Cloning and Nucleotide Sequence Analysis of a tuf Gene from Loofah Witches’ Broom Phytoplasma

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ABSTRACT: Using two primers and the enriched genomic DNA of a loofah witches’ broom (LfWB) phytoplasma as the template, a 1651-bp DNA fragment was amplified in a PCR reaction. The DNA fragment contained two open reading frames (ORFs), one coding for part of the elongation factor EF-G (fus gene) and the other for the full-length EF-Tu (tuf gene). The tuf gene contained 1191 nucleotides encoding a 396-amino acid protein with a predicted molecular mass of 43.8 kDa. The sequence displayed a characteristic of low G+C content of mollicutes and had a G+C content of 33.42%. It had a high degree of amino acid sequence homology, from 53.9 to 61.8%, with those of other bacteria except two archaea (lower than 26%). There was only one tuf gene locus on the genome. The phylogenetic relatedness of LfWB phytoplasma to other prokaryotes revealed by tuf DNA sequences was consistent with the result reported by 16S rDNA sequences.

KEY WORDS: Phytoplasma, tuf gene, Phylogenetic relatedness.

INTRODUCTION

Phytoplasmas previously were known as mycoplasma-like organisms, MLOs. In 1992, the Subcommittee on the Taxonomy of Mollicutes, the International Committee on Systemic Bacteriology suggested to change the name to phytoplasmas. The analyses of 16S rRNA and several ribosomal protein genes indicated that they were phylogenetically closer to acholeplasmas and anaeroplasmas, than to mycoplasmas (Tully, 1993). The organization of tRNA genes downstream the 5S rRNA gene also suggested that they were closer to acholeplasmas, and both evolved from Bacillus subtilis (Ho et al., 2001).

Phytoplasmas are one group of plant bacterial pathogens. They are wall-less and obligate parasites that reside in the phloem of plants, and have a small genome of 500 – 1700 kbp and a relative low G+C content (Razin et al., 1998). The failure of culturing these organisms in vitro makes it very difficult to study their biological and biochemical properties, and results in the uncertainty in their taxonomy. Although they are classified as a member of Class Mollicutes, they still cannot form a genus due to the lack of enough information (Sears and Kirkpatrick, 1994).

Elongation factor EF-Tu protein is considered to be one of the good molecular markers for studies of taxonomy and phylogeny because of its highly conservative nucleotide and amino acid sequences (Sela et al., 1989; Kamla et al., 1996). In addition, the copy number of the tuf gene is different in various bacteria. Gram-negative bacteria usually have two copies per genome, while most gram-positive bacteria such as B. subtilis and some Clostridium

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strains have only one copy (Sela et al., 1981). Exceptions are some Clostridium strains and Streptomyces ramocissimes. The former has more than one copy (Sela et al., 1989) and the latter has three copies (Vijgenboom et al., 1994). Mycoplasma gallisepticum, Mycoplasma genitalium and Mycoplasma pneumoniae also only have one copy (Inamine et al., 1989; Loechel et al., 1989; Yoge et al., 1990). The context of the tuf gene on genome is also different in bacteria. Escherichia coli and most bacteria have a context of 5'-rps12-rps7-fus-tuf-3' (Cousineau et al., 1992). However, the tuf and fus genes of M. genitalium (Loechel et al., 1989), M. pneumoniae (Yoge et al., 1990), Sulfolobus acidocaldarius, Pyrococcus woesei and Desulfiuscoccus mobilis (Ceccarelli et al., 1995) are separated on genome.

In this report, the nucleotide sequence of a loofah witches’ broom (LfWB) phytoplasma tuf gene was presented and used as a basis to discuss the phylogenetic relatedness of this organism.

MATERIALS AND METHODS

Bacteria and plant

LfWB phytoplasmas were maintained by graft inoculation in periwinkle (Chen and Ho, 1997). Diseased periwinkle was provided by Dr. C.-P. Lin, Professor of Department of Plant Pathology, National Taiwan University, Taiwan.

Plant and phytoplasma DNA isolation

The healthy or diseased plant DNA was isolated using the procedures described previously by Ho et al (2001): Fifteen grams of plant branches were disinfected with 1% sodium hypochloric acid, rinsed with distilled water, and then dried by kimwipes briefly. Liquid nitrogen was added to a pre-cooled mortar containing plant tissues. The tissues were ground by a pestle and then by a coffee grinder. The powder was suspended in 15 mL of extraction buffer (500 mM NaCl, 100 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% N-lauroyl sarcosine) and incubated at 55°C for 2 h. After centrifuging at 10,000 xg at 4°C for 20 min, the supernatant was mixed with 0.6 volumes of isopropanol. The mixture was chilled at -20°C for 30 min. The DNA was precipitated at 10,000 xg at 4°C for 20 min. The pellet was then resuspended in 5 mL of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 0.5% SDS and 500 µg proteinase k. After incubation at 37°C for at least 1 h, 875 µL of 5 M NaCl and 700 µL of CTAB/NaCl solution (10% CTAB, 0.7 M NaCl) were added. The incubation was continued for another 10 min at 65°C. The mixture was extracted 3 times with chloroform/isoamyl alcohol (24:1), and then twice with phenol/chloroform/isoamyl alcohol (25:24:1). The supernatant was mixed with 0.6 volumes of isopropanol, chilled at -20°C for 1 h, and then spun at 12,000 xg at 4°C for 20 min. The pellet was rinsed with 75% alcohol, air dried and then resuspended in 1 mL of TE.

The cesium chloride (CsCl)-bisbenzimide density gradient centrifugation was used to separate phytoplasma DNA from host plant DNA as following: The gradient contained DNA solution (in TE), CsCl (gram) and bisbenzimide (0.5mg/mL in TE) in a ratio of 9.5:10.5:1. After centrifugation at 55,000 rpm in p65VT3 rotor (Hitachi Koki Co., Ltd., Japan) at 20°C for 16 h, the phytoplasma DNA was visualized under UV light as the uppermost band in the
gradient of DNA from the diseased plant and was collected with a syringe. This DNA fraction was purified one more time through the gradient and extracted 3 times by water-saturated butanol. After the addition of three volumes of sterile distilled water, eight volumes of absolute alcohol and chilled at -20°C for 1 h, the DNA was precipitated. The DNA pellet was rinsed with 75% alcohol, dried and then dissolved in 500 μL of TE.

**Polymerase chain reaction (PCR)**

Two primers (Fus-1: 5'-AGATGTTAGAGCTATTTTATTTGATG-3' and Tuf-1: 5'-TCATTCTAAATATCTCGATAACTGTTC-3') were synthesized based on our primary result (Chen and Ho, 1998) to amplify a DNA fragment using LfWB phytoplasma DNA as the template in a PCR reaction: The first cycle of the 100 μL PCR reaction was as follows: denaturation at 95°C, 5 min; annealing at 50°C, 1 min; extension at 72°C, 2 min. The reaction continued for 35 cycles as follows: denaturation at 95°C, 1 min; annealing at 50°C, 1 min; extension at 72°C, 2 min plus 3 sec/cycle. Finally, the reaction was performed at 72°C for another 5 min.

**Blot hybridization analysis**

Three micrograms of DNA were digested with different restriction enzymes including Dral, EcoRI, EcoRV and HindIII, and subjected to Southern blot analysis as previously described (Ho et al., 1992) using the full-length tuf DNA as probe. The hybridization was carried out at 42°C in the presence of 50% formamide and 0.1% SDS. Filter was washed in 0.1X SSC (1X SSC: 15 mM NaCl, 15 mM sodium citrate) containing 0.1% SDS at 50°C. The hybridized bands were detected by exposing the filter to a phosphorimager screen (Phosphor Imager 425, Molecular Dynamics, USA).

**DNA sequence determination and analysis**

For DNA sequencing, the PCR product was cloned into pGEM-T easy vector (Promega). The sequences of DNA were determined by a DNA automated sequencer (ABI Prism Model 377, v. 3.0; Applied Biosystems) using a step-by-step procedure in which synthetic primers for forward sequencing were designed from sequences obtained previously. DNA sequence analysis was performed using DNAStar software (DNASTAR).

**RESULTS AND DISCUSSION**

**Cloning strategy**

A 1651-bp PCR DNA fragment was amplified on the LfWB phytoplasma DNA template using the primers Fus-1 and Tuf-1, and cloned into pGEM-T easy vector. The DNA fragment contained two open reading frames (ORFs), one coding for part of the elongation factor EF-G (fus gene) and the other for the full-length EF-Tu (tuf gene) (Fig. 1).

**Analysis of the nucleotide sequence of tuf gene**

The tuf gene contained 1191 nucleotides encoding 396 amino acids with a predicted isoelectric point of 6.42, and a molecular mass of 43.8 kDa. The gene had a G+C content of 33.42%, which was in agreement with the common characteristic of low G+C content of mollicutes, 31.13% to 44.6%, (Berg and Seemuller, 1999; Schneider et al., 1997; Loechel et al., 1989; Yoge et al., 1990) and B. subtilis, 43.91%, the suspected ancestor of mollicutes (Weisburg et al., 1989).
Fig. 1. Nucleotide sequences of the partial fus and the full-length tuf genes, and their deduced amino acid sequences. The sequences for GTP-binding were boxed. •, the amino acid residues of phosphate group of GTP binding; *, the amino acid residues of formation of a small pocket fitting the guanine ring; ○, β-phosphate of GDP binding; ▼, the amino acid residues interacting with tRNA; ▲, the amino acid residues interacting with EF-Ts; △, the amino acid residues of antibiotic kettromycine binding (Ludwig et al., 1990; Yogev et al., 1990). Two termination codons were underlined.
The low G+C content of LfWB phytoplasma tuf gene led to a preference for A or T in the second and third positions of the codon: 64.73% (second position) and 85.39% (third position), respectively. Of the 61 codons, 13 codons were not used including the one (UGG) for Trp. The use of UGA as a tryptophan codon, an unusual feature of mycoplasmas (Razin et al., 1998), was not found in the LfWB phytoplasma tuf gene and the genes encoding an ABC transporter (unpublished data). The LfWB phytoplasma tuf gene contained all the hallmark signatures of an EF-Tu protein, including the amino acid residue(s) interacting with GTP, GDP, tRNA, elongation factor Ts and antibiotic kirromycin (Ludwig et al., 1990; Yogeiv et al., 1990) (Fig. 1).

Comparing the amino acid and nucleotide sequences of LfWB phytoplasma tuf gene with those of other prokaryotic cells listed in the legend of Fig. 3, the results showed that it had high amino acid sequence homology with those of eu-bacteria (53.9 to 61.8%) and much lower homology with those of archaea (lower than 26%). The nucleotide sequences of tuf genes were somewhat divergent. LfWB phytoplasma tuf gene had 38.9 to 65.4% homology with those of eu-bacteria, and 23.5 to 28% with those of archaea.

There were 63 nucleotides between the tuf and its proceeding fus gene with a Shine-Dalgarno (SD) sequence, AGGA, the ribosome binding site (Fig. 1).

**Number of tuf gene locus in LfWB phytoplasma**

In addition to the low G+C content, the copy number of the tuf gene on the genome has been used to support that mollicutes are phylogenetically related to gram-positive bacteria (Woese, 1987). Thus far, only one copy of the tuf gene has been found in the genome of mollicutes (Inamine et al., 1989; Loechel et al., 1989; Yogeiv et al., 1990). In an attempt to find out the number of tuf gene locus, LfWB phytoplasma genomic DNA was digested with each of the following restriction enzymes known not to cleave DNA within the tuf gene: Dral, EcoRI, EcoRV and HindIII, and probed by a 32P-labeled tuf DNA fragment. There was only one single band hybridized in each restricted DNA, suggesting the presence of only one gene locus for EF-Tu (Fig. 2).

The gene context on the genome of mollicutes is variable. The tuf and fus genes are separated in the genomes of M. genitalium and M. pneumoniae (Inamine et al., 1989; Yogeiv et al, 1990). These two genes were linked together in LfWB phytoplasma genome. The same result was reported for several other phytoplasmas (Berg and Seemuller, 1999).

**Phylogenetic relatedness to other prokaryotes**

Recently, the phylogeny of phytoplasmas and their relationship to other prokaryotes were actively investigated using the DNA sequence homology (Sela et al., 1989; Lee et al., 1993; Gunderson et al., 1994; Kamla et al., 1996; Schneider et al., 1997). Because of the nucleotide and amino acid sequence conservation, the tuf gene has been considered as one of the effective markers.
Fig. 3. Phylogenetic trees constructed based on (A) nucleotide sequence of tuf gene, (B) nucleotide sequence of 16S rRNA and (C) amino acid sequence of tuf gene using clustal method with weight table (DNASTAR). The scales in the bottom represent the branch lengths. The abbreviations for organisms are as followings: (a) tuf genes: AAY (L46368); APP (AJ011104); KV (L46369); STOLF (L46370); BSU, B. subtilis (D64127); DMO, D. mobilis (X73582); ECO, E. coli (U00006); MGA, M. gallisepticum (X16462); MGE, M. genitalium (X16463); MHO, M. hominis (X57136); MLU, M. luteus (M17788); MPN, M. pneumoniae (X55768); UUR, U. urealyticum (Z34275); TAC, T. acidophilum (X53866). (b) 16S DNA: AAY (X68373); ACLR (X68383); APP (AF24858); LFWB (AF251150); PAPAYA (Y08173); STLL (Y17055); AAB, Anaeroplasma bactoelasticum (M25050); ABA, Anaeroplasma bactoelasticum (M25049); APA, Acholeplasma palmae (L33734); APO, Acholeplasma polakii (AF031479); AVA, Anaeroplasma varium (M23934); BSU, B. subtilis (M106069); DMO, D. mobilis (M36474); ECO, E. coli (Y00348); MGA, M. gallisepticum (L08896); MGE, M. genitalium (U39693, L43967); MHO, M. hominis (M96660); MLU, M. luteus (U85892); MPN, M. pneumoniae (M21374); UUR, U. urealyticum (L08642); TAC, T. acidophilum (M38637). AAY, ACLR, APP, KV, LFWB, PAPAYA, STOLF and STLL are the strains of phytoplasmas. The numbers in the parentheses are the DNA sequence accession number of the genes used.
in the study of bacterial phylogeny (Ludwig et al., 1993; Yoge et al., 1988). Figure 3A is a phylogenetic tree constructed by DNA sequences of tuf genes of LfWB phytoplasma and several other prokaryotes. It showed that the five phytoplasmas could be closely grouped together, but were separated from other mollicutes (M. galliseptium, M. genitalium, M. hominis, M. pneumoniae and Ureaplasma urealyticum). The data also showed that the mollicutes were closer to B. subtilis than to M. luteus and E. coli, and the two archaea (Thermoplasma acidophilum and D. mobilis) were more diverged. Figure 3B was the phylogenetic tree based on the 16S rDNA sequences. It also showed that the phytoplasmas were closely grouped together, and that phytoplasmas were closer to acholeplasmas and anaeroplasm than to other mollicutes. However, it was surprising that the tuf phylogenetic tree showed that mollicutes were more closely related to B. subtilis. But in the 16S rDNA phylogenetic tree, B. subtilis was grouped closely with M. luteus and E. coli, not with the mollicutes. This may be due to the fact that 16S rDNA sequences are more conserved among the organisms. When the amino acid sequences of the tuf genes were used, a different tree structure was obtained. The LfWB phytoplasma was grouped with apple proliferation (APP) phytoplasma, but not with other phytoplasmas (Fig. 3C). The amino acid sequence of the tuf gene may be too conservative to be used for phylogenetic study on the genus level, although it is still good for reflection of phenotypic and metabolic characteristics within the phytoplasmas (Kamla et al., 1996).

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LITERATURE CITED


絲瓜葉斑病植物菌質體 tuf 基因之選殖及序列分析

何國傑(1)

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摘 要

利用絲瓜葉斑病植物菌質體 DNA 為模板及兩個引作 PCR 反應，產生一段具 1651 個
塩基對的 DNA 片段。經核甘酸序列分析發現此 DNA 片段含有兩個譯讀框架，其中一
個為合成部分之延長因子 EF-G (fus 基因)的譯讀框架，另一個為合成全長之延長因子
EF-Tu (tuf 基因)的譯讀框架。tuf 基因含有 1911 個核甘酸，能合成一個具有 396 個氨基酸
酸，分子量為 43.8 千道爾頓的蛋白質。此基因之核甘酸序列的 G +C 百分比為 33.42，
合乎膜柔細之生物的特性。與其他細菌比較，在氨基酸序列上有高達 53.9 到 61.8%的相
似性，而與古生菌的相似性則低於 26%。基因組上，只存有一個 tuf 基因版本。在親緣
關係方面，由 tuf DNA 序列分析所構成的親緣樹或是利用 16S rDNA 序列分析構成的親
緣樹，顯示絲瓜葉斑病植物菌質體與其他菌種的親緣關係是相當一致的。

關鍵詞: 植物菌質體、tuf 基因、親緣關係。

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