Improved Method for the Isolation and Propagation of Defective Tobacco Mosaic Virus Mutants

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ABSTRACT

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A method is described for rapid isolation and screening of mutant virus strains which yields a high frequency of defective mutants. This method also can be employed to rescue defective mutants from infected plants contaminated by competing revertant wild-type virus strains. To successfully transfer and recover defective mutant isolates, it is necessary to use, as inoculum, lesions at the first sign of their appearance on the leaf surface. Transferable infectivity is rapidly lost with increasing lesion age.

If purified tobacco mosaic virus (TMV) is inactivated by HNO_2 to 0.1% survival (8), inoculated to a local lesion host plant, recovered by excising individual lesions, and these are used to inoculate individual systemically responsive host (eg, *Nicotiana tabacum* 'Samsun') plants, 30% of the latter fail to become infected (the "miss" phenomenon [9]). However, if similarly treated TMV is inoculated at limit dilution to Samsun tobacco, approximately 50% of the plants initially appear to be uninfected, but eventually some of those become infected. By using this screening method, a number of defective TMV mutants with nonfunctional coat proteins were isolated and the lesions they induced contained either no, or very low levels of, transferable infective agent (Table 6-2, in ref. 11) as measured by established techniques. The infectious entity of these mutants is a naked, unprotected, RNA molecule, and the coat protein is found in an aberrant form in the cytoplasmic fraction of infected leaf cells (1,3,5,12). The study of some of these mutants (PM2, PM5, and PM6) has contributed to further understanding of TMV virion assembly (1,5,10,13).

In this paper, we describe an improved method for the isolation of defective mutants that grow more slowly than wild-type TMV and discuss possible applications of this method in plant virus studies.

MATERIALS AND METHODS

Necrotic local lesions were induced on tobacco (*N. tabacum* 'Xanthi-nc') plants by brushing the leaves with a homogenate prepared by grinding a 2- to 3-cm-square piece of leaf tissue infected with a defective mutant of TMV, in ice-cold TP buffer containing 0.05 M Tris, 0.05 M Na_2HPO_4 and adjusted to pH 8.6 with HCl (7). Celite was added at 50 mg/ml as an abrasive. The resulting lesions were excised as soon as they appeared (about 60 hr under our greenhouse conditions) and were individually ground between two sterile glass paddles in two or three drops of ice-cold TP buffer. The homogenate was rubbed immediately onto the leaves of Xanthi or Samsun tobacco plants. Necrotic lesions or symptoms of systemic infection, respectively, will appear on these newly rubbed leaves indicating successful transfer of the infective agent. Transfer of infective agent from lesions induced by defective mutants was highly efficient.

This method has enabled us to devise a new screening technique for defective mutants that is rapid and yields a higher frequency of mutants than the limit dilution method. TMV was treated to 0.1%

TABLE 1. Infectivity of inoculum derived from lesions[a] induced by the PM4 strain of TMV harvested at various times after their appearance

	Lesion age in days[b]				
	0	1	2	3	4
Number of lesions[c]	3.7	3.6	0.4	1.3	0.3

[a] Local lesions of various ages induced by inoculating cultivar Xanthi-nc tobacco plants with PM4 (3) on five consecutive days were used as inocula to test their infectivity on Xanthi-nc tobacco plants in half-leaf experiments using a 5×5 Greco-Latin square. Procedure was described in Methods.

[b] Lesion age is considered to begin as soon as any detectable necrotic local lesions appear.

[c] Average number of lesions per half leaf induced on 10 half-leaves by homogenates of selected lesions. These data represent one typical experiment.

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TABLE 2. Frequency of occurrence of PM6R-type revertants of TMV in PM6-infected tobacco leaves

PM6-infected plants[a] (no. observed)	Observation period for PM6R symptoms (days)	PM6R-type isolates recovered (no.)	Isolates examined for amino acid composition (no.)	Isolates with amino acid composition identical to PM6R[b] (no.)
216	60–90	49	22	21

[a] Data from plants used for 24 mo of routine maintenance of strain PM6; approximately five to seven new plants were infected with PM6 each month. None of these virus stock plants was kept longer than 3 mo. PM6R exhibits wild-type symptoms.
[b] Methods were those previously described (4,5).

survival with HNO_2 (9) and brushed onto Xanthi tobacco. As soon as lesions appeared the small ones were individually excised, ground in ice-cold TP buffer, and rubbed onto separate Samsun tobacco plants. Only the smaller (petite) lesions were used because defective TMV mutants induce a statistically greater number of petite lesions than does wild-type TMV ([8] and J. J. Hubert, *unpublished*).

RESULTS AND DISCUSSION

It was important to use local lesions caused by defective mutants as an inoculum source as soon as they were visible because the transferable infective agent in such lesions, as shown in experiments with defective mutant PM4, decreased 10-fold within a few days after their appearance (Table 1). Reasons for the loss of infectivity are unknown, but it is plausible that the TMV-RNA in these lesions, which is unprotected by a protein coat, is inactivated by nucleases at the onset of necrosis during lesion maturation.

In a typical experiment in which this method was used, PM6R (a revertant wild-type TMV strain derived from the defective mutant PM6 [5]) was treated to 0.1% survival with HNO_2 and then brushed on Xanthi tobacco leaves as previously described. From among the resulting lesions, 100 petite lesions were selected and the homogenate of each was inoculated separately to a single leaf of a young Samsun tobacco plant (two- to three-leaf stage). After 1 wk, 45 of the plants showed wild-type systemic TMV symptoms in their terminal leaves and were discarded; the remaining plants were held for further observation. Nine of these eventually developed small, slowly expanding, yellow-colored infection sites that are typical of a defective mutant. All of these isolates appeared to be defective mutants by previously established criteria (12). Aberrant coat protein rods, but no virus, could be seen in electron micrographs of leaf extracts from plants infected with these isolates. These protein rods were typical of those previously observed (see Fig. 2D, ref. 5).

The 46 remaining plants that were held for observation failed to develop TMV infections and all were serologically negative for soluble or insoluble coat protein (2). Failure to establish TMV infections in this latter group could have been due to lack of transfer of infectious agent in many of the lesions that were used as inoculum or to the senescence of some of the inoculated leaves before defective mutant infections could become established. It is important that none of these plants developed a systemic, wild-type TMV infection, or any other detectable symptom of virus infection. Consequently, it can be concluded that up to 55% of the petite lesions selected as inoculum sources in this study had the low infectivity properties characteristic of defective strains of TMV. It is possible that some of these lesions that failed to produce TMV symptoms in cultivar Samsun (46%) were a result of defective mutant infections in cultivar Xanthi-nc even though the infective agent could not be transferred to the systemic host. This proportion of unproductive transfer of infective agent is larger than the 30% "miss" phenomenon reported by Siegel (9), although it is acknowledged that PM6R and wild-type TMV virions probably are not identical in stability and infectivity (5). PM6R contains a single amino acid exchange in its coat protein that appears to result in a loose encapsidation of PM6R-RNA by PM6R coat protein (5). This mutant may thus be predisposed to increase deamidation by HNO_2, although other workers would disagree (6). Nevertheless, this method of transferring infectivity from immature lesions appears to increase the probability of detecting defective mutants over the previously employed techniques where only one defective mutant (PM6) was found after inoculation of 100 plants with HNO_2-treated virus by using the limit dilution method (4). Clearly, by using rapid transfer of young lesions, the isolation, examination, and characterization of a large number of HNO_2-induced defective TMV mutants should be possible.

Our method was developed to transfer the infective entity in local lesions incited by defective mutants. It also is useful for rescuing these same mutants as pure isolates when their hosts become superinfected with wild-type TMV strains. The occurrence of wild-type and defective mutants as mixed infections in the same plant occurs frequently (Table 2), particularly in PM6-infected plants, because PM6 is unstable and can give rise to a wild-type mutant, PM6R, by reversion (5). The methods described here also are potentially useful for isolating mutants of plant viruses other than TMV that are defective for virus assembly and can produce necrotic or localized lesions on host plants.

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