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Observation of Cells Not Showing Hypersensitive Reaction in the Central Part of Tobacco Mosaic Virus (TMV)-Induced Local Lesions Developing in Detached Leaves of *Datura stramonium* L.

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It is established that the central area of TMV-induced local lesions developed in detached *Datura stramonium* leaves, along with the completely collapsed cells (types I and II), contains cells (type III) conserving to a certain degree integrity of their structural components. A characteristic of the type III cells was the accumulation of considerable amount of virus and formation of TMV-specific granular and tubular inclusions. The study of lesion development showed that a proportion of the collapsed cells and cells of type III did not essentially change in the period up from 3 to 5 days after infection of the leaves. These data suggest that the disease development in cells of type III does not lead to a hypersensitive response and is very similar to that in the systemically infected cells.

Keywords: *Datura stramonium* leaves, tobacco mosaic virus, local lesions, ultrastructure of cells.

The development of a hypersensitive reaction (HR) to viruses in host plants is known to lead to the formation of necrotic local lesions at the sites of virus entry. According to existing data (Hayashi and Matsui, 1965; Milne, 1966; Allison and Shalla, 1974; Favali et al., 1974; Appiano et al., 1977; D'Agostino and Pennazio, 1985), all cells in the lesion centre appeared completely collapsed so that it was nearly impossible to identify discrete structures with the exception of thylakoidal elements, starch grains and ribosomes.

In this report we demonstrate that the central area of TMV-induced local lesions in detached leaves of *Datura stramonium* L., along with cells whose contents are transformed into amorphous electron-opaque matrix in which no pre-existing cell components can be distinguished, contains cells conserving in some degree their structural integrity.

Materials and Methods

The investigations were made on mature detached leaves of 6-week-old greenhouse-grown plants of *Datura stramonium* L. As inoculum, the sap from leaves of *Nicotiana tabacum* L. var. Samsun infected with a common strain of TMV was used. The leaves of *D. stramonium* L. were dusted with Carborundum and infected by mechanical

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inoculation. The comparable *D. stramonium* leaves dusted with Carborundum and rubbed with sap from *N. tabacum* healthy leaves were used as a control. The processed leaves were washed with water and placed in a moisture chamber.

At different time points, 3, 4, 5, 7 and 10 days after infection of the leaves, small tissue pieces including the central and peripheral areas of the lesions were cut. Similar tissue pieces were cut from the control leaves. The tissue patterns were fixed first for 3 h in 6.5% glutaraldehyde prepared in phosphate buffer, pH 7.4, and then for 2 h in 1% osmium tetroxide. The material was dehydrated in a graded alcohol and acetone series and embedded in Araldite. The cross leaf sections were cut with a glass knife using a Reichert Om-U3 ultramicrotome and mounted on slot grids coated with formvar film stabilized with carbon. Using the slot grids, we were able to see under electron microscope all the cell panorama between, and including, the upper and lower epidermis, from the centre of a lesion to its edge, without a loss of any cells or cell parts. This enabled to accurately count the number of cells in the sections. The sections were stained with uranyl acetate and lead citrate and examined with a JEM-7A electron microscope.

Results

Ultrastructural study of local lesions developing in detached leaves of *D. stramonium* L. mechanically infected with TMV showed that reactions of cells to virus invasion in the central area of lesions are essentially not uniform. Some cells demonstrate an acute HR and undergo rapid and complete necrotization while the other cells conserve to a certain degree integrity of their structural components. Analysis performed by us testifies to the fact that cells in the central area of lesions can be divided into at least three types.

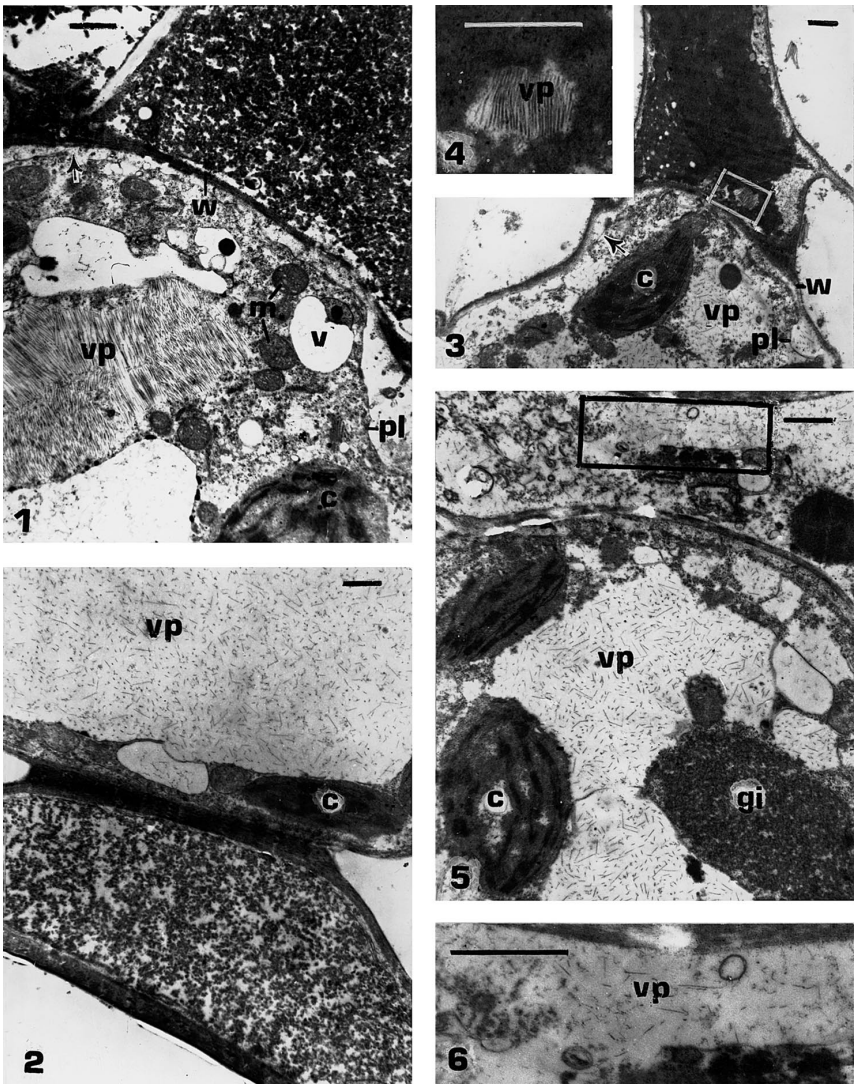
First of all, one may see heavily collapsed cells in which the discrete components are perfectly undiscernible *(Figs 1–3).* Similar cells (type I) were most frequently found in the upper epidermis and less frequently in the parenchyma and lower epidermis *(Table 1).* They were observed in all studied stages of lesion development (3, 4, 5, 7 and 10 days after inoculation of the leaves). The virus particles were not seen or were sometimes observed as small aggregates in the sections of such cells *(Fig. 4).*

Table 1

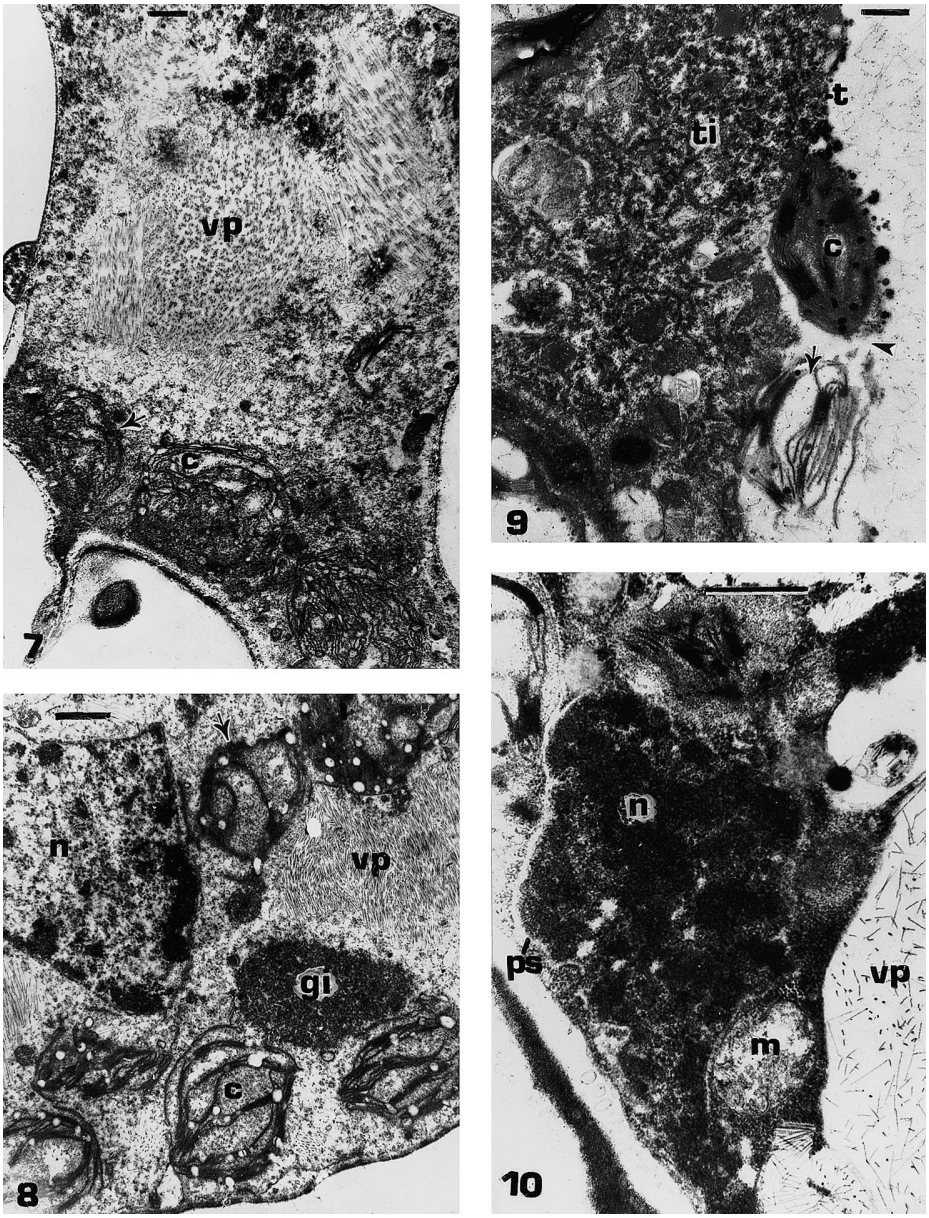
The amount of collapsed (a) cells (types I, II) and cells of type III (b) in the central area of TMV-induced local lesions developing in detached *D. stramonium* leaves*

Tissue	Number of days after infections					
	3		4		5	
	a	b	a	b	a	b
Upper epidermis	65/82.3	14/17.7	89/84.8	16/15.2	101/78.9	27/21.1
Palisade parenchyma	27/12.9	183/87.1	28/10.1	248/89.9	41/12.1	297/87.9
Spongy parenchyma	76/27.8	197/72.2	77/21.3	285/78.7	147/33.3	295/66.7
Lower epidermis	36/38.7	57/61.3	50/40.3	74/59.7	53/34.9	99/65.1

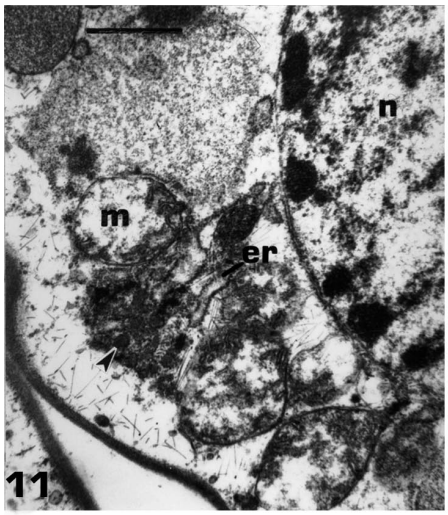
*The numerator shows the amount of cells counted on 10 sections. Every section was cut from a separate local lesion and mounted on a separate slot grid. The denominator shows % from the total amount of observed cells.

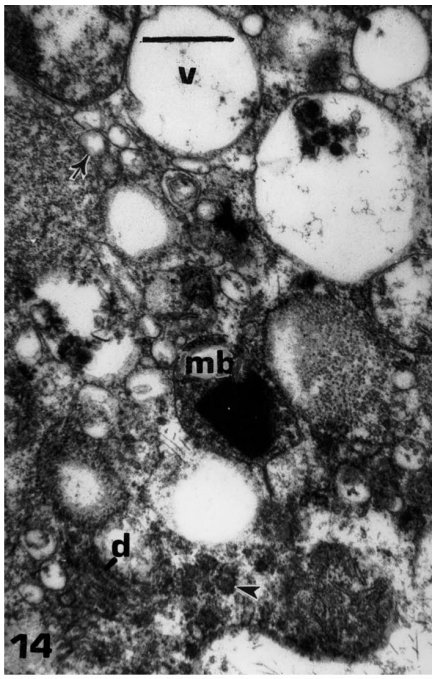


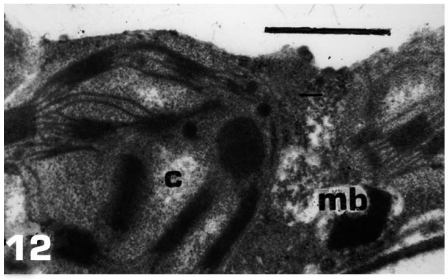
Figs 1–6. Parts of cells in the central area of TMV-induced local lesions developing in detached *D. stramonium* leaves. *Fig. 1.* A completely collapsed cell of the upper epidermis (type I) and a palisade parenchyma cell (type III) 4 days after inoculation of the leaf. *Fig. 2.* A type III cell of the spongy parenchyma and a completely collapsed cell of the lower epidermis (type I) 4 days after infection of the leaf. *Fig. 3.* Two cells of spongy parenchyma one of which is completely collapsed (type I) and another one showing type III features 3 days after inoculation of the leaf.
Fig. 5. A type II cell of upper epidermis and a type III palisade cell 5 days after infection of the leaf.
Figs 4 and 6. Enlargements of the cell areas limited by the frames in Fig. 3 and Fig. 5, respectively.
vp – virus particles, c – chloroplast, m – mitochondrion, v – vacuole, w – cell wall,
pl – plasmalemma, gi – granular inclusion. Arrows in Figs 1 and 3 indicate a rupture
of the plasmalemma. All bars = 500 nm

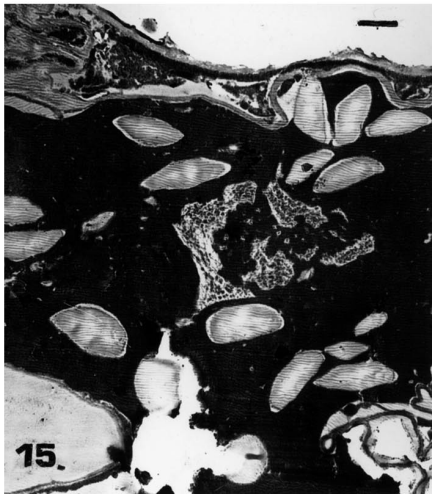


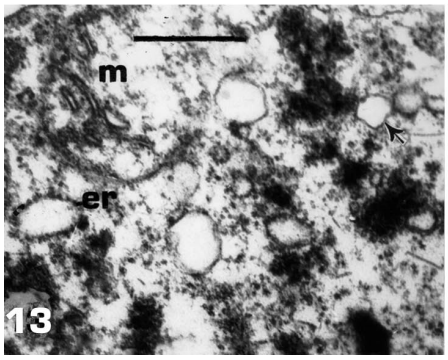
Figs 7–14. Parts of the type III palisade cells in the central area of TMV-induced local lesions forming in detached *D. stramonium* leaves in different stages of lesion development: *3 (Figs 8 and 12), 4 (Fig. 10), 5 (Figs 11, 13 and 14), 7 (Fig. 9) and 10 (Fig. 7)* days after inoculation of the leaves. In *Figs 7 and 8,* the arrows indicate a rupture of the outer chloroplast membrane. In *Fig. 9,* the arrow points to a chloroplast undergone complete disintegration and the arrowhead shows a rupture of the tonoplast. The electron-transparent vesicles *(Figs 13 and 14)*











and vesicles containing a dark staining material *(Figs 11 and 14)*
are shown by the arrows and arrowheads, respectively.
vp – virus particles, n – nucleus, ps – perinuclear space, c – chloroplast, m – mitochondrion,
er – endoplasmic reticulum, d – dictyosome, mb – microbody, t – tonoplast, gi – granular inclusion,
ti – tubular inclusion. *Fig. 15.* Collapsed cells of a healthy leaf dryed with
the help of an electric lamp. All bars = 500 nm

Not infrequently, it was possible to observe collapsed cells in which, however, the discrete structural components else could be distinguished. Such cells (type II) were mainly found in the upper epidermis *(Fig. 5).* Occasionally, they contained some amount of virus particles *(Fig. 6).*

Finally, cells (type III) whose reaction to TMV essentially differed from HR and was like to that of leaf cells systemically infected with TMV were found in all stages of lesion development *(Figs 1–3, 5, 7–14).* Such cells were especially often observed in the palisade parenchyma and most seldom in the upper epidermis (*Table 1).* A characteristic of these cells was the accumulation of considerable amount of virus. In late stages of infection development, the number of virions in the cells decreased. However, some cells contained a large amount of virus in these infection stages *(Fig. 7).* Along with virus particles, granular *(Figs 5 and 8)* and tubular *(Fig. 9)* inclusions, showed earlier to accompany TMV reproduction (Esau, 1968), were seen in the cells.

During pathogenesis, the cells of type III suffered different morphological changes. Nuclei in such cells in early stages of lesion formation were more or less normal in appearance *(Fig. 8).* However, as the infection developed, conspicuous morphological abnormalities were found. In some cases, the condensation of chromatin with its transformation into dense amorphous mass was observed *(Fig. 10).* The irregular light areas interspersed among the condensed chromatin and the extended perinuclear space were also seen *(Fig. 10).* In other cases, a characteristic of nuclear pathological changes was the gradual clearing of nucleoplasm *(Fig. 11).*

Chloroplasts in cells of type III undergo various abnormal changes such as the sticking of thylakoids and grana transformation into dense amorphous formations *(Figs 1–3, 5, 9 and 12),* disturbance of integrity of the outer membrane and formation of vesicles associated with the thylakoid system *(Figs 7 and 8),* complete disintegration *(Fig. 9).*

The amount of mitochondria in type III cells noticeably increased compared to the healthy control. In early stages of infection development in such cells, these organelles still appeared morphologically intact *(Fig. 1).* As infection proceeded, they lost crystae and became electron-transparent in appearance *(Figs 10, 11 and 13).* Occasionally, the outer membrane of the mitochondria suffered a disruption and complete destruction of the organelles occurred *(Fig. 13).*

Many cells of type III contained endoplasmic reticulum (ER) cisternae *(Figs 11 and 13),* dictyosomes *(Fig. 14),* microbodies *(Figs 12 and 14),* small vacuoles *(Figs 1 and 14),* and different vesicles *(Figs 11, 13 and 14).* All these cell components markedly increased in number in comparison with those of the control cells. The vesicles appeared electron-transparent *(Figs 13 and 14)* or contained a dark staining material *(Figs 11 and 14).* The ER cisternae often swelled to become vacuoles *(Fig. 13).* Most of the microbodies contained crystalloids dipped into clean *(Fig. 12)* or densy *(Fig. 14)* matrix.

Occasionally, cells of type III bordering with the collapsed cells demonstrated ruptures of the plasmalemma and its withdrawing from the cell wall *(Figs 1 and 3).* In some type III cells, it was possible to find a ruptured tonoplast *(Fig. 9).* Taken together, the development of infection in cells of type III caused a decrease in the number of ribosomes, clearing of cytoplasm, and a gradual destruction and elimination of organelles.

During studies of lesion development we found that the proportion of collapsed cells (types I and II) and cells of type III in the period up from 3 to 5 days after infection of the leaves did not essentially change *(Table 1).* It is important to note that in our experiments virus particles were observed in different cells of the local lesions including the cells of the lower epidermis already 3 days after inoculation of the upper epidermis. On the 7th and 10th days of lesion development the amount of completely collapsed cells in the central lesion area increased and the cells of type III, respectively, decreased. Supposing that it might be conditioned to a considerable degree by loss of cell water, we investigated the cell ultrastructure of a healthy leaf carefully dryed with the help of an electric lamp. As can be seen in *Fig. 15,* a loss of water by leaf cells actually causes their complete collapse.

Discussion

The results obtained show that the central area of local lesions induced by TMV in *D. stramonium* leaves, along with the completely collapsed cells, contains cells of type III conserving to a certain degree morphological intactness of their structural components. The cells similar to the latter, according to evidence of several authors (Hayashi and Matsui, 1965; Milne, 1966; Da Graca and Martin, 1975; D'Agostino and Pennazio, 1985), occurred only on the periphery of virus-induced local lesions and were infected for a comparably short time not enough to undergo collapse.

Our observations are not in line with the concept that the cells of type III do not show the HR because of late infection. Thus, virus particles were observed by us in different cells of the local lesions including the cells of the lower epidermis already 3 days after inoculation of the upper epidermis. The proportion of collapsed cells and cells of type III in the lesions 3, 4 and 5 days after infection of the leaves did not essentially change. An increase in the number of collapsed cells on the 7th and 10th days of lesion development was not caused by the HR but appeared to be associated with a loss of water. The accumulation of a large number of TMV particles and formation of TMV-specific granular and tubular inclusions in the type III cells show that the disease development in such cells is very similar to that in systemically infected cells (Esau, 1968; Reunov, 1999). The tubular inclusions, as it is known (Esau, 1968), are formed in comparably advanced stages of TMV reproduction. Based on these observations, we suppose that the absence of HR in cells of type III cannot be explained by an early infection stage and probably testifies to the fact that these cells possess a certain degree of tolerancy of the virus.

An increase in type III cells of the number of microbodies with crystalloids is of certain interest. Such microbodies in cells are thought to play an adaptation role and, in particular, inhibit the generation of hydrogen peroxide (Belitser, 1978). It is known that reactive oxygen species such as H_2O_2 may trigger HR cell death (Jacobsen, 1996; Pennell and Lamb, 1997). Therefore, the increased formation of crystalloid-containing microbodies in the cells of type III possibly testify to the development of mechanisms preventing membrane damage through lipid peroxydation and, as a consequence, cell collapse. If so, this provides additional evidence that such cells "are not destined" to HR cell death.

It should be noted that, according to Israel and Ross (1967), the development of TMV-induced local lesions in leaves of *Nicotiana tabacum* L. var. Samsun NN leads to collapse of the mesophyll cells while the epidermis cells preserve intactness. Our results, however, show that namely the cells of the epidermis, especially the upper epidermis cells, respond to the virus by the most acute HR. On the contrary, the parenchyma cells, first of all those of the palisade, undergo collapse considerably more seldom than the epidermis cells.

Different ultrastructural features observed by us in the cells of type III testify to the development of intracellular lytic processes. In accordance with some data (Vasiliev, 1972; Wilson, 1973; Belitser, 1978), such features are the increase in number of dictyosomes, swelling of ER cisternae, formation of vesicles and vacuoles, disruption of tonoplast and plasmalemma, clearing of cytoplasm, destructive changes of organelles.

In the course of lytic processes, the virus particles in the cells of type III may apparently undergo destruction. This is evident from a decrease of the amount of virus particles in the local lesions observed by us here and in earlier studies (Reunov et al., 1996). It seems that the development of lytic processes in type III cells is similar to that in the systemically infected cells (Reunov, 1999) and does not lead to rapid degradation of organelles as in the cells responding to virus infection by HR (Ragetli, 1967; Favali et al., 1974; Vögeli-Lange et al., 1988; Mittler and Lam, 1995; Reunov, 1999).

In conclusion, the results presented here raise the question why some cells in the central area of TMV-induced local lesions developing in *D. stramonium* leaves do not undergo the HR although they contain considerable amount of the virus and are in contact with completely collapsed cells. To answer this question further investigations are needed.

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