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# Phytoplasma-associated Shoot Proliferation and Leaf Yellowing in Lettuce

**Bong Nam Chung*, Jeong Soo Kim and Seung Ryong Cheong**

*National Horticultural Research Institute, Rural Development Administration, Suwon 440-310, Korea*

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**Phytoplasma was identified from leaf lettuce (*Lactuca sativa*) cultivated in commercial green-house in Korea. Diseased leaf lettuce revealed proliferation of shoots, and yellowing and shrinking of leaves (lettuce proliferation-K). Polymerase chain reaction (PCR) with universal primer pair P1/P6, and aster yellows (AY) specific primer pair R16F1/R1 amplified 1.5 kb and 1.1 kb length of DNA fragments, respectively. Nucleotide sequences of 16S rRNA gene were determined (GenBank accession no EF489024). Phylogenetic analysis of 16S rDNA showed the closest relationship with AY phytoplasma (GenBank accession no. AY389822 and AY389826), indicating that lettuce proliferation-K is a member of AY. Phytoplasma bodies were detected in phloem sieve tubes of diseased lettuce by transmission electron microscopy. The structures had round or pleomorphic shapes with a diameter of 130-300 nm. Phylogenetic analysis of 16S rRNA gene, microscopic observation of phytoplasma bodies and symptomatology indicated that lettuce proliferation-K is caused by phytoplasma in the AY group. This is the first report of phytoplasma disease in lettuce in Korea.**

***Keywords*** **:** *Lactuca sativa*, phytoplasma, shoot proliferation, yellowing

The genus *Lactuca* L. (*Compositae*) comprises about 100 species (De Vries, 1997). *Lactuca sativa* (lettuce) is a popular leaf vegetable, and is consumed all year round. Mostly leaf lettuce (loose-leaf type) is cultivated in Korea.

Phytoplasmal disease in lettuce has been reported since 1991. Lettuce collected from Oklahoma revealed yellow symptom by infection with aster yellows (AY) phytoplasma (Errampalli and Fletcher, 1991). AY-infected lettuce was also observed in Ohio (Zhang et al., 2004). Five distinct strains were characterized based on symptoms: Chlorosis of emerging leaves and abnormally upright growth of leaf petioles (AY-BW); yellowing and leaf distortion (AY-BD2); horizontal growth (AY-SG); yellowing and wilting (AY-WB); and stunting, leaf clustering and phyllody (AY-S). Recently reported lettuce phyllody observed in Iran was grouped into pigeon pea withches'-broom phytoplasma (Salehi et al., 2006).

Damages of leaf lettuce revealing shoot proliferation accompanied by leaf yellowing occurred in commercial green-houses in Seoul and Seongju, Korea. Incidence rate was about 1-10% according to leaf lettuce cultivars. In this study we identified AY phytoplasma from the diseased lettuce by determination of nucleotide sequences of 16S rDNA and the observation of phytoplasma bodies by electron microscopy.

## Materials and Methods

**Source of diseased lettuce.** In November 2006, leaf lettuce (*Lactuca sativa*) with unusual growth (Fig. 1) was collected from commercial green-houses in Seoul and Seongju, South Korea. Those symptomatic plants were designated as lettuce proliferation-K. They were maintained in a green-house in National Horticultural Research Institute in Suwon, and directly used for electron microscopy examination and molecular biological studies.

**DNA isolation and primers for polymerase chain reactions (PCR).** DNA was prepared from leaf midribs by a method described previously (Lee and Davis, 1983). Two pairs of primers were used in PCR. A universal primer pair P1/P6, located in the 16S rDNA of the phytoplasma (Deng and Hiruki, 1991), was employed in direct PCR to prime a DNA fragment of 1.5 kb expected size. Primer pair R16F1/R1 (specific for AY group phytoplasma) (Lee et al., 1994) was used in nested PCR.

The PCR reaction mixture contained 20 ng of template DNA, 1×PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 1 mM of each dNTP, 1 μl of 10 pmole of each primer, 2.5 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 45°C for 50 sec and extension at 72°C for 1.5 min. The last cycle was extended for an additional 3 min at 72°C.

*Corresponding author.
Phone) +82-31-290-6236, FAX) +82-31-290-6259
E-mail) chbn7567@rda.go.kr

**Cloning of PCR products and nucleotide sequencing.** PCR product amplified with primer pair P1/P6 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).

**Phylogenetic analysis.** The 16S rRNA gene sequences were aligned using ClustalW using DNASTAR version 7.0 (Madison, WI. USA), and compared phylogenetically to other phytoplasma sequences in GenBank (www.ncbi.nlm.nih.gov).

**Electron microscopy.** Presence of phytoplasma was examined with the leaf midribs. Ultra-thin sections (75 nm) were prepared according to a method described previously (Chung et al., 2005), and observed under a Carl Zeiss LEO 906 transmission electron microscope (Electron Microscopy Science, Washington, PA).

## Results

**Symptom.** Diseased lettuce showed proliferation of shoots (Fig. 1A, B), and yellowing and shrinking of leaves (Fig. 1C, D). Early symptom of diseased lettuce was formation of abnormal multiple shoots on stem, and later symptom was chlorosis and shrinking of leaves.

**Detection of phytoplasma 16S rRNA gene from diseased lettuce by PCR.** Using a universal primer set, a 1.5 kb DNA fragment was amplified from diseased lettuce (Fig. 2A). In the nested PCR assays, the expected DNA fragment

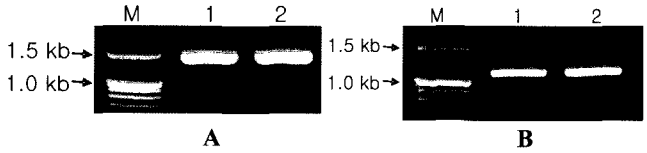


**Fig. 2.** Amplification of a 16S rRNA gene sequences from two lettuce plants infected with lettuce proliferation-K. A: Universal primer pair P1/P6 (lane 1-2). B: AY phytoplasm specific primer pair R16F1/R1 (lanes 1-2). Lane M: 1 kb DNA ladder. PCR products were separated by electrophoresis through a 1.5% agarose gel.

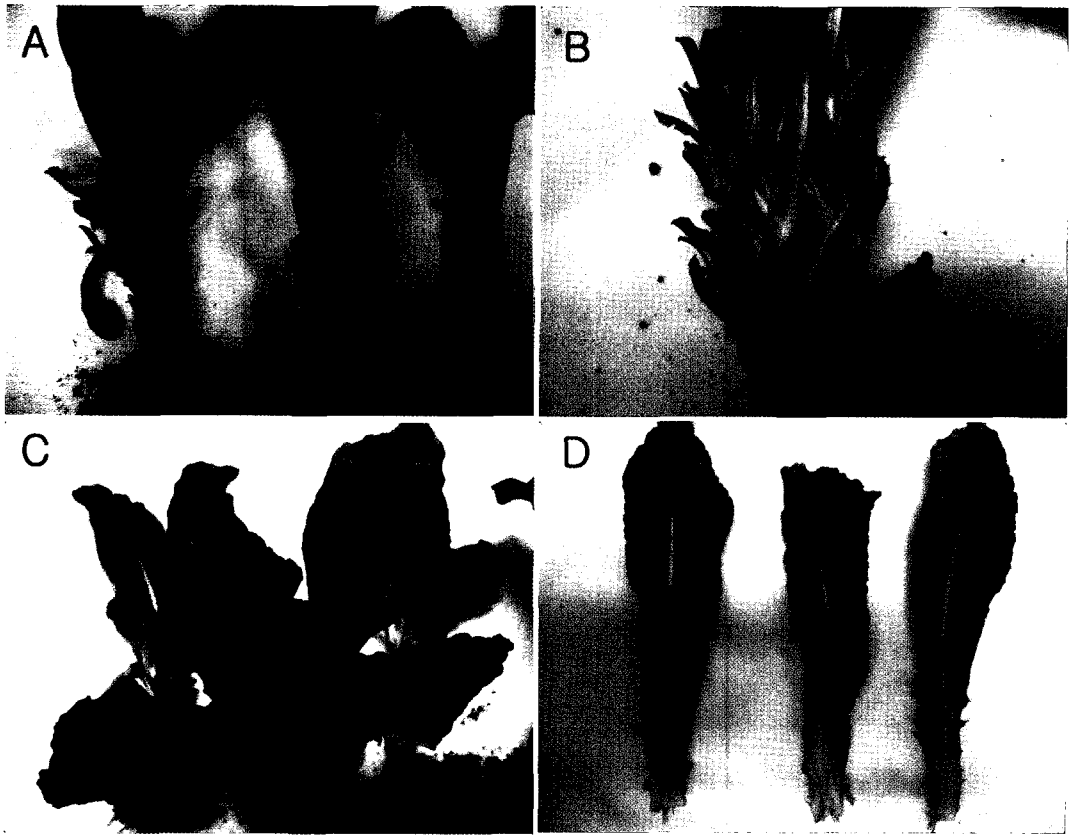


**Fig. 1.** Field collected lettuce infected with phytoplasma isolate lettuce proliferation-K. Source lettuce of sequence analysis of 16S rRNA and microscopy. A and B: Shoot proliferation; C and D: Yellowing and shrinking of leaves.

of 1.1 kb was amplified with the AY specific primer pair R16F1/R1 (Fig. 2B).

**Sequence analyses.** The nucleotide sequences of the cloned 16S rRNA gene have been deposited in the GenBank database under the accession no. of EF489024. Phylogenetic analysis of 16S rDNA of lettuce proliferation-K showed the closest relationship with AY phytoplasma AY389822 and AY389826 (Fig. 3).

**Visualization of phytoplasma.** In the ultra-thin sections of the leaf midribs phytoplasma-like structures were observed (Fig. 4A, B). The structures had round or pleomorphic shapes, with a diameter of 130-300 nm and were limited to phloem sieve tubes. Fine fibrils were observed inside of the phytoplasma bodies (Fig. 4B).

## Discussion

Sequence analysis of evolutionarily conserved genes such as the 16S rRNA has provided a detailed picture of phylogenetic relationships of prokaryotes (Olsen et al., 1994). Lee et al. (1998) grouped 34 phytoplasma strains into 14 major groups and 32 sub-groups based on RFLP analyses of 16S rDNA PCR products.

Lettuce proliferation-K revealed the closest relationship with AY phytoplasma isolates AY389822 and AY389826 (Zhang et al., 2004). According to sequence homology percent of the 16S rDNA (data not shown), lettuce proliferation-K of our study shared 99.6% and 99.7% homology with AY389822 and AY389826, respectively; It showed 99.1% homology with an AY phytoplasma Korean isolate Chrysanthemum witches' broom (EF529494; Chung et al., 2001), and 86.7% homology with U18763, included in Pigeon pea withches'-broom phytoplasma. Nucleotide sequences of 16S rDNA supported the lettuce proliferation-K can be classified in AY group.

Symptoms of shoot proliferation and yellowing observed in lettuce proliferation-K was a typical symptom of phytoplasma infection, and also those symptoms were reported in early reports (Beanland et al., 2005; Errampalli and Fletcher, 1991; Zhang et al., 2004). Phytoplasma strain AY-

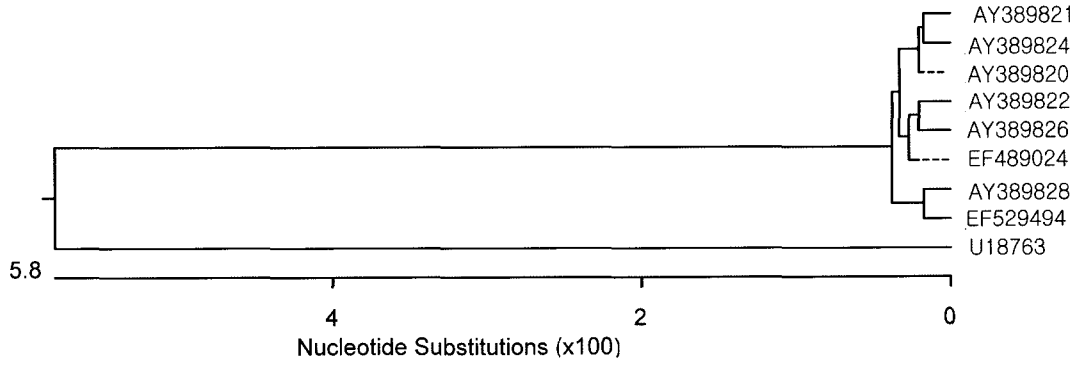


**Fig. 3.** Phylogenetic tree constructed by comparing 16S rDNA sequences of lettuce proliferation-K (EF489024) with other phytoplasmas obtained from GenBank (www.ncbi.nlm.nih.gov), using ClustalW method of DNASTAR version 7.0 (Madison, WI. USA). The scale refers to the similarity index. Dotted lines indicate a negative branch length.

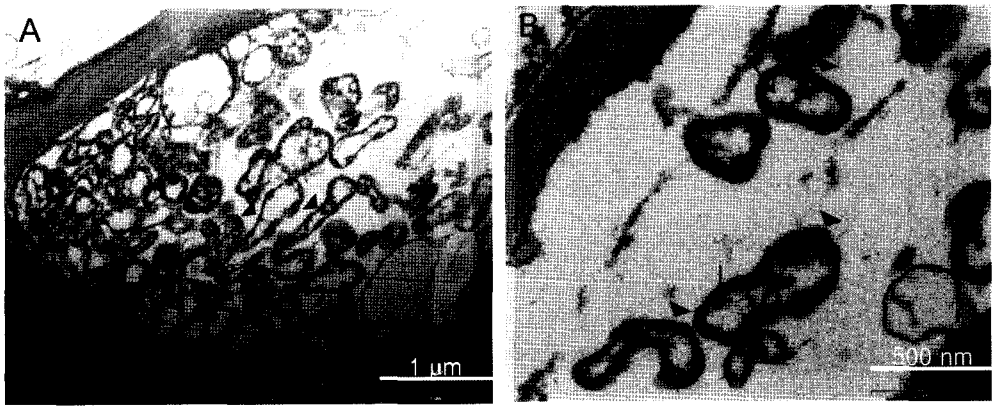


**Fig. 4.** Electron micrographs of phloem sieve tubes of midrib of lettuce infected with lettuce proliferation-K phytoplasma. Pleomorphic phytoplasma structures are shown in A and B (arrow heads). Fine fibrils were observed inside the phytoplasma bodies in B (arrows).

BD2 (AY389822 and AY389826) caused yellowing and leaf distortion in lettuce (Zhang et al., 2004). Similarly lettuce proliferation-K also showed yellowing and leaf distortion and/or leaf shrinking. AY-infected lettuce showing clustering symptom was reported in Ohio (Beanland et al., 2005), and in Oklahoma (Errampalli and Fletcher, 1991).

Several hundreds of yellows-type diseases have been identified on a variety of economic crops worldwide families (McCoy et al., 1989). AY phytoplasma is transmitted by the leafhopper *Macrostelles facifrons* to more than 200 plant species belonging to different botanical families (McCoy et al., 1989). Aster leafhopper (*Macrosteles quadrilineatus*) has also been known as the primary vector of the AY phytoplasma (Beanland et al., 2005).

Lettuce plants infected with lecttuce proliferation-K were observed in two distance places of Seoul and Seongju at the similar time of the year. Coincidently symptom of diseased lettuce collected from those two different places was identical. The plant to plant spread of phytoplasmas in nature is mediated by phloem-feeding insects, leafhoppers, planthoppers, and psyllids (Weintraub and Beanland, 2006). Even though nursing lettuce was done inside of the green-house, doors should have been opened in summer. Phytoplasma is widespread in Korea. Up to 2001, it caused 61 various diseases in 31 plants (Lee, 2004). AY phytoplasma has been reported in chrysanthemum and aster in Korea (Chung et al., 2001, 2005). We inferred from the cultivation environment in summer and prevalent phytoplasma sources around cultivation area that insect vectors may be responsible for the phytoplasma infection of lettuce. Thus, nursery plants should be kept under insecticide treatments or under insect-proof facilities.

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