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Occurrence of Petunia Flattened Stem Caused by Phytoplasma

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This study describes a phytoplasmal disease occurring in *Petunia* leaves grown in the glasshouse of the National Horticultural Research Institute, Suwon, Korea. Abnormal growth like flattened stem with flower malformation or phyllody was observed from the plant. The DNA extracted from the diseased leaves was amplified using a universal primer pair of P1/P6 derived from the conserved 16S rRNA gene of Mollicutes giving the expected polymerase chain reaction (PCR) product of 1.5 kb. In the nested PCR assays, the expected DNA fragment of 1.1 kb was amplified with the specific primer pair R16F1/R16R1 that was designed on the basis of aster yellows (AY) phytoplasma 16S rDNA sequences. The 1.1 kb PCR products were cloned and nucleotide sequences were determined, and the sequences of the cloned 16S rRNA gene were deposited in the GenBank database under the accession no. of EU267779. Analysis of the homology percent of the 16S rDNA of PFS-K showed the closest relationship with Hydrangea phyllody phytoplasma (AY265215), Brassica napus phytoplasma (EU123466) and AY phytoplasma CHRY (AY180956). Phytoplasma isolated from the diseased *Petunia* was designated as Petunia flat stem phytoplasma Korean isolate (PFS-K) in this study. Flattened stem occurring in *Petunia* was confirmed as infection of AY group of phytoplasma by determination of 16S rRNA gene sequences of phytoplasma and microscopic observation of phytoplasma bodies. This is the first report on the phytoplasmal disease in *Petunia* in Korea.

Keywords **:** flat stem, malformation, petunia, phytoplasma

Phytoplasmas (previously called mycoplasma-like organisms) are phloem-limited plant pathogenic prokaryotes. They are characterized by their lack of a cell wall, a pleiomorphic or filamentous shape, normally with a diameter less than 1 micrometer (Hopkins, 1977). They are known as the causal agents of yellowing, stunting, phyllody and witches'-broom diseases in various plants (Bertaccini et al., 1990; McCoy et al., 1989). Phytoplasmal disease in *Petunia* was firstly reported in 1964 from *Petunia* showing stunt or yellow symptom (Doi et al., 1967). Recently 16S ribosomal RNA gene (rRNA) sequences of Petunia flat stem phytoplasma (AY283186) were reported from China (PFS-C) (not published).

Abnormal growth like flattened stem with flower malformation or phyllody was observed in *Petunia* plants grown in a glasshouse in the National Horticultural Research Institute, Suwon, Korea. They were sown in February and were cultivated inside the glasshouse with windows kept open during the daytime since early March. Phytoplasmas were identified from those *Petunia* plants by sequence analysis of 16S rRNA gene and electron microscopic observation of phytoplasmal bodies from the sieve tube elements of phloem tissues.

Materials and Methods

Source of diseased *Petunia*. *Petunia* showing abnormal growth flattened stem (Fig. 1) was collected from the glasshouse raising *Petunia* breeding lines in the National Horticultural Research Institute, Suwon, Korea.

DNA isolation and primers for PCR. DNA was prepared from leaf midribs by a method described previously (Lee and Davis, 1983). Two pairs of primers were used for PCR. A primer pair P1/P6 (Deng and Hiruki, 1991), located in the 16S rDNA, were employed in direct PCR to prime a DNA fragment of 1.5 kb expected size. Primer pair R16F1/R16R1 (specific for aster yellows (AY) group phytoplasma) (Lee et al., 1994) were used in nested PCR. PCR was conducted as previously described (Chung et al., 2007).

Cloning of PCR products and nucleotide sequencing. PCR product amplified with primer pair R16F1/R16R1 was cloned using pGEM-T easy vector (Promega, USA) according to the manufacture's instruction. The ligation mixture was used to transform competent cells of *Escherichia coli* JM 109. Recombinants were screened by blue and white screening method (Sambrook et al., 1989). Nucleotide sequences were determined using ABI Prism BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems, USA).

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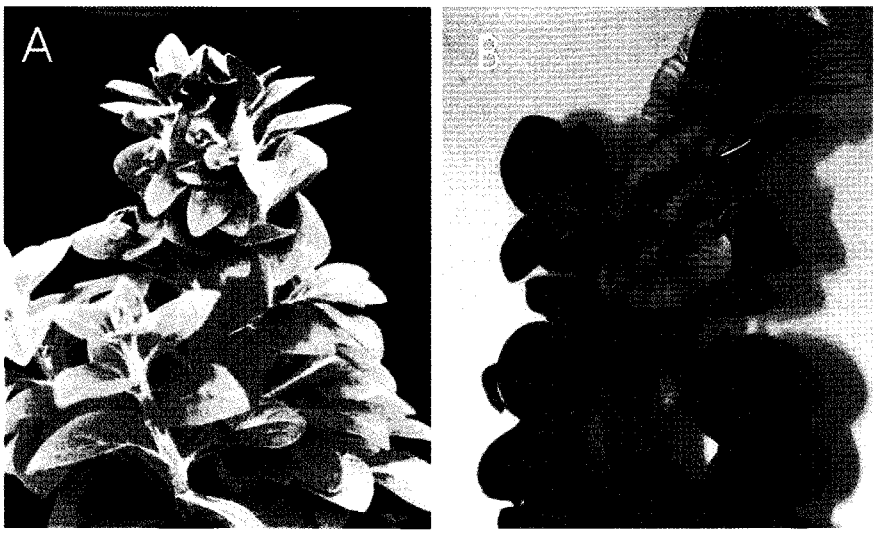

Fig. 1. *Petunia* seedlings infected by Petunia flat stem phytoplasma Korean isolate (PFS-K). (A) Flattened stem with phyllody, (B) Flattened stem with flower distortion.

Phylogenetic analysis. The 16S rRNA gene sequences were aligned using CLUSTAL W using DNASTAR version 7.0 (Madison, WI. USA), and compared phylogenetically to other phytoplasma sequences grouped in 16S rRNA grouped I and II deposited in GenBank (Firrao, 2004).

Electron microscopy. Small pieces from the leaf midribs of PFS-K infected plants were prefixed in 1% Karnovsky's fixative solution, postfixed in 1% osmium tetroxide in cacodylate buffer, pH 7.2, and dehydrated in an ethanol series of 50, 75, 90, 95 and 100% for 30 min each step. Embedding was conducted in Spurr resin (Electron Microscopy Science, Washington, PA). Ultrathin sections were prepared with ultramicrotome, stained with 2% uranyl acetate and 0.08 M lead citrate buffer, pH 12.0. The grids were examined with a Carl Zeiss LEO 906 transmission electron microscope.

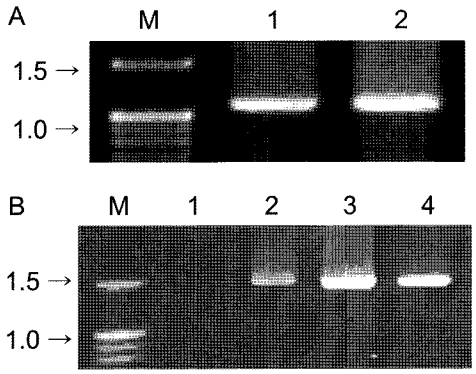

Fig. 2. Amplification of a 16S rDNA sequences from *Petunia* showing symptom of flattened stem using a universal primer pair P1/P6 (A) and a AY specific primer pair 16F1/R1 for nested PCR (B). (A) Lane 1, healthy *Petunia*; lanes 2-4, diseased *Petunia*; (B) lanes 1-2, nested PCR. Lane M, 1 kb DNA ladder. PCR products were separated by electrophoresis using 1.5% agarose gel.

Results

Detection of phytoplasma 16S rRNA gene from plants by PCR. Using the universal primer set, a 1.5 kb DNA fragment was amplified from the diseased *Petunia* (Fig. 2A). In the nested PCR assays, the expected DNA fragment of 1.1 kb was amplified with an AY specific primer pair R16F1/R16R1 (Fig. 2B).

Sequence analysis. The nucleotide sequences of the cloned 16S rRNA gene have been deposited in the GenBank database under the accession no. of EU267778. Analysis of the homology percent of the 16S rDNA from this study showed the closest relationship with AY phytoplasmas of *Hydrangea phyllody* phytoplasma (AY265215), *Brassica napus* phytoplasma (EU123466) and AY phytoplasma CHRY (AY180956) (Table 1; Fig. 3). PFS-K, designated in this study, showed 98.7% sequence identity in 16S rRNA gene with Petunia flat stem phytoplasma (AY283186) reported from China (PFS-C) (Table 1).

Electron microscopy. In the ultra-thin sections of the leaf midribs irregularly globous or amorphous phytoplasmal bodies of 230 nm to 650 nm in size were present in sieve tube elements of phloem tissue (Fig. 4).

Discussion

Petunia is grown by sowing in early February and transplanting in early March into pots in glasshouses. From early

Table 1. Sequence identity percent of the 16S rDNA from this study with other phytoplasmas grouped in 16S rRNA I and II

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1		99.1	99.4	99.4	99.2	99.4	99.8	99.8	99.4	90.0	90.1	90.0	90.6	99.2
2			99.6	99.6	98.2	99.7	99.1	99.0	98.9	89.8	89.9	89.8	90.3	99.5
3				100.0	98.3	99.5	99.5	99.3	99.2	89.9	90.1	89.8	90.3	99.7
4					98.3	99.5	99.5	99.3	99.2	89.9	90.1	89.8	90.3	99.7
5						98.3	98.7	98.6	98.6	89.1	89.2	89.1	89.6	98.7
6							99.1	98.5	99.2	84.6	89.8	85.7	85.1	99.7
7								99.7	99.4	89.7	89.9	89.6	90.1	99.2
8									99.4	84.3	89.9	85.9	80.9	99.2
9										90.2	90.3	90.1	90.6	99.0
10											99.5	97.9	98.5	89.7
11												98.2	98.7	89.8
12													99.0	89.6
13														90.1

1, AF177384(I); 2, AF268403(I); 3, AY180956(I); 4, AY265215 (I); 5, AY283186(I); 6, EU123466(I); 7, M30790(I); 8, M86340(I); 9, U96616(I); 10, AB026155(II); 11, AF028813(II); 12, AF200718(II); 13, U15442(II); 14, EU267779(PSF-K).

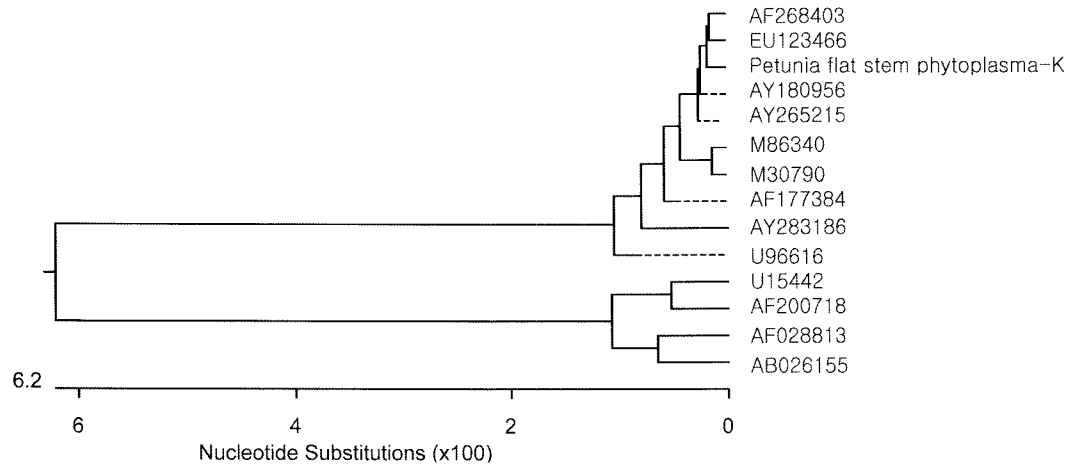

Fig. 3. Phylogenetic tree constructed by CLUSTAL method using DNASTAR software version 7.0 (DNASTAR, Madison, WI. USA), comparing 16S rRNA gene sequences of Petunia flat stem phytoplasma-Korean isolate (PFS-K) and other phytoplasmas registered in GenBank (www.ncbi.nlm.nih.gov). The scale refers to the similarity index.

March, glasshouse windows are usually kept open during the daytime without insect proof facilities. It means insect vectors of phytoplasmal diseases could invade *Petunia* during seedlings, because plenty of phytoplasma sources are present in Korea (Lee, 2004). So far, 52 phytoplasmal diseases have been reported in various plants (Lee, 2004). Accordingly, flattened stem of *Petunia* was assumed to be transmitted by vectors like leaf hoppers that got into inside the glasshouse. Further studies are required about insect vectors transmitting phytoplasmal diseases to *Petunia*.

French scientists have identified two genes in *Arabidopsis* that, when mutated, cause fasciation (development of flattened organs, usually stems) (Reboredo and Silvares, 2007), although a number of other factors can cause this kind of symptom that looks like a mutation. Fasciation has been experimentally produced using X-rays or chemical mutagens (Reboredo and Silvares, 2007). In nature, it has been attributed to infection with various disease agents or insect infestation. Of the disease-causing agents, the most commonly associated pathogen is phytoplasma (Reboredo and Silvares, 2007). Fasciation was observed from *Lilium* Oriental hybrids (Chung and Jeong, 2003) and from maple (Sinclair et al., 1987) by infection with phytoplasmas. Flattened stem observed in *Petunia* in this study was also a kind of fasciation caused by a phytoplasma.

Symptoms of *Petunia* infected with PFS-K were very similar to PFS-C but their similarity of 16S rRNA gene sequences (98.7%) was less than with *Hydrangea phyllody* phytoplasma, *Brassica napus* phytoplasma or AY phytoplasma CHRY strain, suggesting that source *Petunia* plant

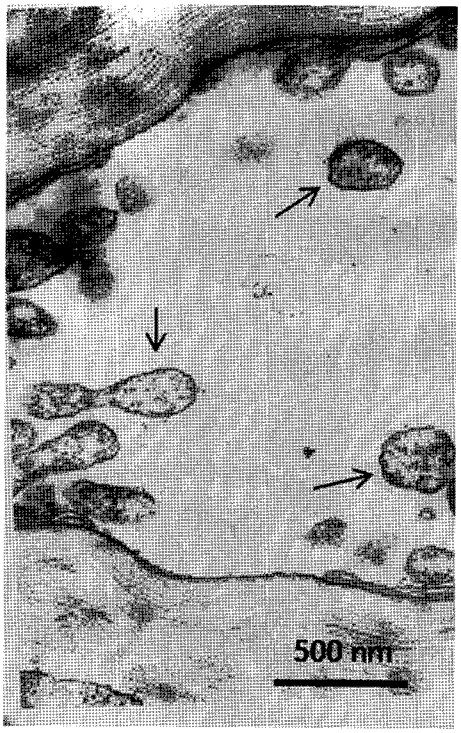

Fig. 4. Electron micrograph of the sieve elements of leaf midrib of *Petunia* infected with Petunia fat stem phytoplasma-Korean isolate (PFS-K), showing severe membrane-bound structures measured shorter than 1 μm (arrows).

of PFS-K and PFS-C may not be the same phytoplasma strain. A different phytoplasma strain could cause the similar symptom in the same plant species. Symptomatology of phytoplasma infection on plants did not always agree with the similarity percentages of the sequences of the 16S rRNA gene (Chung et al., 2007).

Phytoplasma can be classified based on the 16S rRNA gene sequences, because the 16S rRNA gene is universal in prokaryotes and possesses both conserved and variable regions that make it useful for taxonomic studies (Seemüller et al., 1994). PSF-K showed the closest relationship with AY phytoplasmas of *Hydrangea phyllody* phytoplasma strain (AY265215), *Brassica napus* phytoplasma (EU123466) and AY phytoplasma CHRY strain (AY180956) grouped in 16SrI. They showed 99.7% sequence similarity. Sequence homology percent of 16S rDNA concluded that PSF-K should be grouped into 16SrI, AY group.

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