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Two Groups of Phytoplasma from Chrysanthemum (*Dendranthema grandiflorum*) Distinguished by Symptoms and 16S rRNA Gene Sequence in Korea

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Two groups of phytoplasma were identified in chrysanthemum (*Dendranthema grandiflorum*) cv. Chunkwang showing distinct symptoms. Isolate Ph-ch1 showed symptoms of dwarf, witches'-broom, rosette and root death. The other isolate, Ph-ch2, revealed symptoms of dwarf, yellowing, leaf cupping, vein clearing and root death. The presence of phytoplasma structures in chrysanthemum leaf tissue was confirmed by transmission electron microscopy. The 16S rRNA gene was amplified from isolates Ph-ch1 and Ph-ch2 by PCR and cloned, and the nucleotide sequences were determined. In RFLP analysis, isolate Ph-ch2 showed profiles identical to Ph-ch1, except with restriction enzymes *Hha*I and *Mse*I. The sequence data showed that isolate Ph-ch1 was most closely related to the aster yellows (AY) phytoplasma, and isolate Ph-ch2 was more closely related to stolbur phytoplasma than to AY phytoplasma. This is the first reported observation of stolbur phytoplasma in chrysanthemum species.

Keywords : aster yellows, chrysanthemum, *Dendranthema grandiflorum*, phytoplasma, stolbur

Phytoplasmas (previously called mycoplasma-like organisms) are phloem-limited plant pathogenic prokaryotes (Hopkins, 1977). They are known as the causal agents of yellowing, stunting and witches'-broom diseases in various plants. Since the discovery of phytoplasmas in 1967 (Doi et al., 1967), several hundreds of yellows-type diseases have been identified on a variety of economic crops worldwide (McCoy et al., 1989).

Chrysanthemum yellows (CY) disease, classified into aster yellows (AY) phytoplasma group, has been reported in *Chrysanthemum carinatum* and *C. frutescens* (Appiano et al., 1983; Bertaccini et al., 1992; Bertaccini et al., 1990). Ultrastructural observation on *C. carinatum* infected by CY is conducted (Appiano et al., 1983). PCR detection, dot blot and southern blot analysis (Bertaccini et al., 1992; Bertaccini et al., 1990) in *C. carinatum* and *C. frutescens* infected with CY were done. But the sequences of 16S rRNA gene for those pathogens have not been published. Symptoms induced by AY phytoplasma infection include stunt, yellowing, virescences and phyllody in *C. frutescens* (Bertaccini et al., 1992; Bertaccini et al., 1990; Conti et al., 1988), witches'-brooms in *C. carinatum* (Conti et al., 1988), and vein clearing, dwarfing and abnormal production of secondary shoots in aster (Kunkel, 1926).

Chrysanthemum plants are grown in all Provinces of Korea and are sold as cut flower or as pot flower. It accounts for about 29 percent of total cut-flower cultivation area of 2,606 ha in Korea (Ministry of Agriculture & Forestry, 2002). Because of most of the chrysanthemums cultivated in Korea have been propagated by cutting for many years, various virus-like diseases are occurring and problematic in fields.

Here we report two groups of phytoplasma identified from chrysanthemum cultivar 'Chunkwang' naturally infected with those agents. Nucleotide sequence determination of 16S rRNA gene revealed that isolate Ph-ch1 was included in AY group and the other isolate, Ph-ch2, was classified into stolbur group.

Materials and Methods

Phytoplasma source. In 1998, chrysanthemum plants with phytoplasma like-diseases were collected from two geographically distinct locations in Korea. Three chrysanthemum plants, cultivar 'Chunkwang', showing symptoms of dwarf, witches'-broom and rosette (Fig. 1A) were collected from two different farms in Ilsan, Kyeonggi Province, a Northern part in Korea. The other chrysanthemum plant, cultivar 'Chunkwang', showing symptoms of dwarf, yellowing, leaf cupping and vein clearing (Fig. 1C) was collected from a farm in Masan, Kyeongnam Province, a Southern part in Korea. Phytoplasma isolated from chrysanthemum

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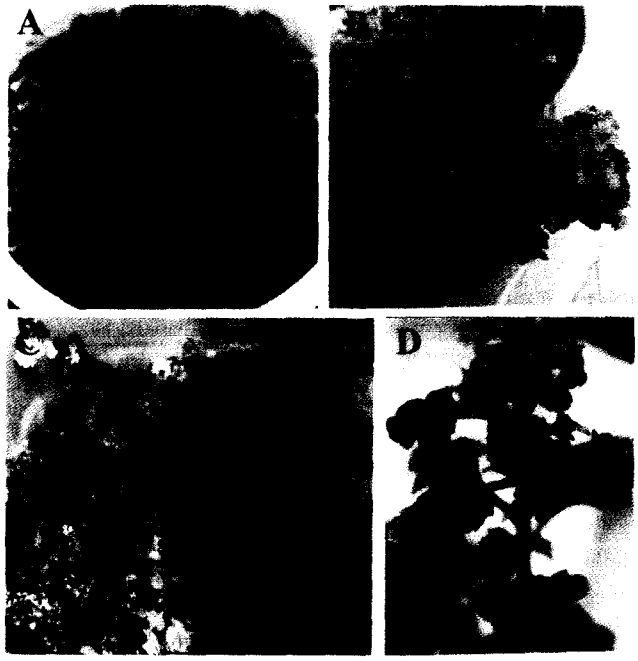

Fig. 1. Chrysanthemum plants infected with isolate Ph-ch1 (A, B) or isolate Ph-ch2 (C, D). (**A**) field-infected source chrysanthemum of isolate Ph-ch1 showing severe dwarf, witches'-broom and rosette, (**B**) witches'-broom symptom 3 months after graft-inoculation, (**C**) field-infected source chrysanthemum of isolate Ph-ch2 showing dwarf, yellowing, leaf cupping and vein clearing, and (**D**) dwarf, yellowing, vein clearing and leaf cupping 6 weeks after graft-inoculation.

of Ilsan was designated as the isolate Ph-ch1 and maintained by grafting healthy chrysanthemum seedlings on infected chrysanthemum plants. The other phytoplasma pathogen isolated from chrysanthemum of Masan was designated as the isolate Ph-ch2 and maintained by grafting healthy chrysanthemum seedlings on infected chrysanthemum plants.

Aster yellows (AY)-infected periwinkle DNA was provided by Abdul Hameed Khadhair (Alberta Research Council, Vegreville, Canada).

Grafting assay. For bioassay purpose, healthy chrysanthemum seedlings were insertion-grafted onto source chrysanthemum plants of Ph-ch1 and Ph-ch2 isolates collected from Ilans and Masan regions. Grafted plants were potted and grown under the glasshouse (25-35°C). Six weeks after grafting, scion plants were cut and potted. After rooting, those plants were pinched to induce branches, which were subject to symptom scoring.

Ultrastructure. Small pieces from the leaf midribs of Ph-ch1 or Ph-ch2 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, and examined with a Carl Zeiss LEO 906 transmission electron microscope.

DNA extraction. DNA was extracted from chrysanthemum infected with isolate Ph-ch1 and from isolate Ph-ch2 individually using a method described previously (Lee and Davis, 1983).

PCR. Three primer sets (P1/P6, R16F1/R1, R16F1/R1-S) were synthesized for amplification of phytoplasma DNA. A 16S rDNA universal primer pair P1/P6 was previously designed based on the nucleotide sequence of phytoplasma 16S rRNA gene (Deng and Hiruki, 1991). The second primer pair, R16F1/R1 previously designed to specifically amplify 16S rDNA sequences from the AY group of phytoplasmas, was used in nested-PCR to amplify DNA fragment of 1.1 kb (Lee et al., 1994).

The sequence of the forward primer (R16F1) was 5'-TAAAAGACCTA GCAATAGG-3' and that of the reverse primer (R1) was 5'-CAATCCGAACT GAGACTGT-3'. The third primer pair R16F1/R1-S was designed based on the nucleotide sequence of a stolbur phytoplasma (GenBank accession no. X76427) (Lee et al., 1998) to amplify 16S rDNA sequences from the stolbur group of phytoplasma in nested-PCR. The sequence of the forward primer was the same as the forward primer of the second primer pair and that of the reverse primer R1-S was 5'-CAATCCGAAC-TGAGACTGC-3'. The PCR and nested-PCR reaction were conducted as described previously (Chung and Jeong, 2002).

RFLP analysis of PCR products. The amplified nested-PCR products of 1.1 kb were digested with each restriction endonuclease, *Sau*3A I, *Hha* I, *Alu* I, *Rsa* I, *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 6% polyacrylamide gel in 0.5 × TBE buffer. Nucleic acids separated in polyacrylamide gel were visualized by silver staining (Promega, USA).

Nucleotide sequence determination of 16S rDNA. Nested-PCR products of 1.1 kb DNA was gel purified with the GENECLEAN III KIT (Q-BIO gene, USA) and ligated into the 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 as described previously (Chung and Jeong, 2003).

Phylogenetic analysis. Nucleotide sequences of the cloned PCR products were determined using ABI Prism™ Terminator Cycle Sequencing Ready Reaction Kit and ABI Prism 377 Genetic Analyzer (Perkin Elmer, USA). The 16S rRNA gene sequences from the isolates Ph-ch1 and Ph-ch2 were aligned and phylogenetic analysis with 42 phytoplasmas, derived from GenBank, was conducted using CLUSTAL Method of DNASTAR software version 5.1 (DNASTAR, Madison, WI. USA).

Results

Grafting assay. Healthy seedling developed narrowing and yellowing when it was grafted onto a source chrysanthemum plant of Ph-ch1. Symptoms were advanced in lateral branches when the cuttings were pinched. New expanded leaves on lateral branches were dwarf and rosette (data not shown). Three months later, the lateral branches developed witches'-broom (Fig. 1B). The other graft-inoculated cuttings developed dwarf, yellowing, leaf cupping and vein clearing symptoms 6 weeks after grafting when the healthy seedlings were grafted onto a source chrysanthemum plant of isolate Ph-ch2 (Fig. 1D).

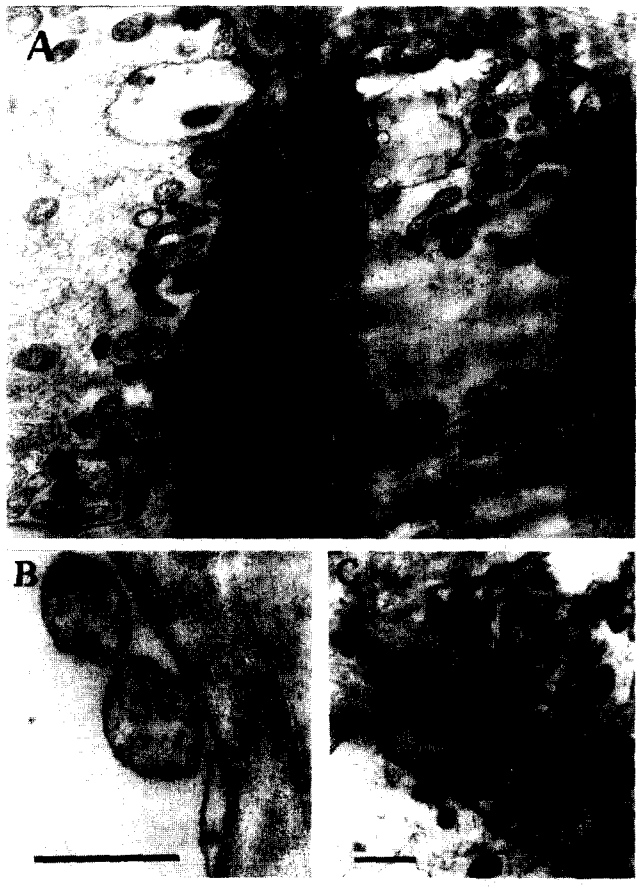

Fig. 2. Ultrastructures of sieve element of chrysanthemum midrib infected with isolate Ph-ch1. (**A**) Sieve tubes filled with pleiomorphic phytoplasma bodies, (**B**) phytoplasma bodies containing fine fibrils (arrow), and (**C**) phytoplasma bodies passing through a sieve pore (arrow). CW: cell wall. Bars represent 500 nm.

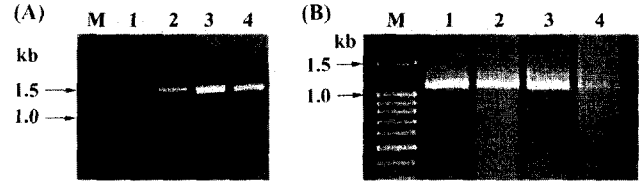

Fig. 3. Amplification of a 16S rDNA from chrysanthemums inoculated with phytoplasma isolates of Ph-ch1 and Ph-ch2 using a universal primer pair P1/P6 (A) and specific primer pair R16F1/R1 and R16F1/R1-S (B). (**A**) Lane 1, healthy chrysanthemum; lane 2, Alberta AY phytoplasma-infected periwinkle; lane 3, Ph-ch1; lane 4, Ph-ch2, (**B**) lane 1, Ph-ch1 with R16F1/R1; lane 2, Ph-ch1 with R16F1/R1-S; lane 3, Ph-ch2 with R16F1/R1-S; lane 4, Ph-ch2 with R16F1/R1. PCR products were separated by electrophoresis through 1.5% agarose gel and stained with ethidium bromide for 30 min. M denotes DNA size marker

Ultrastructure. Electron microscopy of ultrathin sections of the leaf midribs showed numerous round structures resembling typical phytoplasma bodies. The structures had pleiomorphic shapes with lengths ranging from 70 to 700 nm, bounded by membranes, and they were localized in the sieve tubes (Fig. 2A). Sometimes fine fibrils were observed inside of the phytoplasma bodies (Fig. 2B). They were often observed passing through a sieve pore (Fig. 2C).

RFLP analysis of PCR products. A universal primer pair P1/P6 amplified a 1.5 kb DNA fragment of phytoplasma 16S rDNA from isolate Ph-ch1 or isolate Ph-ch2 (Fig. 3A). In a nested-PCR assay with a specific primer pair (R16F1/R1 for Ph-ch1 and R16F1/R1-S for Ph-ch2) produced a

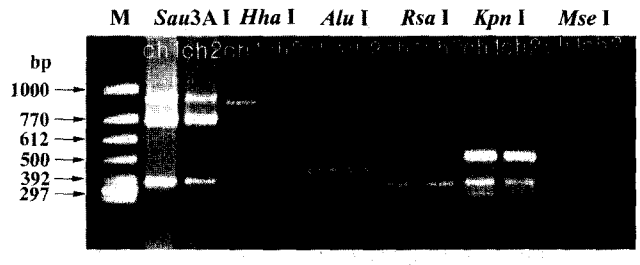

Fig. 4. Restriction endonuclease analysis of the 16S rDNA of isolate Ph-ch1 and isolate Ph-ch2 after PCR amplification with primer pair R16F1/R1 and R16F1/R1-S, respectively. ch1: Ph-ch1; ch2: Ph-ch2. Electrophoresis was carried out in 1.5% agarose gel and the gel was stained with ethidium bromide for 30 min. M denotes 1 kb DNA ladder (Promega, USA).

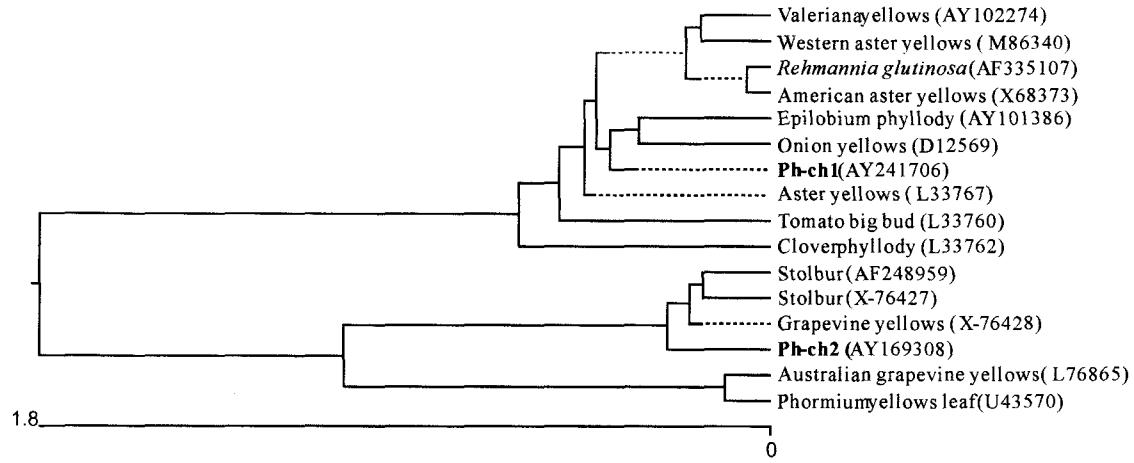

Fig. 5. Phylogenetic tree constructed using Cluster Method analysis, using DNASTAR software version 5.1 (DNASTAR, Madison, WI. USA), of 16S rDNA from Ph-ch1 and Ph-ch2, and 42 phytoplasmas derived from GenBank. The scale refers to the similarity index. Parentheses indicate GenBank accession number. M denotes 1 kb DNA ladder (Promega, USA).

DNA fragment of 1.1 kb out of the 1.5 kb PCR product. Though primer pair R16F1/R1 also amplified a 1.1 kb DNA fragment from isolate Ph-ch2 and R16F1/R1-S from isolate Ph-ch1, each PCR product was less than in comparison with R16F1/R1 for Ph-ch1 and R16F1/R1-S for Ph-ch2 (Fig. 3B) RFLP profiles of isolate Ph-ch1 revealed a different pattern from that of Ph-ch2 after *Hha* I and *Mse* I digestion (Fig. 4).

Nucleotide sequence of 16S rRNA gene. The nucleotide sequences of 16S rRNA gene amplified from both Ph-ch1 and Ph-ch2 were determined and have been deposited in the GenBank database under the accession no. of AY241706 and AY169308, respectively. The homology between two isolates was 95.2%. Isolate Ph-ch1 exhibited 99.5% homology with Japanese AY phytoplasma strains of Onion yellows (GenBank accession no. D12569). The isolate Ph-ch2 showed 98.9% homology with a stolbur phytoplasma (GenBank accession no. X76427) and 94.8% with American AY (16SrI-B) (GenBank accession no. X68373). Accordingly, isolate Ph-ch2 is more closely related to stolbur group than to AY.

Phylogenetic analysis. Ph-ch1 was closely related with Onion yellows, Epilobium phyllody, *Rehmannia glutinosa* var. Purpurea, Valeriana yellows, Western AY, American AY, AY type strain, Tomato big bud and Clover phyllody (Fig. 5). Ph-ch2 had a close relationship to two Stolbur phytoplasma isolates (GenBank accession no. AF248959 and X76427) and Grapevine yellows (Fig. 5).

Discussion

Phytoplasma has been known to cause CY disease in chrysanthemum species: *Chrysanthemum frutescens* and *Chrysanthemum carinatum* (Bertaccini et al., 1990; Conti et al., 1988).

In this study two different groups of phytoplasma, AY (Ph-ch1) and Stolbur (Ph-ch2) were detected in infected *Dendranthema grandiflorum* cv. Chunkwang plants showing symptoms of severe dwarf, leaf cupping, yellowing, witches'-broom, rosette and vein clearing. Chrysanthemum root infected with Ph-ch1 or Ph-ch2 was prolific, thin and short, and eventually led to early death of the plant. These observations are consistent with the symptoms reported previously in chrysanthemum species (Bertaccini et al., 1990, 1992; Conti et al., 1988).

There were some distinctions in symptom development between Ph-ch1 and Ph-ch2. Isolate Ph-ch1 produced more sever symptoms of rosette and witches' broom symptom than isolate Ph-ch2. As a result of severe retardation of growth point Ph-ch1-infected plants did not reach flowering stage, whereas Ph-ch2-infected plants showed stunted growth and produced at least one flower.

Restriction patterns obtained in isolate Ph-ch1 after digestion with restriction enzymes *Sau*3A I, *Hha* I, *Alu* I, *Rsa* I, *Mse* I and *Kpn* I corresponded to the patterns calculated for Maryland aster yellows AY1 phytoplasma (16SrI-B) (Lee et al., 1998), suggesting that the Ph-ch1 might be assigned to 16SrI-B. However, RFLP profiles of isolate Ph-ch2 revealed a different pattern from that of Ph-ch1 after *Hha* I and *Mse* I digestion (Fig. 4). This result suggests that Ph-ch1 and Ph-ch2 be classified in different groups.

Homology percentages of the 16S rDNA nucleotide sequence suggest that the isolate Ph-ch1 belongs to AY phytoplasma, subgroup B, and the isolate Ph-ch2 can be placed in stolbur phytoplasma, subgroup A (16SrVII-A), as

a type strain stolbur based on the classification scheme of Lee et al. (Lee et al., 1998). AY diseases have been reported in *C. frutescens* and other chrysanthemum species from Japan and Italy (Lee et al., 1998). However, there was no report on the stolbur phytoplasma disease in chrysanthemum species worldwide. This is the first reported observation of stolbur phytoplasma in chrysanthemum. Interestingly, nucleotide sequence of 16S rRNA of a stolbur phytoplasma isolate identified from *Lilium* Oriental hybrids, showing stem flattening, multiple meristem and fasciculation, was the same as Ph-ch2. Accordingly, isolate Ph-ch2 was assumed to result from transmission of phytoplasma agent from *Lilium* spp, because stem flattening symptom is widespread in *Lilium* spp., and previously reported in *Lilium martagon* from Czech (Voráckova et al., 1998) as well.

Seemüller et al. (1994) divided 17 phytoplasma strains into five clusters by performing a sequence analysis of 16S rRNA gene. In those grouping both stolbur and grapevine yellows strains were clustered to AY strain. It means that AY and stolbur phytoplasma are evolutionarily close. Also, according to the report stolbur strain (16Sr XII-A) and grapevine yellows strain (16Sr XII-A) exhibit levels of homology of about 97% with the typical AY phytoplasma (16Sr I). Likewise, the two isolates Ph-ch1 and Ph-ch2 were classified into two different groups, AY phytoplasma and stolbur phytoplasma in this study. AY phytoplasma could be distinguished from stolbur phytoplasma by the restriction enzymes *Mse* I and *Hha* I, even though they exhibited high levels of homology between them.

Acknowledgments

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