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นางสาวสิรัส สุลัญชุปกร

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EXPRESSION OF NODULATION AND NITROGEN FIXATION GENES IN 6 STRAINS OF Bradyrhizobium japonicum UNDER DIFFERENT TEMPERATURES

Miss Siras Sulanchupakorn

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science Program in Industrial Microbiology Department of Microbiology

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ในปัจจุบันประเทศไทยผลิตถั่วเหลืองได้เพียง 15% ของที่ใช้ในประเทศ ส่วนที่เหลืออีก 85% เป็นถั่วเหลืองที่ น้ำเข้า เป็นที่ทราบว่าปุ๋ยชีวภาพไรโซเบียมเพิ่มผลผลิตถั่วเหลือง ของบรรจุปุ๋ยชีวภาพไรโซเบียมสำหรับถั่วเหลืองต้อง เก็บในที่เย็นหรือตู้เย็น ดังนั้น ถ้าพัฒนาสายพันธุ์ไรโซเบียมถั่วเหลืองให้ทนร้อน ก็จะประหยัดค่าใช้จ่ายและพลังงาน ใน งานวิจัยนี้เลี้ยงไรโซเบียมถั่วเหลือง Bradyrhizobium japonicum จำนวน 6 สายพันธุ์ ในอาหารสูตรสกัดจากยีสต์-แมนนิทอล และ ในอาหารสูตรสกัดจากยีสต์-กลิซีโรล ภายใต้อุณหภูมิต่างๆ (25 °C-40°C) นอกจากนี้ยังทำ viable plate counts บนจานเพาะเชื้อที่มีอาหารแต่ละสูตรดังกล่าวข้างต้น ที่อุณหภูมิ 30°C และ 40°C ผลการทดลองพบไร โซเบียมถั่วเหลืองทั้ง 6 สายพันธุ์เป็นไรโซเบียมถั่วเหลืองที่ทนร้อน ผลการทำลายพิมพ์ดีเอ็นเอโดยวิธี RAPD-PCR โดย ใช้ RPO1 หรือ CRL-7 เป็นไพร์เมอร์ พบว่า *B. japonicum* ที่ใช้ในการทดลองมีลายพิมพ์ดีเอ็นเอต่างกันจึงเป็นต่าง สายพันธุ์ ผลการทำ RT-PCR โดยใช้ไพรเมอร์ที่เฉพาะเจาะจงต่อ nodD,, nodA, nodB, nifH และ nifD พบการ เปลี่ยนแปลงระดับการแสดงออกของยืน nodD1 เมื่อเลี้ยง B. japonicum สายพันธุ์ SK4, SK26 หรือ SK28 ภายใต้ อุณหภูมิ 25°C, 30 °C, 35 °C และ 40 °C โดย nodD1 ของ B. japonicum SK26 มีการแสดงออกของยืน nodD, เพิ่ม เมื่อเลี้ยงเขลล์ภายใต้อุณหภูมิ 35 °C และ 40 °C ส่วนการแสดงออกของ nodD, ใน B. japonicum SK4 และ SK28 ลดลงเมื่อเลี้ยงเซลล์ที่อุณหภูมิสูงได้แก่ 35 °C และ 40 °C ผลการทดลองได้ตรวจพบการแสดงออกในระดับต่างกันของ ยืน nodB ของ B. japonicum สายพันธุ์ S76 ซึ่งมีการแสดงออกของ nodB เพิ่มขึ้นเมื่อเลี้ยงเซลล์ภายใต้อุณหภูมิ 35°C และ 40°C ผลการทดลองไม่พบการแสดงออกที่แตกต่างกันของ nodD, ของ B. japonicum สายพันธุ์ SK3, S76 และ S162 และ ไม่พบการแสดงออกที่แตกต่างกันของ nodA, nodB, nifH, และ nifD ของ B. japonicum แต่ละ สายพันธุ์ภายใต้สภาวะการทดลอง RT-PCR นอกจากนี้ได้ผลิตภัณฑ์ PCR เมื่อใช้ RNA ทั้งหมดจากเขลล์ของ B. japonicum แต่ละสายพันธุ์ที่เลี้ยง ณ อุณหภูมิ 45°C เป็นเวลา 5 ชั่วโมง ในการทำ RT-PCR

จุฬาลงกรณมหาวทยาลย

ภาควิชา จุลชีววิทยา สาขาวิชา จุลชีววิทยาทางอุตสาหกรรม ปีการศึกษา 2549 ลายมือชื่อนิสิต สิรัส สุลัญชุปกร ลายมือชื่ออาจารย์ที่ปรึกษา การ การป # #4772605323 : MAJOR INDUSTRIAL MICROBIOLOGY

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SIRAS SULANCHUPAKORN : EXPRESSION OF NODULATION AND NITROGEN FIXATION GENES IN 6 STRAINS OF *Bradyrhizobium japonicum* UNDER DIFFERENT TEMPERATURES. THESIS ADVISOR : ASSOC. PROF. KANJANA CHANSA-NGAVEJ, Ph.D. 56 pp. ISBN : 974-14-2603-8

At present 15% of domestic soybean consumption are grown locally. The rest 85% of soybean consumed are imported. Rhizobial biofertilizers have been known to increase soybean productivity. Packages of soybean biofertilizers need to be kept in cool places or in refrigerators. Therefore, if a high nitrogen-fixing, thermotolerant soybean rhizobial strain(s) is developed, it will save cost and energy. In this research, six soybean rhizobial strains of Bradyrhizobium japonicum SK3, SK4, SK26, SK28, S76 and S162 were grown in yeast extract mannitol broth and in yeast extract glycerol broth under different temperatures from 25°-45°C. Viable plate counts on both medium at 30°C and 40°C were also carried out. Results showed all the six B. japonicum strains were thermotolerant. RAPD-PCR fingerprints using either RPOI or CRL-7 were different for the six strains of B. japonicum, hence, the six B. japonicum constituted distinct strains. Results of RT-PCR with each set of primers specific for nodD, nodA, nodB, nifH, nifD showed differential expression of nodD1 when B. japonicum SK4, SK26 or SK28 was grown under 25°C, 30°C, 35°C and 40°C, nodD1 expression increased when SK26 was grown at high temperatures (35°C, 40°C). On the other hand, expression of nodD1 of SK4 and SK28 was found to decrease when growth temperatures were 35°C and 40°C. Differential nodB expression was observed when B. japonicum S76 was grown at 25°C, 30°C, 35 °C and 40°C with high expression at 35°C and 40°C. No differential gene expression was observed for nodD, of B. japonicum SK3, S76 and S162 and for nodA, nodB, nifH and nifD of every B. japonicum strain under all experimental conditions. In addition, RT-PCR products were obtained when total RNA from each B. japonicum strain grown at 45 °C for 5 h was isolated and used for RT-PCR.

Department Microbiology Field of Study Industrial Microbiology Academic Year 2006 Student's Signature...K. Sulanchu pakorn Advisor's Signature...K. Chantenpavej

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CHAPTER I

INTRODUCTION

Soybean rhizobia are bacteria which fix nitrogen in root nodules of soybeans which have been recognized as one of the most important plant sources of proteins for human consumption. Domestic consumption of soybeans in Thailand has increased from 1,397,960 metric tons in 2000 to 1,761,016 metric tons in 2005 [http://www.feedusers.com]. However, in 2005 the country was able to produce only 15% (240,428 metric tons) of the domestic consumption. The remaining 85% of the soybean consumed (1,520,588 metric tons) were imported. There are several socio-economic and technological factors which contribute to relatively high soybean import. One of which is the low cost of imported soybeans which is made possible because of large-scale mechanization in soybean plantations as well as soybean rhizobial biofertilizer utilization in countries which are leading soybean exporters such as the USA and Brazil. The use of soybean rhizobia biofertilizers in Thailand is very limited because there are not many local soybean biofertilizer manufacturing factories. One of the major soybean biofertilizer manufacturers is the Soil Microorganisms Research group affiliated with the Department of Agriculture which manufactures and markets soybean rhizobia biofertilizers. At present, soybean productivity in Thailand is 241 kg per rai which is relatively low when compared to the USA (458 Kg.rai¹[http://www.feedusers.com]). One way to increase soybean productivity is to select super nitrogen-fixing rhizobia for the development of soybean rhizobia biofertilizers. At present, soybean growers need to keep soybean biofertilizers in cool places or in refrigerators. Therefore, if heat-tolerant, high nitrogen-fixing soybean biofertilizers could be developed, it would save cost and energy. The aim of this research is to employ RAPD-PCR fingerprinting technology to show that six Bradyrhizobium japonicum isolates are different strains. Then RT-PCR (Reverse Transcription-Polymerase Chain Reaction) will be employed to determine if there is differential gene expression in nodulation genes and nitrogen fixation genes. The results obtained will lead to better understanding of the effects of temperature on nodulation and nitrogen fixation gene

expression which will eventually lead to the development of high nitrogen-fixing, heat tolerant strains for the development of biofertilizers.

Rationale for research

1.1 RAPD-PCR fingerprinting technology

RAPD-PCR stands for Random Amplified Polymorphic DNA-Polymerase Chain Reaction. The advantage of employing RAPD-PCR is sequences of the whole genome need not be known in order to use PCR method to amplify DNA fragments. In addition, only one primer is used in RAPD-PCR as opposed to the use of two primers. In this research, either RPO1 or CRL-7 primer has been chosen based on the finding by Schofield and Watson in 1985 that RPO1 is a conserved sequence in the promoter region of *nifHDK* of three *Rhizobium* spp. *nifHDK* encodes the enzyme nitrogenase which catalyses nitrogen fixation or reduction of atmospheric nitrogen to ammonia. If DNA of an isolate yields a fragment(s) when RPO1 is used in RAPD-PCR, it may be inferred that the isolate contains nitrogenase enzyme for nitrogen fixation. Hence the isolate(s) may be soybean rhizobia.

CRL-7 primer is chosen as the other primer due to the fact that the primer is an arbitrary primer (5'GCCCGCCGCC3') [Mathis & McMillin, 1996]. In 2006 Ly and Chansangavej reported that soybean rhizobial strains with more than 15 PCR product fragments might be heat tolerant because the more GC rich areas might confer heat tolerant since it needed more energy to break the three hydrogen bonds between nitrogenous bases C and G compared to lesser energy needed to break the two hydrogen bonds between nitrogenous bases A and T in DNA molecules. Information obtained on the extent of GC rich areas as reflected in CRL-7 PCR fingerprints will aid in strain selection for the development of heat-tolerant strains for production of biofertilizers with a long shelf-life which do not require refrigeration during transportation and storage.

1.2 RT-PCR technology

RT-PCR technology refers to the Reverse Transcriptase catalysed conversion of mRNAs to cDNAs and amplification of selected cDNAs with the use of forward and reverse primers which are specific to the amplification of the chosen cDNA template(s). RT-PCR has widely been used in the studies of differential gene expression under various conditions. Expression of GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) gene is usually concurrently carried out in conjunction with the amplification of chosen cDNAs to control the amounts of total RNAs loaded onto the agarose gel for electrophoresis. Equal intensity of Ethidium bromide (EtBr) stain for amplified GAPDH fragments is taken to imply that equal concentrations of total RNAs have been loaded in the RT-PCR experiment. Hence, unequal intensity of EtBr-stained gene fragments implied differential gene expression by the organism(s) under the experimental conditions.

RT-PCR is a rapid method which is far more convenient to use compared to other methods that have been used in the study of differential gene expression. For example, another method used to detect differential gene expression at the transcriptional level is Northern Blotting. This method requires construction of a labeled probe which may be either ³²P-labeled or labeled with non-radioactive chemical including biotinylated probes. In addition, the hybridization step of the experiment is time-consuming. Another method has been used to detect differential gene expression at the translational level. This method requires promoter-gene-reporter gene fusions which is also time-consuming because many steps must be carried out before the final step of detection of differential gene expression. In order to use gene fusions to study differential gene expression, one needs to select promoters and reporter genes for use in the construction of recombinant plasmids. Moreover, suitable methods to introduce the recombinant plasmids into suitable host cells must be selected. Recombinant plasmids may be introduced into host cells and expressed as multicopy plasmids or they may be integrated onto chromosomal DNA and expressed as a single copy gene. Finally, assays of the reporter genes must be carried out to monitor gene expression. Table 1.1 contains information on widely used reporter genes in the study of gene expression at the translational level.

Table 1.1 Widely used reporter genes in the study of gene expression at the translational level (Forsberg et al., 1994).

Reporter genes	Detection assay methods					
lacZ from E.coli	β -galactosidase activity with the use of IPTG inducer					
cat	Chloramphenicol acetyl transferase activity					
luxAB from Vibrio harveyi	Luciferase activity					

In addition, Forsberg et al. (1994) reported that the use of *luxAB* as the reporter gene had to be used with caution because comparisons of results obtained from the use of *luxAB* and the use of *cat* reporter genes had shown that the use of *luxAB* in gene fusion resulted in interference of the expression caused by intrinsically curved DNA segment in the 5' coding sequence of the *luxA* gene. This DNA topology at the 5' coding sequence had been shown to either activate or repress gene expression depending on the types of promoters. Thus, care must be taken when interpreting gene expression results when *luxAB* is used as the reporter gene. Therefore, RT-PCR seems to be the technique of choice for the study of differential gene expression.

1.3 Need for heat-tolerant soybean rhizobia biofertilizers

In Thailand soybeans are grown mainly in the northern, north-eastern, and central parts. Rainy season soybean crops are obtained twice per year as follows : The first crop is planted during April and May with harvesting during July and August. The second crop is planted in July-August with harvesting in October to November. Summer crop is planted in December and January and harvested in March to April. Thus, the months when soybean seeds germinate and soybean rhizobia make initial contact with soybean roots are April, May, Jaly, August, December, and January. The average maximum temperatures in these months are shown in Table 1.2

Areas				Ave	erage n	naximum	tempe	ratures	(°C)			
	Jan	Feb	Mar	Apr	May	June	July	Aug	Sept	Oct	Nov	Dec
Northern part (20 provinces)	31.6	35.7	35.8	37.0	36.2	28.8	32.9	31.4	31.9	31.4	32.5	31.5
North-eastern part (16 provinces)	31.5	35.9	34.6	36.4	35.8	29.4	33.1	33.0	32.5	32.9	31.9	30.0
Central part (9 provinces)	32.2	35.1	35.0	36.1	35.8	30.0	33.1	33.0	32.5	32.9	31.9	30.0
Eastern part (10 provinces)	31.7	33.6	33.7	34.8	34.5	29.9	32.3	32.4	32.0	32.7	32.2	30.1
Southern part (18 provinces)	31.0	33.0	33.5	34.7	33.7	No Data Available	32.6	33.0	32.3	31.2	30.7	28.9

Table 1.2 Average maximum temperatures in Thailand in 2005.

Source : Weather reports 551.506.1, Meteorological Department, Bangkok (2005).

The recorded average maximum temperatures as shown in Table 1.2 indicated that atmospheric temperature could be 37°C in April.

With the global warming phenomenon the average maximum temperatures in each region of Thailand are bound to increase. Therefore, if the global warning trend is still taking place, there is a need to develop both heat-tolerant soybean cultivars and symbiotic soybean rhizobial strains with high nitrogen fixation capability. At present, a lot of information is known about heat shock protein gene regulation in the slow-growing soybean rhizobia, *Bradyrhizobium japonicum* (Fischer et al., 1993; Babst et al., 1996; Minder et al., 1997; 2000; Münchbach et al., 1999; Nocker et al., 2001) However, relative little information is known on the effects of temperature on nodulation and on nitrogen fixation gene expression. Therefore, the aim of the present study is to employ RT-PCR to find out if temperatures have effects on nodulation genes (*nodD*₁, *nodA*, *nodB*) and nitrogen genes (*nifH*, *nifD*) expression in 6 strains of *B. japonicum*.

CHAPTER II

LITERATURE SURVEY

2.1 Soybean production and consumption in Thailand

Soybeans have been used in Thailand for the production of soybean cooking oil and soybean-based products such as tofu, soy sauce, and soy meal for animal feeds. However, soybean production in Thailand does not meet the demand for domestic consumption. Table 2.1 shows Thailand produced between 230, 516 metric tons to 260, 696 metric tons in the past five years (2001-2005) with up to 241 kg.rai productivity. In order to meet local demand, Thailand relies heavily on import of soybeans. Table 2.2 shows soybeans imported to Thailand have increased from 1,363,224 metric tons in 2001 to 1,607,784 metric tons in 2005 which was approximately 85% of domestic soybean consumption.

Year	Soybea	Soybean		
	Summer crop	Rainy season	Tatal	productivity
	(Nov-Apr)	crop (May-Sep)	Total	(kg. <i>rai</i> ⁻¹)
2001	149, 729	110, 967	260, 696	226
2002	148, 534	111, 329	259, 863	230
2003	142, 856	87, 660	230, 516	240
2004	150, 996	89, 432	240, 428	237
2005	155, 775	90, 732	246, 507	241

Table 2.1 Soybean production in Thailand in the past five years (2001-2005).

Source : The Office of Agricultural Economics (2006).

Year	Soybean import (metric tons)
2001	1,363,224
2002	1,528,557
2003	1,689,649
2004	1,435,801
2005	1,607,784

Table 2.2 Soybean import in the past five years (2001-2005).

Source : The office of Agricultural Economics in co-operation with The Customs Office (2006).

Table 2.1 shows local soybean productivity is relatively low compared to those of countries where soybean rhizobium biofertilizers are used such as Argentina, Brazil and USA (http://www.feedusers.com). Soybean productivity could be increased by several means including the utilization of biofertilizers which consist of efficient high nitrogen fixing strains of soybean rhizobia. Efficient soybean biofertilizers must be able to both nodulate soybean roots and fix nitrogen in soybean root nodules. Therefore, the biofertilizers must express nodulation and nitrogen fixation genes.

Figure 2.1 shows genes involved in nodulation and in nitrogen fixation in *B. japonicum* used in this study.

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Figure 2.1 Whole genome sequence of *B. japonicum* USDA110. Each interval represents 100,000 bp. All the genes are color-coded according to their functions. Positions of nodulation and nitrogen fixation genes as well as GAPDH gene used in RT-PCR in this study are shown in the Figure. (http://gib.genes.nig.ac.jp/single/index. php? spid =Bjap_USDA110).

2.2 Genes involved in nodulation

There are several genes, the products of which are involved in successful nodulation process. Chief among these genes includes $nodD_1$ and nodYABC.

Nodulation genes (*nodD*, *nodA*, *nodB*, and *nodC*)

Nodulation genes of *B. japonicum* include $nodD_1$ and nodYABC. $nodD_1$ encodes NodD_1 protein which forms complexes with flavonoids Genistein or Diadzein secreted by soybean (*Glycine max*) root nodules (Kosslak et al., 1987). NodD_1-flavonoid complexes bind to $nodD_1$ box which consists of two 9 bp repeat sequences in the promoter of $nodD_1$. Wang and Stacey (1991) reported that the 9 bp repeat sequences of $nodD_1$ box are

<u>GCGCGTCTA</u> ATTGCTTTT binding of NodD₁-flavonoid complexes to $nodD_1$ box activates transcription of $nodD_1$. The transcriptional start site of $nodD_1$ lies 44 bp downstream of $nodD_1$ box as shown in Figure 2.2

The promoter of *nodYABC* contains *nodY* box which is made up of four 9 bp repeats as follows :

ATCCATCGT GTGGATGTA TTCT ATCGAAACA ATCGATTTT ACCAGAT

The consensus sequence of nod boxes are $A_{74}T_{90}C_{88}G_{85}A_{93}T_{89}T_{71}G_{74}T_{74}$ (Wang and Stacey., 1991).

Wang and Stacey (1991) stated that promoters of $nodD_1$ and of nodYABC overlapped with transcriptional start sites of $nodD_1$ and of nodYABC lying in the *nod* box of the opposing transcript as shown in Figure 2.2.



Figure 2.2 Diagramatic representation of a DNA segment of *B. japonicum* nodulation genes showing promoters of $nodD_1$ and nodYABC are overlapped with transcriptional start sites of nodD and of nodYABC lying in the *nod* box of the opposing transcript (Wang and Stacey , 1991).

Expression of *nodYABC* is also activated by binding of NodD₁-flavonoid to the *nodY* box (Loh and Stacey, 2003). In addition, *nodD*₁ and *nodYABC* are activated by a two-component system encoded by *nodVW*. NodV is a kinase which autophosphorylates and transfers the phosphate group to NodW. Phosphorylated NodW activates transcription of *nodD1* and *nodYABC* possibly by influencing DNA bending as in the case of the activation mechanism of NodD1-flavonoid complexes (Loh and Stacey, 2003).

Expression of $nodD_1$ and nodYABC is repressed by $NodD_2$ which is encoded by $nodD_2$. NoIA product from *noIA* regulates the expression of $nodD_2$. Figure 2.3 summarizes the activation and repression of nodulation gene expression.



Figure 2.3 NodD₁-flavonoid complexes bind to $nodD_1$ box and nodY box in the promoters of $nodD_1$ and nodYABC to activate transcription of $nodD_1$ and nodYABC. A protein product NodV autophosphorylates then transfers the phosphate group to NodW. Phosphorylated NodW-P activates the expression of $nodD_1$ and nodYABC. NolA regulates the expression of $nodD_2$ whose protein product, NodD₂, represses the expression of $nodD_1$ and nodYABC (Loh and Stacey, 2003).

At present, the function of *nodY* protein product is not known. However, protein products of *nodA*, *nodB*, *nodC* are known to be enzymes involved in the synthesis of Nod factors which are lipo-chitooligosaccharide signals involved in the initial steps of nodulation process. The exact function(s) of Nod factors is not known although it has been reported to play a role in root hair curling, infection sac and infection thread formation and possibly the induction of root cortex cell division resulting in nodule formation (Long, 1996).

Nod factor of *B. japonicum* is made up of 5 N-acetylglucosaminyl units with substitutions. The synthesis of Nod factors is catalyzed by these enzymes ; nodC, NodB, and NodA as shown in figure 2.4. Genes *nodC*, *nodB*, and *nodA* encode enzymes NodC, NodB, and NodA respectively. NodC is N-acetyglucosaminyl transferase which catalyses the transfer of N-acetylglucosamine. NodB is N-deacetylase which catalyses the removal of an acetyl group from the N-acetyl glucosaminyl unit at the non-reducing end. NodA is

N-acyltransferase which catalyses the transfer of a C18:1 acyl group to the non-reducing N-acetylglucosaminyl unit.



Figure 2.4 Synthesis and structure of Nod factor of B. japonicum (Stacey, 1995).

2.3 Early study on the role of Nod factors

Bypassing the chemotoxic responses to flavonoid gradients, Pueppke (1984) submerged roots of germinating soybean (*Glycine max* [L.]) Merr. cv Hardee seeds to sterilized 15 ml Jensen's nitrogen free nutrient solution containing 10⁴ cells of each of the following rhizobia : *Rhizobium japonicum* 138, *Rhizobium* sp. 3G4b16, *Rhizobium* sp.229, *Rhizobium lupini* 96B9, *Rhizobium meliloti* 102F51, Determinations of cell numbers adsorbed per 2 cm distal root segment after incubation at room temperature for 2 h showed higher number of rhizobia adsorbed did not always lead to formation of infection thread in soybean roots as shown in Table 2.3

Ctraina	Notural Lloot	Soybean			
Strains	Natural Host	Adsorption	Infectivity		
Rhizobium lupini 96B9	Lupinus polyphillus	384 ± 31	-		
Rhizobium sp.229	Vigna unguiculata	127 ± 7			
R. japonicum 138	Glycine max	120 ± 4	+		
<i>R. meliloti</i> 102F51	Medicago sativa	107 ± 22	-		
Rhizobium sp. 3G4b16	Glycine max	56 ± 7	+		

Table 2.3 Relationship between cell numbers of rhizobia to roots and formation of infection threads at room temperature (27°C) (Pueppke, 1984).

The results indicated that although *Rhizobium lupini* 96B9, with the natural host *Lupinus phyphillus*, adsorbed the most cell number to soybean roots, the adsorption of the rhizobia did not lead to infection thread formation. On the other hand, *R. japonicum* 138 and *Rhizobium* sp. 3G4b16 with intermediate and low adsorbed cell numbers led to successful infection thread formation. The results seemed to indicate that ablility to adsorb to root hair alone could not lead to infection thread formation process (Pueppke, 1984). Most interesting finding reported by Pueppke (1984) was adsorption of *R. japonicum* 138 to soybean roots at 4°C and 37°C decreased to 20 ± 10 cells and 50 ± 90 cells/root segment respectively. Hence, temperature seemed to have an effect on adsorption of soybean roots.

2.4 Nitrogen fixation genes (*nifH*, *nifDK*)

Nitrogenase consists of two proteins : The Fe-protein and the MoFe-protein which are 64 Kda and 220 Kda respectively. The Fe-protein is a dimer of identical subunits, each of which is encoded by *nifH*. The MoFe-protein consists of $\alpha_2\beta_2$ subunits. *nifD* encodes the α subunit while *nifK* encodes the β subunit. Table 2.4 summarizes the known information of the genes and their protein products.

Table 2.4 Genes and protein products for nitrogenase

Genes	Protein products
nifH	Subunit of the Fe-protein
nifD	lpha subunit of the MoFe-protein
nifK	eta subunit of the MoFe-protein

In the slow-growing *B. japonicum, nifH* and *nifDK* are on separate operons in the symbiotic gene cluster (Elkan & Bunn, 1992). A ribbon representation of nitrogenase of *Azotobacter vinelandii* which is made up of an Fe-protein and a MoFe-protein is shown in Figure 2.5



Figure 2.5 A ribbon representation of *Azotobacter vinelandii* nitrogenase which consists of an Fe-protein (light blue) and a MoFe-protein. Metal clusters are shown in yellow (Moffat, 1992).

Each subunit of the Fe-protein consists of two ATP binding sites and one 4Fe-4S cluster while each $\alpha\beta$ subunit of the MoFe-protein consists of one P-cluster and one FeMo-cluster as shown in Figures 2.6 and 2.7.



Figure 2.6 Each subunit of the Fe-protein consists of two ATP-binding sites and one 4Fe-4S cluster. Each $\alpha\beta$ subunit of the MoFe-protein consists of one P-cluster and one FeMo-cluster (http://www.rcsb.org/pdb/molecules/pdb26_3.htm).



Figure 2.7 The P-cluster and the FeMo cofactor (cluster) of the MoFe-protein are attached the Cysteine, Histidine, and Glutamine residues of the MoFe-protein (Kim and Rees, 1992).

Nitrogenase catalyses the reduction of atmospheric N_2 to ammonia by an electron transfer system as indicated in Figure 2.8. The reduction process requires energy in the form of ATP molecules which bind to the Fe-protein subunit. Electrons are transferred through the 4Fe-4S clusters of the Fe-protein to the P-cluster and the FeMo-cluster of the MoFe-protein and finally to a nitrogen molecule which binds to the MoFe-protein. The electron transfer system is shown in Figure 2.8



Figure 2.8 Electron transfer system in the nitrogen fixation process-catalysed by nitrogenase (Voet and Voet, 1995).

In nitrogen fixation reaction, hydrogen is always a by-product of the reaction. Ammonia produced by nitrogen fixation by rhizobia is utilized by host plants to form amino acids via the first enzyme, Glutamine synthase, which catalyses the formation of Glutamine with Ammonia and one molecule of Glutamic acid as shown in the following equations :



Glutamine formed from the incorporation of a molecule of Ammonia to one molecule of Glutamic acid is used in an amino transferase (transaminase) reaction with α ketoglutarate as another substrate to form two molecules of Glutamic acids which enter the amino acid synthesis pathway catalysed by appropriate amino acid transferases (Voet and Voet, 1995).

2.5 Effects of temperature on nodulation and nitrogen fixation

In 1978 Day et al. inoculated each of the five *rhizobium* sp. strains onto cowpea (*Vigna unguiculata*) seeds in 15 cm diameter pots containing sand, grit, and soil (6:2:1 by volume). The pots were placed in water baths which were put in a greenhouse maintained between 30°C (day), 20°C(night). The water baths were maintained at 30°C, 36°C, 38°C, 40°C, 42°C or 44°C. Each set of plants were subjected to 30°C continuously or to the higher temperatures for 5 hours per day from sowing to harvesting at 40 days. The results showed that mean nodule dry weight as well as nitrogenase activity as determined by the acetylene reductase at high temperatures. The results indicated that high soil temperatures reduced the extent of nodulation and nitrogenase activity of the rhizobial strains used in the experiments as shown in Table 2.5

Table 2.5 Effect of *Rhizobium* strains and root temperature on growth, nodulation and nitrogenase activity (Day et al., 1978)

			Rh	izobium stra	in	*	Marra	
Root temperature (°C)*	Uninoculated	R5029	R.5009	R5018	CB756	R 5030	(inoculated)	
			Mean	dry wt plan	t (g)			
30	2.39	8.09	6-93	6-14	5-31	4.62	6-22	
36	2.31	4-06	5-64	4.07	4-85	5-09	4-72	
38	1.72	3-80	3-79	1-43	2.53	3-26	2-96	
40	1-23	1-58	1.67	1.75	1-48	2.01	1.70	
42	1.31	1-64	1-53	1.46	1.34	1-45	1-48	
44	1-17	1-47	1-31	1.14	1-21	1.20	1-27	
			Mean dry	wt nodules (mg/plant)			
30	0	500	463	595	395	380	467	
36	0	440	530	430	370	385	431	
38	0	530	580	30	295	295	346	
40	0	35	15	0	0	150	40	
42	0	0	0	0	0	0	0	
		Nitroger	ase activity	(umoles C ₂ H	l ₄ /g dry wt n	odule/h)		
30		227	166	129	185	198	181	
36		55	115	86	151	129	107	
38		68	53	80	144	116	92	
40		23	50	0	0	48	24	
42		0	0	0	0	0	0	

* Plants exposed to temperatures in range 36-44 °C for 5 h/dav then returned to 30 °C; harvested after 40 days.

CHAPTER III

MATERIALS AND METHODS

3.1 Bacterial strains

The following six slow-growing soybean rhizobia strains were used in the experiments :

Bradyrhizobium japonicum SK3, SK4, SK26 and SK28 isolated from Wang Muang district, Saraburi province by Somchoke Kala (2003) and S76, S162 isolated from Kao Kaw district, Petchaboon province by Suwat Saengkerdsub (1999). The cultures were deposited at MIRCEN Microbiological Resources Center, Bangkok, with MIRSEN codes as shown in Table 3.1. Cell cultures were stored in yeast extract mannitol agar slants (YMA) with 25µg.ml⁻¹ congo red at 4°C until use.

			Sources	
Strain	MIRCEN code	Root nodules of	Soil sample	References
		soybean cultivar	collection sites	
SK3	1596	SJ4	3	Somchoke Kala (2003)
SK4	1597	SJ4	Ta Muang District,	Somchoke Kala (2003)
SK26	1598	SJ4	Saraburi Province	Somchoke Kala (2003)
SK28	1599	SJ4		Somchoke Kala (2003)
S76	1537	SJ5	Kao Kaw District,	Saengkerdsub (1999)
S162	1539	SJ5	Petchaboon Province	Saengkerdsub (1999)

Table 3.1 Soybean rhizobial strains used.

Strains were maintained at 4°C on yeast extract-mannitol agar slants (Appendix A).

3.2 Growth curves of six Bradyrhizobium japonicum strains

3.2.1 Growth curves at mid-log phase

Cells in YMA slants were activated by plating onto YMA containing 25µg.ml⁻¹ congo red agar plates and incubated at 25°C for 4 days. One loopful of cells of each strain was inoculated into 50 ml of either yeast extract mannitol broth (YMB) or yeast extract glycerol broth (YGB), incubated at 30°C, 200 rpm for 4-6 days. Growth was followed by optical density readings at 660 nanometer. Time to reach mid-log phase for each culture was determined from the growth curves.

3.2.2 Growth curves at different temperatures

Cells were grown to mid-log phase for seeding. 5 ml of mid-log phase cells were added to 45 ml of YMB or YGB then incubated at 25°C, 30°C, 35°C, 40°C and 45°C, 200 rpm for 4-6 days. Composition of YMB and YGB was given in Appendix A. Growth was followed by optical density readings at 660 nanometer.

3.3 PCR fingerprinting of *Bradyrhizobium japonicum* SK3, SK4, SK26, SK28, S76 and S162

3.3.1 DNA Extraction

Cells grown in 50 ml yeast extract glycerol broth (YGB) until mid log phase were harvested by centrifugation at 7,000 rpm for 5 minutes, washed with 0.85% normal saline twice before addition of 100µl 2.5mg/ml lysozyme in saline EDTA and incubation at 37° C for 1 hour. Cells were frozen and thawed at -20° C for 5 minutes and at 80° C for 5 minutes 4 times. 250µl DNAzol[®] (Invitrogen) were added to the cells before centrifugation at 10,000 rpm, 4°C for 10 minutes. Supernatant was transferred to a new eppendorf tube and DNA was precipitated with 500µl absolute ethanol at -70° C for 15 minutes. Precipitated DNA was collected by centrifugation at 12,000 rpm, 4°C for 10 minutes, washed with 1,000µl 70% ethanol and air dried in a laminar flow hood. 20µl high-purity distilled water was used to dissolve DNA. Quantity of isolated DNA was determined by absorbance at 260 nm and quality of the isolated DNA was checked by OD₂₆₀/OD₂₈₀ ratios and 0.8% agarose gel electrophoresis by standard methods (Sambrook & Russel, 2001).

3.3.2 PCR fingerprinting

Sequences of RPO-1 and CRL-7 were as reported by Richardson et al (1995) and Mathis & McMillin (1996) as follows :

RPO-1 : 5'AATTTTCAAGCGTCGTGCCA3'

CRL-7:5'GCCCGCCGCC3'

RPO-1 and CRL-7 primer was used in RAPD-PCR fingerprinting in the following mixture:

Mixture			<u> </u>	Program	
10x PCR buffer	2.5	μΙ	95°C	15 seconds)
50 mM MgCl ₂	0.8	μΙ	55°C	30 seconds	5 cycles
10 mM dNTPs	0.5	μΙ	72°C	90 seconds	
10 µM primer	5.0	μΙ	95°C	15 seconds)
DNA template (60-100 ng)	1.0	μl	60°C	30 seconds	25 cycles
<i>Taq</i> polymerase (5U. μl ⁻¹)	0.2	μΙ	72°C	90 seconds	
High quality double distilled water	15.0	μΙ	72°C	10 minutes	
Total	25.0	μΙ			

PCR products were separated by 1.25 % agarose gel electrophoresis by standard method (Sambrook and Russel, 2001). RAPD-PCR fingerprints were viewed and photographed on a UV transilluminator (Bio-rad).

3.4 RT-PCR

3.4.1 Extraction of total RNA

Total RNA was extracted with Trizol[®] (Invitrogen) according to the manufacturer's instruction. Log phase cells from 50 ml YGB were broken by incubation with 700µl Trizol[®] for 5 minutes at room temperature. After centrifugation at 10,000 rpm, 10 minutes, 4^oc, supernatant was transferred to a new Eppendorf tube. 200µl chloroform was added to the supernatant followed by incubation at 15-30^oC for 2-3 minutes. The upper aqueous phase was transferred to a new Eppendorf tube. RNA was precipitated by 500µl isopropyl alcohol at 15-30^oC for 10 minutes, collected by centrifugation at 8,000 rpm, 10 minutes, 4^oc, washed with 1 ml 75% ethanol, then air-dried. 20µl DEPC-treated distilled water was added to the dried RNA preparation. Quantity and quality of RNA preparation

were checked by OD_{260}/OD_{280} and 0.8% agarose gel electrophoresis with RNA markers from Promega according to the manufacturer's instruction.

3.4.2 RT-PCR

Primers for the amplification of *nodD*₁, *nodA*, *nodB*, *nifH*, *nifD* and *GAPDH* were designed by multiple alignments as shown in Appendix C. Primers for the amplification of *nodC* could not be obtained because there were not sufficient conserved sequences as shown in Appendix C. Sequences chosen for using as primers had been checked that they did not self anneal.

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nodD_1
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Forward primer 5' AAAATGGCAGCAGYTCGAA 3' Reverse primer 5' CAACATCAATCTGAGCCAG 3' nodA Forward primer 5' GGTGGAGCGGACGCGAATGC 3' Reverse primer 5' GCTCGCCGATCATGCCG 3' nodB Forward primer 5' AGCACAATTGCWCCCGGGCG 3' Reverse primer 5' GCAACATCGGGTCCCCGCGA 3' nifH Forward primer 5' AGCCACCGCAAACAACGTCG 3' Reverse primer 5' ATCGGCAAGTCCACCACTTC 3' nifD Forward primer 5' TCMAGCAGAATTCGCGA 3' Reverse primer 5' AACTATTACGTTGGCAC 3' GAPDH Forward primer 5' YTCGTTGTCGTACCAG 3' Reverse primer 5' CTGCACSACSAACTGC 3'

*nodD*₁, *nodA*, *nodB*, *nifH*, *nifD* and *GAPDH* were used as specific primers for RT-PCR by using Maxime RT-PCR PreMix Kit (iNtrRON Biotechnology) in the following mixture:

<u>Mixture</u>

Total RNA		100-2000	ng
Forward primer		5-20	pmol
Reverse primer		5-20	pmol
RNase-free water		up to 20	μl
	Total	20 11	

Total 20 µl

<u>Program</u>

Reverse transcription reaction	45°C	30-40 minutes
Inactivation of RTase	94°C	5 minutes
Denaturation	95°C	15 seconds
Annealing		47-58 °C >30 seconds
30-40 cycles		J
Extension	72°C	90 seconds

RNA Template	Primer concentration		Reverse	Annealing
(ng)	Primers	pmol	Transcription (min)	Temp (°C)
2000	nodD ₁ / GAPDH	20 / 5	40	54
100	nodA / GAPDH	20 / 5	30	58
100	nodB / GAPDH	20 / 5	30	55
100	nifD / GAPDH	20 / 5	30	47
100	nifH / GAPDH	10 / 10	30	55

PCR products were separated by 1.25 % agarose gel electrophoresis by standard method (Sambrook and Russel, 2001). RT-PCR products were viewed and photographed on a UV transilluminator (Bio-rad).

CHAPTER IV

RESULTS

4.1 Turbidity profiles of B. japonicum

Since 6 B. japonicum strains produce extracellular polysaccharides, it is likely that changes in turbidity as measured by absorbance at 660 nm may be due to changes in polysaccharide contents and not due to changes in cell numbers. In order to find out if growth of *B. japonicum* could be monitored by changes in turbidity, absorbance readings at 660 nm as well as viable plate counts of samples of cells at different time intervals were obtained. Figures 4.1(A) and 4.1(B) showed changes in turbidity as measured by optical density at 660 nm over incubation time of the 6 B. japonicum strains grown at 30°C, 200 rpm, in yeast extract glycerol medium and in yeast extract mannitol medium respectively. The results showed turbidity of cultures of strains SK3, SK4, SK26, and SK28 increased at the same rate in both media up to 4 days after which increase in turbidity seemed to level off when SK3, SK26 and SK28 were grown in yeast extract mannitol broth (Figure 4.1B).. The strain S162 showed lower rate of increase in turbidity when grown in yeast extract glycerol medium. B. japonicum S76 culture showed slower rate of increase in turbidity when grown in yeast extract mannitol medium The overall results indicated that cultures of SK3, SK4, SK26, SK28 and S76 increased in turbidity when grown in yeast extract glycerol medium more than when grown in yeast extract mannitol medium. However, culture of S162 become more turbidity when grown in yeast extract mannitol medium.

The results showed that the numbers of days needed for cells to grow to mid-log phase were 2.75 days for SK3, SK4, SK26, SK28 and S76 and 4.5 days for S162 when grown in yeast extract glycerol medium and 2.25 days for SK3, SK4, SK26 and SK28, 3.25 days for S76 and 4.5 days for S162. Mid-log phase cells were used as inoculum for the next experiments on RAPD-PCR fingerprinting and the effects of temperature on growth and gene expression.



Figure 4.1 Changes in turbidity of growth cultures of 6 *B. japonicum* strains grown in (A) yeast extract glycerol medium (B) yeast extract mannitol medium, at 30°C, 200 rpm.



Figure 4.2 Turbidity of 6 *B. japonicum* strains grown in (A) yeast extract glycerol medium (B) yeast extract mannitol medium, at different temperatures, 200 rpm.



Figure 4.3 Viable plate counts of each strain of *B. japonicum* SK3, SK4, SK26, SK28, S76 and S162 grown in (A) YGB at 30°C (B) YMB at 30°C (C) YGB at 40°C (D) YMB at 40°C.

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4.2 Effects of temperature and growth medium on turbidity and viable plate counts of *B. japonicum* cultures

When the six strains of *B. japonicum* were grown in either yeast extract glycerol broth or yeast extract mannitol broth at different temperatures from 25° C to 45° C, the results as shown in Figure 4.2(A) and 4.2(B) indicated that turbidity increased uniformly up to OD₆₆₀ of 1.4 in 4 days when cells were grown in yeast extract glycerol broth(YGB) at 25° C to 45° C. Culture of strain S76 seemed to be less turbid when grown in YGB at 40° C. Increased turbidity of the six *B. japonicum* culture tended to be less uniform when cells were cultured in yeast extract mannitol broth(YMB) with maximum turbidity less than OD₆₆₀ of 1.4 for cultures of strains S162 and S76. One remarkable finding was the increase in turbidity of cultures of strains SK3 and SK26 at 45° C when cells were grown in YMB as opposed to no increase in turbidity when cells were grown in YGB at the same temperature. When grown under 25° C - 45° C cultures seemed to increase rapidly then maintain turbidity of OD₆₆₀ at 0.4.

The viable plate counts results as shown in Figure 4.3 reflected the same extent of turbidity when cultures were spread on either YMA agar or YGM medium.



4.3 RAPD-PCR fingerprinting of 6 isolated strains

Figure 4.4 showed RAPD-PCR fingerprints of the 6 *B. japonicum* strains used in this research. The results indicated that the 6 strains were different because they were found to have different sets of fingerprints when either RPO1 or CRL-7 was used as the primer. There seems to be two groups of *B. japonicum*. The first group with 4-6 DNA fragments in RPO1 PCR fingerprints of *B. japonicum* SK3, SK4, SK26 and SK28 isolated from Ta Muang district, Saraburi province. The second group with one RPO1 PCR product consisted of S76 and S162 which were isolated from Nern Mahatsajan, Phetchaboon province.



Figure 4.4 PCR fingerprinting of *B. japonicum* SK3, SK4, SK26, SK28, S76 and S162 when either RPO1 or CRL-7 was used as the primer.



4.4 RT-PCR

Table 4.1 showed sequences and properties of primers designed for RT-PCR of several nodulation genes and nitrogen fixation genes as well as those for reverse-transcription amplification of gene for GAPDH.

Table 4.1 Nucleotide sequences and properties of primers designed for RT-PCR of nodulation genes and nitrogen fixation genes as well as gene for GAPDH.

Primor	Nucleotide sequences	PCR product	N/GG	T (°C)*
	(5'	sizes (bp)	%GC	$T_m(C)^*$
nodD ₁				
Forward primer	5 [,] AAAATGGCAGCAGYTCGAA 3 [,] (19 bases)	317	10 10/	54.2
Reverse primer	5 [,] CAACATCAATCTGAGCCAG 3 [,] (19 bases)	517	42.170	54.2
nodA				
Forward primer	5. GGTGGAGCGGACGCGAATGC 3. (20 bases)		70.0%	67.0
Reverse primer	5 [,] GCTCGCCGATCATGCCG 3 [,] (17 bases)	414	70.5%	62.8
nodB				
Forward primer	5. AGCACAATTGCWCCCGGGCG 3. (20 bases)	204	65.0%	64.9
Reverse primer	5 [,] GCAACATCGGGTCCCCGCGA 3 [,] (20 bases)	304	70.0%	67.0
nifH				
Forward primer	5 [,] AGCCACCGCAAACAACGTCG 3 [,] (20 bases)	262	60.0%	62.9
Reverse primer	5 [,] ATCGGCAAGTCCACCACTTC 3 [,] (20 bases)	303	55.0%	60.8
nifD				
Forward primer	5 [,] TCMAGCAGAATTCGCGA 3 [,] (17 bases)		52.9%	55.5
Reverse primer	5 [,] AACTATTACGTTGGCAC 3 [,] (17 bases)	445	41.1%	50.7
GAPDH				
Forward primer	5 [,] YTCGTTGTCGTACCAG 3 [,] (17 bases)	409	58.8%	57.9
Reverse primer	5 [,] CTGCACSACSAACTGC 3 [,] (17 bases)	498	64.7%	60.4

*T_m use formula T_m = $63.3 + 0.41 \times GC\%$ - 500/length (Pastorino et al., 2003)

(Y = C or T; W = A or T; M = A or C; S = C or G)

	SK3	SK4	SK26	SK28	S76	S162
nodD ₁	500 400 300	500 400 300	500 400 300	500 400 300	500 400 300	500 400 300
nodA	500 400	500 400	500 400	500 400	500 400	500 400
nodB	500 400 300	500 400 300	500	500 400 300	500 400 300	500 400
nifH	500 400 300	500 400 300	500 400 300	500 400 300	500 400 300	500 400 300
nifD	500 400	500 400	500 400	500 400	500 400	500 400

Figure 4.5 Expression of $nodD_1$, nodA, nodB, nifH, and nifD of *B. japonicum* strains SK3, SK4, SK26, SK28, S76 and S162 grown in yeast extract glycerol medium at 25°C (lanes 2), 30°C (lanes 3), 35°C (lanes 4) and 40°C (lanes 5), 200 rpm. Lanes 1 are molecular size standards. GAPDH bands are 498 bp.

	SK3	SK4	SK26	SK28	S76	S162
nodD ₁	500	500	500	500	500	500
	400	400	400	400	400	400
	300	300	300	300	300	300
nodA	500	500	500	500	500	500
	400	400	400	400	400	400
nodB	500 400 300	500 400 300	500 400	500 400 300	500 400 300	500 400
nifH	500	500	500	500	500	500
	400	400	400	400	400	400
	300	300	300	300	300	300
nifD	500	500	500	500	500	500
	400	400	400	400	400	400

Figure 4.6 Expression of $nodD_1$, nodA, nodB, nifH, and nifD of *B. japonicum* strains SK3, SK4, SK26, SK28, S76 and S162 grown in yeast extract glycerol medium at 45° C for 5 h, 200 rpm. Lanes1 are molecular size standards. GAPDH bands are 498 bp.

Figure 4.5 showed RT-PCR results when each total RNA of the six strains of *B. japonicum* was used with primers designed to be specific for amplification of $nodD_1$, nodA, nodB, nifH and nifD. The results indicated differential gene expression in only four out of the 30 combinations of 6 strains and 5 sets of primers. These four combinations with differential gene expression when cells were grown at different temperatures were $nodD_1$ of SK4, $nodD_1$ of SK26, $nodD_1$ of SK28 and nodB of S76. $nodD_1$ expression in SK26 increased when cells were grown at 35 °C and 40 °C. $nodD_1$ in SK4 and SK28 expression was lower when cells were grown at 35 °C and 40 °C (Figure 4.5). No differential gene expression of $nodD_1$ was

observed when other *B. japonicum* strains SK3, S76, and S162 were grown at different temperatures.

Differential *nodB* gene expression was also observed when S76 was grown under 25° C, 30 °C, 35 °C and 40 °C. The expression was found to increase when growth temperatures were 35 °C and 40 °C. No differential *nodB* expression was observed when the other five *B. japonicum* strains were grown at different temperatures.

No differential gene expression was observed for $nodD_1$, nifA, nifH, and nifD genes when the six strains of *B. japonicum* were grown at different temperatures from 25 °C to 40 °C.

Figure 4.6 showed $nodD_{1}$, nodA, nodB, nifH, and nifD of all *B. japonicum* strains were expressed in cells grown at 45 °C for 5 h. It is remarkable that in SK28 cells grown at 45 °C for 5 h there was no $nodD_{1}$ gene expression. The result seemed to be in line with those given in Figure 4.5 where cells of SK28 grown at 40 °C showed relatively little $nodD_{1}$ gene expression.

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CHAPTER V

DISCUSSION

5.1 Growth and RAPD-PCR fingerprints of *B. japonicum*

The experimental results as shown by turbidity profiles and viable plate counts (Figures 4.2 and 4.3) seemed to indicate different degrees of thermotolerance in B. japonicum. It was found out that, in both YGB and YMB medium, B. japonicum SK3, SK4, SK26 and SK28 were definitely more thermotolerant than S76 and S162. Therefore the six B. japonicum strains used in this research seemed to be grouped, based on degree of thermotolerance, into two groups of more thermotolerant (SK3, SK4, SK26 and SK28) and less-thermotolerant strains (S76 and S162). RAPD-PCR fingerprints also indicated the strains used in this research were of two groups: The first group consisted of the more thermotolerant strains (SK3, SK4, SK26 and SK28) with 4-6 PCR products when RPO1 was used as the primer. The second group consisted of the less-thermotolerant strains (S76 and S162) where the number of PCR products was one when RPO1 was used as the primer. In addition, the more thermotolerant strains were found to have more GC rich areas as reflected by the higher number of PCR products when the GC rich arbitrary primer CRL-7 was used. It remains to be seen if heat tolerance properties could be included in polyphasic taxanomic determination at species level of soybean rhizobia. In 2003 Thomas-Oates used polyphasic taxonomy to determine 15 isolates of rhizobia isolated from different leguminous hosts. Symbiotic properties, production of extracellular enzymes for breakdown of 41 synthetic substrates, antibiotic resistance properties, two-primer RAPD PCR patterns as well as patterns of low molecular weight RNA molecules and a phylogenetic tree based on 16S rDNA sequences were used to group the 15 rhizobial isolates into Rhizobium tropici and R. gallicum. Perhaps, in the case of polyphasic taxonomy of B. japonicum to identify the organisms to the species level, apart from symbiotic properties, degree of heat tolerance could be included in the polyphasic taxonomy. Once B. japonicum SK3, SK4, SK26 and SK28 were established as being more thermotolerant than S76 and S162, interpretation of differential nodulation and nitrogen fixation gene expression could be made based on the degree of heat tolerance property.

5.2 Effects of temperature on nodulation and nitrogen fixation gene expression

The results on the effects of temperature on nodulation and nitrogen fixation gene expression were discussed in terms of growth temperatures from 25° C to 40° C and the effect of 45° C on gene expression. The reason was because log phase cells were obtained when cells were grown in YGB and YMB medium at 25° C - 40° C but not much growth was obtained when cells were grown at 45° C. (Figures 4.2 and 4.3) cells were grown at 45° C for 5 h before harvesting for RT-PCR experiments. In addition, attempts were made to discuss the results in terms of gene expression responses to temperatures in less-thermotolerant *B. japonicum* strains S76 and S162 and in term of the more thermotolerant *B. japonicum* strains SK3, SK4, SK26 and SK28.

5.2.1 Differential nodulation gene expression

The expression of $nodD_1$ was found to be the lowest because for RT-PCR experiments of other gene expression, the concentration of RNA template of 100 ng was sufficient for the detection of gene expression, but, for $nodD_1$, the concentration of the template was 2000 ng, for the detection of $nodD_1$ expression. The finding was as expected because normally, with no induction of $nodD_1$ by the soybean flavonoids (Genistein and Daidzein in slow-growers; Daidzein and Coumestrol in fast growers, Bellato et al.(1997a,b), the constitutively expressed level of $nodD_1$ was low. The finding that $nodD_1$ expression increased with increase in growth temperatures in the heat-tolerant *B. japonicum* SK26 strain used in this study was interesting. Heat seemed to induce $nodD_1$ expression in the other heat-tolerant strain SK26 although it was found to reduce $nodD_1$ expression in the other heat-tolerant strains (SK4 and SK28). It is speculated that heat may change promoter topology enabling RNA polymerase to bind more firmly in SK26 and to bind more loosely in SK4 and SK28. It is interesting to note that there was no expression of $nodD_1$ in cells of *B. japonicum* SK28 grown at 45°C for 5 h. The result agreed well with the finding that under 35°C and 40°C there was relatively little *nodD*, gene expression in this strain (Figures 4.5, 4.6).

The effects of temperature on expression of *nodA* and *nodB* was far less marked when compared to differential *nodD*¹ expression. Most strains were found to express *nodA* in similar levels when grown under different temperatures (Figure 4.5). However, it is interesting to note that *nodB* expression in the less heat-tolerant *B. japonicum* S76 was found to decrease with increase in growth temperatures. Since *nodB* encodes N-deacetylase activity to remove N-acetyl group of N-glucosaminyl unit at the non-reducing end of the Nod-factor, the activity did not seem to be crucial to the less heat-tolerant strain S76.

5.2.2 Differential nitrogen fixation gene expression

No differential *nifH* and *nifD* gene expression responses to growth temperature was observed in all heat-tolerant *B. japonicum* strains used in the study, (Figure 4.5). The results seemed to imply that mRNAs of *nifH* and *nifD* of thermotolerant *B. japonicum* were stable at $30^{\circ}C-40^{\circ}C$ and at $45^{\circ}C$ for 5 h.

It is suggested that nitrogen fixation potential in terms of shoot dry weight as well as activity of deactylase enzyme (encoded by *nodB*) should be carried out to verify the results obtain by RT-PCR.

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CHAPTER VI

CONCLUSION

The experimental results indicated that *B. japonicum* SK3, SK4, SK26 and SK28 were more thermotolerant than strains S76 and S162 when vialble plate count experiments were conducted on yeast extract glycerol and yeast extract mannitol medium incubated at 30° C and 40° C. RAPD-PCR fingerprints with either RPO1 or CRL-7 as the primers showed the six *B. japonicum* were different strains which could be divided into two groups based on the number of RAPD-PCR product bands. Group I consisted of the more heat tolerant SK3, SK4, SK26 and SK28 strains with 4-5 RPO1-PCR products and more than 3 CRL-7 PCR products. Group II consisted of the less-heat tolerant strains, S76 and S162, with one RPO1 PCR product and 1-2 CRL-7 PCR products. RT-PCR experiments revealed the more thermotolerant strains SK4, SK26 and SK28 showed differential *nodD*₁ gene expression while the less thermotolerant strain S76 showed high *nodB* gene expression when cells were grown at 35° C and 40° C. It is suggested that nitrogen fixation potential in terms of shoot dry weight as well as activity of deactylase enzyme (encoded by *nodB*) should be carried out to verify the results obtain by RT-PCR.

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REFERENCES

- Babst, M., Hennecke, H. and Fischer H.M., 1996. Two different mechanisms are involved in the heat shock regulation of chaperonin gene expression in *Bradyrhizobium japonicum*. <u>Mol. Microbiol</u>. 19(4) : 827-839.
- Bellato, C.M., Krishnan, H.B., Temprano, F. and Pueppke, S.G. 1997a. The soybean cultivar specificity gene *nolX* is present, repressed in a *nodD*-dependent manner, of symbiotic significance in cultivar-nonspecific strains of *Rhizobium* (*Sinorhizobium*) fredii. <u>Microbiology</u> 143 : 1381-1388.
- Bellato, C.M., Pueppke, S.G., and Krishnan, H.B. 1997b. Regulation of the expression of the *nod* box-independent nodulation gene, *nolX*, in *Sinorhizobium fredii*, a nitrogen-fixing symbiont of legume plants. <u>FEMS Microbiol. Lett</u>. 157 : 13-18.
- Day, J.M., Roughley, R.J., Eaglesham, A.R.S., Dye, M., and White, S.T. 1978. Effect of high soil temperatures on nodulation of cowpeas, *Vigna unguiculata*. <u>Proceedings of the</u> <u>Association of Applied Biologists</u>. p 476-481.
- Elkan, G.H., and Bunn, C.R. 1992. The Rhizobia. In Balows, A., Truper, H.G., Dworkin, M., Harder, W., Schleifer, K-H (eds). The Prokaryotes. 2nd Edition. Chapter 107. New York : Springer Verlag.
- Fischer, H.M., Babst, M., Kasper, T., Acuna, G., Arigoni, F., and Hennecke, H. 1993. One member of a groESL-like chaperonin multigene family in Bradyrhizobium japonicum is co-regulated with symbiotic nitrogen fixation genes. <u>EMBO J</u>. 12 : 2901-2912.
- Forsberg, A.J., Pavitt, G.D., Higgins, C.F. 1994. Use of transcriptional fusions to monitor gene expression: a cautionary tale. <u>J.Bacteriol</u>. 176(7) : 2128-2132.
- Göttfert, M. 1993. Regulation and function of rhizobial nodulation genes. <u>FEMS Microbiol.</u> <u>Rev</u>. 104 : 39-64.
- Jordan, D.C. 1984. Family III. Rhizobiaceae In Krieg, N.R., Holt, J.G. (eds). <u>Bergey's Manual</u> <u>of Systematic Bacteriology</u>. Baltimore : Williams & Wilkins. p. 234-244.

- Kim, J., Rees, D.C. 1992. Structural models for the metal centers in the nitrogenase molybdenum-iron protein. <u>Science</u>. 257 : 1677-1682.
- Kosslak, R.M., Bookland, R., Barkai, J., Paaren, H.E., and Applebaum, E.R. 1987. Induction of *Bradyrhizobium japonicum* common nod genes by isoflavones isolated from *Glycine max*. <u>Proc. Natl. Acad. Sci USA</u>. 84: 7428-7432.
- Loh, J., Stacey, G. 2003. Nodulation gene regulation in *Bradyrhizobium japonicum* : a unique intergration of global regulatory circuits. <u>Appl. Environ. Microbiol</u>. 69(1) : 10-17.
- Long S.R. 1996. *Rhizobium* Symbiosis: Nod Factors in Perspective. <u>The Plant Cell</u>. 8 : 1885-1898.
- Ly, K.P. and Chansa-ngavej, K. 2006. Correlation between RAPD PCR fingerprints, heat responses, and nitrogen fixation potential of soybean rhizobia. Report for the UNESCO Inter-University Training Course in Biotechnology. 10pp.
- Mathis, J.N., and McMillin, D.E. 1996. Detection of genetic variation in *Bradyrhizobium japonicum* USDA 110 variants using DNA fingerprints generated with GC rich arbitrary PCR primers. <u>Plant and Soil</u>. 186 : 81-85.
- Minder, A.C., Fischer, H.M., Hennecke, H., and Narberhaus, F. 2000. Role of HrcA and CIRCE in the heat shock regulatory network of *Bradyrhizobium japonicum*. J. Bacteriol. 182 : 14-22.
- Minder, A.C., Narberhaus, F., Babst, M., Hennecke, H. and Fischer, H.M. 1997. The *dnaKJ* operon belongs to the ³²**σ**-dependent class of heat shock genes in *Bradyrhizobium japonicum*. <u>Mol. Genet</u>. 254 : 195-206.
- Moffat, A.S. 1992. An intimate look at nitrogen's bio-partner. Science 257 : 1624-1625.
- Münchbach, M., Nocker, A. and Narberhaus, F. 1999. Multiple small heat shock proteins in rhizobia. <u>J. Bacteriol</u>. 18 (1) : 83-90.
- Nocker, A., Hausherr, T., Balsiger, S., Krstulovic, N-P., Hennecke, H., and Narberhaus, F. 2001. A mRNA-based thermosensor controls expression of rhizobial heat shock genes. <u>Nucleic Acids Res</u>. 29 (23) : 4800-4807.

- Pueppke, S.G. 1984. Adsorption of slow- and fast-growing rhizobia to soybean and cowpea roots. <u>Plant Physiol</u>. 75 : 924-928.
- Richardson, A.E., Viccars, L.A., Watson, J.M., and Gibson, A.H. 1995. Differentiation of *Rhizobium* strains using the polymerase chain reaction. <u>Soil Biol. Biochem</u>. 27 : 515-524.
- Saengkerdsub, S., 1999. Effects of initial pH on hydrogenase activity and protein patterns of acid-tolerant *Bradyrhizobium japonicum*. M.Sc.thesis. Industrial Microbiology Program. Chulalongkorn university.
- Sambrook, J., and Russel, D.W. 2001. Molecular Cloning: A Laboratory Manual, 3rd Ed. New York: Cold Spring Harbor Laboratory Press. Book1.
- Schofield, P.R., and Watson, J.M. 1985. Conservation of *nif* and species-specific domains within repeated promoter sequences from fast-growing *Rhizobium* species. <u>Nucleic Acids Res</u>. 13 (10) : 3407-3418.
- Somchoke Kala, 2003. Determination of heat shock proteins in *Bradyrhizobium japonicum*. Senior Project Report. Department of Microbiology, Faculty of Science Chulalongkorn University. (in Thai).
- Stacey, G., 1995. *Bradyrhizobium japonicum* nodulation genetics. <u>FEMS Microbiol. Letters</u>. 127 : 1-9.
- Voet, D., Voet, J.G. 1995. Biochemistry. Second Edition. New York : John Wiley & Sons. p. 776 781.
- Wang, S-P., and Stacey, G. 1991. Studies of the *Bradyrhizobium japonicum nodD1* promoter : A repeated structure for the *nod* box. <u>J. Bacteriol</u>. 173 (11) : 3356-3365.

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APPENDICES

APPENDIX A

BACTERIAL GROWTH MEDIA

Preparation of all bacterial growth media is as described by Somasegaran and Hoben (1994) unless otherwise stated.

Yeast Extract Mannitol Broth (YMB)

Mannitol	10.0 g
K ₂ HPO ₄	0.5 g
MgSO ₄ .7H ₂ O	0.2 g
NaCl	0.1 g
Yeast extract	0.5 g
Deionized water	1.0 g

pH of medium was adjusted to 6.8 with 0.1 N NaOH. The medium was autoclaved at 121°C for 15 min.

Yeast Extract Mannitol Agar (YN	IA)
YMB	1 liter
Agar	15 g

Agar was added to 1 liter of YMB. The solution was shaken to suspend the agar then autoclaved at 121°C for 15 min. After autoclaving, the medium was shaken to ensure even mixing of melted agar with medium before pouring onto petridishes and left to solidify.

YMA with Congo Red

Congo Red stock solution: 250 mg of Congo Red dissolved in 100 ml of deionized water. 10 ml of Congo Red stock solution were added to 1 liter of YMA. The final Congo Red concentration was 25 μ g.ml⁻¹. The medium was autoclaved at 121°C for 15 min.

Yeast Extract Glycerol Broth (YGB)

Glycerol	10.0 ml
K ₂ HPO ₄	0.5 g
MgSO ₄ .7H ₂ O	0.2 g
NaCl	0.1 g
Yeast extract	0.5 g
Deionized water	1.0 g

pH of medium was adjusted to 6.8 with 0.1 N NaOH. The medium was autoclaved at 121°C for 15 min.



APPENDIX B

CHEMICALS AND SOLUTIONS

1. Solutions for DNA extraction

Saline-EDTA solution

15 mM NaCl, 10 mM EDTA, pH 8.0

0.9 g NaCl, 0.29 g EDTA were added to distilled water. The final volume was made to 100 ml. 0.1 N NaOH was used to adjust pH to 8.0 before autoclaving at 121°C for 15 min.

DNAzol

2.

DNAzol solution (Gibco BRL) was used according to manufacturer's instruction.

Solutions for RINA extraction		
RNA loading buffer		
Glycerol	500.0	μΙ
Bromophenol blue	2.0	hð
Xylene Cyanol	2.0	hà
0.5 M EDTA	2.0	μΙ
The final volume was made to 100 ml.	by deionize	ed distill water
25x MOPS buffer		
MOPS	41.82	g
Sodium acetate	6.80	g
EDTA	0.38	g
The final volume was made to 100 ml.	by deionize	ed distill water
RNA Sample buffer		
25x MOPS	40.0	μΙ
di-Formamide	500.0	μΙ
di-Formaldehyde	55.0	μΙ

The final volume was made to 1,000 ml. by deionized distill water

Trizol

Trizol solution (Invitrogen) was used according to manufacturer's instruction.

TriReagent

TriReagent solution (Gibco BRL) was used according to manufacturer's instruction.

 3. 50X TAE buffer (pH8.0)

 Tris-base
 24.20 g

 Na2EDTA2H2O
 1.86 g

 Acetic acid
 5.71 ml

The final volume was made to 100 ml. by deionized distill water



APPENDIX C

Nucleotide sequences for design of primers

1. $nodD_1$ forward and reverse primers are in boxes

	2.63		331	1 11			342	151	
Bjapo2 Djapo110 Bjapu1 Belka94								c	ATCCC ATGCG ATCACATGCG ATGCG
BSD B.japoCom	ACTTCAGATT	AATTAAGCGC	TTTCTAACGA	TTTGCATAAT	TGATCGTTCG	GATGACAACC	ATCCGCACTG	GGATAGACTC TGGATTCGCC	ACAACATGCG AGAAC <mark>ATGCG</mark>
	370	1		481	413	421	430	Re	everse primer
Djapo2 Djapo110 Bjapo1 Belka94 BSD B.japoCom	GTTCAAGGGA GTTCAAGGGA GTTCAAGGGA ATTCAAGGGA ATTCAAGGGA TTTTAAGGGC	CTTGATCTAA CTTGATCTAA CTTGATCTAA CTTGATCTAA CTTGATCTAA CTTGATCT <mark>C</mark> A	ATCTTCTCGT ATCTTCTCGT ATCTTCTCGT ACCTTCTCGT ACCTTCTCGT ATCTCCTCGT	TGCGCTCGAC TGCGCTCGAC TGCGCTCGAC TGCGCTCGAT TGCGCTCGAC	GCCGTGATGA GCCGTGATGA GCCGTGATGA GCTCTGATGA GCTCTGATGA GCACTGATGA	CCCCCCCCAA CCCCCCCCCAA CCCACCCCCCAA CCCACCCCCAA CCCAACCCCAA	CCTCACAGCG CCTCACAGCG CCTCACAGCG CCTCACAGCG CCTCACTGCG ACTCACGCGC	GCGGCTCGCA GCGGCTCGCA GCGGCTCGCA GCGGCGCGCGC GCGGCACGCA GCTGCACGCA	AAATCAATCT AAATCAATCT AAATCAATCT AAATTAACCT AAATCAATC
	450	4/3	41	4.91			9.23	1.11	1 341
Djapo2 Bjaµv110 Bjapo1 Belka94 Rsp B.japoCom	GAGCCAGECT GAGCCAGECT GAGCCAGECT GAGCCAGECC GAGCCAGECC GAGCCAGECC	GCTATGAGCG GCTATGAGCG GCTATGAGCG GCCATGAGCG GCCATGAGCG GCCATGAGCG	CTGCGATCGC CTGCGATCGC CTGCGATCGC CTGCGATCGC CTGCGATCGC CACCGATCAC	ACCOUNTRECCO ACCOUNTRECCO ACCOUNTRECCO CCCCUNTRECC CCCCUNTRECC	ACCTATTICC ACCTATTICC ACCTATTICC ACCTATTICC ACCTATTIC ACCTATTIC ACCTATTICC	GCGATGAACT GCGATGAACT GCGATGAACT GCGATGAATT GTGATGAATT GCGATGACCT	CTITACTATG CTITACTATG CTITACTATG CTITACTATG CTITACTATG ATITACCATC	AGAGGTCGCG AGAGGTCGCG AGAGGTCGCG AGAGGTCGCG AGAGGTCGCG A <mark>AT</mark> CCTCCCG	AACTCGTCCC AACTCGTCCC AACTCGTCCC ACTCGTCCC ACCTCGTCCC AACTTCTACC
	550	563	571	581	593	631	£10 	620 	611
Bjapu2 Bjapo110 Bjapo1 Belka94 Bsp B.japoCom	GACACOTOGO GACACOTOGO GACACOTOGO GACACOCOGO AACACOCOGO AACACOCOGO AACTOCOCOGA	GCGGAAGCGC GCGGAAGCGC GCGGAAGCGC GCGGACGCGC GCGCACGCGC CC <mark>ACAACCCC</mark>	TTGCACCTCC TTGCACCTCC TTGCACCTCC TTGCCACCTCC TTGCCACCCC T <mark>GCC</mark> ACCCCC	GGTTCGCGAG GGTTCGCGAG GGTTCGCGAG CGTTCGCGAG TGTCCCCGAG ACTCCCCCGA	GCCCTGCTGC GCCCTGCTGC GCCCTGCTGC GCCCTGCTGC GCCCTGCTGC GCCCTGCTGC	ACATCCAACT ACATCCAACT ACATCCAACT ACATCCAACT ACATCCAACT ACATCCATC	CTCAATCATA CTCAATCATA CTCAATCATA CTCCATCATT CTCCATCATC CTCCATCATC	TCGCGGGACG TCGCGGGACG TCGCGGGACG TCTCGGGATG TCAACGCACG TCAACGCACG TCATCCCATC	CGCTCGACCC CGCTCGACCC CGCTCGACCC CGTTCGACCC CGTTTGACCC CGTTGACCC
Bjapo2 Bjapo110 Bjapo1 Belka94 Bøp B.japoCom	IGCTCAATCC IGCTCAATCC IGCTCAATCC CACCCTCAATCC AACTCACTCC ACCCCACTCA	ACCCACGOT ACCCGACGOT ACCCGACGOT ACCCGACGOT AACCCACCCT CATCCCACGT	TCAGGGTCAT TCAGGGTCAT TCAGGGTCAT TCAGGGTCAT TCACCATCAT TCACCATCAT	TCTCTCAGAT TCTCTCAGAT TCTCTCAGAT TCTCTCAGAT TCTCTCCCAT TCTTTCCCAC	TTCATGACGA TTCATGACGA TTCATGACGA TTCATGACAA TTCATCACCC	ICCULTITI ICCULTITI ICCULTITI ICCUCTITITI ICCUCTITITI ICCUCTITITI	CCGCAGAATT CCGCAGAATT CCGCAGAATT TCCCAGAATT CCGAGAATT	GTGGACCECA GTGGACCECA GTGGACCECA GTGGACCECA GTGGTCATCETA	ICECCEAAGA ICECCEAAGA ICECCEAAGA ICECCEACACEA ICECCECCEA
	TEL	Forw	ard primer		77	р V С ₇₆₁	1 b 1 792	BOS BOS	I BLI
Bjapo2 Bjapo110 Bjapo1 Belka94 B <i>e</i> p	ACCCCCCCCCC ACCCCCCCCCC ACCCCCCCCCC CCCCCC	CTGCCCTTCC GTGCCCTTCC GTGCCCTTCC GTGCCCTTCC GTGCCCTTCC	AACTGCTGCC AACTGCTGCC AACTGCTGCC AACTGCTGCC AATTGCTGCC	ATTTTCTGAT ATTTTCTGAT ATTTTCTGAT ATTTTCCGAT	GAACCGGATG GAACCGGATG GAACCGGATG GAACCGGGTG GAACCGGGTG	ACCTRETERS ACCTRETERS ACCTRETERS ACCTRETERS	GUUGUUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGU	GTCGACTTTC GTCGACTTTC GTCGACTTTC GTCGACTTTC GTCGATTTTC	TEATTETGEE TEATTETGEE TEATTETCEE TEATTTTECE
D.japoCom	AGCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC	GTCAGT TTCC	AGTTGCTGCC	GTTTTCCGAT	<u>GACCCAGATG</u>	ACCTTCTCCC	GCGTGGTGAT	GTCGATTTCC	TCATCCTACC
Rjapo2 Bjapo110 Bjapo1 Belka94 Dop	CIAACUTTTC CAACUTTTC CAACUTTTC CAATUCTTC	ATETECACE ATETECACE ATETECACE ATETECACE ATETECACE	сселсстал Сселсстал Сселсстал Сселсстал Сселтстал	GREGACIETTG CCCCACCETC CCCCACCETC CCCCACCETA	TTEGACGAGA TTEGACGAGA TTEGACGAGA TTEGACGAGA TTEGATGAGA	СССТЕСТАТС СССТССТАТС СССТССТАТС СССТССТАТС	CETCEPATEC CETCEPATEC CETCEPATEC CETCEPATEC	EGCGCGAACA CCCCCCGAACA CCCCCCGAACA CCCCCCGAACA	AREAGETATE AGEACETATE AGEACETATE AGEACETATE
B.japoCom	AGAAATCTTC	ATGTCGCACA	CGCATCCCAG	AGCGAAGCTG	TICGANGAGA	GATTCGTCTC	CGTGACTTCC	CAACGAACC	<u>ACAACCTA</u> CC

Djapo2 Bjapo110 Bjapo1 Belka94 Bsp D.japoCom	CCGCCACCTT CCCCCACCTT CCCCCACCTT CCCACCACTT GCCCCCACCTC	ACGTTCCAAC ACGTTCCAAC ACGTTCCAAC ACATTCCACA TCCATCCACA	ААТАСАТСТС ААТАСАТСТС ААТАСАТСТС ААТАСААТТС АСТАТСТАТ	GATGGGGCAC CATGGGGCAC CATGGGGCAC AATCCCCCAC AATGGGGCAT	GTTACTGCCA GTTACTGCCA GTTACTGCCA GTCACCCCCA GTTGCGGCCCC	ACTTCCCACC ACTTCCCACC ACTTCCCACC ACTTCCCACC RATTCCCCCC	CGCACTEAEA CGCACTEAEA CGCACTEAEA CCCCCTEAEC AAGEAECEG	CCGAACCTCC CCGAACCTCC CCGAACCTCC CCCAACCTCC CCTTCCGTCC	алсаатсстт Алсаатсстт Алсаатсстт Алсаатсстт Ассастсстт Ассаатссст
Bjapo2 Djapo110 Bjapo1 Belka94 Døp B.japoCom	TTTECTTEAC TTTECTTEAC TTTECTEAC CTTECTEAC ATTECCEAC	CACGGCCTGA CACGGCCTGA CACGGCCTGA CACGGCTIGA CACGGACTGC	GCACACCAAT GCACACCAAT CCACACCAAT AAAGCCCCAT CAACACCCCA	TGACGTCCTC TGACGTCCTC TGACGTCGTC CGAGGTCGTT CGAAGTCGCC	GTGCAGGGCT GTGCAGGGCT GTGCAGGGCT GTGCAGGGCT GTGCCGGGCTT	TTACCCTGAT TTACCCTGAT TTACCCTGAT TTACCCTGAT TTACCATGAT	TCCCCCCCTG TCCCCCCCTG TCCCCCCCTG TCCCCCCCTG TCCCCCCCTTG TCCCCCCTTG TCCCCCCTTG	TTECTAPACA TTECTAPACA TTECTAPACA ITETICEACA TTETICECACA	GAGCCGTAT GAGCCGTAT GAGCCCTAT GGGGCCGCAT GGGGCCGCAT
Djapo2 Bjapo110 Bjago1 Belka94 Bsp D.japoCom	CECCACCANG CECCAUCANG CECCACCANG ACCAACAANG ACCAACAANG AECGACCCNC	CCCTTACGAC CCCTTACCAC CCCTTACCAC CCCTTACCAC CCCTTACCAC	Tercacea Tercacea Tercacea Tercaca Tercaca	CTTCCAAAAG CTTCCAAAAG CTTCCAAAAG CTTCCAAAAC CTTCCGCAAAA	CCCATCCCGT CCCATCCCCT CCCATCCCCT TCCATCCCCC GCCATTCCCCC	TGCGGATCAT TGCGGATCAT TGCGGATCAT TGCCGATCAC	ССЛАССС ССЛАССС ССЛАССС ССЛСССЛ ССЛСССЛ	CCCTTCCCC CCCTTCCCC CCCTCCCCC CCACTCCCAC CAACCCATTT	Toccoacatt Tocccacatt Tocccacatt Tocccacatt Tocccacatt
Rjapo2 Bjapo110 Bjapo1 Belka94 Dop B.japoCom	CACAGAGGGC CACAGAGGGC CACAGAGGCC TACCGAGGCC CACCGAGGCC	CTCCAGTOGC CTCCAGTOGC CTCCACTCCC GTCCAGTOGC	CTUATTCEA CTOATTCEA CTEATTCEA CTEATTCEA CTEATTCEA	CAATACCEAC CAATACCEAC CAATACCEAC CAATACCEAC CAATACCEAT CACCACTEAT	CECCCARCA CCCCCCACCA CCCCCCACCA CCCCCCACCA CCCCCC	TETEBATECE TETEGATECE TETEGATECE TETEGATECE TETEGATECE	TCCGATATTG TCCGATATTG TCCGATATTG GCCGATATTG CCAGATATTT	CTCCAGGAAA CTCCAGGAAA CTCCACCAAC TTCCACCAAC TTCCACGACCACC CTACACCACC	LATUCAAUAT GATOCAACAT GATOCAACAT GATOCAACAT GATOCAACAT
Djapo2 Bjapo110 Bjapo1 Delka94 Esp B.japoCom	COCATCINEGE CCCATCINEGE CCCATCINEGE CCCATCINEGE CCCATCINEGE TCAATTINC	CRCCACCACC CACCACCACC CRCCACCACC CRCCACCACC CRCCACCACC CRCCACCACC CRCCACCACCACC CRCCACCACCACCACCACCACCACCACCACCACCACCACC	CTOCAACTCC CTOCAACTCC CTOCAACTCC TACCAACCCC TTOGGCC	CACCCCTCT CACCCCTCT CACCCCCTCT CACCCCCTGT CACCCCCTGC CATGCTCTAT	тёс тёс тёс тёс сётсатстса	ATTGCCTACA	TGCCTCTAAA	GCCGACGGCG	CAACTCGCCA

สถาบันวิทยบริการ จุฬาลงกรณ์มหาวิทยาลัย



2. nodA forward and reverse primers are in boxes

3. nodB forward and reverse primers are in boxes



4. nodC

Belkanii94n	10 • • • • • • • • • • • • • • тг -	20 ••••• •••• GATOTGOTCO	30 • • • • • • • • • • • • • • • • • •	40 • • • • • • • • • • • • • • • • • • • • •	50 • • • • • • • • • • • • • • • • • •	60 • • • • • • • • • A TROBO TG O	70 ••••• •••• ПСТСБАВСАТ
Bjapo110nod Bjapo Symnod	ATG- AGCACA <mark>ATG</mark> C	GACCTGCTCG GA <mark>GCG</mark> GCCAA	CGACGACCAG ATTCACCGGT	TGCTGCCGCC TTTCGCG <mark>GCC</mark>	GTTTCATCTT GCTGCGCCTT	- ATGCGCTCC TG <mark>TGCGC</mark> GAC	TATCGACGAT TG <mark>C</mark> GAT
Belkanii94n Bjapo110nod	80 I I 	90 GCGCAGGCGC GTGCAAGCGC	100 ····I···I TTT <mark>AC</mark> GCTCA TTTATGCTCA	110 GC - CG A C C A A GC - CG G C C A A	120 ••••• TCCC <mark>T</mark> CATC <mark>G</mark> CAACTCATCG	130 ••••• ••• ••• ••••••••••••••••••••••	140 • • • • • • • • • A T <mark>C</mark> T C G A G <mark>C A</mark> A C C T T G G A C A
Bjapo Symnod	<mark>ст</mark> б-б <mark>аб</mark> сат 150	GCGGACCTAT	CCCAAGCTTA	CCTCTACAGG 180	TCGA <mark>T</mark> G <mark>ATC</mark> A 190	CCGGTGATCC 200	G C <mark>C</mark> G C A A T <mark>C A</mark> 210
Belkanii94n Bjapo110nod Bjapo Symnod	AGCCGGGGTG AGCCGAGGTG AGCCGAGGTG A TGGCATT	GTTCTGCCCA GTCGTTCCTG GAATCACGCA	GCGTGGATGT CTGTGGACGT AATTTCGAGG	AATCGTGCCC GATCGTGCCG GATCAAATCT	TGTTTCAATG TGCTTCAACG CGTTCAGGCA	AGGAC <mark>C</mark> CAAA AGAATCCGAA TATCT <mark>C</mark> GCCG	C A C A C - T C T C C A C A C - T C T C C A C A C - T C G C C G G A T A T G A C
Belkanii94n	220 · · · · I · · · · I CGAAT <mark>G</mark> C	230 CTCGCGTCGA	240 • • • • • • • • • T T <mark>G C</mark> A <mark>A G T C</mark> A	250 AGACTACG <mark>C</mark> C	260 • • • • • • • • • • • • • • • • • • •	270 ••••• ••••• GA <mark>GT</mark> CAT <mark>TG</mark> T	280 ••••••••••••••••••••••••••••••••••••
Bjapo110nod Bjapo Symnod	с даат<mark>д</mark> т сдааа<mark>д</mark>таас	CTGGAGTCGA CTGGAGGCAA	TTGCCAGTCA C-GCGAGCCG	AGACTACG <mark>C</mark> C CATACGTT <mark>C</mark> G	GGAAA <mark>G</mark> ATGC CATG <mark>A</mark> ACCAA	AG <mark>GTATATG</mark> T TC <mark>GT</mark> CCC <mark>TG</mark> C	GGTCGAT <mark>G</mark> A- GCGATGC <mark>GA</mark> A
	290	300	310	320	330	340	350
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Belkanii 94 n	TGGATCC -						
Belkanii94n Bjapo110nod	TGGATCC- CGGATCGG	CAAACCGCGA	CGTTGTCGCG	CCT-GTACAC	CGGATATATG	CGAGCGATCC	GAGATTCAGT
Belkanii94n Bjapo110nod Bjapo Symnod	TGGATCC- CGGATCGG CCTGAGTGGG	CAAACCGCGA ATATCGCCGT	CGTTGTCGCG TCCAGGCCAG	ССТ-GTACAC ТТТСGТАААG	CGGATATATG GTGGACTTTT	CGAGCGATCC CGGGCGCTAA	GAGATTCAGT GATAACCACT
Belkanii94n Bjapo110nod BjapoSymnod	TGGATCC- CGGATCGG CCT <mark>G</mark> AGT <mark>GGG</mark>	CAAACCGCGA ATATCGCCGT	CGTTGTCGCG TCCAGGCCAG	ССТ-БТАСАС ТТТСБТАААБ	CGGATATATG GTGGACTTTT	CGAGCGATCC CGGGCGCTAA	GAGATTCAGT GATAACCACT
Belkanii94n Bjapo110nod Bjapo Symnod	TGGATCC- CGGATCGG CCTGAGTGGG 360	CAAACCGCGA ATATCGCCGT 370	CGTTGTCGCG TCCAGGCCAG 330	CCT-GTACAC TTTCGTAAAG 390	CGGATATATG GTGGACTTTT 400	CGAGCGATCC CGGGCGCTAA 410	G A G A T T C A G T G A T A A C C A C T 420
Belkanii94n Bjapo110nod Bjapo Symnod		CAAACCGCGA ATATCGCCGT 370 	CGTTGTCGCG TCCAGGCCAG 330	ССТ- G Т А С А С Т Т Т С G Т А А А G 390	CGGATATATG GTGGACTTTT 400	CGAGCGATCC CGGGCGCTAA 410	G A G A T T C A G T G A T A A C C A C T 420
Belkanii94n Bjapo110nod BjapoSymnod Belkanii94n	TGGATCC- CGGATCGG CCTGAGTGGG 	CAAACCGCGA ATATCGCCGT 	CGTTGTCGCG TCCAGGCCAG 	CCT-GTACAC TTTCGTAAAG 390	CGGATATATG GTGGACTTTT 400	CGAGCGATCC CGGGCGCTAA 410 	G A G A T T C A G T G A T A A C C A C T 420
Belkanii94n Bjapo110nod BjapoSymnod Belkanii94n Bjapo110nod		CAAACCGCGA ATATCGCCGT 370 TGGCGAACAA	CGTTGTCGCG TCCAGGCCAG 380 TGTGGGAAAG	CCT-GTACAC TTTCGTAAAG 390 CGCAAGGCGC	CGGATATATG GTGGACTTTT 400 	CGAGCGATCC CGGGCGCTAA 410 GATACGCAGC	G A G A T T C A G T G A T A A C C A C T 420 T C A T C C G G T G
Belkanii94n Bjapo5ymnod Belkanii94n Bjapo5ymnod Bjapo5ymnod		CAAACCGCGA ATATCGCCGT 370 	CGTTGTCGCG TCCAGGCCAG 330 TGTGGGAAAG CGACCGTTGT	CCT-GTACAC TTTCGTAAAG 390 	CGGATATATG GTGGACTTTT 400 	CGAGCGATCC CGGGCGCTAA 410 	G A G A T T C A G T G A T A A C C A C T 420 I T C A T C C G G T G C A G C C G C G C G
Belkanii94n Bjapo5ymnod Bjapo5ymnod Belkanii94n Bjapo110nod Bjapo5ymnod		CAAACCGCGA ATATCGCCGT 370]] TGGCGAACAA CATTTTCGC	CGTTGTCGCG TCCAGGCCAG 380 TGTGGGAAAG CGACCGTTGT	CCT-GTACAC TTTCGTAAAG 390 CGCAAGGCGC CGGGGACTGC	CGGATATATG GTGGACTTTT 400 11 AGATCGCAGC AGGTCGCTCT	CGAGCGATCC CGGGCGCTAA 410 II GATACGCAGC GGAGGGGCGG	GAGATTCAGT GATAACCACT 420
Belkanii94n Bjapo5ymnod Bjapo5ymnod Belkanii94n Bjapo110nod Bjapo5ymnod	TGGATCC- CGGATCGG CCTGAGTGGG 360 	CAAACCGCGA ATATCGCCGT 370 11 TGGCGAACAA CATTTTTCGC 440	CGTTGTCGCG TCCAGGCCAG 380 	CCT-GTACAC TTTCGTAAAG 390 	CGGATATATG GTGGACTTTT 400 	CGAGCGATCC CGGGCGCTAA 410 II GATACGCAGC GGAGGGCCGG 480	G A G A T T C A G T G A T A A C C A C T 420 II T C A T C C G G T G C A G C C G C G C G 490 I
Belkanii94n Bjapo5ymnod Bjapo5ymnod Belkanii94n Bjapo5ymnod Bjapo5ymnod	TGGATCC- CGGATCGG CCTGAGTGGG 360 I TTTATCTTGT TTCGAGCCAC 430 I	CAAACCGCGA ATATCGCCGT 370 	CGTTGTCGCG TCCAGGCCAG 330 	CCT-GTACAC TTTCGTAAAG 390 11 CGCAAGGCGC CGGGGACTGC 460 11	CGGATATATG GTGGACTTTT 400 II AGATCGCAGC AGGTCGCTCT 470 II	CGAGCGATCC CGGGCGCTAA 410 II GATACGCAGC GGAGGGCCGG 480 II	G A G A T T C A G T G A T A A C C A C T 420 II T C A T C C G G T G C A G C C G C G C G 490 II
Belkanii94n Bjapo Symnod Belkanii94n Bjapo 110nod Bjapo Symnod Belkanii94n Bjapo 110nod	TGGATCC - CGGATCGG CCTGAGTGGG 360 	CAAACCGCGA ATATCGCCGT 370 11 TGGCGAACAA CATTTTTCGC 440 11 CAACGTCGAT	CGTTGTCGCG TCCAGGCCAG 330 11 TGTGGGAAAG CGACCGTTGT 450 11 TCCGATACGA	CCT-GTACAC TTTCGTAAAG 390 11 CGCAAGGCGC CGGGGACTGC 460 11 TACTTGCTGC	CGGATATATG GTGGACTTTT 400 	410 CGAGCGATCC CGGGCGCTAA 410 II GATACGCAGC GGAGGGCGG 420 II ACGAAGCTTG	G A G A T T C A G T G A T A A C C A C T 420 II T C A T C C G G T G C A G C C G C G C G 490 II T A T T G A A G A T
Belkanii94n Bjapo5ymnod Belkanii94n Bjapo110nod Bjapo5ymnod Belkanii94n Bjapo110nod Bjapo5ymnod	TGGATCC- CGGATCGG CCTGAGTGGG 360 	CAAACCGCGA ATATCGCCGT 370 	CGTTGTCGCG TCCAGGCCAG 330 	CCT-GTACAC TTTCGTAAAG 390 	CGGATATATG GTGGACTTTT 400 	410 CG AG CG AT CC CG GG CG CT A A 410 G AT AC GC AG C GG AG GG GC GG 460 ACG A AG CT TG G A AG GG TC A A	G AG A T T C AG T G A T A A C C A C T 420 11 T C A T C C G G T G C AG C C G C G C G 490 11 T A T T G A AG A T C C T G A C T G C T
Belkanii94n Bjapo5ymnod Bjapo5ymnod Bjapo110nod Bjapo5ymnod Bjapo110nod Bjapo110nod Bjapo5ymnod	TGGATCC - CGGATCGG CCTGAGTGGG 360 	CAAACCGCGA ATATCGCCGT 370 	CGTTGTCGCG TCCAGGCCAG 380 	CCT-GTACAC TTTCGTAAAG 390 11 CGCAAGGCGC CGGGGACTGC 460 11 TACTTGCTGC CGGTGATCAG	CGGATATATG GTGGACTTTT 400 	CGAGCGATCC CGGGCGCTAA 410 11 GATACGCAGC GGAGGGGCGG 450 11 ACGAAGCTTG GAAGGGTCAA	G A G A T T C A G T G A T A A C C A C T 420 II T C A T C C G G T G C A G C C G C G C G 490 II T A T T G A A G A T C C T G A C T G C T
Belkanii94n Bjapo Symnod Belkanii94n Bjapo 110nod Bjapo Symnod Belkanii94n Bjapo Symnod	T G G A T C C - C G G A T C G G C C T G A G T G G G 	CAAACCGCGA ATATCGCCGT 370 	CGTTGTCGCG TCCAGGCCAG TCCAGGCCAG 380 	C C T - G T A C A C T T T C G T A A A G 390 11 C G C A A G G C G C C G G G G A C T G C (G G G G G A C T G C 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1	CGGATATATG GTGGACTTTT 400 	410 410 410 410 410 410 410 410	G A G A T T C A G T G A T A A C C A C T 420 T C A T C C G G T G C A G C C G C G C G G 1 T A T T G A A G A T C C T G A C T G C T
Belkanii94n Bjapo110nod BjapoSymnod Bjapo110nod BjapoSymnod Belkanii94n BjapoSymnod	T G G A T C C - C G G A T C G G C C T G A G T G G G 360 T T T A T C T T G T T T C G A G C C A C 430 A T C T G G T T C T A A C C A T C A A G 	CAAACCGCGA ATATCGCCGT 370 	CGTTGTCGCG TCCAGGCCAG TCCAGGCCAG 330 II GTGGGGAAAG CGACCGTTGT 450 II TCCGATACGA GAATTCTCAT 520 II	CCT-GTACAC TTTCGTAAAG 390 11 CGCAAGGCGC CGGGGACTGC CGGGGACTGC CGGGGATCAG CGGTGATCAG S30 11	CGGATATATG GTGGACTTTT 400 	410 CGGGGCGCTAA 410 II GATACGCAGC GGAGGGGCGG 480 II ACGAAGCTTG GAAGGGTCAA 550 II	G AG A T T C AG T G A T A A C C A C T 420 11 T C A T C C G G T G C A G C C G C G C G C A G C C G C G C G 11 T A T T G A A G A T C C T G A C T G C T 11
Belkanii94n Bjapo110nod BjapoSymnod Belkanii94n Bjapo110nod BjapoSymnod Belkanii94n BjapoSymnod	T G G A T C C - C G G A T C G G C C T G A G T G G G 	CAAACCGCGA ATATCGCCGT 370 	CGTTGTCGCG TCCAGGCCAG 330 	CCT-GTACAC TTTCGTAAAG 390 11 CGCAAGGCGC CGGGGACTGC CGGGGACTGC 11 TACTTGCTGC CGGTGATCAG 530 11	CGGATATATG GTGGACTTTT 400 	CGAGCGATCC CGGGCGCTAA 410 11 GATACGCAGC GGAGGGGCGG 480 11 ACGAAGCTTG GAAGGGTCAA 550 11	G A G A T T C A G T G A T A A C C A C T 420 11 T C A T C C G G T G C A G C C G C G C G C A G C C G C G C G 11 T A T T G A A G A T C C T G A C T G C T 11
Belkanii94n Bjapo110nod BjapoSymnod Belkanii94n BjapoSymnod Belkanii94n BjapoSymnod BjapoSymnod BjapoSymnod BjapoSymnod	T G G A T C C - C G G A T C G G C C T G A G T G G G 360 T T T A T C T T G T T T C G A G C C A C 430 A T C T G G T T C T A A C C A T C A A G 500 G C A T G A C C C G	CAAACCGCGA ATATCGCCGT 370 	CGTTGTCGCG TCCAGGCCAG TCCAGGCCAG 330 	CCT-GTACAC TTTCGTACAC TTTCGTAAAG 390 	CGGATATATG GTGGACTTTT 400 	CGAGCGATCC CGGGCGCTAA 410 11 GATACGCAGC GGAGGGGCAGC GGAGGGGCAGG 11 ACGAAGCTTG GAAGGGTCAA 550 11 S50	GAGATTCAGT GATAACCACT 420 II TCATCCGGTG CAGCCGCGCG 490 II TATTGAAGAT CCTGACTGCT 560 II 560 I

	570	520	590	600	610	620	630
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Belkanii94n							
Bjapo110nod	AGGCTGATCG	ACATGGAATA	TTGGCTCGCG	TGCAACGAAG	AGCGCGCGGC	ACAGGCGCGC	TTCGGTGCCG
Bjapo Symnod	AAACCTCTTT	GGTGCTCGTT	GGCGTGCGCG	GCCCACGAGA	CGCTGGGCGG	CGCGCCTTCC	GAGATCCGAA
	640	650	660	670	680	690	700
				1			
Belkanii94n							
Bjapo110nod	TCATGTGTTG	стосоосса	TGTGCCATGT	ATCGGCGTTC	сосостсосс	ттөсттсттө	АТСААТАТВА
Bjapo Symnod	CGCGCCACTA	ттттббсбтт	CAAAAAGCGG	AGTAAGAAGA	TGCATCTGAC	AGGTCTTTCC	GCAGGCAAGG
	710	720	770	740	750	760	770
Belkanii 94 n							
Bjapo110nod	AGCCCAATTC	TTTCGTGGGA	AGCCGAGCGA	TTTCGGCGAG	GACCGCCACC	TAACGATACT	CATGCTCAAG
Bjapo Symnod	CGTTCATCCG	GGAGGTCGCG	AGAACAGGTC	AGACGATTGG	AACGGTCCAC	ACTCTCGGCG	CCCTGCATCT
	780	790	200	810	820	830	840
Belkanii94n							
Biapo110nod	GCGGGGGTTTC	GAACCGAATA	COTTOCOGAC	GCCATAGCAG	CCACAGICGI	CCCGCACAGT	CIICGGCCAI
Biapo Sumnod	TEGECATECA	GAGCIGATIA	GAAGAGCGGC	ATTGGAGAAT	GATGTCGCGA	TCGTCACAGT	GTACCCGAAC
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	250	860	870	880	890	900	910
Relkanii94n							
Biano110nod	4.1.0.1.1.1.1.1.1.1.1.1.1.1.1.1.1.1.1.1.	0.0000000000000000000000000000000000000	TEGECOCCAA	GIACCITICG	AGATACGITI	CTIGCITEGC	0001001000
Bispo Samod	**********	TTAGACCAGG	TEEEAECTAC	GATTTCAATT	TERACEATEA	TATTEGACTT	SCCCTACETT
Djapo Dyninod	ANGAICCAGC	TINGACCAGG	IGGGAGCIAC	GATTICAATT	100×00×10×	IXIIOGXCII	GCCCIACGII
	920	930	940	950	960	970	920
DallanüQdu	••••					· · · · · · · ·	••••
Deixanii 34n							
Bjapo'l'lunod	AGAGCICGAI	GGIIAIIIGA	CGCTAGACGT	IAICGGGCAA	AAICICGGCC	CATIGCICCI	CGCCATTICA
Bjapo Symnod	CAGGAGCAAC	IGCCGIGATI	ICGICGAGCG	ACACCGAAAI	GITCCCGGTC	GGUTATUGAA	CGIAIGIAIC
	990	1000	1010	1020	1030	1040	1050
Belkanii94n							
Bjapo110nod	TCACTTGC	TGCGCTCGCA	CAGCTCCTGA	TCGATGGCTC	TATACCCTGG	TGGACGGGAT	TGACGATTGC
Bjapo Symnod	GCAAGGTGAC	TGTGATTTGC	GTTTAGGGGG	GCCTGAAACT	TATTTGAAGG	AAGCCGTTAC	TGGAGCAATC
	1060	1070	1020	1090	1100	1110	1120
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Belkanii94n							
Bjapo110nod	TGCAATGACT	ACGGTCCGGT	GCTGTGTGGC	AGCGCTTCGT	GCCCGCGAGC	TGCGGTTTAT	CGGCTTCTCG
Rispo Samod	COCTOGATOT	GCTATTCGAG	GCCAACTCGC	AGTTACTTCG	GCCTG444G4	TATAGGTCAG	0010014000

	1130	1140	1150	1160	1170	1180	1190
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Belkanii94n							
Bjapo110nod	CTCCACACGC	CGATCAATAT	стотстстта	CTGCCTTTGA	AGGCCTATGC	GCTTTGTACA	TTGAGCAATA
Bjapo Symnod	TAAAGCGCGC	CGTCGCAGAC	CTGCTTCTCG	ATTGCGAGAT	AAGGTTTGTG	CCGACCGTGA	GATACAAGAA

	1200	1210	1220	1230	1240	1250	1260
						$\cdots \cdots + \cdots +$	
Belkanii94n							
Bjapo110nod	GCGATTGGCT	ATCT <mark>CGGAAA</mark>	GTCACCGATA	TGCCGACGGA	AGAGGGGAAA	CAGCCTGTCA	TCCTGCACCC
Bjapo Symnod	CGGAGTGCCC	ATTTCGTCGC	GCTTGAGGCG	ATTTAACCGT	TCCGAGCTTG	ATGAGCTGTC	стөтстсттс

	1270	1280	1290	1300	1310	1320	1330
	$\cdots \cdots + \cdots +$	· · · · · · · · · · · ·		· · · · I · · · · I	\cdots	$\cdots \cdots \mid \cdots \mid \cdots \mid $	\cdots
Belkanii 94 n		····					
Bjapo110nod	GAATGCCGGA	CGAAGTCCTG	CTGGTGTAGG	GGGGCGCCTG	CTCCTATTCG	TAAGGCGGCG	TTATCGCAGC
Bjapo Symnod	ACGGCACTCA	ACCGTTCAAG	AGAAGAGATC	GCCCGAGGAG	CGTCAAGCGT	GAAGGATCTG (бтсбсбтсбб

	1340	1350	1360	1370	1380	1390	1400
	$\cdots \cdots + \cdots + \cdots$	<mark>.</mark>		· · · ·] · · · ·]		$\cdots \cdots + \cdots + \cdots$	[]
Belkanii94n		· · · · · · · · · · · · · · · · · · ·					
Bjapo110nod	CTCCATCGAG	сстввсввсв	ACGGAGAGTG	TTTCCGGTCG	CGATCGTTCG	ACTGTCTACA	AATAAGTGGT
Bjapo Symnod	CGACATCTCG	GATAGAGTTT	CGACAGTTTA	GACTCGAGTT	TGTTCGGATT	GTCGAC-GCT	GATAATTTCA

	1410	1420	1430	1440	1450	1460	1470
					· · · ·] · · · ·]	[]	[]
Belkanii94n							
Bjapo110nod	CGGCTGATGA	CTCAGGACGA	AAACCATCAG	TTATTAGAGC	GAGAGTTGGC	TGTCGACGAC	CCGTGGCGCC
Bjapo Symnod	AAGATTTAGA	GCAGGTCAAG	CTCCCATTCT	TAATTATTGG	GGCTGCAATG	GCTCATGGCC	TCACGGT

	1480	V A A	
	$\cdots \cdots + \cdots + \top$		
Belkanii 94 n			
Bjapo110nod	TCGACACTAG		
Bjapo Symnod			

5. *nifH* forward and reverse primers are in boxes

					Reverse p	orimer		
Bjaposymni Bjapo110ni Belkanii94	10	20 TAAGACAAAT TAAGACAAAT 	30 CGCCTTCTAC CGCCTTCTAC	40 GGGAAGGGCG GGGAAGGGCG	50 GAATCGGCAA GAATCGGCAA	60 GTCCACCACT GTCCACCACT	70 TCGCAGAACA TCGCAGAACA TCGCAGAACA TCCCAGAATA	8(CGCTAGCGGC CGCTAGCGGC CGTT <mark>G</mark> GCGGC
Bjaposymni Bjapo110ni Belkanii94	90 GCTGGCAGAG GCTGGCAGAG GCTGGC <mark>C</mark> GAG	100 ATGGGTCAGA ATGGGTCAGA ATGGGTCAGA	AGATCCTGAT AGATCCTGAT AGATCCTGAT A <mark>A</mark> ATCCTGAT	TGTAGGGTGC TGTAGGGTGC TGTAGGGTGC CGTGGGATGC	GATCCGAAAG GATCCGAAAG GATCCGAAAG GATCCTAAGG	CGGACTCGAC CGGACTCGAC CGGACTCGAC CGGACTCGAC	TCGCCTTATT TCGCCTTATT TCGCCTTATT CCGCCTGATC	16 CTGCACGCCA CTGCACGCCA CTGCA <mark>T</mark> GCCA
Bjaposymni Bjapo110ni Belkanii94	17: Aggctcaaga Aggctcaaga Aggc <mark>g</mark> ca <mark>g</mark> ga	CACGATTTTG CACGATTTTG CACGATTTTG CACGATTTTG) 190 AGTCTTGCCG AGTCTTGCCG AG <mark>C</mark> CTTGC <mark>A</mark> G	CGAGCGCCGG CGAGCGCCGG CGAGCGCCGG	CAGCGTGGAG CAGCGTGGAG CAGCGTGGAG) 220 GATCTAGAGC GATCTAGAGC GA <mark>CCT</mark> CGA <mark>A</mark> C	TCGAGGACGT TCGAGGACGT TCGAGGACGT TCGAGGACGT	24 AATGAAGGTT AATGAAGGTT G <mark>ATGAAGGT</mark> C
Bjaposymni Bjapo110ni Belkanii94	250 GGCTACCAGG GGCTACCAGG GGCTAC <mark>A</mark> AGG	D 260 ACATTCGCTG ACATTCGCTG ACATTCGCTG ACATCCGCTG	CGTTGAGTCC CGTTGAGTCC CGTTGAGTCC CGT <mark>G</mark> GAGTCC	GGTGGCCCTG GGTGGCCCTG GGTGGCCCTG GG <mark>C</mark> GG <mark>T</mark> CCTG	AGCCAGGTGT AGCCAGGTGT AGCCAGGTGT AGCC <mark>G</mark> GGTGT	CGGCTGCGCC CGGCTGCGCC CGGCTGCGCCC	0 310 GGCCGCGGGTG GGCCGCGGGG GGCCGCGGG	32 TCATCACCTC TCATCACCTC TCATCACCTC
Bjaposymni Bjapo110ni Belkanii94	330 GATCAATTTT GATCAATTTT GATCAATTT <mark>C</mark>	D 340 CTTGAAGAGA CTTGAAGAGA CTGGAGGAA	ACGGAGCCTA ACGGAGCCTA ACGGAGCCTA ACGG <mark>C</mark> GC <mark>T</mark> TA	CGAGAACATT CGAGAACATT CGAGAACATT T <mark>GAGG</mark> ACATT	9	CTTACGATGT CTTACGATGT CTTACGATGT CCTACGACGT	Forw, 390 GCTTGGCGAC GCTTGGCGAC GCTCGGTGAC	ard primer 40 GTTGTTTGCG GTTGTTTGCC GT
Bjaposymni Bjapo110ni Belkanii94	410 GTGGCTTTGC GTGGCTTTGC	0 420 GATGCCAATC GATGCCAATC	0 430 CGCGAAAACA CGCGAAAACA	0 440 AGGCGCAGGA AGGCGCAGGA	0 450 GATCTACATC GATCTACATC	0 460 GTGATGTCTG GTGATGTCTG	0 470 GTGAAATGAT GTGAAATGAT	48 GGCAATGTAT GGCAATGTAT
Bjaposymni Bjapo110ni Belkanii94	49(GCCGCAAACA GCCGCAAACA 	D 500 ATATTTCCAA ATATTTCCAA) 510 GGGGATCCTG GGGGATCCTG	D 520 AAATACGCGA AAATACGCGA	D 530 ACTCAGGTGG ACTCAGGTGG	GGTGCGGTTG GGTGCGGTTG GGTGCGGTTG) 550 GGCGGCCTGA GGCGGCCTGA	56 TCTGCAACGA TCTGCAACGA
Bjaposymni Bjapo110ni Belkanii94	57(GCGGCAGACC GCGGCAGACC 	D 580 GACAAGGAAT GACAAGGAAT) 590 TGGAACTGGC TGGAACTGGC	GGAAGCGTTG GGAAGCGTTG GGAAGCGTTG	GCCAAGAAGC GCCAAGAAGC GCCAAGAAGC	D 620 TTGGCACTCA TTGGCACTCA	0 630 ACTGATCTAC ACTGATCTAC	64 TTCGTGCCGC TTCGTGCCGC
Bjaposymni Bjapo110ni Belkanii94	650 GTGACAATGT GTGACAATGT 	D 660 GGTGCAGCAT GGTGCAGCAT	GCAGAGCTGC GCAGAGCTGC GCAGAGCTGC	D 680 GTCGCATGAC GTCGCATGAC	GGTGCTTGAA GGTGCTTGAA	D 700 TATGCACCCG TATGCACCCCG	D 710	72 GGCTGATCAC GGCTGATCAC
Bjaposymni Bjapo110ni Belkanii94	730 TATCGGAAAC TATCGGAAAC	0 740 	O 750 GGTTCACAAT GGTTCACAAT	D 760 AATGGCGGCA AATGGCGGCA	D 770 AGGGCATCAT AGGGCATCAT	0 780 CCCGACCCCG CCCGACCCCG	0 790 ATCTCAATGG ATCTCAATGG	80 ATGAGCTCGA ATGAGCTCGA
Bjaposymni Bjapo110ni Belkanii94	810 GGACATGCTG GGACATGCTG 	0 820 ATGGAGCATG ATGGAGCATG) 830 GCATTATAAA GCATTATAAA	0 840 GGCCGTGGAT GGCCGTGGAT	0 850 GAATCAATCA GAATCAATCA	D 860 	D 870 CGCCGCCGAA CGCCGCCGAA	88 CTCGCAGCCT CTCGCAGCCT
Bjaposymni Biano110ni	CGTAA CGTAA							

Bjapo110ni <mark>CGTAA</mark> Belkanii94 -----

	10						
BjapoSymni Bjapo110ni Belkanii94	ATGAGTCTCG ATGAGTCTCG	CACGACCAA CCACGACCAA CCACGACCAA	30 CAGCGTCGCA CAGCGTCGCA	40 GAAATCAGGG GAAATCAGGG	CTCGCAACAA CTCGCAACAA CTCGCAACAA	AGAGCTGATC AGAGCTGATC	GAGGAGGTGC GAGGAGGTGC GAGGAGGTGC GTGC
BjapoSymni Bjapo110ni Belkanii94	80 TGAAGGTCTA TGAAGGTCTA TGAAGGTCTA TGAAGGTCTA	90 TCCGGAGAAA TCCGGAGAAA TCC <mark>AGAGAA</mark> G	100 ACCGCGAAAA ACCGCGAAAA ACCGCGAA <mark>G</mark> C) 110 GGCGTGCCAA GGCGTGCCAA GGCGTGCCAA) 120 GCACCTCAAC GCACCTCAAC GCA <mark>T</mark> CTCAAC) 130 GTGCACCAAG GTGCACCAAG GT <mark>TCACCA</mark> GT	D 140 CAGGTAAGTC CAGGTAAGTC CAGGTAAGTC CCGG <mark>G</mark> AAGTC
BjapoSymni Bjapo110ni Belkanii94	150 GGACTGCGGG GGACTGCGGG CGA <mark>T</mark> TGCGG <mark>A</mark>	GTGAAGTCCA GTGAAGTCCA GTGAAGTCCA GTCAAGTCCA	170 ACATCAAATC ACATCAAATC ACAT <mark>A</mark> AAATC	CATACCCGGC CATACCCGGC CATACCCGGC CATACCTGGT	D 190 GTGATGACGA GTGATGACGA GTGATGAC <mark>A</mark> A	D 200 TAAGAGGGTG TAAGAGGGTG TAAGAGGGTG T <mark>C</mark> AGAGG <mark>C</mark> TG	CGCCTATGCA CGCCTATGCA CGCCTATGCA CGCCTATGCA
BjapoSymni Bjapo110ni Belkanii94	220 GGGTCGAAGG GGGTCGAAGG GG <mark>A</mark> TC <mark>C</mark> AAGG	GGGTGGTCTG GGGTGGTCTG GGGTGGTCTG GGGTGGTCTG	GGGACCAATC GGGACCAATC GGGACCAATC GGG <mark>G</mark> CCGATC	AAGGACATGG AAGGACATGG AAGGACATGG AAGGACATGG	TTCATATTAG	CCATGGCCCG CCATGGCCCG CCATGGCCCG CCA <mark>C</mark> GGCCCG	GTTGGCTGCG GTTGGCTGCG GTTGGCTGCG GTTGGCTGCG
BjapoSymni Bjapo110ni Belkanii94	290 GCCAATATTC GCCAATATTC GCCAATATTC GCCA <mark>G</mark> TATTC	ATGGGGCTCG ATGGGGCTCG ATGGGGCTCG GTGGGGCTCG	CGGCGCCAACT CGGCGCCAACT CGGCGCCAACT	ATTACGTTGG ATTACGTTGG ATTACGTTGG ATTACGTTGG	CACCACGGGC CACCACGGGC CACCACGGGC CACAAACGGGC) 340 ATCGATAGCT ATCGATAGCT ATCGATAG <mark>T</mark> T	D 350 TCGTGACTCT TCGTGACTCT TTGTAACCCT
BjapoSymni Bjapo110ni Belkanii94	360 GCAGTTCACC GCAGTTCACC GCA <mark>A</mark> TTCACC	D 370 TCCGACTTCC TCCGACTTCC TCCGA <mark>T</mark> TTCC) 380 AGGAAAAGGA AGGAAAAGGA AGGA <mark>G</mark> AAAGGA) 390 TATCGTATTT TATCGTATTT CATCGTATTC	GGCGGCGACA GGCGGCGACA GGCGGCGACA GG <mark>T</mark> GGCGACA) 410 Agaaactgga Agaaactgga Agaa <mark>g</mark> ctgg <mark>t</mark>) 420 саалатсстт саалатсстт саала <mark>с</mark> тсстт
BjapoSymni Bjapo110ni Belkanii94	430 GATGAAATCC GATGAAATCC GA <mark>C</mark> GAAATCC	AAGAGCTGTT AAGAGCTGTT AAGAGCTGTT A <mark>G</mark> GAGCT <mark>T</mark> TT	0 450 TCCACTCAAC TCCACTCAAC CCC <mark>CCCCCAC</mark>	0 460 AACGGCATTA AACGGCATTA AACGGCATTA C <mark>ACGGCAT</mark> C <mark>A</mark>	CGATACAATC CGATACAATC CGATACAATC C <mark>C</mark> ATCCAATC	0 480 AGAGTGCCCG AGAGTGCCCG GGA <mark>A</mark> TGCCCG	0 490 GTAGGGTTGA GTAGGGTTGA AT <mark>C</mark> GGATTGA
BjapoSymni Bjapo110ni Belkanii94	500 TCGGTGACGA TCGGTGACGA TCGG <mark>G</mark> GACGA	D 510 TATCGAGGCG TATCGAGGCG CATCGAGGCC	GTGTCAAGGG GTGTCAAGGG GTGTCAAGGG GTGTCAAGG	0 530 CGAAATCCAA CGAAATCCAA CGAAATCCAA) 540 Agaatatgga Agaatatgga G <mark>gaatatgg</mark> t) 550 GGCAAGACCA GGCAAGACCA GGCAAGACCA	D 560 TCGTGCCGGT TCGTGCCGGT TCGTGCC <mark>T</mark> GT
BjapoSymni Bjapo110ni Belkanii94	570 CCGTTGTGAG CCGTTGTGAG CCG <mark>C</mark> TGTGAG	GGCTTTCGGG GGCTTTCGGG GGCTTTCGGG GGCTTTCG <mark>C</mark> G	GTGTGTCGCA GTGTGTCGCA GTGTGTCGCA G <mark>C</mark> GTGTCGCA	GTCACTAGGC GTCACTAGGC GTCACTAGGC GTC <mark>GCT</mark> TGGC	0 610 CATCACATTG CATCACATTG CATCACATTG CA <mark>C</mark> CACAT <mark>C</mark> G	D 620 CAAACGATGC CAAACGATGC CAAACGACGC CCAACGACGC	GGTACGCGAT GGTACGCGAT GGTACGCGAT GGT <mark>G</mark> CGCGAT
BjapoSymni Bjapo110ni Belkanii94	640 	D 650 GGCATATCGA GGCATATCGA ACA <mark>A</mark> GC <mark>TCGA</mark>	0 660 	0 670 AAACCAAAGT AAACCAAAGT AAACCAAAGT	D 680 TCGAGCCGAC TCGAGCCGAC TCGAGCCGAC	0 690 ACCATACGAT ACCATACGAT ACCATACGAT G <mark>CCC</mark> TACGAT	GTTGCGATCA GTTGCGATCA GTTGCGATCA
BjapoSymni Bjapo110ni Belkanii94	710 TCGGAGACTA TCGGAGACTA TTGGTGACTA	D 720 CAATATCGGC CAATATCGGC CAATATCGGC) 730 GGCGATGCTT GGCGATGCTT GGCGA <mark>C</mark> GC <mark>C</mark> T	Forwar 740 GGTCATCGCG GGTCATCGCG GGTCATCGCG	d primer 750 AATTCTGCTT AATTCTGCTT AATTCTGCTG) 760 GAAGAGATGG GAAGAGATGG GAGGAAATGG	GACTACGGGT GACTACGGGT GACTACGGGT G <mark>C</mark> CT <mark>G</mark> CGGGT
BjapoSymni Bjapo110ni Belkanii94	780 AATCGCGCAG AATCGCGCAG G <mark>ATTGC<mark>ACAG</mark></mark>	TGGTCCGGCG TGGTCCGGCG TGGTCCGGCG TGGTCCGGCG	ACGGTTCACT ACGGTTCACT ACGGTTCACT ACGGTTCACT	GGCCGAGCTC GGCCGAGCTC GGCCGAGCTC CGCCGAGCTC	GAAGCAACGC GAAGCAACGC GAAGCAACGC GAAGCAACGC) 830 CGAAGGCAAA CGAAGGCAAA CGAAGGC <mark>G</mark> AA	GCTCAACATT GCTCAACATT GCTCAACATT GCTCAACATT

6. *nifD* forward and reverse primers are in boxes

BjapoSymni Bjapo110ni Belkanii94	850 CTGCATTGCT CTGCATTGCT CTGCATTGCT CTGCATTGCT	860 ACCGTTCCAT ACCGTTCCAT ACCGTTC <mark>G</mark> AT	870 GAACTATATC GAACTATATC GAACTA <mark>C</mark> ATC	880 TCACGCCACA TCACGCCACA TCACGCCACA TC <mark>G</mark> CG <mark>T</mark> CATA) 890 TGGAAGAGAA TGGAAGAGAA T	D 900 GTTCGGCATC GTTCGGCATC	910 910 CCTTGGTGCG CCTTGGTGCG
BjapoSymni Bjapo110ni Belkanii94	920 AGTACAACTT AGTACAACTT 	930 CTTCGGACCT CTTCGGACCT	940 TCAAAGATCG TCAAAGATCG	950 CGGACTCACT CGGACTCACT) 960 GCGCAGGATT GCGCAGGATT	970 GCGGGTTATT GCGGGTTATT) 980 TTGACGACAA TTGACGACAA
BjapoSymni Bjapo110ni Belkanii94	990 Gatcaaggaa Gatcaaggaa	1000 GGCGCCGAGC GGCGCCGAGC	D 1010 GAGTGATCGA GAGTGATCGA GAGTGATCGA	D 102 GAAGTATCAG GAAGTATCAG	0 103 CCGCTGGTGG CCGCTGGTGG	0 104 ACGCCGTGAT ACGCCGTGAT	0 1050 TGCAAAATAT TGCAAAATAT
BjapoSymni Bjapo110ni Belkanii94		D 1070 TCGAGGGCAA TCGAGGGCAA) 1080 GACGGTGATG GACGGTGATG) 109 CTGTACGTCG CTGTACGTCG	0 110 GCGGCCTTCG GCGGCCTTCG	0 111 TCCGCGTCAT TCCGCGTCAT	0 1120 GTGATTGGCG GTGATTGGCG
BjapoSymni Bjapo110ni Belkanii94	1130 CGTACGAGGA CGTACGAGGA 	D 1140 CCTCGGGATG CCTCGGGATG	D 1150 GACGTCATTG GACGTCATTG GACGTCATTG	GCACTGGCTA GCACTGGCTA GCACTGGCTA	0 117 CGAGTTCGGT CGAGTTCGGT	0 118 CACAACGACG CACAACGACG CACAACGACG	0 1190 ACTATCAGCG ACTATCAGCG
BjapoSymni Bjapo110ni Belkanii94	1200 CACAGCTCAG CACAGCTCAG 	1210 CACTACGTGA CACTACGTGA) 1220 AAGACAGCAC AAGACAGCAC	CCTCATCTAT	0 124 GATGACGTCA GATGACGTCA	0 125 ATGGCTATGA ATGGCTATGA	0 1260 GTTCGAGCGC GTTCGAGCGC
BjapoSymni Bjapo110ni Belkanii94	1270 TTCGTCGAAA TTCGTCGAAA 	AACTCCAGCC	D 1290 TGATCTTGTC TGATCTTGTC) 130 GGCTCAGGCA GGCTCAGGCA	0 131 TCAAGGAAAA TCAAGGAAAA	0 132 GTACGTTTTC GTACGTTTTC	0 1330 Caaaagatga Caaaagatga
BjapoSymni Bjapo110ni Belkanii94	1340 GTGTGCCGTT GTGTGCCGTT 	1350 CCGGCAGATG CCGGCAGATG	D 1360 CATTCGTGGG CATTCGTGGG) 137 ACTATTCGGG ACTATTCGGG	0 138 TCCATATCAC TCCATATCAC	0 139 GGTTATGACG GGTTATGACG	0 1400 GCTTTGCGAT GCTTTGCGAT
BjapoSymni Bjapo110ni Belkanii94	1410 CTTCGCGCGC CTTCGCGCGC	1420 GACATGGACA GACATGGACA	D 1430 TGGCCGTCAA TGGCCGTCAA	D 144 CTCGCCAATT CTCGCCAATT	0 145 TGGAAAAGAA TGGAAAAGAA	0 146 CGAAAGCTCC CGAAAGCTCC	0 1470 CTGGAAGGAA CTGGAAGGAA
BjapoSymni Bjapo110ni Belkanii94	1480 GCGCCGAGCG GCGCCGAGCG) 1490 CCAAGCTCCA CCAAGCTCCA) 1500 GGCTGCAGAA GGCTGCAGAA) Таа Таа 			

7. GAPDH forward and reverse primers are in boxes

Bjapo110GAP	10 	20 ••••• ••••	30 	40 	50 	60 	70
EcoliGap	GATCAAACAG	TGATATACGC	CGTCACGCTT	GTTATGCAGT	AAACGACCCG	TAAATGGCGG	CTCTGTCCCA
Bjapo110GAP	80 • • • • • • • • • • • • • • • • • • •	90 • • • • • • • • • • • • • • • •	100 	110) 120) 130 	140
EcoliGap	TGATTCTGCG	TCACGTAAAA	CTGCATCTCG	GACAAATTTT	TTTTCAGTTC	TTCTGCCGAA	GTTTATTAGC
Bjapo110GAP EcoliGan	150 		170) 190 	200 	210
rcorroap	CATTIOCICA	CATCICACIT	IAAICOIOCI	CACATIACOI	GACIGATICI	AntiAnantai	IMUNULANU
Bjapo110GAP EcoliGap	220 TGGCAAAATT	230 TTGTCCTAAA	240 CTTGATCTCG	250	260 TGCACCTAAA	270 TCGTGATGAA	280 AATCACATTT
_	200	200	210	220	220	240	250
Bjapo110GAP EcoliGap	TTATCGTAAT	TGCCCTTTAA	AATTCGGGGGC	GCCGACCCCA	TGTGGTCTCA	AGCCCAAAGG	AAGAGTGAGG
	360 • • • • • • • • •	370	380		400 •••••) 410 ••••• •••••	420 · · · · · · · · · · · · · · · · · · ·
Bjapo110GAP EcoliGap	CGAGTCAGTC	GCGTAATGCT	TAGGCACAGG	ATTGATTTGT	CGCAATGATT	GACACGATTC	GCTTGACGCT
Biano11067P	430 • • • • • • • •	440	450	460 •••••	470 ••••	9 480 •••••	490
EcoliGap	GCGTAAGGTT	TTTGTAATTT	TACAGGCAAC	CTTTTATTCA	стаасааата	gctggtggaa	TAT <mark>ATG</mark> ACTA
	500	510	520	530	540	550	560
Bjapo110GAP EcoliGap	TCCGCGTTGG TCAAAGTAGG	AATCAACGGT TATCAACGGT	TTTGG <mark>T</mark> CGTA TTTGG <mark>C</mark> CGTA	TCGGCCGCAA TCGG <mark>T</mark> CGCA <mark>T</mark>	CGTCCTGCGG TGTTTTCCGT	GCGATTGCAG GCTGC <mark>T-CAG</mark>	AGTCCGGCCG AAACG
	570	580 • • • • • • • • • •	590		610 	620 ••• <u>••</u> •••	630
Bjapo110GAP EcoliGap	CAAG <mark>GAT</mark> ATC TTCT <mark>GA</mark> C <mark>ATC</mark>	GAG <mark>GTGGTC</mark> G GAG <mark>AT</mark> CGTTG	GCATCAACGA CA <mark>ATCAACGA</mark>	CCTCGGCCCG CCT <mark>GTTAGAC</mark>	GTCGAGACCA G <mark>CTGA</mark> TTA <mark>CA</mark>	at <mark>gc</mark> ccatct Tg <mark>gc</mark> at <mark>a</mark> cat	GCTCCGTTTC GCT <mark>GAAAT</mark> AT
Bjapo110GAP EcoliGap	640 	650 •••••	660 	670 	680	₀ 690 •• <u>•</u> • <u> </u>	700 •••• <u>• </u>
	GACAGCGTTC GACTCCACTC	ACGGCCG <mark>C</mark> TT ACGGCCG <mark>T</mark> TT	CCCCGGCACC CGA <mark>CGGT</mark> ACC	GTCACCGTCG GTTGAA <mark>GT</mark> GA	ACEGTEATTC AACACEGTCA	GATCAGCCTC TCTGATCGTT	gga gg cggca aac <mark>gg</mark> taaa <mark>a</mark>
	710	720	730	740	750	760	770
Bjapo110GAP EcoliGap	AG <mark>ATC</mark> AAGGT AA <mark>ATC</mark> CGT <mark>GT</mark>	GACCGC <mark>CGA</mark> G TACCGCTGAA	CGCGATCCCT CGTGATCCGG	CGAAGCTGCC CT <mark>AA</mark> CCTGAA	CTGGAAGGAT ATGGGACGAA	CTCGGCGTCG GTTGGTGTTG	ACATCG-CGC ACGTTGTCGC
	780	790	800	810	820	830	840
Bjapo110GAP EcoliGap	TGGAATGCAC TG <mark>AA</mark> GCA-AC	CGGCATCTTC TGGTC <mark>TGTTC</mark>	acctcgaagg ctgactgacg	acaag <mark>gc</mark> ctc aaa <mark>ctgc</mark> tcg	CGCACATCTG TAA <mark>ACA</mark> CA <mark>T</mark> C	accec <mark>ceec</mark> accec <mark>teet</mark> e	CCAAGCGCGT CG <mark>AAG</mark> AAAGT
	850	860 	870	880 • • • • • • •) 890 	900 •••••	910 •••••
Bjapo110GAP EcoliGap	GCTGGTCTCC GGTTATGACT	GCG <mark>CC</mark> CGCCG GGT <mark>CC</mark> GTCTA	ACGGCGCCGA AAGACAACAC	CGCCACCATC TCCGATGTTC	GTCTACGGCG GTTAAAGGCG	TCAACCACGA CT <mark>AAC</mark> TTCGA	CA <mark>CGCTGAC</mark> C CA <mark>AATATGC</mark> T



สถาบนวทยบรการ จุฬาลงกรณ์มหาวิทยาลัย

Biography

Miss Siras Sulanchupakorn was born on October 7, 1982. She obtained a Bachelor of Science Degree in Microbiology from King Mongkut's University of Technology Thonburi, Bangkok, Thailand, in 2004.

Publication

 Chansa-ngavej, K., Chongfuengprinya, W., Ly Kim Pheng, Emampaiwong,D., Sulanchupakorn,S., and Sawangdee,S. 2006. Use of RAPD-PCR fingerprints to monitor changes in DNA and field distribution of soybean rhizobia biofertilizers. Proceedings of The 14th World Fertilizer Congress. Held at Lotus Kad Suan Kaew Hotel, January 23-27, 2006. Chiangmai, Thailand. 6 Pages (in press).

Presentation at Scientific Conferences

- สิรัส สุลัญชุปกร และ กาญจนา ชาญสง่าเวช. 2549. การเปรียบเทียบโพรไฟล์ของโปรตีน ภายในเซลล์ของ Bradyrhizobium japonicum 6 สายพันธุ์ ที่เลี้ยง ณ อุณหภูมิต่างๆ. หนังสือ รวมบทคัดย่อการประชุมวิชาการ คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย. ครั้งที่ 14 : หน้า 13.
- Sulanchupakorn, S. and Chansa-ngavej, K. 2005. Expression of nodulation and nitrogen fixation genes in thermophilic *Bradyrhizobium japonicum* SK3, SK4, SK26, SK28 and non-thermophilic *Bradyrhizobium japonicum* S76 and S162 under different temperatures. Abstract Book, The 2nd AgBiotech Graduate Conference II. May 16-17, 2005. Bangkok, Thailand. Page 87.
- Chansa-ngavej, K., Chongfuengprinya, W., Ly Kim Pheng, Emampaiwong, D., Sawangdee, S., Sulanchupakorn, S., and Yamada, M. 2006. RAPD-PCR fingerprinting , strain selection and greenhouse-scale testing of thermotolerant soybean rhizobial biofertilizers. Abstract Book. The 5th JSPS-NRCT Joint seminar on Development of Themotolerant Microbial Resources and Their Application. November 7-10, 2006. Pattaya. Thailand (in press).