Biological and Molecular Characterization of *Apple chlorotic leaf spot virus* Causing Chlorotic Leaf Spot on Pear (*Pyrus pyrifolia*) in Taiwan

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Abstract. Pear plants (*Pyrus pyrifolia* var. Hengshen) showing symptoms of chlorotic spots on leaves were observed in orchards in central Taiwan in 2004. The sap of diseased leaves reacted positively to *Apple chlorotic leaf spot virus* (ACLSV) antiserum. A purified virus isolate (LTS1) from pear was characterized by host range, electron microscopy, phylogenetic analyses, serological property, and back-inoculation experiments to pear. Fifteen of 28 species of tested plants were susceptible to this virus after mechanical inoculation. Pathogenicity of ACLSV isolate LTS1 was verified by back-inoculating to pear seedlings. Filamentous virions of $\approx 12 \times 750$ nm were observed in the preparations of purified virus. Virus particles accumulated in the cytoplasm were observed in the ultrathin sections of LTS1-infected pear leaf tissue. Sequence analyses of the coat protein (CP) gene of LTS1 and the CP gene of LTS2, which originated from a distinct symptomatic pear sample, shared 81.4% to 92.6% nucleotide and 87.6% to 98.4% amino acid identities with those of the CP of 35 ACLSV isolates available in GenBank. ACLSV isolates were grouped into four clusters, i.e., Asia I, II, III, and Europe, and isolates LTS1 and LTS2 were classified as members of cluster Asia II and Asia I, respectively, based on phylogenetic data. Moreover, the variability of amino acid sequences of the CP gene of 37 ACLSV isolates showed geographically associated clustering in the phylogenetic tree. To our knowledge, this is the first study on the characterization of ACLSV causing the leaf chlorotic spot disease of pear in Taiwan. This study also provides the phylogenetic relationships among ACLSV populations based on amino acid sequences of CPs, which are correlated with their geographic origins.

produce high-quality pear fruits by grafting flower bud-containing pear scions imported from Japan onto the local dominant low-chilling required cultivar, *P. pyrifolia* var. Hengshen, during November and January every year. The major pear production area was located in central Taiwan where 71.9% and 19.1% of the total yields were produced from Taichung County and Miaoli County, respectively. The popular Japanese pear cultivars of Shinko, Kosui, Hosui, and Seiki are sold in Taiwan and exported to international markets, including Hong Kong, Singapore, Malaysia, and Vietnam.

Pear is a natural host for several graft-transmissible pathogens of economical importance (Motoshima et al., 1983; Nemeth, 1986; Pfaltzer, 1962). Viral diseases had not been reported in Taiwan until 2003 (Jan et al., 2003). In our previous works (Jan et al., 2003), *Apple stem grooving virus* (ASGV) and *Apple chlorotic leaf spot virus* (ACLSV) were detected on the pear leaves showing virus-like symptoms by reverse transcription–polymerase chain reaction (RT-PCR). However, the virus isolation and identification including back-inoculation to pear plants were not accomplished.

ACLSV is one of the most economically important graft-transmissible pathogens in fruit trees worldwide (Nemeth, 1986; Yoshikawa, 2001). ACLSV belongs to *Trichovirus* in the family *Flexiviridae* (Martelli et al., 1994, 2007) and it is made of filamentous particles of ≈ 640 to 760×12 nm in size. ACLSV can be transmitted by mechanical inoculation and grafting, but the natural dissemination in woody plant species is limited as a result of ineffective vectors (Martelli et al., 2007; Yoshikawa, 2001). ACLSV causes severe yield loss of many crop species. It infects various rosaceous fruit trees (Nemeth, 1986; Yoshikawa, 2001), including almond (Spiegel et al., 2005) and quince (Mathioudakis et al., 2007; Rana et al., 2007). ACLSV induces symptoms of deformation, reduced size, chlorotic leaf spots, and ring pattern mosaic on leaves of the susceptible cultivars of apple and pear (Nemec, 1967; Yoshikawa, 2001). It also causes apple russetting and top-working diseases (Yanase et al., 1979). Several virulent isolates can cause symptoms of bark split and pseudopox in some plums or peaches and graft incompatibility in apricots or cherries (Nemeth, 1986; Yoshikawa, 2001).

Recently, a pear plant showing symptoms of small leaves and leaf distortion in Taiwan has been identified to be caused by a pear isolate of ASGV (Wu et al., 2010). Another pear disease, consisting of chlorotic spots on the leaves of the pear cultivar Hengshen, had been observed in the orchards in central Taiwan. The etiology of this chlorotic spot disease of pear has not been clarified. The objective of this study was to identify and characterize the causal agent of this pear disease. We report here the isolation, serological and molecular characterizations of the causal agent, ACLSV, of the chlorotic spot disease of pear in Taiwan. We also performed sequence analyses by using the amino acid

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Pear (*Pyrus* spp.) is cultivated worldwide and it is one of the economically important fruit crops in Taiwan with ≈ 8300 ha of cultural areas, which account for cash value of 3.6 billion NT dollars in 2008, just next to citrus, pineapple, and mango production. The good taste of Japanese pear (*P. serotina* Rehder) fruits has made it a very popular fruit in Taiwan. However, the Japanese pear cultivars required high-chilling temperature and produced no fruits in the low elevation area of Taiwan. The farmers invented unique top-working cultivation (Lin et al., 1990) to

sequences of coat protein (CP) genes of this virus isolate and those of other ACLSV isolates available in GenBank to gain the information on the molecular variability and phylogenetic relationship among ACLSV populations.

Materials and Methods

Virus isolation and maintenance. Leaves of the pear cultivar Hengshen (*Pyrus pyrifolia* var. Hengshen) showing symptoms of chlorotic spots (Fig. 1A) were collected for virus detection from the orchards in the Taichung County of central Taiwan in Mar. 2004. The possible presence of viruses was tested by an enzyme-linked immunosorbent assay (ELISA) using commercial antisera, including ASGV, ACLSV, and *Apple stem pitting virus* (ASPV) (Bioreba, Nyon, Switzerland), *Tomato ringspot virus*, *Tomato bushy stunt virus*, *Peach rosette mosaic virus* (ADI, LLC., Fayetteville, AR), *Apple mosaic virus*, *Plum pox virus* (ADGEN Ltd., Scotland, UK), and the POTY monoclonal antibody for potyviruses (ADI, LLC.). A virus isolate, designated as LTS1, was isolated from diseased pear through triple single-lesion isolations on *Chenopodium quinoa* by mechanical inoculation using 10 mM potassium phosphate buffer (pH 7) (Jan et al., 2000), and the virus culture was propagated on *C. quinoa* for subsequent analyses.

Host range and pathogenicity tests. Twenty-eight species representing nine families of plants were used for host range tests. All plants were mechanically inoculated using 30-fold dilution (w/v) of the inoculum prepared by grinding LTS1-infected *C. quinoa* leaves as described (Jan et al., 2000) and were maintained in a greenhouse for symptom observation for at least 2 months. The pear seedlings for pathogenicity tests were obtained from seeds and were mechanically inoculated as mentioned previously for all tested plants except a boost inoculation was carried out 2 weeks after the first inoculation. The symptom development on tested plants was observed for at least 2 months and the presence of virus in the inoculated plants was confirmed by indirect ELISA using polyclonal antiserum against the isolate LTS1. Two plants each of tested herbaceous species and two pear seedlings were mechanically mock-inoculated with potassium phosphate buffer as the negative controls. LTS1-infected

C. quinoa samples were used as positive controls for virus infection and ELISAs.

Virus purification and antiserum production. Viral particles were isolated from LTS1-infected *C. quinoa* leaves and purified as described by de Sequeira and Lister (1969). The purified virus was used as an antigen to immunize a New Zealand white rabbit for the production of antiserum against isolate LTS1 using the protocol described by Jan and Yeh (1995). The purified virions were emulsified with Freund's complete adjuvant (Difco Laboratories, Detroit, MI) and injected intramuscularly into the hind legs of rabbit. The antigens, emulsified with Freund's incomplete adjuvant (Difco Laboratories), were then administered three times at weekly intervals. The rabbits were weekly bled after the fourth injection. The titers of the antisera were determined by sodium dodecyl sulphate (SDS)-immunodiffusion tests (Jan and Yeh, 1995).

Serological analyses. Indirect ELISA tests were performed as described by Clark and Adams (1977) with some modifications. Leaf samples were extracted with coating buffer (0.05 M sodium carbonate, pH 9.6, 0.02% sodium azide) at 50-fold dilution and coated in polystyrene microtitration plates (Greiner Bio-One, Frickenhausen, Germany). The Pab against LTS1 was used at 5000-fold dilution in conjugate buffer (phosphate-buffered saline, pH 7.4, containing 0.05% Tween 20 and 0.2% ovalbumin) to react with testing samples followed by the addition of alkaline phosphatase (AP) conjugated goat antirabbit immunoglobulin (Jackson Immuno Research Laboratory Inc., West Grove, PA) at 5000-fold dilution in conjugate buffer. Color-developing solution was prepared by dissolving the AP substrate tablets (Sigma-Aldrich, St. Louis, MO) in substrate buffer (9.7% diethanolamine and 0.02% sodium azide, pH 9.8) to a final concentration of 1 mg·mL⁻¹. Absorbance values were measured at 405 nm using a Labsystems Multiskan EX microplate reader (Labsystems, Vantaa, Finland) after the addition of substrate solution.

Western blotting was performed as described by Jan and Yeh (1995). Leaf tissues of healthy and LTS1-infected pear plants or purified virions were ground in 3 vol. (v/w) of sample buffer [50 mM Tris-HCl (pH 6.8), 2% SDS, 100 mM dithiothreitol, 10% glycerol, 0.1% bromophenol blue] and boiled. The samples were centrifuged and the dissociated

proteins in the supernatant were loaded and separated by 10% SDS-polyacrylamide gel electrophoresis and then transferred to the polyvinylidene difluoride transfer membrane (Perkin Elmer, Waltham, MA). The membrane was incubated with the generated polyclonal antiserum against ACLSV LTS1 at 5000-fold dilution in TSW buffer (10 mM Tris base, 0.9% sodium chloride, 0.25% gelatin, 0.1% Triton X-100, and 0.02% SDS) followed by incubation with 5000-fold dilution of AP conjugated goat antirabbit immunoglobulin in TSW buffer. The color was developed by treatment with chromogenic substrates (NBT/BCIP) (Amresco, Solon, OH).

Electron microscopy. The purified virus preparations were negative-stained with 2% uranyl acetate (pH 4.2) and examined with a JEOL 200CX transmission electron microscope (JEOL Ltd., Tokyo, Japan). As described by Jan and Yeh (1995), the samples fixed in glutaraldehyde, post-fixed in osmium tetroxide (OsO₄), and dehydrated in ethanol were embedded in LR white acrylic resin and sectioned. Ultrathin sections stained with uranyl acetate followed by lead citrate were examined with a JEOL 1200 EXII transmission electron microscope (JEOL Ltd.).

Molecular cloning and sequence analyses. Total RNAs were extracted from healthy and virus-infected leaves of *C. quinoa* or pear plants as described by Napoli et al. (1990). The segment of ACLSV genome corresponding to the CP gene was amplified by RT-PCR with designed primers based on the reported nucleotide sequences of different ACLSV isolates in the National Center for Biotechnology Information (NCBI) GenBank database. The RT-PCR with the upstream primer FJJ05-7 (5'-CTGGATTAGGTCCCAG-3') and downstream primer FJJ05-8 (5'-GTAGTAAATATTTAAAGTCT-3') was performed as described by Jan et al. (2000). The first strand cDNA was synthesized from the total RNAs using the *Moloney murine leukemia virus* reverse transcriptase kit (Epicentre, Madison, WI) according to the manufacturer's instructions. The subsequent PCR was performed using *Taq* DNA polymerase to amplify the expected product of 0.9 kb. The amplified RT-PCR products were cloned into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The inserted DNA from selected clones was sequenced on an automatic DNA sequencer ABI PRISM 3730 (Applied Biosystems, Hammon, NJ) at the Biotechnology Center, National Chung Hsing University, Taiwan.

The CP gene sequences of the virus isolates collected from infected pear plants were compared with those of 35 ACLSV isolates available in the NCBI GenBank (Table 1). Sequence comparisons were performed using the DNASTAR Lasergene software (DNASTAR, Inc., Madison, WI). Multiple alignments of nucleotide and amino acid sequences were performed by the CLUSTAL W algorithm (Thompson et al., 1994). Phylogenetic analyses using the neighbor-joining method (Saitou and Nei, 1987) was performed with the MEGA

Fig. 1. Symptoms of infected pear plant in the field (A) and inoculated pear plant in the greenhouse (B). (A) The infected pear plant (*Pyrus pyrifolia* var. Hengshen) showing symptoms of chlorotic spots on leaves. (B) Pear seedling displayed chlorotic symptoms on newly emerging leaves after inoculation with *Apple chlorotic leaf spot virus* (ACLSV) LTS1. (C) No systemic symptoms were observed in mock-inoculated pear plant as a negative control. Bars represent 3 cm.

(Molecular Evolutionary Genetics Analysis software) Version 4.0 (Tamura et al., 2007), and the resulting data were tested by 1000 bootstrap replicates.

Results

Virus isolation. During the field surveys of pear viruses, pear leaves showing symptoms of chlorotic spots (Fig. 1A) consistently reacted positively only to the commercial ACLSV antiserum in ELISA tests. A virus culture, designated as LTS1 (HM352768), was isolated from a ACLSV-positive pear sample through triple single-lesion passages in *C. quinoa*. The inoculated leaves of *C. quinoa* displayed symptoms of chlorotic and necrotic local lesions 3 to 5 d after inoculation. The LTS1-infected leaf samples of *C. quinoa* only reacted positively with ACLSV antiserum in ELISA (data not shown).

Host range and symptomatology. Of the 28 inoculated plant species, *Tetragonia tetragonoides* (Aizoaceae), *Gomphrena globosa* (Amaranthaceae), *C. amaranticolor*, *C. murale*, and *C. quinoa* (Chenopodiaceae) showed reactions of local infection to LTS1, whereas *Celosia argentea* (Amaranthaceae), *Lactuca sativa* (Asteraceae), *Capsicum annuum*, *Nicotiana benthamiana*, *N. occidentalis*, *N. tabacum* cv. Vam-Hicks, *N. tabacum* cv. Hicks, *N. tabacum* cv. Xanthi, *N. tabacum* cv. Samsun, and *Physalis alkekengi* (Solanaceae) were systemically infected by LTS1. The systemic symptoms were mostly mosaic, mottling, and/or foliar distortion. Thirteen plant species, including *Amaranthus gangeticus* (Amaranthaceae), *Brassica chinensis* (Brassicaceae), *Cucumis melo*, *C. sativas*, *Cucurbita pepo* L. var. zucchini (Cucurbitaceae), *Ocimum basilicum* (Lamiaceae), *Datura stramonium*, *N. glutinosa*, *N. edwardsonii*, *Solaum esculentum* (Solanaceae), *Phaseolus vulgaris*, *Vigna unguiculata*, and *V. radiata* (Leguminosae), were not infected by LTS1.

The virus isolate LTS1 was back-inoculated onto seedlings of pear cultivar Hengshen by mechanical inoculation. Sixteen of 22 inoculated seedlings were infected by LTS1 and displayed similar symptoms of chlorotic spots on the newly emerging leaves (Fig. 1B) as observed in the field ≈ 23 d after inoculation. Symptoms of chlorosis and mosaic appeared on the newly emerging leaves at the early stage of infection. Typical symptoms of chlorotic spots then developed on the entire infected pear plants ≈ 36 d after inoculation. The mature leaves displayed severe symptoms of more chlorotic spots and reduced leaf size 2 months after inoculation. No symptoms were observed on the mock-inoculated pear plants (Fig. 1C). The presence of the virus in these 16 symptomatic pear seedlings were confirmed by positive reactions in ELISA tests using the antiserum against isolate LTS1. Of these 16 ELISA-positive pear plants, the ACLSV infection was confirmed by RT-PCR, whereas no infection of ASGV and ASPV was confirmed (data not shown).

Electron microscopy and serological analyses. The purified virus preparations

contained filamentous particles $\approx 12 \times \approx 750$ nm in size (data not shown). The aggregated and scattered virion particles similar to that observed in the cytoplasm were frequently observed in the ultrathin sections of the infected leaf tissues (data not shown). Electrophoretic analysis of SDS-dissociated virus revealed the presence of one structural protein with relative molecular mass of ≈ 21 kDa (Fig. 2A). The presence of the 21 kDa protein in the LTS1-infected pear samples was detected with LTS1 antiserum in Western blots (Fig. 2B).

Molecular characterization of Apple chlorotic leaf spot virus isolate from Taiwan. A cDNA fragment of ≈ 900 bps, consisting of the CP gene and flanking regions, was amplified from the total RNA of LTS1-infected *C. quinoa* tissues by RT-PCR with the primers FJJ05-7 and FJJ05-8 designed for amplifying

the CP gene-encoding region of ACLSV. The RT-PCR product was cloned and sequenced. The LTS1 CP gene (HM352768) is 579 bp in length and encodes a predicted protein of 193 amino acids with a calculated molecular mass of 21.5 kDa similar to that of the dissociated peptide of LTS1 in protein electrophoresis assay (Fig. 2A). Another CP clone, designated LTS2 (HM352769), was obtained from a natural infected-pear sample from a different area of Taichung County, Taiwan. Comparisons of the CP genes of LTS1 and LTS2 with those of 35 ACLSV isolates available in NCBI GenBank revealed 81.4% to 92.6% of nucleotide and 87.6% to 98.4% of amino acid identities. The CP gene of LTS1 shared 86.3% of nucleotide and 91.8% of amino acid identities with that of LTS2. These data indicated that both LTS1 and LTS2 are isolates of ACLSV.

Table 1. GenBank accession numbers and their origins of *Apple chlorotic leaf spot virus* isolates used in this study.

GenBank accession no.	Host	Country	GenBank accession no.	Host	Country
CAM56872	Almond	India	CAM97377	Apple	India
ABK62735	Apple	China	CAM56871	Apricot	India
AB060958	Apple	Japan	CAM56874	Apricot	India
BAA03643	Apple	Japan	ABL63752	BR1	Brazil
BAF64464	Apple	Japan	CAM56870	Cherry	India
BAF64467	Apple	Japan	X99752	Cherry	Hungary
BAF64461	Apple	Japan	AAU93348	Peach	China
CAL64067	Apple	India	CAM56873	Peach	India
CAL68578	Apple	India	CAM56876	Peach	India
CAM35605	Apple	India	AAT75238	Pear	China
CAM35606	Apple	India	CAO99325	Pear	India
CAM35607	Apple	India	LTS1	Pear	Taiwan
CAM35608	Apple	India	LTS2	Pear	Taiwan
CAM35610	Apple	India	AAF67188	Plum	Poland
CAM35611	Apple	India	CAO99326	Plum	India
CAM35612	Apple	India	CAB46654	Plum	Germany
CAM35613	Apple	India	M58152	Plum	France
CAM35614	Apple	India	CAM56875	Quince	India
CAM97376	Apple	India

Fig. 2. SDS-PAGE and Western blot analyses of *Apple chlorotic leaf spot virus* (ACLSV) LTS1. (A) Coat protein subunit of ACLSV (21 kDa) (Lane 1) and bovine serum albumin (BSA) (68 kDa) (Lane 2) were observed in sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Lane M in A is protein markers (low-molecular-weight SDS Calibration Kit; Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). (B) Western blot showing the 21 kDa protein from crude saps of ACLSV LTS1-infected pear plant (Lane 2) but not in healthy pear (Lane 1) after probed with antiserum against ACLSV-LTS1. Lane M in B is prestained protein markers (ProSieve color protein markers; BioWhittaker, Rockland, ME).

Phylogenetic analyses indicated that the CP genes of 37 ACLSV isolates can be grouped into four clusters, i.e., Asia I, Asia II, Asia III, and Europe clusters (Fig. 3). The Asia I cluster contains 15 isolates from India (apple, cherry, peach, plum, and quince), Japan (apple), and Taiwan (pear, LTS2) (Fig. 3). Comparisons of CP genes of 15 ACLSV isolates in Asia I group revealed 94.8% to 100% of amino acid identity, suggesting their close genetic relationship. Asia II cluster consists of three isolates from Japan (apple), China (Kuerle pear), and Tai-

wan (pear, LTS1), which shared 92.7% to 93.3% of amino acid identity in CP sequences. The other 14 isolates consisting of isolates from India (almond, apple, apricot, peach, and pear), Japan (apple), China (apple), and Brazil (BR1) formed the Asia III cluster with 96.9% to 100% of amino acid identity. The remaining five isolates including France (plum), Germany (plum), Hungary (cherry), China (peach), and Poland (plum) shared 89.1% to 96.4% of amino acid identity. They were grouped into the European cluster (Fig. 3). This phylogenetic

tree also revealed geographical differentiations. Several isolates from the same location, like Japan or India, were closely related and formed separated sub-branches in the phylogenetic tree (Fig. 3).

Sequence variability of Apple chlorotic leaf spot virus coat protein genes. Phylogenetic analyses revealed the geographic grouping among ACLSV isolates of Asian and European origins, and the Asian isolates can be further divided into three clusters. To evaluate the genetic variability of the ACLSV

Fig. 3. Phylogenetic relationships of the coat proteins of *Apple chlorotic leaf spot virus* (ACLSV) of Taiwanese isolates (LTS1 and LTS2) and other 35 ACLSV isolates. Four major clusters, labeled as Asia I, Asia II, Asia III, and Europe, are observed in the phylogenetic tree constructed by CLUSTAL W and neighbor-joining method with 1000 bootstraps using the MEGA 4.0 software. The scale bars represented the genetic distances indicated at the left bottom. The following descriptions were used to indicate accession numbers, hosts and abbreviations of geographic origins, respectively. FR = France; GM = Germany; HN = Hungary; Ind = India; JP = Japan; C = China; PL = Poland; TW = Taiwan.

population, sequence alignment of the 37 ACLSV CP genes and estimation of the intra- and intergroup amino acid identities of the clusters were conducted. The intragroup values of average amino acid identity between ACLSV isolates of clusters Asia I and III were 97.9% and 98.5%, respectively, which were higher than those in clusters Asia II (93.1%) and Europe (92.3%). Among three Asian groups, the intergroup values were 91.2% to 94.3% between isolates of clusters Asia I and Asia III, 89.6% to 92.7% between isolates of Asia I and Asia II, and 89.6% to 93.3% between isolates of clusters Asia II and Asia III, respectively. The European cluster revealed 87% to 92.2%, 87.6% to 93.3%, and 89.6% to 96.9% intergroup identities with the clusters Asia I, II, and III, respectively. These results revealed a closer relationship between ACLSV clusters Asia I and Asia III than that between cluster Asia II. According to the alignment data, the N-terminal regions of CP seemed more variable than that of C-terminal region (data not shown). Interestingly, based on two individual amino acids at the positions 40 and 75 of CPs, ACLSV isolates can be classified into two groups, i.e., Group A (Ala⁴⁰-Phe⁷⁵) and Group B (Ser⁴⁰-Tyr⁷⁵) (Fig. 4). Group A contains the isolates of the cluster Asia I, whereas Group B consists of those of the clusters Asia II, Asia III, and Europe.

Discussion

Our results demonstrated that the virus (LTS1) isolated from the diseased Hengshen pear bearing chlorotic spot symptoms is a strain of ACLSV. Sequence analyses showed that

this isolate shared more than 87% amino acid identity with those of other 35 documented ACLSV isolates. The pathogenicity of isolate LTS1 to pear plants has been proven by back-inoculations, indicating that the ACLSV LTS1 is the causal agent of the pear disease observed in the fields (Fig. 1). This is the first characterization of ACLSV infecting pear plants in Taiwan.

ACLSV infections by mechanical inoculation have been reported in apple (Lister et al., 1965; Saksena and Mink, 1969), pear (Nemec, 1967; Pfaeltzer, 1962), plum and hawthorn (Sweet, 1980), cherry (Everett et al., 1993; Rana et al., 2008b), almond (Spiegel et al., 2005), and quince (Mathioudakis et al., 2007; Rana et al., 2007). It seems that the host range of ACLSV LTS1 is different from that of other reported ACLSV isolates. Among them, *C. annuum*, *G. globosa*, *L. sativa*, and *N. tabacum* cv. Vam-Hicks can be infected by LTS1 but not by other reported ACLSV isolates. In contrast, *P. vulgaris* can be infected by several ACLSV isolates (Yoshikawa, 2001), including the pear isolates (Nemec, 1967; Pfaeltzer, 1962), but not by an almond isolate (Spiegel et al., 2005) and pear isolate LTS1.

Individual and aggregated virus particles of ACLSV had been observed in the cytoplasm of phloem parenchyma and mesophyll cells in ultrathin sections of the infected *C. quinoa* (Ohki et al., 1989; Yoshikawa, 2001), but not observed in tissues of pear plants. In this study, virus particles were observed in the ultrathin sections of LTS1-infected leaf tissues (data not shown). Elongated, flexuous particles appeared as aggregates in the cytoplasm and sporadically scattered throughout

in the cytoplasm. Previously, virus particles were reportedly observed in plant cells infected with several virus species of the *Flexiviridae*, mainly belonging to *Foveavirus* (ASPV) (Koganezawa and Yanase, 1990) or *Potexvirus* (*Pepino mosaic virus*; Caladium virus X) (Rivas et al., 2005).

The biological and serological properties of ACLSV have been well characterized during past decades; however, few studies of molecular variability among ACLSV isolates have been conducted previously, but not including isolates from Taiwan. ACLSV isolates collected from European and Mediterranean countries can be classified into three groups based on the electrophoretic mobility of the CP (Krishbai et al., 2001; Pasquini et al., 1998). However, no correlation was observed between the CP migrations and phylogenetic relationship. ACLSV isolates from different hosts and geographic locations have been separated into two major phylogenetic groups in phylogeny based on the CP sequence analyses (Al Rwahnih et al., 2004). A similar phylogenetic grouping has also been described by Candresse et al. (1995), suggesting that these separate clusters were host- and geographically associated grouping (Al Rwahnih et al., 2004). In this study, results of phylogenetic tree revealed that there was geographically associated clustering among 37 ACLSV isolates (Fig. 3) irrespective of their host species. A similar phylogenetic result has also been described by Ulubas and Rosner (2006), suggesting that ACLSV isolates from regions of Europe and the Far East revealed a correlation between nucleotide sequence divergence of the CP genes and the geographic origins of ACLSV isolates irrespective of the hosts (Ulubas and Rosner, 2006). However, there were two exceptional isolates, one from Brazil (ABL63752) and the other from China (AAU93348), that were grouped into cluster Asia III and cluster Europe, respectively. This may be partly attributed to the virus sources or their amino acid sequence variations.

Isolates LTS1 and LTS2 were originated from the same geographic area but were grouped in different clusters in phylogenetic analysis. Isolate LTS1 shared 93.3% amino acid identity (data not shown) with the pear isolate (AAT75238 from China) or apple isolate (BAF64467 from Japan). Isolate LTS1 seemed to be phylogenetically closer to the pear isolate from China than apple isolate from Japan (Fig. 3). Isolate LTS2 shared 98.8% amino acid identity (data not shown) with the apple isolate (BAF64461 from Japan) or cherry isolate (CAM56870 from India) in Asia I cluster. It was reported recently that Japanese apple isolates of ACLSV (almost in the same geographic region) were grouped into two clusters (P205 and B6) in phylogenetic analysis (Yaegashi et al., 2007), and similar observations were also reported in the sequence analyses of Indian isolates (Rana et al., 2008a). In addition to Japan, China is another source for the importation of the flower bud-bearing pear scions. This may explain why the two Taiwanese isolates, LTS1 and LTS2, were in two

Fig. 4. Multiple alignment of the coat protein (CP) amino acid sequences of 37 *Apple chlorotic leaf spot virus* (ACLSV) isolates from different host and geographic origins. Asterisks (*) indicate the two amino acids that show inerratic combinations between ACLSV isolates at positions 40 and 75, Ala⁴⁰-Phe⁷⁵ for Group A, and Ser⁴⁰-Tyr⁷⁵ for Group B.

different phylogenetic categories, Asia II and Asia I, respectively.

As previously described for ACLSV (Al Rwahnih et al., 2004; Candresse et al., 1995; German-Retana et al., 1997), higher genetic variations were observed in the N-terminal part of CP genes in sequence alignment of 37 ACLSV isolates (Fig. 4). It was noticed that the inerratic combinations of amino acids at positions 40 and 75 (Fig. 4) could divide the ACLSV isolates into Groups A and B (Fig. 4). Groups A and B corresponded, respectively, to the phylogenetic clusters of P205 and B6 type, as described by Yaegashi et al. (2007). The amino acid changes in CP at positions 40, 59, 75, and 130 has been reported (Rana et al., 2008a; Yaegashi et al., 2007), and the CP-required virus replication affected negatively by the inerratic combination of amino acids at positions 40 and 75 was suggested (Yaegashi et al., 2007). These amino acid substitutions in CP gene might be useful in the phylogenetic grouping of ACLSV populations, but their roles in pathogenicity of ACLSV remain to be elucidated.

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