International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 14 (4), pp. 001-007, April, 2020. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Transposon mutant of *Vigna radiata*-nodulating *Bradyrhizobium* sp. impaired in both resistance to stress conditions and symbiotic performance

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Accepted 16 September, 2019

Rhizobial strains that nodulate *Vigna radiata* (mungbean) were examined for their resistance to several stress conditions. One strain of mungbean rhizobia was selected to construct a collection of transposon mutants based on resistance to high temperature (40°C), alkalinity (pH 8.0) and several heavy metals. One mutant that is deficient in resistance to high temperature, alkalinity and zinc was selected to estimate the relation between resistance to stress conditions and symbiotic performance. Southern hybridization analysis showed a single transposon insertion in the mutant strain. The impaired symbiotic performance was observed in the host plants inoculated with the mutant strain as compared with the wild-type strain. This result suggested that genes involved in stress resistance affected the symbiotic ability. The 16S rDNA sequence analysis revealed that the strain is a member of *Bradyrhizobium* with 98% identity.

Key words: *Bradyrhizobium*, mutant, transposon, stress tolerance, symbiosis.

INTRODUCTION

Nodulation and the subsequent nitrogen-fixing symbioses in leguminous plants are responses to infection by specific groups of bacteria, collectively called “rhizobia”. These bacteria are of enormous agricultural and eco-nomic value with an emphasis on the nitrogen supply to the host plants. “Symbiotic performance”, referred to the ability of rhizobia to nodulate plants and fix nitrogen, is determined by both plant and bacterial genes. In general, bacterial genes involved in symbiotic performance are divided into two categories including nitrogen-fixation genes and nodulation genes. Nitrogen fixation, that is a reduction of nitrogen gas into ammonia, is catalyzed by nitrogenase. Nitrogenase is a complex enzyme consisting of two components. The ∝ and β apo-subunits of component I are encoded by *nif*D and *nif*K, respectively; whereas component II is encoded by *nif*H. Full assembly of nitrogenase requires the products of other *nif* genes (Kaminski et al., 1998). In addition to *nif* genes, *fix* genes which are essential for the regulation of symbiotic nitrogen fixation have been characterized (David et al., 1988; Batut et al., 1989). Nodulation genes are defined as rhizobial genes which play a role in nodulation or which are coordinately regulated with such genes. In chronological order of discovery, they are designated as *nod*, *nol* and *noe* genes (Schlaman et al., 1998). DNA sequence analysis and complementation studies have revealed that some nodulation genes are conserved in all rhizobia, whereas others are restricted to a few or a single species or strain. Thus nodulation genes are designated as “common” or “host specific” (*hsn*), respectively (Kondorosi et al., 1984; Schlaman et al., 1998). Besides nitrogen-fixation genes and nodulation genes, previous studies have characterized some other characteristics and genes which affect the efficiency of symbiotic performance. It is interesting that some characteristics and

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and genes essential for the adaptation of rhizobia to stress conditions are also involved in enabling rhizobia to efficiently nodulate their host legumes. Takuji et al. (1998) have found the relation between hydrogen peroxide tolerance and nodulation efficiency in *Rhizobium fredii* USDA191. Vinuesa et al. (2003) have characterized *atvA* genes required for acid-tolerance and involved in nodulation competitiveness of *Rhizobium tropici* CIAT899. Kiss et al. (2004) have reported that *typA* gene in *Sinorhizobium meliloti* 1021 has a dual function: house keeping functions and functions for the growth of *S. meliloti* under certain stress conditions including low temperature, low pH, and in the presence of sodium dodecyl sulfate (SDS), as well as the establishment of a nitrogen-fixing symbiosis with certain host plants. However, it has been hypothesized that *typA* is not specific to symbiosis and only mediates adaptation to certain stress responses that are encountered both under free-living state as well as in symbiosis with some host plants. The role of sigma factors RpoH1 and RpoH2 on stress tolerances and symbiotic performance of *Rhizobium etli* has been reported by Martínez-Salazar et al. (2009). The *rpoH1* gene is essential for tolerance to heat shock, sodium hypochlorite and hydrogen peroxide. The *rpoH2* gene is involved in tolerance to NaCl, sucrose and osmotic pressure. Both sigma factors partly contribute to nitrogen fixing efficiency and nodule occupation. The relation between tolerance to some other stress conditions and symbiotic performance is possibly found in different species of rhizobia. Thus, we were particularly interested in investigating this aspect in a rhizobial strain that is resistant to high temperature, alkalinity and heavy metals. In this work, one strain of mungbean rhizobia was selected based on the resistance to high temperature, alkalinity and several heavy metals. The 16S rDNA sequence was analyzed to identify the strain to the genus and estimate phylogenetic relationship. A collection of mutants was constructed by using a transposon mutagenesis approach. The symbiotic performance and enzymatic patterns were compared between high temperature, alkaline, heavy metals resistant wild-type and high temperature, alkaline, heavy metal sensitive mutant.

MATERIALS AND METHODS

Bacteria and culture conditions

The 10 strains of mungbean rhizobia used in this study include DASA 02007, DASA 02008, DASA 02009, DASA 02010, DASA 02011, DASA 02068, DASA 02074, DASA 02077, DASA 02087 and DASA 02095. These strains were isolated from root nodules of mungbean (*Vigna radiata*) grown in 5 provinces of Thailand. The strains were profiled using randomly amplified polymorphic DNA (RAPD) analysis and verified as enable the nodulation of *V. radiata* by Pongsilp and Nuntagij (2007). The strains have been deposited in the collection of Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand. Rhizobial strains were grown at 28°C in Yeast-Mannitol (YM) medium (Keele et al., 1969). *Escherichia coli* strains harboring Tn3Hogus transposon donor plasmid (Km^R, Tc^S) and helper plasmid pRK2013 (Km^R, Tc^S) were grown at 37°C in Luria-Bertani (LB) medium supplemented with 200 µg ml^{-1} kanamycin. Arabinose-gluconate (AG) medium (Sadowsky et al., 1987) was used for triparental mating. Antibiotics kanamycin and tetracycline were added at final concentrations 200 and 50 µg ml^{-1}, respectively.

Selection of a rhizobial strain resistant to high temperature, alkalinity and heavy metals

Each rhizobial strain was examined for resistance to kananycin and tetracycline. Rhizobial culture was spread on YM agar plates and antibiotic discs (Oxoid, Basingstoke, UK) were placed on the agar surfaces. Antibiotic susceptibility was observed as clear zones around antibiotic discs. The growth of each strain in the presence of either kanamycin or tetracycline was also measured. Each strain was grown at 28°C in YM liquid medium with 200 rpm shaking for 5 days and used as inoculum. The total cell counts of inoculum was examined by the standard plate count method and the inoculum was inoculated into YM liquid medium supplemented with either 200 µg ml^{-1} kanamycin or 50 µg ml^{-1} tetracycline. Stock solutions of kanamycin and tetracycline were prepared at 10 mg ml^{-1} and 1 mg ml^{-1}, respectively, and filter sterilized before being added to the sterilized YM medium. The initial cell density of each strain was 1.00×10^5 colony forming units (CFU) ml^{-1}. After 8 days cultivation, the cell number was measured by the standard plate count method. The strains sensitive to kanamycin and resistant to tetracycline were selected to study their resistance to stress conditions.

For selection of a rhizobial strain resistant to high temperature, alkalinity and heavy metals, the experiments were prepared as described above except that each strain was cultured in the following conditions: 1) in YM liquid medium, pH 6.8 at 40°C; 2) in YM liquid medium, pH 8.0 at 28°C; 3) in YM liquid medium, pH 6.8 containing 200 µg ml^{-1} of each heavy metal ion: copper (Cu), cobalt (Co), zinc (Zn), cadmium (Cd), mercury (Hg) and arsenic (As) at 28°C. The pH of medium was controlled by using HEPES buffer. Stock solutions of heavy metals $CuCl_2$, $CoCl_2.6H_2O$, $ZnCl_2$, $CdCl_2.2H_2O$, $HgCl_2$ and $AsCl_3$ were prepared at 100 mg ml^{-1} and filter sterilized before they were added separately to the sterilized YM medium to obtain each heavy metal at a final concentration of 200 µg ml^{-1}. One strain of rhizobia was selected based on resistance to stress conditions and used for sequence analysis of partial 16S rDNA and transposon mutagenesis.

Sequence analysis of partial 16S rDNA and phylogenetic analysis

Partial 16S rDNA of the selected strain was amplified using universal primers UN16S 926f (5' AAACTYAAAKGAATTGACGG 3') and UN16S 1392r (5' ACGGGCGGTGTGTRC 3') (Lane, 1991). PCR reactions were carried out and the products were purified as described by Pongsilp et al. (2002). The purified PCR products were sequenced by Bio Basic (Markham, ON, Canada). The nucleotide sequences were aligned using BLASTN program (http://www.ncbi.nim.nih.gov/). Reference 16S rDNA sequences were selected and downloaded from GenBank (http://www.ncbi.nim.nih.gov/). Phylogenetic tree for the DNA sequences was constructed using the Neighbor-Joining method (Saitou and Nei, 1987). Sequences were taken together in the calculations of levels of sequence similarity using CLUSTALW 1.74 (Higgins et al., 1992). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in units of the number of base substitutions per site. All positions containing alignment

gaps and missing data were eliminated only in pairwise sequence comparisons. The Phylogenetic tree was conducted in the MEGA4 suite of program (Tamura et al., 2007).

Transposon mutagenesis

Transposon mutagenesis was done by a triparental mating. *E. coli* donor strain harboring Tn3Ho*gus* constructed in the laboratory of Brian Staskawicz, University of California, Berkeley, USA (Borthakur et al., 2003) and *E. coli* helper strain harboring pRK2013 (Figurski and Hekinski, 1979) were grown overnight on LB agar plates supplemented with 200 µg ml^{-1} kanamycin. Rhizobial strain was grown on AG medium for 3 days. Three very heavy lapfuls of donor, helper and recipient strains were mixed into the center of AG agar plate. The cultures were swirled together and incubated at 28°C for 3 days. The cultures were washed off with 5 ml of 0.85% NaCl containing 0.01% Tween 80. The mixtures were used to make serial dilutions and plated on AG medium without yeast extract supplemented with 200 µg ml^{-1} kanamycin and 50 µg ml^{-1} tetracycline. The mixtures were incubated at 28°C until colonies developed. Single colonies were picked and streaked onto YM agar plates containing 25 µg ml^{-1} Congo red, 200 µg ml^{-1} kanamycin and 50 µg ml^{-1} tetracycline.

Screening of high temperature, alkaline and heavy metals sensitive mutants

Rhizobial mutants carrying Tn3Ho*gus* insertions were firstly screened for loss of the ability to grow on YM agar plates at 40°C, pH 8.0 and in the presence of heavy metals. Then the total cell numbers of the wild-type strain and mutants at each condition were compared as described above. One mutant sensitive to high temperature, alkalinity and heavy metals was selected for southern hybridization and plant test.

Southern hybridization

The insertion of transposon Tn3Ho*gus* into the selected mutant was analyzed by Southern hybridization. Total genomic DNA from the wild-type and mutant strains was isolated by the method for megaplasmid isolation as previously described (Pongsilp and Nuntagij, 2009). DNA was digested with *Eco*RI and restriction fragments were separated by 1% agarose gel electrophoresis. DNA fragments were transferred to Nytran® SPC nylon transfer membranes (Whatman Schleicher and Schuell, Sanford, ME) as described (Sambrook et al., 1989). Plasmids were extracted from *E. coli* cultures by using a GF-1 plasmid extraction kit (Vivantis, Malaysia) according to the manufacture's instruction and digested with *Eco*RI. The restriction fragments were observed in 1% agarose gel electrophoresis. An approximate 9-kb fragment containing transposon was eluted and purified using a QIA Quick Gel Extraction kit (Qiagen, Valencia, CA) and used as a probe. The probe was labeled with digoxigenin-dUTP by using DIG DNA labeling and detection kit (Roche, Germany) and Southern hybridization was done according to the manufacture's instruction. Prehybridization was carried out at 60°C for 2 h and hybridization was performed at 60°C overnight.

Enzymatic patterns

To examine enzymatic patterns, cells of the wild-type and mutant strains were cultured at 28°C in YM broth until they reached mid-log phase. Five-ml of cell cultures were centrifuged at 6,000 rpm for 10 min. Cell pellets were resuspended in distilled water to prepare suspensions with a turbidity of 5 to 6 McFraland. Nineteen different enzymes were detected with the Api®Zym enzymatic quantification test (BioMérieux, Marcy-L'Etoile, France) using 65 µl of cell suspensions in each capule of the Api-Zym® strips. The strips were incubated at 37ºC for 4½ h. The detection was done according to the manufacturer's instructions and the intensity of color was developed within 7 to 10 min. After addition of the reagents, they were scored from 0 to 5 as follows: a value of 0 corresponded to a negative reaction, values of 1 to 2 corresponded to weak positive reactions, and values of 3 to 5 corresponded to strong positive reactions. However, all tests were conducted in duplicate.

Nodulation and nitrogen fixation assays

Seeds were scarified and surface sterilized with 3% hydrogen peroxide as described by Somasegaran and Hoben (1994). The seeds were laid on a moistened cotton plate and incubated at 25°C in the dark for 1 to 2 days. The germinated seeds were planted in leonard's jars amended with sterile sand. The seeds were inoculated with bacterial suspensions of either the wild-type or mutant strain. The seedlings were fertilized with N-free nutrient solution (Broughton and Dilworth, 1971). Uninoculated controls were included in the experiment. After 4 weeks cultivation, nodule numbers were determined. Nodule dry weight and plant shoot dry weight were determined by drying in an oven for approximately 72 h at 80°C until constant weight was obtained. The nitrogen fixing ability was measured by using the acetylene reduction method (Somasegaran and Hoben, 1994).

Statistical analyses

Experimental data was compared by using the SPSS program (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Selection of a rhizobial strain resistant to high temperature, alkalinity and heavy metals

In the determination of resistance of rhizobial strains to high temperature, alkalinity and heavy metals, 6 out of 10 strains of mungbean rhizobia were selected based on sensitivity to kanamycin and resistance to tetracycline. The cell numbers grown at different stress conditions were assessed. The strains showed different levels of stress resistance. Results in Table 1 show the cell numbers of each strain in YM liquid medium at 40°C, pH 8.0 and in the presence of each heavy metal. When compared with the initial cell number 1.00×10^5 CFU ml^{-1}, all strains were able to grow at 40°C. All strains except DASA 02074 were able to grow at pH 8.0. All strains except DASA 02007 were resistant to arsenic, but no strains were resistant to mercury. The strain DASA 02011 exhibited the resistance to high temperature, alkalinity, copper, cobalt, zinc, cadmium and arsenic. The highest cell numbers at 40°C, pH 8.0 as well as in the presence of copper, cobalt, zinc, cadmium and arsenic were observed with the strain DASA 02011, which differed significantly from other strains. Therefore, this strain was selected for further analysis based on the stress

Table 1. Total cell counts of rhizoibial strains grown in liquid medium under different stress conditions.

Strain	Growth under different conditions (log CFU ml^{-1})									
	Antibiotics[a]		Heavy metals[b]						Temperature 40°C	pH 8.0
	Km	Tc	Cu	Co	Zn	Cd	Hg	As		
DASA 02007	0.00 ± 0.00	6.70 ± 0.15	4.05 ± 0.02	4.10 ± 0.05	3.68 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	1.53 ± 0.01	7.16 ± 0.13	7.97 ± 0.02
DASA 02008	0.00 ± 0.00	6.89 ± 0.03	3.32 ± 0.04	5.31 ± 0.07	4.43 ± 0.02	2.58 ± 0.03	1.45 ± 0.06	6.27 ± 0.04	7.10 ± 0.08	8.30 ± 0.11
DASA 02009	0.00 ± 0.00	8.74 ± 0.04	3.76 ± 0.05	4.14 ± 0.09	4.36 ± 0.08	2.67 ± 0.05	2.45 ± 0.03	6.56 ± 0.08	7.36 ± 0.06	6.68 ± 0.11
DASA 02010	0.00 ± 0.00	8.20 ± 0.05	4.73 ± 0.04	3.64 ± 0.03	4.10 ± 0.07	1.43 ± 0.01	0.00 ± 0.00	6.83 ± 0.05	6.38 ± 0.08	6.28 ± 0.09
DASA 02011	0.00 ± 0.00	9.62 ± 0.06	9.58 ± 0.03	7.12 ± 0.09	7.29 ± 0.06	6.32 ± 0.04	0.00 ± 0.00	9.66 ± 0.04	7.71 ± 0.04	5.77 ± 0.07
DASA 02074	0.00 ± 0.00	7.38 ± 0.04	6.31± 0.05	1.77 ± 0.03	2.66 ± 0.03	3.31 ± 0.04	0.00 ± 0.00	5.43 ± 0.08	7.08 ± 0.13	3.24 ± 0.13
Mutant DASA 02011	9.05 ± 0.08	9.13 ± 0.05	9.39 ± 0.06	6.31 ± 0.04	2.28 ± 0.09	6.27 ± 0.02	0.00 ± 0.00	9.33 ± 0.11	3.71 ± 0.04	1.45 ± 0.01

The values shown are the mean of three replicates ± standard deviations. [a]Km, kanamycin at a concentration of 200 µg ml^{-1}; Tc, tetracycline at a concentration of 50 µg ml^{-1}. [b]Heavy metal ions Cu, Co, Zn, Cd, Hg and As at final concentrations of 200 µg ml^{-1}. Gray background highlights the comparisons of the total cell numbers between the wild-type and mutant strains under stress conditions.

resistance.

Sequence analysis of partial 16S rDNA and phylogenetic analysis

The selected strain DASA 02011 was analyzed for the partial 16S rDNA sequence. Analysis of sequence by BLASTN revealed that the strain was closely related to members of the genus *Bradyrhizobium* including *Bradyrhizobium* sp., *B. japonicum* and *B. liaoningense* with 98% identity. The partial 16S rDNA sequence of DASA 02011 is available from the GenBank database under accession number JF290368. A phylogenetic tree based on the 16S rDNA sequences was constructed (Figure 1). Reference 16S rDNA sequences of *Bradyrhizobium* that nodulate 3 species of *Vigna* plants were selected for estimating the phylogenetic relationship. *Bradyrhizobium* strains in different species and from different host plants were claded together. *B. japonicum* that nodulate cowpea (*Vigna unguiculata*) is the closest neighbor to *V. radiata*-nodulating *Bradyrhizobium* DASA 02011.

Transposon mutagenesis and screening of high temperature, alkaline and heavy metals sensitive mutants

High temperature, alkaline and heavy metals sensitive mutants was firstly screened for the inability to grow at 40°C on the selective YM agar plates buffered to pH 8.0 and containing 200 µg ml^{-1} of Cu, Co, Zn, Cd and As. Colonies grew well on YM agar plates supplemented with kanamycin and tetracycline but did not grow on the selective YM agar plates were picked. The screened mutants were examined for the loss of stress resistance by measuring the total cell counts under stress conditions. One of the mutant strains (designated DASA 02011 MuI) was selected based on the most decrease in resistance to high temperature, alkalinity and zinc. Table 1 shows the comparisons of the total cell numbers between the wild-type strain and the selected mutant strain of DASA 02011 under different stress conditions.

Southern hybridization

The selected mutant with a defect in resistance to high temperature, alkalinity and zinc was further characterized. Using Southern hybridization analysis, it was shown that the mutant strain carried a single Tn3Ho*gus* insertion (Figure 2).

Enzymatic patterns

The production of 19 enzymes was examined for the wild-type and mutant strain of *Bradyrhizobium* sp. DASA 02011. Both strains gave strong positive results for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine acrylamidase, valine acrylamidase, crystine acrylamidase, trypsin and napthol-AS-BI-phosphohydrolase, as well as negative results for lipase (C14),

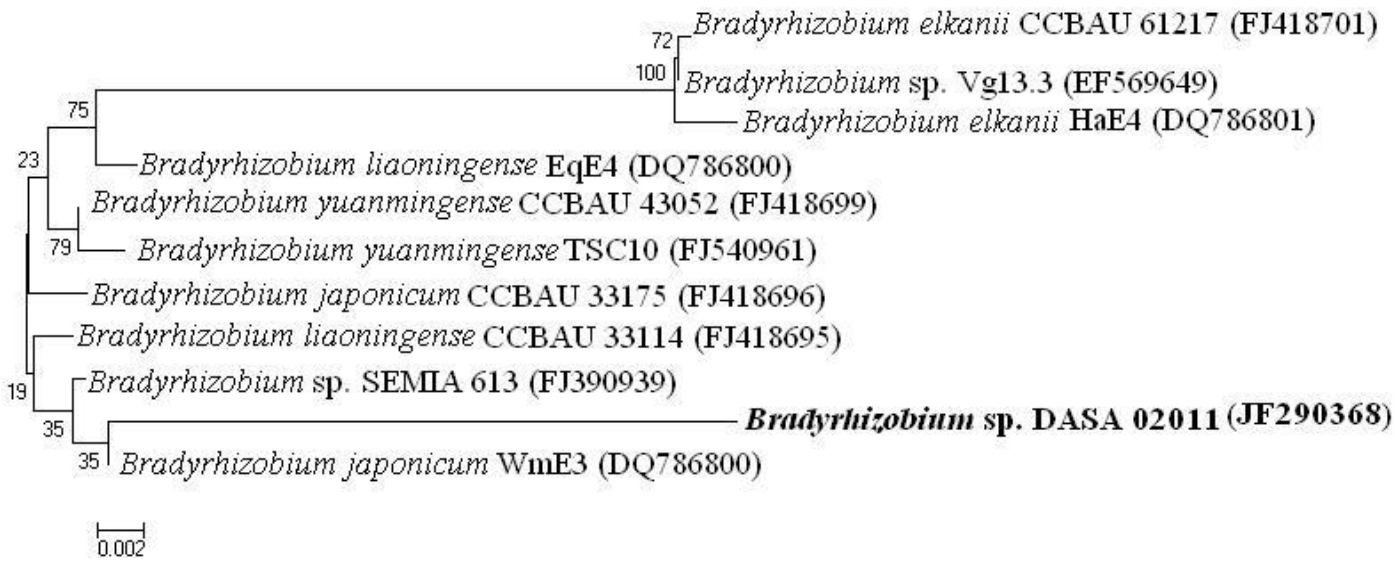

Figure 1. Phylogenetic tree based on 16S rDNA sequences. The numbers at the node are bootstrap values based on 1,000 re-sampling. *Bar* Mutations per sequence position.

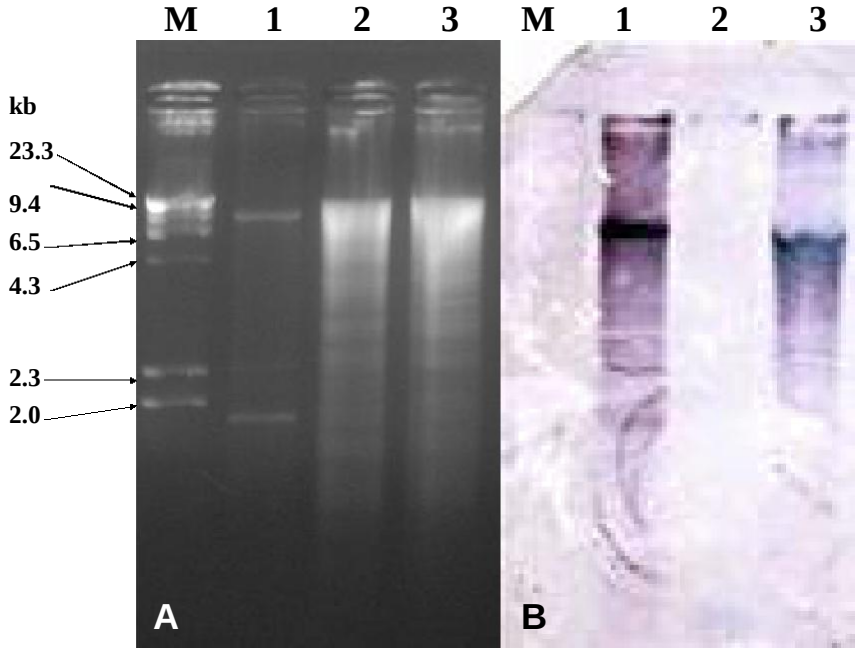

Figure 2. Southern blot analysis of the wild-type and the selected mutant strains of *Bradyrhizobium* sp. DASA 02011. A: *Eco*RI digestion patterns of total genomic DNA of the wild-type and mutant strains; B: Southern hybridization of total genomic DNA of the wild-type and mutant strains to a gene probe carrying Tn3H*ogus*. Lane M, λ/*Hin*dIII DNA ladder; 1; plasmid carrying Tn3H*ogus*; 2; total genomic DNA of the wild-type strain; 3; total genomic DNA of the mutant strain. The numbers on the left side refer to size marker (in kb).

α-chymotrypsin, α-galactosidase, β- galactosidase, β-glucoronidase, α-glucosidase, β- glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. The slight difference in enzymatic patterns was observed with acid phosphatase that has weak positive result by the wild-type strain but negative result by the mutant strain.

Table 2. Symbiotic performance of *V. radiata* inoculated the wild-type and mutant strains of *Bradyrhizobium* sp. DASA 02011.

Plants inoculated with strains	Symbiotic performance			
	Nodule number	Nodule dry weight (mg/plant)	Shoot dry weight (mg/plant)	ARA (µmol ethylene/plant/h)
Uninoculated control	0.00 ± 0.00	0.00 ± 0.00	65.50 ± 14.20	0.65 ± 0.19
Wild-type DASA 02011	13.75 ± 4.19	67.30 ± 7.80	369.00 ± 49.60	2.56 ± 0.63
Mutant of DASA 02011	5.75 ± 0.96	20.30 ± 1.70	188.50 ± 43.50	1.30 ± 0.27

The values shown are the mean of four replicates ± standard deviations.

Nodulation and nitrogen fixation assays

The wild-type and mutant strains were examined for their symbiotic performance with the original host known as *V radiata*. Both strains were found to form effective nodules on *V. radiata*, although the impaired symbiotic performance was observed in plants inoculated with the mutant strain. In Table 2, all parameters including nodule numbers, nodule dry weight, shoot dry weight and ARA of plants inoculated with the wild-type strain were highly significantly from those inoculated with the mutant strain. Nodule numbers, nodule dry weight, shoot dry weight and ARA of plants inoculated with the mutant strain decreased approximately 42, 70, 49 and 49%, respectively, as compared with plants inoculated with the wild-type strain. This result suggests that genes involved in stress resistance affected the symbiotic ability of *V. radiata*-nodulating *Bradyrhizobium* sp. DASA 02011. A correlation between stress tolerance and symbiotic performance has been reported in the previous studies. These stress tolerance includes tolerance to low temperature, low pH, SDS (Kiss et al., 2004), hydrogen peroxide (Takuji et al., 1998; Martinez-Salazar et al., 2009), heat shock, sodium hypochlorite, NaCl, sucrose, osmotic pressure (Martinez-Salazar et al., 2009) and cationic antimicrobial peptides (Vinuesa et al. 2003).

In conclusion, we have found the relation between symbiotic efficiency and resistance to stress tolerance including high temperature, alkalinity and heavy metal of *V. radiata*-nodulating *Bradyrhizobium*. The mutant exhibited 42, 70, 49 and 49% of nodule number, nodule dry weight, shoot dry weight and ARA, respectively, as compared with the wild-type strain.

ACKNOWLEDGEMENTS

This work was supported by the Thailand Research Fund, the office of the Higher Education Commission (grant MRG5080327) and Faculty of Science, Silpakorn University. Authors thank Rujirek Noisangiam, Monchai Manassila and Panlada Tittabutr for providing donor and helper strains.

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