

Enhancement of Tobacco Mosaic Virus Spread in Mechanically Inoculated Leaves by Pre-incubation at a Nonpermissive Temperature

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ABSTRACT

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In tobacco leaves mechanically inoculated with TMV and pre-incubated at temperatures nonpermissive for virus synthesis, the rate of TMV synthesis upon shift to a permissive temperature was markedly greater than that in control leaves. Pre-incubation of infected leaves at 10-12 C inhibited virus synthesis most effectively and subsequent transfer to 25 C produced the most rapid rate of virus multiplication. The optimum duration of pre-incubation was 10 days. In leaves that were pre-incubated at 12 C for 10

days after mechanical inoculation, infectivity increased exponentially from the time of the temperature shift until about 16 hours at 25 C and linearly from 16 hours until 3-4 days at 25 C when the maximum level of virus was attained. The maximum level of virus accumulated in control leaves only after 10 days. Pre-incubation at 12 C enhanced cell-to-cell spread of virus either while at the nonpermissive temperature or immediately after the temperature shift.

We recently described a system that synchronized tobacco mosaic virus (TMV) synthesis in intact tobacco leaves by controlling systemic infection of young tobacco leaves by differential temperature treatment (1, 2). Mechanically inoculated lower leaves were maintained at a temperature permissive for TMV synthesis while upper leaves of the same plants were maintained at a low temperature that was restrictive for replication, but permissive for systemic infection. When the upper leaves were shifted to a temperature permissive for replication, synthesis began in all infected cells simultaneously. The TMV growth curve for these young leaves that became systemically infected (systemically inoculated) at 5 C was almost identical to that in synchronously infected tobacco protoplasts (5) and the time-courses of sensitivity to different inhibitors of virus synthesis occurred sequentially (1), as would be expected in a synchronous infection.

The similarity of the TMV growth curve in leaves systemically inoculated at 5 C and shifted to a permissive temperature to that in synchronously inoculated protoplasts suggests that few events of virus replication occurred at 5 C. However, some early steps of TMV replication did occur at the nonpermissive temperatures. Examination of the time-courses of action of different inhibitors demonstrated that an actinomycin D-sensitive step occurred at the restrictive temperature, 5 C, while at 12 C, both an actinomycin D-sensitive step and a 2-thiouracil-sensitive step occurred, although synthesis of infectious viral RNA and virus particles was blocked at both temperatures (1). After the shift to the permissive temperature, TMV synthesis began earlier in leaves systemically inoculated at 12 C than in leaves systemically inoculated at 5 C in a manner suggesting that some specific steps of virus replication occurred at 12 C that did not occur at 5 C (2).

Mechanical inoculation results initially in infection of

relatively few of the cells of a leaf, usually much less than 0.1% (4). In this paper, we examine the replication of TMV in mechanically inoculated leaves incubated at restrictive low temperatures, and report that, in mechanically inoculated leaves maintained at 12 C, some events occur that greatly facilitate the spread of the virus from cell-to-cell either before or immediately after the leaves are shifted to a permissive temperature. This results in rapid rates of virus multiplication at the permissive temperature that approach synchrony.

MATERIALS AND METHODS

Fully expanded leaves of greenhouse-grown tobacco plants (*Nicotiana tabacum* L. 'Xanthi') were mechanically inoculated with 0.5 mg/ml of TMV, strain U1, in G-P buffer (0.05 M glycine, 0.03 M K₂HPO₄, pH 9.2, 100 µg/ml bentonite, and 1% Celite) using a cheesecloth pad. The leaves then were rinsed with distilled water and the plants were put into a plant growth chamber at constant temperatures as specified in the Results section of this paper. The permissive temperature for TMV replication was 25 C, in a plant growth chamber with a 14-hour photoperiod of 20,000 lx.

Infectivity of TMV was assayed by the half-leaf method on primary leaves of Pinto beans (*Phaseolus vulgaris* L.) with 6-12 replications of each inoculum in a randomized block design. Samples from mechanically inoculated leaves were removed with a cork borer and stored at -20 C. After all samples had been collected, the frozen samples were ground with mortars and pestles in G-P buffer and further diluted with G-P buffer to produce 10-150 lesions per half-leaf.

Tobacco mosaic virus was purified and TMV-RNA was extracted and assayed as described previously (2).

Time-courses of inhibition by actinomycin D (50 µg/ml), 2-thiouracil (1.0 mM), and cycloheximide (10 µg/ml) were determined as described previously (1). Ten

7-mm diameter disks that were removed from MI-12C leaves at intervals after the shift to 25 C were vacuum infiltrated with an inhibitor solution at 0 C, allowed to dry 10-15 minutes on paper towels, and then floated continuously on the inhibitor solution in petri dishes at 25 C. Control disks were treated with distilled water. At 72 hours after the MI-12C leaves were shifted to 25 C, all disks were frozen at -20 C, and assayed for infectivity.

RESULTS

We have previously demonstrated that low temperatures inhibit TMV synthesis (2). Less than 0.1% as much infectivity was present in mechanically inoculated tobacco leaves incubated at 12 C for 15 days as in similar leaves incubated 15 days at 25 C. Experiments were designed to examine whether, in mechanically inoculated tobacco leaves maintained at low temperatures that inhibit synthesis of complete virus, early events of TMV replication occur that affect the rate of synthesis when the leaves are shifted to a permissive temperature. Fully expanded tobacco leaves were mechanically inoculated with TMV and incubated at 5 C, 8 C, 10 C, or 15 C for 10 days, after which they were transferred to a 25 C growth chamber. Beginning at the time the plants were shifted to the permissive temperature, infectivity was monitored in each treatment and compared to that in control leaves that were mechanically inoculated and put directly into the 25 C environment. Rapid increases in infectivity occurred in leaves preincubated at 15 C, 12 C, or 10 C (Fig. 1). After lag periods of less than one day, infectivity rapidly increased and attained the maximum level after 4-5 days at 25 C as compared to about 10 days in the control leaves. Infectivity increased in leaves pre-incubated at 5 C identically to that in leaves that were mechanically inoculated and directly incubated at 25 C. The infectivity increase in leaves pre-incubated at 8 C was variable and intermediate between that in leaves pre-incubated at 10 C and control leaves not pre-incubated at a low temperature.

Infectivity of TMV in leaves pre-incubated at 15 C (MI-15C leaves) increased slightly earlier than in leaves pre-incubated at 12 C (MI-12C leaves) or 10 C (MI-10C leaves). However, 15 C did not inhibit virus synthesis as effectively as 10 C or 12 C. At the time of the temperature shift, after 10 days at 15 C, 0.4-0.5% of the maximum yield of infectivity produced after 4-5 days at 25 C was present in the MI-15C leaves. Smaller amounts of infectivity accumulated in MI-12C leaves during the 10-day incubation at 12 C; it ranged between 0 - 0.1% of the maximal level and was usually near 0.05%. The amount of infectivity in the total phenol-extracted RNA from MI-12C leaves at the time of the step-up to the permissive temperature was more than that of encapsulated RNA infectivity, but usually less than 0.2% of maximal infectivity levels.

The optimum temperatures to pre-incubate mechanically inoculated leaves in order to produce rapid rates of virus synthesis were in the range of 10-12 C. Pre-incubation at these temperatures prevented appreciable virus accumulation while at the nonpermissive temperature, and upon shift of the leaves to a permissive temperature, rapid virus multiplication resulted.

The time of incubation at 12 C required to produce rapid virus accumulation rates was examined by measuring the infectivity increases in tobacco leaves at 25 C that were mechanically inoculated with TMV and pre-incubated at 12 C for 10, 7, 4, 1, or 0 days before transfer to 25 C. Maximal rates of TMV multiplication occurred in leaves pre-incubated at 12 C for 10 days (Fig. 2). Shorter incubation periods resulted in progressively slower virus synthesis rates. When infected leaves were maintained at 12 C for periods longer than 10 days, the inhibition of virus synthesis by the low temperature became less effective. Although less than 0.1% of maximal infectivity yields was present in leaves maintained at 12 C for 10 days, up to 10% of maximum infectivity accumulated after 30 days of incubation at 12 C.

The rapid rate of TMV multiplication in MI-12C leaves approximates that in leaves systemically inoculated at 12

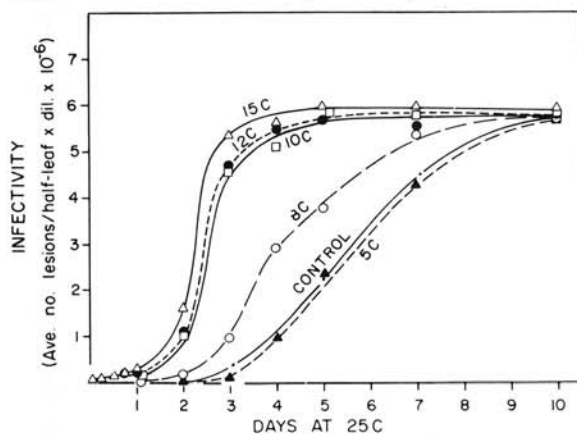


Fig. 1. Accumulation of tobacco mosaic virus (TMV) infectivity in leaves pre-incubated at restrictive temperatures. Tobacco leaves were mechanically inoculated with TMV and incubated at 15 C, 12 C, 10 C, 8 C, or 5 C for 10 days, after which they were incubated at 25 C. Control leaves were incubated at 25 C immediately after mechanical inoculation. Infectivity was determined at intervals after the shift to 25 C.

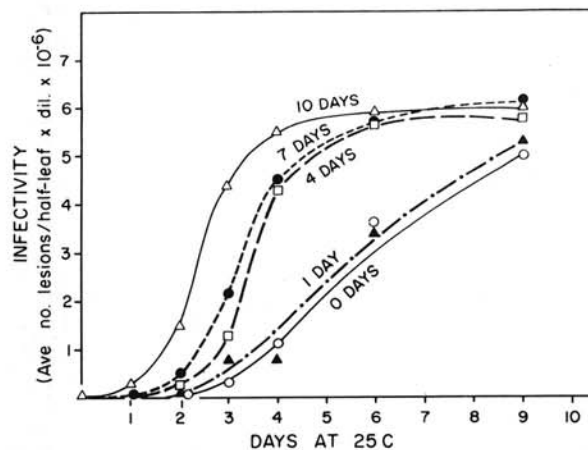


Fig. 2. Accumulation of tobacco mosaic virus (TMV) infectivity in tobacco leaves mechanically inoculated with TMV and maintained at 12 C for different periods before shifting the leaves to 25 C. Infectivity was determined at intervals after the shift to 25 C.

C by the differential temperature procedure in which TMV replicates synchronously (2). Figure 3 shows a comparison of the time-course of TMV infectivity in MI-12C leaves and in leaves systemically inoculated at 12 C. During the first 16 hours at 25 C, infectivity increased more rapidly in MI-12C leaves, but between 1 and 3 days, the rate of infectivity increase was usually greater in the leaves systemically inoculated at 12 C. Nucleoprotein growth curves of TMV in MI-12C leaves had the same time-course as the infectivity curve.

The initial infectivity increase in MI-12C leaves can be more clearly examined by a logarithmic plot (dashed line in Fig. 3). There was no lag period before the exponential increase in infectivity. Infectivity increased exponentially, beginning immediately upon the temperature shift to 25 C until it began increasing linearly at about 16 hours.

Measurable amounts of free-RNA infectivity occurred in MI-12C leaves during the exponential period. However, in relation to the maximal amount of virus that accumulates after 4-5 days at 25 C, the amounts of infectious free-RNA were minimal. The amounts of free-RNA in MI-12C leaves were substantially the same as that previously reported for leaves systemically inoculated at 12 C by differential temperature treatment (Fig. 7-B of reference 2).

We previously demonstrated that in a synchronous infection, the time-courses of resistance of virus synthesis to actinomycin D (AMD), 2-thiouracil (2TU), and cycloheximide (CX) occurred sequentially, during different time periods (1). Since the growth curve to TMV in MI-12C leaves was similar to that in leaves systemically inoculated at 12 C, in which TMV replicates synchronously, we examined the time-courses of inhibition by these chemicals upon TMV synthesis in MI-12C leaves (Fig. 4). In mechanically inoculated tissues incubated only at permissive temperatures, AMD blocks an early step of virus replication (3). In leaves systemically inoculated at 12 C or 3 C, AMD did not inhibit TMV synthesis (1). This step occurred at the nonpermissive temperature prior to the shift to 25 C. However, AMD inhibited TMV synthesis in MI-12C leaves about 40% when treatment began at the time of the shift from 12 C to 25 C. In leaves systemically inoculated at 3 C, 2TU inhibited synthesis of TMV about 95% when added within the first 3 hours at 25 C. The inhibition then rapidly declined when treatment began at later times, until no inhibition when treatment began at 12 hours or later (1). In leaves systemically inoculated at 12 C, the infection was totally resistant to 2TU when shifted to the permissive temperature. In MI-12C leaves, 2TU initially inhibited TMV synthesis about 70%. This was substantially less than in leaves systemically inoculated at 3 C and more than in leaves systemically inoculated at 12 C. The development of resistance of TMV synthesis in MI-12C leaves to CX was gradual and parallel to that of 2TU. The infection in MI-12C leaves did not become resistant to the inhibitors sequentially as occurred in synchronously infected leaves (1). These data demonstrate that, although the time-course of TMV infectivity was similar to that in synchronous systems, TMV did not replicate synchronously in MI-12C leaves.

DISCUSSION

In tobacco leaves that were pre-incubated for 10 days at

12 C after mechanical inoculation, TMV multiplied much more rapidly when transferred to 25 C than in leaves that were mechanically inoculated and put directly into 25 C. The rate of TMV synthesis in MI-12C leaves was similar to that of synchronously multiplying TMV in leaves that were systemically inoculated at 5 C (2) or in tobacco protoplasts (5; for a linear plot, see Fig. 8 of reference 2).

The shape of the TMV growth curve reflects both the rate of accumulation of virus in individual cells and the cell-to-cell movement of virus and concomitant infection. The upper limit of the number of leaf cells that can be initially infected by mechanical inoculation is about 0.1% (4). If early events of virus replication prior to the production of infectious virus occurred in MI-12C leaves at 12 C in only initially infected cells, the resulting rise in infectivity should occur slightly earlier than in the control, but the shapes of the two curves should be the same. However, not only did the infectivity in MI-12C leaves begin increasing earlier than in control leaves, but also the slope of the curve for MI-12C leaves was much greater. This suggests that the number of cells in MI-12C leaves infected with TMV at the time of the temperature

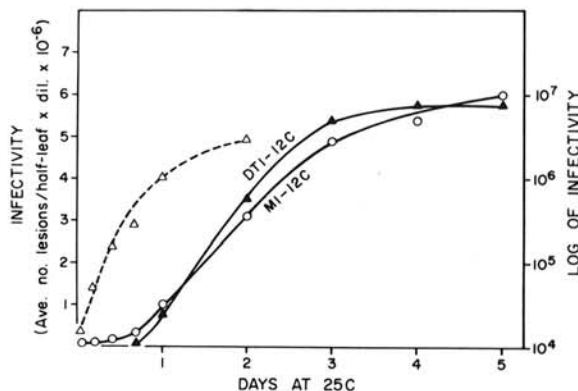


Fig. 3. Comparison of the rate of tobacco mosaic virus infectivity increase in mechanically inoculated leaves pre-incubated 10 days at 12 C (MI-12C) to that in leaves systemically inoculated at 12 C (DTI-12C). Dashed line is the logarithmic plot of the initial infectivity increase in MI-12C leaves.

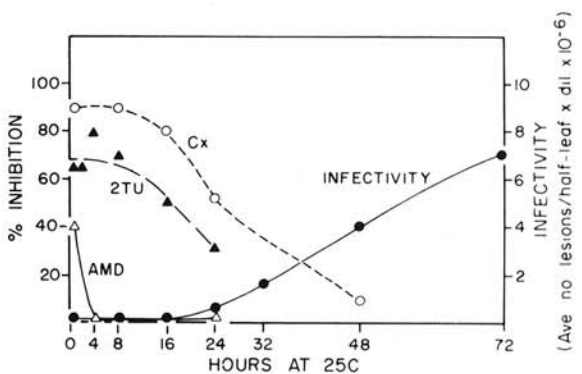


Fig. 4. Time-courses of inhibition of tobacco mosaic virus synthesis in mechanically inoculated leaves pre-incubated 10 days at 12 C. Treatments began at intervals after the shift to 25 C and the disks were treated continuously with one of the inhibitors until 72 hours at 25 C when the infectivity of tissue extracts after each treatment was compared to disks treated with distilled water.

increase or immediately afterwards was much greater than the number of cells initially infected by the mechanical inoculation, that there was cell-to-cell movement of virus and concomitant infection while the leaves were at 12 C, or that events occurred enabling rapid cell-to-cell spread immediately after the temperature shift.

Although little complete virus or viral RNA was synthesized at 12 C or lower temperatures during a period of 10 days or less (2), some events occurred in MI-12C leaves at 12 C that facilitated cell-to-cell movement of TMV. Specific early steps of TMV synthesis were previously shown to occur at the restrictive temperatures in leaves systemically inoculated by differential temperature treatment (1). In systemically inoculated leaves, an actinomycin D-sensitive step occurred at 3 C, and, at 12 C, both the actinomycin D- and the 2-thiouracil-sensitive steps occurred.

In spite of the rapid increase in infectivity, TMV does not appear to replicate synchronously in MI-12C leaves. The time-courses of resistance to different inhibitors did not occur as discrete, nonoverlapping steps as occurs in leaves systemically inoculated by the differential temperature treatment (1). Some infectivity accumulated in MI-12C leaves prior to the temperature shift (up to 0.2% of maximum infectivity levels). There was no lag period before the exponential infectivity increase as occurs in protoplasts (5) and in leaves systemically inoculated at 5 C (2); and, during the exponential infectivity increase, the amount of infectivity was substantially greater than in leaves systemically inoculated by the differential temperature procedure or in inoculated tobacco protoplasts.

The difference between the degree of synchrony of TMV replication in MI-12C leaves as compared to leaves systemically inoculated at 12 C is probably due to the length of time required to infect the cells and to the

duration of incubation of the cells at 12 C after infection. Twelve degrees does not totally inhibit TMV synthesis. Virus synthesis occurs at this temperature upon prolonged incubation (2). In leaves systemically inoculated at 12 C, most cells became infected between the fifth and eighth day at 12 C and thus were incubated at the low temperature less than five days after infection (Dawson and Schlegel, *unpublished*). However, cells in MI-12C leaves became infected over the whole 10-day incubation at 12 C. Many of the cells were incubated for most of the 10 days at 12 C after they became infected whereas some cells only became infected on the tenth day.

Although TMV does not appear to replicate synchronously in MI-12C leaves, the extremely rapid rate of multiplication and the similarity of the virus growth curve to those for synchronous systems make this system suitable for many types of replication experiments. The procedure provides unlimited amounts of tissue and is simple and easy to perform.

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