Botanical Studies (2011) 52: 313-320.

Phytoplasmas of two 16S rDNA groups are associated with pear decline in Taiwan

Shu-Ling LIU[1], Hsiu-Lin LIU[1], Shu-Chen CHANG[2], and Chan-Pin LIN[1,*]

[1]*Department of Plant Pathology and Microbiology, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 10617, Taiwan*
[2]*Applied Zoology Division, Agricultural Research Institute, No.189, Jhongjheng Rd., Wufong, Taichung 41301, Taiwan*

(Received May 28, 2010; Accepted January 7, 2011)

ABSTRACT. Polymerase chain reaction (PCR)-based detection strategies were adopted to examine the etiology and vectorship of pear decline in Taiwan (PDTW). 16S rDNA sequences were amplified from total DNAs prepared from PDTW-affected pear trees *Pyrus serotina* Rehd. cv. Hengshan and pear psyllid *Cacopsylla chinensis* using PCR. According to the sequence analyses, *C. chinensis* carried phytoplasmas of two 16S rDNA groups, PDTW (group 16SrX) and PDTWII (a newly discovered group 16SrII phytoplasma associated with pear decline in Taiwan), that were associated with PDTW-infected pear trees. The 16S rDNA sequences of PDTW and PDTWII phytoplasmas that were amplified from diseased pear trees were identical to those from *C. chinensis*. Transmission trials of phytoplasmas associated with PDTW to healthy pear plants were successfully performed with *C. chinensis*. One of the 17 tested plants was infected with both PDTW and PDTWII phytoplasmas while ten were infected with PDTWII phytoplasma. In grafting tests, both PDTW and PDTWII phytoplasmas were effectively and separately transmitted from diseased pear to periwinkle plants (*Catharanthus roseus*). For detection purposes, specific primers were developed and adopted to detect both PDTW and PDTWII phytoplasmas by nested or semi-nested PCR. Transmission electron microscopic examinations revealed phytoplasma bodies in the sieve tubes and phloem parenchyma cells of diseased pears and in the intestinal wall cells of *C. chinensis* and *C. qianli*.

Keywords: Grafting; Insect transmission; Phytoplasma detection; Transmission electron microscope; Vectors.

INTRODUCTION

The pear decline in Taiwan (PDTW) has posed a major threat to the growth of pear trees (*Pyrus serotina* Rehd. cv. Hengshan) (Chen et al., 2001) since 1994. The leaves of affected trees turn red prematurely and fall early during the autumn. The new leaves remain small and pale throughout the following spring. Symptom severity ranges from mild or slow wilting to quick wilting or death, depending on the weather conditions. The symptoms associated with PDTW resemble those of pear declines caused by phytoplasma elsewhere (Liu et al., 2007a). Since phytoplasmas are non-culturable, PCR amplification of ribosomal RNA genes and the 16S-23S rDNA intergenic spacer region (ISR) have become the conventional means of detecting and identifying them (Bosco et al., 2002; Davies et al., 1995; Seemüller et al., 1998; Smart et al., 1996). Sequences of 16S rDNA have been used to classify various phytoplasmas into groups of phylogenetic systems (Lee et al., 2007). Taxonomically, the phytoplasmas that are associated with pear decline are in group 16SrX, '*Ca*. Phytoplasma pyri', in Europe, North America and South Asia (Davies et al., 1992; Avinent et al., 1997; Seemüller and Schneider, 2004). Based on transmission electron microscopic and molecular analyses from the authors' earlier work, PDTW phytoplasma of the group 16SrX is the causative agent of pear decline in Taiwan (Liu et al., 2007a).

Two species of psyllid, *Cacopsylla pyricola* and *C. pyri*, have been identified as the specific vectors of '*Ca*. Phytoplasma pyri' in the United Kingdom, Italy, France, and Spain (Carraro et al., 1998; Davies et al., 1992; Lemoine, 1991; Seemüller, 1990, 1992; Seemüller and Schneider, 2004; Garcia et al., 2005). Molecular evidence has also revealed that two resident insect vectors in Taiwan, *C. qianli* (Chou and Fang, 1994) and *C. chinensis* that invaded the central part of Taiwan in 2002 (Yang et al., 2004), carry the PDTW phytoplasma, and that *C. qianli* has much higher titers of PDTW phytoplasma than does *C. chinensis* (Liu et al., 2007a). We elected to use *C. chinensis* for the transmission trial for three reasons: the insect transmission trial has not yet been completed, the pear psyllid *C. qianli* is now seldom found in pear orchards in Taiwan, and a relatively high percentage of *C. chinensis* has been found to carry phytoplasmas that are associated

*Corresponding author: E-mail: cplin@ntu.edu.tw; Tel: +886-2-3366-4596; Fax: +886-2-23661980.

with pear decline in Taiwan (Liu et al., 2007b; Yang et al., 2004).

In a study that monitored PDTW phytoplasma in *C. chinensis* from 2003 to 2006, partial rDNA sequences of a phytoplasma of group 16SrII (741 bp) and sequences of PDTW phytoplasma were frequently identified in individual psyllids (Liu et al., 2007b). According to restriction fragment length polymorphism (RFLP) analysis, the causative agent of pear decline in southern Australia is the sweet potato little leaf (SPLL) phytoplasma, which belongs to the 16SrII group (Schneider and Gibb, 1997). The symptoms of phytoplasma-affected pears (*Pyrus communis*) in Australia are not of curling and reddening leaves (Agrios, 2005) but of decline and dieback (Schneider and Gibb, 1997). Whether the sequences of the 16SrII phytoplasma found in *C. chinensis* indicate the existence of group 16SrII-pear decline phytoplasma in Taiwan has thus become a relevant concern.

Leaf redness and curling followed by progressive weakening and wilt of *P. serotina* Rehd. cv. Hengshan, were first observed in Jianshih, Hsinchu County, northern Taiwan in the autumn of 2006. Since sequences of both PDTW and 16SrII phytoplasmas were identified in *C. chinensis* collected from the Jianshih area (Liu et al., 2007b), the pathogen associated with the pear decline there and in other areas of Taiwan needs to be identified. In this study, the presence of a 16SrII phytoplasma in both *P. serotina* Rehd. cv. Hengshan and in *C. chinensis* was confirmed based on sequence analysis, demonstrating the association of this 16SrII phytoplasma with pear decline in Taiwan using transmission electron microscopy. The 16SrII phytoplasma was thus named PDTWII phytoplasma to avoid confusion with the PDTW phytoplasma of the 16SrX group, previously reported (Liu et al., 2007a). This study also verified the capacity of *C. chinensis* to transmit both PDTW and PDTWII phytoplasmas to healthy *P. serotina* Rehd. cv. Hengshan, and completed transmission trials on pear decline phytoplasmas from *P. serotina* Rehd. cv. Hengshan to periwinkle plants (*Catharanthus roseus* cv. Passion) by grafting. Specific PCR primers were designed and utilized to detect both PDTW and PDTWII phytoplasmas.

MATERIALS AND METHODS

Pears and insects used in this study

Pear tree shoot and leaf samples (*P. serotina* Rehd. cv. Hengshan) were collected from pear orchards in the Dungshr and Heping areas of central Taiwan and the Jianshih area in northern Taiwan, from 2004 to 2006. The psyllids, *C. chinensis* and *C. qianli*, were sweep-collected in those orchards from 2005 January to 2006 March.

Identification and detection of pear decline phytoplasma

Total DNA used in this study was purified from plants and insects based on the method of Liu et al. (2007a). To amplify the full length of the 16S rDNA sequence and the 16S-23S rDNA ISR of phytoplasma that is associated with pear decline in Taiwan from plants or the insect vector, *C. chinensis*, a PCR or semi-nested PCR, was performed. In direct PCR or the first amplification of semi-nested PCR, the universal phytoplasma primers P1/ P7 (Deng and Hiruki, 1991; Schneider et al., 1995) were used. In the second amplification of semi-nested PCR, the reverse primer L1n was used (Liu et al., 2007a). The PCR and the semi-nested PCR were performed as described by Liu et al. (2007a). The PCR products were cloned using a TOPO TA cloning kit (Invitrogen, San Diego, CA) and the inserted fragments were sequenced (Mission Biotech, Taipei, Taiwan). The nucleotide sequences of phytoplasmas were then aligned using the nucleotide-nucleotide BLAST program in NCBI (National Center for Biotechnology Information, http: // www.ncbi.nlm.nih.gov/), CLUSTAL X (Thompson et al., 1999) software and analyzed with GeneDoc (Nicholas and Nicholas, 1997) software.

For the detection of PDTW and PDTWII phytoplasmas, some specific semi-nested PCR primers were designed. They were based on the rDNA sequences of PDTW phytoplasma (GeneBank accession no. DQ011588) and that of the PDTWII phytoplasma obtained in this work. The primers fPD2/ rPDS1 (fPD2: Table 1; rPDS1: Liu et al., 2007a), along with the semi-nested primers APf3/ rPDS1 (APf3: Liu et al., 2007b), were used to detect PDTW phytoplasma and primers IIPf1/IIPr1 (Table 1) along with the semi-nested primers IIPf2/IIPr1 (Table 1) were used to detect PDTWII phytoplasma in the plant samples and the insect vectors. Both plant samples and the insect vectors were used in the aforementioned tests or were collected from the orchards in Heping, Dungshr and Jianshih areas in the field survey. The program used in both primary PCR and semi-nested PCR was 30 sec at 95°C, 30 sec at 58°C and 45 sec at 72°C for 35 cycles. Before the aforementioned semi-nested PCR with primers fPD2/ rPDS1 and APf3/ rPDS1 was developed, primers APf2/ L1n (Liu et al., 2007a) along with nested primers APf3/ rPDS1 were used to detect PDTW phytoplasma based on the method of Liu et al. (2007a). To ensure that the phytoplasma identity of various host origins was accurately determined, the full-length 16S rDNA phytoplasma sequence was periodically PCR-amplified using the primers P1/ P7 (direct PCR) or P1/P7 and P1/ L1n (semi-nested PCR), then sequenced and analyzed. The methods of DNA extraction and molecular analyses were described above.

Transmission trials using naturally infected insects

During August 2005, 17 groups of 10-30 *C. chinensis* specimens were sweep-collected from five pear orchards in the Dungshr and Heping areas (Table 2) and transferred to five one-year-old, seven two-year-old and five three-year-old *P. serotina* Rehd. cv. Heng-San pear seedlings for transmission following the method of Garcia-Chapa et al. (2005). Test plants were determined to be free of

Table 1. Sequences of oligonucleotide primers for PCR-based detection of PDTW and PDTWII phytoplasmas.

Primer	Sequence (5' to 3')	Source
f1	AGT GGC GAA CGG GTG AGT AA	Lin and Lin, 1998
r1	CGT CAG TAA AGA CCC AGC AA	Lin and Lin, 1998
P1	AAG AGT TTG ATC CTG GCT CAG GAT T	Deng and Hiruki, 1991
P7	CGT CCT TCA TCG GCT CTT	Schneider et al., 1995
Apf2	GAT GAG TAC TAA GTG TTG GG	Liu et al., 2007a
Apf3	GGG TTA AAC CAG TGC TGA AG	Liu et al., 2007b
L1n	CAA GGC ATC CAC TGT	Liu et al., 2007
fPD2	AAT GAT GGA AAA ATC ATT C	This study
rPDS1	CCA AGC CAT TAT TAA TTT TTA	Liu et al., 2007
IIPf1	GCA AAT GGC GAA CCA TTT GTT	This study
IIPr1	CGA AGA AAA ACT TAG TTG CC	This study
IIPf2	CTA GTA AGT CAG TGG TG	This study

phytoplasma by PCR using the universal phytoplasma primers f1/ r1 (Lin and Lin, 1998) and P1/ P7 before the transmission trial was performed. Inoculated seedlings were covered individually with a cage of fine nylon gauze. Surviving psyllids were recollected 14 days after transmission and the test plants were treated with insecticides. Recollected *C. chinensis* psyllids were analyzed individually and the test plants were analyzed on a monthly basis from the second month after transmission, using nested or semi-nested PCR as described above. PCR products were cloned, sequenced and analyzed as described above to identify the phytoplasmas.

Grafting test

In November 2005, branches of symptomatic *P. serotina* Rehd. cv. Hengshan collected from Heping and confirmed by PCR (described below) to be infected with PDTW or PDTWII phytoplasma, were individually grafted onto five-month-old healthy periwinkle plants (*Catharanthus roseus* cv. Passion). After three weeks of incubation, the leaves

Table 2. Results of PCR-based phytoplasma detection in pear seedlings inoculated by feeding with *Cacopsylla chinensis*.

Pear sample[a]	Insect sources	PDTWII phytoplasma[b]	PDTW phytoplasma
ARI-1	Heping No. 2	+	–
ARI-2	Heping No. 2	–	–
ARI-3	Heping No. 2	–	–
ARI-4	Heping No. 2	+	–
ARI-5	Heping No. 2	+	–
ARI-6	Heping No. 2	–	–
ARI-7	Heping No. 2	–	–
ARI-8	Heping No. 2	+	–
ARI-9	Heping No. 2	+	–
ARI-10	Heping No. 2	+	–
NTU-3	Dungshr	+	–
NTU-6	Heping No. 1	+	–
NTU-7	Heping No. 2	+	+
NTU-8	Heping No. 3	+	–
NTU-14	Heping No.4	+	–
NTU-15	Heping No.4	–	–
NTU-16	Heping No. 1	–	–

[a]ARI, Agricultural Research Institute; NTU, National Taiwan University.
[b]Based on the results of the alignment and analysis of PCR-amplified sequences.

of each periwinkle plant were tested for the presence of phytoplasma. This was done every two weeks using nested or semi-nested PCR as described above. PCR products were cloned, sequenced and analyzed as described above to identify the phytoplasmas.

Transmission electron microscopy (TEM)

Small samples of leaves (1.5 × 3 mm) collected from phytoplasma-infected *P. serotina* Rehd. cv. Hengshan, were prefixed in 1% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer at pH 7.0 for 24 h at 4°C, and then post-fixed in 1% (vol/ vol) osmium tetroxide at room temperature for 1 h. *Cacopsylla chinensis* and *C. quanli* (whole body) samples were prefixed in 2.5% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 for 10 min and then cut with a platinum-chrome knife to separate the head and chest from the abdomen. After fixation, the samples were rinsed in a buffer, dehydrated in ethanol and embedded in LR white resin (Agar Scientific Limited, Cambridge, U.K.). Many serial ultrathin sections of at least 80 samples from plants and insects were stained using 2% (vol/ vol) uranyl acetate and 0.4% (vol/vol) lead citrate, and then observed under a transmission electron microscope (TEM) (JEOL, JEM 1010, Philips Ltd., Eindhoven, The Netherlands) at 80 kV, as described previously (Liu et al., 2007a).

RESULTS

16S rDNA and 16S-23S rDNA ISR sequences of PDTW phytoplasma and PDTWII phytoplasma

Single PCR fragments of 1,761 bp or 1,782 bp, or two PCR fragments of 1,761 bp and 1,782 bp, were amplified by semi-nested PCR with primers P1/ P7 and P1/ L1n, using various DNA templates prepared individually from *P. serotina* Rehd. cv. Hengshan or *C. chinensis* collected from the Jianshih area. The 1,761 bp PCR-amplified rDNA sequence underwent nucleotide-nucleotide BLAST analyses and was 100% identical to that of the crotalaria witches'-broom phytoplasma (GenBank accession no. EU650181) in the NCBI database. The 1,782 bp rDNA sequence of another PCR product was 99% identical to the rDNA sequences of group 16SrII phytoplasmas. The sequence of PDTW phytoplasma was the same as reported previously (Liu et al., 2007a), and five sequences of 16SrII phytoplasma, now called PDTWII phytoplasma, obtained from *P. serotina* Rehd. cv. Hengshan, as well as *C. chinensis* collected from various areas of Taiwan, were analyzed and found to be identical. The rDNA sequence of the aforementioned 1,782 bp fragment of PDTWII phytoplasma was submitted to GenBank with an accession number EF193157.

Transmission trials using field-collected *C. chinensis*

About 27% to 67% of *C. chinensis* psyllids from each group survived transmission. Nested PCR was used to individually detect the recollected psyllids to identify the phytoplasma (semi-nested PCR was not then available). In PCR assays using primers APf2/L1n followed by the nested primer pair APf3/rPDS1, a 16S rDNA fragment of 780 bp was amplified. Approximately 34.3% to 62.5% of the recollected psyllids from each group had been infected by phytoplasma. However inoculated seedling symptom developments were observed repeatedly and phytoplasma was detected using the nested or semi-nested PCR monthly from the second month after transmission. The semi-nested PCR with primers fPD2/ rPDS1 and APf3/ rPDS1, or nested PCR with primers APf2/ L1n and APf3/ rPDs1, can amplify the 16S rDNA from PDTW phytoplasma DNA to yield a fragment of size 780 bp. The semi-nested PCR using primers IIPf1/ IIPr1 and IIPf2/ IIPr1 can amplify the 16S rDNA from PDTWII phytoplasma DNA to yield a fragment of size 1,015 bp. PCR-amplified sequences were first detected in the DNA templates that were prepared from seedlings NTU-3, NTU-7, and ARI-1 three months after transmission, when they still appeared symptom- free. Only the seedling NTU-7 demonstrated infection by both PDTW and PDTWII phytoplasmas; ten other seedlings were infected only by PDTWII phytoplasma, and six others were phytoplasma-free, based on PCR sequence results (Table 2).

Leaves of all 11 diseased seedlings began to exhibit the characteristic upward leaf curling symptoms of pear decline (Liu et al., 2007a) three to six months after transmission. Seedling NTU-7, co-infected by both phytoplasmas, exhibited stem die-back, leaf curling and a quick decline resulting in death after two weeks. Of the rest, three plants exhibited vein-reddening and mild leaf curling, one of which showed the characteristic downward curl associated with pear decline from the leaf tip (Seemüller, 1990, 1992). This symptom was not observed in PDTW phytoplasma-infected pears (Liu et al., 2007a). The remaining seven plants exhibited mild leaf stunting and one exhibited stem die-back and leaf curling. However, none of the ten seedlings that wereinfected only by PDTWII phytoplasma in this test exhibited the reddish foliage, which is the primary symptom of pear decline when caused by PDTW phytoplasma (Liu et al., 2007a).

Grafting trial

PCR-amplified sequences were first detected in the DNA templates that were prepared from grafted periwinkle plants 49 days after grafting. The grafted periwinkles plants began to show symptoms after two to three months. PDTWII phytoplasma-infected periwinkles showed symptoms of reduced and pale yellowish foliage, and unusually small flowers (Figure 1). PDTW phytoplasma-infected periwinkles suffered from a lack of shoot development and generally exhibited quick decline and wilting, before dying in seven to ten days. As described above, the PDTWII phytoplasma-infected or PDTW phytoplasma-infected periwinkle plants were verified based on the results of the PCR sequence analysis.

Figure 1. PDTWII phytoplasma-affected periwinkle, showing symptoms of small flowers, and small and pale leaves (A) and close-up photograph (B).

Field detection of PDTW phytoplasma and PDTWII phytoplasma

Semi-nested PCR using the above-mentioned group-specific primers was also adopted for field detection of PDTW and PDTWII phytoplasmas from September, 2006. Using the DNA templates prepared from *P. serotina* Rehd. cv. Hengshan or *C. chinensis* collected from the Jianshih, Heping and Dungshr orchards, PCR fragments of 780 bp and 1,015 bp were amplified from PDTW and PDTWII phytoplasma, respectively. In addition to the individual infection, a mixed infection of an individual *P. serotina* Rehd. cv. Hengshan (lanes 1, 2, 5, and 6, Figure 2) or *C. chinensis* by PDTW or PDTWII phytoplasma was also revealed. This is the first report that associates PDTWII phytoplasma and the previously identified PDTW phytoplasma with the pear decline in the Heping and Dungshr areas (Liu et al., 2007a).

Transmission electron microscopy

Phytoplasma particles were observed in the sieve tubes of the phloem in the ultra-thin sections of the leaves of diseased pears but not in the sieve tubes of the examined symptomless plants. Phytoplasma particles were also observed in the intestine walls close to the *C. chinensis* and *C. quanli* hemolymph.

DISCUSSION

Since the causative agent of pear decline in Australia is a phytoplasma of group 16SrII (Schneider and Gibb, 1997) and the results of PCR and further sequencing studies showed that both PDTW phytoplasma of the 16SrX group and 16SrII phytoplasma were carried by *C. chinensis* (Liu et al., 2007b), we considered the possibility that a 16SrII phytoplasma may be associated with pear decline in Taiwan. Subsequent studies demonstrated that the PDTW phytoplasma and the 16SrII phytoplasma were transmitted from diseased *P. serotina* Rehd. cv. Hengshan to periwinkle plants by grafting, indicating that phytoplasmas of two 16S rDNA groups inhabit infected plants and insect vectors and can serve as sources of phytoplasmas in pear orchards in Taiwan. To distinguish between the two groups of phytoplasmas that were associated with pear decline in Taiwan, the pear-decline 16SrII phytoplasma is abbreviated to "PDTWII phytoplasma".

This study also demonstrated the transmission of PDTW phytoplasma and PDTWII phytoplasma by field-collected *C. chinensis* to *P. serotina* Rehd. cv. Hengshan in a transmission trial. Psyllidae are insect vectors with high specificity of host plants and phytoplasmas, but this work demonstrated that *Cacopsylla* spp. can transmit phytoplasmas of both group 16SrII and group 16SrX simultaneously. Before this work, only aster leafhoppers and pear psyllids (*C. pyri*) had been identified as being able to transmit more than one group of phytoplasmas (Lee et al., 1998; Goodwin et al., 1999; Križanac et al., 2008). Only a very low population of the other candidate insect vector of PDTW phytoplasma, *C. qianli*, was maintained after 1995 in pear orchards in Taiwan, causing difficulty in the transmission trial using *C. qianli*. In the transmission trial with *C. chinensis*, the leaves of infected pear seedlings exhibited the characteristic upward curling symptom of pear decline (Liu et al., 2007a). Based on PCR detections and symptom developments during transmission trials, pear trees in Taiwan were either infected by PDTW or PDTWII phytoplasma, or co-infected by both, and their infection transmitted by *C. chinensis*.

All diseased pear trees in the orchards exhibited the characteristic upward leaf curling symptom along their longitudinal axes. Singly PDTWII phytoplasma-affected pear trees rarely exhibit the premature reddening of leaves that was evident in most PDTW phytoplasma-infected pear trees (Liu et al., 2007a). Some of the PDTWII phytoplasma-infected pears exhibited the characteristic

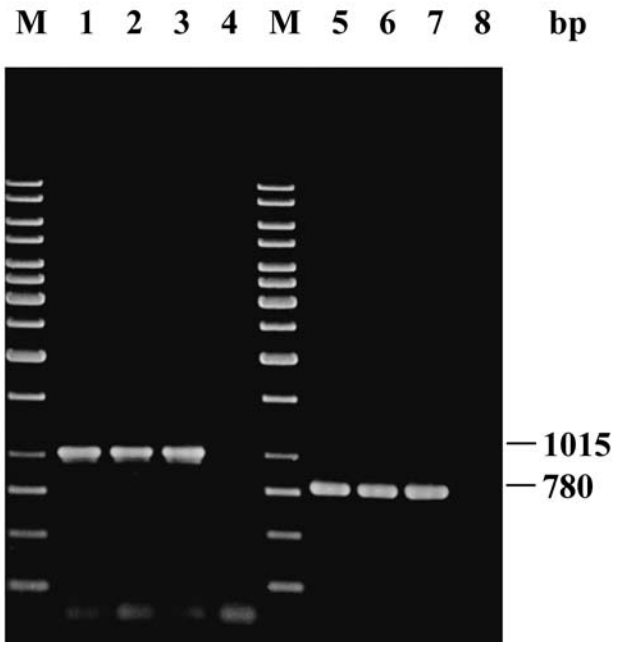

Figure 2. Polymerase chain reaction (PCR) amplification with PDTWII phytoplasma-specific primer IIPf2/ IIPr1 in a semi-nested PCR after a direct PCR using primer IIPf1/ IIPr1 (lanes 1-4), and with PDTW phytoplasma-specific primer APf3/ rPDS1 in a semi-nested PCR after a direct PCR using primer fPD2/ rPDS1 (lanes 5-8) with DNA templates prepared from: lanes 1, 2, 5,and 6, single diseased pear tree from Jianshih area; lane 3, PDTWII phytoplasma-affected periwinkle; lane 7, PDTW phytoplasma-affected pear; lanes 4 and 8, negative control without DNA template. M, GeneRuler^{TM} 1 kb DNA ladder (Fermentas Corp., Burlington, Canada) as molecular weight standards. Sizes (in bp) of PCR products are shown on the right.

downward curling symptom of pear decline from the tips of leaves (Seemüller, 1990, 1992), which was not evident in PDTW phytoplasma-infected pears (Liu et al., 2007a). However, the pear trees that were co-infected by PDTWII and PDTW phytoplasmas exhibited symptoms such as small, sparse foliage, leaf curling, premature foliar reddening, wilting fruit and die-back.

A classification of phytoplasmas, based on the symptoms they cause in periwinkle, yields some similarities to those identified by genetic classification. However, several cases have demonstrated that phytoplasmas that are grouped by symptoms differ genetically. In this work, the pear decline phytoplasmas of group 16SrX and group 16SrII were experimentally transmitted to periwinkles and pears, and they did not cause significant distinguishable symptoms on *P. serotina* Rehd. cv. Hengshan as shown in the field (Liu et al., 2007a). Phytoplasmas associated with sweet potato witches' broom (SPWB) and peanut witches' broom (PnWB) are both group-16SrII phytoplasmas found in Taiwan and can cause witches'-broom symptom in periwinkle plants (Lin and Lin, 1998). However, PDTWII phytoplasma of the same group induced symptoms of small leaves and flowers similar to those caused by sweet potato little leaf (SPLL) in Australia (Schneider and Gibb, 1997) but did not cause obvious symptom of witches' broom in the affected periwinkles. Based on the symptoms induced in periwinkle plants and on the 16S rDNA sequence analysis, PDTWII phytoplasma is considered a new strain of group II phytoplasma in Taiwan.

In field detection performed in 2001 and 2002, the rDNA sequence of the 16SrII phytoplasma had never been amplified using PCR with DNA templates that had been prepared from pear decline-infected trees in Taiwan. A new psyllid, *C. chinensis,* which has been identified as a carrier of PDTWII phytoplasma, invaded the central part of Taiwan in 2002, migrating to the northern part in 2004 (Yang et al., 2004). It is now the dominant pear psyllid species in Taiwan. In 2004, no phytoplasma PCR product was amplified using DNA templates that were prepared from *C. chinensis* or pear trees in the Jianshih area of northern Taiwan. One year after the initial discovery of *C. chinensis* in the Jianshih area, the rDNA sequences of both PDTW phytoplasma and PDTWII phytoplasma were for the first time amplified and sequenced using samples of *C. chinensis* that were collected from the same area in August, 2005. Additionally, in 2006, pear plants began to exhibit red foliage, small developing shoots and hard fruit (Liu et al., 2007b). Phytoplasma-infected *C. chinensis* and pear plants were then detected and identified, and both PDTW and PDTWII phytoplasma have been detected repeatedly in diseased pears and *C. chinensis* since then. In California and Italy, more than one group of phytoplasmas has been found in samples from PD-diseased pears (Kirkpatrick et al., 1994; Lee et al., 1995). The host range of phytoplasmas may depend on their interaction with insect vectors (Seemüller et al., 1998). Individual infection with either PDTWII phytoplasma or the previously identified PDTW phytoplasma (Liu et al., 2007a) and co-infection with both phytoplasmas was also detected in diseased pears in the Heping and Dungshr areas in central Taiwan. Since the orchard population of *C. qianli* has became very low in recent years, *C. chinensis* is now the dominant pear psyllid species in Taiwan. The transmission ability of *C. chinensis* for PDTW and PDTWII phytoplasma was also confirmed. We believe that psyllid *C. chinensis* may now be the major insect vector of PDTW and PDTWII phytoplasma in Taiwan. Psyllid *C. chinensis* may also be responsible for the transmission of both phytoplasmas to pear plants from the central part to the northern part of Taiwan.

In this study, phytoplasmas associated with pear decline in Taiwan were detected in PDTW-affected pear plants and psyllids using PCR and electron microscopy. Previous work using TEM found low populations of phytoplasma randomly distributed in plants (Schneider, 1977; Seemüller, 1992). The observations in this work show that the distribution of phytoplasma in *C. qianli* is highly centralized in abdominal intestines but is uneven in *C. chinensis*. Whether the propagating ability of phytoplasmas in the

two species of pear psyllids is the factor that dominates the transmission efficiency has yet to be revealed. The authors' future work will use real-time PCR to evaluate the relative concentrations of PDTW and PDTWII phytoplasma in multiplex phytoplasma-affected pear trees and insect vectors.

Acknowledgements. The authors would like to thank the Council of Agriculture, Taiwan, R.O.C., for financially supporting this research under project No. 97AS-14.2.3-BQ-B7(5). Dr. C. C. Chen is appreciated for helping with the field survey and for offering technical guidance regarding TEM, Dr. M. M. Yang for identifying the insects, and Dr. T. H. Hung for helpful discussions and comments on the manuscript. Ted Knoy is appreciated for his editorial assistance.

LITERATURE CITED

Agrios, G.N. 2005. *Plant pathology,* 5th edn. Academic Press, San Diego, California.

Avinent, L., G. Llácer, J. Almacellas, and R. Torá. 1997. Pear decline in Spain. Plant Pathol. **46:** 694-698.

Bosco, D., S. Palermo, G. Mason, R. Tedeschi, C. Marzachi, and G. Boccardo. 2002. DNA-based methods for the detection and the identification of phytoplasmas in insect vector extracts. Mol. Biotechnol. **22:** 9-18.

Carraro, L., N. Loi, P. Ermacora, A. Gregoris, and R. Osler. 1998. Transmission of pear decline by using naturally infected *Cacopsylla pyri* L. Acta Hortic. **472:** 665-668.

Chen, C.C., T.D. Liu, C.P. Lin, and K.C. Kuo. 2001. Comments on the disease similar to pear decline in Taiwan. Plant Pathol. Bull. **43:** 1-5.

Chou, L. and S. Fang. 1994. New record of *Psylla qianli* (Homoptera: Psyllidae) from Taiwan. J. Agric. Res. China **43:** 467-468.

Davies, D.L., D.J. Barbara, and M.F. Clark. 1995. The detection of MLOs associated with pear decline in pear trees and pear psyllids by polymerase chain reaction. Acta Hortic. **386:** 484-488.

Davies, D.L., C.M. Guise, M.F. Clark, and A.N. Adams. 1992. Parry's disease of pears is similar to pear decline and is associated with mycoplasma-like organisms transmitted by *Cacopsylla pyricola*. Plant Pathol. **41:** 195-203.

Deng, S. and D. Hiruki. 1991. Amplification of 16S rRNA genes from culturable and nonculturable mollicutes. J. Microbiol. Methods **14:** 53-61.

Garcia-Chapa, M., J. Sabaté, A. Laviña, and A. Batlle. 2005. Role of *Cacopsylla pyri* in the epidemiology of pear decline in Spain. Eur. J. Plant Pathol. **111:** 9-17.

Goodwin, P.H., G.S. Mahuku, H. Liu, and B.G. Xue. 1999. Monitoring phytoplasma in populations of aster leafhoppers from lettuce fields using the polymerase chain reaction. Crop Prot. **18:** 91-99.

Kirkpatrick, B.C., C. Smart, S. Gardner, J.L. Gao, U. Ahrens, R. Maurer, B. Schneider, K.-H. Lorenz, E. Seemüller, N. Harrison, S. Namba, and X. Daire. 1994. Phylogenetic relationships of plant pathogenic MLOs established by 16/23S rDNA spacer sequences. IOM Lett. **3:** 228-229.

Križanac, I., I. Mikec, Z. Budinščak, M.Š. Musić, M. Krajačić, and D. Škorić. 2008. Pomaceous fruit tree phytoplasmas and their potential vectors in Croatia. Acta Hortic. **781:** 477-482.

Lee, I.M., A. Bertaccini, M. Vibio, and D.E. Gundersen. 1995. Detection of multiple phytoplasmas in perennial fruit trees with decline symptoms in Italy. Phytopathology **85:** 728-735.

Lee, I.M., D.E. Gundersen-Rindal, R.E. Davis, and I.M. Bartoszyk. 1998. Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. Int. J. Syst. Bacteriol. **48:** 1153-1169.

Lee, I.M., Y. Zhao, R.E. Davis, W. Wei, and M. Martini. 2007. Prospects of DNA-based systems for differentiation and classification of phytoplasmas. Bull. Insectol. **60:** 239-244.

Lemoine, J. 1991. Dépérissement du poirier. Rôle de *Psylla pyri* dans sa dissémination. Arboric. Fruitière **442:** 28-32.

Lin, T.C. and C.P. Lin. 1998. Evaluation of universal PCR primers for the detection of phytoplasmas. Plant Pathol. Bull. **7:** 33-42.

Liu, H.L., C.C. Chen, and C.P. Lin. 2007a. Detection and identification of the phytoplasma associated with pear decline in Taiwan. Eur. J. Plant Pathol. **117:** 281-291.

Liu, H.L., S.L. Liu, M.M. Yang, and C.P. Lin. 2007b. Studies on the insect vectors and ecology of pear decline in Taiwan. Plant Prot. Bull. **49:** 13-26.

Nicholas, K.B. and H.B. Jr. Nicholas. 1997. GeneDoc: A tool for editing and annotating multiple sequence alignments. Retrieved from http://www.psc.edu/biomed/genedoc

Schneider, B. and K.S. Gibb. 1997. Detection of phytoplasmas in declining pears in southern Australia. Plant Dis. **81:** 254-258.

Schneider, B., E. Seemüller, C.D. Smart, and B.C. Kirkpatrick. 1995. Phylogenetic classification of plant pathogenic mycoplasma-like organisms or phytoplasmas. *In* S. Razin and J.G. Tully (eds.), Molecular and diagnostic procedures in mycoplasmology, Vol. 1. Academic Press, San Diego, California, pp. 369-380.

Schneider, H. 1977. Indicator hosts for pear decline: symptomatology, histopathology, and distribution of mycoplasma-like organisms in leaf veins. Phytopathology **67:** 592-601.

Seemüller, E. 1990. Pear decline. *In* A.L. Jones and H.S. Aldwinckle (eds.), Compendium of apple and pear disease. APS Press, St. Paul, Minnesota, pp. 68-69.

Seemüller, E. 1992. Pear decline. *In* J. Kumar, H. S. Chaube, U. S. Singh, and A. N. Mukhopadhyay (eds.), Plant diseases of international importance, Vol. 3. Prentice Hall, Eaglewood Cliffs, New Jersey, pp. 308-334.

Seemüller, E., C. Marcone, U. Lauer, A. Ragozzino, and M. Göschl. 1998. Current status of molecular classification of

the phytoplasmas. J. Plant Pathol. **80:** 3-26.

Seemüller, E. and B. Schneider. 2004. '*Candidatus* Phytoplasma mali', '*Candidatus* Phytoplasma pyri' and '*Candidatus* Phytoplasma prunorum', the causal agents of apple proliferation, pear decline and European stone fruit yellows, respectively. Int. J. Syst. Evol. Microbiol. **54:** 1217-1226.

Smart, C.D., B. Schneider, C.L. Blomquist, L.J. Guerra, N.A. Harrison, U. Ahrens, K.H. Lorenz, E. Seemüller, and B.C. Kirkpatrick. 1996. Phytoplasma-specific PCR primers based on sequences of the 16S-23S rRNA spacer region. Appl. Environ. Microbiol. **62:** 2988-2993.

Thompson, J.D., F. Plewniak, and O. Poch. 1999. A comprehensive comparison of multiple sequence alignment programs. Nucleic Acids Res. **27:** 2682-2690.

Yang, M.M., J.H. Huang, and F. Li. 2004. A new record of Cacopsylla species (Hemiptera: Psyllidae) from pear orchards in Taiwan. Formosan Entomol. **24:** 213-220.

造成台灣地區梨樹衰弱病之二群植物菌質體之研究

劉淑玲[1] 劉秀玲[1] 張淑貞[2] 林長平[1]

[1] 國立台灣大學 植物病理與微生物學系

[2] 行政院農業委員會農業試驗所 應用動物組

本研究利用聚合酵素連鎖反應之檢測策略 (Polymerase chain reaction (PCR)-based detection strategies) 進行台灣梨樹衰弱病 (pear decline in Taiwan, PDTW) 之流行病學研究及媒介昆蟲之研究。本研究成功利用 PCR 由感染梨衰弱病之橫山梨 (*Pyrus serotina* Rehd. cv. Hengshan) 植株及媒介昆蟲中國梨木蝨 (*Cacopsylla chinensis*) 全 DNA 中增幅出兩種 PCR 產物，經由選殖定序及比對分析，顯示其分別屬於 group 16SrX 之台灣梨衰弱病 (PDTW) 植物菌質體與另一屬於 group 16SrII 之 pear decline phytoplasma (PDTWII) 之 16S rDNA 序列。由罹病梨樹所增幅出之 PDTW 及 PDTWII 植物菌質體之 16S rDNA 序列，分別與由中國梨木蝨 (*C. chinensis*) 上增幅出之兩種植物菌質體序列相同。藉由中國梨木蝨咬食傳菌試驗，證實 PDTW 以及 PDTWII 植物菌質體皆能由中國梨木蝨傳播到梨株。17 株試驗梨樹中，有一株同時感染 PDTW 及 PDTWII 植物菌質體，另有十株感染 PDTWII 植物菌質體。嫁接傳菌試驗亦由田間罹病梨株枝條嫁接 PDTW 以及 PDTWII 植物菌質體至日日春 (*Catharanthus roseus*)。根據上述 PDTW 與 PDTWII 菌質體之 rRNA 基因序列，分別設計出 PDTW 菌質體及 PDTWII 菌質體之專一性引子對，作為後續田間病害檢測及可能媒介昆蟲生態調查之工具。針對罹病梨葉、黔梨木蝨 (*C. qianli*) 及中國梨木蝨進行 TEM 切片觀察，分別於植物篩管、韌皮部薄壁細胞以及蟲體腸壁組織中，皆觀察到植物菌質體。

關鍵詞：嫁接；昆蟲咬食傳菌試驗；植物菌質體偵測；穿透式電子顯微鏡；媒介昆蟲。