator plants yielded negative results under conditions in which such plants reacted positively to authentic samples of these viruses.

DISCUSSION

The results obtained in these experiments extend the basic observation in previous reports that systemic infection of barley by TMV depends on mixed infection with a helper virus. In addition to barley stripe mosaic virus (BSMV) (1, 3), BMV was able to function as a helper virus in TMV infection of barley. Moreover, it appears that the capacity of TMV to establish a systemic infection with BSMV or BMV was not restricted to a particular strain of TMV; two isolates of TMV, one from tomato and the other from systemically infected *C. amaranticolor* also were able to infect barley systemically in a double infection with either BSMV or BMV. The fact that two distinctly different viruses can function as helper viruses for TMV would indicate that the role of the helper virus is a nonspecific one, perhaps affecting the growth and development of the host in some way so as to predispose it to systemic infection by TMV.

The double infection of barley by BMV and TMV appears basically similar to the BSMV/TMV double infection. Tobacco mosaic virus routinely was detected in leaves 3 and 4 of both double infections. In contrast to the BSMV/TMV double infection, TMV always was detected in leaf 2 and by day 21 the amount of extractable TMV was about double that in leaf 1, indicating that enhanced replication was occurring in this leaf. Similar

TABLE 3. Yield of brome mosaic virus (BMV) and tobacco mosaic virus (TMV) in leaves 3 and 4 of doubly inoculated barley after incubation for varying periods of time at 25 C and 31 C

Treatment	Virus yield (mg/g fr. wt.)[a]	
	BMV	TMV
Control 31 C[b]	1.3	3.4
Control 25 C[c]	1.3	0.0
TS ↑ 1[d]	0.7	0.4
TS ↑ 2	1.0	0.4
TS ↑ 3	0.9	0.3
TS ↑ 4	1.1	0.0
TS ↑ 6	1.2	0.0
TS ↓ 1[e]	0.7	0.0
TS ↓ 2	0.6	0.1
TS ↓ 3	0.8	0.6
TS ↓ 4	0.7	0.5
TS ↓ 6	1.0	0.3

[a]Yield of virus from pooled leaves 3 and 4 from 10 plants 20 days after inoculation with a mixture of BMV and TMV.
[b]Inoculated plants were maintained at 31 C constantly.
[c]Inoculated plants were maintained at 25 C constantly.
[d]Inoculated plants were incubated at 25 C and then shifted to 31 C at indicated number of days after inoculation.
[e]Inoculated plants were incubated at 31 C and then shifted to 25 C at indicated number of days after inoculation.

TABLE 4. Amount of brome mosaic virus (BMV), barley stripe mosaic virus (BSMV), and tomato mosaic virus (TMV) in leaves of barley doubly inoculated with TMV and with BMV or BSMV

	Virus yield (mg/g fr. wt.)											
	Leaf 1[a]			Leaf 2[b]			Leaf 3[c]			Leaf 4[d]		
	TMV	Other	Total	TMV	Other	Total	TMV	Other	Total	TMV	Other	Total
BMV		2.7	2.7		4.8	4.8		0.8	0.8		1.2	1.2
BMV + TMV	0.2	2.3	2.5	0.5	4.8	5.3	1.1	1.3	2.4	5.2	2.3	7.5
BSMV[e]		0.2	0.2		0.5	0.5		1.2	1.2		1.0	1.0
BSMV + TMV[e]	0.1	0.2	0.3	0.1	0.5	0.6	2.1	1.4	3.5	4.7	1.0	5.7

[a]Sampled at 7-8 days postinoculation.
[b]Sampled at 10-11 days postinoculation.
[c]Sampled at 14-15 days postinoculation.
[d]Sampled at 20-21 days postinoculation.
[e]From Fig. 1 *in* Dodds and Hamilton, 1972. Virology 50:404-411.

yields of TMV were obtained in the BMV/TMV double infection by day 21 (2.5 and 5.2 mg/g fr. wt. for leaves 3 and 4, respectively) as compared to 2.0 and 4.6 mg/g fr. wt. for the same leaves of the BSMV/TMV double infection (1).

A distinct difference between the two double infections was the apparent absence of genomic masking of TMV-RNA by BMV protein whereas in the BSMV/TMV double infection, approximately 8-10% of the TMV-RNA in leaves 3 and 4 is encapsidated in BSMV protein (2).

It should be noted that the enhanced yield of TMV in leaves 3 and 4 of the BMV/TMV double infection was obtained from leaves from which the yield of BMV was only 20-30% of that in leaf 2; i.e., it appears that BMV replication is repressed in leaves 3 and 4 of both BMV and BMV/TMV infections although these leaves support enhanced replication of TMV. One could conclude from the virus yield data in the BMV infection that leaves 3 and 4 were producing virus at maximum capacity when in fact similar leaves of doubly infected plants were producing twice as much virus, albeit a mixture of both viruses. Clearly, any control mechanisms which were operating to regulate BMV replication were not operating to prevent TMV replication. An analysis of the data for virus yields of the BSMV/TMV infection (Table 4) and of the BMV/TMV infection shows that there was 2-6 times as much virus produced in leaves 3 and 4 of doubly infected plants as there was in the same leaves infected with either BMV or BSMV only. Most of the virus produced was TMV, suggesting that the TMV infection was superimposed upon the infection of BMV or BSMV.

The results of the temperature-shift experiment define some aspects of the systemic infection of barley by TMV. No evidence of TMV infection in noninoculated leaves was obtained in double infections at 20 C (Hamilton and Nichols, *unpublished*) or at 25 C (Table 2); the systemic infection by TMV occurred with BMV or BSMV at 30-31 C, but the interval between 25 C and 30 C has not been critically examined. It is clear that the systemic infection of Black Hulless barley by TMV was temperature-dependent. The temperature-shift data indicate that the first 72 hr is the critical period for the establishment of the systemic infection of TMV in doubly inoculated barley. The fact that TMV did replicate at 25 C, but that only about 20% of the plants became systemically infected in the shifted series would suggest that the low temperature primarily affected the distribution of TMV to the developing primordia of leaves 3 and 4, rather than the replication of TMV in infected leaf primordia. This aspect of the double infection requires more study, especially from a developmental point of view.

The reason for the enhanced replication of TMV in leaves 3 and 4 is not known. Although both BMV and TMV could be routinely found in the same parenchyma cell, there is no proof that replication of TMV in leaves 3 and 4 requires the presence of replicating BMV in the same cell. It would appear likely that BMV infection of leaf 1 may predispose the host to TMV infection of cells in the leaf primordia, but even if that supposition were true it does not mean that the enhanced replication of TMV during cell division and elongation requires the presence of actively replicating BMV in each TMV-infected cell. It is interesting to note that the increasing yields of TMV in leaves 3 and 4 were associated with depressed yields of BMV. Since the yield of BMV in single infections of leaves 3 and 4 also was depressed, it is clear that TMV replication in these leaves did not repress BMV replication. We are currently examining barley protoplasts derived from single and double infections in an attempt to define the role of helper viruses.

The replication of TMV in cereals under controlled laboratory conditions is not without significance to diseases in the natural state. Recent evidence (4) indicates that TMV and soilborne wheat mosaic virus (SbWMV) were isolated from diseased winter wheat in Kansas, and that TMV can be recovered from wheat cover crops that follow TMV-infected flue-cured tobacco in Virginia (S.A. Tolin, *personal communication*). Moreover, a serological relationship between TMV and SbWMV and very similar molecular weights of their respective coat proteins (5) suggests that SbWMV is an unusual strain of TMV. The possibility that such cereal-infecting strains of TMV have evolved as a consequence of the association of TMV with cereal-infecting viruses is amenable to investigation.

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