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Graft Transmission and Cytopathology of Pear Black Necrotic Leaf Spot (PBNLS) Disease

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Graft transmission and cytopathological studies of a severe pear disease, pear black necrotic leafspot (PBNLS), were carried out to determine the causal agent of the disease. No evidence was found that a fungal or bacterial pathogen could be the causal agent of the disease. Attempts to transmit the agent by sap-inoculation to other plants including herbaceous hosts failed. However, the pathogen was readily graft-transmitted from symptomatic diseased pears to healthy pears. Graft transmission of the pathogen was also demonstrated by using an indicator plant, PS-95, developed in the laboratory through various grafting methods. Ultrastructural study of the disease revealed the consistent presence of flexuous rod-shaped virus-like particles (VLP) in the symptomatic leaves of both Niitaka cultivar and indicator pear, PS-95. The particles, approximately 12 nm in diameter with undetermined length, occurred in the cytoplasm of mesophyll parenchyma cells. Cells with VLPs also contained fibril-containing vesicles, which are common in cells infected with plant viruses with ssRNA genome. The vesicles were formed at the tonoplast. Based on the symptomatology, the presence of fibril-containing vesicles, and graft-transmissibility, it is believed that the VLPs that occurred on symptomatic leaves of black necrotic leafspot of pear are viral in nature, possibly those of a capillovirus.

Keywords : capillovirus, fibril-containing vesicles, graft-transmissibility, ssRNA, tonoplast.

During the past two decades, the pear black necrotic leaf spot (PBNLS) has been an epidemic in many pear growing areas in Korea, causing up to 50% yield loss (Hong et al., 1985; Nam and Kim, 1994). The disease, which was then known as abnormal leaf spot of pear, was first observed in 1979 (Hong et al., 1985) occurring sporadically in a number of pear farms.

The disease incidence, however, increased progressively through the years and became a serious pest threatening the country's pear industry (Nam and Kim, 1994; 1995). Because of the importance and economic value of pear as a commercial crop, intensive studies have been undertaken to identify the causal agent of the disease. Reports from a number of the studies on the disease, however, have been widely controversial, with conflicting claims that the disease could have been caused by a fungus or by a bacterium. A strain of fungal pathogen, *Alternaria kikuchiana*, isolated from the symptomatic leaves of PBNLP was presumed to be responsible for the disease by Ki et al. (1984), while a strain of bacterial pathogen, *Erwinia pyrinus*, isolated from the necrotic lesion of PBNLP was claimed to be the causal agent of the disease (Chung et al., 1993).

In a series of follow-up studies on the disease (Nam and Kim, 1995, 1996; Nam et al., 1996), however, no evidence was found that a fungal or a bacterial pathogen could be the causal agent of the disease. Instead, it was demonstrated that the agent was graft transmissible through the use of an indicator plant developed with various grafting methods (Nam et al., 1996).

A disease similar in symptomatology and disease development to the PBNLS of the present study was also observed in the early 1950s in Japan and was named as pear necrotic spot (Noda et al., 1958; Kishi et al., 1972, 1976). It was believed that the causal agent of the disease was a virus rather than a fungus or a bacterium (Kishi et al., 1976). Furthermore, the virus associated with the pear necrotic spot was reported to have a flexuous, rod-shaped particle morphology and related to that of the pear vein yellow and apple stem pitting (Yanase et al., 1988).

This paper reports the graft transmission of PBNLS pathogen and the cytopathological studies of the disease which revealed the consistent presence of flexuous rod-shaped virus-like particles associated with fibril-containing membraneous vesicles common in cells infected with plant viruses with ssRNA genome (Francki, 1987).

Materials and Methods

In the selection of indicator plants for early detection of PBNLS in the fields, 10 out of 102 progenies of pear trees obtained from five

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Table 1. Response of pear seedlings obtained from various cross combinations to the symptom development of black necrotic leaf spot examined by double grafting inoculation using diseased trees and seed-originated virus free stocks

Cross combination (year)	No. of seedlings	
	Inoculated	Symptoms developed
Niitaka Waseaka (1986)	60	6
Niitaka Hosui (1986)	8	–[a]
Niitaka Suhwangbae (1988)	2	1
Niitaka Imamuraaki (1986)	16	1
Niitaka Tama (1988)	6	2
Total	102	10

[a] Not examined.

Table 2. Severity of abnormal leaf spot symptoms developed on seven progenies pre-selected for indicator plants from two parent combinations by double chip budding inoculation method using diseased trees and seed-originated virus free stock in the field in 1994 and 1995, and level of resistance of the progenies to pear black spot disease caused by *Alternaria kikuchiana*

Progeny No.	Severity of abnormal leaf spot[a]		Reaction to *A. kikuchiana*
	1994	1995	
Niitaka × Waseaka			
86-2-2	+++	+++	R[b]
86-2-30	+++	+++	R
86-2-7	+	+	M
86-2-5	+	+	S
86-2-20	+++	++	R
86-7-20	++	+++	R
Niitaka × Imamuraaki			
87-7-137	++	++	R

[a] Observation was made on the basis of 6 plants. + = 1-2 spots/leaf; ++ = 5-10 spots/leaf; +++ = 30-50 spots/leaf.
[b] R = resistant; S = susceptible; M = moderate.

cross combinations of cultivars including Niitaka were pre-selected by using a double grafting method (Tables 1 and 2). These ten candidate progenies were examined in a farmer's field for the incubation period, types of symptoms, severity of symptoms, and level of resistance to the pear black spot disease caused by a fungal pathogen, *Alternaria kikuchiana* (Tables 2 and 3). The symptoms produced by the pear black spot disease resembled that of PBNLS, causing confusion in diagnosing the two diseases (Nam and Kim, 1994). A progeny, 86-2-2, obtained from the cross between Niitaka and Waseaka was finally selected as an indicator plant and named PS-95 (Nam et al., 1996).

For the cytopathological study, specimens were taken from symptomatic leaves of Niitaka pear infected naturally in the fields, as well as, those obtained after graft inoculations. Specimens were also taken from the leaves of PS-95, which demonstrated progressive symptom development. From these leaves, three stages of symptom development were sampled: 1) immediately after the appearance of PBNLP symptoms which was indicated by the presence of lightly chlorotic lesions; 2) immediately after the appearance of the black necrotic spots which were smaller and less in number compared with those in the later stages of infection; and 3) fully symptomatic leaves which were covered with large circular black necrotic spots (Fig. 1).

Table 3. Development of abnormal leaf spot disease on a selected indicator plant 86-2-2, when top-grafted to the cultivar Niitaka at an orchard on April 10, 1995

Tree status	No. trees tested	No. twigs grafted/tree	% diseased leaves	
			June 15	June 30
Diseased Niitaka	8	2	75.4	89.2
Healthy Niitaka	2	2	0	0

In all cases, a small area of the necrotic lesion and surrounding non-necrotic area were included in each specimen sampled. Specimens, 1-2 mm^2 from symptomatic, as well as, from symptomless control leaves were fixed in 2.5% glutaraldehyde in Milonigs phosphate buffer, pH 7.0, for at least 2 hours at room temperature. After three washes with the same buffer solution, the tissues were *en-bloc* stained overnight in 0.5% aqueous uranyl acetate. Tissues were dehydrated through an ethanol series from 30% to 100% and two changes of propylene oxide. The tissues were then embedded in Epon 812. Thin sections were cut with a diamond knife and were double stained with 2% aqueous uranyl acetate and lead citrate before viewing under Zeiz electron microscope using 80 KV.

Results and Discussion

The indicator plant selected in this study, PS-95, has demonstrated to be an excellent tool in diagnosing PBNLS, as well as, in breeding resistant varieties of pears to this disease. It has served very efficiently and accurately in diagnosing the disease in commercial fields throughout the country with the use of a variety of grafting methods such as, double-grafting, tongue-grafting, double chip-grafting, double chip-budding, and common top-grafting (Figs. 2, 3, and 4).

Cytopathological studies of symptomatic leaves of both Niitaka (*Pyrus pyrifolia* Nakai) and indicator pear PS-95 collected from the field and the greenhouse, respectively, revealed a consistent presence of long flexuous rod-shaped virus-like particles in the cytoplasm of mesophyll cells (Fig. 5). Cells in lesions sampled in different stages of symptom development (Fig. 5) all contained the particles, although the early lesions, which appeared as lightly chlorotic spots, showed more cells exhibiting the particles. This may due to the fact that fully developed black spots consisted of many collapsed necrotic cells that were undergoing degradation of cell organelles.

The virus-like particles usually occurred as longitudinally aligned bundles of various sizes and numbers near the nuclei (Figs. 5 and 6). Transverse sections of the particles

Fig. 1. Typical black necrotic leaf spot symptoms appeared in a fully-grown pear tree (Niitaka cultivar) in a commercial orchard.

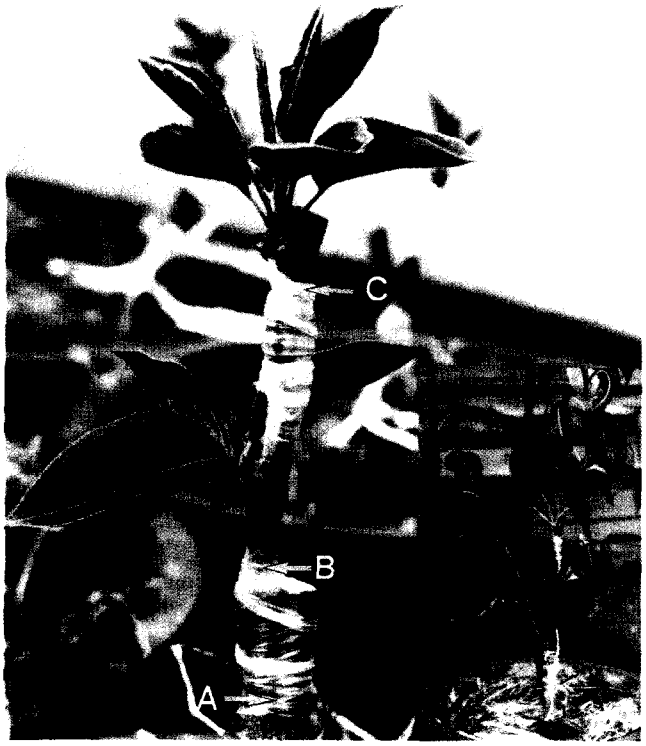

Fig. 2. Double-grafting method used to examine graft-transmissibility. A diseased Niitaka pear (B) was grafted on a healthy root stock seedling (A), and healthy Niitaka pear (C) was then grafted on diseased Niitaka. The healthy Niitaka pear (C) showed symptoms ca. 2 months after double grafting (bottom right).

appeared as electron-dense solid spheres measuring approximately 12 nm in diameter (Fig. 7). Cylindrical or pinwheel inclusions characteristic of potyviruses, and beaded sheath inclusions of potexviruses (Francki et al.,

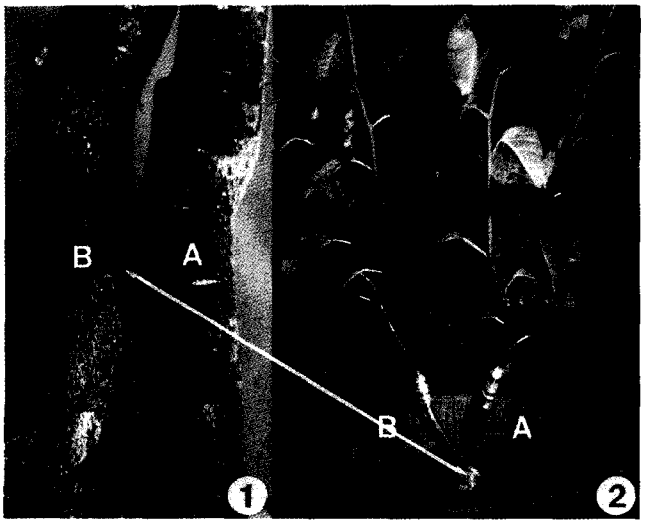

Fig. 3. Black necrotic leaf spots developed 3 months after tongue-grafting. ① Tow health root stock seedlings were tongue-grafted. ② When healthy (A) and diseased (B) Niitaka pears were grafted on each of a healthy rootstock seedling, leaves of healthy Niitaka pear grafted showed typical symptoms.

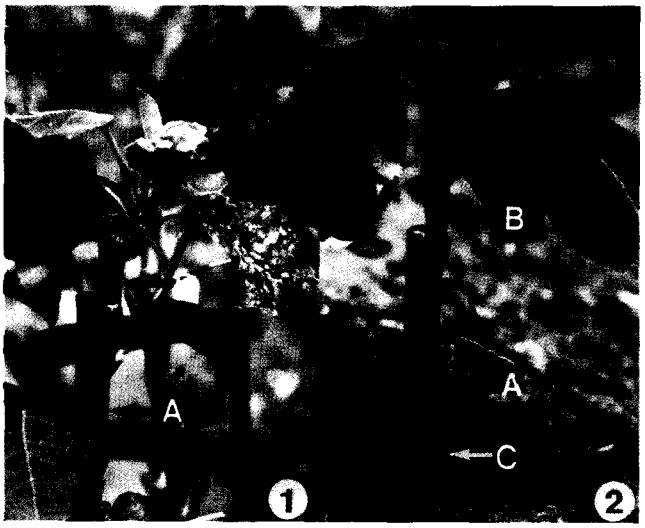

Fig. 4. Detection method of the disease used in commercial orchard. ① Top grafting. An indicator pear PS-95 (B) was grafted on a disease-suspicious Niitaka pear tree (A). ② Double chip grafting. PS-95 (B) was grafted on a rootstock seedling (C), and then, a bud (A) from a disease-suspicious pear tree was chip-grafted to the rootstock seedling. Typical symptom of black necrotic spots usually appeared in PS-95 2 months after grafting.

1985) were not observed in any cell of the symptomatic pear leaves used in this study, despite the intensive search. This suggests that the particles present in the symptomatic leaves of PBNLP are not representing one of the two flexuous rod-shaped viruses mentioned. The shape and general appearance of virus particles in the cytoplasm infected with another group of flexuous rod-shaped virus,

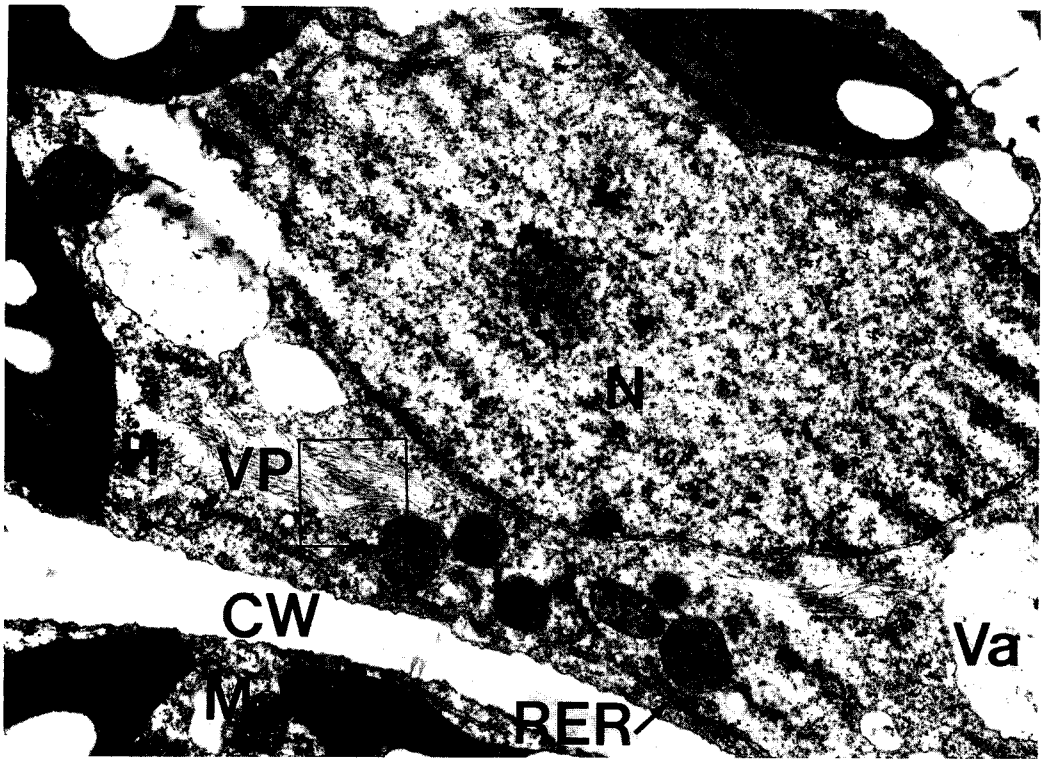

Fig. 5. A representative palisade mesophyll cell from symptomatic leaf infected with black necrotic leaf spot disease of pear, which was transmitted by graft inoculation (×20,000). This cell contains two bundles of longitudinally sectioned long flexuous rod-shaped virus-like particles (VP) in cytoplasm near the nucleus (N). CW = Cell wall; ER = Rough endoplasmic reticulum; M = Mitochondria; Va = Vacuole; CH = Chloroplast.

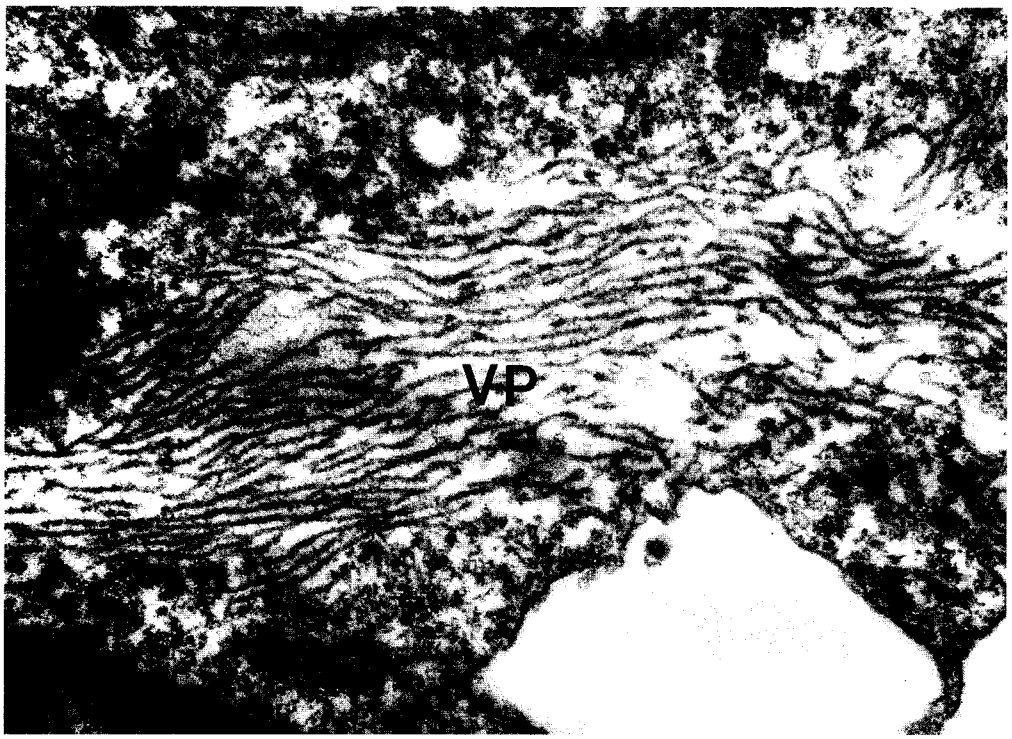

Fig. 6. Higher magnification of virus- like particles (VP) in the square area Fig. 5 in exhibiting relative lengthsand their highly flexuous nature (× 90,000).

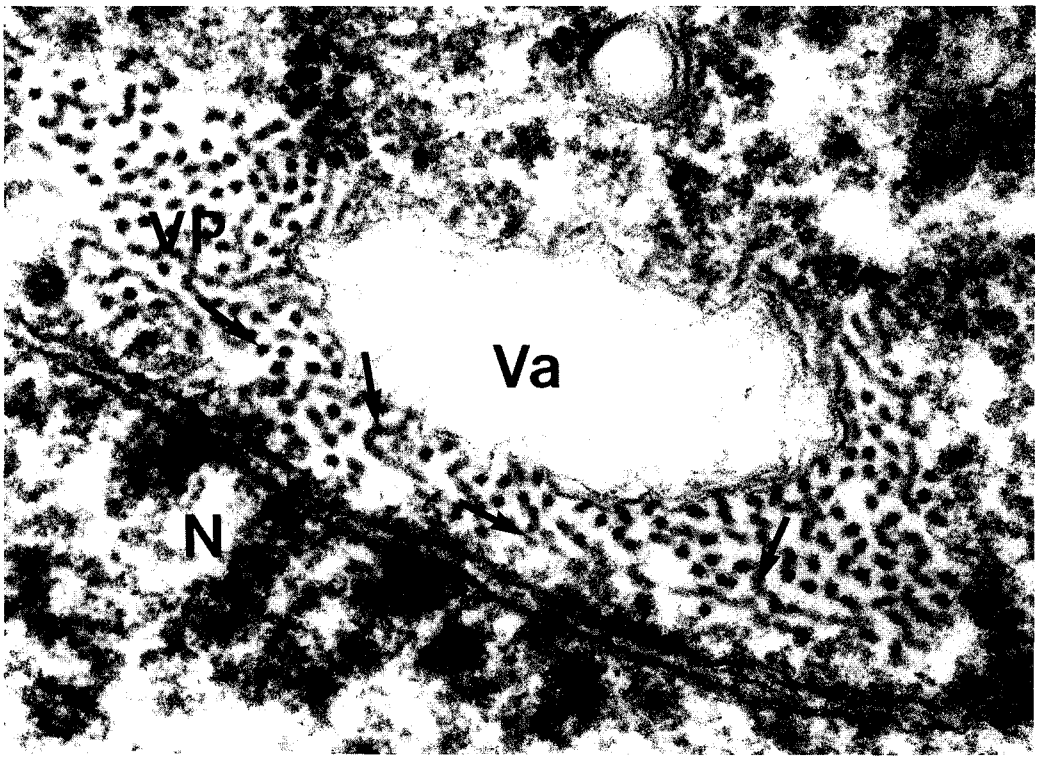

Fig. 7. Higher magnification of virus-like particles (VP) in Fig. 5 showing details. Some particles which are obliquely sectioned (arrows) are also present (×150,000). N=Nucleus; Va=Vacuole.

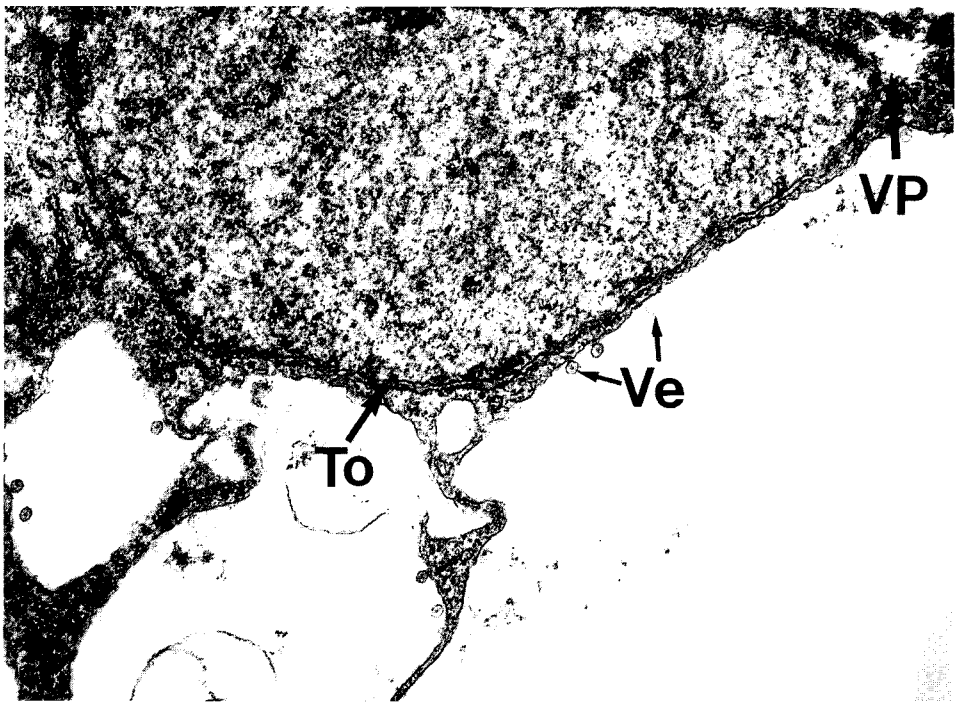

Fig. 8. A palisade mesophyll cell similar to those in Figs. 5 and 6 containing virus-like particles (VP) also contains small membraneous vesicles (Ve) with fine fibrils which are considered to be a diagnostic feature for plant viruses with ssRNA genome (×36,000). Depending on the viruses, the vesicles can be associated with membranes of various cell organelles such as the endoplasmic reticulum, mitochondria, peroxisomes, chloroplasts and tonoplasts. The vesicles in pear black necrotic leaf spot iseased cells of the present study are associated with tonoplasts (To).

carlaviruses, were also different from those observed in the pear cells of this study (Atkinson and Cooper, 1976; Brunt et al., 1976). It appeared that the bundles of flexuous rod-shaped particles present in the pear cells were morphologically similar to those in cells infected with some of the closteroviruses (Larsen et al., 1991; Zee et al., 1987), or the capilloviruses (Murphy et al., 1995), two other groups of flexuous rod-shaped plant viruses in terms of their association with fibril-containing vesicles.

Cells in pear lesions containing the bundles of virus-like particles also contained small membranous circular vesicles of approximately 50-100 nm in diameter formed at the tonoplast (Fig. 8). The vesicles contained electron-dense fine fibrils in the center (Fig. 8). Among the cytopathic effects, the fibril-containing vesicles are significant in establishing the viral etiology of black necrotic leaf spot of pears, since these vesicles have been demonstrated to be a common cytopathological structure in cells infected with plant viruses with ssRNA genome (Francki, 1987) and have contributed greatly in diagnosing many diseases with uncertain etiology (Kim et al., 1989; Larsen et al., 1991; Zee et al., 1987). Therefore, these vesicles have been considered to be as an important ultrastructural marker for diagnosing viruses with ssRNA genome, and are called as "virus vesicles" (Francki, 1986). In some virus infections, the vesicles have been demonstrated to be the site of viral RNA replication, and the fibrils within the vesicles represent viral double-stranded RNA (Assink et al., 1973; Lafleche et al., 1972; Van Kammen, 1984).

The presence of virus vesicles and associated flexuous rod-shaped virus-like particles along with phloem limitedness has been especially important in diagnosing many closteroviruses. This is because symptomatology and host range studies are of limited value since these viruses are not readily sap transmitted from their original host, and symptoms are not always distinctive (Francki et al., 1985; Larson et al., 1991; Lister and Bar-Joseph, 1981).

The virus vesicles induced by most closteroviruses have a characteristic feature and have been referred to as "BYV type" (Esau and Hoeferi, 1981; Francki et al., 1985) because they are all similar to those induced by beet yellows virus, the type member of the closteroviruses. The origin of the BYV-type vesicles has not been fully established because of the lack of spatial association with any particular organelle. It has been postulated, however, that the vesicles originate "de novo" as receptacles of the fibrils that might be the viral RNA (Atkinson and Cooper, 1976). In addition to BYV-type, mitochondria and tonoplasts have been reported to be involved in the formation of closterovirus-induced virus vesicles. Vesicles induced by dendrobium vein necrosis and grapevine leafroll virus were formed at the mitochondria (Kim et al., 1989), whereas, those induced by Diodia vein necrosis virus, a whitefly-transmitted closterovirus (Larsen et al., 1991), were formed at the tonoplasts of vacuolated phloem parenchyma cells.

Unlike most closteroviruses, however, virus particles and associated virus vesicles induced by PBNLS of the present study were not phloem-limited occurring many mesophyll parenchyma cells. In lesions of PBNLS-affected leaves, no virus particles were present in sieve elements, the most common and diagnostic site for location of the particles of closteroviruses. Virus particles of capilloviruses such as, lilac chlorotic leaf spot (Brunt and Stace-Smith, 1978) and apple stem grooving viruses (Ohki et al., 1989), which have similar particle morphology to those of clostoroviruses, are also not phloem-limited, but occur in mesophyll and phloem parenchyma cells excluding sieve elements (Murphy et al., 1995). The virus vesicles reported to be induced by a capillovirus, lily chlorotic leaf spot (Brunt and Stace-smith, 1978), were also not BYV-type, but were formed at the endoplasmic reticulum.

It must be noted that capilloviruses have formerly been considered to be clostero-like or closteroviruses based primarily on the same typical flexuous rod-shaped particle morphology (Francki et al., 1985; Candresse, 1993). Later studies, however, revealed that the sequence of the coat protein, genome organization, and strategy of gene expression between the two groups of viruses were different enough to classify them into two separate genera, the genus capillovirus and the genus closterovirus (Murphy et al., 1995). It is of interest to note that most closteroviruses are phloem-limited, while capilloviruses are not, but occur primarily in mesophyll and other parenchymatous cells.

Based on the symptomatology, graft transmissibility, and the presence of fibril-containing virus vesicles which have been considered to be a characteristic cytopathic marker for ssRNA plant virus infections, it is believed that the flexuous rod-shaped virus-like particles observed in symptomatic leaves of PBNLS represent virions of ssRNA virus, most likely a capillovirus. Fibril-containing virus vesicles observed in PBNLS have not been reported in other capillovirus infections

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