

Characterization of a Defective Mutant of the Dahlemense Strain of Tobacco Mosaic Virus

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Tobacco Mosaic Virus, Defective Mutant, Induced Mutation

A mutant of tobacco mosaic virus has been isolated after treating the dahlemense strain with nitrous acid and called D 16y. Like dahlemense it produces necrotic lesions on java-type tobacco but induces a strong chlorosis on samsun tobacco, in which it spreads systemically. Yield of virus from infected plants is significantly lower than that of dahlemense and the virus particles are unstable *in vitro*, and probably also *in vivo*. It is serologically closely related to dahlemense, its coat protein is somewhat less electronegative in weakly alkaline medium and it is inactivated at elevated temperatures more easily than dahlemense. Its coat protein contains a histidine residue, that is not present in dahlemense. The poor biological activity and the instability of D 16y toward storage and high temperatures are properties that are common with many defective mutants. D 16y is possibly the first induced mutant of dahlemense and with its several altered characters is expected to be very useful for comparative studies on the structure and action of TMV.

Introduction

The nucleoprotein particles of a majority of the chemically induced mutants [1] of tobacco mosaic virus (TMV) have a stable configuration and they spread in samsun tobacco systemically. In contrast to these “normal” variants of TMV others have been found, which can be considered “defective” with respect to the stability of their particles *in vivo* and *in vitro* and their reduced capacity for spreading rapidly within their host. The defective mutants are useful for studies on the protective action of the coat protein on the viral genome [2–4], biosynthesis of the coat protein under altered temperature conditions [5, 6] and also for investigations on host-virus interactions [7–10]. The starting material for the isolation of induced mutants of TMV has been almost always the common strain vulgare [11–14]. However, if defective mutants could be raised from a different naturally occurring strain of TMV, it might be very helpful for comparative studies on the structure and function of TMV. Two arguments led us to choose the strain dahlemense [15] for this purpose: 1. Dahlemense has contributed significantly to the elucidation of the structure of TMV [13, 15–17] and 2. in spite of a large difference in the amino acid composition [18, 19] the coat protein of dahlemense is capable of associating with the vulgare protein to give rise to mixed aggregates [20].

Here we describe the properties of a defective mutant isolated after treatment of the dahlemense strain of TMV with nitrous acid. We call the new mutant D 16y, as it originated from dahlemense, had the protokoll no. 16, and, in contrast to dahlemense, produces a bright yellow symptom on samsun tobacco.

Materials and Methods

Virus and host

The TMV variants were vulgare (= common strain), dahlemense [15, 21] and D 16y. *Nicotiana tabacum* L. var samsun and *N. tabacum* L. var Xanthi nc. were used as the systemic and local lesion hosts, respectively. Other host plants are mentioned in Table I.

Isolation of the mutant D 16y

A highly purified preparation of dahlemense was subjected to the action of nitrous acid according to the method of Mundry and Gierer [11]. The reaction mixture contained (final concentrations) 13.6 mg TMV/ml, 1 mM NaNO₂ and 0.25 M sodium acetate. The pH was maintained at 4.6 with 2 N acetic acid and the reaction was stopped after 3 hrs by adding 10 vols of 0.1 M tris-phosphate buffer of pH 8.8 (TP-buffer: [22]). This solution was used to inoculate several leaves of Xanthi nc. tobacco and the tiny local lesions that were detectable after two days were cut

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out singly, homogenized in ice-cold TP-buffer of pH 8.8 and used to inoculate individual young samsun plants. Out of the 77 local lesions transferred in this way only the sample no. 16 produced a strong chlorosis on samsun tobacco. This mutant, D16y, was passed twice through the local lesion host Xanthi nc. and increased in samsun tobacco.

Purification of virus

D16y was extracted from primary-inoculated samsun leaves one week after inoculation using 0.067 M phosphate buffer of pH 7.0, by emulsification with a mixture of chloroform and *n*-butanol and differential centrifugation, essentially as described by Steere [23]. Virus concentration was estimated using the formula: $E_{260\text{nm}}^{1\text{cm}} = 1.0 = 0.365 \text{ mg TMV/ml}$. The purity of the virus preparations was checked by ultracentrifugation and electron microscopy.

Host range study

The symptoms produced by D16y were compared with those of vulgare and dahlemense on several kinds of host plants grown in a greenhouse under controlled conditions. Control plants were mock inoculated with phosphate buffer only.

Measurement of particle length in the electron microscope

Dip preparations [24] were made using freshly cut primary inoculated samsun leaves and contrasted with 2% phosphotungstic acid of pH 6.0.

Preparation of viral coat protein

Purified virus was dissociated either in 67% acetic acid [25] or in glycine-NaOH buffer of pH 10.5 [26]. The protein in acetic acid was dialysed exhaustively against distilled water, allowed to aggregate at pH 5.5, pelleted by centrifugation and dissolved in 0.067 M phosphate buffer, pH 7.0. The protein prepared by the alkali-method was incubated with a mixture of pancreatic ribonuclease and T_1 -RNase (both at a final conc. of 1 µg/ml) at 34 °C for ½ hr at pH 6.5, dialysed exhaustively against glycine-NaOH buffer at pH 9.5 and then against 0.067 M phosphate buffer of pH 7.0. It was purified by passing through a column filled with DEAE-Sephadex A-50 (coarse) and elution with phosphate buffer with increasing concentrations of NaCl (up to 0.5 M).

Stability of virus at elevated temperatures in vitro

Sap from infected samsun leaves as well as purified preparations of virus in 0.05 M phosphate buffer of pH 7.0 were subjected to different temperatures for 10 min each, rapidly cooled and tested for infectivity on Xanthi nc. tobacco.

Gel-electrophoresis of viral protein

Electrophoresis in 7% polyacrylamide gel were performed according to the methods of Weber and Osborn [27] and Davies and Stark [28] (as described in the technical bulletin no. MWS-877 of Sigma Chemical Co., St. Louis, Mo., USA) in 10 cm long Perspex tubes having an internal diameter of 6 mm. The gels were run for 16 hrs at 2 mA per tube, stained with Coomassie blue and destained in 7.5% aqueous acetic acid containing 5% methanol.

Serological test

Ouchterlony double-diffusion tests were performed in 0.8% agar dissolved in 0.85% NaCl of pH 7.0.

Amino acid composition of the coat protein of D16y

Viral coat protein, prepared by the acetic acid method [25], was hydrolysed with 6 N HCl in sealed tubes for different periods (20 h, 40 h and 60 h) and the hydrolysates were concentrated and analysed in a Beckman automatic amino acid analyser in the Max-Planck-Institut für Biologie, Tübingen.

Results

Host range

Three or four days after inoculation with D16y the samsun leaves developed small chlorotic spots which increased in area and spread over the whole leaf in about 10 days (Fig. 1a). After 3–4 weeks characteristic chlorotic areas with rather sharp borders were visible on systemically infected younger leaves (Fig. 1b). On Java tobacco and on samsun-EN [29] D16y produced only local necrotic lesions just like dahlemense. The symptoms produced on a few other hosts tested are summarized in Table I. The symptoms of D16y have some similarity to those produced by "luridum", a spontaneous mutant of dahlemense, isolated by Melchers [21] and partially



Fig. 1a. Chlorotic symptom produced by the TMV mutant D16y on samsun tobacco, 10 days after mechanical inoculation of the leaf.



Fig. 1b. Systemic symptom of D16y on samsun tobacco, 19 days after inoculation.

Table I. Comparison between the primary and systemic symptoms of vulgare, dahlemense and D16y on different hosts*.

Test plant	Virus vulg.	dahl.	D16y
<i>N. tabacum</i> L.	pr. gm or n	ma	ch
var samsun	sek. gm, mo, d	gm, mo	ym, ok
var Xanthi nc.	pr. ll	ll	ll
	sek. (—)	(—)	(—)
var samsun EN	pr. ma	ll	ll
	sek. gm	(—)	(—)
var White Burley	pr. gm	ma	ch
	sek. gm, mo, d	gm, d, cr	ym
<i>Physalis flori- dana</i> Rydb.	pr. ma, ll	ma, ll	ma, ll
	sek. gm, d	gm	gm, ym
<i>Nicandra physalo- ides</i> (L.) Gaertn.	pr. ch	ma	ch, ne
	sek. ma	(—)	(—)
<i>Datura stramonium</i> L.	pr. ll	ll	ll
	sek. (—)	(—)	(—)
<i>Gomphrena globosa</i> L.	pr. ll	ll	ll
	sek. (—)	(—), ma?	(—), ma?
<i>Chenopodium urbicum</i> L.	pr. ll	ll	ll
	sek. gm, mo, d, epi, ne	gm, mo, d, epi, ne	gm, mo, d, epi, ne

* Explanation of abbreviations and symbols:

- pr. = symptom on leaf inoculated directly by rubbing.
 sek. = symptom on leaf infected due to a systemic spread of the virus in the host.
 (—) = no visible symptom and no detectable virus.
 ch. = chlorosis.
 cr. = leaf crinkle.
 d. = deformation of systemically infected leaves.
 epi. = epinasty of leaf.
 gm. = green mosaic.
 ll. = local necrotic lesion.
 ma. = masked.
 mo. = mosaic symptom on young apical leaves.
 n. = no clear visible symptom.
 ne. = necrotic in course of time, in contrast to the primary local lesions.
 ok. = oak leaf pattern.
 ym. = yellow mosaic.

characterized by Aach and his coworkers [18, 30, 31]. The mutant D16y differs from luridum in that it produces a strong chlorosis on primary inoculated samsun leaf and its yield is very low (see below).

Yield of virus and its specific infectivity

From samsun leaves infected systemically with D16y only about 0.1 mg virus could be extracted per gram fresh wt. (see Materials and Methods). Under

identical conditions of growth and extraction procedure the yields of dahlemense (0.7–0.8 mg/g) and vulgare (1.0–1.1 mg/g) were nearly ten-fold. The infectivity per unit weight (specific infectivity) of purified virus was very poor in case of D16y; a 6–10 fold concentration was needed to produce the same number of lesions as those induced by dahlemense. Every single lesion produced by dahlemense or vulgare on *Xanthi* nc. tobacco, when homogenized in phosphate buffer (pH 7.0) and applied to fresh leaves, produced about 300 necrotic lesions. With D16y hardly more than 30 lesions were obtained under identical conditions; indicating that each lesion of D16y contains a lower amount of infective material or that the virus is more quickly inactivated in homogenates.

Heat-inactivation

In contrast to vulgare and dahlemense D16y is inactivated at a lower temperature. An exposure for 10 min to a temperature of 65 °C reduced the infectivity of purified preparations of D16y by 90%. The same degree of inactivation was obtained with dahlemense at 75 °C and with vulgare at 80 °C. The effect of high temperature may be primarily on the coat protein, since isolated coat protein of D16y produces insoluble aggregates already at 30 °C (*cf.* Jockusch [32]).

Virus particle length

Purified preparations of D16y contained many particles shorter than 300 nm (Fig. 2). On centrifugation in a sucrose density gradient D16y produced a broader band than vulgare and dahlemense, indicating a greater degree of inhomogeneity among the particles of D16y. The Schlieren diagram of D16y during sedimentation in an analytical ultracentrifuge was also indicative of polydispersity (Fig. 3). The polydispersity of D16y increases during storage of the virus even at a low temperature. After dialysis for 36 hrs at 4 °C against 0.05 M tris-HCl buffer of pH 8.0 the sedimentation coefficient of D16y fell to nearly 65s, whereas that of dahlemense remained unchanged near 190s. In spite of polydispersity, a sufficient number of particles of a length ~ 300 nm were observed under the electron microscope in leaf-dip preparations, showing that the “normal length” [33, 34] of D16y is the same as that of other strains and mutants of TMV.

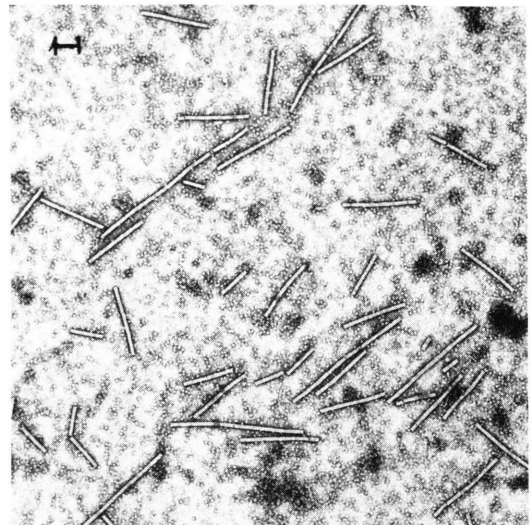


Fig. 2. Partially purified particles of D16y, negativ stain with 2% PTA, pH 6.0. Bar = 100 nm.

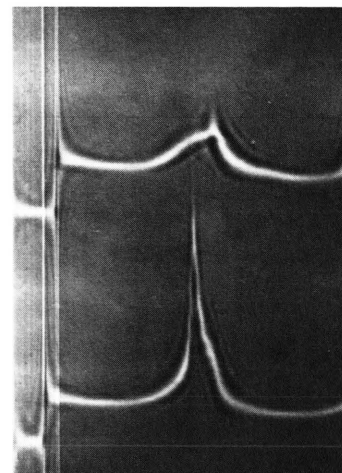


Fig. 3. Schlieren diagram of purified virus in the analytical ultracentrifuge (Beckman-Spinco) at 24,000 rpm, 15 min after reaching speed. Virus conc. = 3.5 mg/ml in 0.067 M phosphate buffer, pH 7.0, temp. 19 °C. D16y in the positive wedge cell (upper fig.), dahlemense in the normal cell (lower fig.). Note that D16y is less uniform than dahlemense. The sharp peaks of both have the same sedimentation coefficient, although D16y appears to move faster. This is due to the fact that the wedge cell contains a smaller volume of the virus suspension.

Electrophoretic mobility of D16y protein

The mobility of the coat protein of D16y in polyacrylamide gel (see Materials and Methods) was

found to be equal to that of *vulgare*, whereas dahlemense protein moved faster [31, 35], indicating that D16y possesses a lower net negative charge as compared to dahlemense.

Serological relationship

As expected, D16y is serologically more closely related to dahlemense than to *vulgare* (Fig. 4).

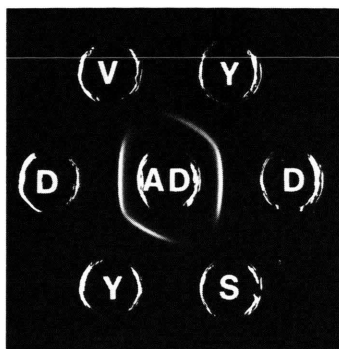


Fig. 4. Ouchterlony double-diffusion test in 0.8% agar dissolved in 0.85% NaCl, pH 7.0. All virus samples were at a conc. of 2 mg/ml containing 0.5% sodium dodecyl-sulfate. D = dahlemense, V = *vulgare*, Y = D16y and S = 0.85% NaCl only. AD = antiserum to dahlemense, titer about 1/256. Dahlemense and D16y are serologically closely related.

Amino acid composition of the coat protein

The coat protein of D16y obviously contains one histidine residue, which appears to have replaced a glutamine or glutamic acid. Histidine is not present in dahlemense.

Discussion

According to the conditions of the incubation with nitrous acid chosen here each particle of dahlemense had received four hits on the average [1]. Among the survivors therefore one can expect to find some chemically induced mutants. However, the possibility cannot be excluded, that the particular mutant D16y did not originate due to the action of nitrous acid, but that it was already present as a spontaneous variant of dahlemense. The origin of histidine, that is not present in dahlemense, and which seems to have been exchanged for a residue of glutamine, is dif-

ficult to reconcile with the known mechanism of mutagenesis with HNO_2 [1, 36]. One should postulate a change from adenine to uracil or guanine to cytosine, if in fact a glutamine has been replaced by histidine (either $\text{CAA} \rightarrow \text{CAU}$ or $\text{CAG} \rightarrow \text{CAC}$). However, with HNO_2 there should occur only the conversions $\text{C} \rightarrow \text{U}$ and $\text{A} \rightarrow (\text{H}) \rightarrow \text{G}$. In other words, either D16y is a spontaneous variant or some other amino acid has been exchanged and replaced by histidine. Amino acid exchanges that cannot be explained on the basis of the action of HNO_2 have been found in other induced mutants too [2, 14, 37–42].

Even if the appearance of histidine cannot be understood from the preliminary analysis of the total amino acid composition, D16y is undoubtedly a mutant of dahlemense: it was isolated after a chemical treatment of highly purified particles of dahlemense, it is serologically indistinguishable from dahlemense and it is also local necrotic on Java tobacco. The yellow symptom produced by D16y and its lower electrophoretic mobility as compared to that of dahlemense under weakly alkaline conditions fit the observation of von Sengbusch [43, 44], who had found that all TMV mutants producing chlorosis had an exchange either from an acidic to neutral or from neutral to basic amino acid in their coat protein. D16y therefore probably confirms the connection between yellow symptom, charge difference of the virus particle, primary structure of the coat protein and temperature-sensitivity proposed by Jockusch [32]. Histidine is present in the coat protein of the Holmes-ribgrass strain [45] and artificial histidine-containing mutants of TMV were reported by Rombauts and Fraenkel-Conrat [38]. However, D16y is probably the first histidine-containing mutant of the dahlemense strain.

The observed instability of D16y at elevated temperatures and its low specific infectivity are characters that speak for considering D16y a defective mutant, although in contrast to the PM-type mutants (Siegel *et al.* [4]) it does produce some intact particles of proper size. A similar partially defective mutant of TMV was isolated by Sehgal [46].

D16y has been used successfully in investigations on the mechanism of cross protection [47] in combination with the truly proteinless defective mutant DT-1G. Further studies are necessary to determine the amino acid sequence of the coat protein and to find out how far the mutant D16y can be useful for

comparative studies on the structure and function of TMV *in vivo* and *in vitro*.

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