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Lettuce Leafroll Mosaic-a New Lettuce Disease Caused by Caulimovirus-like Agent in Taiwan

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ABSTRACT

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Asparagus lettuces (*Lactuca sativa* L. var. *asparagina* Bailey) with mosaic, leafroll, and stunting were discovered in the field of Taichung, Taiwan. These lettuce plants were infected by a caulimovirus-like agent. The isometric particles of the viral agent, which were about 50 nm in diameter, formed characteristic inclusions of caulimoviruses in infected cells. The particles were transmitted by means of mechanical inoculation or by aphids (*Myzus persicae* Sulzer) in a nonpersistant or semipersistant manner to *Lactuca scariola* var. *sativa*, *L. sativa* var. *asparagina*, *L. sativa* var. *crispa*, and *L. sativa* var. *capitata* only. This is the first report of a caulimovirus infection on lettuces in nature.

Key words: asparagus lettuce, caulimovirus, inclusion, *Lactuca sativa* var. *asparagina*.

INTRODUCTION

Asparagus lettuce (*Lactuca sativa* L. var. *asparagina* Bailey), a lettuce with large edible stem, is a commercially important winter vegetable in Taiwan. In 1991, during a field survey for lettuce mosaic virus (LMV) isolates from lettuce at Taichung area, some asparagus lettuce plants with viruslike symptoms-typically leafroll, mosaic and stunting, were discovered. The disease symptoms resembled that of LMV except that showed downward leafroll in lower leaves and no tiny necrotic spots in young leaves. When investigated by electron microscopy, only several spherical virus-like particles (VLPs) about 50 nm in diameter were found in the preparations from the diseased leaf sap.

Caulimoviruses were known to be isometric particles which were approximately 50 nm in diameter (11). The virus can be transmitted mechanically or by aphids in a non-persistant or semi-persistant manner in nature. One of the characteristics of this viral infection is that it forms spherical or ellipsoidal cytoplasmic inclusions in infected cells. The inclusion bodies consist mainly a finely granular matrix with several electron-transparent area of varied sizes. The host ranges of caulimoviruses are generally relatively narrow (11).

Up to now, about eleven definitive and six tentative members of caulimovirus have been reported (9,12). Two caulimoviruses, dahlia mosaic virus (DMV) (2) and thistle mottle virus (ThMoV) (8), infected plant family Compositae. DMV is world-wide distributed, which only infected *Dahlia* spp. in nature, and can be transmitted to some plant genera in family Amaranthaceae, Chenopodiaceae, Compositae, and Solanceae by mechanical inoculation and by aphids experimentally. However, it did not infect lettuce (1,3). ThMoV exists only in certain restricted regions in Europe (11).

The virus-like particles (VLPs) were isolated from the above described diseased asparagus lettuces. In this report, some properties of this VLPs were described. The known physical and biological characteristics indicated that it is possibly a new member of the Caulimovirus group.

MATERIALS AND METHODS

Virus source and maintenance

The VLPs were isolated from naturally infected asparagus lettuce plants collected in Taichung. Because no local lesion hosts were found, and the possible contaminating LMV have lower thermal inactivation points than caulimoviruses (11,24), we treated the tissue homogenate of infected asparagus lettuce by heating (75 C for 10 min) in a water bath and isolated the VLPs via whole plant assay to assure the identity of the isolate. The VLPs were maintained in asparagus lettuce or garden lettuce (*Lactuca scariola* L. var. *sativa* Bisch.) by mechanical inoculation. Inoculum for mechanical inoculation was prepared by homogenizing 1 g (fresh

weight) of infected lettuce leaves with 10 ml of 0.1 M potassium phosphate buffer (KPB), pH 7.2, containing 1% sodium sulfite. Inoculation was carried out by rubbing the inoculum on leaves of healthy lettuces previously dusted with carborundum (400 mesh). All plants used for the performed experiment were grown in a greenhouse.

Host range test

Thirty-eight species of plants belonging to 8 different plant famalies were inoculated with tissue homogenates obtained from infected lettuce plants or with aphids (*Myzus percicae* Sulzer) which acquired VLPs from infected lettuce plants. Appearance of observal symptoms was monitored daily for about 6 weeks. Symptomless plants were mechanically back-inoculated to asparagus lettuces and examined for the existance of inclusion bodies by light microscope about 6 wk after inoculation.

Effect of physical factors on infectivity of tissue homogenates

Tissue extracts obtained from apparently disease-affected *L. sativa* var. *asparagina* by homogenization in KPB were used for the determination of the dilution end point (DEP), the thermal inactivation point (TIP) and the longevity in vitro (LIV) assay using the *L. sativa* var. *asparsgina* as an bioassay host. A 1:10 dilution of homogenate was used for the TIP and LIV tests; and a ten-fold dilution series was used for the DEP tests. All the performed assay were carried out by mechanical inoculation as described above.

Light microscopy

Epidermal strips were removed from the underside of the apparently infected or the healthy appearing lettuce leaves with forceps, stained with a mixture of 5 volumes of 1.25% (W/V) Azure A and 1 volume of 0.2 M disodium phosphate for 15 min, destained with 95% ethanol for 3–5 min, and treated with 2-methoxyethyl acetate for 10 min. The strips were mounted in LR White resin for light microscope examination (5,17).

Electron microscopy

For negative staining, leaf extracts obtained by grinding small pieces of infected lettuce leaves in KPB were floated on copper grids covered with Fomvar for 2 min, washed with distilled water, and then stained with 2% uranyl acetate, pH 4.2 (6). For ultrathin sectioning, infected leaf tissues were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4 C for 4 hr, then postfixed with 1% osmium tetroxide in the same buffer for 2 hr. After fixation, the tissues were dehydrated in a gradual series of ethanol followed by infiltration with LR White resin and embedded in LR White (16). Specimens were cut with a diamond knife and were double stained with 2% uranyl acetate and 0.4% lead citrate (25). Ultrastructural examination was carried out with a JOEL Model 200 CX electron microscope.

Transmission

The mechnical transmission was performed by the method described above. Asparagus lettuce, garden lettuce, cabbage lettuce (*L. sativa* var. *capitata* L.) and crinkle garden lettuce (*L. sativa* var. *crispa* L.) were used as assay host to illustrate the differential susceptibility among the lettuce varieties.

As for insect transmission, virus free aphids, *Myzus persicae* Sulzer and *Aphis gosspii* Glover, were reared on tobacco (*Nicotiana tabacum* cv. Van Hicks) and cucumber (*Cucumis sativus* L.) plants, respectively. In the transmission efficiency tests, the aphids were first transferred to Petri dishes and starved for 2 hr. These aphids were later allowed to feed for 1 to 20 min on infected lettuce leaves and then transferred to healthy lettuce plants. The aphids were caged around the midrib of a young leaf on each healthy plant with a cage which has an open-end covered with nylon sieve. The feeding lasted until next morning. One to 15 aphids were used for each test plant.

In the tests for retention time determination, *M. persicae* were starved and allowed to acquire viruses as described above. After fed on first test plant for 10 min, the aphids were transferred to the second test plant. Repeated transfers at the interval of 30 min were then followed upto 240 min. Five aphids per plant were used.

For seed transmission test, the seeds were collected from apparently infected asparagus lettuces and planted in plastic pots for a continual observation on the symptom development. All the seedlings were also examined by light microscopy for the presence of virus associated inclusions.

RESULTS

Symptoms and host range

Naturally infected asparagus lettuce plant (Fig. 1-A) generally showed mosaic on upper leaves, downward leafroll and vein chlorosis on lower leaves. And all the affected plants were severely stunted. Symptoms on lettuces inoculated mechanically or by aphids in the greenhouse with the VLPs varied with the varieties. *L. sativa* var. *asparagina* initially showed vein clearing and mosaic on young leaves, the leaves then became downward leafrolling, vein chlorosis and vein banding (Fig. 1-B). *Lactuca scariola* var. *sativa*, also showed vein clearing and mosaic on young leaves first, then the leaves became rugose and showed downward leafrolling (Fig. 1-C). *L. sativa* var. *crispa* L. showed mosaic and vein clearing on young leaves, the leaves were reduced

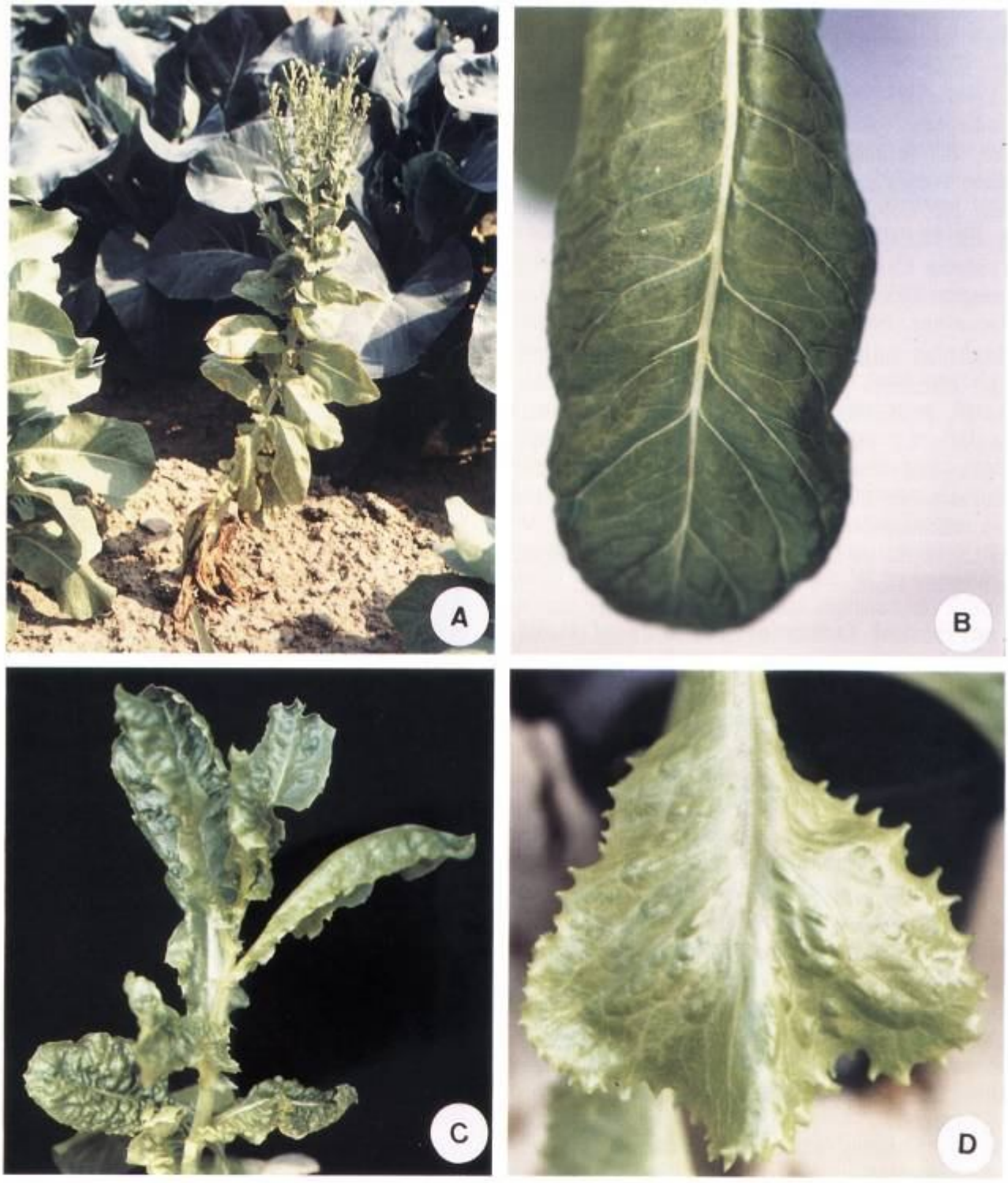

Fig. 1. Symptoms of lettuces caused by caulimovirus-like agent. A. An infected asparagus lettuce in the field, showed mosaic, downward leafroll, and stunting. Uninfected plant showed part of healthy leaves on the left. B, C, and D. Symptoms of *Lactuca sativa* var. *asparagina, L. scariola* var. *sativa,* and *L. sativa* var. *crispa,* respectively, after mechanical inoculation with caulimovirus-like agent.

in size as compared with leaves on uninfected plants (Fig. 1-D). No other symptoms were observed on this variety. All inoculated lettuce plants showed stunting symptom. Incubation period of symptom development on plants varied with season. Symptoms generally appeared about 14 days after inoculation in summer and about 30 days in winter.

Among 38 species of plants that had been inoculated mechanically or by aphids, the VLPs infected only *Lactuca* spp. and showed systemic symptoms. All the other tested plants showed no detectable symptoms. The following plant species were not infected by test VLPs, and their virus-free status were confirmed by back-inculation to healthy lettuces

and by examination for the absence of inclusion bodies under light microscope: Amaranthaceae – *Amaranthus mangostanus* L., *Gomphrena globosa* L.; Caryophyllaceae – *Dianthus caryophyllus* L.; Chenopodiaceae – *Chenopodium amaranticolor* Coste & Reyn., *C. quinoa* Willd., *Spinacia oleracea* L.; Compositae – *Ageratum houstonianum* Mill., *Calendula officinalis* L., *Centaurea cyanus* L., *Chrysanthemum coronarium* L., *Crassocephalum ranben* S. Moore, *Dahlia pinnosa* Cav., *Wedlia chinensis* (Osbeck) Merr., *Zinnia elegans* Jacq.; Cruciferae – *Brassica chinensis* L., *B. oleracea* var. *botrytis* L., *B. oleracea* var. *capitata* DC., *Raphanus sativus* L.; Cucurbitaceae – *Cucumis sativus* L., *Cucurbita pepo* L.; Leguminosae – *Arachis hypogaea* L., *Phaseolus angularis* Wight, *P. vulgaris* L., *Pisum sativum* L., *Vigna unguiculata* (L.) Walp. subsp. *unguiculata;* and Solanaceae – *Capsicum annuum* L., *Datura stramonium* L., *Lycopersicon esculentum* Miller, *Nicotiana benthamiana* Domin., *N. clevelandii* Gray., *N. glutinosa* L., *N. rustica* L., *N. tabacum* L. cv. Van Hicks, *Petunia hybrida* Hort..

Effect of physical factors on infectivity of tissue homogenates

Virus-like particles in tissue homogenate appeared to retain infectivity after being diluted to 10^{-3}, but not to 10^{-4}; after being stored at 20 C for 1 day, but not for 2 days; and after being heated at 85 C for 10 min, but not at 90 C.

Light microscopy

When stained with Azure A, spherical vacuolated inclusions (Fig. 2). were observed in the leaf epidermal strips of infected lettuces. These inclusions were light purple in color, 3–10 μm in diameter, present within the cytoplasm of epidermal and guard cells (Fig. 2-A) and mostly located beside or near but not inside the nucleus. Most examined cells contained only single inclusion with few exceptions that contained more than one (Fig. 2-B).

Electron microscopy

In negative staining of leaf extract from infected lettuce, the VLPs appeared to be isometric and were about 50 nm in diameter (Fig. 3–A). The ultrathin section profile revealed that most inclusions were spherical or ellipsoidal. All the inclusions consisted of a finely granular, electron dense material in which electron transparent areas of variable sizes were found scattered throughout the matrix (Fig. 3–B). In the ground substance and electron transparent area of the inclusions, variable numbers of isometric VLPs about 45 nm in diameter were often observed. The number of the VLP seems to be a function of the developmental states of the inclusions. There were no discernible membrane surrounding the inclusions; but the surface

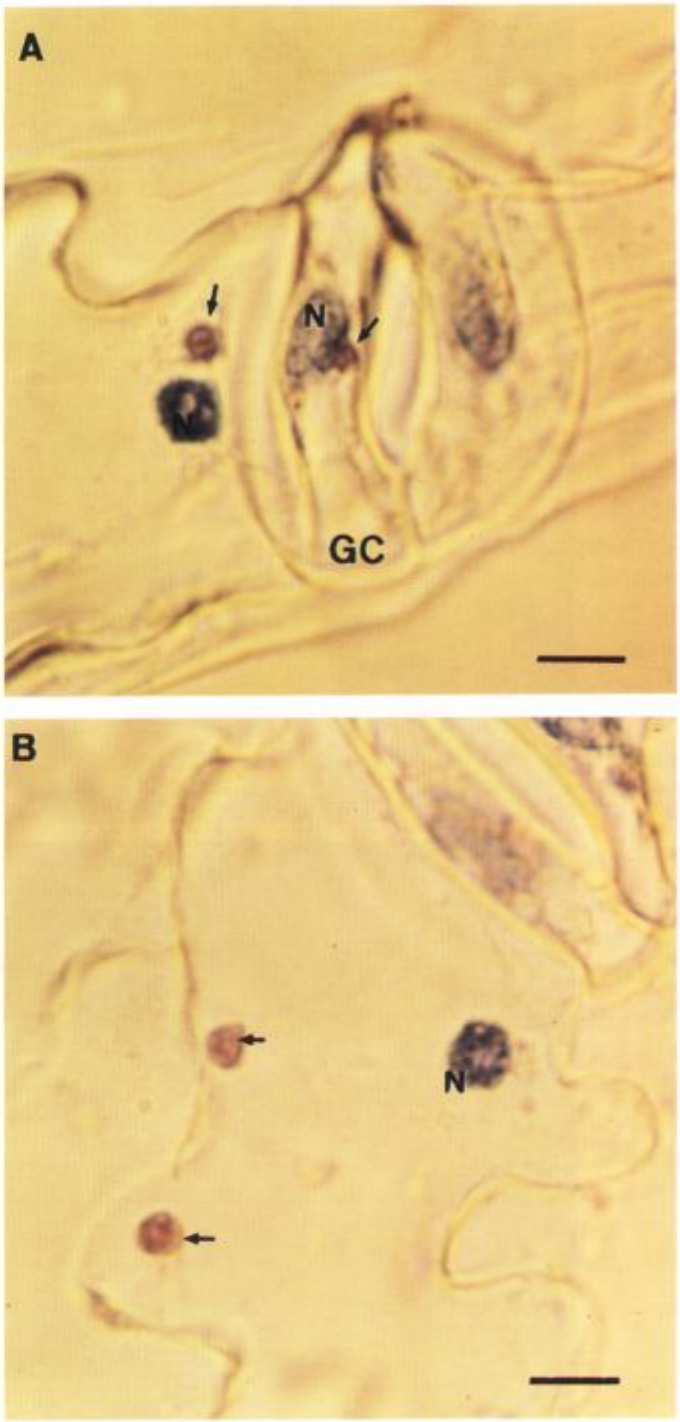

Fig. 2. Inclusion bodies (arrow) in epidemis of *Lactuca sativa* var. *asparagina*. A. Inclusion bodies within epidemic and guard cells. B. An epidemic cell containing two inclusion bodies. N: nucleus, GC: guard cell. Bars = 10 μm.

of inclusions was always covered by ribosome-like structures. The infection seemed not causing any substantial changes in regard to the shape of nucleus and other organelles.

Trasnmission

The VLP was transmitted by mechanical inoculation and by *Myzus persicae*, but not transmitted

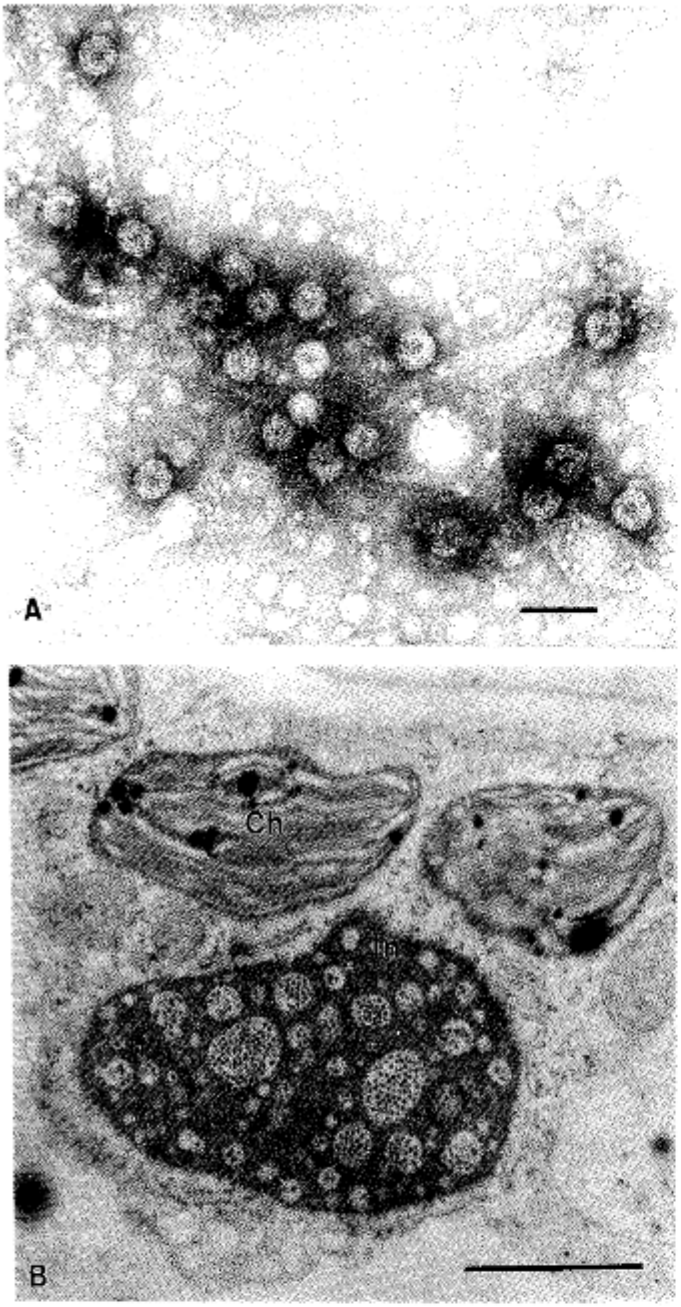

Fig. 3. Electron micrographs of particles and inclusion of caulimovirus-like agent. A. Particels from leaf extrats of infected *Lactuca sativa* var. *asparagina*, Bar = 100 nm. B. Thin sections of caulimovirus-like agent infected asparagus lettuce leaves, showing an inclusion (In) composed mainly of viruslike particles, embedded in an electron-dense matrix with several electron-lucent areas where the particles are well defined. Ch = chloroplast, Bar = 2 μm.

by *Aphis gossipii*. In mechanical inoculation tests, susceptibility varied with the lettuce varieties. *L. scariola* var. *sativa* was most susceptible, *L. sativa* var. *asparagina* and *L. sativa* var. *crispa* were moderately susceptible, whereas *L. sativa* var. *capitata* was resistant (Table 1). In aphid transmission tests, one aphid per plant could not transmit the VLPs to the lettuces, but transmission efficiency could be easily increased by increasing the number of the vector (Table 2). *M. persicae* transmitted the VLPs in a nonpersistant and semipersistant manner. The retention time lasted for 60 min, but not for 90 min (Table 3).

Among 250 seeds collected from infected asparagus lettuces, 187 germinated. None of the plants showed disease symptoms or have cellular inclusions which were considered as a characteristic of the infection by the test virus.

DISCUSSION

Caulimoviruses are known to have restricted host ranges which are confined largely to a few closely related plants in nature, and appear to be little overlap among host ranges of the individual viruses within the group (22). The lettuce leafroll mosaic VLPs have a restricted host range, narrower than those of other reported caulimoviruses. From the results of the host range test, it is apparent that the VLPs were not dahlia mosaic virus which had been reported in Taiwan (17), since it did not infect dahlia, and neither did DMV infect lettuce (1,3).

The inclusion bodies associated with the caulimovirus infection appeared to have a well-defined unique structure as revealed by observation of ultrathin sections with the electron microscope. The bulk of a typical inclusion consists of an electron-dense, granular matrix in which virons are embedded. Throughout the matrix, in an apparent random distribution, are transparent, vacuole-like areas (2,4,10,13,14,17,18, 20,21,23). This vacuolated, conglomerative type of inclusion body is known to be unique to caulimoviruses and provides a reliable characteristic for distinguishing caulimoviruses from other viral agents (22). The inclusions observed in studied leafroll mosaic VLPs affected lettuces (Fig. 3-B) clearly showed the characteristics which are typical of caulimoviruses infection.

The phenomenon including the existence of virus-particle in and the channel enlargement and wall

TABLE 1. Influence of lettuce varieties on susceptibility to caulimovirus-like particles isolated from asparagus lettuce (*Lactuca sativa* var. *asparagina*)

Lettuce varieties	Plants infected/ plants inoculated		
	Test 1	Test 2	Test 3
Lactuca sativa var. *asparagina*	7/10	15/20	28/42
Lactuca sativa var. *capitata*	1/10	2/20	3/42
Lactuca sativa var. *crispa*	7/10	13/20	27/42
Lactuca scariola var. *sativa*	9/10	19/20	39/42

TABLE 2. Influence of insect number and acquisition time of *Myzus persicae* on transmission of caulimovirus-like particles isolated from asparagus lettuce (*Lactuca sativa* var. *asparagina*)

No. of aphid(s) per plant	Acquisition time (min)	No. of plants infected[1]		
		Test 1	Test 2	Test 3
1	1	0	0	NT[2]
	5	0	0	NT
	10	0	0	NT
	15	0	0	NT
	20	0	0	NT
5	1	1	0	2
	5	5	4	5
	10	6	6	5
	15	6	6	6
	20	7	6	7
10	1	6	6	8
	5	10	10	10
	10	10	10	10
	15	10	10	10
	20	10	10	10
15	1	8	10	9
	5	10	10	10
	10	10	10	10
	15	10	10	10
	20	10	10	10

[1] Ten plants were used for each test.
[2] NT: Not tested.

TABLE 3. Influence of retention time on aphid (*M. persicae*) transmission of caulimovirus-like particles isolated from asparagus lettuce (*Lactuca sativa* var. *asparagina*)

Retention time (min)	No. of plants infected out of five inoculated plant		
	Test 1	Test 2	Test 3
10	5	5	5
30	5	5	5
60	1	0	1
90	0	0	0
120	0	0	0
150	0	0	0
180	0	0	0
210	0	0	0
240	0	0	0

thickenings of transformed plasmodesmata are common in plants infected with known caulimoviruses (4,7,15,19,23). However, these characteristics were not found in the preparation of lettuce infected by the studied VLPs. Moreover, the light and electron microscopic profiles (Fig. 2 and 3), transmission manners, and physical properties of VLPs described here were also similar to those of the caulimovirus members (2,10,11,13,18,20,21,23).

At this moment, we do not have data regarding to the serological relationships and nucleic acid properties to conclude that this VLP is a definitive member of the caulimovirus group. However, among 35 groups of plant viruses (9), caulimoviruses are the only member that have isometric particles with diameters of about 50 nm and induce inclusions consisting of an electron-dense matrix with vacuolar regions, which can be easily seen with the light microscope or in ultrathin sections with the electron microscope. These two discernible characteristics mentioned above suggest that the lettuce leafroll mosaic-asociated viral agent is a possible member of caulimoviruses.

Because the VLPs were discovered only in a few samples from the field, its distribution was probably limited. However, the presence of the VLPs might be overlooked since the symptoms induced by the VLPs are similar to those of LMV. The possible impact of this virus disease on lettuce production thus deserve great attention. Continued efforts are aimed to purify and characterize the viral agent, and to develope serological or nucleic probes for the exploration of it's epidemiological aspects.

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摘　　要

陳煜焜、陳脉紀．1994．萵苣捲葉嵌紋病 —— 一種由疑似花椰菜嵌紋病毒群成員之病毒引起之新病害．植病會刊3:209-215．(台中市　國立中興大學植物病理學系)

本研究由台中地區栽植嫩莖萵苣(*Lactuca sativa* L. *asparagina* Bailey)之田間發現一種具嵌紋、捲葉及矮化等病徵之病株，經系列研究證實其爲由一疑似花椰菜嵌紋病毒群成員之病毒所致之病毒病害。電子顯微鏡鏡檢顯示該病毒顆粒爲球形，直徑約50 nm。光學顯微鏡鏡檢及超薄切片觀察則發現病毒可導致病組織內形成花椰菜嵌紋病毒群成員特有之內含體(inclusion)。病毒顆粒可經汁液機械接種或經蚜蟲以非永續性或半永續性方式傳播至特定之健康寄主，在總共8科37種供試植物中，本研究發現其僅能感染平葉萵苣(*Lactuca scariola* var. *sativa*)、嫩莖萵苣(*L. sativa* L. *asparagina*)、皺葉萵苣(*L. sativa* var. *crispa*)及結球萵苣(*L. sativa* var. *capitata*)。本文爲似花椰菜嵌紋病毒群病毒感染萵苣之首次報導。

關鍵詞：嫩莖萵苣、花椰菜嵌紋病毒群、內含體。