Behaviour of a Temperature Sensitive Strain of Tobacco Mosaic Virus in Tomato Leaves and Protoplasts

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SUMMARY

A temperature-sensitive (ts) strain, LsI, was isolated from a culture of L, a tomato strain of tobacco mosaic virus (TMV). LsI caused smaller necrotic local lesions than L in leaves of hypersensitive tobacco plants. A temperature shift treatment (22 °C for 3 days, 32 °C for 2 days and 22 °C for 1 day) allowed L to produce necrotic lesions surrounded by a collapsed area, whereas LsI caused necrotic lesions without a collapsed surrounding area, suggesting that LsI did not spread outside the lesions at 32 °C. In tomato leaf discs at 22 °C, infectivity of LsI increased in parallel with that of L, but at 32 °C the increase in infectivity of LsI was negligible and contrasted with the large increase of infectivity of L. Few mesophyll cells from LsI-inoculated discs incubated at 32 °C were stained by fluorescent antibody but many of those incubated at 22 °C were stained. In protoplasts at 32 °C, however, LsI and L infected and multiplied similarly. When leaves inoculated with LsI were kept at 20 to 25 °C for 24 h, and discs were then prepared and cultured for 12 h at 32 or 22 °C, the proportion of mesophyll cells containing virus antigen did not increase at 32 °C but infectivity increased greatly. This suggests that LsI multiplied readily at 32 °C in already infected cells. At 22 °C the proportion of infected cells and the infectivity of leaf extracts increased rapidly. The growth curve of LsI in leaf discs at 32 °C resembled the so-called ‘one-step growth curve’ of LsI or L in protoplasts, and not the growth curve of L in discs, in which the virus could spread from cell to cell. These results suggest that LsI is a ts strain that multiplies normally at the non-permissive temperature but has a malfunction in cell-to-cell movement.

INTRODUCTION

It was reported that a severe tomato strain (strain L) of tobacco mosaic virus (TMV) can be differentiated from its attenuated isolate (strain L11A) by the type of necrotic local lesion produced in inoculated Xanthi-NN tobacco leaves after incubation in defined conditions that include a specific temperature shift (Nishiguchi & Oshima, 1977). In this case inoculated plants were first kept at 22 °C for 3 days, transferred to 32 °C for 2 days and then maintained at 22 °C for 1 day. This temperature shift treatment differs only slightly from those described by Jockusch (1968) and Peters & Murphy (1975) and can also be used to select temperature-sensitive (ts) mutants in cultures of wild type virus. Using this procedure we found an aberrant lesion, which morphologically resembled that of a non-coat protein mutant (Jockusch, 1968), among typical lesions in a leaf inoculated with strain L. Virus
from this lesion was designated Ls1. This paper describes the symptoms caused by Ls1 in several host plants and the behaviour of Ls1 in mesophyll protoplasts and leaf discs of tomato.

METHODS

Plants and viruses. Plants used for comparison of symptoms were: *Nicotiana tabacum* cv. Xanthi-nc, cv. Xanthi-NN, cv. Bright Yellow and cv. Samsun, *N. sylvestris*, *N. glutinosa*, *Lycopersicon esculentum* cv. Fukuju no. 2 and *Phaseolus vulgaris* cv. Gintebo. They were grown in a greenhouse where the temperature ranged from 22 to 30 °C. Plants were inoculated when 2 months old for Xanthi-nc, Xanthi-NN and *N. glutinosa*, 7 weeks old for Bright Yellow, Samsun and *N. sylvestris*, 3 weeks old for tomato and 2 weeks old for *P. vulgaris*.

Two strains of TMV, L and Ls1, were used. L is the common tomato strain of TMV (Oshima et al. 1964, 1971) and Ls1 was isolated from an aberrant lesion among lesions produced on Xanthi-NN by L after the temperature shift treatment. Ls1 was passed through a series of six single lesions in Xanthi-nc and was propagated in Samsun. Viruses were purified as previously described (Moytoyoshi & Oshima, 1975) and the purified virus suspensions (1 mg/ml in 0.01 M-sodium phosphate buffer, pH 7.0) were stored at −18 °C until use. Sap of infected leaves of Samsun tobacco diluted tenfold with 0.01 M-sodium phosphate buffer, pH 7.0, was used to inoculate the range of plants listed above. Purified virus suspension at appropriate concentrations was used to inoculate leaf discs and protoplasts.

Preparation, inoculation and culture of discs. Fully expanded leaves at the 7 to 8 leaf stage of tomato and the 5 to 6 leaf stage of Samsun tobacco were used. Before inoculation the leaves were washed with tap water and blotted dry with filter paper. The leaves were dusted with 600-mesh Carborundum and inoculated, using a glass spatula, with virus suspension at 500 μg/ml in 0.01 M-sodium phosphate buffer, pH 7.0. The leaves were washed with tap water about 3 min after inoculation and discs, 14 mm in diam., were cut out with the aid of a cork borer. Ten discs in each treatment were floated on incubation medium in a Petri dish, 6 cm in diam. The composition of this medium and the conditions of culture were the same as previously reported (Motoyoshi & Oshima, 1975).

Preparation, inoculation and culture of protoplasts. Preparation, inoculation and culture of protoplasts were as previously reported (Motoyoshi & Oshima, 1975, 1976). The concentrations of virus and of poly-L-ornithine were 1 μg/ml.

Detection of virus antigen in protoplasts. Percentages of protoplasts infected were determined by fluorescent antibody staining as described by Motoyoshi & Oshima (1975). The fluorescent antibody from rabbit antiserum to strain L (Motoyoshi & Oshima, 1975) was used to stain antigens of both L and Ls1.

Virus assay. The concentration of virus in discs and protoplasts was assayed by local lesion tests on Xanthi-nc tobacco. Discs were homogenized with 0.01 M-sodium phosphate buffer, pH 7.0. The homogenate was appropriately diluted with the same buffer and inoculated on ten half-leaves of two Xanthi-nc plants. The standard virus suspension, strain L at 0.1 μg/ml, was inoculated on the opposite half-leaves. The inoculum from protoplasts was prepared by the methods of Motoyoshi & Oshima (1975).
RESULTS

Symptoms on test plants

Both Ls1 and L caused only local necrotic lesions in the inoculated leaves of Xanthi-nc, Xanthi-NN and Bright Yellow tobacco, *N. glutinosa* and *N. sylvestris*. Local lesions of Ls1 were, however, much smaller than those of L (Fig. 1). Ls1 induced yellow mottle in systemically infected Samsun tobacco leaves, whereas L caused a mosaic in them. Both strains produced a systemic mosaic in tomato cv. Fukuju no. 2 and neither induced symptoms in *P. vulgaris*.

When half-leaves of Xanthi-nc tobacco were inoculated with Ls1 and the opposite halves with L and the plants were given the temperature shift treatment described by Nishiguchi & Oshima (1977), L caused lesions having small necrotic centres with a surrounding collapsed area, whereas lesions of Ls1 lacked the collapsed area (Fig. 2). A similar morphological difference between the lesions of the two strains was also observed in Xanthi-NN tobacco, *N. glutinosa* and *N. sylvestris*.

Multiplication of virus in discs and protoplasts

Leaf discs of tomato inoculated with either Ls1 or L were incubated at either 22 or 32 °C and after 3 days the discs were harvested and the infectivities of their homogenates determined. Infectivity was high with either strain at 22 °C or with L at 32 °C, but very low with Ls1 at 32 °C (Table 1). Similar results were obtained using leaf discs of Samsun tobacco. Thus Ls1 is obviously temperature-sensitive in leaf discs.
Table 1. Growth of strains LsI and L in leaf discs of tomato and tobacco

<table>
<thead>
<tr>
<th>Plant species</th>
<th>TMV strain</th>
<th>Temperature (°C)</th>
<th>Assay dilution</th>
<th>Total no. of lesions</th>
<th>Relative no. of lesions†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato</td>
<td>LsI</td>
<td>32</td>
<td>$10^{-4}$</td>
<td>10</td>
<td>$7.2 \times 10^2$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22</td>
<td>$10^{-5}$</td>
<td>961</td>
<td>$1.6 \times 10^3$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control‡</td>
<td>$10^{-2}$</td>
<td>342</td>
<td>$4.5 \times 10^2$</td>
</tr>
<tr>
<td>Tomato</td>
<td>L</td>
<td>32</td>
<td>$10^{-6}$</td>
<td>271</td>
<td>$2.2 \times 10^3$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22</td>
<td>$10^{-5}$</td>
<td>1217</td>
<td>$1.2 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control‡</td>
<td>$10^{-2}$</td>
<td>419</td>
<td>$8.7 \times 10^2$</td>
</tr>
<tr>
<td>Tobacco</td>
<td>LsI</td>
<td>32</td>
<td>$10^{-2}$</td>
<td>386</td>
<td>$8.6 \times 10^2$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22</td>
<td>$10^{-5}$</td>
<td>937</td>
<td>$8.8 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control‡</td>
<td>$10^{-2}$</td>
<td>667</td>
<td>$6.7 \times 10^2$</td>
</tr>
<tr>
<td>Tobacco</td>
<td>L</td>
<td>32</td>
<td>$10^{-6}$</td>
<td>77</td>
<td>$1.2 \times 10^3$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22</td>
<td>$10^{-5}$</td>
<td>1084</td>
<td>$1.1 \times 10^6$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control‡</td>
<td>$10^{-2}$</td>
<td>316</td>
<td>$9.5 \times 10^2$</td>
</tr>
</tbody>
</table>

* Leaf discs (14 mm in diam.) were prepared from leaves inoculated with TMV at 500 µg/ml in 0.01 M-phosphate buffer, pH 7.0, and incubated for 3 days in culture medium under continuous illumination.

† Relative number of lesions defined as:

\[
\text{Relative number of lesions} = \frac{\text{Total number of lesions} \times \text{dilution factor}}{\text{Total number of lesions with standard suspension of L at 0.1 µg/ml}}
\]

‡ Samples taken before incubation.

Fig. 3. Growth patterns of LsI and L in tomato protoplasts. ••–••, LsI at 32 °C; ○–○, LsI, at 22 °C; ▲–▲, L at 32 °C; △–△, L at 22 °C.
Table 2. Effect of temperature on virus multiplication and movement from cell to cell in tomato leaf discs

<table>
<thead>
<tr>
<th>TMV strain</th>
<th>Temperature for incubation of discs (°C)</th>
<th>% infected mesophyll cells*</th>
<th>Assay dilution</th>
<th>Total no. of lesions</th>
<th>Relative no. of lesions†</th>
</tr>
</thead>
<tbody>
<tr>
<td>LsI</td>
<td>32</td>
<td>0.8</td>
<td>10^-3</td>
<td>1482</td>
<td>737</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>11.2</td>
<td>10^-3</td>
<td>1044</td>
<td>672</td>
</tr>
<tr>
<td></td>
<td>Control†</td>
<td>1.4</td>
<td>10^-3</td>
<td>34</td>
<td>29</td>
</tr>
<tr>
<td>L</td>
<td>32</td>
<td>9.4</td>
<td>10^-4</td>
<td>1293</td>
<td>7457</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>11.8</td>
<td>10^-4</td>
<td>291</td>
<td>1471</td>
</tr>
<tr>
<td></td>
<td>Control†</td>
<td>1.6</td>
<td>10^-3</td>
<td>26</td>
<td>23</td>
</tr>
</tbody>
</table>

* The design of this experiment and the procedure for estimating % infected mesophyll cells are described in the text.
† Defined as:

\[
\frac{\text{Total number of lesions produced by homogenate of discs} \times \text{dilution factor}}{\text{Total number of lesions produced by standard suspension of L at 0.1 μg/ml}}
\]

‡ Samples taken before incubation.

In another experiment, virus-inoculated discs were incubated for 2 days at 22 or 32 °C. Half of the discs were used for infectivity assay and mesophyll protoplasts were isolated from the other half and stained with fluorescent antibody. None of the protoplasts from discs inoculated with LsI and incubated at 32 °C were stained, suggesting that no virus accumulated in leaf mesophyll cells. By contrast, 33% of the protoplasts from LsI-inoculated discs incubated at 22 °C were stained as compared with 65 to 66% of those from discs inoculated with L and kept at either temperature. Infectivity assays gave results very like those in Table 1. These findings suggest that the high temperature greatly limited multiplication of LsI in mesophyll cells of leaf discs.

Tomato mesophyll protoplasts were inoculated with LsI or L and incubated at 22 or 32 °C for periods up to 2 days. As shown in Fig. 3, the infectivities and percentages of protoplasts infected did not essentially differ between LsI and L at either temperature and were higher at 32 °C than at 22 °C. It was apparent that incubation at 32 °C, which limited growth of LsI in leaf discs, favoured infection and growth of LsI in protoplasts. Virus recovered from homogenates of LsI-infected protoplasts after incubation at 32 °C for 2 days did not differ in lesion morphology from standard LsI in Xanthi-nc tobacco given the temperature shift treatment, suggesting that the progeny virus in protoplasts incubated at 32 °C was LsI.

From the result of the above experiment, it might be assumed that the temperature-sensitivity of LsI involved some very early event in protoplast infection that occurred before inoculated protoplasts reached 32 °C. In one experiment, however, no significant difference in infectivities and percentages of protoplasts infected was observed between LsI- and L-infected protoplasts which had been inoculated and washed at 32 °C, followed by being cultured at either 32 or 22 °C for 2 days. This result suggests that the temperature-sensitivity of LsI is not related to any early event in protoplast infection. Thus it was concluded that the temperature-sensitivity of LsI was not expressed in protoplasts.
Fig. 4. Growth patterns of LsI and L in leaf discs and protoplasts at 32 °C. Intact leaves on tomato plants were inoculated either with LsI or with L and kept at 20 to 25 °C in a greenhouse for 24 h. Discs were then prepared from the leaves, protoplasts were isolated from some of the discs, and the remaining discs and the protoplasts were incubated simultaneously at 32 °C. •——•, LsI in discs; ○——○, LsI in protoplasts; △——△, L in discs; △——△, L in protoplasts.

Fig. 5. Growth patterns of LsI and L in tomato leaf discs at 22 and 32 °C. Discs were prepared as described in Fig. 4. •——•, LsI at 22 °C; •——•, LsI at 32 °C; △——△, L at 22 °C.

Virus movement from cell to cell

Tomato leaves attached to plants were inoculated with LsI or L in the morning and the plants were kept in a greenhouse at 20 to 25 °C under natural light for 24 h to allow the infection to establish. Leaf discs were cut from these infected leaves. The discs infected with LsI or L were divided into three batches, each of which consisted of 40 discs. One of the three batches was immediately harvested, and the other two were incubated at 22 and 32 °C, respectively, in the incubation medium and after 12 h they were harvested. Ten of 40 discs in each batch were homogenized immediately after harvest and used for infectivity assay. The rest of the discs were used for isolating mesophyll protoplasts. The protoplasts were cultured at 25 °C for a further 2 days to allow virus to multiply and sufficient antigen to accumulate to be detected by fluorescent antibody staining. Percentages of mesophyll cells which were infected at the time of harvest of the discs were estimated from percentages of fluorescing protoplasts thus obtained. The percentages of mesophyll cells infected with LsI and with L were 1.4 and 1.6, respectively, at the start of incubation of discs. During the 12 h incubation period, the percentage of LsI-infected mesophyll cells increased eightfold at 22 °C but did not increase at 32 °C (Table 2). With L, percentages of infected mesophyll cells increased about six- to sevenfold at both 22 and 32 °C.

Although the percentage of mesophyll cells infected with LsI did not increase at 32 °C, infectivity increased by 25 times (Table 2). This increase factor is lower than that of 324 times obtained with L at 32 °C, but as the proportion of mesophyll cells infected with LsI was only about one-tenth of those with L, the virus yield of each strain per infected cell was rather similar. The yield per infected cell of LsI and L at 22 °C was less than at 32 °C. These results suggest that LsI multiplies normally at 32 °C but does not move from cell to cell.
Comparison of growth curves of virus in leaf discs and protoplasts

If Ls1 does not move from cell to cell at 32 °C (Table 2), its pattern of growth in discs at this temperature should be similar to that in protoplasts, because the possibility of secondary infection of protoplasts by progeny virus is negligible (Takebe, 1975).

Tomato leaves attached to plants were inoculated and the plants were kept for 24 h in a greenhouse at 20 to 25 °C under natural light. Discs were then prepared from the leaves. Some of the discs (ten discs in each treatment) were incubated at 32 or 22 °C and protoplasts were isolated from the other discs (30 discs in each treatment) and incubated. At various times, discs and protoplasts were harvested and infectivities were assayed. The pattern of Ls1 growth in discs at 32 °C resembled those of Ls1 and L in protoplasts (Fig. 4). The pattern of Ls1 growth in discs at 22 °C was similar to that of L and clearly different from that of Ls1 at 32 °C (Fig. 5). These findings further support the conclusions already mentioned, with respect to the behaviour of Ls1 at 32 °C.

DISCUSSION

Strain L, a common tomato strain of TMV, produces mosaic on tomato plants, necrotic local lesions on N. tabacum cv. Bright Yellow and N. sylvestris, and no symptom on P. vulgaris cv. Gintebo. The reactions of these test plants to Ls1 are similar to those with L, except that the lesions are smaller. It is possible that Lsi is a mutant produced from L by a spontaneous mutation.

Jockusch (1968) described two types of ts mutant of TMV, one of which had coat malfunction and the other replicative defects. The first type causes local lesions of normal size without producing infectious particles, and the second type, e.g. Ni 2519, causes small local lesions in leaves of Xanthi-nc tobacco at 23 °C, but these stop spreading when the temperature is raised to 32 °C. The behaviour of Ls1 on Xanthi-nc tobacco is similar to that of the second ts type described by Jockusch (1968). In spite of this apparent resemblance, Ls1 can replicate at 32 °C as rapidly as a wild type strain in isolated protoplasts, and in mesophyll cells of leaf discs of tomato if the virus has already entered these cells, whereas Ni 2519 produced coat protein but not intact virus particles at the non-permissive temperature (Bosch & Jockusch, 1972; Oishi et al. 1975). As regards its replication, Ls1 is not temperature sensitive.

One of the ts mutants, II-27, selected by Peters & Murphy (1975) also resembles Ls1 in its behaviour in Xanthi-nc tobacco. II-27 caused small lesions at 23 °C and, when exposed to the temperature shift procedure, formed necrotic lesions without a halo. From this lesion morphology Peters & Murphy assumed that the mutant had a defect in its ability to move from cell to cell. Our results show more definitely that Ls1 lacks the ability to move from cell to cell at the high temperature.

In parenchymatous tissue virus particles probably move from cell to cell through plasmodesmata (Gibbs, 1976; Weintraub et al. 1976), but it is also likely that non-encapsidated TMV-RNA can move through them (Siegel et al. 1962). Ls1 may therefore have a ts ability to move through plasmodesmata, either in the form of particles or RNA. Two of the possible causes of the inability of Ls1 to move from cell to cell are the following: (i) the host plants may be induced to produce material that blocks virus movement through plasmodesmata. Callose production is known to be induced in tissue that responds hypersensitively to virus infection, with the result that virus movement through plasmodesmata is blocked (Allison & Shalla, 1974). No tests, however, have yet been made on whether callose production is induced by Ls1 in leaf discs of tomato and Samsun tobacco at the
high temperature. (2) The blocking of virus movement from cell to cell may be caused by a functional defect in a virus-coded protein. There is no direct evidence for such a protein but it is not improbable that genetic information for a protein controlling cell to cell movement is carried by the virus genome. From this point of view it is intriguing that, besides coat protein and the possible replicase, TMV produces at least one kind of protein with no known function (Zaitlin & Hariharasubramanian, 1972; Sakai & Takebe, 1974).

Some ts mutants of TMV and cowpea chlorotic mottle virus (CCMV) have been studied using protoplast systems (Oishi et al. 1975; Dawson et al. 1975). Oishi et al. (1975) reported that tobacco protoplasts could be infected with Ni 118 or Ni 2519, two ts mutants isolated by Jockusch (1968). Each mutant produced progeny virus at 24 but not at 33 °C. When protoplasts were inoculated with Ni 2519 and incubated at 33 °C, large inclusion bodies unusual in shape were produced and these could be stained with fluorescent antibody (Oishi et al. 1975) although no infectivity was detected in protoplast extracts (I. Takebe, personal communication). A ts mutant of cowpea chlorotic mottle virus (CCMV) could infect tobacco protoplasts and multiply at 25 °C, but at 35 °C no encapsidation occurred, possibly because of a functional defect in its coat protein; the mutant RNA however could be encapsidated with the coat of wild type CCMV at 35 °C when the protoplasts were infected simultaneously with ts and wild type virus (Dawson et al. 1975). In contrast to these ts mutants, the high temperature did not cause any detectable effect differentiating LsI from L in the protoplast system. The present study thus suggests that protoplast systems are especially useful for classifying and characterizing different types of ts mutant of plant viruses.

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REFERENCES


Behaviour of a ts strain of TMV


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