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(*Received April 4, 2011 and accepted May 9, 2011*)

Sunn hemp is a tropical or subtropical plant of the legume family, and used as green manure and ligni-fied fiber. Chlorotic leaves, internodes shortening, reduced sized leaves and shoot proliferation symptoms were observed on sunn hemp in a number of fields around Yezin area, Myanmar. To determine possible association of a phytoplasma with the disease, DNA amplifications were conducted by polymerase chain reaction (PCR) using a universal phytoplasma primer pair, SN910601/SN011119 that amplified 1.8 kb fragments encoding the entire 16S rDNA, the 16S–23S intergenic spacer region and the beginning of 23S rDNA. Phytoplasmal rDNA was amplified in the PCR, indicating that phytoplasma was associated with the disease and designated sunn hemp witches' broom (ShWB) phytoplasma. On the basic of nucleotide sequence analysis of 16S rDNA and phylogenetic analysis, the ShWB phytoplasma was closely related to strains SUNHP from diseased sunn hemp in Thailand which are classified in 16SrII group. Results of putative restriction fragment length polymorphism maps showed that the ShWB phytoplasma belonged to subgroup 16SrII–A. This report is the first observation and molecular characterization on sunn hemp witches' broom phytoplasma in Myanmar.

INTRODUCTION

Phytoplasmas are wall–less prokaryotes that live as obligate parasites. Phytoplasmas normally inhabit the phloem of plants at low concentrations and have an uneven distribution in woody plants especially in monocots. These characteristics make their detection and identification difficult (Seemüller *et al*., 1998). Plants associated with phytoplasma including yellowing, decline, witches' broom, leaf curl, floral virescence and phyllody are the most conspicuous symptoms, and sometimes infected plants are asymptomatic. The association of phytoplasma in these unique symptoms have been detected by using fluorescent microscopy, transmission electron microscopy, serological tests such as enzyme–linked immunosorbent assay and molecular techniques based on polymerase chain reaction (PCR) (Lee *et al*., 2000). Among these techniques, PCR assays provide a much more sensitive means than others and facilitated detection of low titers of phytoplasmas that were not readily detected by other method.

Although phytoplasmas have not yet been grown *in vitro*, phylogenetic analyses based on various conserved genes have confirmed that they represented a distinct, monophyletic clade within the class *Mollicutes*. The restriction fragment length polymorphism (RFLP) and sequences from the genes of 16S rRNAs, ribosomal protein (rp) and elongation factor TU (*tuf*) are now widely used to differentiate various groups and subgroups of phytoplasmas (Seemüller *et al*., 1998; Marcone *et al*., 2000). Recently, all available phytoplasma sequence accessions were classified into 28 16Sr RFLP groups and around 100 subgroups according to the computer–simulated RFLP analysis of 16S rDNA (Wei *et al*., 2007).

Sunn hemp, originated in India, is a member of Leguminosae and economically is the most important species of genus *Crotalaria*, which consists of about 550 species. It is the most widely grown legume and has been utilized as a green manure, cover crop and bast fiber crop in the tropic and subtropics. It is widely used because not only it is adapted to a wide range of soils and it performs better on poor sandy soils than most crops but also its rapid growth and relatively short growing season requirement (NRCS, 1999). It is also famous for fiber crop and its resistance to root–knot nematode, and has advantage of being a soil–improving crop via nitrogen fixation. Sunn hemp contains pyrrolizidine alkaloid, a serious liver toxin, and it has been shown to be poisonous to most livestock (Wang *et al*., 2008). In Myanmar, it has been grown mainly as summer crop after rice to maintain soil fertility and suppress plant–parasitic nematodes. In recent year, symptoms of general stunting, floral virescence, yellowing of leaves and proliferation of axillary shoots with reduced sized leaves were observed on large number of sunn hemp fields around Yezin area. General stunting and proliferation of shoots are suspected as the symptoms of the involvement of phytoplasmas. This disease causes detrimental effect on seed production that leads to seed shortage problem for next plantation because the seeds are used as sources for the following season. Therefore, this paper presents the results of PCR–based phytoplasma identification on diseased sunn hemp plants. RFLP analyses of PCR–amplified 16S rDNA, the 16S–23S intergenic spacer region (SR) and the beginning of 23S rDNA were used to characterize Myanmar phytoplasma associated with this disease and also to study its phylogenetic relatedness to other phytoplasmas.

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MATERIALS AND METHODS

Source of phytoplasmas and nucleic acid extraction

Samples were collected from naturally infected sunn hemp plants showing phyllody, chlorosis, proliferation and little leaves symptoms from different fields in Yezin area, Myanmar, 2010. Asymptomatic plants were also collected to use as healthy control. Water dropwort witches' broom phytoplasma reported by Jung *et al.* (2002) served as a reference strain. Total nucleic acids were extracted from leaf tissues (0.3 g) of each infected and healthy sample. The DNA was isolated from each sample according to a previously described protocol (Namba *et al.*, 1993). All samples were ground using a mortar and pestle in 0.9 ml of cetyl trimethyl ammonium bromide (CTAB) buffer, incubated at 65 °C for 30 min, and centrifuged ($20,000 \times g$, 5 min, 4°C). An equal volume of chloroform–isoamyl alcohol (24:1) was added to the supernatant and centrifuged ($20,000 \times g$, 5 min, 4 °C). Then DNA was precipitated with 80% volume of cold isopropanol, incubated at room temperature for 10 min, and centrifuged ($20,000 \times g$, 5 min, 4 °C). The pellet was washed with cold 70% ethanol and dried using evaporator for 10 min. The final nucleic acid pellets were dissolved in TE buffer and DNA concentration was determined on 0.7% agarose gel compared to a 100 bp Plus DNA ladder marker.

Primers and polymerase chain reaction (PCR) analysis

The universal phytoplasma primer pair, SN910601/SN011119 was used to amplify parts of ribosomal operon (1.8 kb) consisting the 16S rDNA, SR and the beginning of the 23S rDNA of phytoplasma from phytoplasma infected samples (Jung *et al.*, 2003). The oligonucelotide sequences of the primers are: SN910601 (5'–GTT TGA TCC TGG CTC AGG ATT–3') and SN011119 (5'–TCG CCG TTA ATT GCG TCC TT–3').

PCR amplification was performed in a thermal cycler 9700 (Applied Biosystems) with the following condition: one cycle of 94 °C for 2 min followed by 35 cycle reaction profile involving 30s of denaturation at 94 °C, 30s of annealing at 55 °C and 90s of extension at 72 °C, with a final elongation step 7 min at 72 °C. Each PCR reaction mixture (20 μl) contained 2 μl of total nucleic acid (100 ng) plus 2 μl of each primer (10 pmols each), 0.4 μl of 10 mM dNTP, 2 μl of 10x PCR buffer and 0.2 μl (1.0 unit) of *Taq* DNA polymerase (SolGent Co., Korea). The reaction products were analyzed by 1% agarose gel electrophoresis in 1x Tris–acetate EDTA buffer containing ethidium bromide. DNAs were visualized and photographed using a UV transilluminator.

Sequencing and phylogenetic analysis

The PCR products of ShWB phytoplasma were purified with ExoSAP–IT (USB Co., USA). The resultant products were then sequenced by ABI 3730 XL DNA sequencer (SolGent Co., Korea) with 7 primers (350F, 350R, 788F, 920R, 1505F, SN910601 and SN011119) that have been used to sequence phytoplasma 16S rDNA, SR and the beginning of 23S rDNA (Jung *et al.*, 2003).

Sequence editing and analysis was performed using the DNASTAR program. The sequences were visually inspected for logical placement of gaps and manually adjusted where necessary. The sequence data for ShWB reported in this study was deposited to the DNA Data Bank of Japan (DDBJ) and assigned as accession number AB558143. Sequence similarities were evaluated for phytoplasma strains using GENETYX software homology search option. The phylogenetic analyses of 16S rDNA and SR sequence isolated from ShWB phytoplasma in this study and others known 28 representatives of the genus *Candidatus* Phytoplasma obtained from BLAST search of NCBI website was performed using the ClustalW program (Thompson *et al.*, 1994). Phylogenetic tree was generated based on the neighbour–joining method. *Acholeplasma palmae*, a *Mollicute* that is phylogenetically related to the phytoplasmas was employed as the outgroup to root the tree. Bootstrap analysis (100 times) was performed to estimate stability and support for the inferred clades.

RFLP analysis and putative RFLP map

The PCR products were digested with each of the restriction endonucleases: *Alu*I, *Hae*III, *Hha*I, *Mse*I, *Msp*I and *Rsa*I in buffers supplied by the manufacturer. Digestion mixtures were incubated for 2 hrs at 37 °C for all the restriction enzymes except *Mse*I which was incubated for 2 hrs at 65 °C. Fragments were then analyzed on a 5% acrylamide gel with 10x Tris–borate–EDTA as running buffer and visualized after staining with ethidium bromide by UV transillumination. The restriction patterns of ShWB phytoplasma were compared with those described previously (Lee *et al.*, 1998).

Since nucleic acid was not available from many phytoplasmas that were closely related to ShWB, based on phylogeny, a putative RFLP was carried out on 5 sequences from Genbank in comparison to ShWB. Phytoplasmas employed were peanut witches' broom (PnWB), sunn hemp witches' broom (SUNHP), lime witches' broom (WBDL), faba bean phyllody (FBP) and sweet potato little leaf (SPLL) phytoplasmas. A putative RFLP map was then constructed on each sequence separately as restriction sites of 6 endonuclease enzymes (mentioned above) using the MapDraw program of the DNASTAR software package and manually aligned.

RESULTS AND DISCUSSION

Various disease symptoms were observed on large number of sunn hemp fields including yellowing, malformation of vegetative and floral parts. Profuse axillary shoots bearing reduced size leave of the diseased plants (Fig. 1A) are the most conspicuous symptom of this disease. Floral parts are noticeably appeared as small green leaf like structures (Fig. 1B) while yellow color on normal flowers (Fig. 1C). The affected plants can be easily seen in the field as growth retarded plants with small leaves (Fig. 1D) among the normal healthy plants (Fig. 1E). Such variation of disease symptoms on affected

Fig. 1. Sunn hemp witches' broom disease symptoms; shoot proliferation with reduced size leaves and chlorotic leaves (A); abnormal (virescence) flowers (B); normal flowers (C); phytoplasma affected plant (D); healthy plants in the field (E).

plants is related to different levels of phytoplasma concentration and stages of host plant at the time of infection. However, these abnormalities especially leaf yellowing symptoms make often confused with viral diseases, such as yellow leaf syndrome of sugarcane associated with sugarcane leaf virus and sugarcane yellows phytoplasma (Naz *et al.*, 2009). To clear this problem, PCR analysis was performed on several symptomatic and asymptomatic plant samples.

The presence of phytoplasma in the affected samples was confirmed by amplified DNA fragments with expected size of 1.8 kb and those also in the positive control, while no amplification were found in the asymptomatic samples. This amplification by a universal primer pair, SN910601/SN011119 indicated that the diseased sunn hemp plants were infected by a phytoplasma and naming as sunn hemp witches' broom (ShWB) phytoplasma.

As a sequence result, the amplified fragments were 1,818 bp long encoding 16S rDNA, SR and the partial of 23S rDNA. All sequences obtained from various diseased symptoms were 100% similarity to each other. The highest sequence identity was found between the ShWB phytoplasma (AB558143) and those of crotalaria witches' broom (EU650181), sweet potato witches' broom (DQ777762), alfalfa witches' broom (AB259169), peanut witches' broom (L33765) and eggplant phyllody (FN257482), all are members of peanut witches' broom group phytoplasmas. Homology search showed that the ShWB phytoplasma had the greatest identity with that of sunn hemp witches' broom (SUNHP) from Thailand (99.9% identity, EF193358) (Martini *et al.*, 2007). Results of phylogenetic analysis on 16S rDNA plus SR indicated that ShWB isolates from Myanmar are closely related to phytoplasmas belonging to the 16SrII group (Fig. 2).

RFLP analyses were consistent with PCR results, confirming that the ShWB phytoplasma was associated with sunn hemp samples. The restriction of PCR products with 6 endonucleases (Fig. 3) resulted similar 16S rDNA RFLP profiles for phytoplasmas that previously classified to group 16SrII. Lee *et al.* (1998) described 4 subgroups based on RFLP analysis of 16S rDNA by 17 restriction enzymes for 16SrII group; for instance, 16SrII–A: peanut witches' broom (PnWB) and sunn hemp
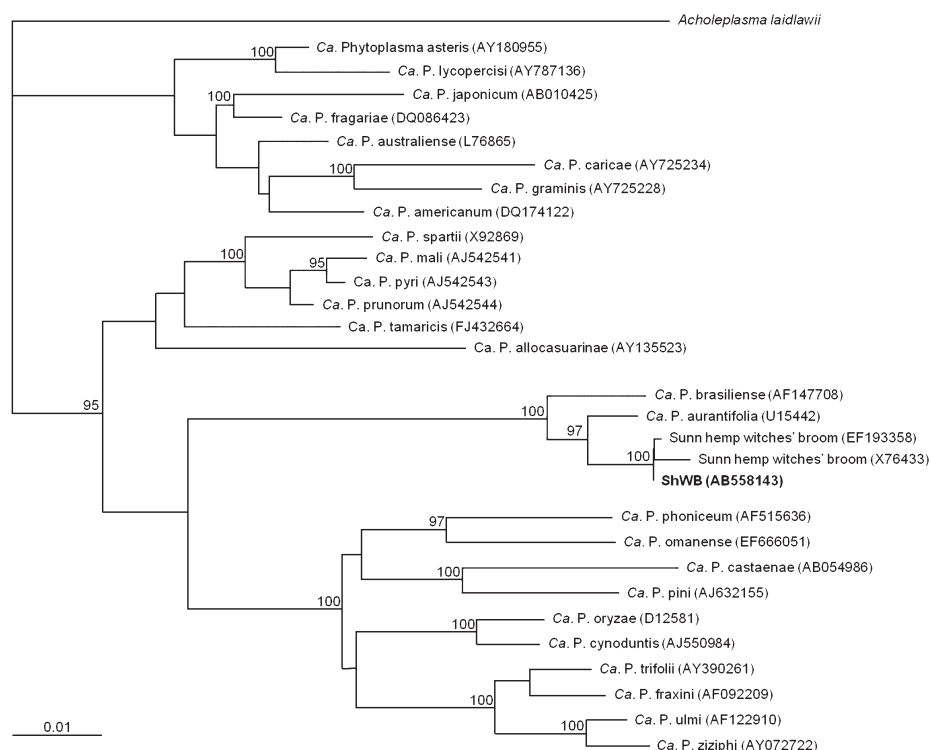

Fig. 2. Phylogenetic tree constructed by the neighbour–joining method of 16S rDNA sequences from ShWB phytoplasma and previous described of *Candidatus* Phytoplasma sp. *Acholeplasma laidlawii* was employed as the outgroup. Numbers on branches are confidence values obtained from 100 bootstrap replicates (only values above 80%) are shown.
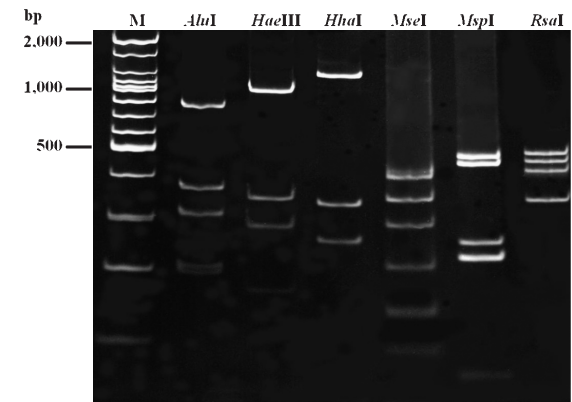
Fig. 3. RFLP analysis of 1.8 kb 16S rDNA sequences amplified in PCR primed by SN910610 and SN011119 from sunn hemp witches' broom (ShWB) phytoplasma digested with restricted endonucleases *Alu*I, *Hae*Ⅲ, *Hha*I, *Mse*I, *Msp*I and *Rsa*I. Lane M– 100 bp plus DNA ladder.

witches' broom (SUNHP), 16SrⅡ–B: lime witches' broom (WBDL), 16SrⅡ–C: faba bean phyllody (FBP) and 16SrⅡ–D: sweet potato little leaf (SPLL). By using above data, the relationship between ShWB and PnWB group phytoplasmas were determined by exploiting putative restriction maps (Fig. 4). This result indicated that ShWB phytoplasma had identical restriction sites patterns with PnWB (L33765) as well as SUNHP (X76433) and clearly different from other subgroups at two *Mse*I recognition sites (about 150 bp and 550 bp). The actual RFLP pat-

terns were in agreement with the results of putative RFLP maps. Based on the putative RFLP results, the ShWB phytoplasma was classified into 16SrⅡ–A. Therefore, RFLP analysis using PCR–amplified 16S rDNAs has provided a simple and reliable means of differentiation and classification of many unknown phytoplasmas in a relatively short period of time (Lee *et al.*, 1998). Also, a putative restriction site analysis is a valuable tool for classification when closely related reference phytoplasma strains were readily not available in certain condition.

Phytoplasma diseases in sunn hemp have been reported in Thailand (Seemüller *et al.*, 1994), Brazil (Amaral Mello *et al.*, 2004) and China (Wang *et al.*, 2008). Interestingly, these phytoplasma are found to be attributed in two groups; Thai isolates (X76433, EF193358) and Chinese isolates (EF656453, EF656454) belong to 16SrⅡ group of phytoplasmas while Brazilian isolate belongs to 16SrV group. The disease symptoms found in Myanmar are similar to those found in China and Brazil, and the most characterized symptom is proliferation of little leaves and shoots with shortened internodes. However, the same host could be naturally infected by phytoplasmas belonging to different groups which could induce similar symptoms. These phenomenon were occurred in big bud diseases of tomato that have been associated with at least four distinct phytoplasmas (16SrI–A, 16SrⅡ–E, 16SrVI–A and 16SrXⅡ–A) strains worldwide (Lee *et al.*, 1998; Marcone *et al.*, 1997; Davis *et al.*, 1997; Shaw *et al.*, 1993). Hence, the phytoplasmas associated on sunn hemp in Asia could be consid-
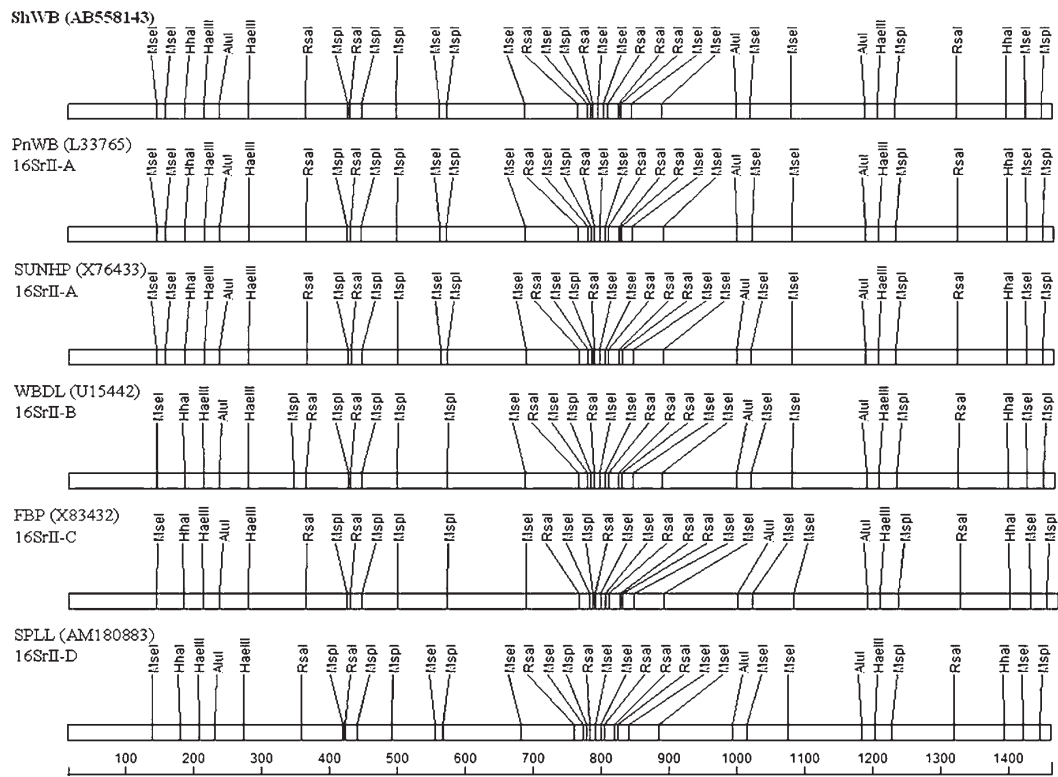

Fig. 4. Comparative analysis of putative restriction sites in 16S rDNA sequences of sunn hemp witches' broom (ShWB) and other 16SrⅡ group phytoplasmas: PnWB, Peanut witches' broom; SUNHP, Sunnhemp witches' broom; WBDL, Witches' broom of lime; FBP, Faba bean phyllody; SPLL, Sweet potato little leaf. Sequences were extracted from GenBank.

ered as a high relationship phylogenetically. The distribution of phytoplasmas in various geographical areas has more facilitated their molecular classification into groups and subgroups (Lee *et al.*, 1998; Semüller *et al.*, 1998). More importantly, the association of phytoplasma on sunn hemp plants becomes a threat to other important crops because it can serve as reservoirs for phytoplasmas although information on transmission of this phytoplasma disease by insect vectors is still untouched. This study represents the first evidence of phytoplasma association on sunn hemp plants related to *Ca.* Phytoplasma which belongs to 16SrII–A group in Myanmar. This is basis information for future study concerned with epidemiology aspects of the disease, host range study and identifying possible insect vectors through molecular techniques.

ACKNOWLEDGEMENTS

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