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First Report on the Witches' Broom in Annual Statice (*Limonium sinuatum*) in Korea

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In 2003 typical phytoplasma symptoms of witches' broom and flower malformation were observed on statice (*Limonium sinuatum*) plants grown at commercial greenhouses in Busan, South Korea. The DNA extracted from the infected leaves was amplified using universal primer pair of P1/P6 derived from 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 16F1/R1 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. The sequences were identical to that of Onion yellows OY phytoplasma (GenBank accession no. D12569) isolated from Onion in Japan. Electron microscopy of thin sections of leaf veins showed phytoplasma bodies in the phloem. Statice witches' broom symptom occurred on statice in commercial greenhouses in Korea was confirmed as infection of AY phytoplasma by transmission electron microscopy observation, and by determination of 16S rRNA gene sequences of phytoplasma.

Keywords **:** *Limonium sinuatum*, statice, witches' broom

Statice (*Limonium sinuatum*) belongs to the family of the *Plumbaginaceae*. It is a floricultural crop grown for both fresh and dried flowers. Most of them are perennials and a very few are annuals. They are propagated by division of root. Statice plants grown in South Korea occupied 70.5 ha in 2004 (Ministry of Argiculture and Forestry, 2004). In 2004, the sales of statice in Korea totaled 3.4 million US dollars.

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.

Phytoplasma bodies were found in phloem cells of *L. sinuatum* in Poland (Kamińska et al., 1996), Germany (Muller et al., 1973), the USA (Baker et al., 1983) and Japan (Wakibe and Guo, 1998). The symptoms included leaf reddening, premature leaf abscission, abnormal production of secondary shoots and flower malformation (Kamińska et al., 1996) and witches' broom with yellowing (Wakibe and Guo, 1998). They detected various shapes of phytoplasma bodies in sieve tube of infected statices. Statice witches' broom occurred in statice in Japan was identified as transmission by *Macrosteles striifrons* (Wakibe and Guo, 1998). Ultrastructural changes in AY phyto plasma-affected statice were studied (Rudzińska-Langwald and Kamińska).

Statice witches' broom symptom has been occurred since 1997 in commercial fields of statice in Korea (Hahm et al., 1998), but the causal agents has not been identified. The objective of the present study was to identify pathogenic agents associated with witches' broom symptom on statice by transmission electron microscopy observation, and by determination of 16S rDNA sequences of phytoplasma.

Materials and Methods

Source of diseased plants. In 2003 statice cultivars of 'Yellow', 'Lavenda', 'Gold' and 'Purple' showing witches' broom with flower malformation symptoms (Fig. 1) were collected from commercial greenhouses in Busan, South Korea. Those symptomatic plants were directly used for electron microscopy examination and molecular studies.

DNA isolation and primers for polymerase chain reactions (PCR). Nucleic acid for use as a template in PCR was extracted from leaf midrib by a method described previously (Lee and Davis, 1983). Two pairs of primers were used in PCR. Primer pair P1/P6 (Deng and Hiruki, 1991) was used to prime phytoplasma-universal amplification of 16S rRNA sequences in PCR. Primer pair R16F1/R1 (specific for group 16SrI phytoplasma rDNA) (Lee et al., 1994) was used in AY group-specific nested PCR.

The PCR reaction mixture contains 20 ng of template DNA, 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 1 mM of each dNTP, 1 ul of 10 pm each primer, 2.5

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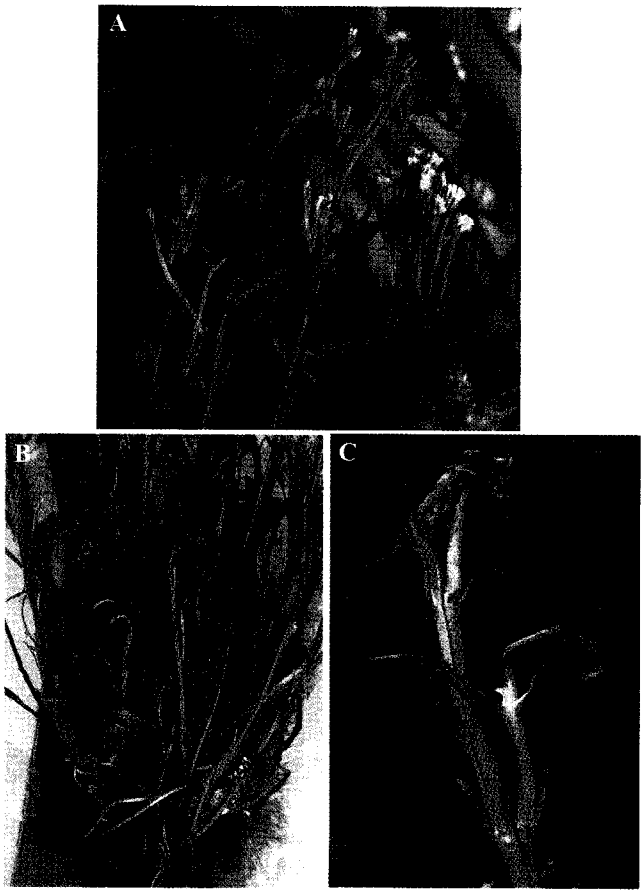

Fig. 1. Naturally phytoplasma-infected statice plants. (A) witches' broom symptom, (B) malformation of flowers with enlarged yellowish stem, (C) severely reduced flowers.

mM $MgCl_2$ and 2.5 U Taq DNA polymerase (Applied Biosystems, USA). Thirty-five PCR cycles were conducted in PTC-0220 Perlitier Thermal Cycler (MJ Research, MA, USA). The thermal conditions were as follows: denaturation at 94°C for 30 sec (2 min for the first cycle), annealing at 65°C for 50 sec (45°C for reactions using 16F1/R1) and extension at 72°C for 1.5 min. The last cycle was extended for an additional 3 min at 72°C.

Cloning of PCR products and nucleotide sequencing. We cloned PCR amplified phytoplasma 16S rDNA, which had been amplified in nested PCR primed by R16F1/R1, using pGEM-T easy vector (Promega, USA) according to the manufacture's instruction. The ligation mixture is 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).

Phylogenetic analysis. The 16S rRNA gene sequences were aligned using CLUSTAL Method of DNASTAR software version 5.1 (Madison, USA).

Electron microscopy. Presence of phytoplasma was examined with the midribs of leaves. The segments were cut into small pieces by hand dissection and fixed in 2% Karnovsky's fixative solution, pH 7.2 for 4 h. Postfixation in 1% osmium tetroxide in cacodylate buffer, pH 7.2 for 2 h at 4°C. The material was dehydrated in concentration gradients of ethanol (50%, 75%, 90%, 95% and 100% for 30 min each step) and embedded in Spurr resin. Ultrathin sections were prepared with ultramicrotome (RMC-MTX), 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 (Electron Microscopy Science, USA).

Results

Detection of phytoplasma 16S rRNA gene from plants by PCR. Using a universal primer set P1/P6, a 1.5 kb DNA fragment of the 16S rRNA gene was amplified from infected plants (Fig. 2). In the nested PCR assays, the expected DNA fragment of 1.1 kb was amplified with the specific primer pair R16F1/R1 that was designed on the basis of AY phytoplasma 16S rDNA sequences (Fig. 2).

Sequence analyses. The nucleotide sequences of the cloned 16S rRNA gene of the phytoplasma observed in statice have been deposited in the GenBank database under the accession no. of DQ192513. The nucleotide sequences were identical to those of phytoplasma from Ash trees witches' broom (GenBank accession no. AY566302) reported in Korea (unpublished). They were also identical to Onion yellows OY phytoplasma (GenBank accession no. D12569) isolated from onion in Japan (Namba et al., 1993). Meanwhile, it shared 99.1% and 93.9% homology with American aster yellows AAY (GenBank accession no. X68373) and Clover phyllody CPh (GenBank accession no. L33762), respectively (Table 1).

Visualization of phytoplasma. In the ultra-thin sections of

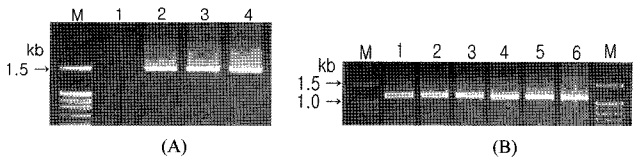

Fig. 2. Amplification of a 16S rDNA sequence using universal primer pair P1/P6 (A) and AY specific primer pair R16F1/R1 (B). (A) lane 1, healthy statice; lane 2-4, diseased statice; (B) all lanes, diseased statice. Lane M, 1 kb DNA ladder.

Table 1. Sequence homology percent of the 16S rDNA of statice witches' broom with other phytoplasmas[a]

	Ash WB	OY	AAY	SWB
CPh	93.9	93.9	93.0	93.9
Ash WB		100.0	99.1	100.0
OY			99.1	100.0
AAY				99.1

[a]Ash WB: Ash witches' broom (AY566302); OY: Onion yellows (D12569); AAY: American aster yellows (X68373); SWB: Statice witches' broom (DQ192513); CPh: clover phyllody (L33762).

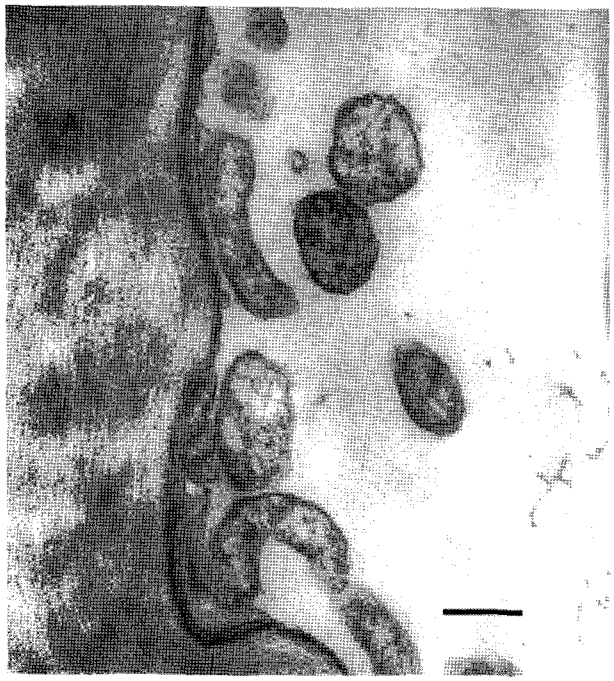

Fig. 3. Electron micrograph of phloem cells of phytoplasma-infected statice leaf showing pleomorphic phytoplasma structures. Bar indicates 200 nm.

the leaf midribs phytoplasma-like structures were observed (Fig. 3). The structures had round or pleomorphic shapes, with a diameter of 130-300 nm and were limited to phloem cells. Fine fibrils were observed inside of the phytoplasma bodies.

Discussion

In 2003, typical phytoplasma infection symptom of witches' broom on statice cultivars of 'Yellow', 'Lavender', 'Gold' and 'Purple' were abundantly occurred in commercial greenhouses in Busan, South Korea. The witches' broom symptom of statice had been found in Gangwon alpine areas (Hahm et al., 1998), and in Namwon, Jeolla Province (Lee, 2004), South Korea. The present study was undertaken to identify the causal agent of witches' broom in statice plants in Korea. The results were based on electron-microscopic examination and nucleotide sequence analysis of 16S rRNA gene.

Phytoplasma disease were reported in statice in Poland (Kamińska et al., 1996), Japan (Wakibe and Guo, 1998) and USA (Baker et al., 1983). Phytoplasma caused leaf and flower discoloration and malformation, shoot dieback and seed sterility (Kamińska et al., 1999), and witches' broom with yellowing (Wakibe and Guo, 1998) in statice. Symptoms of witches' broom and flower malformation revealed in phytoplasma-affected statice in this study were similar to those described in above reports.

The nucleotide sequences determined in present study were identical to those of phytoplasma from Ash trees witches' broom (GenBank accession no. AY566302) reported in Korea. They were also identical to Onion yellows OY phytoplasma (16SrI-B) (GenBank accession no. D12569) isolated from onion in Japan (Namba et al., 1993). The Onion yellows OY phytoplasma was isolated from filed-collected onions. *Macrosteles striifrons* transmitted phytoplasma disease in statice in Japan, and the same vector also transmitted phytoplasma diseases to tomato, onion and turnip (Wakibe et al., 1996). We inferred from the report that phytoplasma pathogen infecting statice might be the same one with that infected onion, resulting in same nucleotide sequence.

Sequence similarity of Statice witches' broom with phytoplasma grouped in AY indicated symptom of witches' broom in statice plants was caused by phytoplasma infection involved in AY was group according to classification of Lee et al. (1998). In Poland, they grouped the causal phytoplasma of statice as AY, subgroup B by restriction fragment length polymorphism (RFLP) analysis of PCR products of 16S rDNA (Kamińska et al., 1999). Nucleotide sequence analysis of phytoplasma found in statice has never been conducted. This study proved the witches' broom symptom in statice was caused by phytoplasma disease by determination of nucleotide sequences of 16S rRNA gene.

The witches' broom symptom on statice occurred in Korea was proved as infection of AY phytoplasma by transmission electron microscopy observation, and by determination of 16S rRNA gene sequences of phytoplasma. This study provided the first evidence of association of AY phytoplasma with statice witches' broom in Korea.

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