Morphology and Intracellular Appearance of Orchid Fleck Virus

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Abstract

A bacilliform virus, designated orchid fleck virus (OFV), was isolated from six genera of orchid plants including Anguloarea, Cymbidium, Dendrobium, Odontoglossum, Oncidium, and Pescatorea. The virus causes systemic leaf symptoms of chlorotic or necrotic flecks in orchid plants. The virus was transmitted to Dendrobium, tobacco, Nicotiana glutinosa, Chenopodium amaranticolor, and three other plants, by sap inoculation when the temperature was higher than 30°C. The virus particles could be detected in preparations obtained by the modified direct negative staining method devised by the present authors. The virus was partially purified, and the preparation contained numerous virus particles and was infectious to Dendrobium plants. The virus particle is approximately 40 × 150 nm in negatively stained preparations, but 32–35 × 100–140 nm in thin sections. The particle had no envelope and consisted of a helical structure with a pitch of 4.5 nm. In thin sections of OFV-infected tissues, a large number of virus particles were found in the nuclei and in the cytoplasm, and the viroplasm development was also observed in the nuclei of OFV-infected cells. A series of electron micrographs suggest that OFV particles are formed in the viroplasm and disperse along the membrane system into the cytoplasm after passing through the nuclear envelope. From these observations it is concluded that OFV is a virus belonging to a new virus group different from the rhabdovirus group. Dendrobium virus (Petzold, 1971), Phalaenopsis virus (Lesemann and Begtrup, 1971), ringspot virus of coffee (Kitajima and Costa, 1972), citrus leprosis virus (Kitajima and Costa, 1972) may be identical with, or closely related to, OFV.

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Introduction

A bacilliform virus, designated orchid fleck virus (OFV), was first described from Cymbidium by Doi et al. in Japan3). They examined thin sections of Cymbidium leaves with necrotic flecks under electron microscope, and demonstrated bacilliform virus particles of approximately 32 × 120 nm in the nuclei and in the cytoplasm. Subsequently Chang et al. detected OFV in several other orchid genera and succeeded in its mechanical transmission and partial purification, and described its intracellular appearance and localization1). Possibly the same virus has been reported in Germany. Petzzold detected a bacilliform virus, 30 × 100 nm, in thin sections of Dendrobium hybrids8). Later Lesemann and Begtrup reported a similar virus, 30 × 110 nm, found in thin sections from Phalaenopsis lueddemanniana leaves with leaf spot symptoms, which was tentatively named Phalaenopsis virus7). The latter

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authors observed virus particles in the nucleus and in the cytoplasm of a few parenchyma cells. OFV has been further investigated by the present authors and a more detailed study is described in this paper.

**Materials and Methods**

**Diseased plants:** Two orchid plants, *Cymbidium* and *Odontoglossum*, both of which showed leaf symptoms of necrotic flecks, were used as virus source throughout this study. The former was collected at the Ofuna Flower Center of Kanagawa Prefecture in 1966, and the latter at the Kemigawa Arboretum, University of Tokyo, in 1972. Both plants were maintained in a greenhouse.

**Mechanical inoculation:** Diseased orchid leaves were macerated in 0.01M phosphate buffer (pH 7.2), and crude sap was expressed. Young seedlings of several plant species and healthy orchid plants were inoculated by rubbing with the expressed sap, or with partially purified virus preparation described below.

**Dip-negative staining method:** Dip-negative stained preparations were obtained by a modification of the direct negative staining method\(^4\). The procedure was as follows: a small piece of an OFV-infected leaf was cut several times with a razor blade in a drop of 2% osmium tetroxide on a clean slide glass. A carbon-coated grid was touched to the surface of the drop and was air-dried for a few seconds. The grid was then dipped in a drop of 2% PTA, air-dried, and examined in an electron microscope.

**Partial purification:** Diseased orchid leaves were homogenized at 4°C in 0.1M phosphate buffer (pH 7.0) containing 0.1M sodium diethyldithiocarbamate, 0.1% L-ascorbic acid, 5% Triton X-100, and 0.5% sodium deoxycholate. The homogenate was centrifuged at 5,000 rpm for 15 min, and the supernatant was centrifuged at 30,000 rpm for 2 hr. The pellet was suspended in 0.1M phosphate buffer, followed by two more cycles of differential centrifugation. The virus was further purified by sucrose density gradient centrifugation. The density gradient was formed by two layers of 10 and 40% sucrose in 0.01M phosphate buffer (pH 7.0). The partially purified virus was negatively stained with PTA, or shadowed with chromium, and then examined under electron microscope.

**Thin-sectioning:** Small pieces of leaves from OFV-infected plants showing leaf symptoms of chlorotic or necrotic flecks (or spots) were fixed in 5% glutaraldehyde in 0.1M phosphate buffer containing 0.25M sucrose at pH 7.0 for 3 hr, and postfixed in 1% osmium tetroxide in the same buffer at pH 7.0 for 1 hr before being dehydrated through a graded series of ethanol and embedded in Epon 812. Sections were cut on a LKB ultramicrotome with glass knives, and stained with uranyl acetate and lead citrate. The specimens were examined under a Hitachi HU-12 electron microscope. Samples from healthy plants were prepared in the same way and examined as controls.

**Results**

**Mechanical inoculation with OFV**

OFV was transmitted to a number of plant species by sap inoculation with the expressed juice from diseased orchid leaves, or with the partially purified virus preparation. The successful transmission, however, was obtained only in summer when the temperature in a greenhouse was higher than 30°C. Chlorotic spots appeared on the inoculated leaves of *Chenopodium amaranticolor, C. quinoa, Petunia hybrida,*
Nicotiana glutinosa, and four varieties of N. tabacum, namely Bright Yellow, White Burley, Xanthi nc, and KY-57 (Figs. 1 and 2). Dendrobium plants produced chlorotic or necrotic spots, sometimes flecks, on the inoculated leaves and later on the upper leaves. The incubation period was usually two or three weeks.

**OFV-particles in negatively stained dip-preparation**

Bacilliform particles, although the majority of particles were bullet-shaped, were easily detected in dip-preparations from diseased plants of six orchid genera including Angulorea, Cymbidium, Dendrobium, Odontoglossum, Oncidium, and Pescat-

![Image of local chlorotic spots on leaf of N. tabacum var. Xanthi nc caused by OFV.](image1)

![Image of local chlorotic spots on leaf of N. glutinosa caused by OFV.](image2)

![Image of systemic necrotic flecks on Cymbidium leaves infected with OFV.](image3)

Fig. 1. Local chlorotic spots on leaf of N. tabacum var. Xanthi nc caused by OFV.

Fig. 2. Local chlorotic spots on leaf of N. glutinosa caused by OFV.

Fig. 3. Systemic necrotic flecks on Cymbidium leaves infected with OFV.

Symptoms of these diseased orchid plants were systemic leaf symptoms of chlorotic or necrotic flecks (Fig. 3). The same particles were also detected in experimentally infected plants, but not in healthy plants. The particles were approximately 40 nm wide and 150 nm long (Plate I–A, D). They had no envelope, and showed helical structures with a pitch of about 4.5 nm (Plate I–B, C). The rounded end of individual particle usually attached to membrane apparently depressed by the particle (Plate I–A). The particle have never been found in preparations prepared by the usual direct negative stain-ing method which lacks osmium-prefixation. This suggests that the particles may be easily broken down in PTA.

**Partial purification of OFV**

The virus was partially purified from diseased leaves of Cymbidium and Odontoglossum. The partially purified preparation contained numerous bacilliform particles (Plate I–E). Their particle morphology was the same as described above. In addition, the preparations were infectious by sap inoculation to healthy plants of Dendrobium. From these results it is concluded that these bacilliform particles are OFV-particles.

**Electron microscopy of thin sections from OFV-infected tissues**

In thin sections of OFV-infected tissues a large number of OFV particles were seen both in the nuclei and in the cytoplasm of infected cells (Plate II). Plate I–F and Plate II show the particles in longitudinal and transverse sections respectively. The particles were bacilliform, being uniform in size and shape. The particles were morphologically similar to those detected in negatively stained preparations or
partially purified preparations, although their dimensions were slightly smaller. The particles in thin sections measured 32-35 nm wide and 100-140 nm long.

The most striking feature of OFV-infected cells was the presence of masses of electron low-dense material of granular or fibrous appearance in the nuclei. These structure, named hereafter 'viroplasm' by analogy with those observed in association with other viruses\(^2,9,10\), were of various sizes and often found to occupy most area of the nucleus. As shown in Plate III, these viroplasms were observed frequently near the nucleoli. Virus particles were observed consistently in and around the viroplasm (Plate III-C, D and Plate IV-E, F). A series of selected pictures of nuclei suggested a hypothetical course of viroplasm formation. First, virogenic areas appear within the nucleoplasm, followed by increase of their number (Plate III-A, B). Then virus particles appear in and around viroplasms (Plate III-C). Next, the viroplasms increase their size (Plate III-D), and virus particles accumulate around the viroplasm (Plate IV-E). Subsequently virus particles move to the nuclear envelope (Plate IV-F), and as a result, projections of the nuclear envelope into the cytoplasm are observed in some places (Plate IV-G, arrows). Finally, the nuclei deform and nuclear membrane evaginate to produce cytoplasmic vesicles enclosing virus particles (Plate IV-G, H). In conclusion, OFV-particles are thought to be formed in viroplasms which occupy most area of the nucleus.

In addition to the accumulation of virus particles, dense vacuolated nuclei, swollen or amoeboid chloroplasts, and swollen mitochondria were observed in some of OFV-infected cells.

In the nuclei, virus particles existed in the chromatin area singly or in side-by-side arrangement, showing crystalline arrays in cross section (Plate I-F and Plate II). In addition, virus particles were commonly found to attach vertically to the inner nuclear membrane (Plate V-A, B). Occasionally, virus particles were seen in the distended area between the outer and inner membranes of the nuclear envelope. In Plate V-B, a small number of virus particles surrounded by the inner membrane are seen to be present in such areas. This may be explained by the dispersion of virus particles along the membrane system into the cytoplasm (Plate V-A). A number of virus particles surrounded by the inner membrane often showed a structure like a spoke wheel. Such virus groups were frequently observed at the periphery of the nucleus and also in the cytoplasm of OFV-infected cells (Plate II and Plate VI-C). These structures are thought to be cross sections of nuclear envelope projections in the cytoplasm.

In the cytoplasm, virus particles were found to associate with, or to be surrounded by, the membrane system, sometimes showing a structure like a spoke wheel as described above (Plate VI-C). Some particles were found to associate or attach to the endoplasmic reticulum (Plate VI-A). Rarely, partially enveloped particles were found to be enclosed with a single membrane (Plate VI-B). These cytoplasmic membrane systems were thought to originate from the nuclear envelope.

From these observations it is considered that OFV is a bacilliform particle with distinct capsid structure, but with no envelope, and that the primary site of virus assembly is the nucleus.

Discussion

Our results suggest that OFV is widespread in orchid plants in Japan. The virus is transmitted by inoculation of sap, but natural infection may occur in orchid plants by transmission of some unknown vector or vectors. The modified direct
negative staining method always revealed the presence of bacilliform particles in
lesion parts of leaves from plants naturally or artificially infected with OFV and
their absence in healthy leaf tissues of control plants. The virus was partially
purified, and the preparation contained numerous bacilliform particles and showed
to be infectious. From these results it is concluded that these bacilliform particles
are OFV particles. OFV causes leaf symptoms of chlorotic or necrotic flecks in
orchid plants.

OFV particle is approximately $40 \times 150 \text{ nm}$ in negatively stained preparations,
but $32-35 \times 100-140 \text{ nm}$ in thin sections. The particle has no envelope and shows
helical structure with a pitch of 4.5 nm. If we apply the usual direct negative
staining method, we cannot detect OFV particles in diseased leaves, indicating that
OFV is of a labile nature. These morphological features led us to the conclusion
that OFV is a virus belonging to a new virus group different from rhabdovirus group.

OFV particles were found in the nuclei and in the cytoplasm, and the viroplas-
sm development was also observed in the nuclei of OFV-infected cells. A series of
our electron micrographs suggest that OFV particles are formed in the viroplasms
and disperse along the membrane system into the cytoplasm after passing through
the nuclear envelope.

Dendrobium virus$^8$), Phalaenopsis virus$^7$), ringspot virus of coffee$^6$), and
citrus leprosis virus$^5$) are very similar to OFV in several features. Although satisf-
actory evidences are still lacking, the former four viruses may be identical with, or
closely related to, the latter virus.

Literature cited

   (Abstr.).
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Explanation of Plates

Plate I  (A) Virus particles in negatively stained preparation from OFV-infected Cymbidium. Note their association with membrane system (arrows). Inset shows a high magnification.
(B and C) Bullet-shaped particles which have lost one of their ends. Note cross striations.
(D) Complete bacilliform particle.
(E) Virus particles in partially purified preparation of OFV shadowed with chromium. Inset shows a high magnification.
(F) Leaf cells of N. tabacum var. Xanthi nc infected with OFV. Virus particles (arrows) in nucleoplasm.

Plate II Spongy parenchyma cell of N. glutinosa infected with OFV. Note side-by-side arrangements of virus particles in nucleus and membrane encircled virus particles in cytoplasm (arrows). c, cytoplasm; ch, chloroplast; cw, cell wall; m, mitochondrion; ne, nuclear envelope; v, virus; vac, vacuole; vp, viroplasm.

Plate III Hypothetical course of viroplasm development in nuclei of OFV-infected cells.
(A) Appearance of viroplasm near nucleolus in nucleoplasm (N. glutinosa).
(B) A high magnification of the inset in (A).
(C) Appearance of virus particles in and around viroplasm (N. tabacum var. Xanthi nc).
(D) Enlarged viroplasm (N. glutinosa).

Plate IV (E) Accumulation of virus particles around viroplasm (N. glutinosa).
(F) Movement of virus particles to nuclear envelope (arrows) (N. glutinosa).
(G and H) Partial evagination of nuclear envelope and formation of vesicles enclosing virus particles. Note projections of nuclear envelope (arrows) (N. glutinosa).

Plate V Leaf cells infected with OFV.
(A) Dispersion of virus particles along membrane system into cytoplasm (N. glutinosa).
(B) Virus particles surrounded by inner nuclear membrane in area between inner and outer nuclear membrane (N. tabacum var. Xanthi nc).

Plate VI (A) Virus particles associated with endoplasmic reticulum in leaf cell of N. tabacum var. Bright Yellow infected with OFV (arrows).
(B) Partially enveloped virus particles (arrows) surrounded by a single membrane in cytoplasm of leaf cell of N. glutinosa infected with OFV.
(C) A number of virus particles surrounded by membrane system in cytoplasm of leaf cell of Dendrobium.

c, cytoplasm; ch, chloroplast; cw, cell wall; m, mitochondrion; v, virus; vac, vacuole.
Plate I
Plate III
Plate V
Plate VI

A

vac

er

c

ch

cw

0.5 μ

B

m

A

C

ch

c

v

v

0.5 μ