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Biological Assay and Cytopathological Characteristics of *Grapevine leafroll-associated 3 virus* (GLRaV-3) and *Grapevine fanleaf virus* (GFLV)

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Grapevine leafroll-associated 3 virus **(GLRaV-3) and *Grapevine fanleaf virus* (GFLV) are important viral diseases of grapevine in the world. In this study, the most reliable woody indicator plants were selected for virus indexing. Two grapevines, LN33 (Couderc 1613 x *Vitis berlandieri*) and *Vitis riparia* Gloire, were selected for GLRaV-3 and GFLV graft indexing, respectively. The specific characteristics of *Closterovirus* isolated from grapevines cultivated in Korea were identified. Filamentous virus-like particles only existed in the phloem parenchyma cell. In particular, the vesiculation of mitochondria was observed. This mitochondrial vesiculation was considered to be one of the most reliable cytopathic features of *Closterovirus*. During observation of GFLV-infected *Chenopodium quinoa* sections, virus-like particles arranged consistently were found forming several layers in cytoplasm. Moreover, virus-like particles in tubules were observed and were associated with plasmodesmata in cytoplasm. This is the first report on cytopathological characteristics of *Closterovirus* and *Nepovirus* identified from grapevines in Korea.**

Keywords : biological assay, *Closterovirus*, cytopathology, grapevine, *Nepovirus*.

Grapevine leafroll-associated virus (*Closterovirus*) and *Grapevine fanleaf virus* (GFLV, *Nepovirus*) are important viral diseases in grapevine worldwide. The etiology of grapevine leafroll disease has not been determined conclusively, but seven distinct phloem limited viruses are associated with the disease (Choueiri et al., 1996; Zimmerman et al., 1990). Grapevine leafroll-associated viruses (GLRaV) belong to the genus *Closterovirus*. All GLRaV are graft transmissible, and in recent years, numerous reports have shown that flexuous, filamentous *Closterovirus*-like particles ranging from 1400 to 2200 nm in length are associated with grapevine leafroll disease (Faoro et al., 1981; Milne et al., 1984; Namba et al., 1979; Zimmermann et al., 1990). These are serologically distinct, thus, have been designated as GLRaV-1 to -7. GLRaV-3 is the best characterized *Closterovirus* associated with leafroll disease. Of the seven GLRaVs, GLRaV-3 has been reported to be transmitted by vectors such as mealybugs.

In Korea, GLRaV-3 was the most prevalent viral disease, but GFLV has not been detected in cultivated grapevines so far (Kim, 2000). Although leafroll symptom is not usually lethal, it causes erratic bearing, and reduces sugar content to about 25-50% than fruits from healthy vine (Goheen, 1970; Goheen and Cook, 1959; Hewitt, 1968). Flexuous, filamentous closterovirus-like particles ranging from 1400 to 2200 nm in length and 12 nm in diameter are closely associated with grapevine leafroll diseases (Faoro et al., 1981; Hu et al., 1990).

There is no suitable herbaceous host for the viruses. In the spring, leaves of diseased and healthy grapevines appear to be similar, but as the season progresses, the diseased leaves turn yellowish or reddish, depending on the cultivar. Symptoms begin to appear on the lower leaves near the base of infected vines and become more intense with time. In fall, the interveinal area of the leaf blade may be bright yellow or red, depending on the anthocyanin pigments present in the cultivar, while the major veins in the leaf remain green (Goheen et al., 1958).

GFLV is a very damaging virus, causing reduced yields due to poor berry set. The reduction in yield can be over 80% in some varieties (Bovey, 1973). Symptoms include characteristic fan-like distortions of leaves, as well as, ringspots, line patterns, vein banding, yellowish mottling, and yellow mosaic pattern in the early growing season. The virus is transmitted by a nematode (*Xiphinema index*) and can infect all *Vitis* species.

This paper reports on the cytological characterization of leafroll or fanleaf-diseased grapevine leaves such as the presence of virus-like particles and cytopathic effects characteristic of *Closterovirus* or *Nepovirus* infection.

Materials and Methods

Leafroll and fanleaf virus isolates. Virus infected vines were

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selected through ELISA using antiserum purchased from BIOREBA Co. (Switzerland). The leafroll isolate was *Vitis vinifera* cv. Rubi Okuyama, and the fanleaf isolate was 7120CL rootstock cultivar selected from the germplasm of the National Horticultural Research Institute, Suwon, Korea.

Biological indexing. Graft inoculation was done using the following indicators: LN33 (Couderc 1613 x *Vitis berlandieri*), *Vitis rupestris* St. George, *Vitis berlandieri* x *V. riparia* Kober5BB, *V. vinifera* cv. Cabernet franc, *Vitis berlandieri* x *V. rupestris* 110R, and *V. riparia* Gloire. Inoculated plants, as well as, non-inoculated healthy controls were grown in plastic pots under field condition. The vines were observed for symptom expression for three consecutive growing seasons.

Electron microscopy. Leafroll and reddening leaves were collected from virus inoculated 'LN33' vines reacting positively to GLRaV-3 and used as source of *Closterovirus*. Meanwhile, GFLV sap inoculated *Chenopodium quinoa* leaves were used as *Nepovirus* source. For thin-section electron microscopy, leaf specimen (1-2 mm^2) was taken from earlier symptomatic leaves and fully progressed leaves including the midrib of plants showing leafroll and reddening symptoms. GFLV infection was taken from severe mottling leaves of *Chenopodium quinoa*. Similar samples were taken from healthy plants. Specimens were fixed in Karnovsky's fixative solution overnight at 4°C. After three times washing for 20 minutes with 0.05M cacodylate buffer (pH 7.2) solution, the tissues were postfixed in 1% osmium tetraoxide solution for 2 h, and pre-stained with 0.5% uranyl acetate overnight at 4°C (Kim et al., 2001). Tissues were dehydrated in an ethanol series (30-100%) and 100% propylene oxide, and then, embedded in Spurr's resin. Thin sections cut with a diamond knife were double stained with 2% uranyl acetate and lead citrate before examination under a transmissible electron microscope (CarlZeiss LEO 906).

Results

Biological and cytopathological characteristics of GLRaV-3. Leafroll and reddening symptoms typically appeared in 'LN33' indicator plant. Leafroll symptom initially appeared 2 months after inoculation, but typical diagnostic symptoms were observed in younger and mature leaves the following year. These symptomatic leaves showed positive reactions to GLRaV-3 antiserum by ELISA (Table 1). Leafroll and reddening were observed on lower leaves of vines from August and became more intense, progressing to the upper leaves. At the early stage, the leaf blades started to become bright red with downward rolling of the leaf margins (Fig. 1A). Other symptoms such as rolling of upper leaves and dark reddish leaves with green main veins also appeared (Fig. 1B).

Observation of these symptomatic leaves through electron microscopy showed long, flexuous, filamentous particles, which were absent in healthy leaves. Virus-like particles were also found only in sieve elements and

Table 1. Symptoms on leaves and stems of woody indicators inoculated with GLRaV-3 and GFLV

Inoculated viruses	Indicators	Symptoms[a]	Symptom severity[b]	ELISA reaction[c]
GLRaV-3	Cabernet franc	S	–	–
	LN33	LR, Rd	S	+
	Vitis riparia Gloire	Sp, M	L	–
	110R	S	–	–
	St. George	S	–	–
	Kober5BB	M	L	+/–
GFLV	Cabernet franc	mM	L	+
	LN33	LR	L	+
	Vitis riparia Gloire	CS, YM, Fl	S	+
	110R	CS	M	+
	St. George	mM, Fl	M	NT
	Kober5BB	Mf, M	L	NT

[a]S: symptomless, LR: leafroll, Rd: reddening, Sp: spot, M: mosaic, mM: mild mosaic, CS: chlorotic spot, YM: yellowish mottling, Fl: fanleaf, Mf: malformation.
[b]L: light, M: moderate, S: severe
[c]+: positive reaction, –: negative reaction, +/–: positive limit absorbency, NT: not tested.

Fig. 1. Symptoms of grapevine leaves infected with *Grapevine leafroll-associated 3 virus* and *Grapevine fanleaf virus* (GFLV). **A**: leaf downward rolling and reddening on LN33 (Couderc 1613 x *Vitis berlandieri*). **B**: leaf reddening and rolling on *Vitis vinifera* cv. Rubi Okuyama. **C**: fanleaf and yellowish mottling on *Vitis riparia* Gloire. **D**: Systemic mottle on *Chenopodium quinoa* leaves sap inoculated with GFLV.

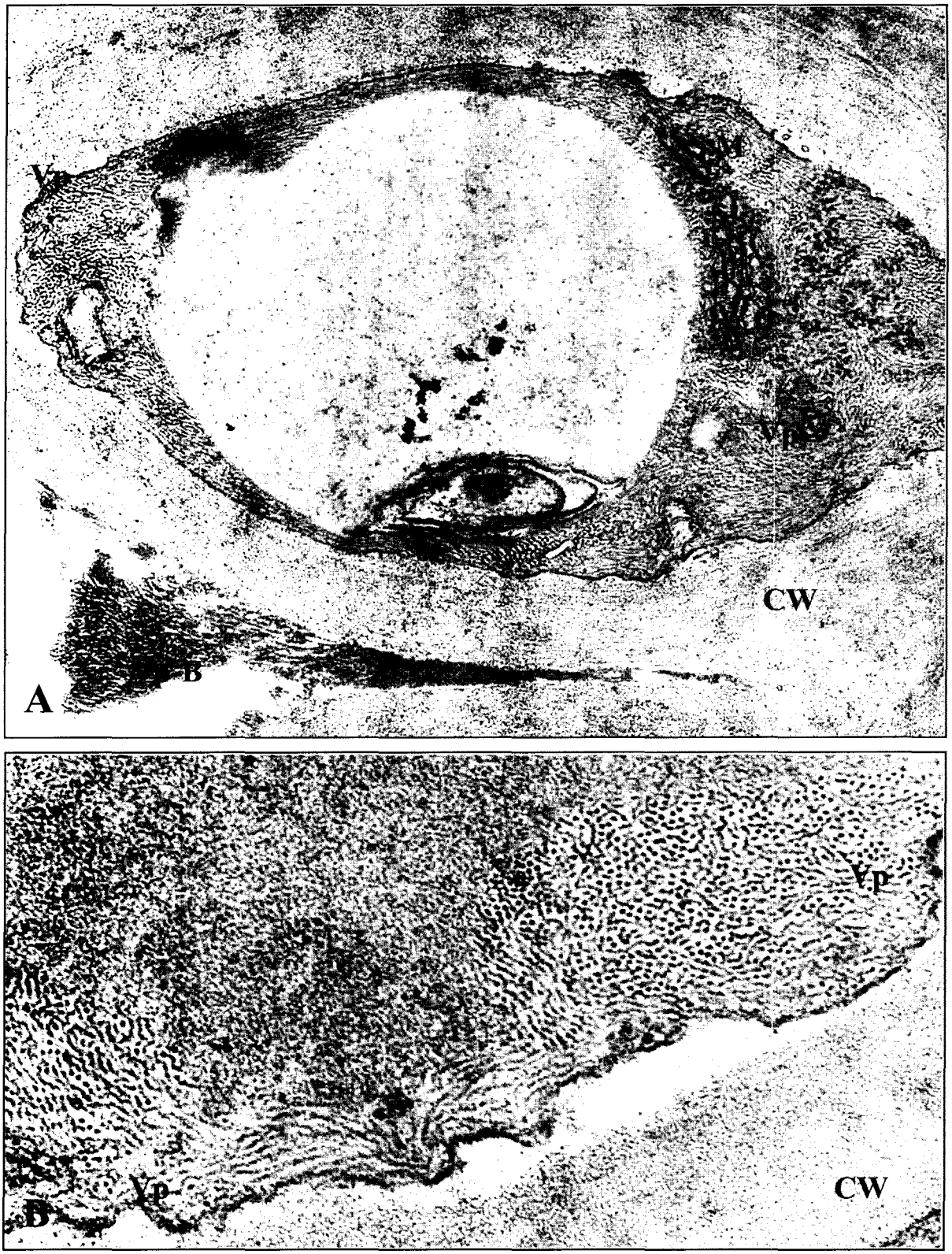

Fig. 2. Electron micrographs of grapevine leaf infected with GLRaV-3. **A**: Phloem parenchyma cell containing masses of flexible filamentous virus-like particles (Vp) in the cytoplasm. Electron densely packed bundles of virus-like particles (Vp-B) in parenchyma cell. M; mitochondria, **B**: Virus particles sectioned transversely and longitudinally, CW: cell wall.

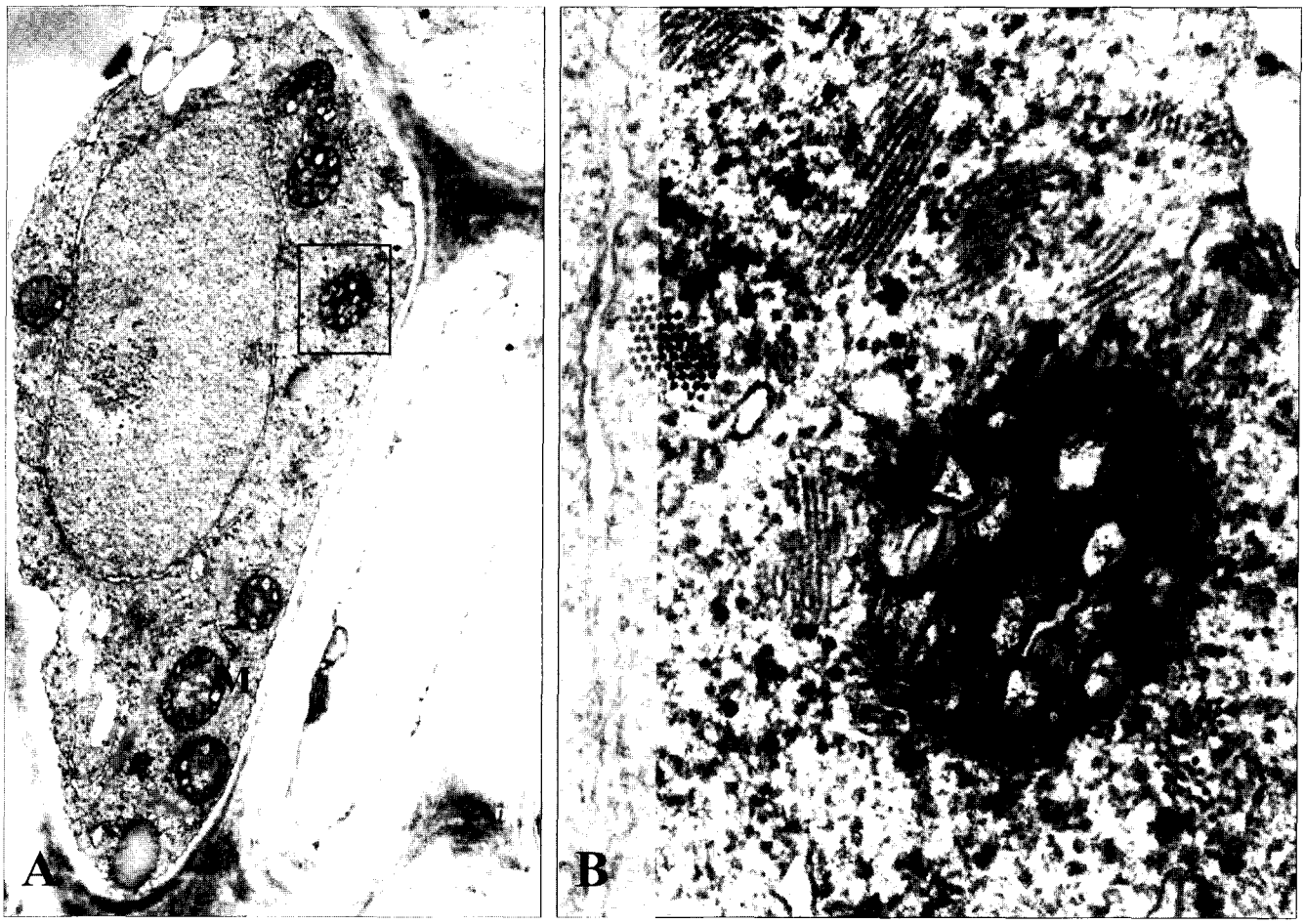

Fig. 3. Electron micrographs of grapevine leaf infected with GLRaV-3. **A**: A companion cell containing several vesiculated mitochondrias (M) in sieve element. **B**: A magnification of squared area of panel A.

parenchyma cells of the vascular bundles. Masses of virus-like particles and several lipids were observed in phloem parenchyma cell of leaves infected with GLRaV-3. Electron densely packed aggregates of virus-like particles were observed in parenchyma cell (Fig. 2A, B). A specific mitochondrial vesiculation was found in the samples (Fig. 3). This mitochondrial vesiculation was reported as a specific cytopathic feature of *Closterovirus* (Kim et al., 1989; Zee et al., 1987). Therefore, these isolates were identified as *Closterovirus* through cytology characteristics such as presence and morphology of virus-like particles.

In the reddened leaf, several inclusions assumed to be associated with anthocyanin pigments were scattered in sieve tubes, and the number of inclusions increased according to the degree of leaf reddening (Fig. 4A, B). Starch accumulation and several lipid grobules in chloroplast were examined in reddening leaves, which differed from the healthy leaves. Eventually, phloem parenchyma cell necrosis was observed in fully reddened leaves (Fig. 4C, D).

Biological and cytopathological characteristics of GFLV. Among the GFLV inoculated vines, *V. riparia* Gloire expressed the most severe symptoms. Chlorotic spot was observed 40 days after inoculation in several indicators. However, younger and mature leaves of *V. riparia* Gloire during the following sprouting season showed more reliable symptoms such as fanleaf and yellowish mottling (Table 1, Fig. 1C). Sap inoculated *C. quinoa* leaves showed systemic severe mottling and vein clearing (Fig. 1D). Apical necrosis was also observed. Virus-like particles were arranged consistently forming several layers in cytoplasm of GFLV sap inoculated *Chenopodium quinoa* leaves (Fig. 5A, B). Virus-like particles in a tubular membrane were investigated in cytoplasm and were associated with plasmodesmata. An unusual number of vesicles were observed in the cytoplasm of GFLV infected cell (Fig. 5C, D).

Discussion

GLRaV-3 and GFLV are important viral diseases of

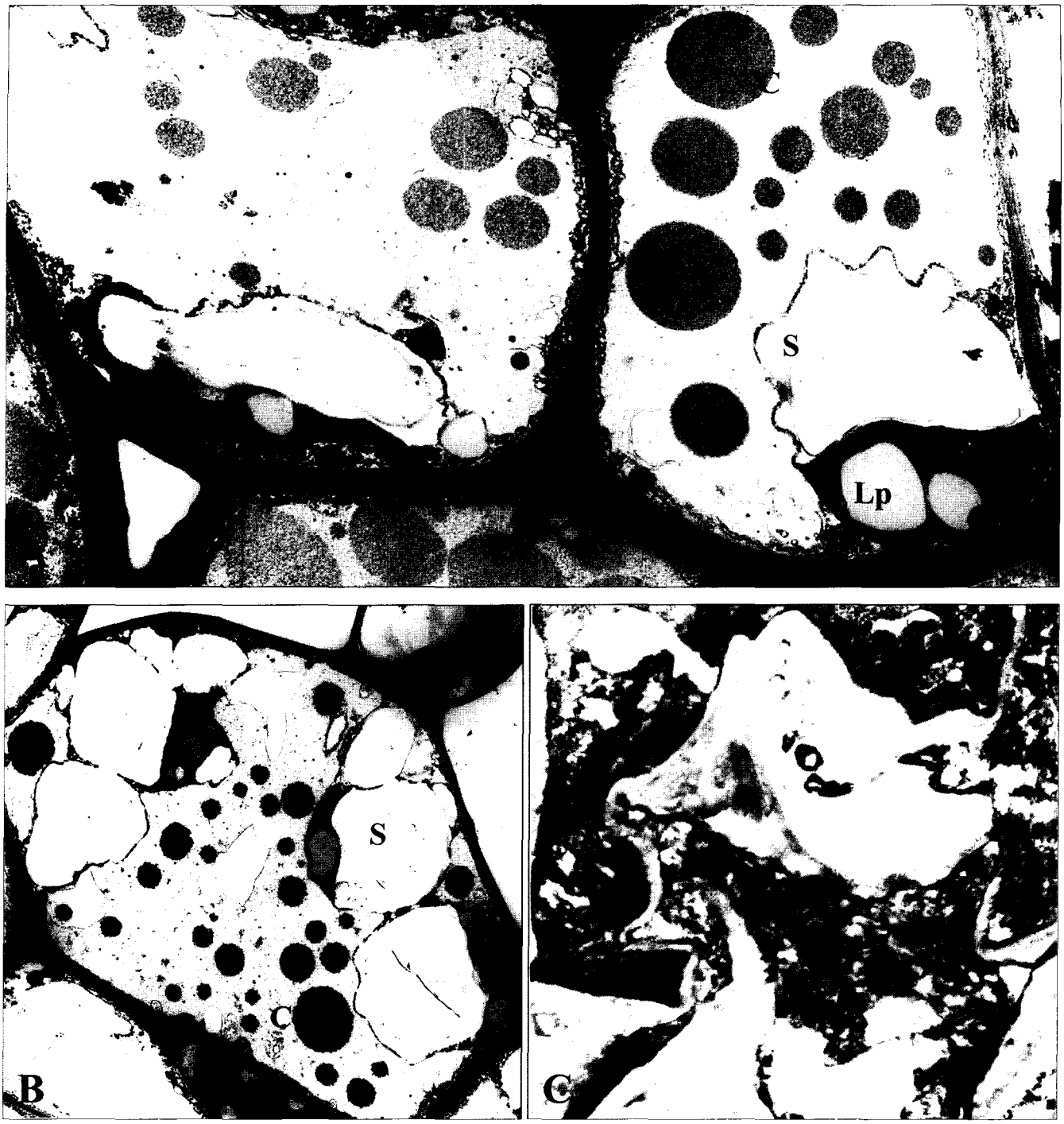

Fig. 4. Electron micrographs of grapevine leaf infected with GLRaV-3. **A**: Sieve tube showing several inclusions (C) assumed to be associated with anthocyanin in early reddening leaves. **B**: Starch (S) and lipid (Lp) accumulation in chloroplast and many inclusions in cytoplasm of sieve element in reddening leaves. **C**: Phloem parenchyma cell necrosis at fully reddened leaves.

grapevine worldwide. While GFLV has not been observed in Korea, GLRaV-3 was one of the most widespread viral diseases in the country (Kim, 2000). However, most cultivars such as 'Kyoho' and 'Campbell early' did not express typical viral symptoms on the vines. Thus, biological assay using hosts sensitive to each virus was an essential procedure. Several indicators have been used for viral disease indexing in other countries, but in Korea, results are not available until now. In this study, the most reliable woody indicator plants were chosen for the two

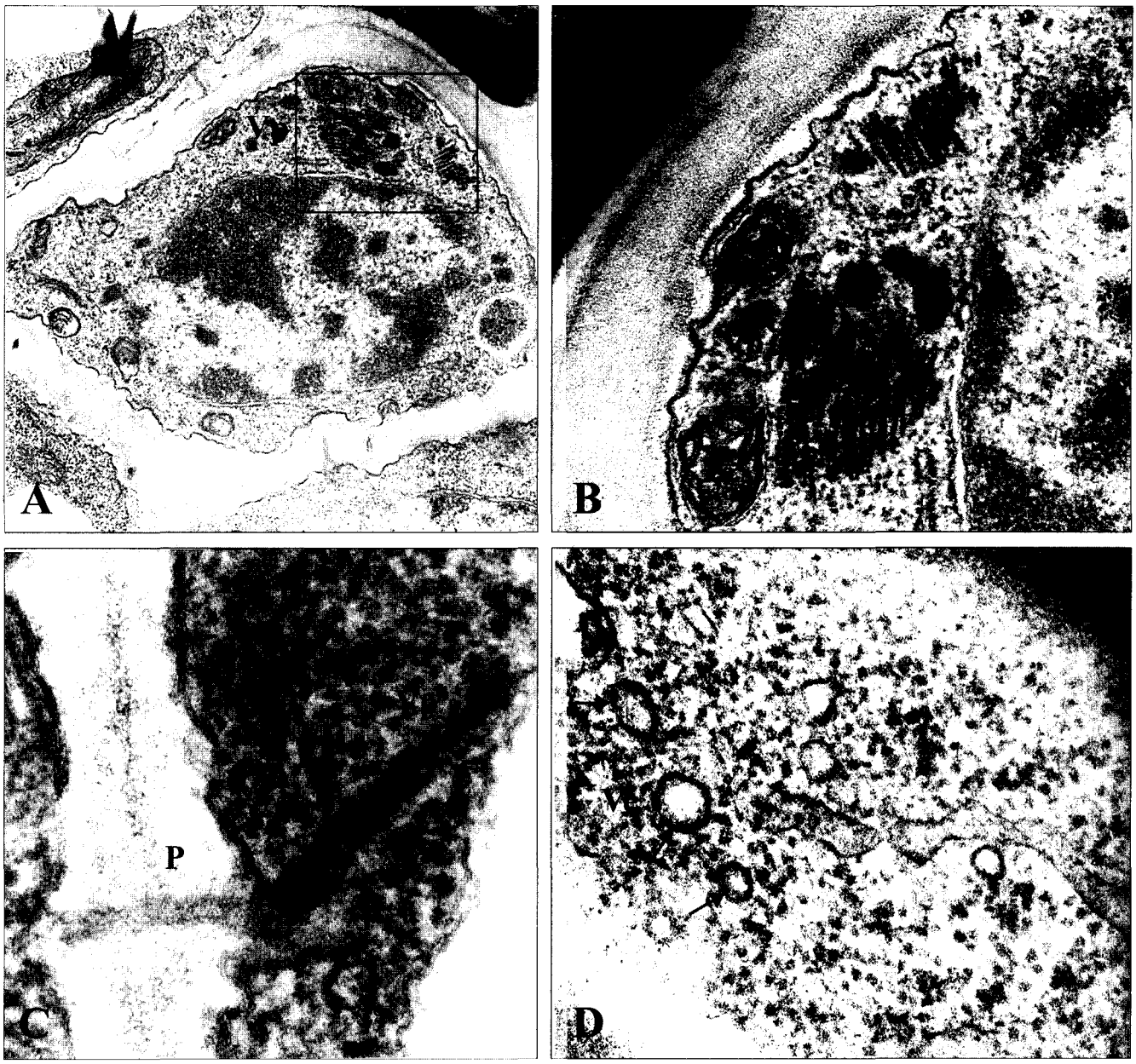

Fig. 5. Electron micrographs of *Grapevine fanleaf virus* infected *Chenopodium quinoa* leaves. **A**: Spherical virus-like particles (Vp) arranged consistently forming several layers in cytoplasm of mesophyll cell. **B**: Higher magnification of squared area of panel A, **C**: Spherical virus-like particles in tubular membrane associated with plasmodesmata (P) in cytoplasm, **D**: Several vesicles (Ve) formed in cytoplasm.

viruses indexing. LN33 (Couderc 1613 x *Vitis berlandieri*) and *Vitis riparia* Gloire were selected for GLRaV-3 and GFLV graft indexing, respectively.

This study found the specific characteristics of *Closterovirus* in virus-infected grapevine leaves. Filamentous virus-like particles only existed in the phloem parenchyma cell, and were never seen in the mesophyll cell. Virus-like particle morphology and phloem-limited cytopathic effects of ultra-structural characteristics were similar with those occurring in other known *Glosterovirus* infection (Francki et al., 1985; Lister and Bar-Joseph, 1981).

In particular, vesiculation of mitochondria was observed. This mitochondrial vesiculation has been reported previously in grapevine with leafroll symptoms, and is considered to be one of the most reliable cytopathic features (Zee et al., 1987; Kim et al., 1989). Cytoplasmic membranous vesicles induced by most *Closteroviruses* have a characteristic morphology. The BYV-type vesicle has also

been reported in most *Closterovirus* infected samples (Esau and Hoefert, 1971; Francki et al., 1985).

The recognition of *Nepovirus* particles in thin-sectioned cells presents some difficulties, because of their resemblance to ribosomes (Francki et al., 1985). Virus particles and ribosomes could not be easily distinguished. However, virus-like particles in tubules, associated with plasmodesmata in cytoplasms, were found. Cytoplasmic tubules containing *Nepovirus* were first observed in CLRV infected *Nicotiana rustica* thin section (Walkey and Webb, 1968). Based on the results of these specific cytopathological characteristics, *Closterovirus* and *Nepovirus* in grapevines can be identified.

References

Bovey, R. 1973. Maladies a virus et a mycoplasmes de la vigne. Station federale de recherches agronomiques de Changins. p. 76, Suisse.

Choueiri, E., Bosica, D., Digiaro, M., Castellano, M. A. and Martelli, G. P. 1996. Some properties of hitherto undescribed filamentous virus of the grapevine. *Vitis*. 35:91-93.

Esau, K. and Hoefert, L. L. 1971. Cytology of beet yellows virus infection in Tetragonia. I. Parenchyma cells in infected leaf. *Protoplasma* 72:255-273.

Faoro, F., Tornaghi, R., Fortusini, A. and Belli, G. 1981. Association of a possible closterovirus with grapevine leafroll in northern Italy. *Rev. Pathrl. Veg. Ser.* IV 17:183-189.

Francki, R. I. B., Milne, R. G. and Hatta, T. 1985. *Nepovirus* and *Closterovirus* group. In: Atlas of Plant Viruses. Vol. II. CRC Press. Boca Raton. FL.

Goheen, A. C. 1970. Virus and virus-like diseases of the grapevine. pp. 209-212. In: Virus diseases of small fruits and grapevines. N.W. Frazier, ed. University of California, Berkeley.

Goheen, A. C. and Cook, A. J. 1959. Leafroll (Red-leaf or Rougeau) and its effects on vine growth, fruit quality, and yields. *Am. J. Enol. Vitic.* 10:173-181.

Goheen, A. C., Harmon, F. N. and Weinberger, J. H. 1958. Leafroll (white emperor disease) of grapes in California. *Phytopathology* 48:51-54.

Hewitt, W. B. 1968. Viruses and virus-like diseases of the grapevine. *Rev. Appl. Mycol.* 47:433-455.

Hu, J. S., Gonsalves, D. and Teliz, D. 1990. Characterization of *Closterovirus* like particles associated with grapevine leafroll diseases. *J. Phytopathol.* 128:1-14.

Kim, H. R. 2000. Molecular biological characteristics of Grapevine Leafroll-associated 3 *Closterovirus* and establishment of virus-free stock production system. *Ph.D thesis. Kyungpook National University.* pp. 32-33.

Kim, J. S., Cho, J. D., Choi, H. S. and Kim, K. S. 2001. Ultrastructural aspects of the mixed infections with Turnip mosaic virus and Ribgrass mosaic virus in oriental cabbage. *Plant Pathol. J.* 17:201-204.

Kim, K. S., Gonsalves, D., Teliz, D. and Lee, K. W. 1989. Ultrastructure and mitochondrial vesiculation associated with closteroviruslike particles in leafroll-diseased grapevines. *Phytopathology* 79:357-360.

Lister, R. M. and Bar-Joseph, M. 1981. *Closterovirus*. pp. 809-844. In: Handbook of plant virus infection and comparative diagnosis. E. Kurstak, ed. Elsevier/North Holland Biomedical Press, New York.

Milne, R. G., Conti, M., Lesemann, D. E., Stellmach, G., Tanne, E. and Cohen, J. 1984. *Closterovirus*-like particles of two types associated with diseased grapevines. *Phytopathol. Z.* 110:360-368.

Namba, S., Yamashita, S., Doi, Y., Yora, K., Terai, Y. and Yano, R. 1979. Grapevine leafroll virus, a possible member of *closteroviruses*. *Ann. Phytopathol. Soc. Japan* 45:479-502.

Walkey, D. G. A. and Webb, M. J. W. 1968. Virus in plant apical meristems. *J. Gen. Virol.* 3:311.

Zee, F., Gonsalves, D., Goheen, A., Kim, K. S., Pool, R. and Lee, R. F. 1987. Cytopathology of leafroll-diseased grapevines and the purification and serology of associated closterovirus-like particles. *Phytopathology.* 77:1427-1434.

Zimmerman, D., Bass, P., Legin, R. and Walter, B., 1990. Characterization and serological detection of four closterovirus-like particles associated with leafroll disease in grapevines. *J. Phytopathol.* 130:205-218.