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Detection and Molecular Characterization of a Stolbur Phytoplasma in *Lilium* Oriental Hybrids

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Stolbur phytoplasma was detected from *Lilium* Oriental hybrids showing flattened stem and flower clustering. The presence of phytoplasma was demonstrated using polymerase chain reaction (PCR) assays with phytoplasma-universal (P1/P6) and stolbur phytoplasma-specific 16F1/R1-S primer pairs amplifying phytoplasma 16S rDNA regions. Nucleotide sequences of the phytoplasma 16S rDNA were determined. Nucleic acid extracted from lily amplified 1.5 kb DNA with a phytoplasma universal primer pair. In nested PCR, 1.1 kb PCR product was obtained using specific primer pair, indicating an isolate of stolbur phytoplasma. Nucleotide sequence of phytoplasma 16S rDNA reported in this study showed 99.5% and 99.1% identities with two known stolbur phytoplasmas (16Sr XII-A). Also, it exhibited a sequence homology of 98.0% with phormium yellow leaf (16Sr XII-B), and 97.9% with Australian grapevine yellows (16Sr XII-B). Meanwhile, it showed 98.1% identity with strawberry green petal phytoplasma, (16SrI-C), and 94.7 % with American aster yellows (16SrI-B). Homology percentage of the 16S rDNA nucleotide sequence suggests that this phytoplasma could be classified into the stolbur phytoplasma, subgroup A (16Sr XII-A), as a type strain stolbur.

Keywords **:** *Lilium*, phytoplasma, rDNA, stolbur.

In 1954, Brierley and Smith described symptoms of lily rosette on *Lillium longiflorum* 'Croft' and 'Georgia'. The affected plants showed stunting and rosette-like symptoms. Bertaccini and Marani (1982) described flower and leaf malformation and discoloration in lily hybrid 'Pink Perfection' in Italy associated with multiple infection of *Lily mottle virus* (LMoV) and *Lily symptomless virus* (LSV), and the presence of phytoplasmas. Phormium yellow leaf phytoplasma, a lethal disease of the large tufted monocotyledon New Zealand flax (*Phormium tenax*), which is a member of the family *Liliaceae*, was phylogenetically assigned to the stolbur phytoplasma (16Sr XII-A) and German grapevine yellows (16Sr XII-A) based on 16S rRNA gene sequences (Liefting et al., 1996). Phytoplasma infection was reported in *L. martagon* showing flattened stems based on RFLP analysis of PCR amplification of 16S rDNA. This phytoplasma was classified as aster yellows (AY) phytoplasma (16Sr), subgroup C, with clover phyllody phytoplasma as a type strain (Voráckovà et al., 1998). Recently, presence of AY phytoplasma (AY I-B) was reported from plants of three groups – *L. longiflorum* and Oriental and Asiatic lily hybrids – showing stunting and flower bud deficiency (Kaminska and Korbin, 2002).

The objective of this study was to report on the occurrence of phytoplasma in lily grown in commercial greenhouses in Korea. The presence of phytoplasmas in the plants examined was tested by PCR and nucleotide sequence determination of 16S rRNA gene.

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Materials and Methods

Plant material. *Lilium* Oriental hybrid 'Conamore' showing flattened stem was collected from commercial greenhouses in Paju, Kyonggi Province. Lily plants showing stem flattening were designated as Ph-lily in this study. The incidence of phytoplasma disease was surveyed by visual inspection on 11 commercial farms in the main cultivation regions of Hwaseong, Icheon, Paju and Jeju.

DNA extraction. DNA was extracted from the petioles and the leaf vein of lilies using the method of Gibb and Padovan (1994).

PCR. The presence of phytoplasmas in lily showing flattened stems was demonstrated using PCR assays with universal (P1/P6) and specific R16F1/R1-S primer pairs amplifying phytoplasma 16S rDNA regions. P1/P6 was previously designed based on the common 16S rDNA sequences of phytoplasma (Deng and Hiruki, 1991). The sequence of the forward primer (P1) was 5'-AAGAGTTTGATCCTGGCTCAGGATT-3' and that of the reverse primer (P6) was 5'-TGGTAGGGATACCTTGTTACGACTTA-3'. R16F1/R1-S was designed in this study to amplify 16S rDNA sequences based on the nucleotide sequence of a stolbur group of phytoplasma (GenBank accession no. X76427), and was used in nested-PCR to amplify DNA fragment of 1.1 kb.

The sequence of the forward primer (R16F1) was 5'-TAAAAGACCTAGCAATAGG-3' and that of the reverse primer (R1-S) was 5'-CAATCCGAACTGAGACTGC-3'.

The PCR reaction mixture contained 20 ng of nucleic acid, 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 100 mM of each dNTP, 0.4 mM of each primer, 2.5 mM $MgCl_2$, and 2.5 U *Taq* DNA polymerase (Perkin Elmer, Roche, Branchbug, NJ, USA). Amplification was performed in a DNA Thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, USA). The thermal conditions for the primer set P1/P6 included 35 cycles of denaturation at 94°C for 30 seconds (2 minutes for the first cycle), annealing at 65°C for 50 seconds and extension at 72°C for 1.5 minutes. The last cycle was extended for an additional 3 minutes at 72°C. The amplified nucleic acid was precipitated by adding 0.1 volumes of 3 M sodium acetate, pH 5.2 and 2.5 volumes of cold ethanol, and standing at −70°C for 1 hour. Nucleic acid precipitated was recovered and used as template in nested-PCR.

In nested-PCR, approximately 10 ng of the precipitated nucleic acid was used as template. A total of 35 thermal cycles were carried out consisting of denaturation at 94°C for 1 minute (4 minutes for the first cycle), annealing at 55°C for 1 minute and extended at 72°C for 1.5 minutes, and extended again for 7.5 minutes in the last cycle. The PCR product was separated by electrophoresis on 1.5% agarose gel in 0.5× TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.0) (Sambrook et al., 1989) at 100 V for 20 minutes, and then visualized by staining in ethidium bromide solution under a UV transilluminator.

RFLP analysis of PCR products. The amplified nested-PCR product of 1.1 kb was precipitated by adding 0.1 volumes of 3 M sodium acetate, pH 5.2, and 2.5 volumes of cold ethanol. Approximately 100-200 ng of the precipitated nucleic acid (1.1 kb) was digested with each of the restriction endonuclease *Sau*3A, *Hha*, *Alu*, *Rsa*, *Mse*I, and *Kpn*I according to the manufacturer's instructions (Takara, Shiga, Japan). Nucleic acids (1.1 kb) treated with each endonuclease were separated by electrophoresis on 1.8% agarose gel in 0.5× TBE buffer (44.5 mM Tris-HCl, 44.5 mM boric acid, 1 mM Na2-EDTA, pH 8.3).

Nucleotide sequence determination of 16S rDNA. Nested-PCR products of 1.1 kb DNA was gel purified with the Promega GeneClean III kit (BIO 101, Carlsbad, CA) and ligated into the pGEM-T easy vector (Promega) according to the manufacturer's instruction. The ligation mixture was used to transform competent cells of *Escherichia coli* JM 109. Recombinants were screened for white and antibiotic resistant colonies on LB media containing 100 mg/mL ampicillin, 80 mg/mL X-Gal, and 0.5 mM IPTG. Plasmid DNA was extracted by alkaline lysis methods (Sambrook et al., 1989).

Nucleotide sequences of the cloned PCR products were determined using ABI PrismTM Terminator Cycle Sequencing Ready Reaction Kit and ABI Prism 377 Genetic Analyzer (Perkin Elmer, USA). Alignment of nucleotide sequence and the phylogenetic analysis were conducted using DNASTAR program (DNASTAR Inc. WI. USA).

Classification of Ph-lily was based on the revised classification scheme of Lee et al. (1998).

Results

Disease incidence. Lilies showing flattened stem have been observed in commercial greenhouses (Fig. 1A). They formed multiple meristems at the end of the stems (Fig. 1B). The most heavily affected plants showed shortening of internodes and witches' broom symptom (Fig. 1C). Numerous flowers remained attached, but some flowers were torn while some stayed unopened (Fig. 1D). Bulb growth was much poor (Fig. 1E). The incidence of lily showing those symptoms ranged from 0.1 to 40.0% (Table 1). The incidence rate varied depending on the cultivars and investigation region (Table 1). These symptoms were observed on a large scale in 'Conamore'.

RFLP analysis of PCR products. Universal primer pair of P1/P6 amplified the 1.5 kb DNA fragment of phytoplasma 16S rDNA from nucleic acid extracted from lily showing disease symptom (Fig. 2A).

In a nested-PCR assay, the amplification of 16S rDNA from 1.5 kb PCR products produced the expected DNA fragment of 1.1 kb (Fig. 2B). The restriction profiles of nested-PCR products from Ph-lily are shown in Fig. 3. The number of RFLP types obtained with each restriction endonucleases *Sau*3AI, *Alu*I, *Rsa*I, *Hha*I, *Kpn*I, and *Mse*I were 2, 3, 2, 1, 3, and 2, respectively.

Nucleotide sequence determination of 16S rDNA. The nucleotide sequences of Ph-lily phytoplasma have been deposited in the GenBank database under the accession number of AY169309. Nucleotide sequence of phytoplasma 16S rDNA from Ph-lily showed 99.5% and 99.1% homology with two stolbur phytoplasmas (16Sr XII-A) of AF248959 and X76427 (GenBank accession no.), respectively. Also, it exhibited a sequence homology of 98.0% with phormium yellow leaf (16Sr XII-B) (GenBank accession no. U43570), and 97.9% with Australian grapevine yellows (16Sr XII-B) (GenBank accession no. L76865). Meanwhile, it showed 98.1% homology with strawberry green petal (16SrI-C) (GenBank accession no. AJ243044), and 94.7% with American aster yellows (16SrI-B) (GenBank accession no. X68373). Homology percentage of the 16S rDNA nucleotide sequence suggest that the phytoplasma isolated from Ph-lily can be included in stolbur phytoplasma, subgroup A (16Sr XII-A), as a type strain stolbur based on the classification scheme of Lee et al. (1998).

The phylogenetic tree constructed by comparing 16S rDNA sequences of Ph-lily with other phytoplasmas (Lee et al., 1998) obtained from the GenBank is shown in Fig. 4. Ph-lily isolate is most closely related to the stolbur (STOL; 16Sr XII-A).

Discussion

The present study provides evidence that plants of *Lilium*

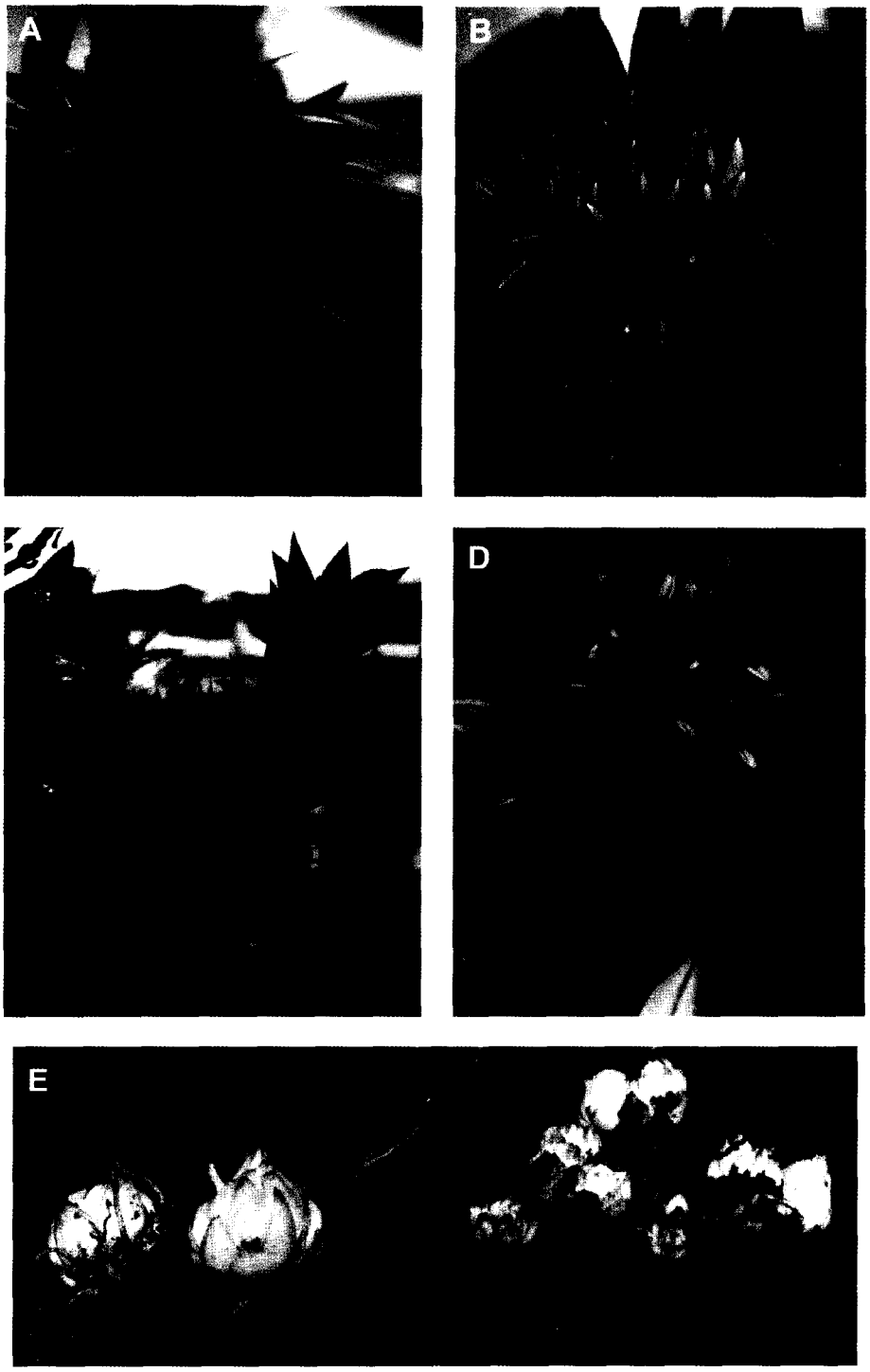

Fig. 1. *Lilium* Oriental hybrids 'Conamore' spontaneously infected with phytoplasma. **A**: Lily showing flattened stem, **B**: multiple meristems, **C**: witches' broom symptom, **D**: tearing of flower leaf and unopened flowers, and **E**: poor bulb growth (left: two bulbs, healthy; right: diseased bulbs).

Oriental hybrids showing flattened stem (Ph-lily) were naturally infected with phytoplasma. The amplification of 1.1 kb DNA in nested PCR with stolbur phytoplasma specific primer pair (16F1/R1-S) from Ph-lily suggests the presence of a stolbur phytoplasma isolate.

Voráckovά et al. (1998) reported the presence of aster yellows (AY) phytoplasma, subgroup C (16SrI-C), from *L. martagon* showing unusual malformation in the shape of a

Table 1. Incidence rate of phytoplasma in *Lilium* Oriental hybrids

Cultivar	Region investigated	No. of farms investigated	No. of plants investigated	Incidence rate (%)
Conamore	Hwaseong	3	160,000	0.6-0.8
	Icheon	5	283,000	0.5-0.9
	Paju	1	80,000	40.0
Renev	Icheon	1	18,000	0.1
Marcopolo	Jeju	1	50,000	0.1

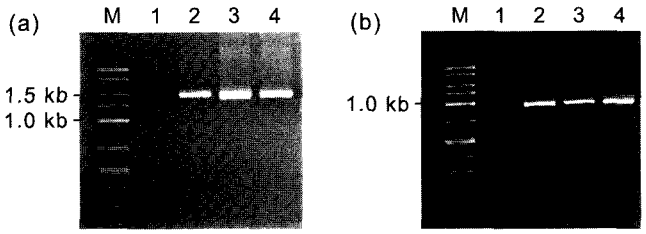

Fig. 2. Electrophoresis of direct (A) and nested (B) PCR products of phytoplasma 16S rDNA sequences from Ph-lily. Lane M: DNA marker (50-2000 bp ladder, FMC). Lane 1 (A, B): healthy plants; lanes 2-4 (**A**): direct PCR performed by using primer pair P1/P6, and lanes 2-4 (**B**): nested PCR performed by using R16F1/R1-S primer pair.

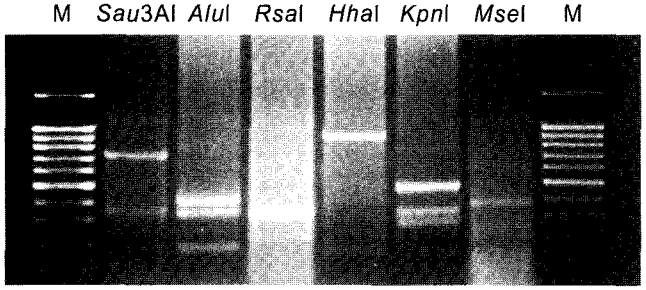

Fig. 3. Restriction endonuclease analysis of 16S rDNA (R16F1/R1-S nested PCR products) from lily showing flattened stem. PCR products were digested with restriction enzymes *Sau*3AI, *Alu*I, *Rsa*I, *Hha*I, *Kpn*I and *Mse*I and separated by electrophoresis through a 1.8% agarose gel. Lane M: 100 bp DNA ladder.

very flattened stem. Since lily reported in this study (Ph-lily) showed similar symptom to that of Voráckovᡠet al. (1998), nucleotide sequence of 16S rDNA was compared with a phytoplasma strain of strawberry green petal phytoplasma grouped in 16SrI-C (GenBank accession no. AJ243044), showing 98.1% sequence homology.

Also, presence of AY phytoplasma, subgroup B (16SrI-B), was reported previously from plants of three groups – *L. longiflorum,* Oriental, and Asiatic lily hybrids – showing stunting and flower bud deficiency (Kaminska and Korbin, 2002), thus, 16S rDNA nucleotide sequence of Ph-lily was compared with an AY phytoplasma, subgroup B (GenBank accession no. 68373). It showed 94.7% sequence identity. According to Seemüller et al. (1994), typical AY strains of American aster yellows, oenothera aster yellows, and severe American aster yellows exhibited levels of sequence homology of more than 99%. Considering this assumption by Seemüller et al. (1994), nucleotide sequence homology between AY phytoplasma and Ph-lily was a little low to classify Ph-lily in AY phytoplasma.

Liefting et al. (1996) phylogenetically assigned the phytoplasma isolated from *Liliaceae Phormium tenax* to stolbur strain (16Sr XII-A) and German grapevine yellows strain (16Sr XII-A) based on 16S rRNA gene sequences. Thus, the nucleotide sequences of 16S rDNA isolated from Ph-lily isolate was compared with those of stolbur phytoplasma isolates submitted in the GenBank (accession nos. AF248959, X76427, L76865, U43570). Ph-lily showed much higher sequence homology with subgroup A strains than that with subgroup B. Homology percentage of the 16S rDNA nucleotide sequence suggests classification of Ph-lily phytoplasma in the stolbur phytoplasma, subgroup A (16Sr XII-A), as a type strain stolbur.

Seemüller et al. (1994) divided 17 phytoplasma strains into five clusters by performing a sequence analysis of 16S rRNA gene. In those grouping, stolbur and grapevine yellows strain were clustered to AY strain. Also, according to the report of Seemüller et al. (1994), stolbur strain (16Sr XII-A) and grapevine yellows strain (16Sr XII-A) exhibited levels of homology of about 97% with the typical AY phytoplasma (16Sr I).

Considering the reports of Liefting et al. (1996) and the results obtained in this study, it was concluded that the phytoplasma isolated from lily (*L. martagon*) showing

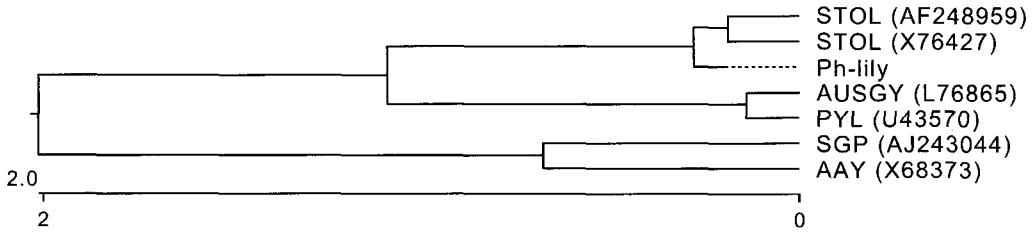

Fig. 4. Phylogenetic tree constructed by comparing 16S rDNA sequences of Ph-lily with other phytoplasmas obtained from GenBank.

flattened stem by Voráckovά et al. (1998) and then classified in AY phytoplasma, subgroup C (16SrI-C), based on RFLP analysis of 16S rDNA sequences should be analyzed by its 16S rRNA sequence. Seemüller et al. (1994) also stated that the RFLP-based division of the members of the apple proliferation cluster also does not fully coincide with the phylogenetic relationships of this organism by sequence analysis of 16S rDNA, and asserted that the sequence of a large molecule such as the 16S rRNA gene, reflects phylogenetic distance more accurately than restriction patterns which depend on significantly fewer genetic characters.

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