การแสดงออกของยีนเกี่ยวกับการสร้างปมและยีนเกี่ยวกับการตรึงไนโตรเจนใน *Bradyrhizobium japonicum* 6 สายพันธุ์ ที่อุณหภูมิต่างๆ

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

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ในปัจจุบันประเทศไทยผลิตถั่วเหลืองได้เพียง 15% ของที่ใช้ในประเทศ ส่วนที่เหลืออีก 85% เป็นถั่วเหลืองที่นำเข้า เป็นที่ทราบว่าปุ๋ยชีวภาพไรโซเบียมเพิ่มผลผลิตถั่วเหลือง ของบรรจุปุ๋ยชีวภาพไรโซเบียมสำหรับถั่วเหลืองต้องเก็บในที่เย็นหรือตู้เย็น ดังนั้น ถ้าพัฒนาสายพันธุ์ไรโซเบียมถั่วเหลืองให้ทนร้อน ก็จะประหยัดค่าใช้จ่ายและพลังงาน ในงานวิจัยนี้เลี้ยงไรโซเบียมถั่วเหลือง *Bradyrhizobium japonicum* จำนวน 6 สายพันธุ์ ในอาหารสูตรสกัดจากยีสต์-แมนนิทอล และ ในอาหารสูตรสกัดจากยีสต์-กลีซีโรล ภายใต้อุณหภูมิต่างๆ (25 °C-40°C) นอกจากนี้ยังทำ viable plate counts บนจานเพาะเชื้อที่มีอาหารแต่ละสูตรดังกล่าวข้างต้น ที่อุณหภูมิ 30°C และ 40°C ผลการทดลองพบไรโซเบียมถั่วเหลืองทั้ง 6 สายพันธุ์เป็นไรโซเบียมถั่วเหลืองที่ทนร้อน ผลการทำลายพิมพ์ดีเอ็นเอโดยวิธี RAPD-PCR โดยใช้ RPO1 หรือ CRL-7 เป็นไพร์เมอร์ พบว่า *B. japonicum* ที่ใช้ในการทดลองมีลายพิมพ์ดีเอ็นเอต่างกันจึงเป็นต่างสายพันธุ์ ผลการทำ RT-PCR โดยใช้ไพรเมอร์ที่เฉพาะเจาะจงต่อ $nodD_1$, *nodA*, *nodB*, *nifH* และ *nifD* พบการเปลี่ยนแปลงระดับการแสดงออกของยีน *nodD1* เมื่อเลี้ยง *B. japonicum* สายพันธุ์ SK4, SK26 หรือ SK28 ภายใต้อุณหภูมิ 25°C, 30 °C, 35 °C และ 40 °C โดย *nodD1* ของ *B. japonicum* SK26 มีการแสดงออกของยีน $nodD_1$ เพิ่มเมื่อเลี้ยงเซลล์ภายใต้อุณหภูมิ 35 °C และ 40 °C ส่วนการแสดงออกของ $nodD_1$ ใน *B. japonicum* SK4 และ SK28 ลดลงเมื่อเลี้ยงเซลล์ที่อุณหภูมิสูงได้แก่ 35 °C และ 40 °C ผลการทดลองได้ตรวจพบการแสดงออกในระดับต่างกันของยีน *nodB* ของ *B. japonicum* สายพันธุ์ S76 ซึ่งมีการแสดงออกของ *nodB* เพิ่มขึ้นเมื่อเลี้ยงเซลล์ภายใต้อุณหภูมิ 35°C และ 40°C ผลการทดลองไม่พบการแสดงออกที่แตกต่างกันของ $nodD_1$ ของ *B. japonicum* สายพันธุ์ SK3, S76 และ S162 และ ไม่พบการแสดงออกที่แตกต่างกันของ *nodA*, *nodB*, *nifH*, และ *nifD* ของ *B. japonicum* แต่ละสายพันธุ์ภายใต้สภาวะการทดลอง RT-PCR นอกจากนี้ได้ผลิตภัณฑ์ PCR เมื่อใช้ RNA ทั้งหมดจากเซลล์ของ *B. japonicum* แต่ละสายพันธุ์ที่เลี้ยง ณ อุณหภูมิ 45°C เป็นเวลา 5 ชั่วโมง ในการทำ RT-PCR

ภาควิชา จุลชีววิทยา ลายมือชื่อนิสิต สิรัส สุลัญชุปกร

สาขาวิชา จุลชีววิทยาทางอุตสาหกรรม ลายมือชื่ออาจารย์ที่ปรึกษา

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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*$_1$, *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*$_1$ 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.

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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.*rai*[1] [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 Chansa-ngavej 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 ^{32}P-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, July, August, December, and January. The average maximum temperatures in these months are shown in Table 1.2

Table 1.2 Average maximum temperatures in Thailand in 2005.

Areas	Average maximum temperatures (°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

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.

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

Year	Soybean production (metric tons)			Soybean productivity (kg.rai^{-1})
	Summer crop (Nov-Apr)	Rainy season crop (May-Sep)	Total	
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

Source : The Office of Agricultural Economics (2006).

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

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

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.

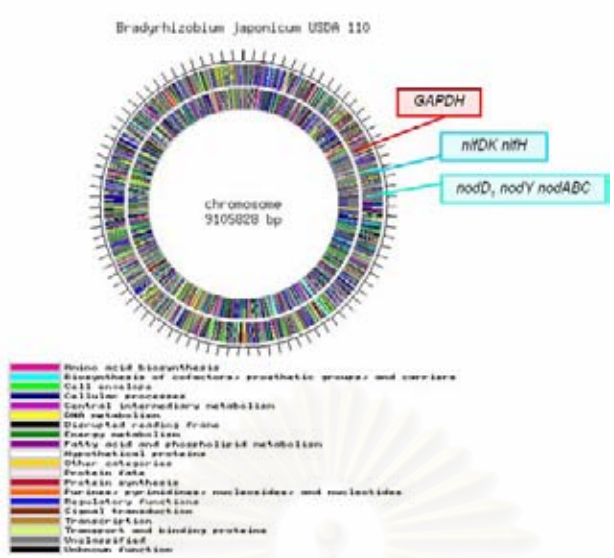


Figure 2.1 Whole genome sequence of *B. japonicum* USDA110. Each interval represents 100,000 bp. All the genes are color-coded according to their functions. Positions of nodulation and nitrogen fixation genes as well as GAPDH gene used in RT-PCR in this study are shown in the Figure. (http://gib.genes.nig.ac.jp/single/index. 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*$_1$, *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

GCGCGTCTA ATTGCTTTT binding of NodD$_1$-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.

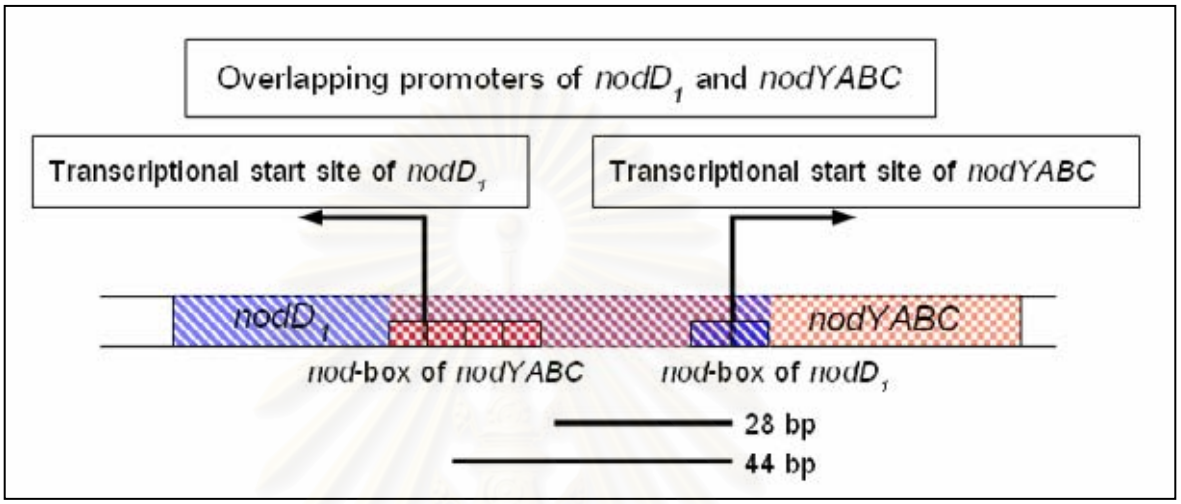


Figure 2.2 Diagramatic representation of a DNA segment of *B. japonicum* nodulation genes showing promoters of *nodD*$_1$ and *nodYABC* are overlapped with transcriptional start sites of *nodD* and of *nodYABC* lying in the *nod* box of the opposing transcript (Wang and Stacey , 1991).

Expression of *nodYABC* is also activated by binding of NodD$_1$-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*$_2$. NolA product from *nolA* regulates the expression of *nodD*$_2$. Figure 2.3 summarizes the activation and repression of nodulation gene expression.

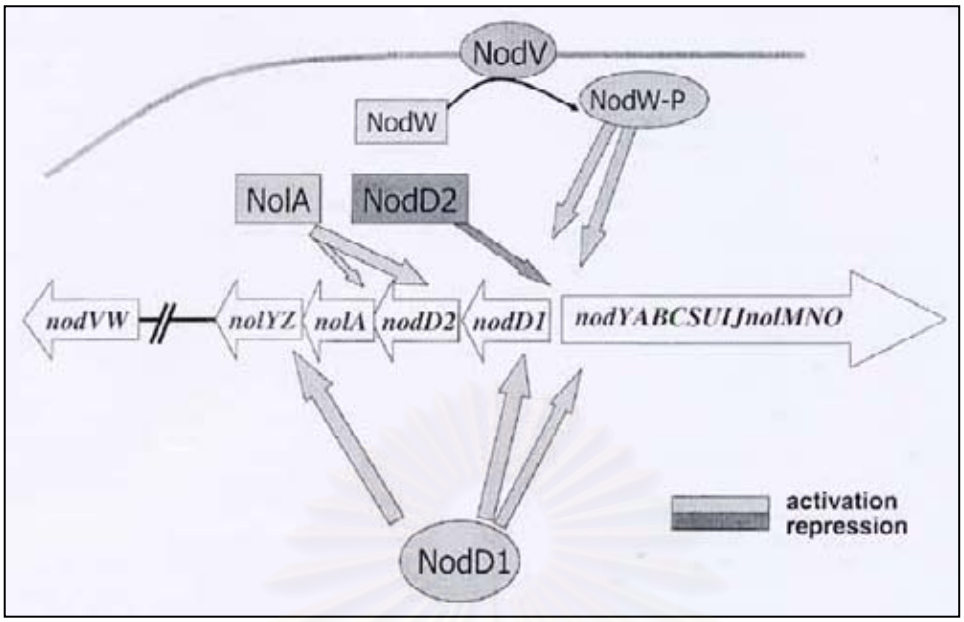
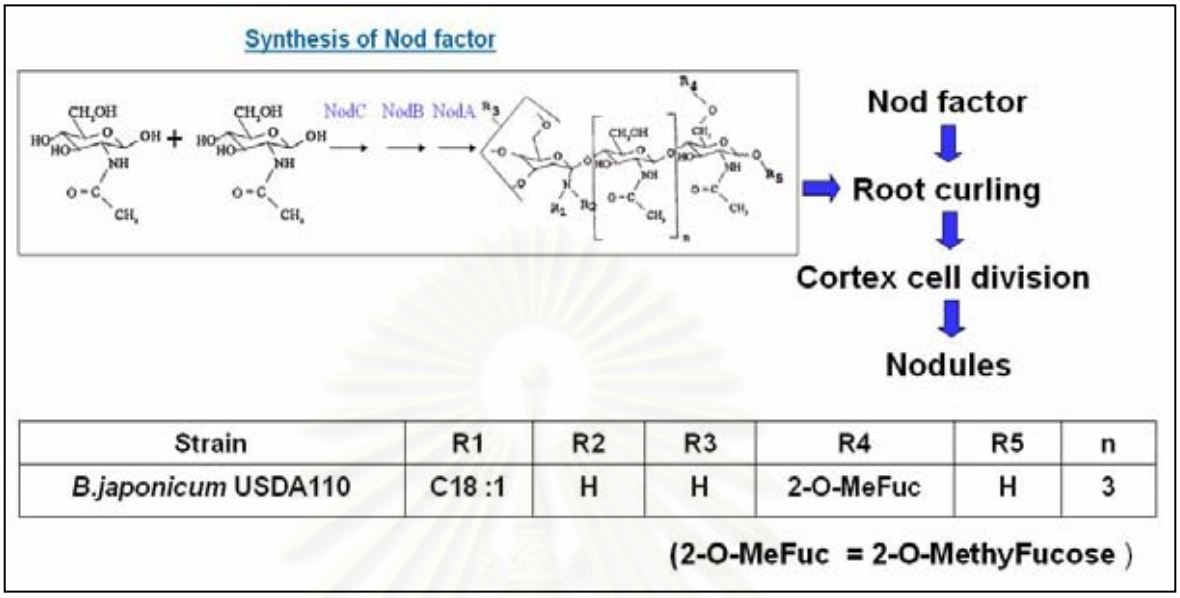


Figure 2.3 $NodD_1$-flavonoid complexes bind to $nodD_1$ box and *nodY* box in the promoters of $nodD_1$ and *nodYABC* to activate transcription of $nodD_1$ and *nodYABC*. A protein product NodV autophosphorylates then transfers the phosphate group to NodW. Phosphorylated NodW-P activates the expression of $nodD_1$ and *nodYABC.* NolA regulates the expression of $nodD_2$ whose protein product, $NodD_2$, 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.



Strain	R1	R2	R3	R4	R5	n
B.japonicum USDA110	C18 :1	H	H	2-O-MeFuc	H	3

(2-O-MeFuc = 2-O-MethyFucose)

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 10^4 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

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

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

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. Nod factors seemed to have a role in the 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 rhizobial cells to 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	α subunit of the MoFe-protein
nifK	β 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

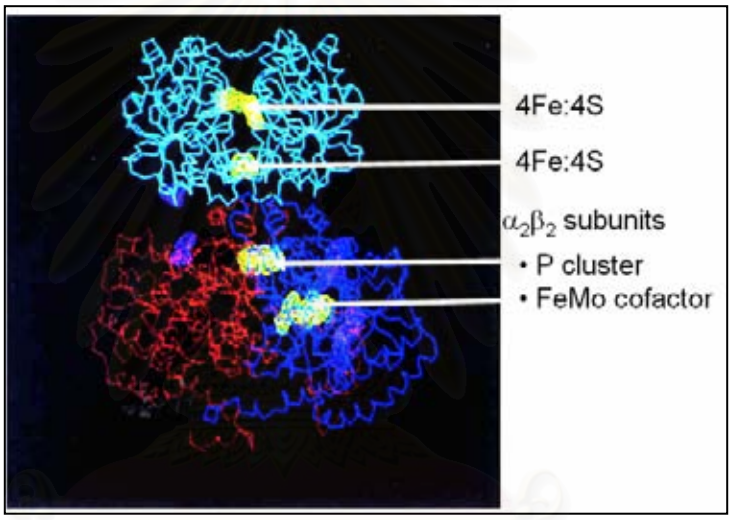


Figure 2.5 A ribbon representation of *Azotobacter vinelandii* nitrogenase which consists of an Fe-protein (light blue) and a MoFe-protein. Metal clusters are shown in yellow (Moffat, 1992).

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.

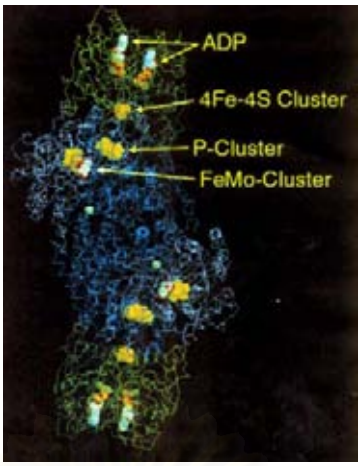


Figure 2.6 Each subunit of the Fe-protein consists of two ATP-binding sites and one 4Fe-4S cluster. Each $\alpha\beta$ subunit of the MoFe-protein consists of one P-cluster and one FeMo-cluster (http://www.rcsb.org/pdb/molecules/pdb26_3.htm).

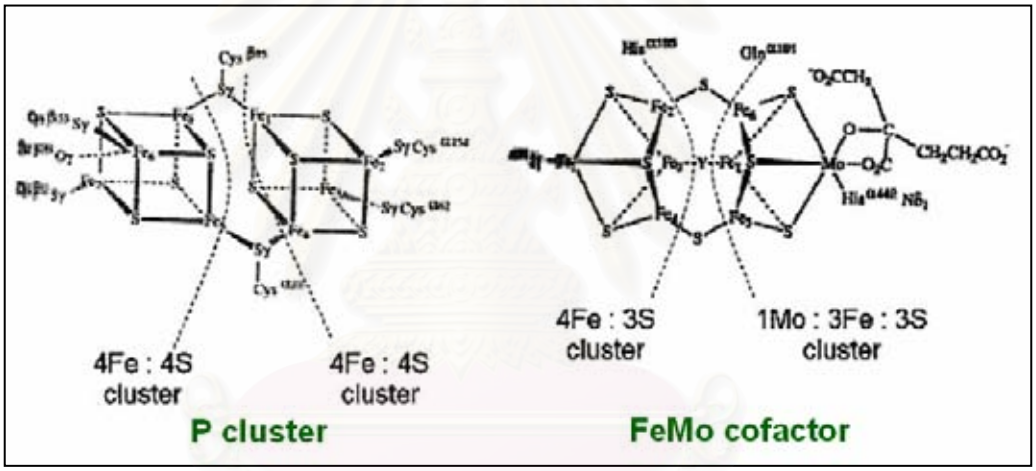


Figure 2.7 The P-cluster and the FeMo cofactor (cluster) of the MoFe-protein are attached the Cysteine, Histidine, and Glutamine residues of the MoFe-protein (Kim and Rees, 1992).

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

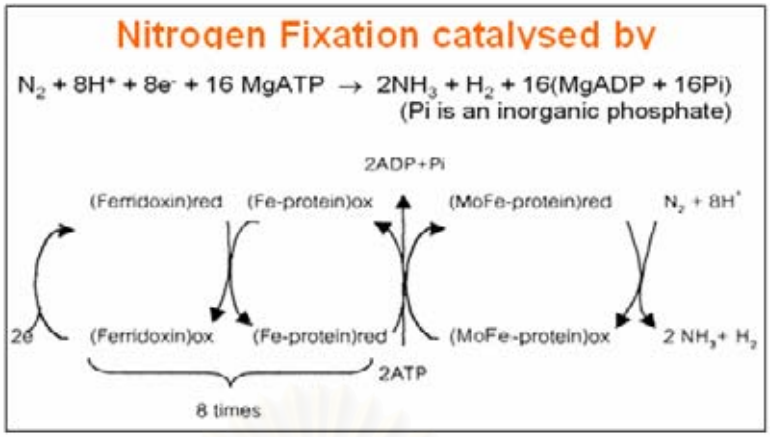
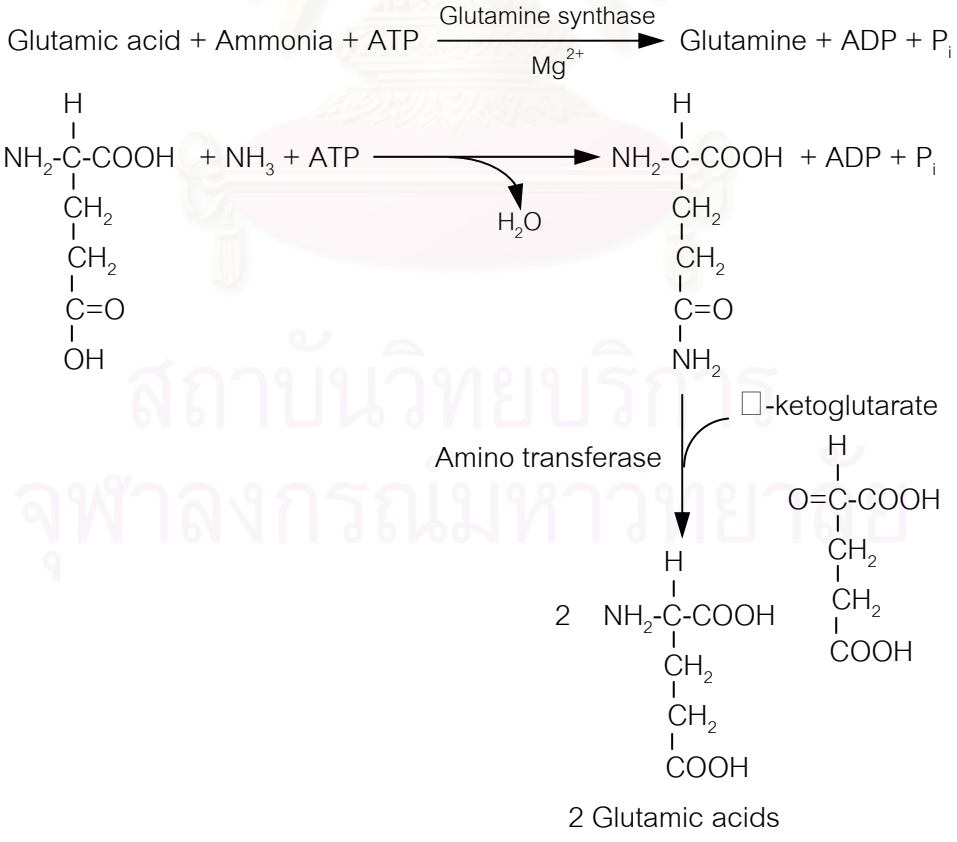


Figure 2.8 Electron transfer system in the nitrogen fixation process-catalysed by nitrogenase (Voet and Voet, 1995).

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)

Root temperature (°C)*	Uninoculated	*Rhizobium* strain R5029	R5009	R5018	CB756	R5030	Mean (inoculated)
		Mean dry wt plant (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
		Nitrogenase activity (μmoles C_2H_4/g dry wt nodule/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^{o}C until use.

Table 3.1 Soybean rhizobial strains used.

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

Strains were maintained at 4^{o}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 OD_{260}/OD_{280} 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			Program		
10x PCR buffer	2.5	µl	95 °C	15 seconds	5 cycles
50 mM $MgCl_2$	0.8	µl	55 °C	30 seconds	
10 mM dNTPs	0.5	µl	72 °C	90 seconds	
10 µM primer	5.0	µl	95 °C	15 seconds	25 cycles
DNA template (60-100 ng)	1.0	µl	60 °C	30 seconds	
Taq polymerase (5U. μl^{-1})	0.2	µl	72 °C	90 seconds	
High quality double distilled water	15.0	µl	72 °C	10 minutes	
Total	25.0	µl			

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°c, supernatant was transferred to a new Eppendorf tube. 200µl chloroform was added to the supernatant followed by incubation at 15-30°C 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°C for 10 minutes, collected by centrifugation at 8,000 rpm, 10 minutes, 4°c, 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.

nodD$_1$

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:

Mixture

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

Program

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				
Extension	72 °C	90	seconds	

RNA Template (ng)	Primer concentration		Reverse Transcription (min)	Annealing Temp (°C)
	Primers	pmol		
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

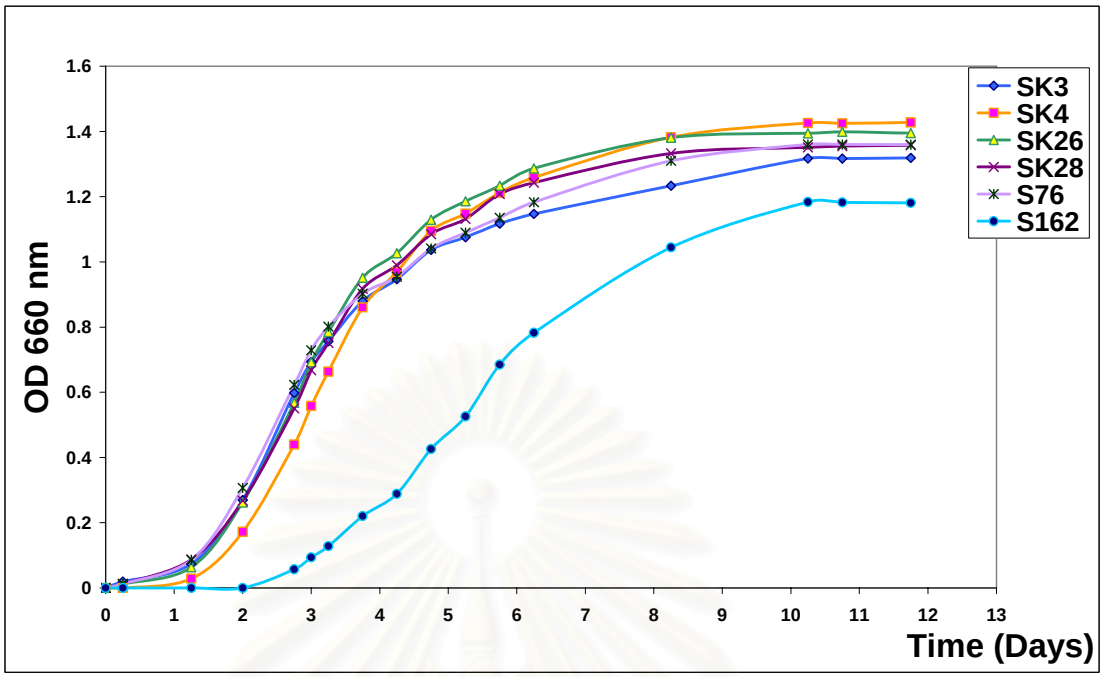
RESULTS

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^{o}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.

(A)



(B)

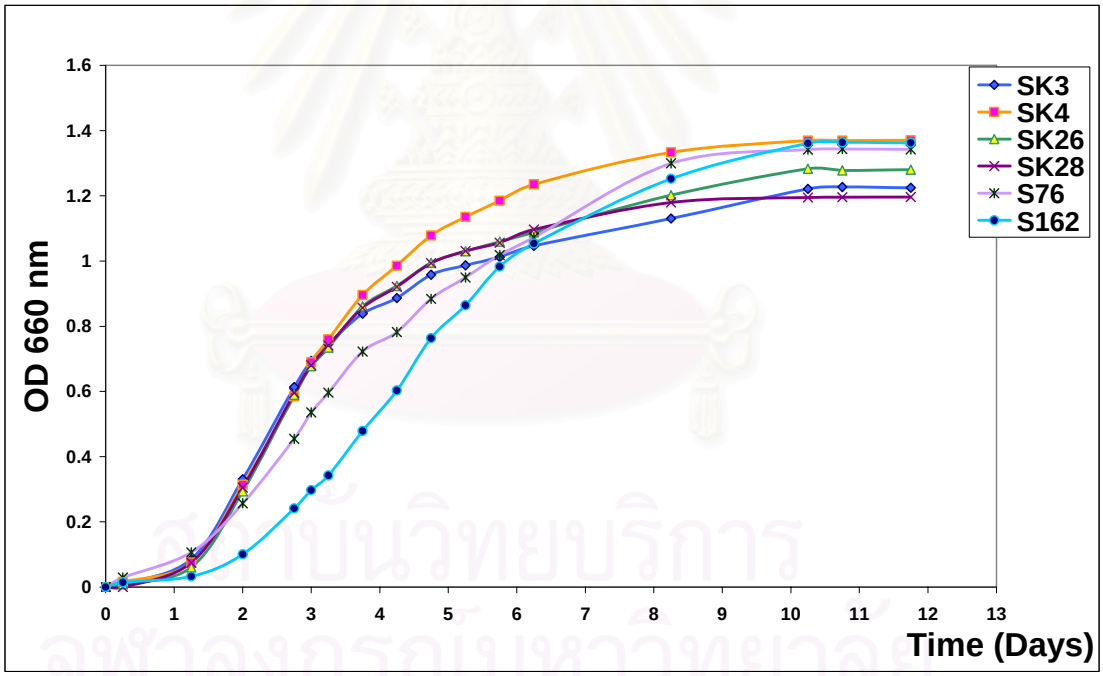


Figure 4.1 Changes in turbidity of growth cultures of 6 *B. japonicum* strains grown in (A) yeast extract glycerol medium (B) yeast extract mannitol medium, at 30°C, 200 rpm.

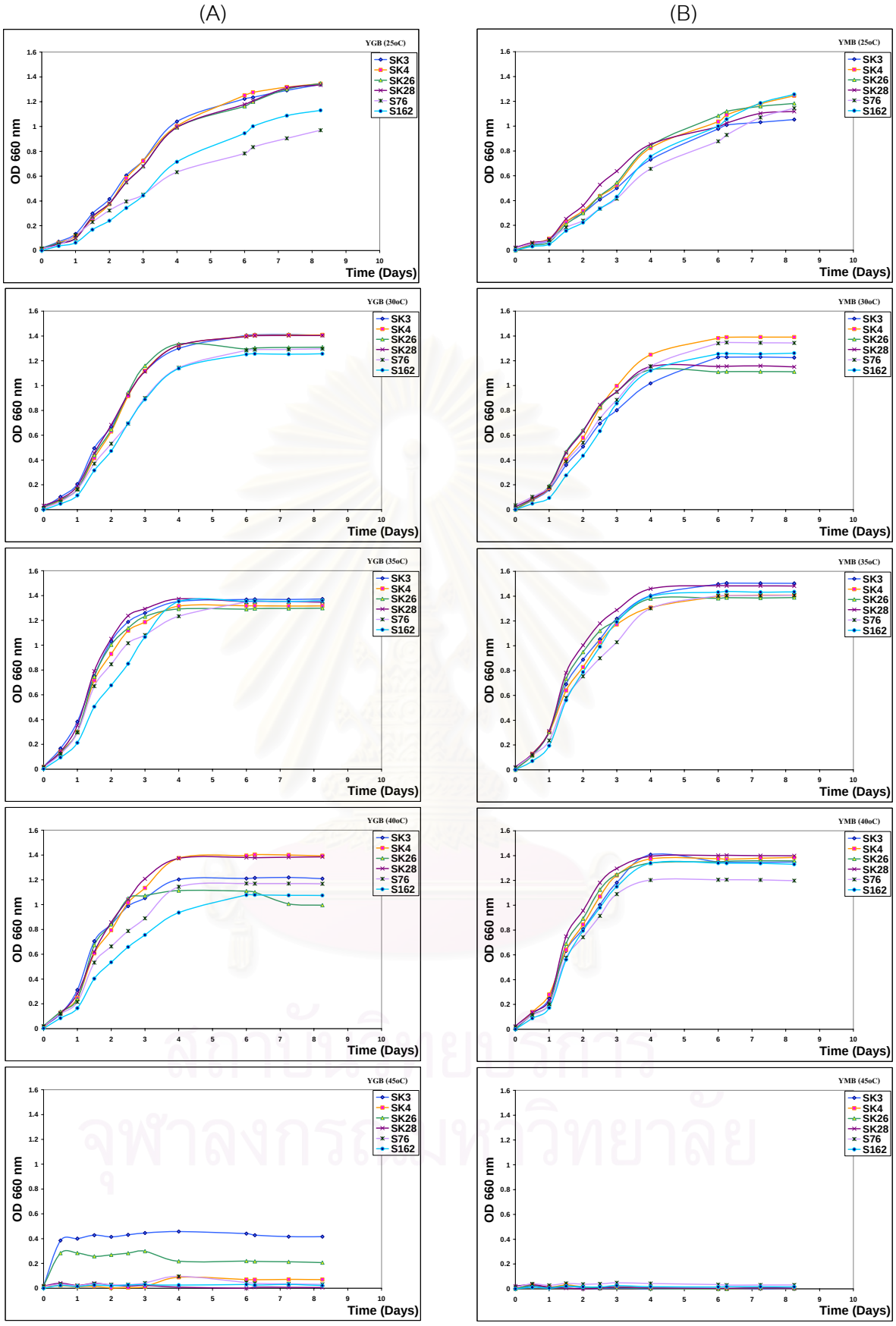


Figure 4.2 Turbidity of 6 *B. japonicum* strains grown in (A) yeast extract glycerol medium (B) yeast extract mannitol medium, at different temperatures, 200 rpm.

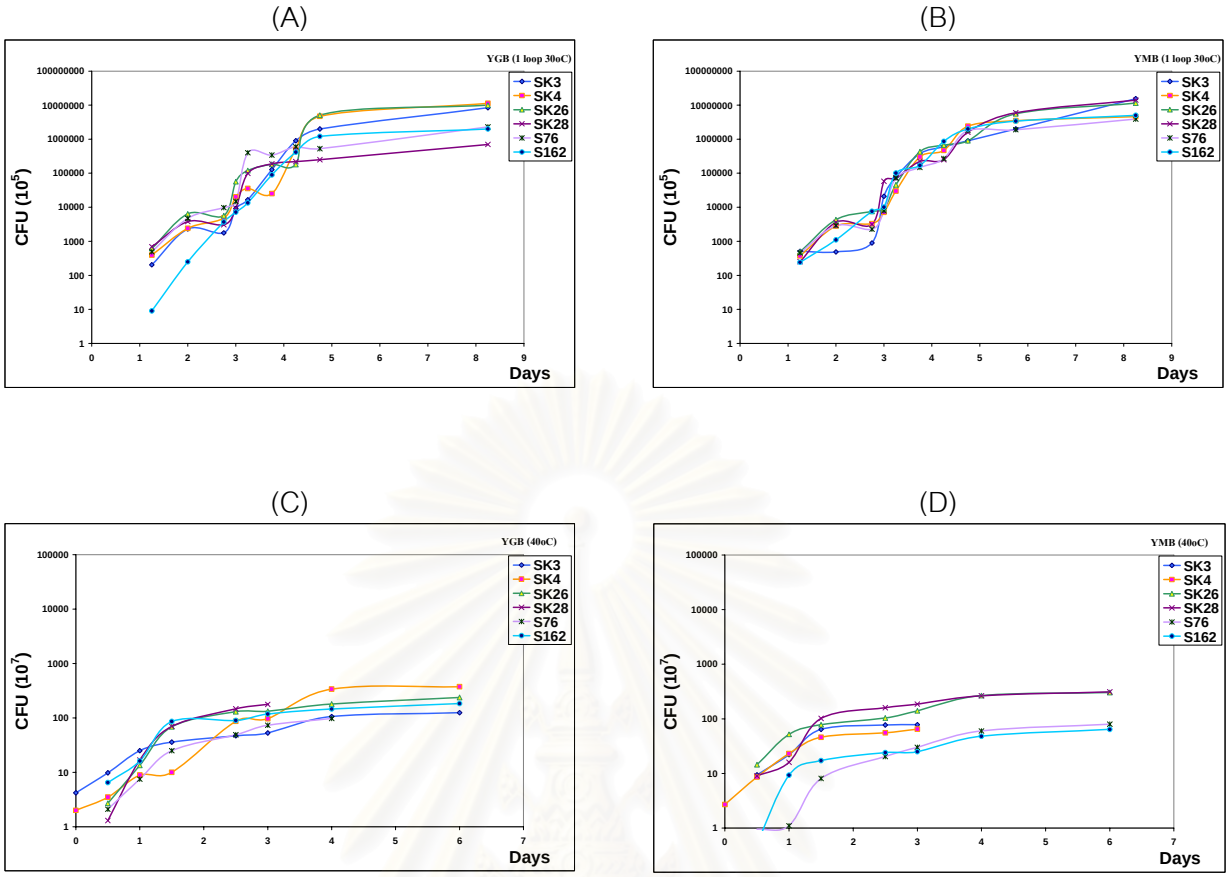


Figure 4.3 Viable plate counts of each strain of *B. japonicum* SK3, SK4, SK26, SK28, S76 and S162 grown in (A) YGB at 30°C (B) YMB at 30°C (C) YGB at 40°C (D) YMB at 40°C.

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 OD_{660} 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 OD_{660} 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 turbidity in YMB while at 45°C cultures of SK3 and SK26 seemed to increase rapidly then maintain turbidity of OD_{660} 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.

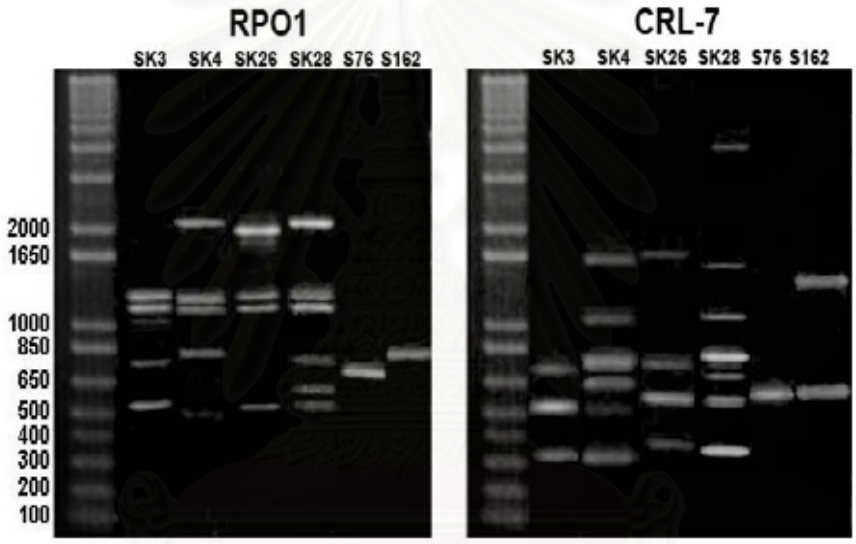


Figure 4.4 PCR fingerprinting of *B. japonicum* SK3, SK4, SK26, SK28, S76 and S162 when either RPO1 or CRL-7 was used as the primer.

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.

Primer	Nucleotide sequences (5' ⟶ 3')	PCR product sizes (bp)	%GC	T_m (°C)*
nodD$_1$				
Forward primer	5' AAAATGGCAGCAGYTCGAA 3' (19 bases)	317	42.1%	54.2
Reverse primer	5' CAACATCAATCTGAGCCAG 3' (19 bases)			
nodA				
Forward primer	5' GGTGGAGCGGACGCGAATGC 3' (20 bases)	414	70.0%	67.0
Reverse primer	5' GCTCGCCGATCATGCCG 3' (17 bases)		70.5%	62.8
nodB				
Forward primer	5' AGCACAATTGCWCCCGGGCG 3' (20 bases)	384	65.0%	64.9
Reverse primer	5' GCAACATCGGGTCCCCGCGA 3' (20 bases)		70.0%	67.0
nifH				
Forward primer	5' AGCCACCGCAAACAACGTCG 3' (20 bases)	363	60.0%	62.9
Reverse primer	5' ATCGGCAAGTCCACCACTTC 3' (20 bases)		55.0%	60.8
nifD				
Forward primer	5' TCMAGCAGAATTCGCGA 3' (17 bases)	445	52.9%	55.5
Reverse primer	5' AACTATTACGTTGGCAC 3' (17 bases)		41.1%	50.7
GAPDH				
Forward primer	5' YTCGTTGTCGTACCAG 3' (17 bases)	498	58.8%	57.9
Reverse primer	5' CTGCACSACSAACTGC 3' (17 bases)		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)

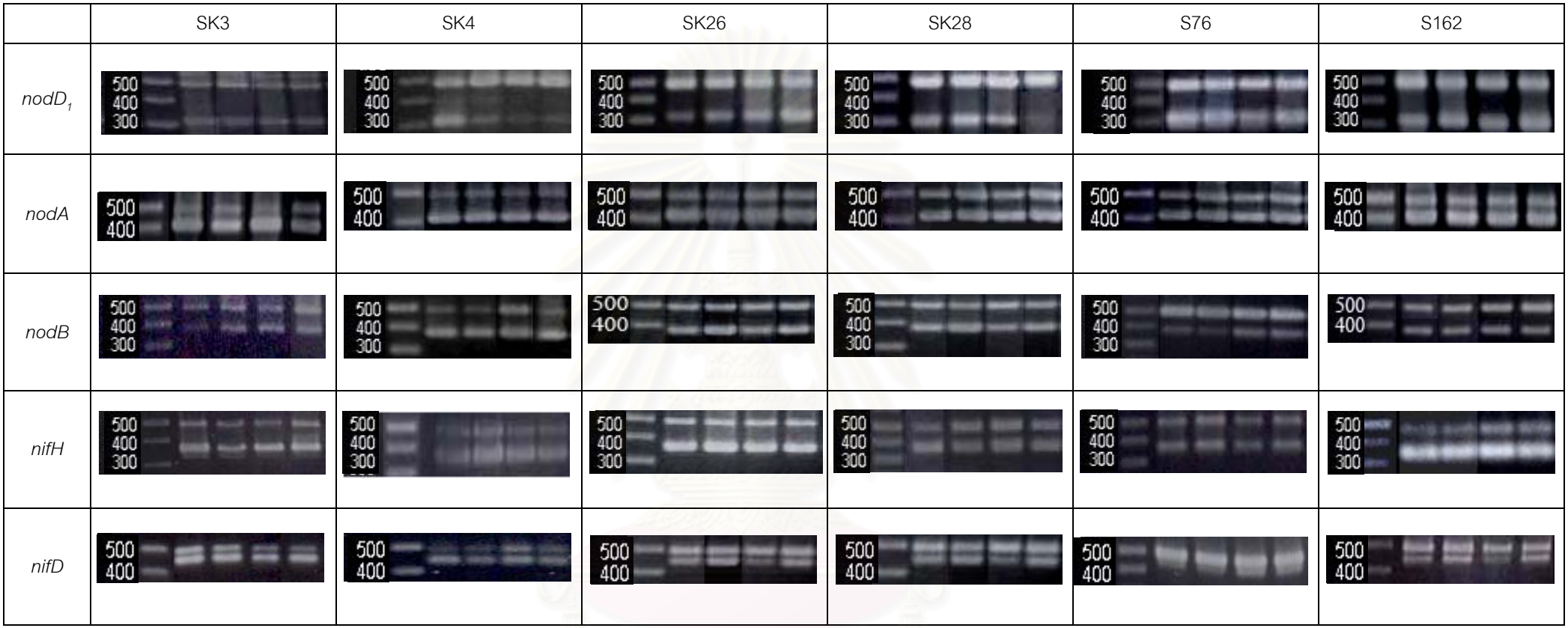


Figure 4.5 Expression of *nodD*$_1$, *nodA, nodB, nifH*, and *nifD* of *B. japonicum* strains SK3, SK4, SK26, SK28, S76 and S162 grown in yeast extract glycerol medium at 25°C (lanes 2), 30°C (lanes 3), 35°C (lanes 4) and 40°C (lanes 5), 200 rpm. Lanes 1 are molecular size standards. GAPDH bands are 498 bp.

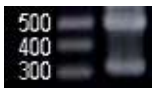
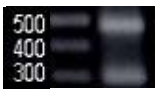
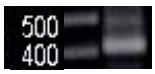
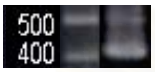
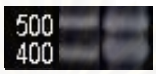
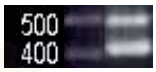
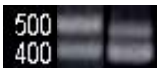
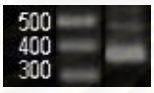
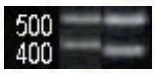
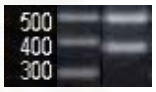
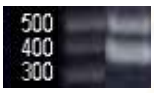
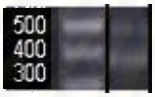
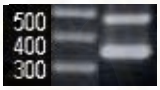
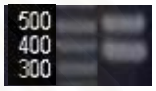
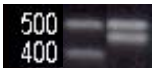
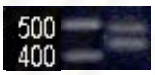
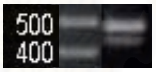
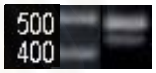
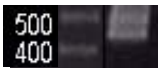
	SK3	SK4	SK26	SK28	S76	S162
nodD$_1$						
nodA						
nodB						
nifH						
nifD						

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.

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 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 $nodD_1$ 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°C–40°C and at 45°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.

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.

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*. Mol. Microbiol. 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*. Microbiology 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. FEMS Microbiol. Lett. 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*. Proceedings of the Association of Applied Biologists. 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. EMBO J. 12 : 2901-2912.

Forsberg, A.J., Pavitt, G.D., Higgins, C.F. 1994. Use of transcriptional fusions to monitor gene expression: a cautionary tale. J.Bacteriol. 176(7) : 2128-2132.

Göttfert, M. 1993. Regulation and function of rhizobial nodulation genes. FEMS Microbiol. Rev. 104 : 39-64.

Jordan, D.C. 1984. Family III. Rhizobiaceae In Krieg, N.R., Holt, J.G. (eds). Bergey's Manual of Systematic Bacteriology. Baltimore : Williams & Wilkins. p. 234-244.

Kim, J., Rees, D.C. 1992. Structural models for the metal centers in the nitrogenase molybdenum-iron protein. Science. 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*. Proc. Natl. Acad. Sci USA. 84: 7428-7432.

Loh, J., Stacey, G. 2003. Nodulation gene regulation in *Bradyrhizobium japonicum* : a unique intergration of global regulatory circuits. Appl. Environ. Microbiol. 69(1) : 10-17.

Long S.R. 1996. *Rhizobium* Symbiosis: Nod Factors in Perspective. The Plant Cell. 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. Plant and Soil. 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 $^{32}\sigma$-dependent class of heat shock genes in *Bradyrhizobium japonicum*. Mol. Genet. 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. J. Bacteriol. 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. Nucleic Acids Res. 29 (23) : 4800-4807.

Pueppke, S.G. 1984. Adsorption of slow- and fast-growing rhizobia to soybean and cowpea roots. Plant Physiol. 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. Soil Biol. Biochem. 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. Nucleic Acids Res. 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. FEMS Microbiol. Letters. 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. J. Bacteriol. 173 (11) : 3356-3365.

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_2HPO_4	0.5 g
$MgSO_4.7H_2O$	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 (YMA)

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 $\mu g.ml^{-1}$. The medium was autoclaved at 121°C for 15 min.

Yeast Extract Glycerol Broth (YGB)

Glycerol	10.0 ml
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	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

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

2. **Solutions for RNA extraction**

RNA loading buffer

Glycerol	500.0	µl
Bromophenol blue	2.0	µg
Xylene Cyanol	2.0	µg
0.5 M EDTA	2.0	µl

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

RNA Sample buffer

25x MOPS	40.0	µl
di-Formamide	500.0	µl
di-Formaldehyde	55.0	µl

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)**

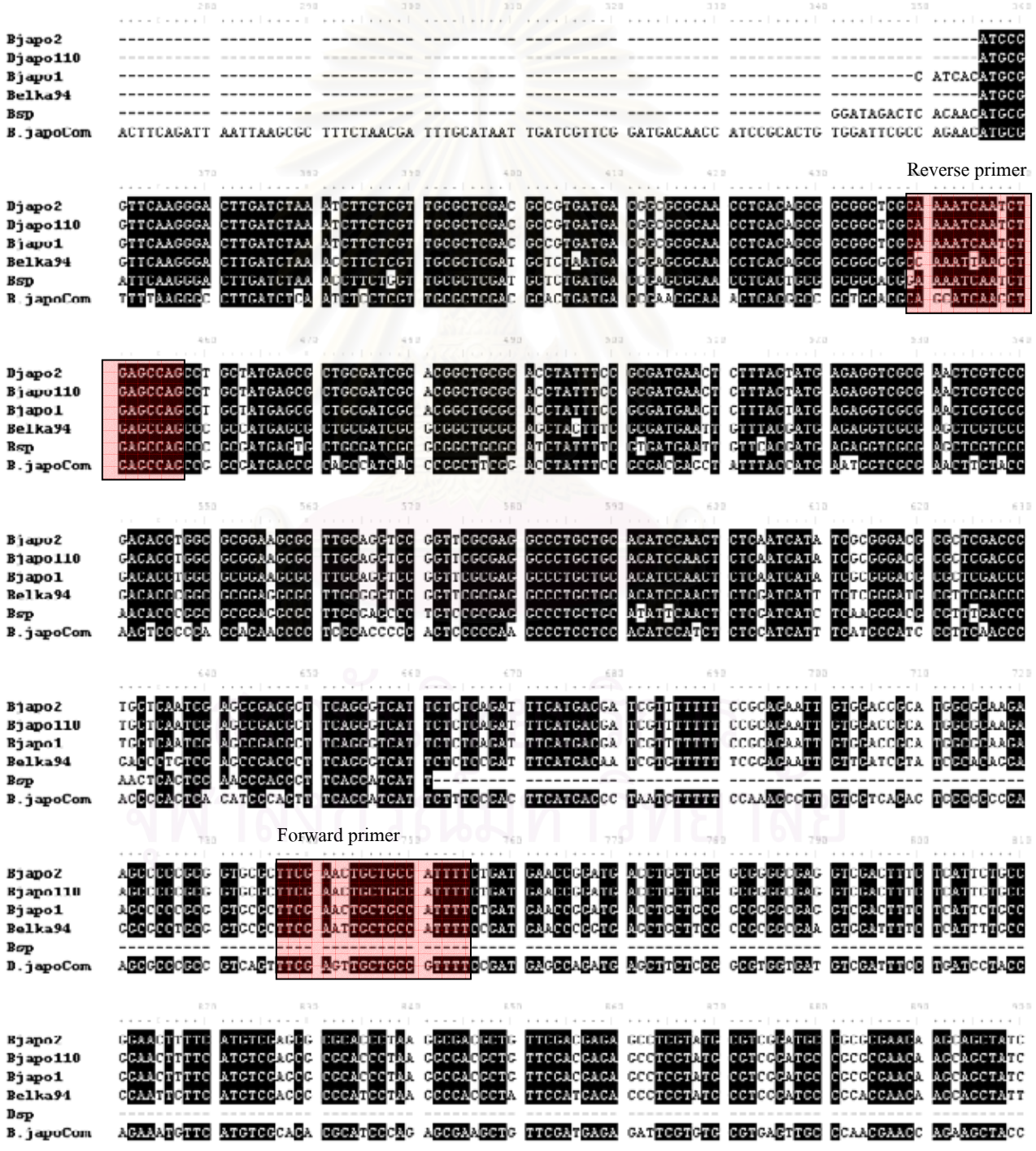
Tris-base	24.20 g
$Na_2EDTA{\cdot}2H_2O$	1.86 g
Acetic acid	5.71 ml

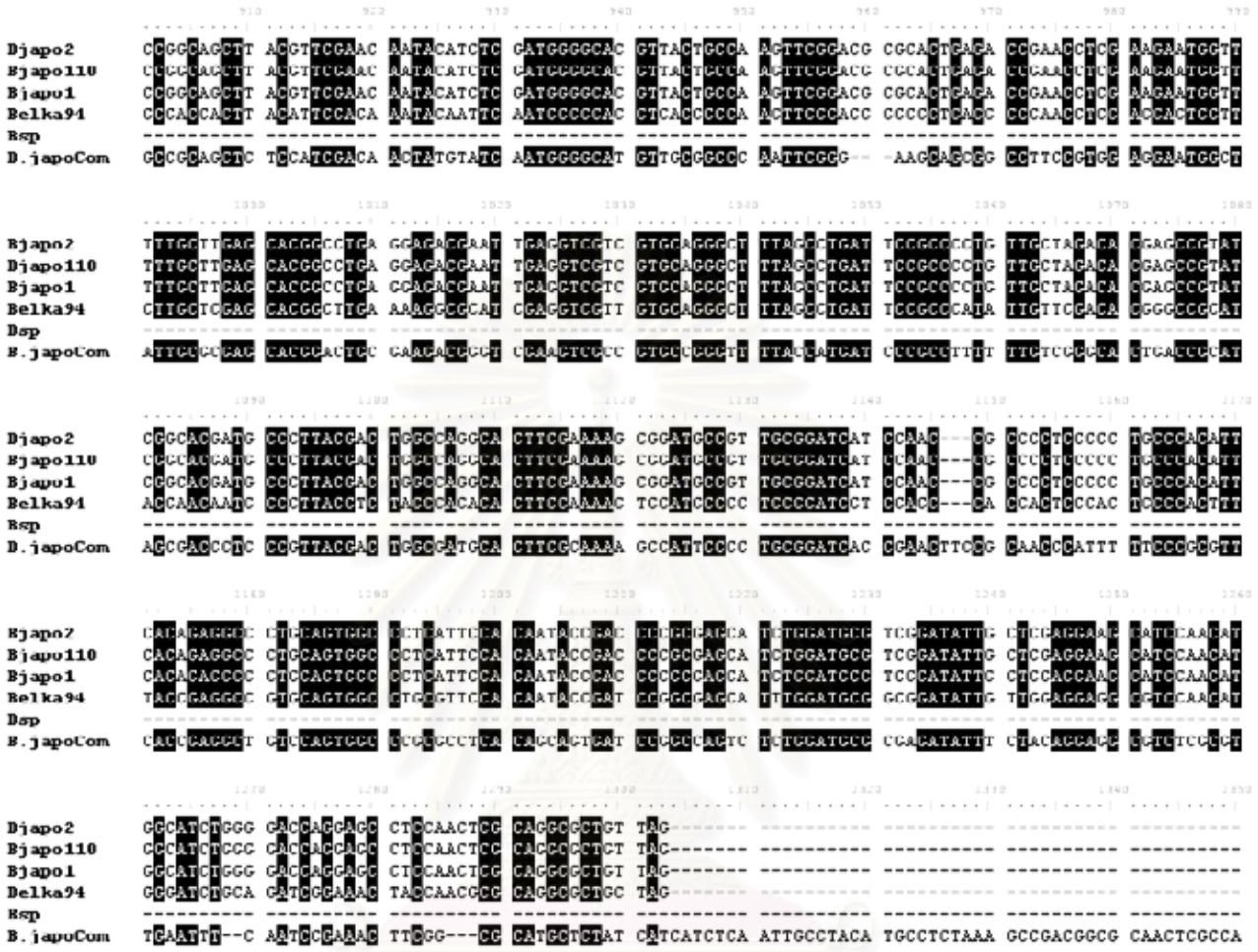
The final volume was made to 100 ml. by deionized distill water

APPENDIX C

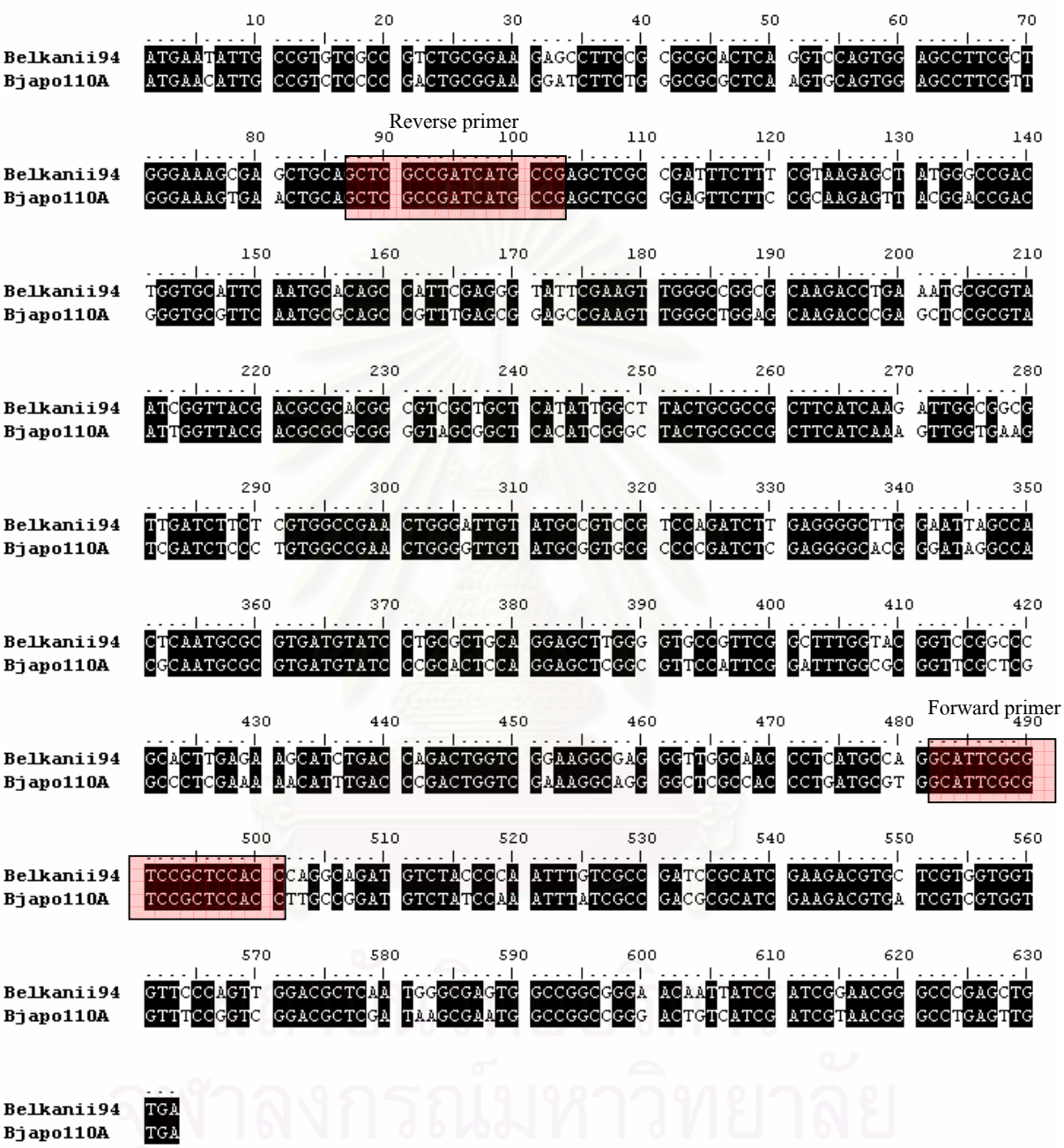
Nucleotide sequences for design of primers

1. *nodD*$_1$ forward and reverse primers are in boxes

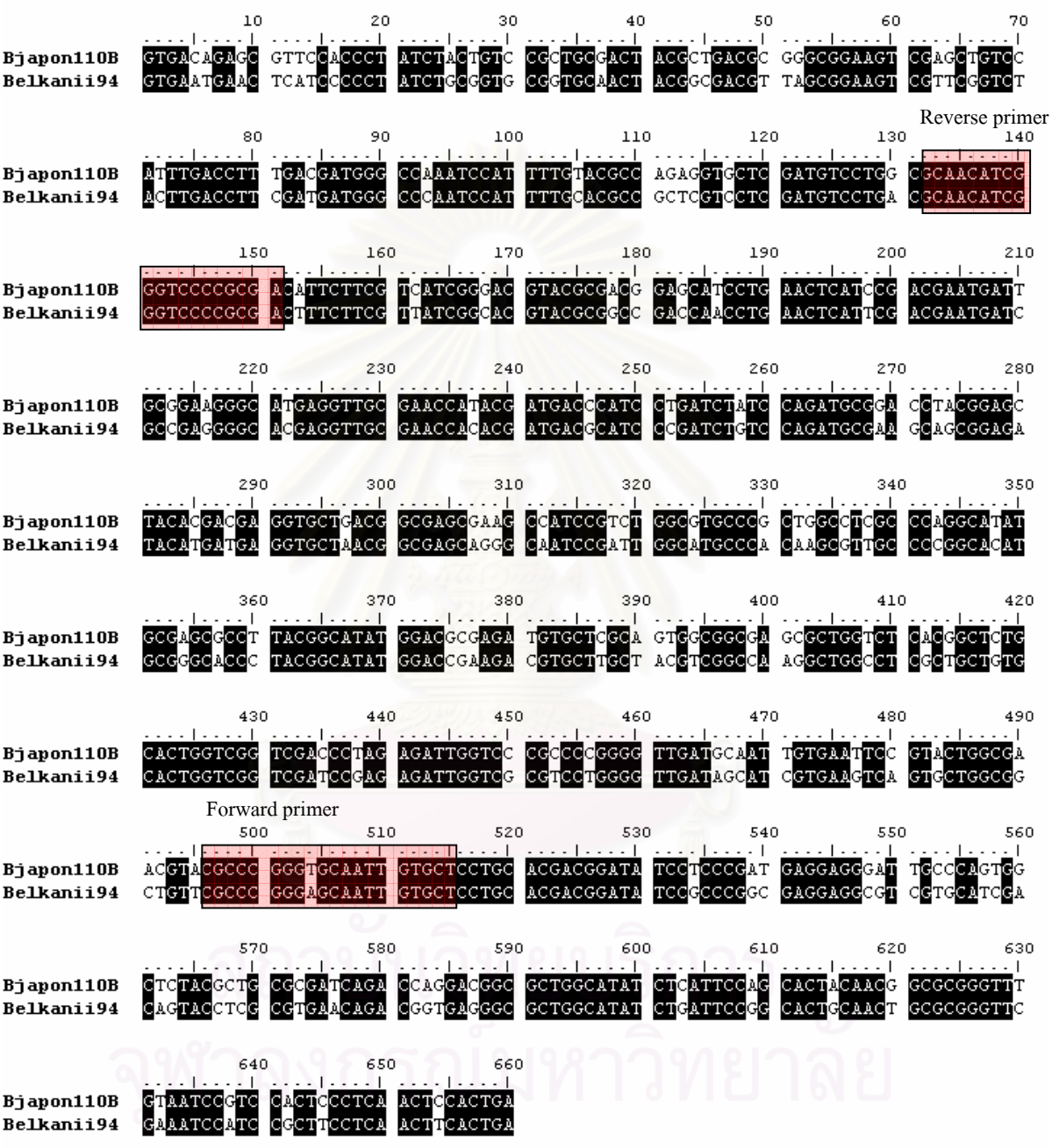




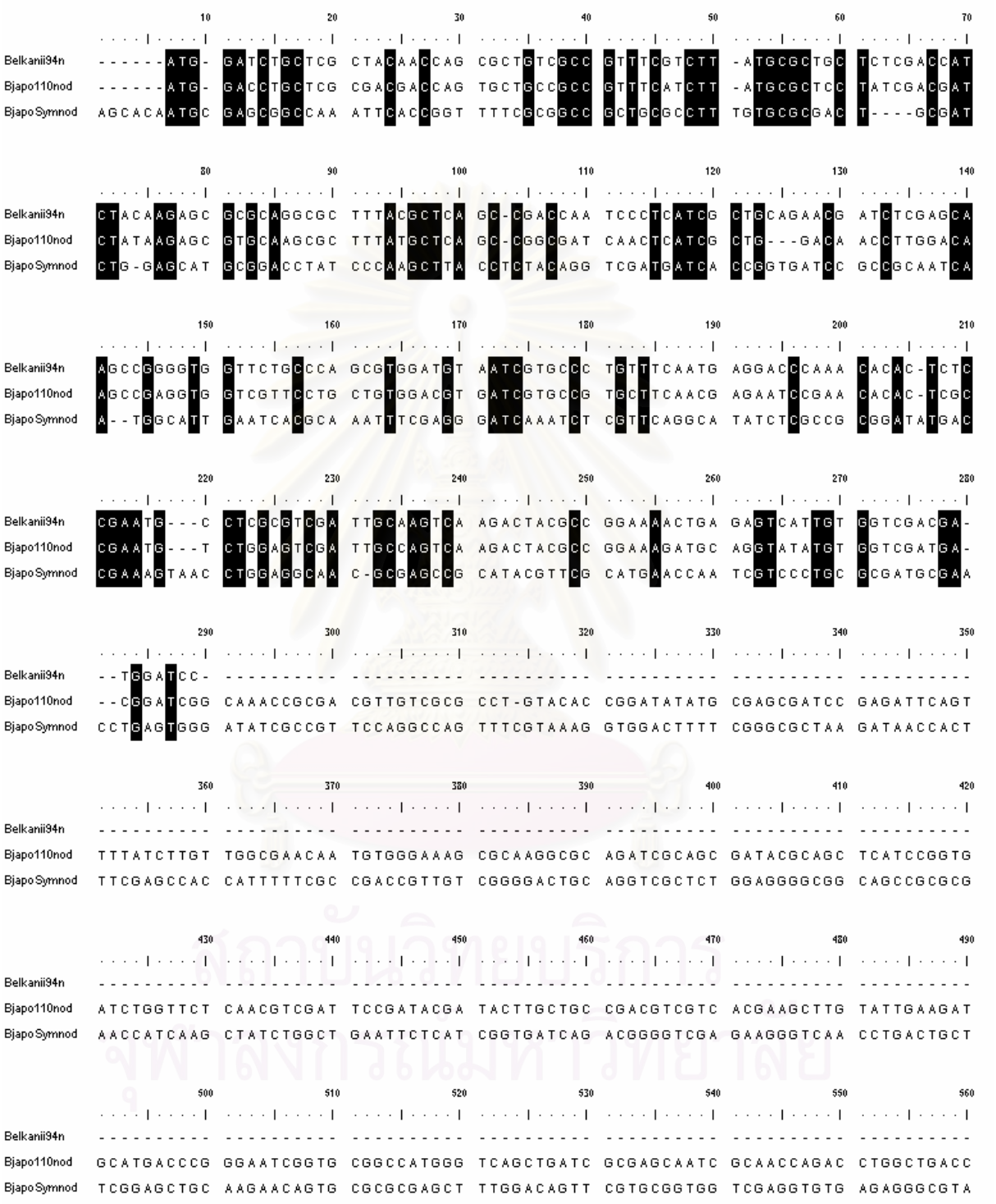
2. *nodA* forward and reverse primers are in boxes

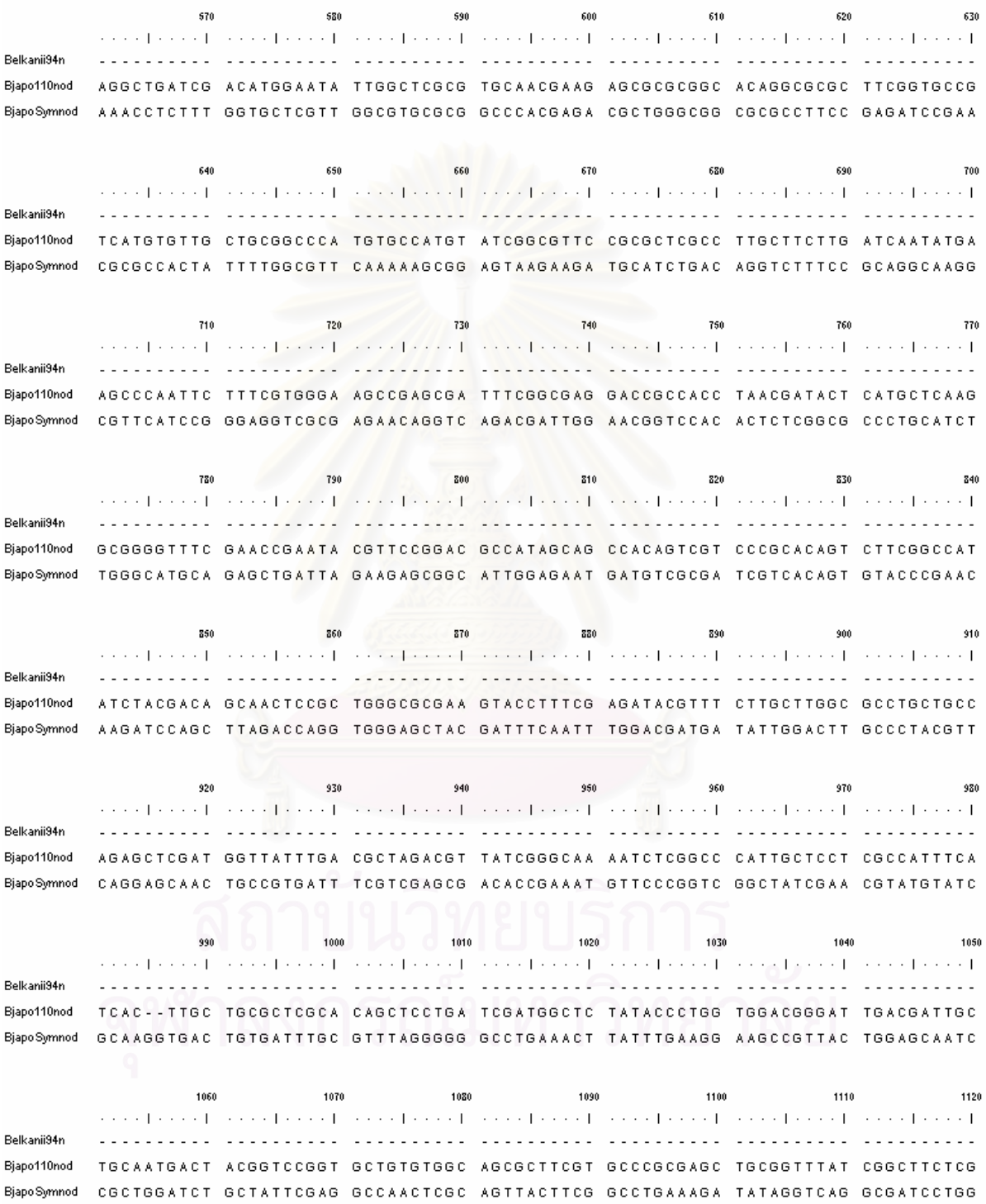


3. *nodB* forward and reverse primers are in boxes



4. *nodC*

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BjapoSymnod AAACCTCTTT GGTGCTCGTT GGCGTGCGCG GCCCACGAGA CGCTGGGCGG CGCGCCTTCC GAGATCCGAA
640 650 660 670 680 690 700
Belkanii94n ---------- ---------- ---------- ---------- ---------- ---------- ----------
Bjapo110nod TCATGTGTTG CTGCGGCCCA TGTGCCATGT ATCGGCGTTC CGCGCTCGCC TTGCTTCTTG ATCAATATGA
BjapoSymnod CGCGCCACTA TTTTGGCGTT CAAAAAGCGG AGTAAGAAGA TGCATCTGAC AGGTCTTTCC GCAGGCAAGG
710 720 730 740 750 760 770
Belkanii94n ---------- ---------- ---------- ---------- ---------- ---------- ----------
Bjapo110nod AGCCCAATTC TTTCGTGGGA AGCCGAGCGA TTTCGGCGAG GACCGCCACC TAACGATACT CATGCTCAAG
BjapoSymnod CGTTCATCCG GGAGGTCGCG AGAACAGGTC AGACGATTGG AACGGTCCAC ACTCTCGGCG CCCTGCATCT
780 790 800 810 820 830 840
Belkanii94n ---------- ---------- ---------- ---------- ---------- ---------- ----------
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BjapoSymnod TGGGCATGCA GAGCTGATTA GAAGAGCGGC ATTGGAGAAT GATGTCGCGA TCGTCACAGT GTACCCGAAC
850 860 870 880 890 900 910
Belkanii94n ---------- ---------- ---------- ---------- ---------- ---------- ----------
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BjapoSymnod AAGATCCAGC TTAGACCAGG TGGGAGCTAC GATTTCAATT TGGACGATGA TATTGGACTT GCCCTACGTT
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Belkanii94n ---------- ---------- ---------- ---------- ---------- ---------- ----------
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BjapoSymnod CAGGAGCAAC TGCCGTGATT TCGTCGAGCG ACACCGAAAT GTTCCCGGTC GGCTATCGAA CGTATGTATC
990 1000 1010 1020 1030 1040 1050
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BjapoSymnod GCAAGGTGAC TGTGATTTGC GTTTAGGGGG GCCTGAAACT TATTTGAAGG AAGCCGTTAC TGGAGCAATC
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BjapoSymnod CGCTGGATCT GCTATTCGAG GCCAACTCGC AGTTACTTCG GCCTGAAAGA TATAGGTCAG GCGATCCTGG

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Belkanii94n         ----------  ----------  ----------  ----------  ----------  ----------  ----------  ----------
Bjapo110nod         CTCCACACGC  CGATCAATAT  CTGTCTCTTA  CTGCCTTTGA  AGGCCTATGC  GCTTTGTACA  TTGAGCAATA
BjapoSymnod         TAAAGCGCGC  CGTCGCAGAC  CTGCTTCTCG  ATTGCGAGAT  AAGGTTTGTG  CCGACCGTGA  GATACAAGAA

                    1200                1210                1220                1230                1240                1250                1260
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Belkanii94n         ----------  ----------  ----------  ----------  ----------  ----------  ----------  ----------
Bjapo110nod         GCGATTGGCT  ATCTCGGAAA  GTCACCGATA  TGCCGACGGA  AGAGGGGAAA  CAGCCTGTCA  TCCTGCACCC
BjapoSymnod         CGGAGTGCCC  ATTTCGTCGC  GCTTGAGGCG  ATTTAACCGT  TCCGAGCTTG  ATGAGCTGTC  CTGTCTCTTC

                    1270                1280                1290                1300                1310                1320                1330
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Belkanii94n         ----------  ----------  ----------  ----------  ----------  ----------  ----------  ----------
Bjapo110nod         GAATGCCGGA  CGAAGTCCTG  CTGGTGTAGG  GGGGCGCCTG  CTCCTATTCG  TAAGGCGGCG  TTATCGCAGC
BjapoSymnod         ACGGCACTCA  ACCGTTCAAG  AGAAGAGATC  GCCCGAGGAG  CGTCAAGCGT  GAAGGATCTG  GTCGCGTCGG

                    1340                1350                1360                1370                1380                1390                1400
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Bjapo110nod         CTCCATCGAG  CCTGGCGGCG  ACGGAGAGTG  TTTCCGGTCG  CGATCGTTCG  ACTGTCTACA  AATAAGTGGT
BjapoSymnod         CGACATCTCG  GATAGAGTTT  CGACAGTTTA  GACTCGAGTT  TGTTCGGATT  GTCGAC-GCT  GATAATTTCA

                    1410                1420                1430                1440                1450                1460                1470
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Belkanii94n         ----------  ----------  ----------  ----------  ----------  ----------  ----------  ----------
Bjapo110nod         CGGCTGATGA  CTCAGGACGA  AAACCATCAG  TTATTAGAGC  GAGAGTTGGC  TGTCGACGAC  CCGTGGCGCC
BjapoSymnod         AAGATTTAGA  GCAGGTCAAG  CTCCCATTCT  TAATTATTGG  GGCTGCAATG  GCTCATGGCC  TCACGGT---

                    1480
                    ....|....|
Belkanii94n         ----------
Bjapo110nod         TCGACACTAG
BjapoSymnod         ----------
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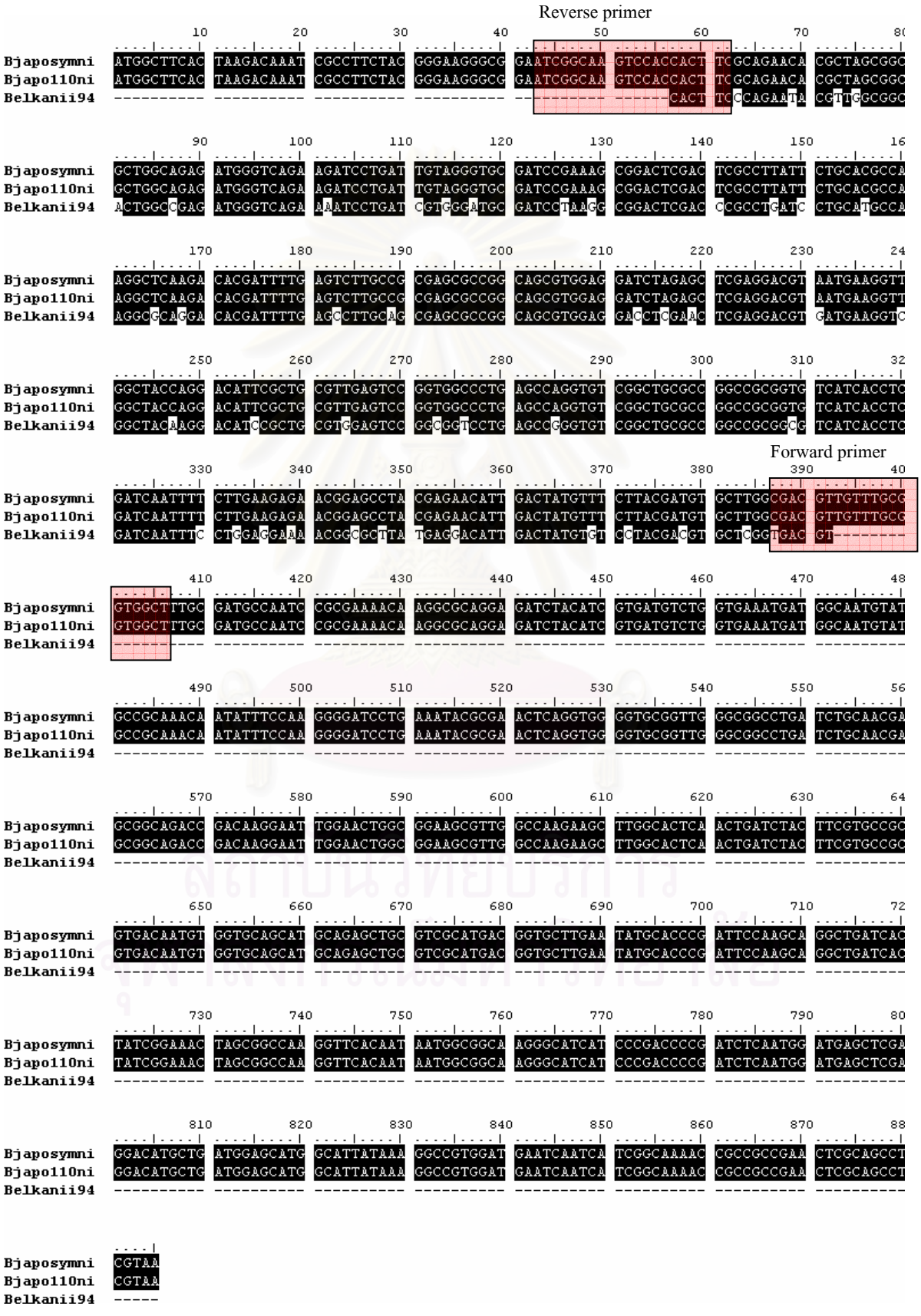
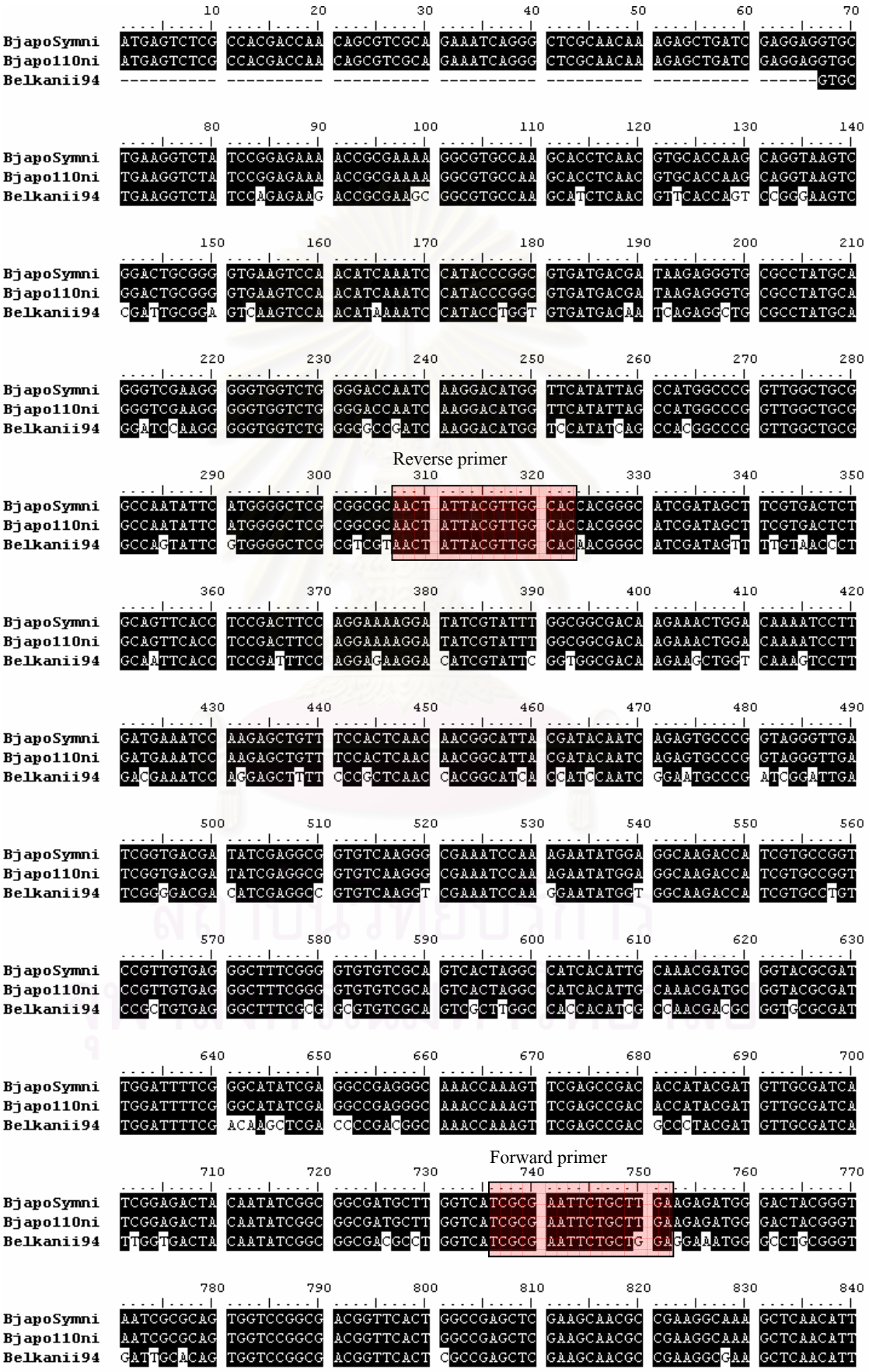
5. *nifH* forward and reverse primers are in boxes



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

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Bjapo110ni CTGCATTGCT ACCGTTCCAT GAACTATATC TCACGCCACA TGGAAGAGAA GTTCGGCATC CCTTGGTGCG
Belkanii94 CTGCATTGCT ACCGTTCGAT GAACTACATC TCGCGTCATA T---------- ---------- ----------
920 930 940 950 960 970 980
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Bjapo110ni AGTACAACTT CTTCGGACCT TCAAAGATCG CGGACTCACT GCGCAGGATT GCGGGTTATT TTGACGACAA
Belkanii94 ---------- ---------- ---------- ---------- ---------- ---------- ----------
990 1000 1010 1020 1030 1040 1050
BjapoSymni GATCAAGGAA GGCGCCGAGC GAGTGATCGA GAAGTATCAG CCGCTGGTGG ACGCCGTGAT TGCAAAATAT
Bjapo110ni GATCAAGGAA GGCGCCGAGC GAGTGATCGA GAAGTATCAG CCGCTGGTGG ACGCCGTGAT TGCAAAATAT
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Belkanii94 ---------- ---------- ---------- ---------- ---------- ---------- ----------
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Bjapo110ni CACAGCTCAG CACTACGTGA AAGACAGCAC CCTCATCTAT GATGACGTCA ATGGCTATGA GTTCGAGCGC
Belkanii94 ---------- ---------- ---------- ---------- ---------- ---------- ----------
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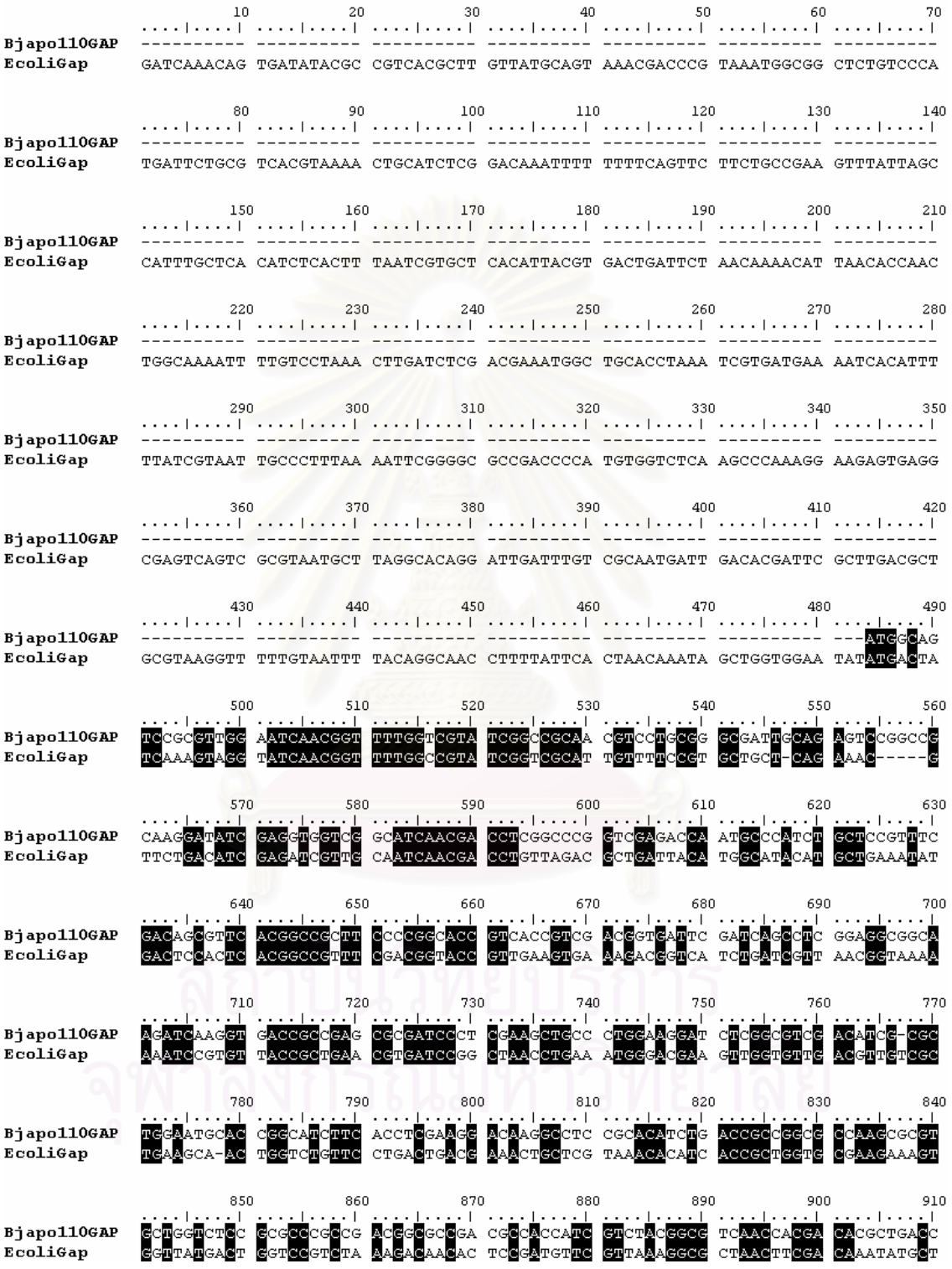
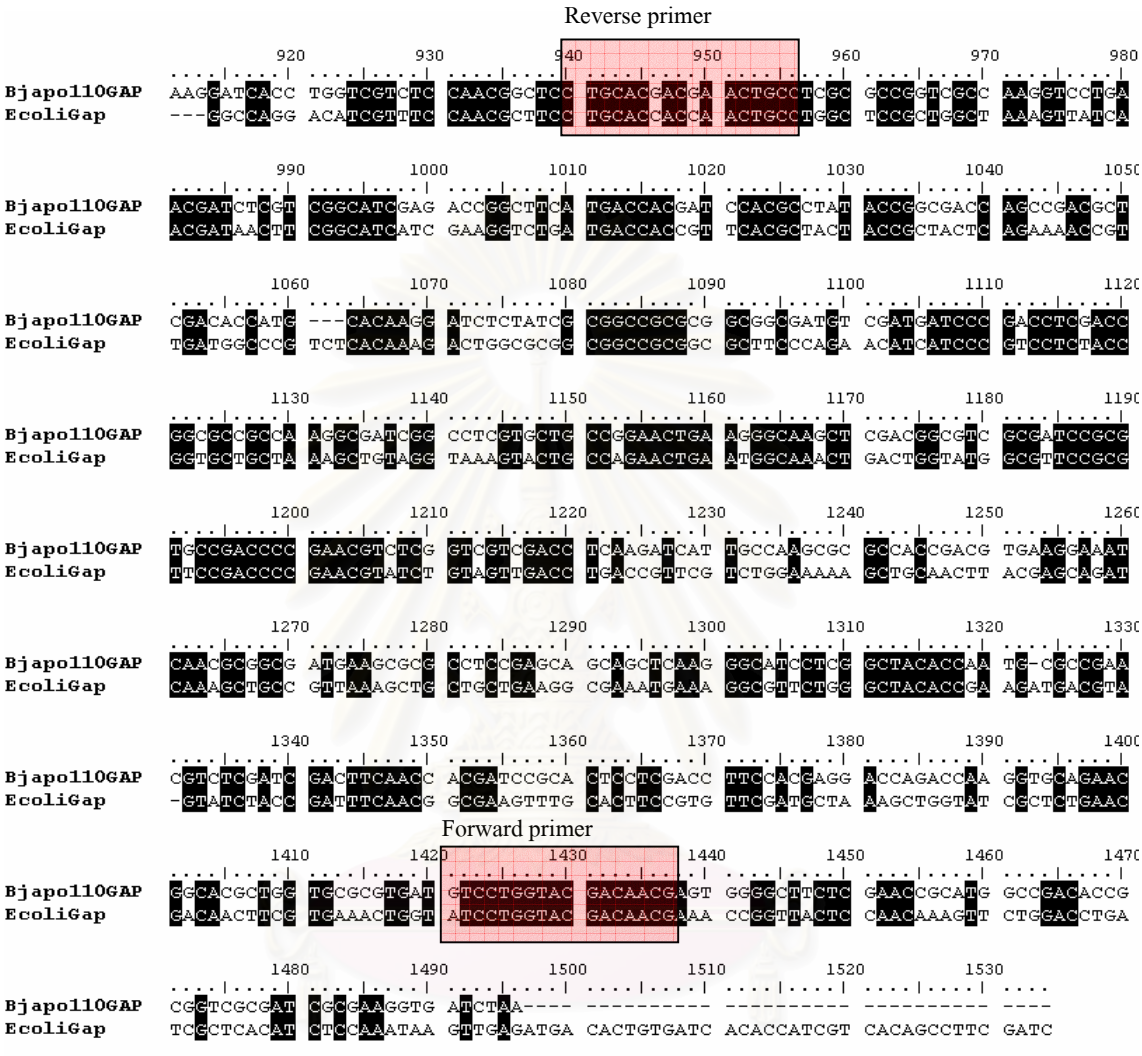
Bjapo110ni GCGCCGAGCG CCAAGCTCCA GGCTGCAGAA TAA
Belkanii94 ---------- ---------- ---------- ---

7. GAPDH forward and reverse primers are in boxes





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

1. 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

1. สิรัส สุลัญชุปกร และ กาญจนา ชาญสง่าเวช. 2549. การเปรียบเทียบโพรไฟล์ของโปรตีนภายในเซลล์ของ *Bradyrhizobium japonicum* 6 สายพันธุ์ ที่เลี้ยง ณ อุณหภูมิต่างๆ. หนังสือรวมบทคัดย่อการประชุมวิชาการ คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย. ครั้งที่ 14 : หน้า 13.
2. 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.
3. 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).