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Chromosomal organization and nucleotide sequence of the genes coding for the molecular chaperones DnaK, DnaJ and GrpE from phytoplasma associated with peanut witches' broom

Keyword: Heat shock protein; *Mollicutes*; nested-PCR

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Abbreviations: PNWB, peanut witches' broom

Abstract

The PCR-based strategy was used for cloning and analysis of *dnaK* gene of phytoplasma associated with peanut witchs' broom. For nested-PCR, two pairs of nucleotide primer were designed based on the conserved regions of bacterial *dnaK* genes. A 750 bp *dnaK* gene fragment was amplified as predicted and further used for screening an *Eco*RI-digested genomic library of PNWB phytoplasma. Four open reading frames were identified in the order of ORF1, *grpE*, *dnaK* and *dnaJ*. Chromosomal arrangement of these genes in phytoplasmas is identical to *Clostridium acetobutylium* and other bacteria phylogenic closed to phytoplasmas. It implies the primitive bacterial molecular chapone HSP70 mechine exsists in phytoplasma and the mechanism it may involve when phytoplasma exposed to stress could be similar to other prokaryotes.

1. Introduction

Once exposed to stress such as heat shock, cold shock, osmobic shock and ultraviolet radiation, the heat-shock genes will be induced and the heat-shock proteins (HSPs) will transiently increased to facilitate to survive in most organisms (Taglicht et al., 1987; Hedyde and Portalier, 1990; Meuty and Kohiyama, 1991). The HSPs, which are mainly composed of two broad class of protein, molecular chaperone and protease, are highly conserved across prokaryotes and eukaryotes (Zeilstra-Ryalls et al., 1991). Based on both size and function, the HSPs can be distinguished into several families, including two groups of molecular chaperone nominated as the HSP 60 mechine (GroEL/ ES), the HSP 70 mechine (DnaK, DnaJ and GrpE), and two guoups of protease, the HSP 90 and the HSP 20 families (Bukau and Horwich, 1998; Mayer and Bukau, 1998). Now HSP60 and HSP70 are known playing essential roles in protein metabolism and protein translocation under both stress and non-stress conditions. In addition to this feature, members of the HSP 70 family of molecular chaperones are known to be involved in a wide variety of different cellular processes (Kelley, 1998), including protein folding and refolding (Hartl and Martin, 1995; Netzer and Hartl, 1998). The major components of the HSP 70 mechine are DnaK, DnaJ and GrpE. In most bacteria, genes encode for these proteins arrange in the order of grpE-dnaK-dnaJ (Narberhaus et al., 1992; Amemura-Maekawa and Watanabe,

1997; Wetzstein et al., 1992, Falah and Gupta, 1997).

Phytoplasma, a group of cell wall free prokaryotes, are nonhelical mollicutes associated with diseases in several hundred plant species (McCoy et al., 1989; Seemuller et al., 1998). Understanding the physiology, biochemistry and molecular biology of phytoplasmas is limited because of their resistance to culturing under axenic conditions (Lee and Davis, 1986), while procedures available for purification of phytoplasmas from disease plants are tedious and unsatisfactory (Clark et. al., 1989; Jiang and Chen, 1987) due to the inevitable contamination of plant component. In the last decade, the molecular biology methods has brought into phytoplasmology. Now phytoplasma DNA can be successfully separated on its extreme AT bias of codon usage using a CsCl-bisbenzimide density gradient centrifugation method (Kirkpatrick et al, 1987; Sears et al., 1989). Consequently genetic information has been the most important resource for phytoplasma study. In this work the genes encoding the primitive bacterial molecular chapone HSP70 are found in phytoplasma. It implies the mechanism it may involve when phytoplasma exposed to stress could be similar to other prokaryotes.

2. Materials and methods

2.1 Source of phytoplasmas

PNWB-phytoplasma-infected *Catharanthus roseus* (L.) G. Don (periwinkle) originally obtained by transmission through *Cuscuta australis* R. Broom (dodder) was provided by I. L. Yang (Taiwan Agricultural Research Institute, Wufang, Taiwan) and was maintained and propagated in periwinkle by side grafting (Shen and Lin, 1993; Ko and Lin, 1994).

2.2 Purification of phytoplasma DNA and genomic library construction

Total DNA was isolated from healthy and phytoplasma-infected plant tissues according to the method described by Ko and Lin (1994). To separate phytoplasma DNA, a CsCl-bisbenzimide density-gradient centrifugation method was applied (Ko and Lin, 1994). Approximately 250ng purified PNWB phytoplasma DNA was digested with the restriction enzyme *Eco*RI and ligated to 2 μg *Eco*RI-cleaved calf-intestine-phosphotase-dephosphorylated lambda ZapII cloning vecor, and then packaged with Gigapack II Gold packaging extract (Stratagene).

2.3 PCR

The phytoplasma is phylogenically closed to gram-positive bacteria and the nucleotide sequence of *dnaK* is highly conserved among most eubacteria. The amino acid sequences of seven *dnaK* gene of four gram positive bacteria, including

Bacillus subtilis (Bs), *Lactococcus lactis* (Ll), *Streptococuss pneumoniae* (Sp), *Clostridium acetobutylicum* (Ca); one gram negative bacteria *Escherichia coli* (Ec); and three mollicutes *Mycoplasma pneumoniae* (Mp), *Mycoplasma capricolum* (Mc), *Mycoplasma genitalium* (Mg) are aligned with CLUSTAL program (Higgins and Sharp, 1988). Three degenerate primers:

KF1: 5'-GAYYTWGGWACYACYAAYTC-3' (forward stream),

KF2: 5'-GCWGTTATTACHGTWCCTGC-3' (forward stream),

KR1: 5'-GWTAAWGGRGTWACRTCYAA-3' (reverse stream)

was designed based on the sequences of ATPase domain to amplify PNWB phytoplasma *dnaK* gene fragment.

The nested PCR method was used. First round PCR was subjected to 35 cycles at the following parameters: denaturation for 30s at 94 ℃, annealing for 60s at 42 ℃ and extension for 30s at 72 ℃, the total DNA from healthy or phytoplasma-infected plant tissues as temple, and KF1 and KR1 as primers in GeneAmp PCR System 2400 (Perkin Elmer, Foster City, CA). The first round PCR product was purified with QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany). The 25 cycles second round PCR used the purified PCR product as template, KF2 and KR1 as primers, and increased the annealing temperature to 50 ℃. The amplified PCR product was predicted to be an approximate 750 bp fragment. The PCR product was

purified, cloned directly into a TA cloning vector (pCR II, Invitrogen) and sequenced.

2.4 Genomic library screening

The amplified 750 bp *dnaK* gene fragments were random priming-labeled with digoxigenin-11-dUTP using DIG DNA labeling and detection kit (Boehringer Mannheim). The digoxigenin-labeled nucleic acid probes was then used to screen the phytoplasma genomic library. Overnight cultures of *Escherichia coli* XL1 Blue grown in LB supplemented with 0.2% maltose and 10mM $MgSO_4$ were harvested and infected with the recombinant phage (10^5 pfu. per plate of 150 mm in diameter) at 37 ℃ for 20 min. Infected cells were then plated on NZY plates (0.5% NaCl, 0.2% $MgSO_4$, 1.5% Bacto agar, pH 7.5) and incubated at 37℃ overnight. The plates were then overlaid with MagnaGraph nylon filter (Micron Separation). Filters were hybridized with digoxigenin-labeled probe following the instructions from Boehringer Mannheim. Plaques giving positive signals were isolated and subjected to secondary and tertiary screenings to insure plaque purification.

For an internal *EcoRI* restriction site in the amplified 750 bp *dnaK* gene fragment, no selected recombinant phagemid contained full-length *dnaK* gene. The 750bp fragment could be cut into one 200bp fragment and one 550bp fragment with *EcoRI.* For cloning complete sequences of *dnaK* gene, the 200 bp fragment also used as probe.

In vivo excision, the recombinant pBluescript SK(-) phagemid containing inserts were selected and excised from the lambda Zap II vector using the EsAssist helper phage system (Stratagene).

2.5 Southern blotting

For southern hybridization, total DNA from healthy periwinkle and PNWB-phytoplasma affected periwinkle were digested with the following restriction enzymes: BamHI, EcoRI and XabI (Boehringer Mannheim, Germany). Three micrograms of the digested DNA were electrophoresed in a 0.8 % agarose gel. After denaturation and neutralization, the DNA fragments were blotted onto a nylon membrane (Micron Separation) by capillary transfer. Nucleic acid probes used in Southern hybridization are the same as the probes used in genomic library screening. Hybridization was performed under low and high stringency at 55 C and 68 C, respectively (Chen and Lin, 1997; Ko and Lin, 1994).

2.6 DNA sequence analysis

Nucleotide sequences were performed with the BigDye Terminator Cycle Sequencing Kit (Perkin Elmer) using ABI PRISM 310 Genetic Analyzer (Perkin Elmer). The entire nucleotide sequence of the selected recombinant was read on both strands. The nucleotide sequences were aligned with the sequences in GeneBank/ EMBL database and the *Mycoplasma genitalium* Gemone Database

(http:// www.tigr.org/ tdb/ mdb/ mgdb/ mgdb. html) using program BLAST and FASTA. For further analysis, the nucleotide sequence was performed with computer program DNASTAR (DNASTAR), including finding ORF, codon usage analysis and secondary structure prediction. The nucleotide and amino acid sequences reported in this paper have submitted to the GeneBank/ EMBL database, and the accession number is: AF160726.

3. Result and Discussion

3.1 Amplify a specific dnaK gene fragment of PNWB-phytoplasma

After first round PCR, no significant fragment was obtained. Thus the second pair of primer were used for amplifying a phytoplsma specific gene fragment. After second round PCR, a specific PCR product (750 bp) was amplified using the purified and diluted first round PCR product as template. The result was shown in the Fig. 1, DNA templates prepared from PNWB-phytoplasma-infected periwinkle plants in first round PCR (lane 2), but no PCR product was obtained using DNA templates prepared from healthy periwinkle plants in first round PCR (lane 1). The 750 bp PCR product matched the expected size was then sequenced and aligned with other prokaryotic dnaK genes. The PCR fragment contains nucleotide sequences identical to highly conserved region, ATPase domain of dnaK gene, and more than 80 % identity of nucleotide sequences with other prokaryotic dnaK genes. This shows that the PCR fragment should be the dnaK gene fragment of PNWB-phytoplasma.

3.2 Cloning and nucleotide sequence analysis

After screening recombinant phages of the PNWB phytoplasma genomic library, five positive clones were obtained and confirmed by Southern hybridization analyses. All of these 5 clones contained a 2.7 kb insert and encodeded the same nucleotide

sequences, containing the *dnaK* gene 5' end. For cloning full length *dnaK* gene, the partial section of amplified PCR fragment, 200 bp, was used as probe for screening. Six positive clones were obtained and all contained a 2.3 kb insert. The nucleotide sequence of 2.3 kb insert was contained the 3' end of *dnaK* gene. The 2.7 kb fragment and the 2.3 kb fragment are combined to be a 5kb fragment containing full length *dnaK* gene. The complete nucleotide sequence of the two cloned fragments of the 4974 bp was determined (Fig. 2). Based on the universal codon usage, four putative ORFs were identified. The ORF1 contains 831 bp (nt 22-852), encodes a polypeptide of 276 a. a. with a calculated MW of 32.4 kD, the ORF2 contains 795 bp (nt 936-1730), encodes a polypeptide of 265 a. a. with a calculated MW of 30.5 kD, the ORF3 contains 1782 bp (nt 1723-3504), encodes a polypeptide of 594 a. a. with a calculated MW of 64.9 kD, and the ORF4 contains 1107 bp (nt 3700-4806), encodes a polypeptide of 369 a. a. with a calculated MW of 41.9 kD. All the four ORFs were starting from the ATG initiation codon and stopping at translation termination codon TAA. Four putative Shine-Dalgano sequence, which is complementary to the3' end sequence of 16S rRNA of phytoplasmas, were found in the upstream of ORFs, but no consensus –10 and –35 sequences were identified.

The base composition of the ORF1 is 36.70 mol% of A, 11.07 mol% of C, 12.39 mol% of G, and 39.83 mol% of T; the ORF2 is 43.52 mol% of A, 9.94 mol% of C,

13.96 mol% of G, and 32.58 mol% of T, the ORF3 is 37.43 mol% of A, 12.85 mol% of C, 18.07 mol% of G, and 31.65 mol% of T, and the ORF4 is 38.30 mol% of A, 11.92 mol% of C, 16.62 mol% of G, and 33.15 mol% of T. A low G+C content (in order of 23.47%, 23.90%, 30.92%, and 28.55%) of the ORFs, a preferential use of A- and T- rich codons, and the high frequency of the use of A or T residues at the 5'-end (1st base) (73.19%, 61.36%, 54.30%, 56.79%) and 3'-end (3rd base) (83.70%, 89.02%, 87.86%, 90.49%) of codons were observed, which are similar to the features of other phytoplasmas (Kirkpatrick et al., 1987; Lim and Sears, 1991; Sears et al., 1989). Except ORF1, AAA lysine codon appeared in other ORFs, which made it the most frequent codon. In the four ORFs, 15 tryptophan residues are encoded by UGG. The result agrees with that of a previous study on the ribosomal protein genes for *Oenothera* phytoplasma (Lim and Sears, 1991). In the class *Mollicutes*, mycoplasmas and spiroplasmas both utilize UGG and UGA triplets are tryptophan codons (Inamine et al., 1990; Lim and Sears, 1992), but *Acholeplasma laidlawii* utilizes UGG only (Tanaka et al., 1989; Lim and Sears, 1992).

3.3 Southern hybridization analysis

In Southern hybridization (shown in Fig. 3), total DNA of PNWB phytoplasma–infected and healthy (lane 1) periwinkle were digested with *Bam*HI (lane 2), *Eco*RI (lane 3) and *Xba*I (lane 4) and hybridized with the PCR amplified 750 bp *dnaK* gene

fragment. No positive signal was observed for the DNA prepared from healthy plants in the Southern hybridization under both low and high stringencies. According to the hybridization patterns, in *Bam*HI (lane 2) and *Xba*I (lane 4) digests, only one fragment, approx. 15.0 kb and 7.0 kb, respectively indicated that the PNWB phytoplasma has only one *dnaK* gene. Two bands, approx. 12.0 kb and 2.7 kb of hybridization signals were detected in *Eco*RI digested lane. The result was matched that there was an internal *Eco*RI site in *dnaK* gene of the PNWB phytoplasma.

3.4 Deduced protein sequence analysis and sequence similarities

The deduced amino acid sequences of the four ORFs were compared with sequences available in the EMBL and GeneBank. The ORF1 showed no significant identity with all known genes but the ORF2, ORF3 and ORF4 were found highly similar with *grpE*, *dnaK* and *dnaJ* genes of many eubacteria. The ORF3, and ORF4 were aligned with the *dnaK* and *dnaJ* genes of several bacteria in Fig. 4 and 5. The ORF3 shares 63% of nucleotide sequence identity and 55.3% of amino acid sequence identity with *dnaK* gene of *Mycoplasma genitalium.* It reveals the gene organization and the nucleotide sequence in conserved region of ORF3 is similar to other *dnaK* genes. The ORF3 contains ATPase domain and substrate binding domain. In addition, PNWB-phytoplasma DnaK has 23-aa deletion in the N-terminus region that is characteristic of gram-positive bacteria. The ORF4 shares 52% of nucleotide

sequence identity and 32% of amino acid sequence identity with *dnaJ* gene of *Mycoplasma genitalium.* The ORF4 contains four regions, J-domain and G/F motif are interacted with DnaK; Zu finger and C-terminal domain are substrate binding region. All of those regions are functional domain and responsible to most biological function of DnaK and DnaJ proteins, thus it suggests that this ORF3 and PRF4 encode DnaK and DnaJ of molecular chaperones of PNWB-phytoplasma.

The ORF2 shares 43.5% of nucleotide sequence identity and 37.8% of amino acid sequence identity with *grpE* gene of *Bacillus subtilis.* The secondary structure of the putative *grpE* gene was also predicted based on deduced amino acid sequence using the method developed by Garnier et al. (Garniel et al., 1978) (Fig. 6). Most of the *grpE* genes could be divided into three regions. Region 1 is unknown function. Region 2 is mainly composed of á helix, while region 3, the DnaK binding domain, is mainly composed of â sheet. The putative secondary structure of phytoplasma *grpE* gene is similar to other *grpE* genes, except á helical region 2.

3.5 Phytoplasma dnaK-dnaJ-grpE linkage in comparision with the chromosomal organization of dnaK-dnaJ-grpE genes in other bacteria

As mentioned above, the PNWB-phytoplasma has the chromosomal arrangement 5'-ORF1 (0.83 kb)-*grpE* (0.80 kb)-*dnaK* (1.72 kb)-*dnaJ* (1.11 kb)-3' (Fig. 7). The organization of these genes in PNWB-phytoplasma is similar to those in several

eubacteria, including *Bacillus subtilis*(Wetzstein et al., 1992), *Clostridium acetobutylicum*(Narberhaus et al., 1992) and *Mycoplasma capricolum*(Falah and Gupta, 1997). Notably, the chromosomal arrangement of these three genes was not conserved in all Mollicutes. In *Mycoplasma genitalium*(Fraser et al., 1995) and *Mycoplasma pneumoniae*(Himmelreich et al., 1996), those genes are located on separate transcription unit. In gram-positive bacteria such as *B. subtilis*, the four genes(*orfA*, *grpE*, *dnaK*, and *dnaJ*)(Wetzstein et al., 1992) and *C. acetobutylicum*, the seven genes (*orfA*, *grpE*, *dnaK*, *dnaJ*, *orfB*, *orfC*, and *orfD*) (Narberhaus et al., 1992) form an operon, whereas in the PNWB-phytoplasma no obvious promoter sequences were detected. In *B. subtilis* and *C. acetobutylicum* a transcription terminator is present immediately downstream of the *dnaK* gene and in the PNWB-phytoplasma the inverted repeat sequences is also found downstream of the *dnaK* gene (Fig. 2).

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Legends of Figures

Fig. 1. Nested-polymerase chain reaction (PCR)-amplified product with KF2/ KR1 primer pair using PCR-amplified products of total DNA of periwinkle infected with PNWB-phytoplasma with KF1/ KR1 primer pair as template. The DNA templates were extracted from: lane 1, healthy periwinkle; lane 2, periwinkle infected with PNWB-phytoplasma; lane 3, ddH_2O control. M, 1kb ladder as molecular weight standards. Size (in bp) of PCR product is shown on the right.

Fig. 2. Nucleotide sequence of the 2.3 kb and 2.7 kb insert DNA of the two selected recombinant plasmid combined into 5.0 kb long and predicted amino acid sequences of the open reading frame. Sequences of ribosome binding sites (RBS) are shown by boldface letters. The stop codon is indicated by asterisk. Inverted repeat sequences of the transcription terminator are indicated by arrows. The DNA sequences of primers KF1, KF2, and KR1 are indicated.

Fig. 3. Southern blot analysis of total DNA prepared from healthy perwinkle digested with *Eco*RI (lane 1) and diseased perwinkle affected with phytoplasma associated PWNB (lanes 2, 3, 4) digested with *Bam*HI (lane 2)、*Eco*RI (lane 3)、*Xba*I (lane 4).

Sizes (in kb) of the hybridization signals are shown on the right.

Fig. 4. Multiple amino acid sequence alignment of the putative *dna*K gene(PWB) and *dna*K gene in *Eacherichia coli* (Ec)、*Bacillus subtilis* (Bs)、*Lactococcus lactis* (Ll)、*Streptococuss pneumoniae* (Sp)、*Clostridium acetobutylicum* (Ca)、*Mycoplasma pneumoniae* (Mp)、*Mycoplasma capricolum* (Mc)、*Mycoplasma genitalium* (Mg) conducted by CLUSTAL sequence analysis program. Predicted functional domains are showed by arrows. Conserved amino acid resdues are labelled by asterisks and amino acid resdues of similar properties are labelled by dots.

Fig. 5. Multiple amino acid sequence alignment of the putative *dna*J gene(PWB) and *dna*J gene in *Escherichia coli* (Ec)、*Bacillus subtilis* (Bs)、*Lactococcus lactis* (Ll)、*Streptococuss pneumoniae* (Sp)、*Clostridium acetobutylicum* (Ca)、*Mycoplasma genitalium* (Mg) conducted by CLUSTAL sequence analysis program. Predicted functional domains are showed by arrows. Conserved amino acid resdues are labelled by asterisks and amino acid resdues of similar properties are labelled by dots.

Fig. 6. Secondary structure of grpE of PNWB phytoplasma predicted by Garnier-Robson method. A: α helix, B: β sheet, T: β turns, C: coiled region.