Subliminal Infection of Cotton by Tobacco Mosaic Virus

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ABSTRACT

Cotton (*Gossypium hirsutum*) seedlings formerly considered immune to tobacco mosaic virus (TMV) infection were subliminally infected as symptomless carriers. The virus content was approximately 1/200,000th of that produced in a systemic host, *Physalis floridana*. The inhibition in vitro of TMV by cotton extract was eliminated by high-speed centrifugation. Cotton apparently does not react in a local lesion manner because the total virus content does not vary proportionally to the concentration of the TMV inoculum, even over a difference of 1,000-fold. Furthermore, detached leaf culture under continuous semidarkness or infiltration of infected leaves with tannic acid greatly increases the TMV content in cotton. The stability of TMV infectivity from infected cotton reached a peak about 2 weeks after inoculation, then diminished rapidly. Phytopathology 60:41-46.

Tobacco mosaic virus (TMV) infects many species of plants. Until 1958, the known host range of the common strain of TMV included 322 species (10). The same report lists 173 species as insusceptible (immune) to TMV infection by mechanical inoculation. Most of these early investigations of the host range of TMV were based on symptoms and on virus recoveries from crude juice inoculations to assay plants. At least 40 species out of the 173, however, were later proven susceptible to TMV. Evidently, lack of symptoms and virus recovery from crude juice are not conclusive in establishing insusceptibility. Environmental factors such as light (3, 7, 8), temperature (13, 17) and the presence of naturally occurring virus inhibitors (2) in crude juice influence success or failure in transmitting TMV to, and recovery from, many plant species. Therefore, whether or not there is true genetic immunity among species reported as insusceptible is uncertain until critical investigations have been made.

It is generally believed that resistance of plants to TMV is represented by a local lesion reaction as compared with the recognized susceptible reaction of mottling and mosaic involving the systemic spread of virus. Many species are symptomless carriers under normal greenhouse conditions, and they may contain quite a wide range of recoverable virus when studied by means of a differential centrifugation procedure (*unpublished data*). Therefore, the severity of symptoms may not adequately represent their relative resistance to TMV. Attempts should be made, however, to correlate resistance with virus-reproducing potential; the net virus gain in host tissue under standardized conditions. Several species have been found in this laboratory to be highly resistant; their virus reproducing potentials are very low. This physiological resistance differs from the local lesion hypersensitive reaction.

This paper concerns some TMV infection studies on cotton, reported to be insusceptible (immune) to TMV by Holmes (9). The term "sublimal infection" (1) is used to describe a low range of virus recovery.

MATERIALS AND METHODS.—Plants of *Gossypium hirsutum* L. 'Acala 4-42' were grown in the greenhouse in 3-inch pots. Fully expanded cotyledons of seedlings were inoculated.

The common strain of TMV was purified from infected leaves of *Physalis floridana* Rydb., and kept in distilled water for use in inoculation. To assay TMV content in cotton, cotyledons were homogenized (Virtis "45" homogenizer) with three volumes of 0.01 M neutral phosphate buffer containing 0.01 M cysteine HCl. The homogenate was filtered through one layer of cheesecloth, clarified at 60 C for 10-20 min, and given two cycles of low- (3,200 g) and high-speed (54,000-80,801 g) centrifugation (Beckman Model L ultracentrifuge). The final TMV pellet was dispersed in a known amount of distilled water for biological assay on cucumber cotyledons.

Cucumis sativus L. 'Chicago Pickling' cucumber seedlings were grown in 3-inch pots in a greenhouse (diurnal temperature range from 29-21 C) fitted with a carbon filter for smog control. Ten to 15 days after seeding, the expanded cotyledons were in a proper stage for inoculation. The cucumber cotyledons were inoculated with dilute TMV preparations from cotton by the air-brush method (12). At least 14 replications were used for each sample. About 7 days after inoculation, the cucumber cotyledons were harvested and then stored 24 hr in a dark moist chamber to remove excess starch. Subsequently, the chlorophyll was removed by heating with 70% ethanol. Starch lesions were developed by placing the bleached cotyledons in an IKI-lactic acid mixture.

Estimation of virus content by means of the assay on cucumber cotyledons provides only approximate comparisons of the range of relative infectivity of these cotton samples. A standard curve of purified TMV was prepared by plotting the spectrophotometrically-determined concentration of TMV against the average number of starch lesions produced on cucumber cotyledons. The standard curve is based on data from different experiments at different times of the year under our greenhouse condition (Fig. 1). Final determination of virus content was based on the average number of starch lesions per sample and its proportion to the lesion count of a standard (0.1 μg/ml TMV) inoculation on the same day under the same conditions. After this correction, the lesion count can then be converted to the value of μg/ml of TMV according to the standard curve.

For vacuum infiltration of cotton seedlings, the plants

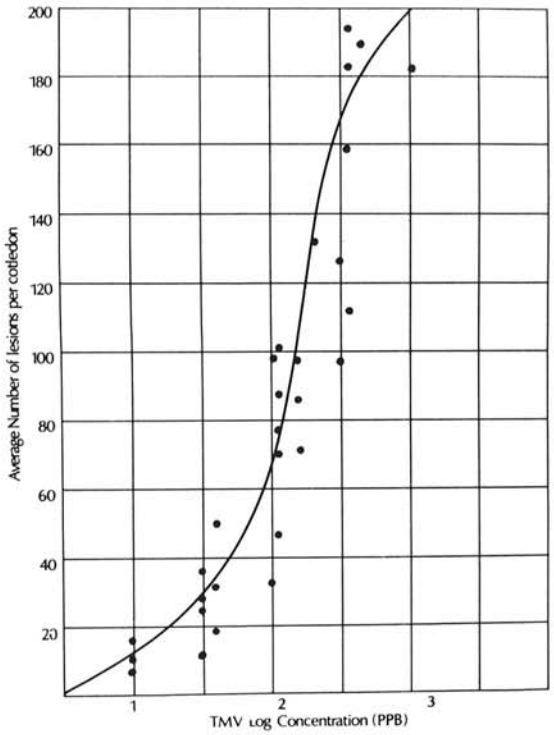

Fig. 1. Dilution curve of purified tobacco mosaic virus (TMV) in parts per billion (PPB) and the number of starch lesion per cotyledon using cucumber as assay plants.

were inverted over beakers in a desiccator with the foliage immersed in the solution. A vacuum of approximately 127 mm of mercury was applied for 3 min.

RESULTS.—*TMV recovery from inoculated cotton seedlings.*—No macroscopic symptoms developed on cotton seedlings inoculated with purified TMV. The inoculated plants were normal in color and size. If inoculated cotyledons were detached, stored in a moist, dark chamber overnight, cleared in alcohol, and stained with IKI-lactic acid mixture, there were no starch lesions.

Fifteen cotton seedlings were inoculated with each of three different concentrations of TMV: 0.5; 2.5; and 12.5 μg/ml, respectively. The 30 cotyledons for each group (about 15 g wt) were harvested 2 weeks after inoculation. The final virus preparation (10 ml) from each group was used for cucumber assay. An average of 3, 18, and 13 lesions/cucumber cotyledon were noted for cotton samples previously inoculated with 0.5, 2.5, and 12.5 μg/ml of TMV, respectively. Eighteen lesions/cucumber cotyledon indicates a TMV concentration in the inoculum at approximately 0.04 μg/ml under these assay conditions. Based on this figure, approximately 0.03 μg of TMV was obtained/g fresh wt of cotton cotyledons 2 weeks after inoculating with 2.5 μg/ml of TMV. This is, however, the minimal estimation, as the portion of TMV particles lost during preparation is not known, and is not included in calculations.

Seventy-two cotton seedlings were divided into two groups: (i) 36 seedlings, each inoculated with 0.1 μg/ml of TMV; and (ii) 36 seedlings, each with 100 μg/ml of TMV. Immediately after inoculation, the cotyledons were rinsed with deionized water. The cotyledon samples harvested from each group 2 weeks later yielded about 24 g of fresh tissue. Samples were frozen, homogenized, and after high-speed centrifugation (80, 801 *g*) suspended in 20 ml distilled water for infectivity assay on cucumber. An average of 31 lesions/ cucumber cotyledon was obtained for the cotton group previously inoculated with 0.1 μg/ml TMV; an average of 22 lesions for the group inoculated with 100 μg/ml TMV. The resulting TMV content was about the same between the two groups, although there was a difference of 1,000 times in original inoculum concentration. The TMV contents were again calculated to be about 0.03 μg/g fresh wt of cotton cotyledon.

Samples for TMV assay were taken at different time intervals following inoculation with TMV concentrations of 0.2 and 2.0 μg/ml. Samples of 20 cotyledons each were taken at 5, 9, 14, and 28 days after inoculation. Each sample at 10 ml for cucumber assay represents total virus infectivity in 20 cotton cotyledons collected at different time intervals after TMV inoculation (Table 1).

One hundred cotton seedlings were inoculated with a 0.25 μg/ml TMV solution. After inoculation, they were thoroughly washed with running tap water. Three hr after inoculation, cotyledons from 50 cotton seedlings were collected, weighed, and frozen. The remaining 50 cotton seedlings were likewise collected, weighed, and frozen 2 weeks after inoculation. Virus contents were collected via high-speed centrifugation. Samples were resuspended in 40 ml of distilled water for infectivity assay. The 3-hr sample produced an average of 0.6 lesion/cucumber cotyledon; the 2-week sample produced an average of 24 lesions/cotyledon, indicating a TMV content of approximately 0.025 μg/g fresh wt of cotton cotyledon tissue. This test eliminated the possibility that the recovery of infectivity from inoculated cotton was due to residual virus inoculum.

A test was conducted to determine if there was an increase of TMV in cotton cotyledons proportional to the inoculum concentration and frequency of inoculation. Three groups of 40 cotton plants each were inoculated with TMV as follows: group 1, 2, and 3 with 0.1 μg/ml; after 3 days, groups 2 and 3 with 1 μg/ml;

TABLE 1. Relative tobacco mosaic virus (TMV) content in 20 cotton cotyledons inoculated with 0.2 μg/ml or 2.0 μg/ml TMV, respectively, determined by cucumber starch lesion assay

Days after inoculation	Avg no. starch lesions/cucumber cotyledon	
	Inoculum at 0.2 μg/ml	Inoculum at 2.0 μg/ml
5	7	5
9	5	6
14	22	25
21	2	0.5
28	0	0

after 3 more days, group 3 with 10 μg/ml. The cotyledons from the three groups were collected for infectivity assay 2 weeks after the final inoculation. There was no relationship of TMV content, determined by infectivity assay, with inoculum concentration or inoculation frequency (Table 2). The differences in TMV content were due to the different time courses of TMV development. Differences were not comparable because samples were collected at different stages of TMV development.

Only limited synthesis of TMV occurred in the cotton cotyledons. The maximum TMV content, as determined by infectivity, occurred 14 days after inoculation; no infective virus was obtained 28 days after inoculation (Table 1). In separate tests, no infectivity was detected 35 days and 45 days after TMV inoculation. In comparison with *Physalis floridana* (a susceptible solanaceous host plant of TMV), an average net gain of 5-6 mg TMV/g fresh wt of leaf (Fig. 2-A) was obtained 3 weeks after inoculation, or about 200,000 times more virus than in the cotton cotyledons. Furthermore, the high level of virus content in inoculated *Physalis* leaf remained almost constant throughout the 100 days of the experiment. TMV biosynthesis in cucumber cotyledon, a starch lesion host, on the other hand, yielded 12 μg TMV/g fresh wt when inoculated with 0.1 μg/ml of TMV. When cucumber cotyledons were inoculated with 0.25 μg/ml of TMV, they yielded about 40 μg virus/fresh wt of cotyledon. Therefore, the total virus content in cucumber cotyledons depends mainly on the concentration of the inoculum; in this case a 2.5-fold increase in inoculum concentration results in a 3.3-fold increase in infective virus content 7 days after inoculation.

Studies on the inhibitory effect of cotton cotyledon extract and recovery of TMV.—Low recovery of TMV activity from infected cotton tissue could be associated with the existence of inhibitors in the cotton plant. Studies were made to assess to what extent cotton extract could affect the quantitative biological assay of TMV-infected cotton tissue.

Twenty-two g fresh wt of healthy and uninoculated cotton cotyledons were homogenized with 2 parts/wt of distilled water. This extract, after passage through one layer of cheesecloth, was designated as the stock strength from which further dilutions, 1:2, 1:4, 1:8, and 1:16 were made. Twenty-five μliters of a purified TMV solution at a concentration of 100 μg/ml was introduced into 20 ml of each cotton extract dilution and into 20 ml of distilled water serving as control. After standing at room temperature for 1 hr, the mixtures were inoculated separately to cucumber cotyledons for infectivity comparisons. Cucumber cotyledons were rinsed immediately after inoculation with deionized water. One week later, the average numbers of starch lesions developed by TMV mixed with 1:1, 1:2, 1:4, 1:8, 1:16 dilutions of cotton extract and with water control were determined to be 13, 16, 56, 108, 117, and 185, respectively. Therefore, cotton extract in stock strength when mixed with TMV and inoculated directly to cucumber cotyledons caused 93% inhibition on TMV infectivity.

Most plant extracts act as inhibitors of infection rather than as inhibitors of virus synthesis or inactivators of virus particles. Differential centrifugation should separate virus particles from the influence of inhibitors in plant extracts. Fifty μliters of a 100 μg/ml-TMV solution were introduced separately into 10 ml of crude cotton cotyledon extract (prepared by mortar and pestle without addition of water) and 10 ml of distilled water. These mixtures, after stirring, were incubated at room temperature overnight. A low-speed centrifugation at 5,000 rpm for 30 min (sediment discarded) was followed by high-speed centrifugation at 35,000 rpm for 90 min (80,801 *g*). The virus pellets from both tubes were resuspended in 10 ml of distilled water for infectivity assay on cucumber cotyledons. The average number of lesions produced by a virus pellet centrifuged from cotton extract was 448; the average number of lesions produced by virus pellet centrifuged from distilled water was 185. Possibly, more active virus were collected (aggregation of virus particles) from cotton extract than from the distilled water by means of high-speed centrifugation. The average lesion number of 448 produced on cucumber cotyledons indicated that there was no inactivating effect of cotton extract to the introduced TMV particles.

After mixing separate aliquots of TMV (50 μl of

TABLE 2. Results of successive inoculations on cotton cotyledons with increasing concentrations of tobacco mosaic virus (TMV) inocula and their relative TMV content

TMV inoculation	Total fresh wt of sample	Avg no. lesions produced from cotton preparation (final volume in 2 ml)			
		1-100 Dilution		1-10 Dilution	
		Cucumber	*Nicotiana glutinosa*	Cucumber	*Nicotiana glutinosa*
Once					
0.1 μg/ml	30.22 g	1.3	2.1	16	27
Twice					
0.1 μg/ml	26.43 g	4.5			
1.0 μg/ml			8	32	43
Thrice					
1.0 μg/ml	27.42 g	5			
0.1 μg/ml			2.6	27	22
10 μg/ml					

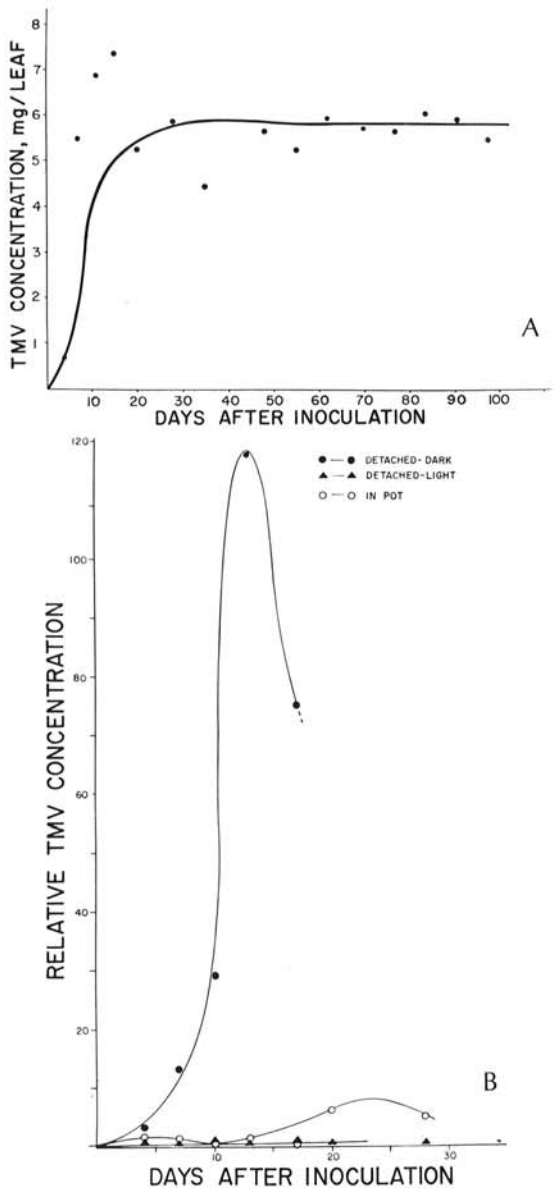

Fig. 2. The growth curve of tobacco mosaic virus (TMV) in **A)** inoculated *Physalis floridana* leaves; **B)** detached cotton cotyledons under different light conditions. The *Physalis* leaves were inoculated with 0.10 μg/ml of TMV, and TMV content was determined by purification and spectrophotometric reading at 260 mμ. Cotton cotyledons were inoculated with 0.25 μg/ml of TMV. Detached cotyledons were then divided and placed into two separate humidity boxes, one under continuous illumination and the other in semidarkness. A portion of the cotton seedlings remained in pots for comparison.

100 μg/ml-TMV solution) with various dilutions of cotton extract, a portion from each mixture was assayed directly on cucumber seedlings for infectivity comparison. The remaining portions were incubated at room temperature overnight, then subjected to differential centrifugation. Resulting pellets were resuspended separately in 10-ml aliquots of distilled water for infectivity comparison on cucumber seedlings. Infectivity increased with increasing dilution of the cotton extract, and the inhibitory effect was removed by centrifugation (Table 3).

Effect of tannic acid treatment on TMV content in cotton cotyledons.—Cheo & Lindner (4) reported that vacuum infiltration of 24-hr, TMV-inoculated cucumber cotyledons with tannic acid caused a systemic increase in TMV content. The effect of infiltration of cotton cotyledons with a 0.05% solution of tannic acid (reagent grade) on TMV development was studied. Tannic acid infiltration was made 24 hr after TMV inoculation (posttreatment), or 24 hr before inoculation (pretreatment). Comparable water infiltration treatments were also included. Samples for TMV content assay were collected 11 days after inoculation.

The results indicate an increase in TMV content in infected cotton cotyledons when they were either pretreated or posttreated with tannic acid. In the pretreatment experiment, the average number of lesions per leaf from control sample of cotton with no infiltration was 1.3; from cotton samples preinfiltrated with water, 0.7; and from cotton samples preinfiltrated with tannic acid, 76. In the posttreatment experiment, the average number of lesions was 26, 32, and 310, respectively, from control cotton samples without infiltration, with water infiltration, and with tannic acid infiltration.

The relative TMV content in detached infected cotton cotyledons under continuous light and semidarkness.—Greenhouse-grown cotton seedlings were inoculated with 0.25 μg/ml TMV and divided into three groups. The first group, grown intact in the greenhouse, served as control. Inoculated cotyledons of the second and third groups were detached and placed on moist filter paper inside Lucite boxes. One box was illuminated continuously during the experimental period at a distance of 76 cm under two rows of white fluorescent lights (cool-white, 40w); the other Lucite box was put under semidarkness continuously during the experimental period. Semidarkness was obtained by wrapping the Lucite box with two layers of Saran screen (80% shade) and placing it under the laboratory bench. The moist filter papers were changed weekly, and the remaining cotyledons were rinsed with running water during the changes. At intervals of 4, 6, 10, 13, 16, 20, and 28 days after TMV inoculation, 20 cotyledons from each group were collected for TMV assay. A large

TABLE 3. Infectivity comparison of tobacco mosaic virus (TMV) when inoculated in conjunction with the cotton extract and when cotton extract was removed by high-speed centrifugation (35,000 rpm or 80,801 *g* for 90 min)

TMV in dilutions of cotton extract	Avg no. starch lesions produced on cucumber cotyledons	
	Direct assay of the mixture	Extract removed by centrifugation
(1-1) + TMV	21	292
(1-2) + TMV	43	266
(1-4) + TMV	88	206
(1-8) + TMV	138	198
Buffer + TMV	415	36

increase in TMV content occurred in detached cotton cotyledons under the semidarkness condition (Fig. 2-B). Under semidarkness, the detached cotyledons turned yellow in about 10 days.

Under darkness, senescence proceeds rapidly in detached leaves, resulting in losses of chlorophyll, proteins, and nucleic acids. Continuous illumination of a detached leaf, on the other hand, retards senescence (5). It appears, then, that senescence favors TMV synthesis in cotton. Further studies are being undertaken to explore the relationship between senescence in continuous darkness and TMV synthesis in various host plants.

DISCUSSION.—A special type of TMV resistance is involved in the subliminal infection of plant species by TMV. Subliminal infection should be investigated, since it constitutes a defense against TMV. Many species reported as insusceptible to TMV may be subliminally infected, even though under normal conditions the infection cannot be detected by ordinary means of assay. Therefore, the word "insusceptible" used in describing the reaction of plant species to an artificial inoculation with TMV may be ambiguous. The word "insusceptible" is used because of convenience, since no critical method has been developed for differentiating an immune species from a highly resistant, subliminally infected species.

The cotton plant can be a subliminally infected host of TMV. Early classification of cotton as insusceptible to TMV was due mainly to its low virus content and to the high inhibitory action of cotton extract. Using larger amounts of infected tissue accompanied by a process of partial purification via differential centrifugation to eliminate inhibitory effects of cotton extract, TMV multiplication in cotton can be demonstrated. These experiments also indicate that cotton does not react in the manner of the local lesion host in virus expression. First, no local lesions are visible, nor are starch lesions present. Second, based on the infectivity assay of virus content, the cotton plant does not react quantitatively to the concentration of TMV inoculum, as is the case of other local lesion hosts. Another possibility may be that cotton cotyledons have a limited number of infection sites for localized TMV infection, and such localized infection is not detectable either visually or with iodine stain. On this possibility, the infection sites were saturated by an inoculum concentration of TMV at 0.1 μg/ml using the airbrush spray method for inoculation, and all of the inocula concentrations above 0.1 μg/ml concentration had no further effect on increasing total infection. However, the understanding of action at the infection site is not complete, and it would be difficult to visualize the infection site as a fixed feature of a particular plant species.

It has also been shown that the TMV content in cotton can be increased 10-100 times by vacuum infiltration with tannic acid or via detached culture in semidarkness (Fig. 2-B). Tannic acid infiltration probably results in a loss of proteins, nucleic acids, and chlorophyll, thus causing the tissue to follow the usual patterns of senescence. Tannic acid infiltration induces an early yellowing of the cotyledons. These observations suggest that rapid senescence favors TMV synthesis in cotton cotyledons, which are otherwise only subliminally infected. The surplus of soluble nitrogenous fractions or breakdown products from proteins and nucleic acids following senescence or infiltration with tannin could be used as raw materials for TMV synthesis. Such quantities may not be available in the normal anabolic state of metabolism. However, TMV synthesis is an anabolic state of metabolism. During rapid senescence in the dark, the rate of total synthesis slows down, and this should include synthesis of TMV. The assumption that the TMV synthesis increases during rapid senescence simply because of the presence of a greater amount of raw materials is not as likely as that some other factor is acting. Resistance to TMV infection in cotton tissue may be correlated with the metabolic state. During senescence, the specialized resistance against TMV synthesis breaks down, resulting in a net gain in TMV content, even though the total rate of synthesis during senescence is comparatively low.

The growth curve of TMV in cotton (Table 1) is different from the growth curve of TMV in *Physalis floridana* (Fig. 2-A). Recoverable infectious virus in inoculated cotton reaches its peak at about 2 weeks after inoculation and later diminishes rapidly. Steere (16) and Goodchild et al. (6), using different methods for TMV measurement, presented growth curves similar to *P. floridana* (Fig. 2-A) from tobacco plants. Kuhn & Bancroft (11) reported a growth curve similar to cotton for alfalfa mosaic virus (AMV) in tobacco, and concluded that as AMV was increasing at a rapid rate; some agent, physical, chemical, or both, was inactivating the virus, presumably by affecting ribonucleic acids. AMV is not as stable as TMV. Ross (14) reported that purified AMV is fairly stable at 4 C, but is rapidly inactivated at room temperatures, and is even much less stable in sap. In contrast, Silber & Burk (15) reported that the infectivity of TMV was not lost after being stored in plant juice for 50 years. Therefore, it is possible that a strong in vivo TMV inactivation system exists in cotton tissue.

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