The Nature of the Resistance of Agrotricums to Wheat Streak Mosaic Virus

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ABSTRACT

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The agrotricums, C.I. 15092, C.I. 15321, and C.I. 15322, possess a hypersensitive type of resistance to wheat streak mosaic virus (WSMV). Following inoculation with WSMV, chlorotic or necrotic local lesions develop on inoculated leaves and increase in size until the leaves die. All three agrotricums are resistant to WSMV in the field, but under greenhouse or growth chamber conditions only C.I. 15092 remains resistant. In C.I. 15092, WSMV replicates in the inoculated leaf and is translocated in both directions within the leaf. Virus can be transferred from the lesions and the titer increases until 5 days postinoculation, then declines as necrosis ensues. Virus was purified from inoculated leaves but was not detected in roots or noninoculated leaves of C.I. 15092 by infectivity assay. Virus particles and pinwheel inclusion bodies were found only in inoculated leaves. Resistance was not affected by light intensity or plant age at inoculation, but was broken at 35 C. The percentage of plants that became systemically infected was influenced by the length of heat treatment and the period that elapsed between inoculation and heat treatment. Resistance also was broken by daily treatment of inoculated plants with tannic acid, acridine orange, cycloheximide, or actinomycin D. Agrotricum C.I. 15092 is a systemic host of foxtail, brome, and barley stripe mosaic viruses. Necrotic local lesions did not develop following infection by those three viruses.

Additional key words: grass viruses, hypersensitivity.

Wheat streak mosaic virus (WSMV) continues to be a serious threat to wheat production in the Great Plains of the USA. Yield reductions by WSMV in Kansas alone were estimated at 5.5, 10.9, and 8.2 metric tons (20, 40, and 30×10^6 bushels) in 1949, 1959, and 1974, respectively (12). The average annual loss in Kansas the past 5 yr has been 3.5 metric tons (13×10^6 bu). Adequate levels of resistance to this virus are not present in agronomically suitable wheat (*Triticum aestivum* L. em. Thell).

Several WSMV-resistant wheat germplasms containing chromosome substitutions or translocations from *Agropyron* spp. (agrotricums) have been released recently. Agrotricums C.I. 15321 and C.I. 15322, disomic substitution and translocation lines from *Agropyron elongatum* (Host) P.B., respectively, (10) were released by the Agricultural Research Service of the U.S. Department of Agriculture and the Oklahoma Agricultural Experiment Station. These agrotricums also possess resistance to the wheat curl mite, *Aceria tulipae* Keifer, the vector of WSMV (10). The South Dakota Agricultural Experiment Station released C.I. 15092, a WSMV-resistant disomic substitution line derived from a cross of 'Carsten V' wheat × *Agropyron intermedium* (L.) S.C. (8). Mites develop readily on C.I. 15092 (10).

These agrotricums, especially C.I. 15322, are being widely used by both public and private wheat breeders. Since widespread use of a single resistance may create genetic vulnerability, it is important to determine the nature of the agrotricum resistance and to define conditions under which it will be effective. We report responses of the agrotricums to WSMV, other viruses, and metabolic inhibitors under various experimental conditions.

In early experiments we found that C.I. 15321 and 15322 always were resistant to WSMV in field tests. However, their response was unpredictable under greenhouse and growth chamber conditions. Therefore, the more detailed experiments were performed only with C.I. 15092.

MATERIALS AND METHODS

Virus culture and detection.—The isolate of WSMV used in these investigations was obtained from wheat near Russell, KS, in 1970. The virus was maintained on Parker wheat with frequent transfers. An extract of infected plants was prepared by grinding leaves with a mortar and pestle at a 1:5 dilution (w:v) in 0.02 M potassium phosphate buffer, pH 7.0 (phosphate buffer). Seven days after planting, test plants were dusted with Carborundum and the leaves were rubbed with the extract. Plants were maintained in growth chambers at 24 C, and 10,800 lux of fluorescent lighting with a 12-hr photoperiod unless otherwise indicated.

Inoculum for field experiments was prepared by grinding leaves of 14-day infected Parker in a 3.8 liter Waring Blendor at a 1:20 (w:v) dilution in phosphate buffer. The extract was filtered through four layers of cheesecloth and Carborundum added to 1.5% (w/v). Seedlings were sprayed at the two-to three-leaf stage with a DeVilbiss EGA-502-394-G sprayer from a distance of

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2.5 cm and at 6.7 kg/cm^{-2}. The extract was agitated to keep the Carborundum in suspension. Four replications were used in a split plot design, with four 3-m rows per plot.

To detect systemic infection by WSMV, a leaf which developed subsequent to inoculation was ground in 1 ml phosphate buffer and rubbed on Parker wheat test plants. Systemic infection of the test plants was judged to have occurred if mosaic symptoms typical of WSMV developed 6 to 10 days postinoculation (PI).

Experimental environments.—To determine the effects of plant age and environmental factors on WSMV resistance, plants were inoculated at different ages and subjected to various light intensities and temperatures. To measure effects of plant age on resistance the agrotricums C.I. 15092, C.I. 15321, C.I. 15322 (hereafter referred to as 092, 321, 322, respectively), and Parker wheat were maintained in a greenhouse and inoculated at different times after seeding. All leaves were inoculated, and the plants were tested for systemic infection 14 days PI.

To measure the effects of light intensity on WSMV resistance 092, 321, 322, and Parker were inoculated and maintained in growth chambers at 27 C with a 12-hr photoperiod and light intensities of 8,600, 21,500, or 37,700 lux. A set of plants also was maintained under January/February greenhouse conditions (natural light with 25 C days and 18 C nights). All plants were tested for systemic infection 14 days PI.

To determine the effects of temperature on WSMV resistance, 092, 321, 322, and Parker were inoculated and maintained in growth chambers at 18, 27, 35 C and 10,800 lux with a 12-hr photoperiod. Plants were maintained at 18 and 27 C for 14 days, but were left at 35 C only 3 days and then at 24 C the remaining 11 days. All plants were tested for systemic infection 14 days PI.

A more extensive temperature study was performed with 092 and Parker. Inoculated plants were placed in a growth chamber at 35 C and 10,800 lux with a 12-hr photoperiod. Heat treatments began the day before and up to 5 days PI. Plants were heated for 24, 48, or 72 hr and then placed in a 24 C growth chamber. All plants were tested for systemic infection 7 days PI.

Metabolic inhibitors.—Infected Parker and 092 were treated daily with metabolic inhibitors. Application began 1 day PI and continued for 5 or 6 days. Inhibitors and concentrations used were: tannic acid (17 mg/ml), acridine orange (100 μg/ml), cycloheximide (10 μg/ml), and actinomycin D (100 μg/ml). All were dissolved in a solution of 0.1% aerosol O.T. (a wetting agent) and applied with a cotton swab. Two days after the final application, all plants were tested for systemic infection.

Additional viruses.—The reaction of 092 to other viruses and the effects of these viruses on WSMV resistance were determined. The primary leaves of 092 or Parker plants were inoculated with barley stripe mosaic virus (BSMV), brome mosaic virus (BMV), or foxtail mosaic virus (FMV). After 7 days, the second leaf was tested for systemic infection. The BSMV and BMV were detected by agar double-diffusion serology (13) and FMV was detected by grinding the second leaf and rubbing the extract on *Chenopodium amaranticolor* Coste and Reyn. Local lesions indicated systemic infection by FMV. The effect of other viruses on the agrotricum response to WSMV was determined by inoculating the primary leaf of 092 with a mixture of WSMV and either BMV, BSMV, or FMV. Systemic infection was determined by serologically specific electron microscopy (5). Grids with parlodion films were carbon-coated and floated on the appropriate antiserum for 30 min. The wheat leaf was ground in buffer 7 days PI. Antiserum-coated grids were floated on the extract for 4 hr, then negatively stained with 2% phosphotungstic acid. A Philips Model 201 electron microscope operating at 60 kV was used to examine the grids.

Ultrastructural studies.—Thin sections were cut from primary leaves of healthy and infected 092 and Parker. At 5 days PI, all material was fixed and embedded as described by Langenberg and Schroeder (7). Samples were sectioned with a Reichert ultramicrotome, placed on grids, and stained for 30 min in 5% uranyl acetate in 50% ethanol followed by a 12-min treatment with Reynolds' lead citrate.

The fluorescence method of Currier and Strugger (4) as modified by Wu et al. (15) was used to detect callose. Sections were stained for 4 hr, 8 hr, or overnight, and observed with a Leitz fluorescent microscope.

Virus replication and translocation.—Parker and 092 were tested daily from 1 to 10 days PI to determine the extent of virus replication. Leaves were ground (1:8, w:v) in buffer and nine successive twofold dilutions were inoculated to Parker. For these experiments the dilution endpoint was considered to have been reached when less than 75% of the test plants became infected. To determine the direction and extent of WSMV translocation, the upper half or lower half of 092 and Parker primary leaves was inoculated. After 5 days, the leaf-halves were separated and each half was tested for infectivity.

Purification of wheat streak mosaic virus.—Primary leaves of 092 and Parker harvested 5 days PI were blended for 90 sec in cold 0.06 M dibasic sodium phosphate and 0.01 M sodium diethyldithiocarbamate, pH 9.2 (1), at a 1:3 (w:v) dilution. The extract was filtered through two layers of cheesecloth and chloroform was added to 8% (v:v). The mixture was stirred 20 min at 4 C then centrifuged 10 min at 13,000 *g*. The supernatant liquid was made to 6% (w:v) polyethylene glycol 6000 and 0.4 M NaCl. The mixture was stirred for 2 hr at 4 C, then centrifuged 10 min at 13,000 *g*. The pellet was resuspended in 0.1 M Tris-0.032 M citrate, pH 6.5, then pelleted through a layer of sucrose (200 mg/ml) for 75 min at 192,000 *g*. The pellet was resuspended overnight in 2 ml of Tris-citrate. The suspension was centrifuged 10 min at 13,000 *g*, and the supernatant liquid was layered on equilibrium gradients (2) of 2 ml each of 400, 500, 600, and 700 mg sucrose/ml. These were centrifuged for 9.5 hr at 205,000 *g*. Gradients were scanned at 254 nm with the ISCOUA-2UV analyzer and Model D density gradient fractionator attached to an external recorder. Fractions were collected and tested for infectivity.

RESULTS

Agrotricum responses to wheat streak mosaic virus.—Under greenhouse and growth chamber conditions, local lesions developed on leaves of 092, 321, and 322 that had been inoculated with WSMV. These lesions enlarged, coalesced, and eventually the inoculated

leaves died. The WSMV was transmitted readily from the lesions on all three lines and from necrotic leaves for a short time after leaf death, but eventually infectivity was lost. Dark, necrotic lesions first appeared on 092 two days PI and enlarged rapidly along the length and then the width of the leaf until the leaf died. Callose was not detected in association with lesions of 092; 321 and 322 were not examined. Lesions first appeared on 321 and 322 four days PI. The chlorotic spots elongated, coalesced, and eventually the entire leaf became necrotic. The development of necrosis was much more rapid in 092 than in 321 or 322.

No symptoms were observed on noninoculated leaves of 092 under greenhouse or growth chamber conditions and WSMV was not transmitted from noninoculated leaves. Owing to its spring habit, 092 was not tested in field plots, but it was resistant to WSMV in Montana field tests (T. Carroll, *personal communication*). Under greenhouse and growth chamber conditions, 321 and 322 often developed mild WSMV symptoms on noninoculated leaves following the death of inoculated leaves. WSMV was readily transmitted both from lesions and from leaves with systemic symptoms. In field tests with the same seed lot, 321 and 322 always were resistant. The virus was restricted to the inoculated leaves. No systemic symptoms developed and no yield reductions occurred in plots of the inoculated plants. This unpredictable difference in response between greenhouse, growth chamber, and field conditions for 321 and 322 caused us to use only 092 in more detailed experiments.

Effects of plant age, light intensity, and temperature.—The resistance of 321 and 322 to WSMV was broken in some plants at almost every plant age, light intensity, and temperature tested (Tables 1, 2, 3). However, 092 remained resistant to systemic infection at all plant ages and light intensities tested (Tables 1,2). Virus was not detected in roots or noninoculated leaves of any 092 plants by infectivity assay. The resistance of 092 was expressed at 18 and 27 C, but was broken at 35 C (Table 3). The percentage of systemically infected plants was influenced by the duration of heat (35 C) treatment and the time between inoculation and heat treatment (Fig. 1). Heat treatment initiated 2 days PI was most effective in breaking resistance. Heating before inoculation broke resistance in only a small percentage of plants; this percentage increased among plants exposed to the 72-hr heat treatment (Fig. 1).

Variously patterned heating regimes were used to identify differences between high temperatures in the field and in growth chambers. Heating effects were cumulative; i.e., a comparable percentage of resistance breakdown resulted from two 12-hr periods of heating or 24 hr of continuous heat. Therefore, when heating occurred only during illumination, more successive days of high temperature were required to break resistance. When either the shoot or root portion was heated (35 C) and the remaining portion was cooled (24 C), resistance was also broken.

TABLE 1. Effect of plant age at inoculation on resistance of Parker wheat and three agrotricum lines to wheat streak mosaic virus under greenhouse conditions

Age at Inoculation (days)	Systemic infection of wheat or agrotricum line			
	Parker (%)	C.I. 15092 (%)	C.I. 15321 (%)	C.I. 15322 (%)
5	100[a]	0	10	61
7	100	0	9	41
10	100	0	24	29
15	100	0	5	58
20	100	0	28	7
25	100	0	10	0
28	100	0	41	9
30	100	0	31	9
35	100	0	39	0

[a]Results of two separate trials, each containing 10-20 plants. Averages are reported.

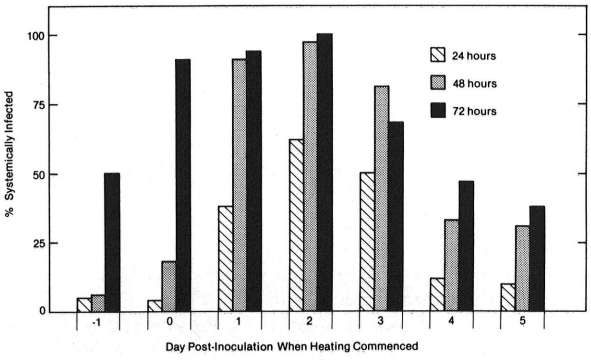

Fig. 1. Effect of heating 3 days at 35 C followed by 11 days at 24 C on resistance to wheat streak mosaic virus (WSMV) in the agrotricum C.I. 15092. Nonheated C.I. 15092 plants were all resistant to WSMV. Correspondingly inoculated Parker wheat plants became 100% infected whether heated or not.

TABLE 2. Effect of light intensity on resistance of Parker wheat and three agrotricum lines to wheat streak mosaic virus

Wheat or agrotricum line	Systemic infection of inoculated plants maintained at a light intensity of:			
	8,600 lux (%)	21,500 lux (%)	37,700 lux (%)	Ambient greenhouse (%)
Parker	100[a]	100	100	100
C.I. 15092	0	0	0	0
C.I. 15321	22	29	62	12
C.I. 15322	40	24	62	12

[a]Results of two separate trials, each containing 23-30 plants. Averages are reported.

Breaking of resistance in 092 by heating caused lesions to appear on leaves which developed after inoculation. This response usually resulted in necrosis of all leaves and death of the plant. The few plants that survived heating and the resulting systemic infection were raised to maturity; their progeny were resistant to WSMV.

Metabolic inhibitors and additional viruses.—Treatments with metabolic inhibitors were less effective than heating for breaking resistance. Daily foliar application of tannic acid, actinomycin D, acridine orange, or cycloheximide broke resistance (Table 4). However, vacuum infiltration of these inhibitors as well as D-threo-chloramphenicol, erythromycin, rifampicin, 6-methyl-purine, and 6-azauracil did not break resistance. Foliar application of D-threo-chloramphenicol (1, 3, or 6 mg/ml) and 6-azauracil (2 mg/ml) did not break resistance.

Both 092 and Parker became systemically infected with BMV, BSMV, and FMV and exhibited mosaic symptoms, but none of those viruses caused lesions on 092. Following inoculation with each of these viruses plus WSMV, WSMV was not recovered from Parker or from the inoculated or noninoculated leaves of 092. The other viruses were readily recovered.

Ultrastructural changes.—Virus particles and pinwheel inclusion bodies were observed in both inoculated and noninoculated leaves of infected Parker wheat, but these were observed only in the inoculated leaves of 092. Virus particles were most readily observed in 092 at the base of lesions fixed 5 days PI. Cells appeared normal at the base of the lesion, but there was increasing cellular disorganization toward the center of the lesion. The first major indication of lesion formation was disruption of the plasmalemma, followed by organelle disorganization and rupture of organelle membranes, and finally cell wall collapse. Virus particles were observed in "normal-appearing" infected cells, cells with a disrupted plasmalemma, and cells with disrupted organelle membranes.

Virus replication and translocation.—In inoculated leaves of 092 WSMV replicated and was translocated basipetally and acropetally within the leaf. Virus titer in 092 increased rapidly until 5 days PI, then declined as necrosis ensued (Fig. 2).

Virus was purified from inoculated leaves of 092 and Parker at 5 days PI. No differences in virus concentration

TABLE 3. Effect of temperature on resistance of Parker wheat and three agrotricum lines to wheat streak mosic virus

Wheat or agrotricum line	Systemic infection of plants maintained at a temperature of:		
	18 C[a] (%)	27 C[a] (%)	35 C[b] (%)
Parker	100[c]	100	100
C.I. 15092	0	0	88
C.I. 15321	5	35	25
C.I. 15322	30	58	43

[a]Plants kept at constant 18 or 27 C for 14 days.
[b]Plants kept at 35 C for three days, then at 24 C for 11 days.
[c]Results of three separate trials, each containing 7-12 plants. Averages are reported.

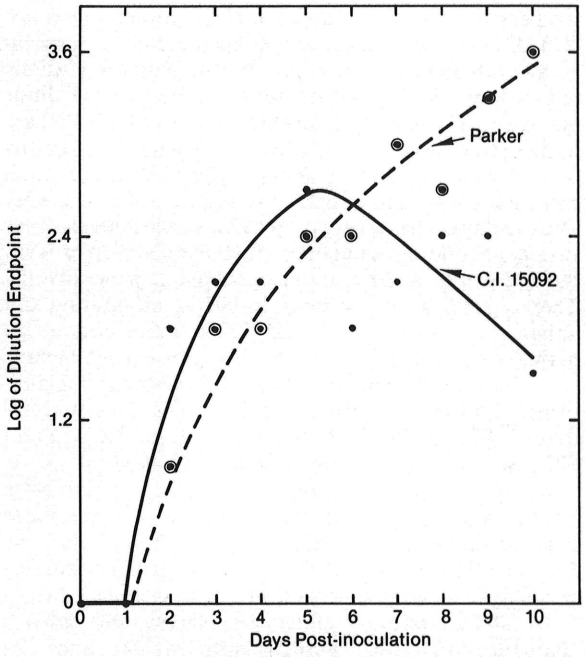

Fig. 2. Multiplication of wheat streak mosaic virus in Parker wheat and the agrotricum C.I. 15092. Assays were performed by grinding inoculated leaves in 0.02 M potassium phosphate buffer, pH 7.0 and using various dilutions to inoculate Parker wheat. The dilution end point was considered to have been reached when less than 75% of the inoculated plants became infected.

TABLE 4. Effect of metabolic inhibitors on resistance of the agrotricum C.I. 15092 to wheat streak mosaic virus

Inhibitor	Concentration (mg/ml)	Number of applications[a]	Systemically infected[b,c] (%)
Tannic acid	17.00	6	87
Actinomycin D	0.10	6	22
Acridine orange	0.10	5	20
Cycloheximide	0.10	5	14
Aerosol O.T.	100.00	6	0

[a]Daily applications initiated one day postinoculation.
[b]Corresponding treatments with Parker wheat were 100% systemically infected.
[c]Results of two separate trials, each containing 5-10 plants. Averages are reported.

could be detected with the described purification method. Virus yields of 1.4 A_{254} units/100 g of tissue were comparable with the 1.2 A_{254} units obtained by Brakke and Ball (2). Only fractions collected from the UV-absorbing peak on density gradients were infectious. Inoculations with a concentration of 1.2 μg/ml of purified virus (based on $E_{260}^{0.1\%} = 3.0$) caused infection in 50% of the test plants.

DISCUSSION

The resistance of 092 to WSMV differs both genotypically and phenotypically from that of 321 and 322. Agrotricum 092 resulted from a cross of *Agropyron intermedium* with wheat, its resistance is more temperature-sensitive (Table 3) and it is not mite-resistant (10); 321 and 322 derive their resistance from *Agropyron elongatum* and are mite-resistant (10). The local lesions induced by WSMV on these agrotricums also differ markedly. Those on 092 develop rapidly (2 days PI), are necrotic and expand rapidly the length of the leaf. Lesions on 321 and 322 appear later (4 days PI), are distinct chlorotic spots, and expand the length of the leaf only after 12 days. The resistance of 092 is stable under a wider variety of conditions but once resistance has been broken, the 092 plants suffer extreme damage and generally die. That is, the loss of resistance in 092 is self-limiting and lethal. Resistance in 321 and 322 is more difficult to define, but when resistance is broken, consequent damage is less because the plants develop a mild systemic infection rather than the systemic necrosis of 092. This suggests that, in addition to the hypersensitive response, 321 and 322 also may possess some tolerance to WSMV.

Responses of the agrotricums to inoculation with WSMV were compared; 092 always was resistant under field, greenhouse, and growth chamber conditions. The 321 and 322 always were resistant under field conditions, but in different experiments the same seed lot varied from 0 to 100% resistant under greenhouse and growth chamber conditions. Some plants of 321 and 322 remained resistant at all temperatures, light intensities, and plant ages tested, but there was no obvious correlation between resistance and these parameters. We were unable to define controlled conditions at which 321 and 322 were totally resistant or susceptible. Both lines were selected originally from plants that were resistant to systemic infection under greenhouse conditions in late fall when inoculated at the two- to three-leaf stage (E. E. Sebesta, *personal communication*). However, we could not identify the critical environmental factor(s) necessary for the uniform expression of resistance. Such information is valuable because a greenhouse screening test for resistance between harvest and planting would permit selection of resistant plants and hasten the development of WSMV-resistant cultivars.

Except in heat- and inhibitor-treated plants, infectivity and virus particles were associated only with the local lesions and inoculated leaves of 092. This type of resistance is termed "localization" (9); it should not be called immunity [as was previously reported (8)] because virus replication and some translocation occurs within inoculated leaves.

The development of local lesions and resistance by 092 was specific for WSMV and was not observed in response to infection by BMV, BSMV, or FMV. This suggests that the resistance of 092 to WSMV is an active process that requires a specific recognition between the virus and the host. We cannot explain our inability to detect WSMV in double-infection experiments with the other viruses. This suggested interference with WSMV infection occurred in both Parker and 092. Attempts to detect WSMV in double infection experiments by infectivity and serologically specific electron microscopy all failed. The second virus was detected readily in all cases of double infection as was WSMV alone in singly infected plants. The results of simultaneous inoculation with both viruses and the lack of relationship among these diverse viruses should preclude cross protection.

The resistance of 092 was broken by high temperatures or metabolic inhibitors. At 35 C, the percentage of plants becoming systemically infected increased with the time of heat treatment. Resistance was most readily broken when heating was begun 2 days PI. Results suggest that the host mechanisms responsible for resistance are initiated about 2 days PI and continue to about 5 days PI (Fig. 1). The percentage of 092 plants that became systemically infected varied significantly ($P = 0.01$, two-way analysis of variance) with both the time of heat treatment and the PI time at which heating commenced. Heating apparently interferes with these mechanisms and resistance is not expressed. Analogous situations have been demonstrated for TMV in tobacco (11) and bean yellow mosaic virus in bean (14). In both of those pathogen-host systems, heating of inoculated plants overcame the normal hypersensitive localization of infection, and systemic infection occurred. Although temperatures frequently reach 35 C in wheat fields and experimental plots, resistance in 092, 321, and 322 is maintained. Since resistance was broken when the shoot or root portion of 092 was heated and the remaining portion was cooled, the cooler soil temperatures in the field probably are not responsible for maintaining resistance under high temperature conditions in the field. Rather, plants may remain resistant under these conditions because they are exposed to high temperatures for a relatively short period, and long, cool nights interrupt the heating cycle.

Although all of the plants treated with metabolic inhibitors did not become susceptible, the results indicate that resistance in 092 is an active phenomenon. Results with the metabolic inhibitors (actinomycin D and cycloheximide, respectively) suggest that transcription and translation are necessary for resistance to be expressed. Tannic acid broke the resistance of 092 more effectively than did the other inhibitors, but its specific effect on cell metabolism is unknown. A much higher concentration of tannic acid was used in comparison to other inhibitors (Table 4). Even at that concentration, tannic acid was less toxic to the plants than the other inhibitors. Higher concentrations of the other inhibitors were tested, but these often killed the plants. Another tannin, chlorogenic acid (6), also broke resistance in 092. Cheo and Lindner (3) report that tannic acid complexes with proteins and RNA. In their experiments, the amount of cellular RNA decreased after treatment with tannic acid, but returned to normal after 24 hr. In 092, tannic acid may complex with proteins and/or RNA synthesized

in response to, and necessary for expression of, resistance to WSMV.

The resistance mechanisms of 092, 321, and 322 to wheat streak mosaic virus need further characterization. They appear to operate by related but distinctly different mechanisms and stem from different origins. Independent use of these sources of resistance would avoid presenting a uniform host genotype to the pathogen and, thus, would reduce genetic vulnerability.

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