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A SOIL-DWELLING *Pseudomonas* SP.593 HARBORING AN EXOGENOUS *hrpZ* GENE ELICITS A TYPICAL HYPERSENSITIVE RESPONSE IN TOBACCO AND SOYBEAN LEAVES

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Abstract- *Pseudomonas* sp.593 is a soil-dwelling bacterium unable to elicit any hypersensitive response (HR) in tobacco or soybean. The *hrpZ* gene, encoding an abundant Type III secretion system (T3SS) dependent protein, was cloned from a phytopathogen *Pseudomonas syringae* pv. *syringae* Van Hall CFCC 1336. The HrpZ harpin expressed in *E. coli* was purified to homogeneity, and used to raise polyclonal anti-HrpZ rabbit serum. The cloned *hrpZ* gene was then introduced into the soil bacterium *Pseudomonas* sp.593 *via* transformation of the plasmid pMEK-hrpZ. Western blotting and HR assay showed that the *hrpZ*-transformed *Pseudomonas* sp.593 was not only able to secret HrpZ harpin but also elicited a strong HR reaction in tobacco and soybean just as *P. syringae* did. In addition, bacterial cells were able to grow and multiply in the HR zone. Our results demonstrate that an avirulent strain can become a virulent-like pathogen, or a pathogen strain can broaden its host range once a single exogenous *hrpZ* gene cloned from a phytopathogen is introduced into a bacterium displaying phylogenetic diversity.

Keywords- *Pseudomonas* sp.593, HrpZ harpin, Type III secretion system, Hypersensitive elicitation

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Introduction

A number of Gram-negative bacterial animal and plant pathogens (e.g. *Escherichia coli, Salmonella typhimurium, Salmonella enterica, Burkholderia mallei, Shigella flexneri, Yersinia spp, Pseudomonas syringae, Xanthomonas campestris, Ralstonia solanacearum* and *Erwinia amylovora*) depend on the type III secretion system (T3SS) for the full bacterial virulence [1-5]. The T3SS is thought to only express and assemble to appre-ciable levels after the bacterium directly contacts with a eukaryotic host or when the bacterium grows in the hrp-inducing minimal medium [6]. The T3SS pilus, a major part of the secretion ap-paratus, forms a direct conduit between the pathogen and its host and injects T3SS effector proteins directly from the bacterial cell into the cytosol of the host [7,8]. The major function of theeffector proteins, delivered into host cells by T3SS, is to suppress the defense response by interfering with signal transduction, which results in cytoskeletal changes or direct cytotoxic effects [9,10]. In resistant plants, some of those effector proteins secreted via T3SS induce the hypersensitive response (HR) characterized by local programmed cell death [11] The HrpZ harpin, an abundant T3SS-dependent protein, was isolated and identified as the product of the *hrpZ* gene in several *P. syringae* pathovars [12,13]. This protein is conserved in all the *P. syringae* patho-vars except in pv. *tabaci* [14], and shares some characteristics with other harpins (eg. HrpF, HrpK and HrpN) found in different Gram-negative phytopathogens, and is also partially homologous with HrpN [15-17]. It is heat-stable, glycine-rich, lacks cysteine and is secreted to the extracellular space, and is the sole *P. syringae* T3SS effector that can induce the host defense response outside host cells. The HrpZ harpin is abun-dantly secreted under T3SS-inducing conditions, and localized around the HrpA pili during the filament pilus extension and within the plant cell wall region [18].

The HrpZ protein is thought to bind to lipid bi-layers and form ion-conducting pores in the host cell membrane, but its function in pathogenesis is still conjectural. The *hrpZ* mutant has no detectable phenotype in virulence on host plants [19] or in defense induction on nonhost plants [20]. The secretory expression of the *hrpZ*psph gene in those transgenic plants is able to elicit hypersensitive cell death [21]. Several different activities associated with HrpZ protein *in vitro* suggested that it could be a multifunctional protein able to interact with several other molecules. For instance, HrpZ is able to insert into patricidal lipid bilayers and to form cation-permeable pores [19,22], harbor a binding site for peptides with a defined consensus sequence and bind proteins of host origin [16]; The peptide-binding activity of HrpZ was localized to a region separate from the region acting as an HR elicitor in tobacco and parsley [23]. The detailed study found that HrpZ forms dimers or higher order oligomers, and interacts only with phosphatidic acid among 15 different membrane lipids [24]. In addition, HrpZ oligomerization is mainly medi-ated by a region near the C-terminus of the protein, and the same region is also essential for membrane pore formation. Phosphatidic acid binding is mediated by two regions separate in the primary structure. The 24-amino-acid fragment residing in the region indispensable for the oligomerization and pore formation functions of HrpZ is responsible for HR elicitor function, and peptide-

binding is mediated by a long region near the N-terminus of the protein[24,25] have recently found that *P. syringae* naturally lacking one or more gene loci of the canonical T3SS are ubiquitous in non-agricultural habitats and phylogenetical diverse. It is inevitable to raise a question whether a nonagricultural habitat with phylogenetical diversity can become a virulent phytopathogen once it acquires the related gene from external environment via certain genetic mechanisms. In this study, the *hrp*Z gene, cloned from a phytopathogen *P. syringae pv. syringae* Van Hall CFCC 1336, was introduced into a soil-dwelling *Pseudomonas* sp. 593 unable to induce HR in tobacco and soybean. The *Pseudomonas* sp. 593 strain harboring an exogenous *hrp*Z gene effectively secreted HrpZ protein, and elicited a typical hypersensitive response in nonhost soybean and tobacco just as the phytopathogen *P. syringae* did. Potential biological significance is also discussed.

Materials and Methods

Strains, Plasmids, Primers and Chemicals

The strains, plasmids and primers used in this study are described in [Table-1]. All reagents for molecular manipulations and DNA marker were purchased from Takara. The DNA gel recovery kit was purchased from Axygen Scientific, Inc., and culture media, antibiotics, inorganic and organic reagents were purchased from Zhong Ke (Shanghai, China).

Table 1- Strains, plasmids and primers used in this study.

Strain, plasmid and primer	Characteristics	Source
Strains		
DH5a	*F-,φ80dlacZ⊿M15,⊿(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rk-,mk+), phoA, supE44, λ-, thi-1, gyrA96, relA1*	Invitrogen
BL21(DE3))pLysS	*F-, ompT, hsdSB(rB-, mB-), gal(λcI857, ind1, Sam7nin5, LacUV5-T7 gene1), dcm(DE3), pLysS*	Stratagene
Pseudomonas.sp 593	*amp r, cm r*	Lab collection
Pseudomonas syringae pv. syringae Van Hall CFCC 1336		CFCC*
Plasmids		
pMD18-T	*amp r, ColE1*	Takara
pET23a	*T7-promoter, amp r, his-Tag, T7-teminator*	Novagen
ptac85	*amp r, tac promoter*	Marsh P [39]
pTRG	*tet r, ColE1*	Agilent technologies
pMEKm12	*amp r, tac promoter, ColE1, ori1606*	Gift from Dr. Cheng
pMEK-hrpZ	*amp r, tet r, ColE1, ori1606, tac promoter*	This study
Primers		
hrpZ-F	5'-AGGATCATATGCAGAGTCTCAGTCTTAACA-3'	This study
hrpZ-R1	5'-AGTGTCGACCTATCAGGCCGCGGCCTGATT-3'	This study
hrpZ-R2	5'-AGTGAATTCCTATCAGGCCGCGGCCTGATT-3'	This study
hrpZGF	5'-KPCKOLMCTKWAOOAOTCGWA -3' ⋆	This study
hrpZGR	5'-TWCTWCAYCTTKAAYAGLTMWTTW -3' ⋆	This study
tet-F	5-AGTCCTGCAGGACCCGCAAGAATTGATTGGGC-3'	This study
tet-R	5'-AGTCAAGCTTGCTATTATCCGCTCACAATTCC-3'	This study
hrcC-F	5'- ggatccatgcgcaaggccttgatg-3'	This study
hrcC-R	5'- aagctttggctttgctcctcgga-3'	This study

Gene Cloning and Expression

Based on the *hrp*Z sequence of *P. syringae pv. syringae* B728a in Genbank (NC-007005.1), the forward and reverse primers hrpZ-F and hrpZ-R were designed and synthesized by Invitrogen, Shanghai. Total DNA of *P. syringae pv. syringae* Van Hall CFCC 1336 was extracted using standard protocol described in Molecular Cloning [26], and used as template. PCR was performed using the following cycle parameters: 5 min. at 94°C, followed by 30 cycles of 30 sec. at 94°C, 1.5 min. at 65°C, 1 min. at 72°C, and a final incubation at 72°C for 5 min. The amplified DNA fragments were separated through 1% agarose electrophoresis, and the desired DNA fragment (1kb) was recovered using DNA gel recovery kit. The recovered DNA fragment was ligated with pMD18-T vector by incubating the mixture at 16°C for 4Hrs., and then the ligation mixture was transformed directly into *E. coli* DH5a by using the $CaCl_2$ method [26]. The transformants ware selected using LB plates supplemented with 100mgml ampicillin. Positive colonies were confirmed by PCR using two primers hrpZ-F and hrpZ-R1, and the cloned *hrp*Z gene was sequenced by Shanghai Sangon Biotech.

The recombinant pMD18-T plasmid DNA containing the cloned *hrp*Z gene was digested with *Nde*I and *Sal*I, and then the *hrp*Z gene fragment was recovered using a DNA gel recovery kit. The *hrp*Z gene was inserted into the expression vector pET23a at the *Nde*I and *Sal*I sites to form recombinant plasmid pET23a-hrpZ. The pET23a-hrpZ plasmid was then transformed into *E. coli* BL21 (DE3) pLysS by using the $CaCl_2$ method [26]. Positive transformants were screened on LB plates containing 100mg/ml ampicillin. Expression of the *hrp*Z gene in *E. coli* BL21 (DE3) pLysS transformants growing at 25°C in LB broth supplemented with 100mg/ml ampicillin was induced by adding IPTG to a final concentration of 0.5mM for 6Hrs. Bacterial cells were harvested by centrifugation at 4,000 rpm at 4° C, the cell pellet was suspended in 50mM phosphate buffer (pH7.4), and the crude extract was finally prepared *via* centrifugation at 12,000 rpm at 4°C after sonication.

Protein Purification

Since the HrpZ protein contains a 6´His-tag at its C-terminus, Ni-affinity chromatography was employed for purification. The crude extract was clarified by centrifugation at 12,000 rpm for 15 min. at 4°C. The Ni-column was equilibrated with 50mM phosphate buffer (pH7.4), and then washed and gradient eluted with 50mM phosphate buffer (pH7.4) containing 0-200mM imidazole after the protein sample was loaded. Protein concentration was estimated by measuring the absorbance at a wavelength of 280 nm and calculated by using its extinction coefficient of 5500 $mol.L^{-1}$ cm^{-1}. Protein purity was routinely monitored by SDS-PAGE, after staining with Coomassie blue R250. The HrpZ protein was purified to homogeneity, and 25mg pure protein was obtained from 1 L of bacterial culture through one step of purification. The biological activity of the puri-

fied HrpZ was confirmed by HR assay.

Polyclonal Antibody Preparation

Polyclonal antibody was generated to the HrpZ harpin based on the method described by Vinogradov, et al. [27] and He, et al. [28]. The HrpZ protein clarified by centrifugation at 12,000 rpm at 4°C for 30 min., and then 0.5mg of the clarified protein was mixed with CFA (complete Freund's adjuvant) (1:1 v/v). Anti-HrpZ serum was raised in rabbits by i.h injection of 100 ml of the protein-CFA mixture each time. After 6 injections, three rabbits were sacrificed and their sera were collected. Antibody titer for anti-HrpZ serum was monitored by ELISA according to the method described by Pantophlet, et al. [29].

Plasmid Construction and Transformation

Construction of the plasmid pMEK-hrpZ is shown in [Fig-1]. The soil bacterium *Pseudomonas* sp. 593 is capable of growing in a medium containing low dosage of kanamycin (~30mg/ml). To obtain positive transformants easily, the *tet*^{r} fragment (1.5kb) with a promoter was first amplified by PCR using tet-F and tet-R as a primer pair and pTRG plasmid DNA as template, and then inserted into pMEKm12 at *pst*I and *Hind*III sites to form a recombinant plasmid pMEKm12-tet^r. The activity of pMEKm12-tet^r against tetracycline was examined by transforming the plasmid into *E. coli* DH5a and growing the bacterium in on an LB plate containing 30mg/ml tetracycline. Then the *hrp*Z gene (1kb), cloned from *P. syringae pv. syringae* Van Hall CFCC 1336 by using the primers pcs-F and pcs-R2, was inserted into the plasmid pMEKm12-tet^r at *Nde*I and *EcoR*I sites to generate a recombinant plasmid named as pMEK-hrpZ. All molecular manipulations were done according to standard protocols [26].

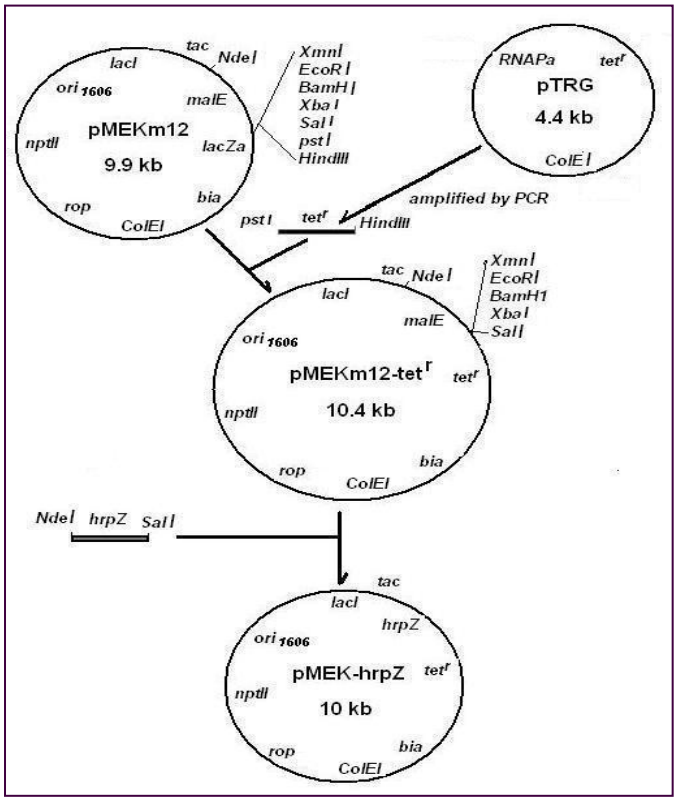

Fig. 1- Construction of the expression vector pMEK-hrpZ

Transformation of *Pseudomonas* sp. 593 was performed according to the methods described by Solaiman [30]. Bacterial cells grown in LB broth to logarithmic phase (OD_{600}=0.6) were collected and pre-cooled on ice for 30 min., and then washed 3 times by centrifugation at 4°C with pre-cooled 100 ml 300mM sucrose. The competent cells were re-suspended in 2 ml 300mM sucrose, and stored at -80° C. The plasmid pMEK-hrpZ was transformed into the strain *Pseudomonas* sp. 593 by electroporation (Electroporator 2510, Eppendorf). Positive colonies were screened by spreading the transformed cells on LB plates supplemented with 30mg/ml tetracycline and incubating at 37°C overnight.

Secretion Test and Immunoblot Analysis

The strain *Pseudomonas* sp. 593 and the transformant containing pMEK-hrpZ were grown to late-exponential phase in 25 ml hrp-inducing minimal medium (50mM potassium phosphate, 7.6mM $(NH_4)_2S0_4$, 1.7mM $MgCl_2$, 1.7mM NaC1, 10 mM fructose and mannitol [14] and induced by adding 0.5mM IPTG at 37°C. Bacterial cultures were centrifuged at 6,000 rpm at 4°C for 15 min., the pellets were resuspended in 50mM phosphate buffer (pH7.4) and washed once with the same buffer to remove any medium residue. Bacterial cells were broken by sonication, and crude extracts were prepared as described above. Bacterial medium solutions obtained from the first centrifugation were centrifuged again at 12,000 rpm at 4°C for 20 min. in order to remove any bacterial residue. The proteins in the supernant were precipitated with 5% trichloroacetic acid, washed with acetone and dissolved in 5mM $MgCl_2$ as described by Preston, et al. [31]. Protein concentration was determined using the Bradford method [32].Cytoplasmic and membrane proteins were prepared according to the methods reported previously [26,33]. All proteins were separated by SDS-PAGE, and then transferred to Immobilon-P transfer membranes (Millipore) according to the standard procedure [26].

The HrpZ harpin or HrcC protein was recognized with rabbit polyclonal antisera raised to the purified HrpZ or HrcC encoded by *hrp*Z or *hrc*C gene of *P. syringae pv. syringae* Van Hall CFCC 1336. Goat-anti-rabbit IgG alkaline phosphate conjugate (Sigma) was used as the secondary antibody. Membrane-bound secondary antibodies were visualized with BCIP/NBT tablets (Sigma).

HR assay

Based on the method reported previously [13,34], tobacco (*Nicotiana tabacum*) and soybean (*Glycine max*) plants were grown to the 6-8 leaf stage in a climatic chamber at 25°C with a 14Hrs. photoperiod and 70-90% humidity. The purified HrpZ protein, the *Pseudomonas* sp. 593 cells transformed with the plasmid pMEK-hrpZ, and the mixture of the HrpZ protein and *Pseudomonas* sp. 593 cells were respectively infiltrated onto the abaxial surfaces of plant leaves using a No. 5 latex stopper with a hole designed to fit tightly around a 0.5ml syringe, thus permitting thumb pressure on the other side of the leaf to provide a tight seal. *Pseudomonas* sp. 593 cells and 5mM $MgCl_2$ were used as control. In each inoculum, 5mg HrpZ protein or $3´10^6$ bacterial cells dissolved or suspended in 10 ml 5mM $MgCl_2$ was applied. All bacterial cells were harvested by centrifugation, washed twice with 5mM $MgCl_2$ and suspended in 5mM $MgCl_2$ after growth in LB broth containing 100mgml ampicillin to an OD_{600} value of 0.2. The plants were observed over 3 days at 25°C for development of hypersensitive response.

Bacterial Counting

Each inoculum zone of the plant leaves was scooped out with a sterile punch within 3 days postinoculation, and then suspended in 1 ml PBS (pH7.2). Bacterial cells were released into the solution by

shaking on a shaker at room temperature for 20 min. A serial 10´dilution was performed with PBS (pH7.2), and the standard plate count method was employed to count bacteria. LB plates containing 100mg/ml ampicillin were used for the bacterial cells from the spots of control groups (eg. HrpZ protein, 5mM $MgCl_2$ and *Pseudomonas* sp. 593), and the LB plates containing 100mg/ml ampicillin and 30mg/ml tetracycline for *Pseudomonas*.sp *593* transformed with the plasmid pMEK-hrpZ. Colony-forming units (CFUs) were counted from triplicates of each dilution, and the number of bacterial cells per milliliter was calculated according to the following formula. Number of bacteria/ml = (number of colonies (CFU) / (dilution´amount plated)

Results

The Product of the *hrpZ* Gene Cloned from *P. syringae* pv. *syringae* Van Hall CFCC 1336 Functions as a HrpZ Harpin

A 1047 bp DNA segment was amplified *via* PCR in which hrpZ-F, hrpZ-R and the genomic DNA of *Pseudomonas syringae* pv. *syringae* Van Hall CFCC 1336 were respectively used as primers and template, and the program TRANSLATE (www. expasy.org) gave it a 1026 bp ORF encoding 341 amino acids [Fig-2].

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atgcagagtctcagtcttaacagcagctcgctgcaaaccccggcaatggcccttgtcctg
 M  Q  S  L  S  L  N  S  S  S  L  Q  T  P  A  M  A  L  V  L
gtacgtcctgaagccgagacgactggcagtacgtcgagcaaggcgcttcaggaagttgtc
 V  R  P  E  A  E  T  T  G  S  T  S  S  K  A  L  Q  E  V  V
gtgaagctggccgaggaactgatgcgcaatggtcaactcgacgacagctcgccattgggc
 V  K  L  A  E  E  L  M  R  N  G  Q  L  D  D  S  S  P  L  G
aaactgctggccaagtcgatggccgcggatggcaaggcgggcggcggtatcgaggatgtc
 K  L  L  A  K  S  M  A  A  D  G  K  A  G  G  G  I  E  D  V
atcgctgcgctggacaagctgatccatgaaaagctcggtgacaacttcggcgcgtctgcg
 I  A  A  L  D  K  L  I  H  E  K  L  G  D  N  F  G  A  S  A
gacagcgcctcgggtaccggacagcaggacctgatgactcaggtgctcaatggcctggcc
 D  S  A  S  G  T  G  Q  Q  D  L  M  T  Q  V  L  N  G  L  A
aagtcgatgctcgatgatcttctgaccaagcaggatggcgggacaagcttctccgaagac
 K  S  M  L  D  D  L  L  T  K  Q  D  G  G  T  S  F  S  E  D
gatatgccgatgctaaacaagatcgcgcagttcatggatgacaatcccgcacagtttccc
 D  M  P  M  L  N  K  I  A  Q  F  M  D  D  N  P  A  Q  F  P
aagccggactctggctcctgggtgaacgaactcaaggaagacaacttccttgatggcgac
 K  P  D  S  G  S  W  V  N  E  L  K  E  D  N  F  L  D  G  D
gaaacggctgcgttccgttcggcactcgacatcattggccagcaactgggtaatcagcag
 E  T  A  A  F  R  S  A  L  D  I  I  G  Q  Q  L  G  N  Q  Q
agtgacgctggcagtctggcagggacgggtgaggtctgggcactccgagcagttttttcc
 S  D  A  G  S  L  A  G  T  G  G  G  L  G  T  P  S  S  F  S
aacaactcgtccgtgatgggtgatccgctgatcgacgccaataccggtcccggtgacagc
 N  N  S  S  V  M  G  D  P  L  I  D  A  N  T  G  P  G  D  S
ggcaataccgtggtgaggcgggcaactgatcggcgagcttatcgaccgtggcctgcaa
 G  N  T  R  G  E  A  G  Q  L  I  G  E  L  I  D  R  G  L  Q
tcggtattggccggtggtggactgggcacacccgtaaacaccccgcagaccggtacctcg
 S  V  L  A  G  G  G  L  G  T  P  V  N  T  P  Q  T  G  T  S
gcgaatggcggacagtccgctcaggatcttgatcagttgctgggcggcttgctgctcaag
 A  N  G  G  Q  S  A  Q  D  L  D  Q  L  L  G  G  L  L  L  K
ggcctggaggcaacgctcaaggatgccgggcaaacaggcaccgacgtgcagtcgagcgct
 G  L  E  A  T  L  K  D  A  G  Q  T  G  T  D  V  Q  S  S  A
gcgcaaatcgccaccttgctggtcagtacgctgctgcaaggcagccgcaatcaggccgcg
 A  Q  I  A  T  L  L  V  S  T  L  L  Q  G  S  R  N  Q  A  A
gcctga
 A  -
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Fig. 2- DNA sequence of the *hrpZ* gene cloned from *Pseudomonas syringae pv.syringae* Van Hall CFCC 1336 and the deduced amino acid sequence

Theoretical calculation gave the deduced protein an apparent molecular weight of 34.7 kD with an isoelectric point of 4.2. Amino acid sequence comparison revealed that the deduced protein shows strong homology to the HrpZ of *Pseudomonas syringae pv. syringae* (ABQ883651) with an identity of 99.71%. Only one amino acid is different at the position 335, where a threonine residue (Thr) in the HrpZ of *Ps syringae pv. syringae* is replaced by a serine residue (Ser) in the deduced protein. Based on the method of Kyte and Doolittle [35], hydropathic calculation for the deduced protein gave a grand average of hydropathicity of -0.247, indicating that this protein is hydrophilic. Analysis of subcellular localization by PSORTb (www.psort.org) predicted that it is an extracellular protein with a localization score of 10. All parameters obtained from the deduced protein are consistent with those for HrpZ harpins encoded by the *hrpZ* genes of *P. syringae pv. syringae* in Genbank, suggesting that the ORF cloned from the genomic DNA of *P. syringae pv. syringae* Van Hall CFCC 1336 appears to encode a HrpZ harpin. The cloned *hrpZ* gene was inserted into an expression vector pET23a to form a recombinant plasmid pET23a-hrpZ, and its expression was controlled under a T7 promoter. The pET23a-hrpZ plasmid was then introduced into *E. coli* BL21 (DE3) pLysS. The *hrpZ* gene in *E. coli* was expressed at 25°C for 6 Hrs. and induced by adding 0.5mM IPTG. [Fig-3a] shows an abundant HrpZ expression as a soluble form in *E. coli*. Since a 6´his-tag was fused at the C-terminus, the HrpZ protein was purified by using Ni-affinity chromatography. The protein was finally purified to homogeneity [Fig-3b].

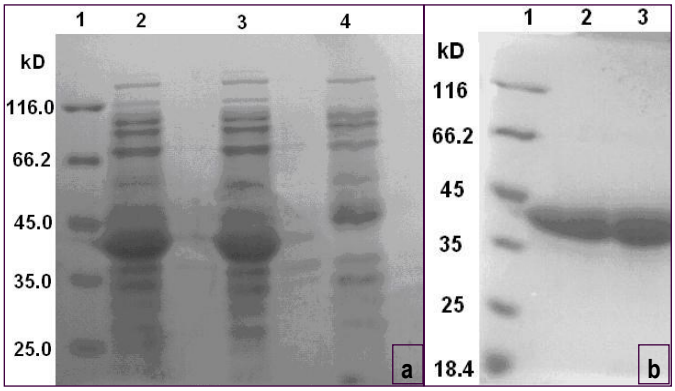

Fig. 3- 10% SDS-PAGE gels showing the expression product of the *hrpZ* gene cloned from *Pseudomonas syringae pv.syringae* Van Hall CFCC 1336 and protein purification. (a) the product of the *hrpZ* gene expressed in *E. coli*. Lane 1: protein standard; lane.2 and 3: the crude extracts obtained from two positive transformants; and lane.4: the crude extract of the transformant containing pET23a plasmid as control. (b) the HrpZ protein purified by Ni-affinity chromatography. Lane.1: protein standard; lane 2 and 3: overloaded pure HrpZ protein

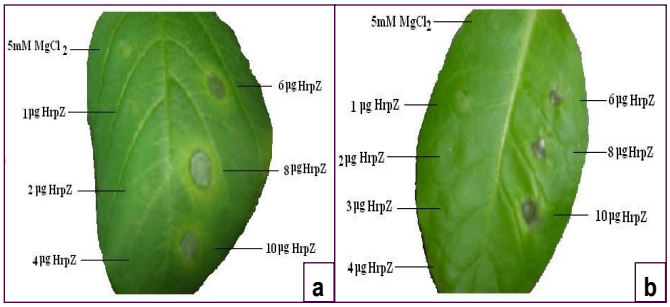

Fig. 4- Tobacco and soybean leaves infiltrated with different concentrations of the purified HrpZ. (a) soybean leaf, and (b) tobacco leaf. Each experiment was repeated three times by using three different leaves under the same condition. 10ml sample was applied in each inoculum, and the results were observed at 3 days postinoculation.

To verify whether the purified protein was the product of the *hrp*Z gene, the purified protein dissolved in 5mM $MgCl_2$ was infiltrated into both tobacco and soybean leaves to elicit HR. As shown in [Fig -4], hypersensitive confluent necrosis was noticeably observed when more than 4mg HrpZ protein was used, and enhanced with the increase of protein concentrations. HR elicitation by the purified protein at both tobacco and soybean leaves demonstrates again that the *hrp*Z gene cloned from *P. syringae* pv. *syringae* Van Hall CFCC 1336 indeed functions as a HrpZ harpin.

Soil Bacterium *Pseudomonas* sp. 593 does not Elicit Hypersensitive Responses in Tobacco and Soybean Leaves

Pseudomonas sp. 593 was previously isolated from soil for other research purposes in our laboratory [36,37]. Sequence analysis of 16S rRNA gene revealed that this soil bacterium belongs to *Pseudomonas* genus, but further classification has not been done. BLAST showed 99% homology among the 16S rRNA gene sequences of *Pseudomonas* sp. 593, *P. nitroreducens* IAM 1439 and *P. knackmussii* B13. In contrast, only less than 95% homology is exhibited between 16S rRNA gene sequences of *Pseudomonas* sp. 593 and *P. syringae*, indicating that the two bacteria may perhaps have a more distant phylogeneic relationship. Unlike *P. syringae pv. syringae*, *Pseudomonas* sp. 593 did not infect tobacco and soybean or elicit any hypersensitive response of two plants when its cells were inoculated to tobacco or soybean leaves [Fig-5].

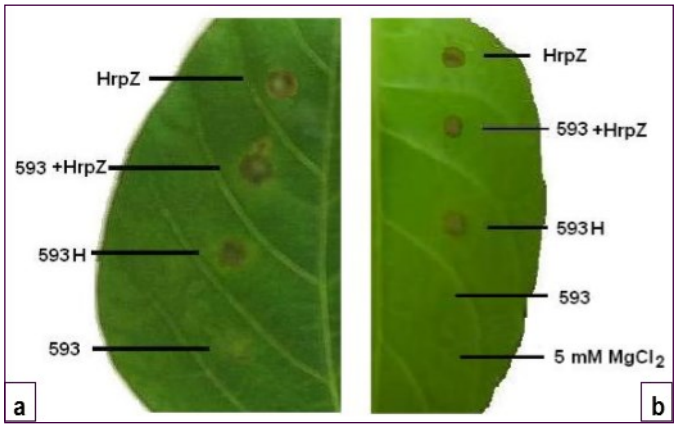

Fig. 5- HR elicitation of tobacco and soybean leaves by the HrpZ harpin and bacterial cells at 3 days postinoculation. (a) soybean leaf, and (b) tobacco leaf. 10ml sample was applied in each inoculum, protein amount was 5mg, and bacterial cells were 10^6. Each experiment was repeated three times by using three different leaves under the same condition. The results were observed at 3 days postinoculation. HrpZ: the purified HrpZ protein; 593: *Pseudomonas.*sp *593*; 593+HrpZ: *Pseudomonas.*sp *593* cells mixed with the HrpZ protein; 593H: *Pseudomonas.*sp *593*H cells.

Obviously, *Pseudomonas* sp. 593 was a HR^- strain incapable of inducing a hypersensitive reaction in tobacco and soybean, implying that it might lack at least one gene in the canonical hrp/hrc locus or the associated conserved effector locus because those gene loci are necessary for function of T3SS. HrpZ is one of those important virulence factors in T3SS. Hence, hrpZ-F and hrpZ-R specific for cloning the *hrp*Z gene of *P. syringae pv. syringae* were used as a primer pair, and PCR was performed to amplify the *hrp*Z gene from the genomic DNA of *Pseudomonas* sp. 593. No DNA band was observed on 0.8% agarose gel (data not shown). The degenerate primers hrpZGF and hrpZGR, designed based on the conserved amino acid sequences of HrpZ proteins of *Pseudomonas* species in the database, were also used in PCR. Different from the control strain *P. syringae pv. syringae* Van Hall CFCC 1336, no DNA band was detected in *Pseudomonas* sp. 593 (data not shown). To further examine whether the soil bacterium has HrpZ protein, we used the polyclonal antibody against the HrpZ protein to hybridize with both cytoplamic and secretory proteins of *Pseudomonas* sp. 593. Western blotting did not show any hybridization signal [Fig-6a]. All those results suggest that the soil bacterium *Pseudomonas* sp. 593 may not have a *hrp*Z gene such as existing in many *Pseudomonas* plant pathogens. We also investigated if *Pseudomonas* sp. 593 contains HrcC protein which is a major component of T3SS in bacterial membrane. Hybridization between the membrane extract of the soil bacterium and the polyclonal antibody against the HrcC protein of *P. syringae pv. syringae* Van Hall CFCC 1336 gave a strong band in a nitrocellulose blotting membrane [Fig-6b]. When the primers hrcC-F and hrcC-R specific for cloning *hrc*C gene of *P. syringae pv. syringae* Van Hall CFCC 1336 were used, *hrc*C gene was sucessfuly amplified by PCR from the genomic DNA of *Pseudomonas* sp. 593, and its corresponding amino acid sequence shared 99% homology with that for *P. syringae pv. syringae* Van Hall CFCC 1336 (data not shown). These results imply that the soil bacterium *Pseudomonas* sp. 593 may perhaps hold a T3SS.

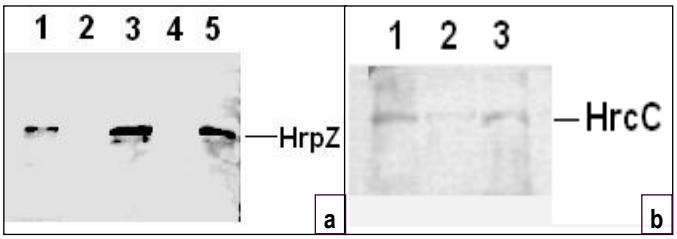

Fig. 6- Western blot analysis. (a) hybridization of the anti-HrpZ polyclonal rabbit serum with the proteins prepared separately from bacterial cells and the cell-free culture. Lane 1: the purified HrpZ; lane 2: the crude extract of *Pseudomonas.*sp *593* cells; lane 3: the crude extract of *Pseudomonas.*sp *593*H cells; lane 4: the cell-free culture in which *Pseudomonas.*sp *593* grew; lane 5: the cell-free culture in which the *Pseudomonas.*sp *593*H grew. (b) hybridization of the anti-HrcC polyclonal rabbit serum to the membrane proteins extracted from *Pseudomonas.*sp *593* strain. Lane 1: the purified HrcC; lane 2-3: the outer membrane extract of *Pseudomonas.*sp *593*. The amount of protein loaded in lane 2 was 60% less than that in lane 3

Soil Bacterium *Pseudomonas* sp. 593 Transformants Containing Exogenous *hrpZ* Gene are Capable of Secreting HrpZ harpin

Given that our speculation described above was correct, *Pseudomonas* sp. 593 containing an exogenous *hrp*Z gene could use its type III pathway to secrete HrpZ harpin from cytoplasm to the medium. We inserted the *hrp*Z gene cloned from *P. syringae* pv. *syringae* Van Hall CFCC 1336 into a expression vector pMEKm12-tet^r to form a recombinant plasmid pMEK-hrpZ as shown in [Fig-1].

The recombinant plasmid was then introduced into *Pseudomonas* sp. 593 cells, and positive colonies were isolated and confirmed by PCR. The positive transformant containing pMEK-hrpZ was named as *Pseudomonas* sp. 593H. A single colony of *Pseudomonas* sp. 593H was inoculated into the hrp-inducing minimal medium supplemented with 0.5% choline, and then incubated at 37°C. The expression of *hrp*Z gene was induced by adding IPTG to a final concentration of 0.5mM. After 12 Hrs., bacterial cells and the medium were separated by centrifugation, and then the cytoplasmic and secreted

proteins were respectively prepared from the cell pellet and the cell-free medium. Finally, the cytoplasmic and secreted proteins wee separately hybridized with the anti-HrpZ polyclonal antibody. As shown in [Fig-6a], both proteins, prepared from the cytoplasmic extract and the cell-free medium of positive transformants, gave a strong band, but those extracted from the control strain *Pseudomonas* sp. 593 did not give any signal. This result clearly demonstrates that *Pseudomonas* sp. 593H harboring the exogenous *hrpZ* gene is able to secret HrpZ protein effectively.

Soil Bacterium *Pseudomonas* sp. 593 Transformant Harboring an Exogenous *hrpZ* Gene is Capable of Eliciting Hypersensitive Responses

Effective secretion of HrpZ harpin implies that *Pseudomonas* sp. 593H could have an ability to infect plants. *Pseudomonas* sp. 593H was grown in the hrp-inducing minimal medium containing 0.5% choline and 0.5mM IPTG at 37°C for 12Hrs. Bacterial cells were collected by centrifugation, and then resuspended in 5mM $MgCl_2$. The suspended cells ware immediately inoculated onto the surface of both tobacco and soybean leaves. Meanwhile, *Pseudomonas* sp. 593, *Pseudomonas* sp. 593 transformed with pMEKm12-tet^r, *Pseudomonas* sp. 593 plus the purified HrpZ protein, the purified HrpZ protein alone, and 5mM $MgCl_2$ were used as controls. [Fig-5] shows all results observed in 3 days postinoculation of tobacco and soybean leaves. As expected, *Pseudomonas* sp. 593 ($3´10^6$ cells), and 5mM $MgCl_2$ did not display any activity of HR elicitation. The *Pseudomonas* sp. 593 transformed with pMEKm12-tet^r ($3´10^6$ cells) did not show any visible HR elicitation (data not shown). The purified HrpZ protein (5mg), *Pseudomonas* sp. 593 ($3´10^6$ cells) mixed with the HrpZ (5 mg) clearly showed HR elicitation. The HR symptom, caused by *Pseudomonas* sp. 593 mixed with the HrpZ, could result from the HrpZ harpin, because *Pseudomonas* sp. 593 alone did not induce any HR reaction. Interestingly, the *Pseudomonas* sp. 593H ($3´10^6$ cells) elicited a typical HR in both tobacco and soybean leaves of inoculum [Fig-5]. This result again verifies that the soil bacterium *Pseudomonas* sp. 593H is able to secret HrpZ harpin. The HR specificity of *Pseudomonas* sp. 593H clearly comes from the HrpZ harpin.

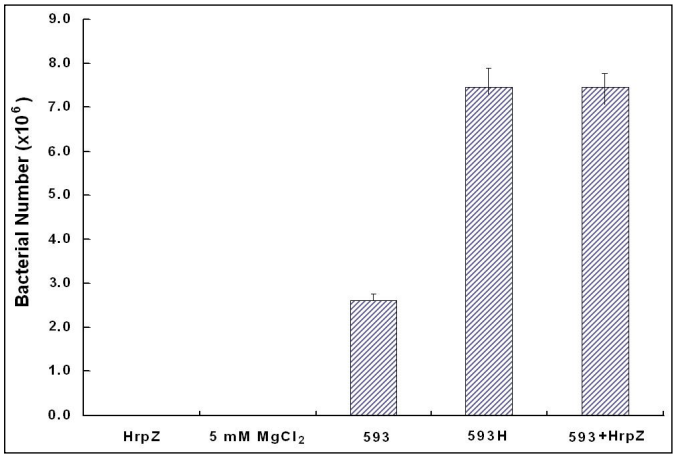

Fig. 7- Bacterial numbers counting from inoculum zones of tobacco leaves as shown in [Fig-5]. The standard plate count method was used as described in experimental section. Bacterial cells were collected within 3 days postinoculation. All data were calculated from three independent experiments. HrpZ: the purified HrpZ protein; 593: *Pseudomonas.*sp *593*; 593+HrpZ: *Pseudomonas.*sp *593* mixed with the HrpZ protein; 593H: *Pseudomonas.*sp *593*H.

Bacterial survival and multiplication in HR zones were also investigated *via* viable bacterial cell count, and the result was summarized in [Fig-7]. As expected, no viable bacterial cell was detected in those regions infiltrated with the solvent (5 M $MgCl_2$) or the hrpZ protein. The zone infiltrated with *Pseudomonas* sp. 593 gave 2.6 $´10^6$ cells, similar to the bacterial amount ($3´10^6$ cells) used initially for infiltration. This soil bacterium could survive on the surface of tobacco leaves in 3 days postinoculation although it did not elicit any HR reaction. In the zones infiltrated with *Pseudomonas* sp. 593 plus the HrpZ protein or *Pseudomonas* sp. 593, viable bacterial count gave $7.5´10^6$ cells. Obviously, bacterial numbers in both cases increased 2.5 fold as compared with that for initial infiltration, suggesting that the bacterial cells not only survived but also multiplied slowly in the HR zones. Bacterial multiplication indicated that bacterial cells could utilize the nutrition released from the cation-permeable pores of plant cells [19,22] after tobacco leaves were infiltrated with either the purified HrpZ or the HrpZ secreted by *Pseudomonas* sp. 593H.

Discussion

The strain *Pseudomonas* sp. 593 was isolated from soil in our laboratory. BLAST search (http://blast.ncbi.nlm.nih.gov) revealed that approximate 1501 bp DNA sequence of *Pseudomonas* sp. 593 16S rRNA gene was ³98% identical to those of *Pseudomonas nitroreducens* strain IAM 1439 (99%), *Pseudomonas knackmussii* strain B13 (99%), *Pseudomonas citronellolis* strain DSM 50332 (98%), *Pseudomonas delhiensis* strain RLD-1 (98%) and *Pseudomonas jinjuensis* strain Pss 26 (98%). *P. syringae pv. syringae* did not appear in the 95% similarity list of 16S rRNA gene sequences. Based on sequence analysis of 16S rRNA gene, *Pseudomonas* sp. 593 and *P. syringae pv. syringae* belong to the same *Pseudomonas* genus but may be different species. Strong hybridization signal between the membrane proteins of *Pseudomonas* sp. 593 and the anti-HrcC polyclonal antibody and effective secretion of HrpZ harpin by *Pseudomonas* sp. 593H illustrate that this soil bacterium man hold an intact type III secretory apparatus in its cell membrane, because the HrcC protein is a major component of type III secretory apparatus. Without HrcC in outer embrane, the *hrc*C mutant accumulated the HrpZ in periplasmic space as a membrane-bound protein although the mutant expressed HrpZ protein as effectively as the wild type [20].

Thus, we postulated that *Pseudomonas* sp. 593 harboring the plasmid pMEK-hrpZ could elicit plant HR reaction. Our results demonstrate that *Pseudomonas* sp. 593H indeed elicits a typical HR in both tobacco and soybean foliage. Recently, Morris's group [25] has reported that *P. syringae* naturally lacking the canonical type III secretion system are ubiquitous in nonagricultural habitats, and display phylogenetic diversity. In the natural environment, gene transfer from a bacterial strain to another may happen *via* different genetic mechanisms, such as bacterial conjugation, DNA transformation or transfection in intraspecies of *P. syringae*. It is also possible that one or more gene transfers could happen in interspecies of *Pseudomonas* via certain genetic mechanisms. Once a *hrpZ*$^-$ soil bacterium holding a T3SS acquires a *hrpZ* gene from external environments, it can become a virulent pathogen. Even though *Pseudomonas* sp. 593 does not elicit any HR reaction in tobacco and soybean, we can not rule out the possibility that it is a pathogen able to infect other plants yet, because some *P. syringae* strains lacking any one gene of the canonical T3SS still cause severe symptoms [25]. When *Pseudomonas* sp. 593 unable to infect tobac-

co and soybean acquired the *hrp*Z gene of *P. syringae pv. syringae*, it not only expressed and secreted HrpZ harpin but also induced a typical HR reaction in tobacco and soybean. These results demonstrate that an avirulent strain can become a virulent-like pathogen, or a pathogen strain can broaden its host range once it obtains *hrp*Z gene from external environments. Nevertheless, further investigation specific for gene transfer in intraspecies and interspecies of bacteria in nature is still required. HrpZ harpin is thought to be very useful in agriculture for inducing plant resistance against various viral or bacterial phytopathogens [38]. Pavli, et al. [38] have recently reported that the HrpZ enhanced resistance to Rhizomaania disease in transgenic tobacco and sugar beet, which suggests that phytobacterial harpins may now offer an opportunity for generating broad-spectrum resistance in plants. Nevertheless, potential health harm caused by transgenic plants has caused public concern. Successful expression and secretion of HrpZ harpin by the soil-dwelling *Pseudomonas* sp. 593 harboring a *hrp*Z gene may perhaps provide another insight into enhancing plant resistance against phytopathogens using the phytobacterial harpins. The avirulent soil bacterium harboring a harpin gene is sowed around plant roots, and then plant resistance will be induced *via* infiltration of the harpin secreted by the avirulent soil bacterium into plant roots. The correlative studies are in progress in our laboratory.

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