Molecular Characterization of White Leaf Phytoplasma Associated with the Graminae in Myanmar

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https://doi.org/10.5109/27351

出版情報：九州大学大学院農学研究院紀要. 58 (2), pp.225-229, 2013-09. Faculty of Agriculture, Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., **58** (2), 225–229 (2013)

Molecular Characterization of White Leaf Phytoplasma Associated with the Graminae in Myanmar

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(*Received March 26, 2013 and accepted May 9, 2013*)

Typical white leaf disease symptoms were observed on Bermuda grass (*Cynodon dactylon*) in Magway and Nyungoo, and goosegrass (*Eleusine indica*) in Kyaukpadaung of Myanmar, 2011. Phytoplasma associations with these grasses were determined by polymerase chain reaction analysis with amplified DNA fragment of 1.8 kbp including nearly full–length of 16S rRNA, 16S–23S spacer region and partial of 23S rRNA gene. The 16S rRNA gene sequence analysis resulted that two Bermuda grass white leaf (BGWL) isolates had the highest similarity (99%) with members of '*Candidatus* Phytoplasma cynodontis'. The goosegrass white leaf (GGWL) phytoplasma showed <98.8% identity with 16S rRNA gene sequences of '*Ca*. P. cynodontis' and sugarcane white leaf phytoplasma (SCWL, AB646271), and shared 99.5% similarity with that of sorghum grassy shoot (SGS, AF509324). Percent homology of 16S–23S spacer region sequences showed that the GGWL isolate was closet (97% identity) to SCWL. Putative restriction site analysis of 16S rRNA gene sequence including spacer region and partial of 23S rRNA gene revealed that the GGWL isolate was distinguished from the BGWL isolate. The phylogenetic analysis proved that the GGWL isolate diverged from the sugarcane white leaf phytoplasmas and closely related with SGS phytoplasma. In addition, the GGWL phytoplasma was distantly related with '*Ca.* P. cynodontis' and SCWL group. This is the first report on the presence of Bermuda grass white leaf and goose grass white leaf phytoplasmas in Myanmar.

Key words: white leaf phytoplasma, graminae, goosegrass

INTRODUCTION

White leaf disease on Bermuda grass (BGWL) was first reported from Taiwan (Chen *et al*., 1972) and is now known to occur in other Asian countries (Zahoor *et al*., 1995; Jung *et al*., 2003; Rao *et al*., 2007), Africa (Daffala & Cousin, 1988), Australia (Tran–Nguyen *et al*., 2000), Europe (Marcone *et al*., 1997) and Cuba (Arocha *et al*., 2005). '*Candidatus* Phytoplasma cynodontis' (BGWL; AJ550984) is a designated novel species for BGWL phytoplasmas belonging to 16SrXIV group (Marcone *et al*., 2004). Most of phytoplasmas associated with Bermuda grass belongs to the '*Ca*. P. cynodontis'. Also, BGWL phytoplasmas belong to other '*Ca*. Phytoplasma' species such as '*Ca*. P. asteris' in Thailand (Marcone *et al*., 2000) and '*Ca*. P. graminis' in Cuba (Arocha *et al*., 2005). Moreover, a number of white leaf diseases of other grasses are associated with phytoplasmas that fall within the '*Ca*. P. cynodontis' including Brachiaria grass (*Brachiaria distachya*), annual blue grass (*Poa annua*), crowfoot grass (*Dactyloctenium aegyptum*) and Delhi grass (*Dichanthium annulatum*) whereas the '*Ca*. P. asteris' associated with common meadow grass (*Poa pratensis*) and tall fescue (*Festuca arundinacea*) (Valiūnas *et al*., 2007), and the '*Ca*. P. oryzae' associated with thatching grass (*Hyparrhenia rufa*) (Obura *et al*., 2011). Recently, the white leaf disease of golden beard grass (*Chrysopogon acicalatus*) was reported as '*Ca*. P. cynodontis' associated disease in Myanmar (Win and Jung, 2012a).

In a 2011 survey of central Myanmar (Mandalay and Magway Division), white leaf disease symptoms on diverse grass species including Bermuda grass (*Cynodon dactylon*) and goosegrass (*Eleusine indica*) were occurred between vegetable fields and uncultivated areas. Especially, white leaf diseased Bermuda grass was constantly found on many places as small white patches. These grasses exhibited small white leaves, bushy growing habit, stunting and death of the plants. Because of these typical symptoms, the white leaf disease of Bermuda grass and goosegrass were assumed to be caused by phytoplasma. Although white leaf disease for golden beard grass was reported before, white leaf diseases for Bermuda grass and goosegrass have not been identified in Myanmar yet. On goosegrass, witches' broom and yellows diseases had been reported and these diseases were associated with '*Ca*. P. asteris' in China and Taiwan (Wang *et al*., 2010; Chen *et al*., 2011). Therefore, in this work, the etiological agent of white leaf diseases of Bermuda and goosegrass were verified by applying molecular analysis whether the associated phytoplasma belongs to '*Ca*. P. cynodontis' or '*Ca*. P. asteris' or other '*Ca*. Phytoplasma' species.

MATERIALS AND METHODS

Plant samples

Naturally infected grasses exhibiting white leaf symptoms of goose grasses in Kyaukpadaung and of Bermuda grasses in Magway and Nyungoo were collected in

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September, 2011. The infected plants produced many small, stiff and white leaves on the goose grasses (Fig. 1A) and completely loss of chlorophyll on the Bermuda grass (Fig. 1B). White leaf samples from three separate plants were collected for both grass species. The apparently healthy leaf samples were also collected around the infected grasses. An isolate from golden beard grass white leaf (GBGWL) disease was used as reference phytoplasma that belongs to '*Ca*. P. cynodontis' (Win and Jung, 2012a).

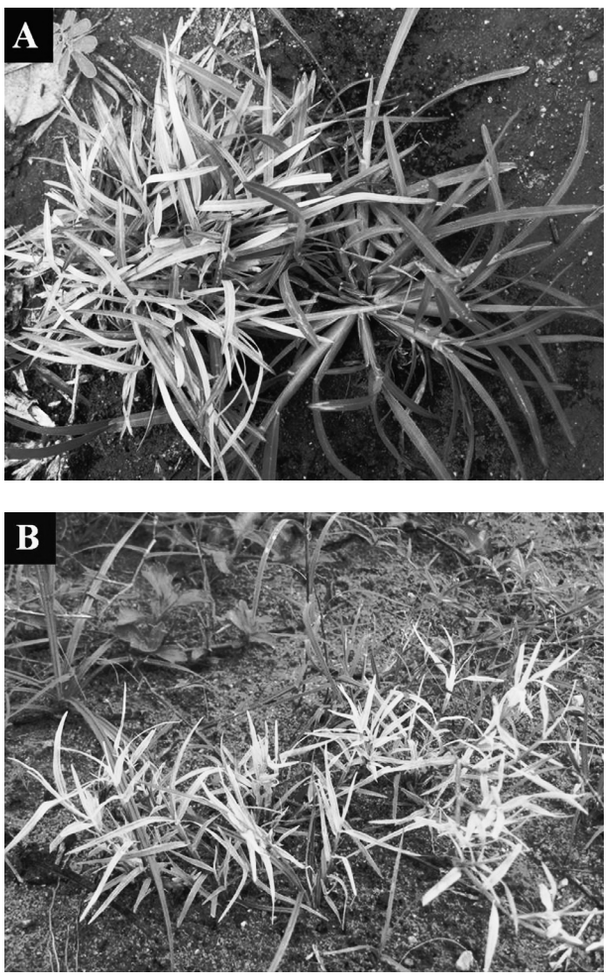

Fig. 1. Symptom of goosegrass white leaf showing bushy, stunting, small and white leaves in Kyaukpadaung (A) and Bermuda grass white leaf exhibiting whitening of above ground plant parts in Magway (B) Myanmar.

Total DNA extraction and polymerase chain reaction analysis

About 0.3 g of leaf sample was used to extract total DNA using cetyltrimethylammoniumbromide buffer method (Namba *et al*., 1993). Polymerase chain reaction (PCR) assays were performed with universal phytoplasma primers SN910601/SN011119 for amplification of the nearly full–length of 16S rRNA gene, 16S–23S spacer region and partial of 23S rRNA gene (Jung *et al*., 2003). The extracted DNA samples were used for direct PCR to detect phytoplasma. The PCR reaction mixture (20 μl) contained 2 μl of total DNA (100 ng), 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* polymerase (SolGent Co., Daejon, Korea). The amplifications were carried out in an automated thermocycler 2720 (Applied Biosystems, Foster City, CA, USA) using the following condition: 2 min at 94˚C as the first denaturation, 35 cycles of denaturation for 30 s at 94˚C, annealing for 30 s at 55˚C and extension for 90 s at 72˚C followed by 7 min at 72˚C as the final step. The PCR products were electrophoresed in 1% agarose gels in Tris EDTA buffer staining with ethidium bromide and visualized by a UV transilluminator.

Sequencing and putative restriction site analyses

The PCR products were purified and directly sequenced with the primers used in PCR and other five primers (350F, 350R, 520R, 788F and 1505F) that used to sequence nearly full–length of 16S rRNA, 16S–23S spacer region and partial of 23S rRNA gene (Jung *et al*., 2003). The full sequences obtained from the goosegrass and Bermuda grass white leaf disease samples were deposited in the GenBank database under accession numbers AB741629 and AB741630 respectively.

Putative restriction site maps of 16S rRNA gene sequences including 16S–23S spacer region and partial of 23S rRNA gene were generated for Bermuda grass white leaf, goosegrass white leaf and golden beard grass white leaf phytoplasma isolates using MapDraw option of DNASTAR program. The isolates were manually aligned to compare restriction sites for the endonucleases *Hinf* I, *Mse* I, *Taq* I, *Hae* Ⅲ and *Sau* 3AI.

Phylogenetic analysis

Multiple sequence alignments with the 16S rRNA gene sequences of phytoplasma belonging to '*Ca*. P. oryzae' and '*Ca*. P. cynodontis' available in GenBank database were performed using Clustal W software. A phylogenetic tree was constructed using neighbor–joining method and the bootstrap test was conducted for 1,000 replications. '*Ca*. P. pruni' was used as the outgroup to root the tree. The tree was viewed using TREEVIEW program.

RESULTS AND DISCUSSION

Several grass species including golden beard grass, Bermuda grasses and goosegrass are growing naturally on almost every soil type in Myanmar. The infected grasses were observed as small to large white patches on the ground. They exhibited whitening of above ground plant parts, reduction of leaf size and stunting (Fig. 1). By PCR analysis, the expected phytoplasma DNA fragments (1.8 kbp) were amplified with the primers SN910601/ SN011119 from total DNA extracted of infected Bermuda grasses and goosegrass. The amplified DNA fragments sizes were the same size with that of reference GBGWL phytoplasma. No DNA amplification was obtained from symptomless healthy plants. Therefore, the white leaf symptoms of Bermuda grass and goosegrass were confirmed as the phytoplasma associated disease.

Then, the associated phytoplasmas with two grass

species were identified by sequencing of 16S rRNA gene including spacer region and partial of 23S rRNA gene. The nucleotide sequences of two BGWL isolates from Magway and Nyungoo regions were 100% identical. However, the BGWL isolates and GGWL isolate shared 98.8% homology each other in 16S rRNA gene sequences. Blast search revealed that 16S rRNA gene of BGWL isolate (1,525 bp) shared highly similarity (99%) with the 16S rRNA gene sequences of golden beard grass white leaf (AB642601), Thai Bermuda grass white leaf (AF248961), Malaysian Bermuda grass white leaf (EU294011) and Italian Bermuda grass white leaf (AJ550984), and all are members of '*Ca*. P. cynodontis'. Unexpectedly, 16S rRNA gene sequence of GGWL phytoplasma (1,462 bp) was closely (99% similarity) related with sorghum grassy shoot (SGS, AF509324) and sugarcane white leaf (SCWL, AB052874), and also, with member of '*Ca*. P. cynodontis' including Bermuda grass white leaf (AJ550984) and Brachiaria grass white leaf (AB052872). Then, percent homology between sequences of 16S rRNA gene and 16S–23S spacer region was further compared. The BGWL isolate (AB741630) shared 99.7–99.8% and 100% similarity to 16S rRNA and 16S–23S spacer region gene of GBGWL and '*Ca*. P. cynodontis' (BGWL–C1) respectively (Table 1 and 2). The GGWL isolate (AB741629) shared 98.8% with BGWL–C1 and 97.7–98.9% similarity with members of '*Ca*. P. oryzae' (RYD, SCWL–Taiwan and SCWL–Myanmar) for 16S rRNA gene sequences while it shared 94.6–97% similarity with 16S–23S spacer region gene sequence of sugarcane white leaf phytoplasmas. Hence, based on the result of 16S–23S spacer region gene similarity, the GGWL isolate could be assumed to be more closely related with sugarcane white leaf phytoplasmas.

Putative restriction site analysis of 16S rRNA gene sequence (~1.8 kbp) including 16S–23S spacer region and partial of 23S rRNA gene revealed that the GGWL isolate was distinguishable from BGWL and GBGWL isolates based on the presence or absent of *Mse* I, *Hae* Ⅲ, *Taq* I, *Sau* 3AI and *Hinf* I restriction sites (Fig. 2). The BGWL and GBGWL were indistinguishable except the presence of *Hinf* I sites at the 5' end of 16S rRNA gene in BGWL which was absent in GBGWL isolate. The restriction sites were more variable in region of 16S–23S spacer gene region between GGWL isolate and BGWL, GBGWL isolates.

In the phylogenetic analysis based on the 16S rRNA gene (Fig. 3), the BGWL isolates clustered with GBGWL and other members of '*Ca*. P. cynodontis'. The BGWL isolate was most closely related with Bermuda grass white leaf (AB05271) and *Digitaria sanguinalis* white leaf (AF248961) phytoplasmas from Thailand. However, the GGWL isolate was separated from the sugarcane white leaf phytoplasmas and formed a new branch clustering with SGS phytoplasma (AF509324). It shared 99.5% sequence similarity with 16S rRNA gene sequence of SGS. Other hand, the GGWL phytoplasma observed in Myanmar was different from goosegrass witches' broom (FJ263620) from China and goosegrass yellows (GU361756) from Taiwan which are members of '*Ca*. P. asteris'.

Percent homology, putative restriction analysis and phylogenetic analysis revealed that BGWL isolate is a member of '*Ca*. P. cynodontis' and possess the unique signature sequence: 5'–AATTAGAAGGCATCTTTTAAT–3' (Marcone *et al*., 2004) for BGWL phytoplasma group whereas the GGWL isolate do not have that unique signature. Similarly, the conserved region, 5'–TATTAGACTA –3', found in '*Ca*. P. oryzae' (rice yellow dwarf group) especially for sugarcane white leaf and BVK phytoplasma (Jung *et al*., 2003) was absent on the nucleotide sequence

Table 1. Percent similarity among white leaf isolates of grasses and other reference phytoplasmas as determined by analysis of 16S rRNA gene sequences

	BGWL AB741630	GBGWL AB642601	GGWL AB741629	BGWL–C1 AJ550984	RYD D12581	SCWL–Thai AB052874	SCWL–Mm AB646271
BGWL		99.8	99.8	99.7	97.7	98.4	98.4
GBGWL			98.6	99.7	97.7	98.3	98.3
GGWL				98.8	97.7	98.9	98.8

Phytoplasma isolate: BGWL, Bermuda grass white leaf, Magway; GBGWL, golden beard grass white leaf, Tatkon; GGWL, goosegrass white leaf, Kyaukpadaung; BGWL–C1, Bermuda grass white leaf, Italy; RYD, rice yellow dwarf; SCWL–Thai, sugarcane white leaf, Thailand; SCWL–Mm, sugarcane white leaf, Myanmar.

Table 2. Percent similarity among white leaf isolates of grasses and other reference phytoplasmas as determined by analysis of 16S–23S rRNA spacer gene sequences

	BGWL AB741630	GBGWL AB642601	GGWL AB741629	BGWL–C1 AJ550984	RYD AY139873	SCWL–Thai AY139874	SCWL–Mm AB646271
BGWL		100	88.9	100	88.2	86.0	88.1
GBGWL			88.9	100	88.2	86.0	88.1
GGWL				88.9	89.8	94.6	97.0

Abbreviations are the same as given in Table 1.

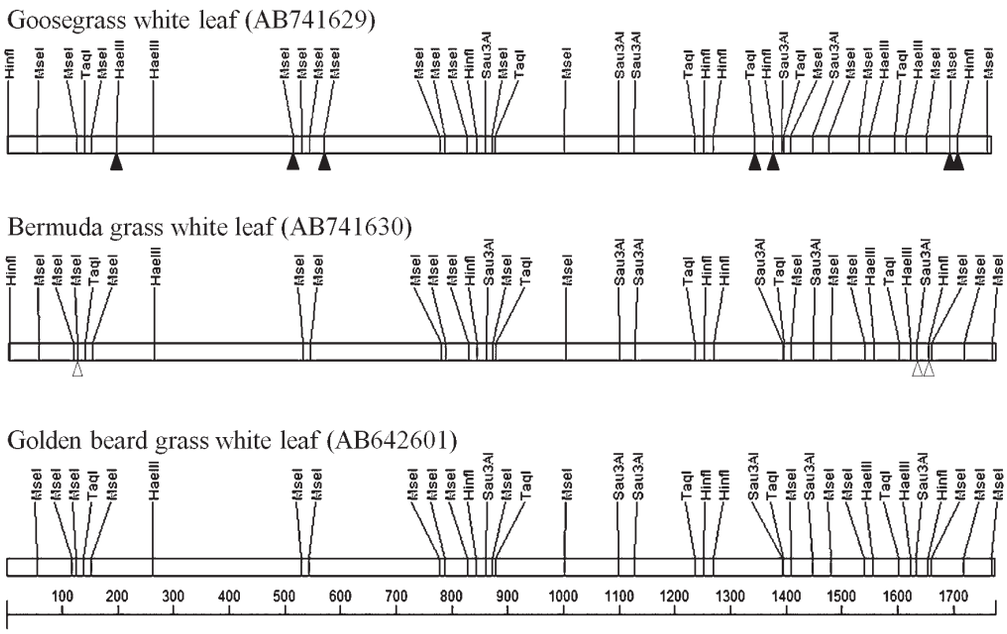

Fig. 2. Putative restriction site maps of 16S rRNA gene sequences (~1.8 kbp) including 16S–23S spacer region and parital of 23S rRNA gene of goosegrass white leaf, Bermuda grass white leaf and golden beard grass white leaf phytoplasmas. Maps were generated using MapDraw option of DNASTAR program. Black triangle (▲) and white triangle (Δ) indicate the presence of additional restriction sites and absent of restriction sites on sequence of goosegrass white leaf phytoplasma compared to Bermuda and golden beard grass white leaf phytoplasmas.

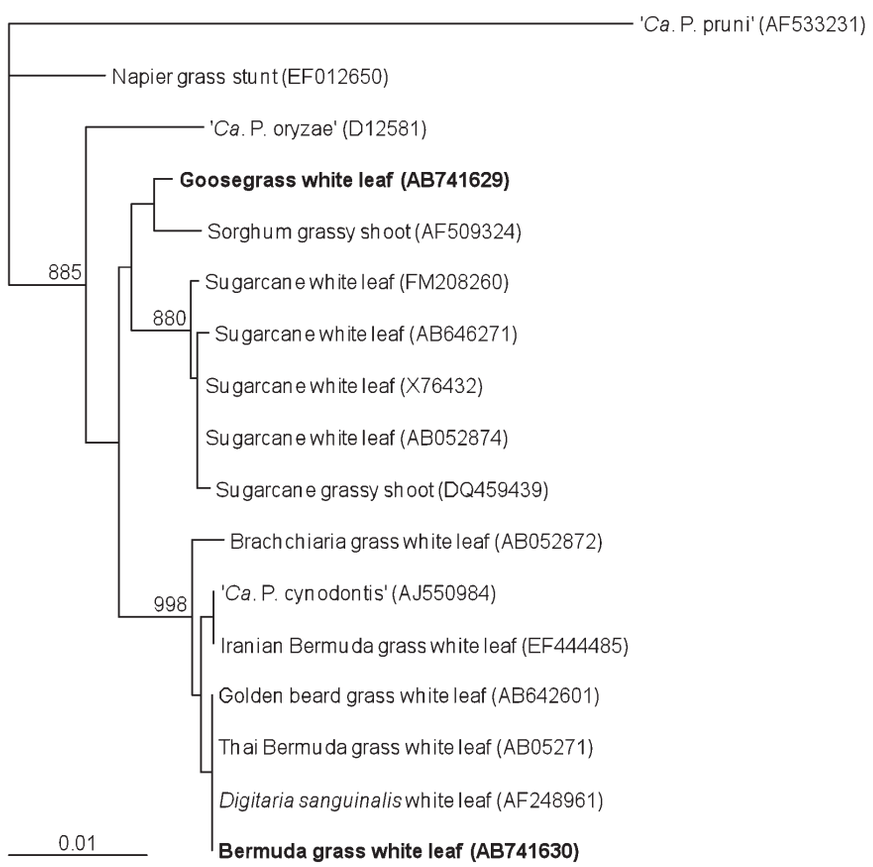

Fig. 3. Phylogenetic tree constructed by the neighbor–joining method from 16S rRNA gene sequences of Bermuda grass white leaf and goosegrass white leaf phytoplasma isolates and other phytoplasma members of '*Ca.* P. oryzae' and '*Ca.* P. cynodontis'. '*Ca.* P. pruni' (AF533232) was used as outgroup. Numbers on branches are confidence value obtained from 1,000 bootstrap replicates (only values above 80% are shown). Bar represents phylogenetic distance.

of GGWL 16S rRNA gene. The GGWL phytoplasma was distantly related with sugarcane white leaf phytoplasma group, '*Ca*. P. oryzae' and '*Ca*. P. cynodontis' among previously described phytoplasma species. Therefore, in this circumstance, the GGWL phytoplasma is difficult to identify into appropriate '*Ca*. Phytoplasma' species that previously described or 16S rRNA group. Due to possessing of distinguish features, the GGWL phytoplasma would have potential to be new phytoplasma species. However, data of other molecular evidence, insect vector and geographical distribution are needed to support as new phytoplasma species.

Phytoplasma diseases in Myanmar have been expanded and two new grass hosts add to the known host species for phytoplasma infection (Win and Jung, 2012b). Too far, goosegrass was the host for '*Ca*. P. asteris' in China and Taiwan (Wang *et al*., 2010; Chen *et al*., 2011), and it could be assumed as alternative host for infection of paulownia witches' broom phytoplasma in China (Wang *et al*., 2010). The Bermuda grass infecting phytoplasmas observed in two different regions are largely identical and represent the same entity, '*Ca*. P. cynodontis'. The grass species would be potential reservoirs for phytoplasma distribution. Further studied are needed to understand possible phytoplasma transmitting from grasses to other important the Gramineae plant species such as sugarcane and sorghum through insect vectors. To our knowledge, this is the first report for the presence of Bermuda grass white leaf and goosegrass white leaf phytoplasma in Myanmar.

ACKNOWLEDGEMENT

This work was supported by Mid–career Researcher Program through NRF grant funded by the MEST (No.2010–0027638).

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