Expression analyses of *Bradyrhizobium japonicum* in the initial interaction with *Glycine max* (L.) merr

Min Wei

Science of Bioresources

The United Graduate School of

Agricultural Science, Iwate University

(Obihiro University of Agriculture and Veterinary Medicine)

To the memory of my father

Declaration of Authenticity

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by other person nor material which has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgement has been made in the text.

Min Wei

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Abstract

Initial interaction between (Brady)rhizobia and legumes actually starts via encounters of both partners in the rhizosphere. To explore this interaction, in this thesis, the global expression profiles of *Bradyrhizobium japonicum* in response to soybean (*Glycine max*) seed extracts (SSE) and genistein, a major soybean-released isoflavone which induce *nod* genes of *B. japonicum*, were monitored with time at common growth (30°C) and low (15°C) temperature.

First, at the common growth temperature, SSE markedly induced four predominant genomic regions within a large symbiosis island (681kb), which includes type III secretion system (TTSS) and various nodulation genes. In addition, SSE-treated cells expressed many genomic loci containing genes for polygalacturonase (cell wall degradation), exopolysaccharide (EPS) synthesis, 1-aminocyclopropane-1- carboxyl ate (ACC) deaminase, ribosome proteins family and energy metabolism even outside symbiosis island. On the other hand, genistein-treated cells exclusively showed one expression cluster including common *nod* gene operon within symbiosis island and six expression loci including multidrug resistance, which were shared with SSE-treated cells. Second, low temperature led to a potentially delayed expression of those SSE-preferentially-induced genome loci including TTSS genes at 30°C compared with those genistein-induced loci relevant to nodulation and multidrug resistance. Finally, NodD2 was found to be a novel component required for the full induction of TTSS genes and then a modified regulatory circuit for the TTSS genes was proposed.

Taken together, this thesis originally captured genome-scale expression profiles of *B. japonicum* in the initial interaction with soybean. The results can provide a profound insight into *B. japonicum*-soybean symbiosis, and also a basic knowledge for screening novel genes relevant to this process.

CHAPTER 1

Introduction

1. 1 Biological Nitrogen Fixation

Nitrogen fixation, a process fixing the abundant unavailable N_2 in the form of ammonium (NH_4) or nitrate (NO_3), is a vital component of the global nitrogen cycles and agricultural practices. Higher plants and animals obtain nitrogen ultimately from biological nitrogen fixation (BNF) (agricultural land, forest, non-agricultural land and sea) or non-biological nitrogen fixation (industrial, combustion and lightning strikes). The total biological nitrogen fixation is estimated to be twice as much as the total nitrogen fixation by non-biological processes. Available soil nitrogen, which originates from decomposing plant residues and microorganisms, is normally deficient for intensive crop production. Increasing pressure on food production and growing concerns about the environment, energy, nutrition, and agricultural sustainability make the need for BNF research more compelling.

All nitrogen-fixing organisms are prokaryotes (bacteria), so far as is known. Some of them are free-living nitrogen-fixing bacteria, including *Azospirillum* (Tarrand *et al.*, 1978), *klebsiella* (Wright *et al.*, 1981), *Azotobacter* and *Beijerinckia* (Evans *et al.*, 1988). Others live in intimate symbiotic associations with plants, including *Rhizobia* (Jordan, 1984) and *Flankia*.

1. 2 *Rhizobia*- Legume Symbiosis

Rhizobia (*Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Azorhizobium* and *Bradyrhizobium*) belongs to the α-proteobacterial *Rhizobiaceae* family and under the condition of nitrogen limitation, they invade the root of leguminous plant, leading to the formation of a highly specialized organ, the nitrogen-fixing root nodule. The body of knowledge generated by studies over last 150 years has revealed that the successful establishment of *Rhizobia*-legume symbiosis is quite complex and shows a high degree of host specificity, e.g. only certain combination of legume and *rhizobia* are compatible for establishing the nitrogen-fixing symbiosis.

1.2.1 Signal Exchanges between *Rhizobia* and Legume

Nodule formation is a complex multistep process with highly coordinated exchange of signals between the symbiotic partners. An early and essential event for the initiation of rhizobia-plant interaction in the rhizosphere involves bacterial chemotaxi toward plant root exudates or wound saps. Different rhizoia strains have been described to be positively chemotactic to the chemical compounds present in plants exudates such as sugars, amino acids and various dicarboxylic acids (Barbour *et al.*, 1991). Chemotaxis seem to not be required for nodulation but have an influence on competition and establishment in the rhizosphere. Subsequently, the rhizobia attaches to the plant root surface. This attachment was proposed to be mediated by the specific binding of bacterial exopolysaccharides (EPS) to host plant lectin (proteins that posses at least one noncatalytic domain that binds reversibly to mono- or oligosaccharides) and might

involve in the specificity of rhizobia-legume symbiosis (Hirsch, 1999).

The symbiotic interaction starts when plant surface-attached rhizobia cause root hair branching, deforming, and curling (Schultze *et al.*, 1998) (Figure 1.1). Plant-derived compounds (usually flavonoids) activate the expression of the rhizobial nodulation (*nod, nol* and *noe*) genes involved in the synthesis and secretion of Nod-factors (NF), lipochito- oligosaccharides (LCO's) that are recognized by the plant (Figure 1.1). Nod -factors together with additional microbial signals such as polysaccharides and secreted proteins allow bacteria to penetrate the root through a tubular structure called the infection thread, which grows towards the root cortex where the nodule primordia is developing. When the thread reaches the primordia, the bacteria are released into the plant cytoplasm, where they differentiate into their endosymbiotic form, the bacteroids. These bacteroids are able to reduce nitrogen into ammonia, which is used by the plant. In return, the bacteria are supplied with carbohydrates in a protected environment.

Rhizobial nodulation genes are host-range determinants and divided into two kinds, common *nodABC* found in all *Azo(Brady)Rhizobia* strain and host-specificity *nod* genes limited to certain species. Enzymes encoded by the common *nodABC* genes direct the synthesis of the lipo-oligosaccharide backbone of the Nod-factors, while host-specificity *nod* genes modify the basic Nod-factor's structure. The induction of *nod* genes requires flavonoids excreted from host plant, the lysR family transcriptional activator NodD and NodD-binding *cis*-regulatory element *nod*-box. *nod* genes frequently involves positive and negative control (Hanin *et al.*, 1999).

1.2.2 Genome of *Rhizobia*

Traditionally, studies involving rhizobia have focused on a limited number of genes involved in root-nodule formation, symbiotic specificity and nitrogen fixation by using mutational and biochemical approaches like transposon mutagenesis, RNA fingerprinting, promoter probe assay and gene fusions to reporter genes (*lacZ, gusA or gfp*) (Leveau *et al.*, 2002). More recently, advance in the genome sequencing technology followed by the genomic analyses have made it possible to define and understand the involvement of whole rhizobia genomes in the symbiotic process ((Ampe *et al.*, 2003; Uchiumi *et al.*, 2004; Sarma and Emerich, 2005, 2006).

The genomes of seven rhizobia, namely *S. meliloti* (Galibert *et al.*, 2001), *M. loti* (Kaneko *et al.*, 2000), *B. japonicum* (Kaneko *et al.*, 2002), *R. leguminosarum bv viciae* (Young *et al.*, 2006), *R. etli* (González *et al.*, 2006) and two photosynthetic *Bradyrhizobium* (Giraud *et al.*, 2007), have already been sequenced. Genome sizes of rhizobia tend to be larger and their genomic architecture varies considerably due to additional genetic requirement presumably imposed by survival in two different niches, in the rhizosphere and in their host plant (MacLean *et al.*, 2007). Symbiotically relevant genes that are likely to be required for Nod factor synthesis (*nod* genes), nitrogen fixation (*fix, nif* genes) and DNA transmission (insertion sequence element and transposes) in rhizbia are often clustered on large plasmid (pSym), or within genomic islands (referred to as symbiosis island), emphasizing the gene acquisition via horizontal gene transfer (HGT) in the adaptive evolution of rhizobia (Dobrindt *et al.*, 2004). Recently, an important genomic component for type III secretion system (TTSS), which was first identified in plant and animal pathogens, have also been found present

in some but not in all rhizobia, suggesting a role for control of host compatibility in the process of symbiotic interaction (Marie *et al.*, 2001). The transcriptomic and proteomic analyses for rhizobia has been examined under a variety of conditions and provided valuable insight into rhizobia-legume symbiosis (Ampe *et al.*, 2003; Uchiumi *et al.*, 2004; Sarma and Emerich, 2005, 2006).

1.2.3 *Bradyrhizobium*-Soybean Interaction

Bradyrhizobium can form nodules on roots of tropical and some temperate zone leguminous plants. *Bradyrhizobium* are featured by slow growth (>8 hour generation time) on yeast extract-mannitol medium and an alkaline reaction in mineral salts-mannitol medium (Bergey's maual). *Bradyrhizobium* have two species, *B. japonicum* (USDA110 and USDA135) and *B. elkani* (USDA61), and can nodulate agronomically important crops, including soybean [*Glycine max* (L.) Merr], peanut [*Arachis hypogaea* (L.)], cowpea [*Vigna unguiculata* (L.) Walp], mungbean [*Vigna raditata* (L.) Wilcek] and pigeon pea [*Cajanus Cajun* (L.) Millsp]. *B. japonicum* USDA 110 was originally isolated from soybean nodule in Florida, USA, in 1957 and has been widely used for the purpose of molecule genetic, physiology, and ecology, because of its superior characteristics regarding symbiotic nitrogen fixation (Kaneko *et al.*, 2002).

Apparently, *Bradyrhizobium*-soybean interactions share some common features with other rhizobia-legume interactions, while they possess unique characteristics with respect to the regulation of nodulation gene and genome structure. For example, soybean produces two major groups of active *nod*-gene inducing isoflavonoids, the 5-hyroxyl series based on genistein and 5-deoxy series of daidzein conjugate. Besides

nodD1 which encodes a positive transcriptional activator and responses to plant-produced isoflavones (genistein and daidzein), *B. japonicum* has other two members of the family two-component regulatory systems involving the regulation of *nod* genes induction. One is *nodVW,* which is essential for full isoflavone-mediated expression of common *nodYABC* operons, and another is *nwsAB* which is homologues and cross-talks to *nodVW* (Sanjuan *et al.*, 1994; Grob *et al.*, 1994). Interestingly, two proteins encoded by the genes adjacent to *nodD1,* NolA and NodD2, form two components in the repression of nodulation genes through feedback as well as quorum regulation (Loh and Stacey, 2003) (Figure 1.2), suggesting that *nodD1nodD2nolA* region plays an important role in fine-tuning expression of *nod* genes which is required for optimal nodulation.

Among rhizobia, *B. japonicum* has the largest chromosome size of approximately 9.2 Mb comprising 8317 potential protein-coding genes. A presumptive symbiosis island 681 kb in length including genes relevant to symbiotic nitrogen fixation (i.e. *nod, fix, nif*) and DNA transmission has been identified (Figure 1.3). In *B. japonicum,* a large genomic element for the TTSS was also found to be located in the symbiosis island and a mode of regulatory cascade controlling expression of the TTSS gene cluster was proposed (Krause *et al.*, 2002). Genome-wide transcript analysis in *B. japonicum* under a variety of conditions such as minimal vs. rich medium, free-living vs. bacteroids, aerobic vs. microaerobic, desiccation vs. osmotic stress has also been recently reported (Chang *et al.*, 2007; Pessi *et al.*, 2007; Cytryn *et al.*, 2007). However, little is known about the comparative effects of SSE and genistein on the genome-wide expression of *B. japonicum* in the initial symbiotic process.

1. 3 Objective of This Study

The objective of this study is to make a contribution to the understanding of the role of *Bradyrhizobium japonicum* in the initial stage of *Bradyrhizobium*-soybean symbiosis by using functional genomic tools.

1. 4 Organization of This Thesis

Chapter 2 provides a research into the comparative global expression profiling of *B. japonicum* USDA 110 in response to soybean-released compounds (SSE and genistein) at 30°C, a common growth temperature. This chapter includes determination of induction conditions, measurement of the genistein contents in SSE, macroarray analyses and validation of novel regulated genes by using quantitative real-time RT-PCR. The results demonstrate that compared with genistein, SSE remarkably and preferentially expresses *B. japonicum* genome loci, including TTSS genes cluster.

Chapter 3 presents a research on the genome-wide expression of *B. japonicum* in the presence of the SSE and genistein at 15°C, a suboptimal root zone temperatures (RZTs) which was reported to be strongly inhibit nodulation, and makes a comparison with results obtained in the chapter 2. The results show that low temperature leads to a potentially delayed expression of SSE-exclusively-induced *B. japonicum* genomic loci including TTSS genes cluster found in the chapter 2 compared with those of genistein-induced *nod* genes.

In *Chapter 4*, based on the results of the chapters 2 and 3, it is described that the induction of TTSS genes occurs simultaneously with that of genes *nolA* and *nodD2* in

the presence of SSE, but not genistein. Another experiment is performed to test the hypothesis that the induction of TTSS genes is positively quorum-regulated. Besides, array analysis is conducted to examine the effect of *nodD2* and *nolA* mutagenesis on the expression of TTSS in the presence of SSE. Then, a new regulation pathway for the induction of TTSS genes is proposed and discussed.

Finally, in *Chapter 5*, the work of previous chapters is summarized and discussed, and then the conclusions of the thesis are presented.

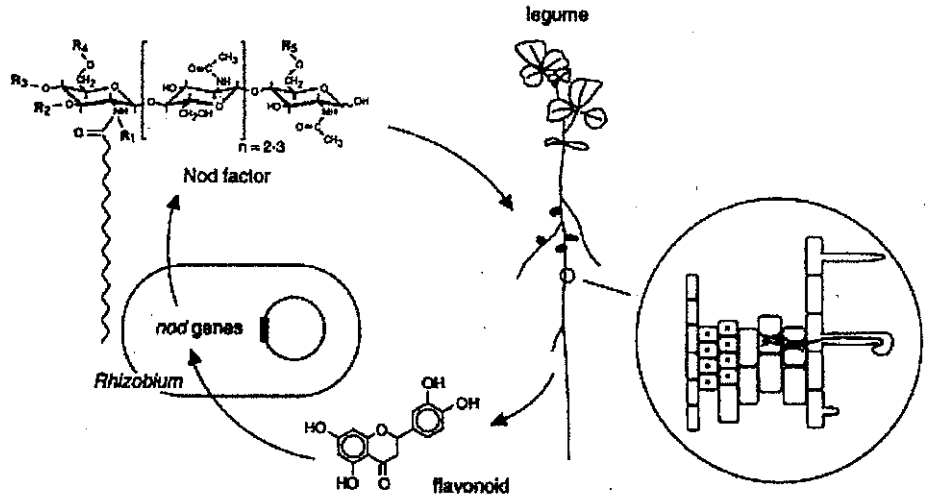

Figure 1.1 Signal exchange in the (Brady)rhizobium-plant symbiosis. Flavonoids, e.g. genistein, induce the rhizobial nod genes. This leads to the production of nodule-inducing (Nod) factors, lipochitooligosaccharides (LCOs), that are differently modified depending on the rhizobium species. Possible substituents (other than hydrogen) are R1: methyl; R2, R3: carbamoyl; R4: acetyl or carbamoyl; R5: sulfate, acetyl, D-arabinose or differently substituted fucose. The insert shows an infection thread passing the root cortex toward a cluster of dividing cells that will become a nodule primordium (Schultze *et al.*, 1998).

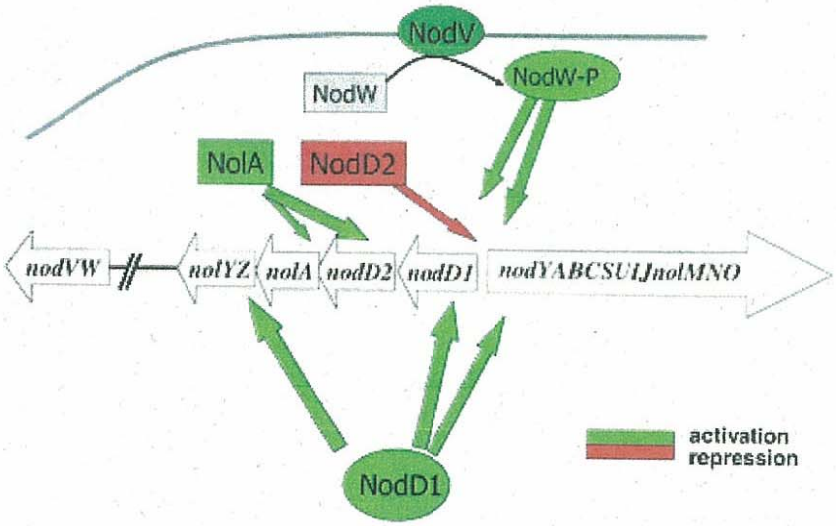

Figure 1.2 Model illustrating the key components involved in *B. japonicum nod* gene regulation. In response to genistein, the nod genes are activated by NodD1 and NodVW, resulting in the synthesis of the Nod factors. Negative regulation of the nod genes is mediated by NolA and NodD2. NolA regulates NodD2, which then represses the *nod* genes (Loh and Stacey, 2003).

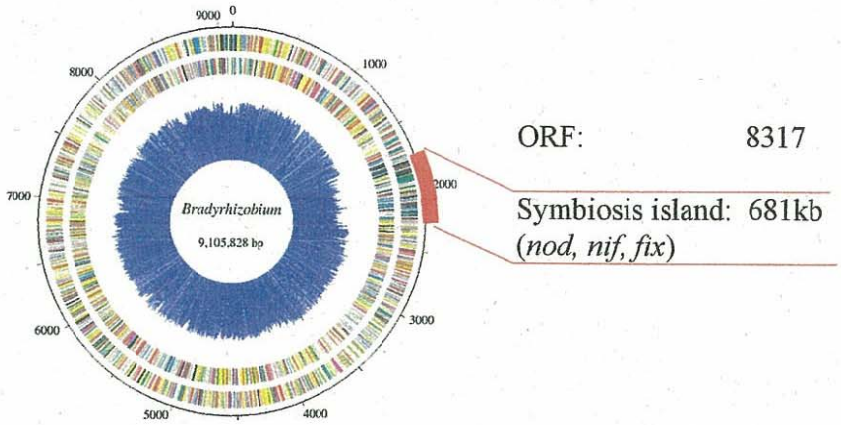

Figure 1.3 Circular representation of the chromosome of *Bradyrhizobium japonicum* USDA 110. The scale indicates the location in bp starting from *Pac I* recognition site. The bars in the first and second outermost circles show the positions of the putative protein-encoding genes in the clockwise and counterclockwise directions, respectively. The innermost circle shows the average GC percent calculated using a window-size of 10 kb. The chromosome comprises 8317 potential protein-coding genes and a presumptive symbiosis island, a 681 kb DNA region of lower GC content at the coordinates 1.68-2.36 Mb, is indicated as a red area in the first outermost circle (Kaneko *et al.*, 2002).

CHAPTER 2

Comparative Expression Analyses of *Bradyrhizobium japonicum* in Response to Soybean Seed Extracts and Genistein at Common Growth Temperature

2. 1 Introduction

The rhizobia are known to inhabit the soil as free-living cells or the nodules as nitrogen-fixing endosymbiont with leguminous plants to convert atmospheric dinitrogen (N_2) into biologically usable ammonia (NH_3). The symbiosis between rhizobia and leguminous plants is an unimaginably elaborated process and the movement of rhizobia to the germinating seed and root of the host plant is considered to be important in the early step of nodulation for colonization in rhizosphere. Both chemotaxis and motility contribute to the colonization in rhizosphere and were reported to correlate with the competition for the nodulation between rhizobia, e.g., non-motile and non-chemotactic mutants required 10- to 30 -fold number of cells to form nodules at the same rate as the wild strain (Caetano *et al.*, 1988). Exudates of soybean seed and root are known to contain a wide range of organic compounds, and some of which showed attractive action to rhizobia (Gaworzewska *et al.*, 1982). Amino acids such as glutamate and dicarboxylic acids such as succinate are reported to have a strong attractive action to *Bradyrhizobium japonicum* cells (Barbour *et al.*, 1991; Kape *et al.*, 1991).

It has been well established that the leguminous plant-released iso/flavonoid signals induce *nod* genes of rhizobia so that they can produce Nod-factors, lipo-chito oligosaccharides (LCOs), that specifically trigger various plant responses and initiation of cell division to form the nitrogen-fixing root nodules (Denarie *et al.*, 1996; Fischer *et al.*, 1994). Genistein and daidzein are isoflavones present in exudates of soybean seed and root and major *nod*-genes inducers in *B. japonicum.* Thus, they are well used as inducers for expression analyses of symbiotic genes by using transcriptional *lacZ* fusion (Kosslak *et al.*, 1987; Banfalvi., *et al* 1988; Smit *et al.*, 1992; Graham, 1991; Philips and Streit, 1996). Unlike the exudates, however, these isoflavones were not chemoattractant of *B. japonicum* cells, although *Rhizobium* cells exhibited chemotaxis to their *nod*-genes inducer (Kape *et al.*, 1991; Aguilar *et al.*, 1988). Besides, the double mutant of two global regulatory families, *nodD1* and *nodW,* was reported to be unable to induce *nodY-lacZ* fusion in the presence of genistein or daidzein, but still showed a two- to three-fold induction in the presence of soybean seed extracts (SSE) (Sanjuan *et al.*, 1994). These reports suggest that the signal exchange in the early stages of *B. japonicum*-soybean symbiosis can not be explained only by a simple paradigm of flavonoid-Nod factors.

The whole genomic sequencing of *B. japonicum* USDA 110 (9.10Mb) has been recently completed, and as in *M. loti* MAFF303099, *B. japonicum* also carries a symbiosis island of 681kb on genome, in which most of the key genes relevant to the symbiotic nitrogen fixation (*nod, nif* and *fix*) are concentrated (Kaneko *et al.*, 2000; Kaneko *et al.*, 2002). A oligonucleotide microarray for genome-wide transcript analysis in *B. japonicum* under a variety of conditions such as minimal vs. rich medium, free-living vs. bacteroids, aerobic vs. microaerobic, desiccation vs. osmotic

stress has also been reported recently (Chang *et al.*, 2007; Pessi *et al.*, 2007; Cytryn *et al.*, 2007). However, little is known about the comparative effects of SSE and genistein on the genome-wide expression of *B. japonicum* in the initial symbiotic process. In this study, I used a DNA macroarray based on the M13 clones of *B. japonicum* USDA 110 genome as a comprehensive tool to monitor the comparative global expression of its genomic loci in response to SSE and genistein. Results revealed that SSE collectively and markedly induced four predominant genomic regions inside symbiosis island with some genomic loci outside, suggesting that the SSE-induced *B. japonicum* genomic loci are relevant to the initial interaction with soybean.

2. 2 Materials and Methods

Design of Genomic DNA Macroarray

A DNA macroarray system of *Bradyrhizobium japonicum* (Figure 2.1) was constructed for monitoring the genome-wide expression profiling throughout the study. To cover the entire genome of *B. japonicum* USDA 110 as widely as possible, a minimally overlapping set of 3,739 clones with an average length of 2.7 kb from 10,718 of M13 candidates, which were automatically extracted from the total 52,000 of M13 clones of genomic libraries by using Phred/Phrap program (Kaneko *et al.*, 2002), was firstly PCR-amplified. Then, 221 DNA fragments of 1.2 kb (on average) were PCR-amplified with 442 PCR primers (http://orca10.bio.sci.osaka-u.ac.jp/array02/) to cover the gaps left on the genome by using cosmid clones as templates. The resultant coverage of total 3,960 clones was more than 98.5% of the entire genome of *B. japonicum* USDA 110.

M13 phage supernatants of the selected clones were used as templates for PCR amplification reactions. PCR was performed using M13 universal primer pairs (5'-GGGTTTTCCCAGTCACGAC and 5'-TTATGCTTCCGGCTCGTAATGTTGTG) in 30 cycles (97 °C for 20 s, 68 °C for 6 min) followed by a 7-min incubation at 72°C. The insert of each clone was amplified by PCR in 100 μl of reaction mixture, precipitated by addition of isopropyl alcohol, and then dissolved in 20 μl 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA. 10 μl of spotting dye solution containing 0.25% bromophenol blue and 60% glycerol was added, and the amplified inserts were singly spotted on nylon filters (80 mm x 120 mm, Biodyne-A, Japan Pall Co. Ltd., Tokyo, Japan) by using a spotting machine Biomek 2000 (BioRad, Tokyo, Japan). In addition, the PCR products of the genes *nodD1*, *nodD2*, *nodAB*, *nifDK*, the lamda and TE buffer were regularly spotted on the array membrane to control the hybridization quality. To normalize signal intensity of spots, the gene *sigA*, one housekeeping gene of *B. japonicum* (Becker *et al.*, 1997), was also PCR-amplified and spotted on the array membranes.

Bacterial Strains and Culture Conditions

B. japonicum USDA 110 was used as a wild type strain for global expression analysis throughout the experiment. *B. japonicum* ZB977 harboring a plasmid (pZB32) with a *lacZ* fusion in *nodY* based on the background of *B. japonicum* USDA 110 (Banfalvi *et al.*, 1988), was used as an indicator strain to determine the induction conditions (Table 2.1). *B. japonicum* USDA 110 and ZB977 were maintained on the yeast extract-mannitol agar (YMA) with chloramphenicol (30 μg/ml) and tetracycline (50 μg/ml) and cultured in yeast extract-mannitol broth (YMB) without

antibiotics for the induction (Jordan, 1984; Loh and Stacey, 2001).

β-Galactosidase Activity Assay

SSE used in the experiment was prepared according to the previous report (Smit *et al.*, 1992), and the genistein content in SSE was quantified by high performance liquid chromatography (HPLC). Mid-log-phase cultures of *B. japonicum* ZB977 were diluted to different cell densities (OD_{600} of 0.025, 0.05 and 0.1) with fresh liquid-YMB containing no antibiotics and induced with SSE of different concentrations (10, 20, 30, and 40 μl /ml culture) at 30℃ for 20 h. Induction was carried out in the 2 ml of diluted culture, and the *β*-galactosidase activity was assayed as described previously (Kosslak *et al.*, 1987).

RNA Preparation, Hybridization and Image Capture

B. japonicum USDA110 cells were pre-cultured in 50 ml of YMB without antibiotics for three days at 30°C and then scaled up to 200 ml of YMB for growing to mid to log-phase (OD_{600}=0.3-0.5). The cells were then diluted with fresh YMB to OD_{600} of 0.1 and induced with SSE (20 μl/ml of culture) or genistein (5 μM) under the conditions determined by *B. japonicum* ZB977. The cells were also induced with 0.5% (v/v) absolute ethanol for checking its influences on expression profiles because it was the solvent of SSE.

After the equal volume of ice-cold 5% (w/v) phenol/EtOH was quickly added into the culture to minimize the degradation of mRNA, the cells were immediately harvested and stored at –80°C (Inaba *et al.*, 2003). Total RNA was isolated according to the protocols provided by the manufacturer of ISOGEN-LS (Nippon Gene Co.,

Tokyo, Japan) and treated with DNase I at 37°C for 30 min. After the mRNAs were enriched with the MICROB*Express*™ Kit (Ambion, Austin, TX, USA), cDNA labeling was carried out as described previously (Uchiumi *et al.*, 2004).

Hybridization, image acquisition, and data analyses were done as described previously (Uchiumi *et al.*, 2004). Briefly, after pre-hybridization in Church's phosphate buffer at 55°C for at least 6 h, hybridization was carried out in the same buffer containing probe cDNA at 55°C for at least 15 h. Then, washing was done three times at room temperature in 2×SSC containing 0.1% SDS for 5 min, twice at room temperature in 0.3×SSC containing 0.1% (w/v) SDS for 10 min, and twice at 55°C in 0.3×SSC containing 0.1% (w/v) SDS for 10 min, in this order. Array membranes were exposed to the PhosphorImager and the hybridized signals were captured as image files by using a BioImaging analyzer (BAS5000, Fuji Film). At least three independent sets of array analysis using duplicate array membranes were performed.

Data Analyses

The signal intensity of each spot was quantified by using ArrayVision software (GE Healthcare Bio-Sci, NJ, USA) after the subtraction of the local background value and normalized based on the total sum of the signal intensities of spots on one array sheet. The relative expression levels of each clone between SSE/genistein-treatment and untreatment were represented by the ratio of the corresponding normalized signal intensity. The value of $\log_{10}$ (expression ratio) for each clone was calculated to construct the genome-wide expression profiles. The $\log_{10}$ (expression ratio) of all 3,960 clones complied normal distribution and the

mean was approximately 0.0, indicating that any expression ratio is significant (95% confidence) if the value of the $\log_{10}$ (expression ratio) is greater or lower than 1.96 standard deviations from the mean (0.0) (Han *et al.*, 1999).

Quantitative Real-Time RT-PCR

The relative intensity of gene expression was validated by quantitative real-time RT-PCR. The primers were designed by Primer 3 (http://fokker.wi.mit.edu/primer3/input.htm) (Rozen and Skaletsky, 2000). Three hundred ng of total RNA were used as template and real-time RT-PCR reactions were performed with MiniOpticon™ Version 3.1(Bio-Rad, Hercules, CA,USA) in combination with the QuantiTect SYBR Green RT-PCR (Qiagen GmbH, Hilden, Germany). Quantification was performed using the Pfaffl method according to the real-time RT-PCR application guide provided by Bio-Rad. The housekeeping gene of *B. japonicum*, *sigA*, was used as an internal reference for quantitative real-time RT-PCR as reported previously (Pessi *et al.*, 2007; Uchiumi *et al.*, 2004). The expression levels of *sigA* gene, which was regularly spotted on the array membranes, were also not significantly changed with the SSE/genistein-treatment in this experimental condition.

Plant Infection Test

Glycine max (L.) cv. Enrei was used as a host plant for the inoculation test. Seeds were surface-sterilized with 70% (v/v) EtOH for 1 min, rinsed with sterilized distilled water for three times, and then immersed in sodium hypochlorite solution (available chlorine, 0.5%) for 5 min. After washing with sterilized distilled water for six times, seeds were put into glass bottles (300 ml) filled with vermiculite

containing nitrogen-free medium (Norris and Date, 1976), and inoculated with 1ml of pre-treated cell suspension with SSE or genistein per seed. For the pre-treatment, *B.japonicum* cells were diluted to an OD_{600} of 0.1 and then treated with SSE (20 µl /ml culture) or genistein (5 µM) for 12 h. After washing with PBS (KH_2PO_4, 0.7 g; Na_2HPO_4, 0.95 g; NaCl, 6.8 g per 1 liter, pH 7.0) for three times, cells population was adjusted to around 10^8 cfu/ml.

Plants were grown in a greenhouse and harvested appropriately for the measurement of nodule number formed in the roots, the fresh weight of above-ground and roots of the plant. To visualize the nodules in earlier stage, the roots were fixed in FAA (70% EtOH: acetic acid anhydride: formalin=90:5:5, v/v/v) and then stained with 0.003% toluidine blue O overnight. The development of nodules was divided into three stages as shown in Figure 2.10, i.e., nodules of stage 1 being very small at the initial formation stage, nodules of stage 3 being complete mature stage and nodules of stage 2 being between stage 1 and 3.

2. 3 Results

Determination of Induction Conditions

The SSE-induced *nodY-lacZ* expressions at the different cell densities were monitored by *β*-galactosidase activity and the results are shown in Figure 2.2. Although the maximal *nodY* expression was detected with 30 µl SSE/ml culture at the cells density of OD_{600}=0.025, taking into consideration of the requirement of enough cells for RNA isolation and the influences of alcohol, which is solvents of SSE, on the expression of genes, initial cell density of OD_{600}=0.1, and 20 µl SSE/ ml

culture were determined as two induction conditions. Then, the induction periods were investigated. As shown in Figure 2.3, the *nodY* expression reached maximal levels at 12 hour post induction (hpi). Thus, three inducing time points during the elevation of *nodY* expression, 0.5, 6 and 12 hpi, were used for RNA isolation.

Since the final concentration of genistein in the SSE-supplemented culture was measured as 4.7 μM (Figure 2.4), a final concentration of 5 μM pure genistein in parallel with SSE was used for induction.

One Giant Expression Region Corresponds to the Symbiosis Island

The genome-wide expression profiles were constructed as described in Materials and Methods. As shown in Figure 2.5, one giant chromosomal region (1.68-2.36 Mb) was markedly and collectively induced with time and reached maximum at 12 hpi in the presence of SSE or genistein. This region showed the similar expression profiles between SSE- and genistein-treated cells. However, the levels induced by SSE were much more conspicuous than those by genistein. The expression profile of cells induced by EtOH, which was a solvent of SSE and genistein, indicated that there were no strongly induced loci except for one locus (covering genes from *bll0330* to *blr0336*) including a probable alcohol dehydrogenase precursor (*bll0333*) (Figure 2.5), suggesting that the expression patterns were mainly obtained by the SSE or genistein in itself.

Surprisingly, we found that this giant SSE-induced chromosomal region at 12 hpi corresponded very well to the symbiosis island. As shown in Figure 2.9B, among 139 significantly up-regulated clones (expression ratio, ≥2.2-fold) on the whole genome, 68 (49%) were located inside the symbiosis island, whereas all significantly

down-regulated clones (expression ratio, ≤0.4-fold) was outside this genomic island. For genistein-treated cells at 12 hpi, however, among 89 significantly up-regulated clones on the whole genome, only 14 (16%) were located inside symbiosis island, and 5 (6%) were significantly down-regulated in this region. The results here show that genes in symbiosis island is induced by both SSE and genistein, but the expression levels were much more intensive for SSE-treatment.

Symbiosis Island Functions as Great Expression Clusters (ECs)

Figure 2.6 shows the expression profiles of symbiosis island in a smaller window size. In consequence, four predominant expression clusters were identified and designated as EC-I, II, III and IV, considering more than ten successively up-regulated (expression ratio, ≥1.0-fold) adjacent clones as a cluster. Among ECs, the expression levels of EC-III and IV were clearly greater than those of EC- I and II by covering more significantly up-regulated clones. A part of EC-I and II were also induced with SSE and genistein at 0.5 and/or 6 hpi. However, their expression levels induced by genistein were considerably weak as compared with those by SSE at 12 hpi. EC-III was strongly induced by SSE at 12 hpi. A similar profile was also found in genistein-treated cells at 12 hpi, but the expression levels were considerably weak. The profiles of EC-IV were very similar between SSE- and genistein-treated cells at 6 and 12 hpi. The expression features of ECs and their covered genes are shown in Figures 2.6, 2.7 and Appendix 1.

EC-I contained 13 clones covering 33 genes from *blr1625* to *blr1657* (at the coordinate 1, 781, 581-1, 815, 640), and most of them encode putative transposase (7 genes) and unknown (or hypothetical) proteins (19 genes). One locus (clone 2-4)

covering 5 genes that encode multidrug resistance protein (*blr1629*), NolK (*bll1630*), NoeL (*bll1631*), NodM (*blr1632*) and NoeD (*blr1633*) was induced by SSE and genistein at 6 hpi and then became weaker at 12 hpi. Downstream of this locus, two other loci (clone 5-7, 11-12) covering 12 genes (*bll1634-bsl1639*, *blr1649-bsl1654*) were exclusively induced by SSE at 12 hpi. However, all these 12 genes were assigned into unknown or hypothetical protein-encoding genes.

EC-II contained twenty-three clones covering genes from *blr1689* to *blr1735* (at the coordinate 1, 845, 670-1, 898, 742), and two loci (clone 7-10 and 15-17) were induced by SSE or/and genistein. The former locus covering 3 genes that encode hypothetical (*bll1703*, *blr1704*) or unknown proteins (*blr1705*) was only induced by SSE at 12 hpi. Whereas, the latter locus covering 4 genes that encode two-component response regulator (*bsl1713*), NodW (*bll1714*), NodV (*bll1715*) and putative transposase (*blr1716*) was induced 2.1-8.6 fold by both SSE and genistein during 12 h. NodWV was identified as a two-component system which is unique to *B. japonicum* and directly induces *nodYABC* operon in response to genistein (Göttfer *et al.*, 1990; Sanjuan *et al.*, 1994; Loh *et al.*, 1997). The long-range expression manner of *nodWV* here supports its important roles in the early symbiotic process.

EC-III contained 29 clones covering genes from *bll1796* to *blr1867* (at the coordinate 1, 953, 234-2, 020, 145) and 28 clones among them (69.0%) were induced 2.4- to 20.7-folds by SSE at 12 hpi. On the other hand, most of the clones (20 among 29 clones) also tended to be up-regulated by genistein, but, only 5 clones (clone 3, 5, 18, 20 and 28) exhibited the significant expression levels, indicating that this cluster was significantly and preferentially induced by SSE (Figure 2.8A). These SSE-induced clones were divided into three loci, the first locus (clone 1-13)

including genes from *bll1796* to *bll1832*, the second (clone 17-21) from *blr1839* to *blr1847* and the third (clone 27-29) from *blr1859* to *bll1864*. A genes cluster (*tts*) encoding recently identified type III secretion system (TTSS) of *B. japonicum*, $RhcC_1C_2JNQRSTUV$ (*bll1811-bll1842-blr1813-blr1816-blr1818-blr1819-bsr1820 -blr1821-blr1822-bll1800*) (Göttfer *et al.*, 2001; Kaneko *et al.*, 2002), was completely included within the former two loci. Recently, it has been reported that mutations within *tts* of *B. japonicum* affected the symbiosis in a host-dependent manner (Krause *et al.*, 2002). Besides, several genistein-inducible TTSS-secreted proteins were identified in *B. japonicum* 110*spc*4, including three hypothetical proteins encoded by genes *blr1649* (covered by clone 11 and 12) in EC-I, *blr1806* (covered by clone 4, first locus) and *bll1862* (covered by clone 27 and 28, third locus) in EC-III (Süβ *et al.*, 2006). This experiment showed that clones covering these genes were induced by SSE (Appendix 1). Among 6 genes covered by the third locus, only 2 genes have putative functions, i.e. putative transposase (*bll1861*) and citrate-proton symporter (*bll1864*, *citA*). However, the presence of a *nod* box located between *bll1862* and *bsr1863* as well as a σ^{54} consensus between *bll1864* and *blr1865* (Göttfer *et al.*, 2001) suggests that the third locus may play a role in symbiosis.

The most strongly induced genes cluster on the genome was EC-IV covering genes from *bsr2010* to *bll2067* (at the coordinate 2, 171, 906-2, 232, 429) (Figures 2.7 and 2.8B). Among the 23 clones covered in EC-IV, 16 clones (clone1-11, 15 and 20-23) (69.6%) were strongly induced by SSE at 12 hpi (2.8-52.3 fold). The induction of EC-IV occurred in advance of EC-III and its expression patterns, particularly those of clone 2, 5-8, and 20, were very similar between SSE- and

genistein- treated cells at 6 and 12 hpi (Figure 2.8B). Clone 1-11 covering 31 genes from *bsr2010* to *bll2040* completely involved a great genes cluster, *nolZY*-[2genes] -*nolA*-[1genes]-*nodD2*-[1genes]-*nodD1YABCSUIJ-nolMNO-nodZ-fixR-nifA-fixA* (*bsl2015-blr2038*) which was reported previously (Kündig *et al.*, 1993; Kaneko *et al.*, 2002). The *nolZY-nolA-nodD2-nodD1YABCSUIJ-nolMNO-nodZ* has been identified as a common *nod* gene operon involved in the biosynthesis, modification and transport of Nod-factor and reported to be essential for the process of nodulation (Loh and Stacey, 2003; Hanin *et al.*, 1999). Downstream of this *nod* genes operon, clone 20 was induced at least 4.5-fold by both SSE and genistein during 12h, suggesting that either or both of its covered two adjacent genes (*bsr2061* and *blr2062*) were induced strongly. *blr2062* has been estimated to encode a nodulation protein, NoeI, although the function of *bsr2061* is still unknown. These results support that *nod* gene operon is an important locus of *B. japonicum* in the initial stage of the symbiotic process, and suggest that the genistein is one major *nod* genes inducer in SSE.

Clone 21 was induced more than 3.2-fold by both SSE and genistein at 6 and/or 12 hpi, suggesting that its covered single gene (*bll2063*, *nrgC*) was induced strongly. *nrgC* was reported to be NifA-regulated but not essential for symbiosis (Nienaber *et al.*, 2000). The loci covered by clone 15 and 22-23 were also induced beyond 2.8 fold by SSE at 12 hpi, but only 3 genes *bll2049*, *bll2065* and *bll2067* have functions as anthranilate phosphoribosyltransferase (*trpD*), carbonic anhydrase and nodulate formation efficiency C protein (*nfeC*).

Clones covering a large gene cluster, *nifDKENX-fer3-nifS-fixU-nifB-frxA-nifZ-nifH-fixBCX* (*blr1743-bsr1775*), which are located between EC-II and EC-III and

likely to be required for nitrogen fixation under microaerobic conditions (Kaneko *et al.*, 2002), showed no potential expression changes in the presence of either SSE or genistein except for one clone covering genes from *bsr1749* to *blr1752* (Figure 2.6). One operon composed of eight ORFs (*blr2077-bsl2084*), which was reported to be likely involved in the biosynthesis of rhizobitoxine-like (RtxA) molecule (Göttfer *et al.*, 2001), as well as another five ORFs (*blr2143-blr2147*) for cytochrome P450-family proteins did not also substantially respond to either SSE or genistein. These results support the previous reports that rhizobitoxine production is confined exclusively to genotype II strains of *B. japonicum* and cytochrome P450 are well expressed in bacteroids (Minamisawa *et al.*, 1992; Sarma *et al.*, 2005).

Exclusively Expressed Genome Loci by SSE

Nine loci covered by 19 clones inside symbiosis island but outside ECs (LISs: LIS 1-9) were exclusively induced by SSE at 12 hpi (Figure 2.6 and Appendix 2). The LIS 7 covered by two clones (brb19974, brb02802) was induced 2.8-12.5 fold, suggesting that either or both of the two adjacent genes encoding polygalacturonase (*blr1993*) and pectinesterase (*blr1994*) were induced strongly. Besides, one clone (brb06413) in LIS 6 was also induced 3.9 fold, suggesting that putative sugar hydrolase (*blr1964*) was likely induced. These results suggest that plant cell wall-degrading enzymes were preferentially induced by SSE. However, the locus covered by clone 13 in EC-I was not induced strongly, suggesting that a gene encoding another putative sugar hydrolase (*blr1656*), which was reported to be strongly induced by the genistein in a *nodW*-dependent manner (Baumberger *et al.*, 2003), did not show the substantial expression change. For other LISs, genes

encoding a TTSS-secreted protein similar to NopP of *Rhizobium* sp. NGR234 (*blr1752* in LIS 3) (Süβ *et al.*, 2006), alanine dehydrogenase (*blr1738* in LIS 3), ferredoxin (*bsr1739*, *bsr1750* in LIS 3), putative bacA (*blr1902* in LIS 5), two NoeE homologs (*blr2073*, *blr2074* in LIS 8) and putative transketolase (*blr2168*, *blr2169* in LIS 9) were also putatively induced. However, around 59.5% (25/42 genes) of the products of putatively-induced genes covered by LISs were unknown or hypothetical proteins.

In addition, nine loci outside symbiosis island (LOSs: LOS 1-6, 13-15) were also induced by SSE (Figure 2.5 and Appendix 3). The LOS 6 covered by 14 clones (from *blr2358* to *bll2381* at the coordinate 2, 561, 229- 2, 595, 246) was located downstream of the symbiosis island and induced by SSE with time. This locus was reported to be likely involved in a gene cluster (*exo*) for exopolysaccharide (EPS) synthesis and deletion mutants within *exo* caused a delayed nodule initiation (Eggleston *et al.*, 1996; Kaneko *et al.*, 2002). LOS 15 (from *bll5380* to *bll5420* at the coordinate 5, 925, 788 to 5, 964, 027) composed of 17 clones and 13 of them were strongly induced with time by SSE. Among 39 putatively induced genes, 27 genes (69.2%) encode ribosomal proteins. On the other hand, another large locus, LOS 19 covered by 16 clones (from *blr6843* to *blr6883* at the coordinate 7, 540, 433-7, 575, 109), was potentially induced not by SSE but by genistein (Figure 2.5 and Appendix 3). Among 15 putatively induced genes, 12 genes (80%) encoded flagella-related proteins. Recently, it has been reported that abundant extracellular proteins from genistein-induced wild type *B. japonicum* were identified as flagellin and the deletion mutant of *bll6865* and *bll6866* had a thick flagellum without thin flagella (Süβ *et al.*, 2006; Kanbe et al., 2007).

Moreover, LOS 2 covered by four clones (from *blr0232* to *bll0242* at the coordinate 228, 013-237, 522) tended to be induced by SSE during 12 h. One clone (brb07072) was induced 1.4-6.9 fold, suggesting that either or both of the two adjacent genes (*blr0240*, *blr0241*) were induced strongly. The product of *blr0241* has been reported to be 80% identical with 1-aminocyclopropane-1-carboxylate (ACC) deaminase of *M. loti* (*mlr5932*), which is likely involved in nodulation enhancement (Uchiumi *et al.*, 2004). Other six loci covering genes related to energy metabolism, transporter and regulator such as cytochrome O ubiquinol oxidase (*blr0149-blr0152* in LOS 1), transporter family (*bll0379-bll0381* in LOS 3), ATP synthase (*bll0439-bll0443* in LOS 4, *bll1185-bsl1189* in LOS 5), NADH ubiquinone oxidoreductase (*bll4904-bll4919* in LOS 13), and transcriptional regulator and dehydrogenase (*bll5275-blr5278* in LOS 14) were also induced by SSE. Contrarily, two loci covering genes encoding two component sensor/regulator (*bll6184-blr6185* in LOS 18, *bll7306-bll7307* in LOS 21) and alcohol dehydrogenase (*blr6207*, *blr6213*, *blr6215* and *bll6220* in LOS 18) were repressed.

Expression Genome Loci Shared between SSE- and Genistein-treated Cells

In addition to genomic loci covering *nod* genes, six loci outside symbiosis island were induced with sharing between SSE- and genistein-treated cells during 12 h (LOS 7, 9-10, 12, 16 and 20) (Figure 2.5 and Appendix 4). Two adjacent clones (brb11706, brb23885) in LOS 12 were induced 2.2-27.6 fold during 12 h, suggesting that either or both of the two adjacent genes (*blr4773*, *blr4774*) were induced strongly. NwsAB (*blr4773* and *blr4774*) was identified as a two-component system

to function with NodWV in a cross-talk way and involved in the quorum regulation of the nodulation genes (Grob *et al.*, 1994; Loh *et al.*, 2002b). Two adjacent clones (brb17502, brb07789) in LOS 10 were also induced strongly during 12 h, suggesting that 3 adjacent genes encoding a putative multidrug resistance protein (*bll4319*), probable RND efflux membrane fusion protein (*bll4320*) and putative outer membrane channel lipoprotein (*bll4321*) may act as an efflux system.

Nine adjacent clones covering LOS 20 (*bll7018-bll7032*) were collectively and strongly induced by genistein, especially at 0.5 hpi (3.8-57.3 fold). Two adjacent clones (brb07276, brb06980) in this locus and 2 adjacent clones (brb06269, brb14218) covering LOS 9 contained 10 putatively-induced genes during 12 h, and 5 among them (50 %) encode transcriptional regulatory protein. Six adjacent genes (*blr2437-blr2442*) covered by LOS 7 encode ABC transporter-related protein, whereas, 5 of 6 putatively-induced genes (*blr5790-blr5795*) in LOS 16 encode unknown or hypothetical proteins.

Contrarily, four loci (LOS 8, 11, 17 and 22) were repressed by both SSE and genistein during 12 h (Figure 2.5 and Appendix 4). Four adjacent genes encoding ABC transpoter-related proteins (blr4553, blr4555-blr4557 in LOS 11), 2 adjacent genes encoding RhtB family transporter and two-component response regulator (*bll7341* and *bll7342* in LOS 22) and putative monooxygenase component (*blr3679* in LOS 8) seemed to be repressed. However, all of six putatively-repressed genes (*blr5841- bll5846*) in LOS17 encode hypothetical proteins.

Validation of Potentially Regulated Genes

The character of macroarray used in the experiment is that some clones are

adjacently overlapped and each clone contains at least one gene. Thus, the putatively regulated genes were screened and validated with real-time RT-PCR according to the following strategies: (i) stringent selection of significantly up-and down-regulated clones which had an expression ratio of more than 2.2 and less than 0.4 fold, respectively. The candidates were selected according to the distribution profiles of scatter plots and the area corresponding to them was shown in Figure 2.9A (Scatter plots for SSE- or genistein-treated cells at 0.5 and 6 hpi are shown in Figure 2.10). (ii) selection of putatively regulated genes from the clones as described before (Uchiumi *et al.*, 2004) and their validation by quantitative real-time RT-PCR.

As a result, 12 representative genes from some substantially regulated genomic loci (5 genes in ECs, 1 gene in LISs and 6 genes in LOSs) were selected and their relative amounts of transcripts were quantified by using real-time RT-PCR (Table 2.2). The primers of these selected genes were designed and confirmed as described in Materials and Methods (*Table 2.3*). Among these 12 genes, genes encoding unknown protein (*blr1649* in EC-I), RhcN (*blr1816* in EC-III) and probable polygalacturonase (*blr1993* in LIS 7) were exclusively up-regulated by SSE at 12 hpi (5.3-18.9 fold). The gene encoding ACC deaminase (*blr0241* in the LOS 2) was also induced by SSE but the levels were maintained higher during 12 h (2.4-4.2 fold). Whereas, a gene encoding TtsI (*bll1843* in EC-III) was induced by both SSE and genistein, but the levels were higher for SSE-treatment at 12 hpi (SSE: 10.1 fold; genistein: 4.1 fold). Genes encoding NodW (*bll1714* in EC-II), NodC (*blr2027* in EC-IV) and probable RND efflux membrane fusion protein (*bll4320* in LOS10) were strongly induced by both SSE and genistein during 12 h, and the expression levels of *blr2027* was maximal at 6 hpi (218.1-252.9 fold). One gene encoding transcriptional

regulatory protein TetR family (*blr7023* in LOS 20) was mainly induced by genistein.

On the other hand, genes encoding a hypothetical protein (*bll5843* in LOS 17) and ABC transporter substrate-binding protein (*blr4553* in LOS 11) were significantly down-regulated by both SSE and genistein, particularly at 6 and 12 hpi. But, a gene encoding rhtB family transporter (*bll7341* in LOS 22) was unsubstantially repressed by using real time RT-PCR, although the clone covering this gene was significantly down-regulated in macroarray analyses. Relative expression levels seem to differ between real-time RT-PCR and macroarray analyses to some extent. Nevertheless, the high correlation (R^2=0.90) of the expression ratio between real-time RT-PCR and macroarray analyses showed the reliability of macroarray analyses with the validity of selection method for putatively induced genes.

2. 4 Discussion

In this experiment, the symbiosis island of *B. japonicum* was collectively expressed as four predominant expression clusters (EC I-IV) in SSE-treated cells (Figures 2.5 and 2.6). The symbiosis island of *M. loti* MAFF303099 was also reported to be collectively expressed in bacteroids and function as clustered expression island (EI) (Uchiumi *et al.*, 2004). However, each symbiosis island functions as different great expression clusters, such as *nod* and *tts* induced by SSE in this experiment, while *nif, fix and fdx* induced in bacteroids of *M. loti* MAFF 303099. Genes cluster *fixK2/fixLJ/fixNOPQ/fixGHIS* (from *bll2754* to *bsr2773* at the coordinate 3,032,744-3,050,922), whose products are the oxygen-sensing cascade

and the high-affinity terminal oxidase that are required for microaerobic respiration and nitrogen fixation in nodules (Nellen-Anthamatten *et al.*, 1998), was not potentially expressed in this experiment (Figure 2.5). Genome-wide expression analyses on these two rhizobia clearly demonstrate that the rhizobia genome is a vehicle for the symbiosis island, which functions in distinctive gene clusters in the infection and differentiation stages of symbisois.

Despite approximately the same final concentration of genistein between SSE- and genistein-induction medium (4.7 and 5.0 μM) (Figure 2.4), SSE-treated cells exhibited a distinctive genome-wide expression profiling as shown by the results that a number of genomic loci were exclusively and strongly induced by SSE, e. g., genomic loci involved in TTSS, polygalacturonase, EPS synthesis, ACC deaminase, ribosome proteins and energy metabolism. Unexpectedly, however, loci related to chemotaxis such as *cheAWYB* (*blr2192~blr2195*) and *cheR1WA* (*bll0390-bll0392-bll0393*) (Kaneko et al., 2002) were significantly repressed by SSE as well as genistein with time in this experiment (Data not shown).

The following plant infection test demonstrated that the number of the mature nodules formed in the root inoculated with SSE pre-treated cells was significantly enhanced (up to 12), compared with those formed with genistein-pretreated (6 of mature nodules) as well as untreated cells (4 of mature nodules) at 13 DAI (Figure 2.11). Besides, SSE-pretreated cells slightly promoted the fresh weight of both above-ground and roots of soybean at 10 and 13 DAI, compared with genistein-pretreated cells (Figure 2.12). It was reported that the infectiousness and competitiveness of *B. japonicum* cells for nodulation were stimulated by the pre-treatment with soybean meal extract and the slow-to-nodulate phenotype of a *B.*

japonicum mutant was reversed by pre-incubation with soybean root extract (Halverson and Stacey, 1984; Lodeiroa et al., 2000). It is likely that these SSE-induced distinctive gene expressions of *B. japonicum* are responsible for such stimulation of nodulation abilities.

It is clear that the expression of EC-III differed considerably between SSE-and genistein-treated cells within symbiosis island and its covered *tts* genes were induced later than common *nod* gene operon (in EC-IV) and *nodW* (in EC-II) (Figures 2. 6 and 2.7). The *tts* genes were first reported to be induced later than most *nod* genes in *R.* sp. NGR234 and recently reported to be highly conserved in all *Bradyrhizobium* strain belonging to genomic group II (Viprey *et al.*, 1998; Mazurier *et al.*, 2006). A model of regulatory cascade for *B. japonicum* has been recently proposed that many *tts* genes, which particularly possess a *tts* box upstream of each of them (Figure 2. 8A), are genistein-inducible under the indirect control of *nodD1nodD2nolA* and *nodW* through a transcriptional activator of the two-component regulatory family (TtsI encoded by bll1843), resulting in the expression of *tts* later than most of *nod* genes. *G. max* was also reported to form similar nodule numbers but exhibited a delay in the nodule development with deletion mutants of several *tts* genes, especially bll1843 (Krause *et al.*, 2002). In this experiment, the expression profile of *nodW* was similar between SSE-and genistein-treated cells (Table 2.2). However, the locus covering gene *nodD1nodD2nolA* (clone 3 and 4 in EC-IV) as well as a gene bll1843 was induced stronger by SSE than genistein at 12 hpi (Figure 2.8B and Table 2.2), suggesting that higher expression of *tts* genes is likely a result of integrated regulation of *nodD1nodD2nolA* rather than *nodW* by some compounds in SSE. It has been reported that the expression of *tts* genes in some pathogenic bacteria is

controlled by environmental factors, such as temperature, nutrition, osmolarity and quorum sensing (Hueck, 1998). These results suggest that the stronger expression of *tts* genes is one reason for the enhanced nodule development of *G. max* inoculated with SSE-pretreated cells.

Gene *blr1993* encoding polygalacturonase, one of the plant cell wall-degrading enzymes, was reported to be strongly induced by genistein in a NodW-dependent manner, although the null mutant of this gene did not impair symbiosis with the host (Baumberger *et al.*, 2003). In this experiment, however, it was not substantially induced by genistein (1.4 fold) but induced strongly by SSE (18.9 fold) at 12 hpi, and its expression occurred later than *nod* gene induction, suggesting that it seems to be tightly regulated. Recently, the expression of genes encoding plant cell wall-degrading enzymes including one polygalacturonase (*pehB*) as well as exopolysaccharide in *Ralstonia solanacearum* was reported to be regulated in concert with type III secretion system (Valls *et al.*, 2006). Another gene, *blr0241*, encoding ACC deaminase was induced by SSE during 12 h. This enzyme is likely involved in the reduction of ethylene concentration by degrading ACC, the precursor of ethylene biosynthesis in plant, during the nodule formation process (Penmetsa *et al.*, 1997; Uchiumi *et al.*, 2004). Recently, *blr0241* was also found to be well-induced in bacterioid (Pessi *et al.*, 2007), implying that this gene plays an important role during symbiotic process. Higher expression of genomic loci relevant to ribosomal protein synthesis and energy metabolism by SSE seems to be reasonable, because SSE is rich in nutrients such as amino acids, organic acids, sugars, vitamins, etc (Philips and Streit, 1996), and advantageous to *B. japonicum* for the colonization in the rhizosphere of soybean.

The SSE-induced loci shared with genistein-treated cells seem to be mainly

caused by genistein in SSE. LOS 10 covering three adjacent genes for the multidrug resistance-related proteins (*bll4319-bll4321*) was induced by both SSE and genistein, in which *bll4320* encoding a probable RND efflux membrane fusion protein was induced 7.4-28.8 fold during 12 h (Table 2.2). Moreover, LOS 10 was also induced by daidzein at 12 hpi (data not shown), suggesting that this locus is specifically involved in the isoflavonoid-inducible resistance of *B. japonicum*. The resistance of *B. japonicum* to phytoalexin such as glyceolin from soybean was reported to be induced by genistein and daidzein (Parniske *et al.*, 1991). In LOS 20, gene *blr7023* encoding transcriptional regulatory TetR family was mainly induced by genistein during 12 h (3.6-8.0 fold). Besides, one clone (BJ7162) covering a single gene for a AcrB/AcrD/AcrF family protein (*bll7019*) was induced 2.6-33.3 fold by both SSE and genistein at 0.5 hpi (Appendix 4), suggesting that this locus responds to genistein at the early stage and is also involved in the multidrug resistance of *B. japonicum*. These two loci are located far away from symbiosis island, but seem to play an important role in the competition of *B. japonicum* in the rhizosphere of soybean.

Since SSE-supplemented medium contained daidzein (around 6.8 μM), the genome-wide expression of *B. japonicum* in response to daidzein (5 μM) at 12 hpi was also monitored (Appendix 1 and 2). Nine clones covering *nod* genes such as *nodWV* (clone 15-16 in EC-II), common *nodYABC* operon (clone 2, 5, 6-8 in EC-IV) and *noeI* (clone 20 in EC-IV) inside symbiosis island were also induced by daidzein as well as genistein, although the induction levels by daidzein tended to be slightly lower than those by genistein. These results indicate that daidzein is also a natural inducer of *nod* genes. It was reported that daidzein induced *nod* genes in *B.*

japonicum (Kosslak *et al.*, 1987). However, no potential regulation of LISs and LOSs except for the LOS 8-10, 12, 16 and 19 was observed for daidzein-treated cells (Appendix 3 and 4), indicating that the exclusively regulated loci by SSE are not caused by daidzein.

In combination with the time course of induction and validation of real-time PCR, the global expression profiles of loci in the presence of SSE were successfully captured in the experiment and it will be useful to identify novel genes relevant to the initial stage of symbiosis because bacterial genome are often organized into functional units. Nevertheless, it is important to keep in mind that the composition of SSE might be modified in the rhizosphere because it is known that microbes inhabiting rhizosphere influence the composition of plant-released compounds (Philips and Streit, 1996). In conclusion, the genome-wide expression analyses in this study reveal that symbiosis island functions as distinctive expression clusters (ECs) in the initial stage of symbiosis, and that there are several loci which are exclusively regulated by SSE or shared between SSE- and genistein-treated cells. These findings will provide an insight into the mechanism of plant-microbe interaction by identifying the novel genes relevant to symbiosis.

All information of the genes and clones described in this study is accessible in the Web database, RhizoBase, at http://www.kazusa.or.jp/rhizobase/, and http://orca10.bio.sci.osaka-u.ac.jp/brady/.

2. 5 Summary

Initial interaction between (Brady)rhizobia and legumes actually start via encounters of both partners in the rhizosphere. In this study, the global expression profiles of *Bradyrhizobium japonicum* USDA 110 in response to soybean (*Glycine max*) seed extracts (SSE) and genistein, a major soybean-released isoflavone for *nod* genes induction of *B. japonicum*, were compared. SSE induced many genomic loci as compared with genistein (5.0 μM), nevertheless SSE-supplemented medium contained 4.7 μM genistein. SSE markedly induced four predominant genomic regions within a large symbiosis island (681 kb), which include *tts* genes (type III secretion system) and various *nod* genes. In addition, SSE-treated cells expressed many genomic loci containing genes for polygalacturonase (cell wall degradation), exopolysaccharide (EPS) synthesis, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, ribosome proteins family and energy metabolism even outside symbiosis island. On the other hand, genistein-treated cells exclusively showed one expression cluster including common *nod* gene operon within symbiosis island and six expression loci including multidrug resistance, which were shared with SSE-treated cells. Twelve putatively regulated genes were indeed validated by quantitative RT-PCR. Several SSE-induced genomic loci likely participate in the initial interaction with legumes. Thus, these results can provide a basic knowledge for screening novel genes relevant to the *B. japonicum*-soybean symbiosis.

2. 6 Figures and Tables

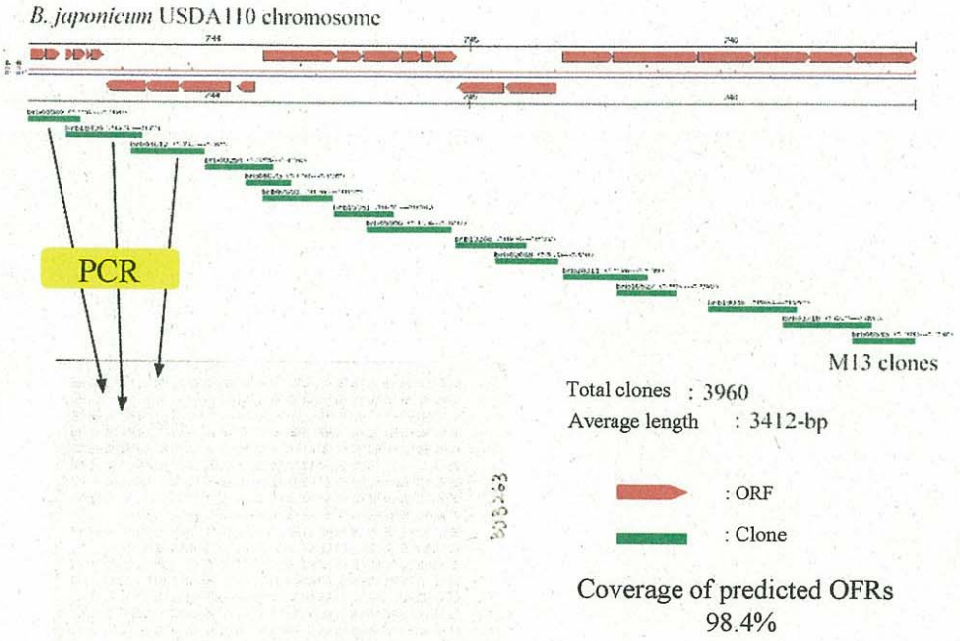

Figure 2.1 A DNA macroarry platform of *Bradyrhizobium japonicum* USDA 110 used throughout this study.

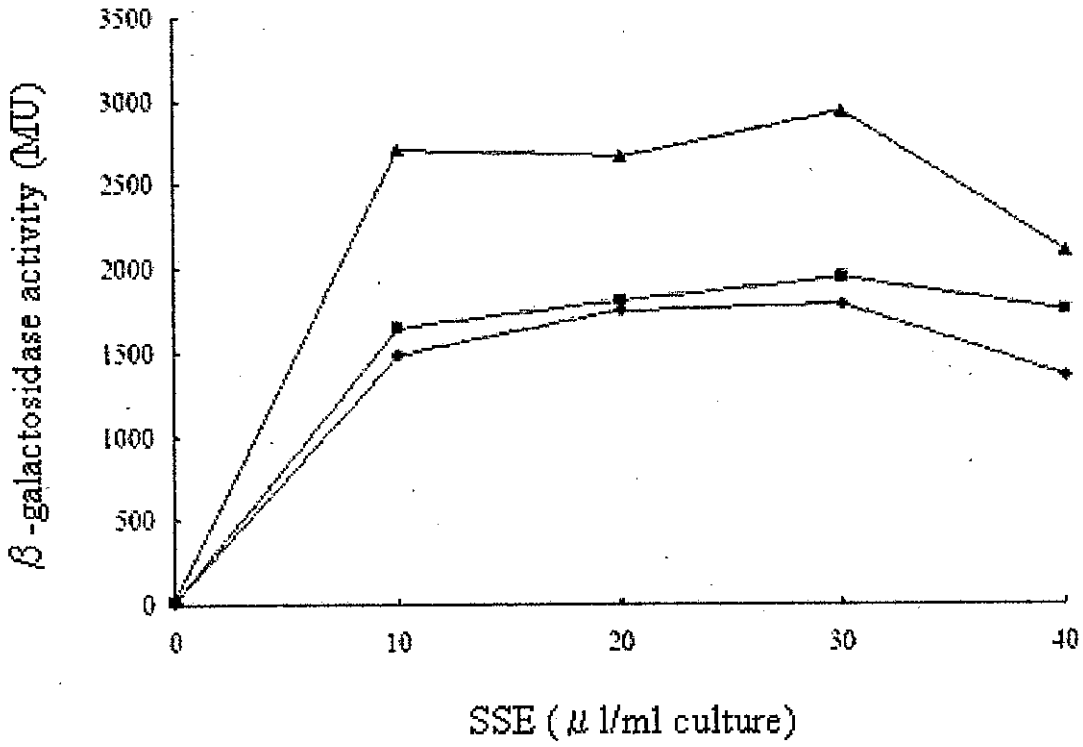

Figure 2.2 Effects of cell densities and SSE concentrations on the *nodY* expression. *B. japonicum* ZB 977 cells grown to the log-phase were diluted to 0.025 (▲), 0.05 (■) and 0.1 (◆) of OD_{600} with fresh YMB culture medium containing no antibiotics and treated with SSE (10, 20, 30, and 40 μl/ml culture) at 30°C for 20 h.

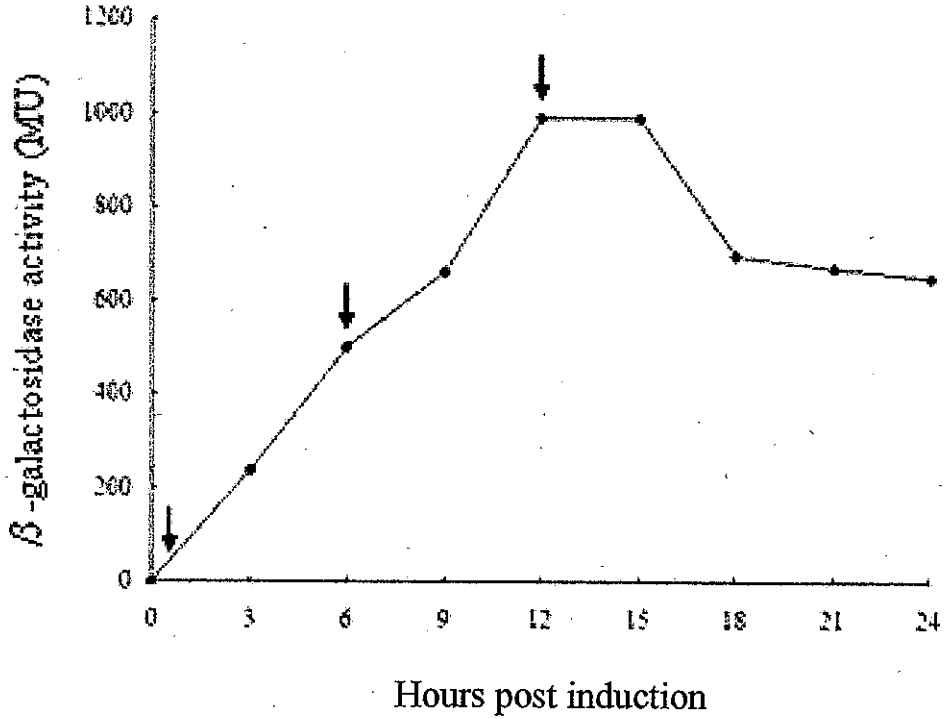

Figure 2.3 Effect of induction periods on the *nodY* expression. *B. japonicum* ZB 977 cells grown to the log-phase were diluted to 0.1 of OD_{600} with fresh YMB culture medium containing no antibiotics and treated with SSE (20 µl/ml culture) at 30°C for 24 h. A portion of the culture was withdrawn every 3 h during incubation and β-galactosidase activity was measured as described previously. Induction periods used for the expression analyses are shown by the arrows (0.5, 6 and 12 hpi).

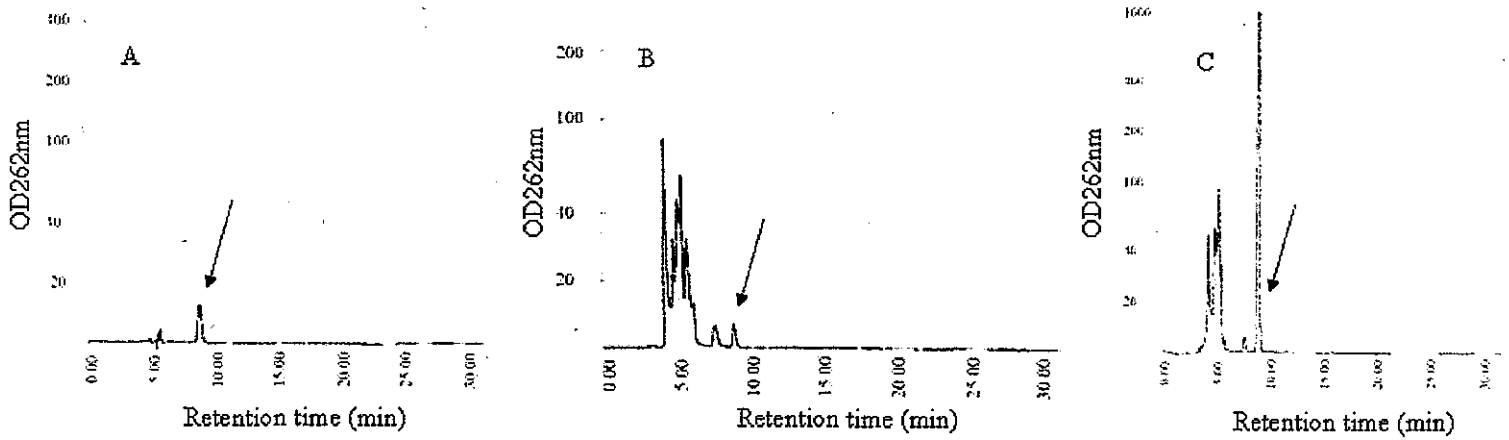

Figure 2.4 Analysis of genistein content in SSE. Genistein present in 100-fold-diluted SSE was measured by high-performance liquid chromatography (HPLC) (panel B). HPLC was done with a Tosoh liquid chromatography system under the following conditions: column, TSK gel ODS-100V (4.6 mm ×250 mm); mobile phase, methanol (0~25min with an isocratic elution of 60% methanol, 25-30 min with a linear gradient from 60 to 76% methanol, 30~50min with an isocratic elution of 76% methanol, 50~52min with a linear gradient from 76 to 100% methanol and 52~60min with an isocratic elution of 100% methanol); flow rate, 0.8 ml/min; temperature, 25°C; detector, Tosoh W8010 (262 nm). Panel A shows HPLC profile of standard genistein (12 μM) (4', 5, 7-trihydroxyisoflavone, from soybean, SIGMA). Panel C indicates the HPLC profiling of mixture of SSE and pure 1mM of genistein (V/V: 24/1).

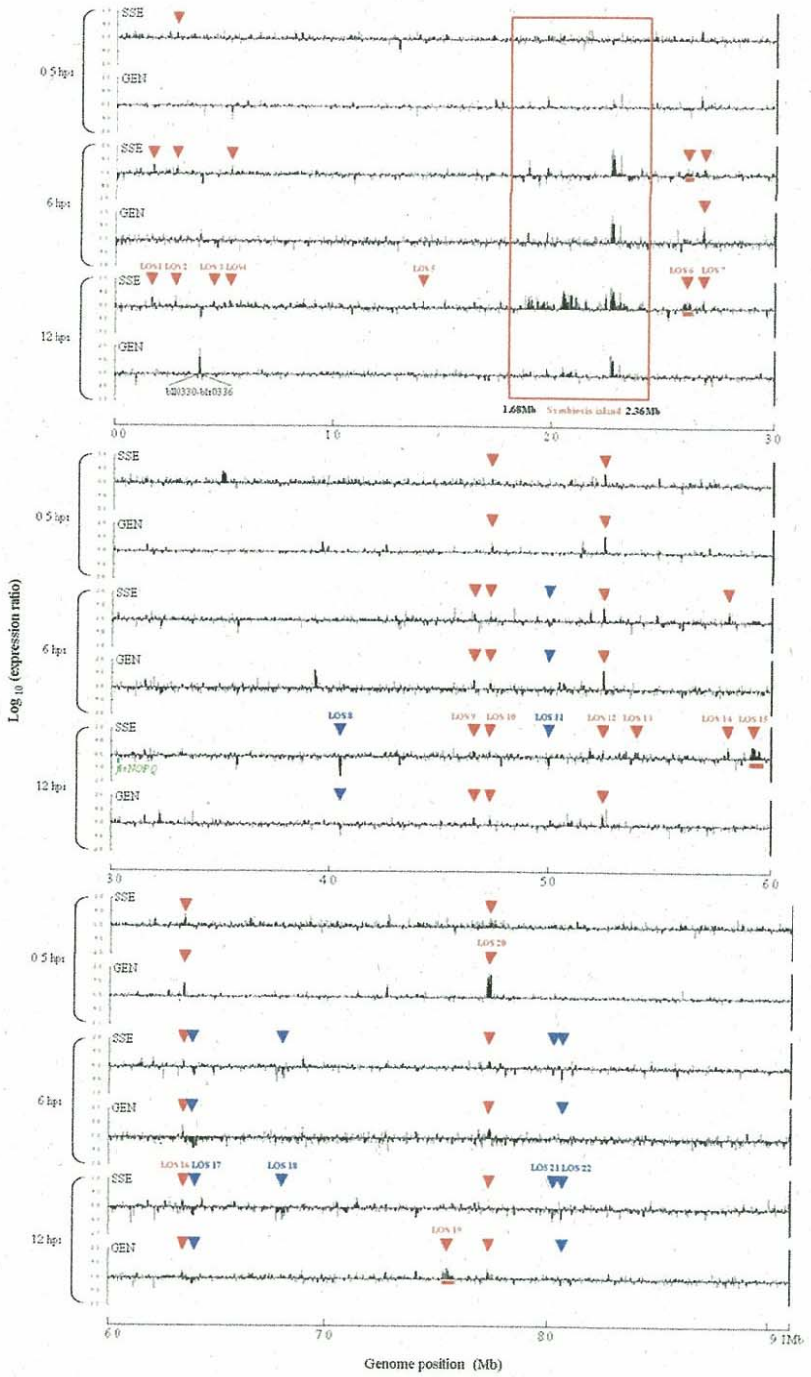

Figure 2.5 Genome-wide expression profiling of *B. japonicum* USDA 110 in response to SSE and genistein (GEN) at 0.5, 6 and 12 h post induction (hpi). Expression profiles were constructed as described in Materials and Methods. Expression levels of each clone were sorted according to the genome coordinate and represented by a single bar. The symbiosis island is located on the chromosome at coordinates 1.68-2.36Mb (Kaneko *et al.*, 2002). Red- and blue-colored arrows indicate some positions of strongly induced and reduced loci outside symbiosis island (LOSs: LOS1-22), which cover genes as shown in Appendix 3 and 4, respectively.

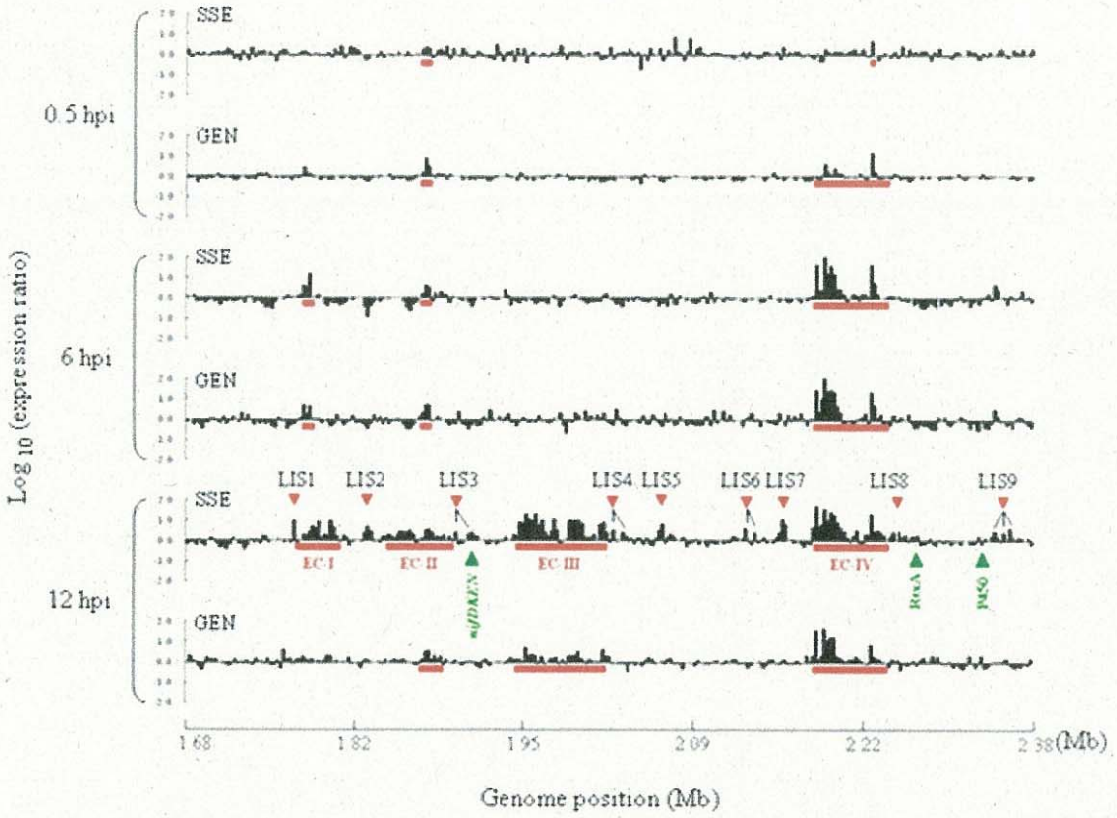

Figure 2.6 Expression profiles of the symbiosis island of *B. japonicum* USDA 110 induced by SSE and genistein (GEN) at 0.5, 6 and 12 hpi. Expression profiles were constructed as the value of $\log_{10}$ (expression ratio) of 276 clones covering symbiosis island as described in Materials and Methods. Expression levels of each clone were sorted according to the genome coordinate andrepresented by a single bar. Four predominant expression clusters, which were designated as ECs (EC-I to IV), are underlined with red bars. The SSE-induced loci inside symbiosis island but outside ECs (LISs: LIS 1-9) at 12 hpi are shown by the red arrow heads. The genes covered by ECs and LISs are shown in Appendix 1 and 2, respectively.

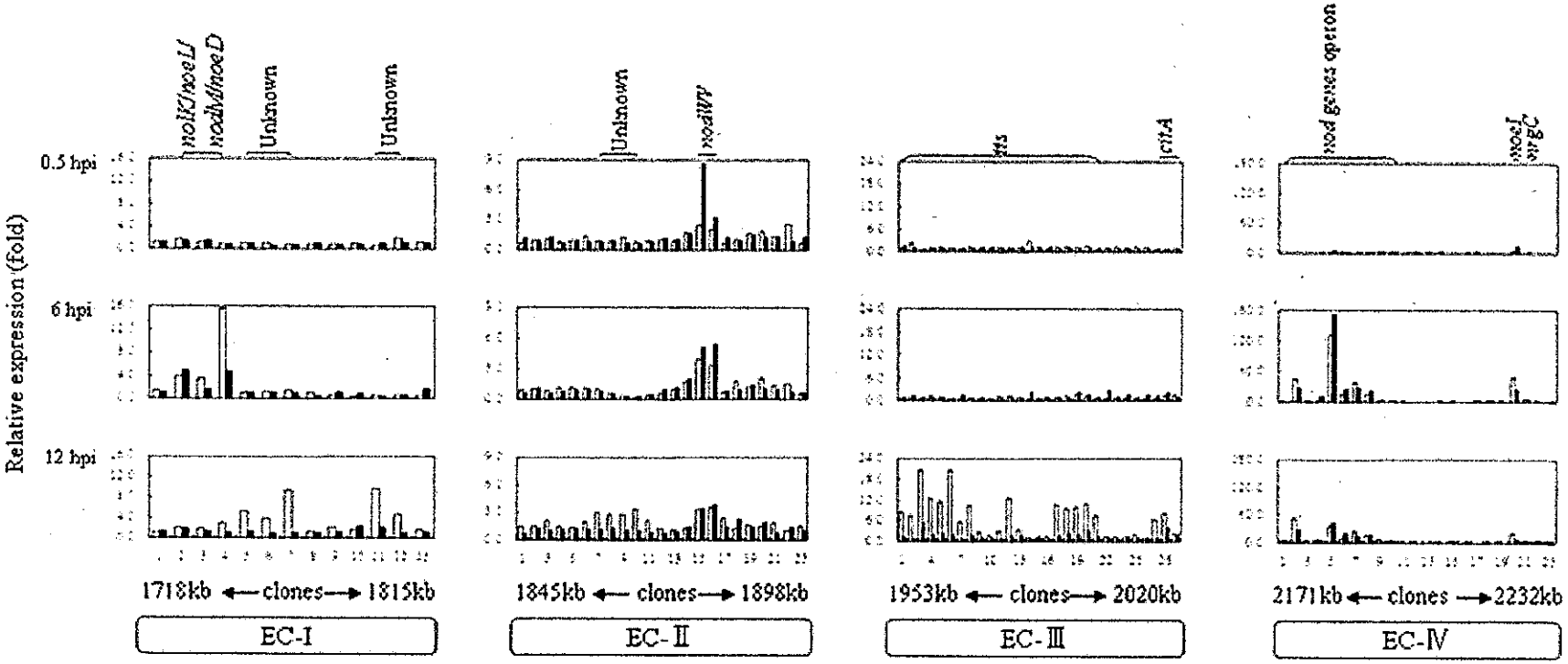

Figure 2.7 Comparative expression profiles of clones and their covered genes within ECs after the treatment of SSE or genistein at 0.5, 6 and 12 hp. *tts* and *nod* genes operon correspond to *rhcC1C2JNQRSTUV* for the type III secretion system and *nolZY-nolA-nodD2-nodD1YABCSUIJ-nolMNO-nodZ* for the nodulation, respectively. Detailed expression profiles of EC-III and IV are shown in Figure 2.8 and Appendix 1. White and black bars indicate the expression levels of clones in response to SSE and genistein, respectively.

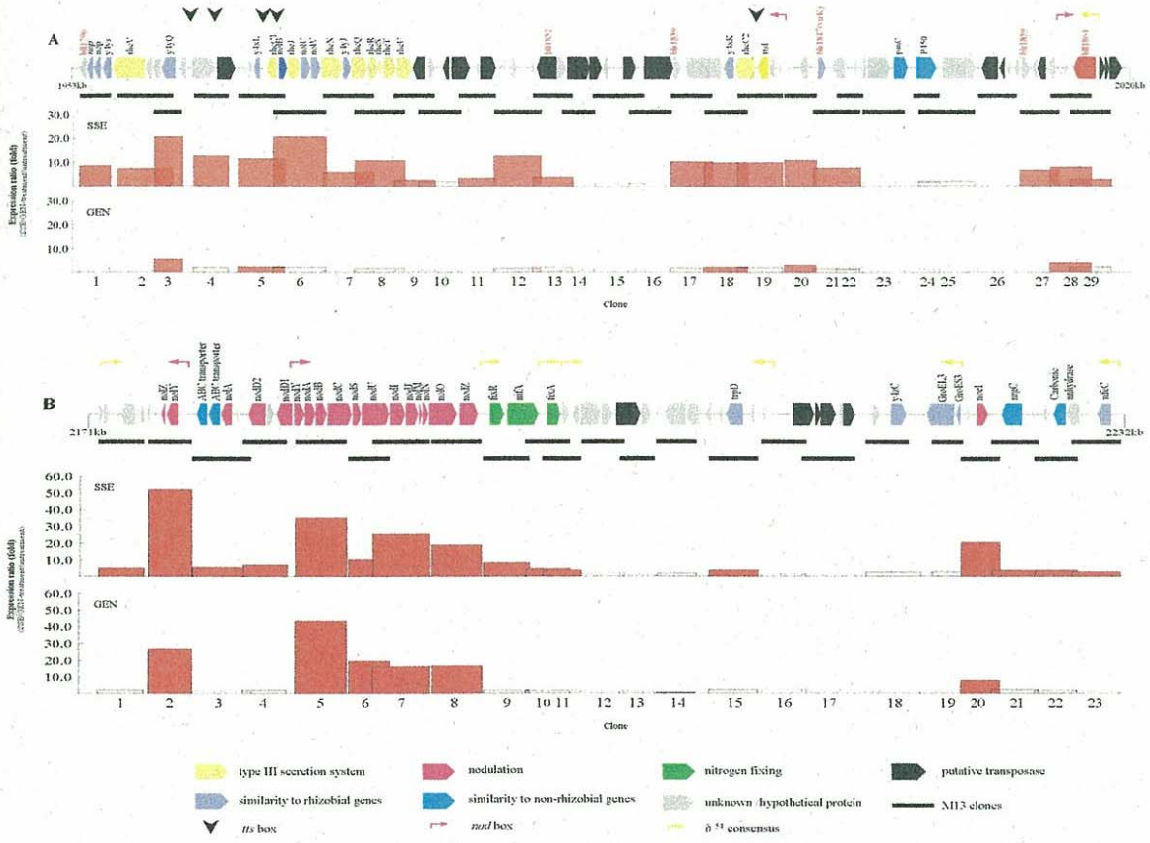

Figure 2.8 Expression profiles of clones and their covered genes in (**A**) EC-III and (**B**) EC-IV at 12 hpi after the treatment of SSE and genistein (GEN). Bars below the gene map indicate M13 clone inserts for macroarray construction and the box charts under M13 clone indicate differential expression of M13 clones in SSE/GEN-treated cells to untreated cells. The solid boxes indicate clones with strong (expression ratio≥2.2-fold, red) and mediate (1.5-fold ≤expression ratio<2.2-fold, pink) hybridization signal, while the dotted boxes indicate weak hybridization signal (expression ratio<1.5-fold). Genes *rhcN* (*blr1816*) and *ttsI* (*bll1843*) in EC-III and *nodC* (*blr2027*) in EC-IV were selected for validation by real-time RT-PCR. *tts* box (tcGTCAGctTNtcGaa AGct-N3-ccNcctA), *nod* box (ATCCA-N7-GATG-N6-ATCCAAACAAT CGATTTTACCAATC) and δ^{54} consensus (TGGCAC-N5-TTGCT/A) are described previously (Göttfert *et al.*, 2001; Süβ *et al.*, 2006)

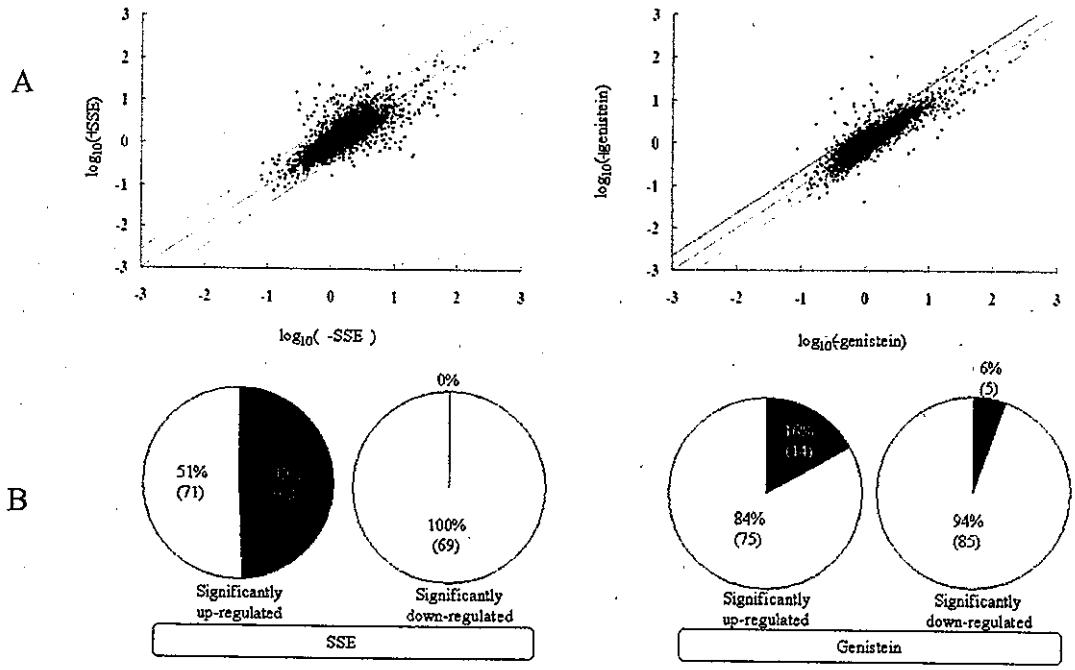

Figure 2.9 Scatter plots for the genome wide expression of *B. japonicum* USDA 110 in response to SSE and genistein at 12 hpi. (A) Log plots of the hybridization signals for macroarry obtained in SSE/genistein-treated cells against those in untreated cells. The x and y axes indicate the $\log_{10}$ values of the spot signal normalized based on sum of the signal intensities. (B) The distribution of clones inside and outside symbiosis island of *B. japonicum* USDA 110, which were significantly up- and down-regulated by SSE and genistein at 12 hpi, respectively. Black and white areas indicate the ratio (%) (number in parentheses) of clones inside and outside symbiosis island, respectively. In this experiment, the thresholds of significantly up- and down-regulated clones had a signal ratio of more than 2.2- and less than 0.4-fold, respectively.

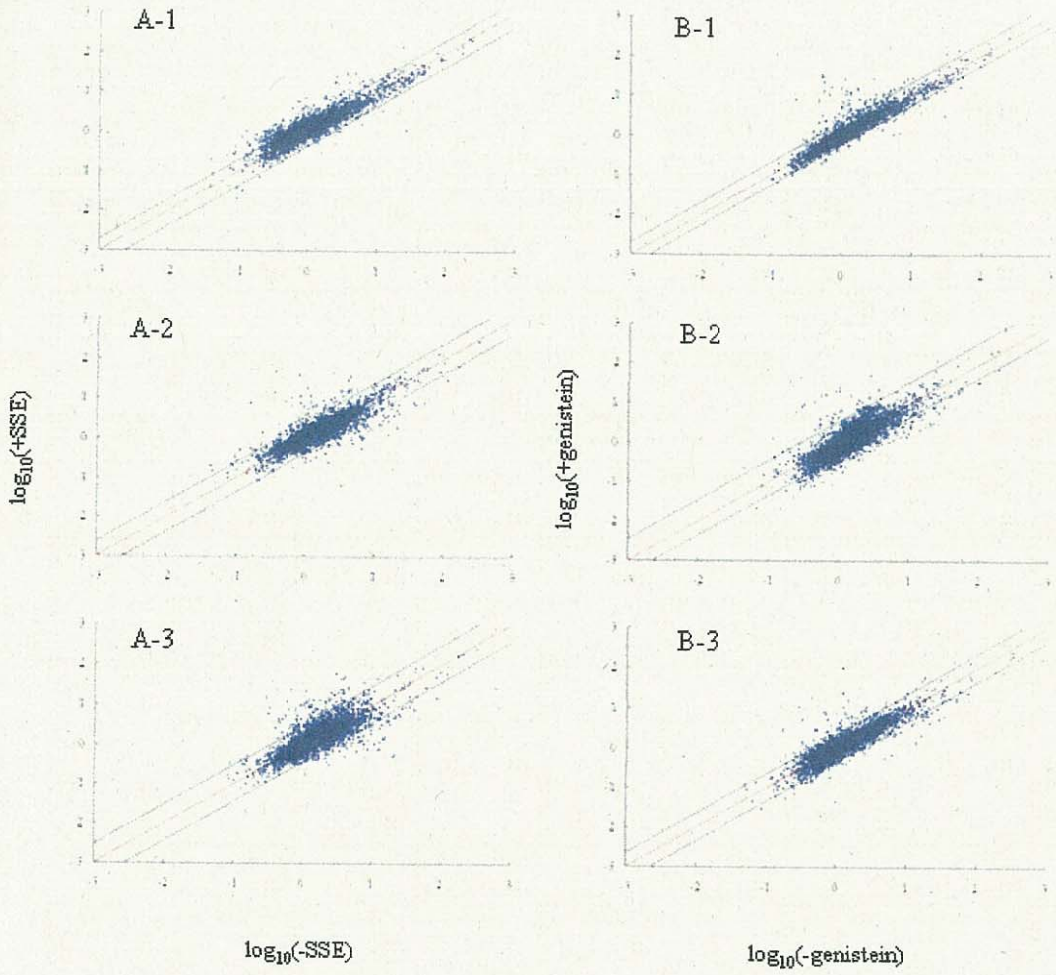

Figure 2.10 Scatter plots for the genome-wide expression of *B. japonicum* USDA 110 in response to SSE and genistein. Log plots of the hybridization signals for macroarray obtained in SSE- or genistein-treated cells against those obtained in untreated cells (panels A-1, A-2 and A-3: treated with SSE for 0.5, 6 and 12 h, respectively; panels B-1, B-2 and B-3: treated with genistein for 0.5, 6 and 12 h, respectively).The x and y axes indicate the log values of the spots signal normalized based on the sum of the signal intensities.

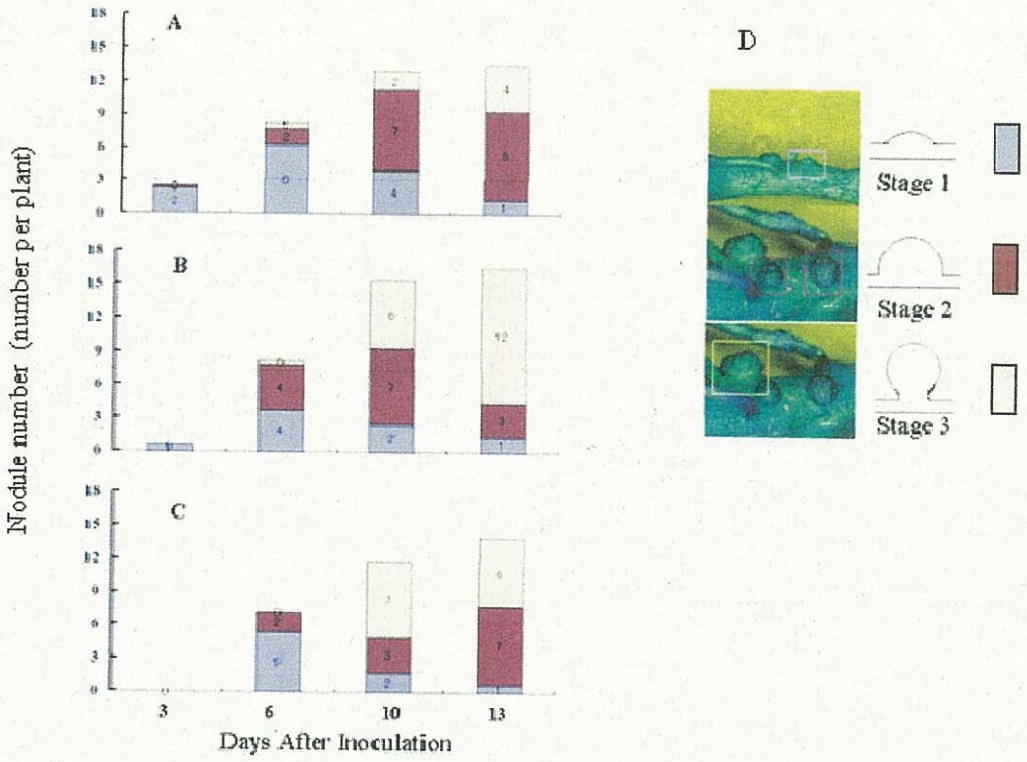

Figure 2.11 Effects of pretreatment of *B. japonicum* USDA 110 cells with SSE or genistein on the development of nodules formed in the root of *Glycine max* (L.) Merr. For the pre-treatment, *B. japonicum* cells were diluted to an OD_{600} of 0.1 and then treated with (B) SSE (20 µl/ml culture) or (C) genistein (5 µM) for 12h. Nodule number without the treatment is shown in panel (A). Each box represents the mean value obtained from the nodule number formed in at least 7 plant roots. To visualize the nodules in earlier stage, the roots were fixed in FAA (70% ethanol: acetic acid anhydride: formaldehyde=90:5:5, v/v/v) and then stained with 0.003% toluidine blue O overnight as described in Materials and Methods.

The development of nodules was divided into three stages, i.e., nodules of stage 1 being very small at the initial formation stage (blue box), nodules of stage 3 being complete mature stage (yellow box) and nodules of stage 2 (red box) being between stage 1 and 3 as shown in panel **(D)**. Nodule number corresponding to each stage was counted under a stereomicroscope.

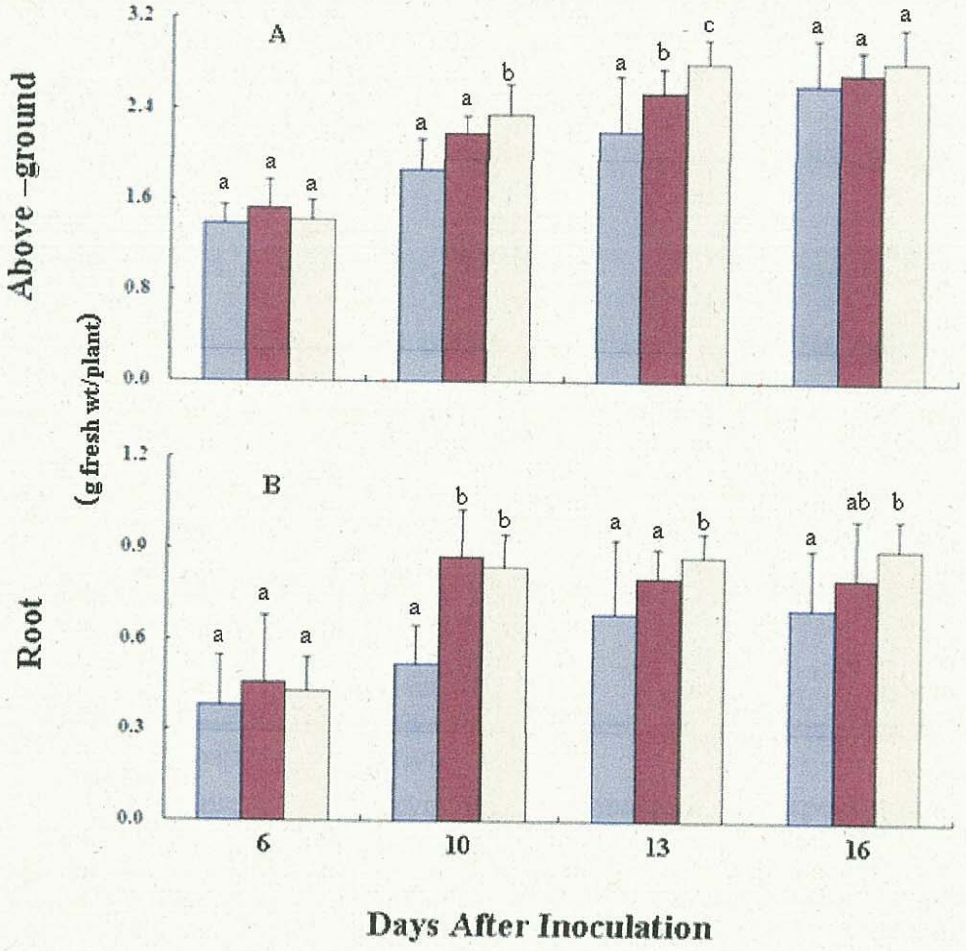

Figure 2.12 Effects of pretreatment of *B.japonicum USDA* 110 cells with SSE or genistein on the fresh weight of (**A**) above-ground and (**B**) root of *Glycine max* (L.). Seeds of Glycine max (L.) cv. Enrei were inoculated with *B. japonicum* USDA 110 cells pre-treated with SSE (20 µl/ml of culture: yellow bars) or genistein (5 µM: red bars) for 12 h. Each bar represents the mean value of at least 7 plants. Weight without the treatment is shown as blue bars. Different letters above bars indicate a statistically significant difference among each treatment at the same day after the inoculation ($p<0.05$).

Table 2.1 Bacterial strains and plasmids used in the study

Strain and plasmid	Relevant characteristics	Reference
B. japonicum		
USDA 110	Wild type, Cm^R	Kaneko *et al.*, 2002
ZB977	USDA 110, pZB 32, Tc^R	Banfalvi *et al.*, 1988
Δ370	*nodD2* deletion mutation, Cm^R, Km^R	Göttfert *et al.*, 1989
BjB3	*nolA* insertion mutation, Cm^R, Sp^R, Sm^R	Garcia *et al.*, 1996
plasmid		
pZB32	*nodY-lacZ* fusion	

USDA: U.S. Department of Agriculture.
Cm: chloramphenicol, Tc: tetracycline, Km: kanamycin, Sp: spectinomycin
Sm: streptomycin

Table 2.2 Validation of putatively up- and down-regulated genes by real-time RT-PCR

Gene	Gene annotation	Location[a] (Clone[b])	Relative expression (fold)					
			SSE			Genistein		
			0.5 h	6 h	12 h	0.5 h	6 h	12 h
Significantly up-regulated genes								
blr0241	1-aminocyclopropane-1-carboxylate deaminase	OUT(LOS 2) (brb07072)	2.9±0.8 1.4	4.2±0.8 3.9	2.4±0.3 6.9	1.1±0.1 0.6	1.5±0.2 1.0	1.5±0.2 0.7
blr1649	Unknown protein	SYM (EC-I) (brb02447)	0.5±0.1 0.7	0.4±0.0 0.6	5.3±1.7 9.6	1.4±0.3 1.1	1.0±0.1 0.5	1.0±0.2 2.1
bll1714	Two component system (NodW)	SYM (EC-II) (brb16383)	3.7±0.2 2.4	8.6±1.6 3.9	2.5±0.6 3.3	6.0±0.1 8.6	8.1±0.1 5.1	3.4±0.0 3.5
blr1816	RhcN protein (RhcN)	SYM (EC-III) (brb05339)	0.7±0.1 1.0	0.3±0.0 0.5	12.9±1.9 20.6	1.3±0.0 1.0	1.1±0.1 0.8	2.0±0.3 2.1
bll1843	Two-component response regulator (TtsI)	SYM (EC-III) brb00710	0.6±0.0 1.3	2.1±0.5 1.5	10.1±0.2 9.7	1.1±0.1 0.9	4.9±1.0 2.0	4.1±1.1 1.9
blr1993	Polygalacturonase	SYM (LIS 7) (brb02802)	0.8±0.2 0.8	1.1±0.0 1.1	18.9±3.0 12.5	0.6±0.1 0.7	1.4±0.1 0.9	1.4±0.1 1.2
blr2027	Chitin synthase (NodC)	SYM (EC-IV) (brb16006)	2.1±0.4 1.8	218.1±11.5 131.3	17.5±4.9 35.0	6.1±2.1 4.0	252.9±15.0 172.1	25.0±1.7 43.6
bll4320	Probable RND efflux membrane fusion protein	OUT(LOS 10) (brb07789)	9.6±0.3 3.5	7.4±0.3 5.5	13.1±3.4 3.6	18.9±5.5 5.7	28.8±6.9 2.9	22.5±2.4 6.1
blr7023	Transcriptional regulatory protein TetR family	OUT(LOS 20) (brb07276)	1.6±0.1 4.6	1.7±0.2 2.8	0.84±0.1 1.4	7.7±2.2 57.3	8.0±2.35 3.4	3.6±0.4 5.7

Significantly down-regulated genes								
blr4553	ABC transporter substrate -binding protein	OUT(LOS 11) (brb05409)	0.6±0.0 1.0	0.19±0.0 0.4	0.1±0.01 0.1	1.9±0.4 0.7	0.3±0.1 0.5	0.4±0.1 0.9
bll5843	Hypothetical protein	OUT(LOS 17) (brb02759)	0.4±0.1 0.9	0.1±0.1 0.3	0.5±0.1 0.3	0.4±0.1 0.9	0.3±0.1 0.2	0.3±0.1 0.3
bll7341	RhtB family transporter	OUT(LOS 22) (brb08591)	0.5±0.1 0.6	0.4±0.0 0.1	0.8±0.1 0.1	0.9±0.2 0.7	0.7±0.1 0.4	1.0±0.2 0.4

[a] Locations of gene: SYM and OUT indicate inside and outside symbiosis island on the chromosome at coordinates 1681-2362kb, respectively. EC-I, II, III, IV indicate great expression clusters inside symbiosis island. LIS indicates genomic loci inside symbiosis island but outside ECs and LOS indicates genomic loci outside symbiosis island as shown in Figures 2.5 and 2.6.

[b] DNA fragments amplified by PCR using M13 clones of the genomic libraries used for the array construction. Their genome position and entirely or partially covered genes can be seen in Appendix 1-4.

Table 2.3 Primers used for the validation of putatively regulated genes by real-time RT-PCR

Gene	Gene annotation	Forward primer (5'-3')	Reverse primer (5'-3')
blr0241	1-aminocyclopropane-1-carboxylate deaminase	CTCCGTGCACAAATATGG	GACCACGATGTAGTCGAACTT
blr1649	Unknown protein	TCCTCAAGGCTGACCATTAC	GTGTCAGCTCATCAAGAGTCAT
bll1714	Two component system (NodW)	GCTGATGAACAAGCAGGTAG	TCGCTCATTCTGATCAAGTC
blr1816	RhcN protein (RhcN)	CTCCATTACGGCCTTCTATACT	AGAGAATAATGTGGCCATCG
bll1843	Two-component response regulator (TtsI)	ACTACCACCTCGTACTGCTG	GCTGAGATCATTGAGACTGC
blr1993	Polygalacturonase	GGACGTGACACTCGAAGAT	AATGCCTTGGAACATACCTC
blr2027	Chitin synthase (NodC)	CCACCTAACGATACTCATGCT	GTAGATATGGCCGAAGACTGT
bll4320	Probable RND efflux membrane fusion protein	GACAATACGGATCGCAAG	TACCGACTCTCCATAGGTGT
blr7023	Transcriptional regulatory protein TetR family	AAGCTCATCGAGGAATCGTT	CGTCGCTTCATTCAAGAGTT
blr4553	ABC transporter substrate-binding protein	TTCCTCAATAAGCTCGGTCT	CTTGAAGGTGATGGTGTGAC
bll5843	Hypothetical protein	GACTACATCACCAGCAACAAC	CTCGAGATTCTCCGAATAGG
bll7341	RhtB family transporter	GCTTCCTGATCAACATCCTC	CGCTCAATTCCAGCATTC
bll7349[a]	Primary sigma factor (SigA)	GAGAACCAGATGTCGCTTGC	TGGATGTCCTGCTCCTGAAG

[a]: a housekeeping gene used as a internal control in this experiment.

CHAPTER 3

Expression Analyses of *Bradyrhizobium japonicum* in Response to Soybean Seed Extracts and Genistein at Low Temperature

3. 1 Introduction

Rhizobium-legume symbiotic interaction is an unimaginably elaborated process controlled by the exchange of molecular signals between two partners. However, this interaction is strongly affected by suboptimal environmental conditions such as salinity, unfavorable soil pH, nutrient deficiency, mineral toxicity, desiccation and temperature extremes (Zahrm, 1999). *Bradyrhizobium japonicum* is a nitrogen-fixing endosymbiont associated with soybean (*Glycine max*). Most optimal soybean-*B. japonicum* symbiosis requires root zone temperatures (RZTs) ranging from 25 to 30°C (Jones and Tisdale, 1921; Dart and Day, 1971). Studies involving *B. japonicum* are usually carried out under laboratory rather than field conditions, typically with a growth temperature of 28°C, at which the bacteria are considered to grow best. In fact, soil temperature in the short-season soybean production areas is often below this range and the bacteria have to adapt and continue to grow under this suboptimal temperature condition.

Root zone temperature, particularly lower than 17.5°C, was reported to strongly inhibit the process of nodulation and nitrogen fixation due to the delayed initiation of infection threads, nodule development, the decreased biosynthesis and secretion of

genistein (Lynch and Smith, 1993; Zhang and Smith, 1994, 1996). Application of genistein to inocula and soil could overcome the low temperature inhibition of soybean nodulation and nitrogen fixation (Zhang and Smith, 1997; Belkheir, *et al.*, 2000; Leibovitch *et al.*, 2001). However, these reports mainly focused on the investigations of the nodule mass, various morphological changes (i.e. root-hair curling and infection thread), the Nod factor production and the selection of low temperature-tolerant strain. Comparatively little is known about the genetic and molecular mechanisms underlying *B. japonicum*-soybean interaction at low temperature.

The accumulating evidences in the genomic analyses of *B. japonicum* suggest that isoflavones (genistein and daidzein)-Nod factor relationship is important but not in full to explain the signal-interaction during the early stage of symbiosis (Broughton *et al.*, 2000; Krause *et al.*, 2002; Becker *et al.*, 1998). Recently, genistein has been also reported to possess a much broader function than mere induction of *nod* genes (Lang *et al.*, 2008). In chapter 2, the global expression profiling of *B. japonicum* genomic loci in response to SSE and genistein were captured with time at common growth temperature (30°C) by using an array system based on the M13 libraries generated by the genome sequencing. The results revealed that compared with genistein, SSE collectively and preferentially express many genomic loci such as TTSS, polygalacturonase within symbiosis island of *B. jaoinucm* as well as some distinctive loci such as EPS synthesis, ribosomal proteins family and ACC deaminase outside the symbiosis island.

In the present chapter, a study was conducted to monitor the genome-wide expression profiles of *B. japonicum* in response to SSE and genistein at 15°C, a suboptimal RZT reported to strongly inhibit infection steps (Zhang and Smith, 1994),

and compared with the results of the chapter 2. The results revealed that low temperature leads to a potentially delayed expression lag of genomic loci preferentially induced by SSE at 30°C, including TTSS gene cluster, whereas nodulation gene loci were strongly and early induced, suggesting that the SSE-preferentially-induced genomic loci might be tightly regulated with integration of a variety of environmental cues for the adaptive capabilities of *B. japonicum*.

3. 2 Materials and Methods

Bacterial Strains and Culture Conditions

B. japonicum USDA 110 was used as a wild type strain for genome-wide expression analyses. *B. japonicum* ZB977, a derivative of *B. japonicum* USDA 110 harboring a plasmid (pZB32) which carries a translational *nodY-lacZ* fusion (Banfalvi *et al.*, 1988), was used as an indicator strain for the determination of the induction conditions at 15°C. *B. japonicum* USDA 110 and ZB977 were maintained on the yeast extract-mannitol agar (YMA) medium with chloramphenicol (30 μg/ml) and tetracycline (50 μg/ml), respectively, and cultured in yeast extract-mannitol broth (YMB) without antibiotics for the induction.

β-Galactosidase Activity Assay

SSE and genistein (4', 5, 7-trihydroxyisoflavone, from soybean) were used as described in the chapter 2. *B. japonicum* ZB977 cells grown to the log-phase were diluted to 0.1 of OD_{600} with fresh YMB containing no antibiotics and induced with 20 μl SSE /ml culture or 5 μM genistein for 24 h at 15°C. Induction was carried out in 50

ml of YMB and a portion of culture (2 ml) was withdrawn at 3 h interval for measuring β-galactosidase activity as described previously (Banfalvi *et al.*, 1988).

RNA Isolation, cDNA Synthesis and Macroarray Analyses

DNA macroarray platform of *B. japonicum* was described in the chapter 2. *B. japonicum* USDA 110 cells were pre-cultured in 30 ml of YMB without antibiotics for three days and then scaled up to 200 ml of YMB for growing to mid- log-phase (OD_{600}=0.3-0.5) at 30°C. The cells were then diluted with fresh YMB to OD_{600} of 0.1 and induced with SSE (20 μl/ml of culture) or genistein (5 μM) for periods determined by *B. japonicum* ZB977 at 15°C. RNA isolation, hybridization, image capture and data analyses were also performed as described in the chapter 2. Briefly, the equal volume of ice-cold 5% (w/v) phenol/ethanol was added into the culture to minimize the degradation of RNA. Total RNA was isolated with ISOGEN-LS (Nippon Gene, Tokyo, Japan) and then treated with DNase I. After removal of rRNA with the MICROB*Express*™ Kit (Ambion, Austin, TX, USA), the cDNAs were made by reverse transcriptase with the incorporation of [α-^{33}P] dCTP and hybridized with DNA array sheets at 55°C overnight. Hybridized signals were captured by using a BioImaging analyzer (BAS 5000, Fuji Film, Tokyo, Japan). The signal intensity of each spot was quantified by using ArrayVision software (GE Healthcare Bio-Sci, NJ, USA) and normalized on the basis of the total sum of the signal intensities of spots on the array membrane and the data analysis was done as described in the chapter 2.

Quantitative Real-Time RT-PCR

Total RNA isolation was performed as described in the chapter 2 and the primers

were designed by Primer 3 (http://fokker.wi.mit.edu/primer3/input.htm) (Rozen and Skaletsky, 2000). One to three hundred ng of total RNA were used as template and real-time RT-PCR reactions were performed with MiniOpticon™ Version 3.1 (Bio-Rad, Hercules, CA, USA) in combination with the QuantiTect SYBR Green RT-PCR (Qiagen GmbH, Hilden, Germany). Quantification was performed using the Pfaffl method according to the real-time RT-PCR application guide provided by Bio-Rad. A housekeeping gene of *B. japonicum, sigA,* which showed no significant change in expression level under the growth conditions in this experiment, was used as an internal reference for quantitative real-time RT-PCR in the experiment.

3. 3 Results

Determination of Induction Conditions at Low Temperature

To compare the genome-wide expression profiling of *B. japonicum* in response to SSE (20 μl/ml culture) and genistein (5 μM) at 30°C, the concentrations of both inducers were remained same, but the induction periods were determined using *nodY* expression by a *nodY-lacZ* transcriptional fusion at 15°C. As shown in Figure 3.1, the *nodY* expression increased with time and reached maximum at 20 hpi in the presence of SSE and genistein at 15°C. Additionally, similar expression patterns were observed between both treatments. Because the *nodY* expression reached maximum at 12 hpi with SSE-treatment at 30°C, and three time points (0.5, 6 and 12 hpi) during elevating phase were chosen for the global expression analyses (Figure 2.2), 12 and 20 hpi were firstly used for RNA isolation at 15°C.

In addition to these two time points determined by the *nodY* expression, a longer

induction time up to 48 hpi was lately chosen for RNA isolation due to the reason which would be subsequently presented.

Nodulation Loci Are Strongly and Early Induced by Both SSE and Genistein at Low Temperature

Firstly, the *B. japonicum* cells were induced with SSE and genistein for 12 and 20 hours at 15°C. The genome-scale expression profiling of *B. japonicum* were captured and constructed with the value of $\log_{10}$ (expression ratio) of each clone (a total of 3960 overlapped clones covering the whole genome), which represent the relative expression levels between SSE/genistein-treated and untreated clones (Figure 3.2).

In the chapter 2, compared with genistein, SSE collectively and remarkably induced symbiois island of *B. japonicum* as four predominant expression clusters (ECs, EC-I~IV) at 12 hpi at 30°C (Figures 2.5 and 2.6). However, such symbiosis island with a similar expression profiling was not found in SSE-and genistein-treated cells at either 12 or 20 hpi at 15°C. Looking closer at the expression profiles of symbiois island revealed that it was very similar between the SSE- and genistein-treated cells at 12 and 20 hpi, and displayed in a portrait of three distinguishably expressed genome loci within it (Figure 3.3). It clearly showed that two of these distinguishably expressed loci were a part of EC-I and II, and one corresponded well with EC-IV. Unexpectedly, EC-III was completely undetectable at 20 hpi at 15°C. The expression portraits of symbiosis island at 12 and 20 hpi at 15°C were similar to those captured at 6 hpi at 30 °C.

Using the cut-off described in the chapter 2 (expression ratio$\geq$ 2.2-fold and $\leq$ 0.4-fold for strongly induced and repressed clones, respectively) identified 25 and 20

strongly induced clones within symbiosis island in SSE-treated cells, while 31 and 19 for genistein-treatment at 12 and 20 hpi, respectively. A comparison with the results that 69 and 14 clones within symbiosis island were strongly induced by SSE and genistein at 12 hpi at 30°C, suggests that within symbiosis island, the expression of many clones preferentially induced by SSE are susceptible to low temperature.

Furthermore, it was found that these three distinguishably expressed genomic loci covered most of the known nodulation genes (Figure 3.3 and Appendix 1), i.e., the first (at the coordinate 1, 781, 581-1, 7943, 82 within EC-I) covering five genes *blr1629/ bll1630(nolK)/bll1631(noeL)/blr1632(nodM)/blr1633(noeD)*, the second (at the coordinate 1,875,786-1,883,701 within EC-II) covering four genes *bsl1713/ bll1714(nodW)/bll1715(nodV)/blr1716*, and the third (at the coordinate 2, 171, 906-2, 232, 429) corresponding well with EC-IV covering genes from *bsl2015* to *blr2035*, which involved a common *nod* genes operon, *nolZY-nolA-nodD2-nodD1YABCSUIJ-nolMNO-nodZ* at the beginning and three genes *bsr2061/blr2062* (*noeI*)/*bll2063* (*nrgC*) at the end.

The expression of clones within the three nodulation loci showed a generally declining tendency with time from 6 to 12 hpi at 30°C and from 12 to 48 hpi at 15°C (Appendix 1). However, the exception was observed for three clones within EC-IV, clone 2 covering genes from *bsl2014* to *bll2016* and clone 3 and 4 from *bll2017* to *bll2023*. The expression of clone 2 covering genes *bsl2014/bsl2015(nolZ)/bll2016(nolY)* was increased with time for both SSE and genistein treatments, while that of clones 3 and 4 enhanced for only SSE-treatment from 6 to 12 hpi at 30°C and 20 to 48 hpi at 15°C. Interestingly, a *bll2019(nolA)/bll2021(nodD2)/bll2023(nodD1)* genes region was found to be

overlapped covered within clone 3 and 4. In *B. japonicum*, NodD1 responds to genistein and functions as a positive transcriptional activator of common *nod ABCSUIJ* operon, whereas NolA and NodD2 form two key components in the feedback regulation as well as quorum regulation of the nodulation genes (Loh and Stacey, 2003). The unusual expression patterns of this regulatory genome region imply that it may be involved in the regulation of some SSE-preferentially-induced genome loci at 12 hpi at 30°C and 48 hpi at 15°C.

Besides, two other loci outside ECs (at the coordinate 1, 940, 649-1, 945, 060 and 2, 328, 495-2, 335, 384) involved genes from *blr1781* to *blr1785* and from *bll2155* to *bll2161* were also strongly induced by SSE and genistein at 12 and 20 hpi (Figure 3.3). Interestingly, these 12 genes encode unknown or hypothetical proteins as well as two identical set of three putative transposase (*blr1781-blr1783*, *bll2159-bll2161*). The induction of these two mobile-like loci simultaneously occurred with that of *nod* genes, implying that they might play an important role in the early stage of symbiosis.

Part of nod Genes Is Expressed Higher at Low Temperature

A comparison of the expression level of these nodulation loci at two time points (6 and 12 hpi) at 30°C with those (20 and 48 hpi) at 15°C demonstrated that clones within loci covering genes *blr1629/ bll1630(nolK)/bll1631(noeL)/blr1632(nodM)/ blr1633(noeD)*, *bsl1713/bll1714(nodW)/bll1715(nodV)/blr1716*, and *bsr2061/blr2062* (*noeI*) had a higher expression level at 20 hpi, but their expression dropped to a very low level at 48 hpi at 15°C for both SSE and genistein treatments (Figures 3.5 and 3.6). A substantially higher expression level was actually observed

for these loci during initial 12 h at 15°C (Appendix 1). Strikingly, clone 20 within EC-IV covering *bsr2061/blr2062* (*noeI*) was the most strongly induced clone on the whole genome of both SSE- and genistein-induced cells at 12 (173- and 200-fold) and 20 hpi (101-and 82-fold) at 15°C, which would suggest that *noeI* likely play an important role in the early stage of *B. japonicum*-soybean symbiosis at low temperature. On the contrary, loci covering common *nod* genes operon *nolZY-nolA-nodD2-nodD1YABCSUIJ-nolMNO-nodZ* showed generally lower expression levels at both 20 and 48 hpi, even as early at 12 hpi at 15°C. These results indicate that the *nod* genes are induced early and strongly at low temperature and part of them was even induced higher level than at 30°C.

Expression of TTSS Loci Is Potentially Delayed at Low temperature

The disappearance of EC-III at 20 hpi at 15 °C led to the hypothesis that the TTSS gene cluster it covered may not be induced at all or has a longer expression lag at 15 °C. Therefore, *B. japonicum* cells were induced with SSE and genistein at various higher temperatures (20, 25 and 30°C) and for a longer time (36, 48 and 72 h) at 15°C, and the expression of TTSS was investigated with three representative genes, *bll1843*, *blr1816* and *blr1649,* which respectively encode a transcriptional activator (TtsI), a core structural (RhcN) and an unknown secreted protein of TTSS (Krause *et al.*, 2002; Süß *et al.*, 2006), by using real-time RT-PCR.

As shown in Figure 3.7, in response to SSE, the expression of these three genes was increased when the temperatures were elevated from 15 to 30°C. Although the expression of these three genes was not detected at 20 hpi at 15°C, they showed a strong induction at 12 hpi at 20°C and were significantly increased from 6 to 12 hpi

at 30°C, suggesting that the elevation of temperature shortened the expression lag of TTSS gene cluster. Interestingly, their induction was increased with time at certain temperature, suggesting that the expression of TTSS genes is most likely cell growth phase-dependent. On the other hand, no such similar temperature-dependent expression manner was observed for genistein-treatment. Although *ttsI* was significantly induced at 20 hpi at 15°C and maintained a higher level at other temperatures, the expression levels of other two genes were not substantially changed.

To determine whether the TTSS gene cluster has a longer expression lag at 15°C, the induction periods was extended up to 36, 48 and 72 hpi. As shown in Figure 3.8, although *ttsI* started to be induced at 36 hpi, three genes were maximally induced by SSE at 48 hpi. On the other hand, *ttsI* was strongly induced by genistein from 36 h, and the level was higher than that of genistein-treatment, However, the expression of *rhcN* and *blr1649* was not substantially changed in genistein-treated cells untill 72 hpi. These data here supported the hypothesis that the induction of TTSS genes was temperature-dependent and-acclimated in the presence of SSE, but not genistein.

Subsequently, the cells were induced with SSE and genistein for up to 48 h at 15°C and the genome-wide expression profiles were constructed by using macroarray (Figure 3.3). Surprisingly, SSE expressed symbiosis island very similar to that at 12 hpi at 30°C, on which EC-III covering TTSS genes was found to be exclusively and strongly induced by SSE (Figure 3.2). Although the EC-III was a strongly induced region on the genome of SSE-treated cells at 48 hpi at 15°C, compared with that at 12 hpi at 30°C, the expression levels of the clones in this region were slightly weaker, especially those of clone 7, 9, 11 and 29. As for genistein treatment, a very lower expression level was observed, however, four

clones (clone 16, 18, 19 and 28) still showed a significant expression level (Figures 2.8 and 3.4) according to the cut-off of the chapter 2 (expression ratio≥ 2.2-fold and ≤ 0.4-fold for strongly induced and repressed clones, respectively). Among these clones, clone 19 covers gene *ttsI*, which has been reported to be essential for the expression of other genes in the TTSS gene cluster in the presence of genistein (Krause *et al., 2002*).

Besides, other SSE-exclusively-induced clones within ECs at 12 hpi at 30°C were also detected at 48 hpi at 15°C (Appendix 1), i.e., clone 7 covering genes *bsl1639*, clone 11 and 12 covering genes from *blr1649* to *bsl1654* within EC-I, clone 9 and 10 covering gene *blr1704* and *blr1705* within EC-II. Although the products of these genes are still unpredictable, three *tts* box motifs have recently been found at the upstream of three genes (*blr1649*, *bsl1652* and *blr1704*) (Zehner *et al.*, 2008), suggesting that these likely TTSS-related gene also showed an intensively delayed expression at low temperature.

These results suggest that low temperature leads to a more intensively delayed expression lag of TTSS gene cluster compared with those of nodulation loci and confirm the results of chapter 2 that an interaction with entire soybean-released compounds (i.e., SSE) rather than a single isofavonois (i.e., genistein) is critical for the full induction of the *B. japonicum* TTSS genes at low temperature. The expression of *Yersinia* spp type III secretion system was also reported to be positively controlled by an increase in growth temperature from 26 to 37°C (Cornelis *et al.*, 1987; Lambert de Rouvroit *et al.*, 1992), likely suggesting that it is widely existed among many bacteria species that the expression of TTSS is positively controlled by temperature.

Other Genomic Loci with Delayed Expression at Low Temperature

Besides SSE-preferentially-expressed EC-III and several clones within EC-I and II as described above, six of nine loci within symbiosis island but outside ECs (LISs: LIS1-5 and 7), which were found to be induced by SSE at 12 hpi at 30°C, including a locus (LIS7) for polygalacturonase (*blr1993*) (Figure 3.3 and Appendix 2) were also detected in the SSE-treated cells at 48 hpi at 15°C. Additionally, almost all SSE-preferentially-induced genome loci outside symbiosis island (LOSs: LOS1-6, 13-15) was also found to be strongly induced at 48 h at 15°C, including those for cytochrome O ubiquinol oxidase (LOS1), ACC deaminase (LOS-2), ATP synthase (LOS4 and LOS 5), synthesis of expolysaccharide (EPS, LOS 6), NADH ubiquinone oxidoreductase (*bll4904-bll4919* in LOS 13) and ribosome proteins family (LOS 15). However, interestingly, a large gene cluster for flagellar-related proteins (LOS 19), which was potentially induced by genistein at 12 hpi at 30°C, was found to be exclusively induced by SSE at 48 hpi at 15°C (Figure 3.2 and Appendix 3). These results strengthen the possibility that the expression of these genomic loci is regulated in concert with TTSS gene cluster.

In contrast, LOSs (LOS-7, 9-10,12, 16 and 20) including genes for multidrug resistant (LOS 10) (Figure 3.2 and Appendix 4), which were induced with sharing between SSE- and genistein-treated cells during 12h at 30°C, were also found to be induced at 12 or 20 hpi at 15 °C. These LOSs, as for *nod* genes, seemed to be mainly induced by the genistein present in SSE and were not so sensitive to low temperature.

Correlation Analysis of Genome-Scale Expression Profiling of B. japonicum between 15 and 30°C

Beside the significantly induced genomic loci as described above, correlation analyses of the relative expression of a total of 3960 clones covering 98.5 % of whole *B. japonicum* genome between 15 and 30 °C in the presence of either SSE or genistein were made (Table 3.1). It clearly demonstrated that compared with genistein-treatment, the correlation coefficient of genome-wide expression profiles of *B. japonicum* cells were intensively dispersed for the SSE-treatment. This suggests that the *B. japonicum* cells upon exposure to SSE are very sensitive to the low temperature. A high correlation coefficient of 0.66 was observed between SSE-treated cells at 48 hpi at 15°C and those at 12 hpi at 30°C, whereas a correlation coefficient of 0.46 found between genistein-treated cells at 20 hpi at 15°C and those at 6 hpi at 30°C, suggesting that it is reasonable to make a comparison of expression at 6 and 12 hpi at 30°C with those at 20 and 48 hpi at 15 °C, respectively.

Regardless of temperature factor, correlation analyses of genome-whole expression were made between SSE- or genistein-treated cells (Table 3.2). The intensively varied correlation coefficient was also observed for SSE-treated cells, suggesting that compared with genistein, addition of SSE stimulates distinguishably functional states of metabolism of *B. japonicum* cells in a view of system biology. A correlation coefficient of 0.35 and 0.38 at 6 hpi at 30°C and 20 hpi at 15°C between SSE and genistein treatment, respectively, was found.

3. 4 Discussion

Previous studies showed that when the RZT dropped from 25 to 17.5°C, infection initiation of soybean was delayed 1 day, while a further decreasing from 17.5 to 15°C delayed infection initiation for another 2 days (Zhang and Smith, 1994), indicating that the early stage of *B. japonicum*-soybean interaction is very sensitive to the RZT below 17.5°C. Thus, in this experiment, the global expression profiles of *B. japonicum* were monitored in response to SSE and genistein at 15°C. In this study, *nod* genes loci were induced strongly and early at 15°C, and part of them even showed a higher expression level than at 30°C, i.e., *blr1629/ bll1630*(*nolK*)/*bll1631*(*noeL*)/*blr1632* (*nodM*)/*blr1633*(*noeD*) and especially *bsr2061/blr2062* (*noeI*). Although it was addressed that the amount of Nod factor of *B. japonicum* USDA 110 was markedly decreased at low temperature (15 and 17°C) (Zhang *et al.*, 2002; Duzan *et al.*, 2006), there are no reports on its structural changes with the exposure to low temperature. *Rhizobium leguminosarum* was reported to produce larger relative amounts of *nodX*-mediated, acetylated LCOs at 12°C than at 28°C (Olsthoorn *et al.*, 2000), the product of *noeI* was suggested to be probably involved in the modification of Nod factor of *B. japonicum* with its 2-O-methylation of the fucosyl group (Göttfert *et al.*, 2001). Therefore, the results here strongly suggest that a modified structure of LCOs of *B. japonicum* mediated by these genes, especially *noeI*, is likely activated for triggering early plant responses such as root hair curling and induction of meristem formation at low temperature.

On the other hand, the expression of genomic loci such as those for TTSS, polygalacturonase and ACC deaminase at common growth temperature was

potentially delayed at low temperature (Figure 3.9). It suggests that these components are very sensitive to low temperature and may be a main reason for the delayed nodule development of soybean at low temperature. Genes for *B. japonicum* TTSS were reported to be maximally induced at 10 and 13 days post inoculation (dpi) (Pessi *et al.*, 2007) and the pretreatment of *B. japonicum* cells with SSE greatly enhanced the number of mature nodule at the same dpi (Figure 2.11), suggesting that the intermediate of nodule development (e.g. infection thread elongation, release into plant cells, and bacterial differentiation into mature bacteriods) is most likely sensitive to low temperature or other environmental stresses such as salinity, unfavorable soil pH, nutrient deficiency, mineral toxicity. Furthermore, the distinctly different expression lag between TTSS and *nod* gene cluster in response to low temperature suggest that the acquisition of these two genes cluster likely occurred through horizontal gene transfer in the different stages during the adaptive evolution of *B. japonicum*.

3. 5 Summary

(*Brady*)*rhizobium*-legume symbiotic interaction is an elaborated process controlled by the exchange of molecular signals between two partners and strongly affected by suboptimal environmental conditions, including low temperature. In the present chapter, an experiment was conducted to assess the effect of low temperature (15°C) on the global gene expression portraits of this strain with time in response to SSE and genistein, and a comparison was made with those at a common growth temperature (30°C). The results showed that a similar symbiosis island in a expression portrait of nodulation (*nod*) loci was detected at 12 and 20 hpi at 15°C between both inducers-treated cells, whereas the SSE-exclusively-induced loci for TTSS and polygalacturonase, were detected at 48 hpi at 15°C, as well with EPS synthesis, ACC deaminase, ribosome proteins family and energy metabolism-related proteins outside symbiosis island. In addition, genome-scale expression profiling showed a higher correlation coefficient of 0.66 between SSE-treated cells at 48 hours at 15°C and at 12 hours at 30°C, while 0.48 between genistein-treated cells at 20 hours at 15°C and at 6 hours at 30°C. Taken together, these results suggest that low temperature leads to a potentially delayed expression lag of SSE-preferentially-induced genomic loci, which may be tightly regulated with integration of a variety of environmental cues for the adaptive capabilities of *B. japonicum*.

3. 6 Figures and Tables

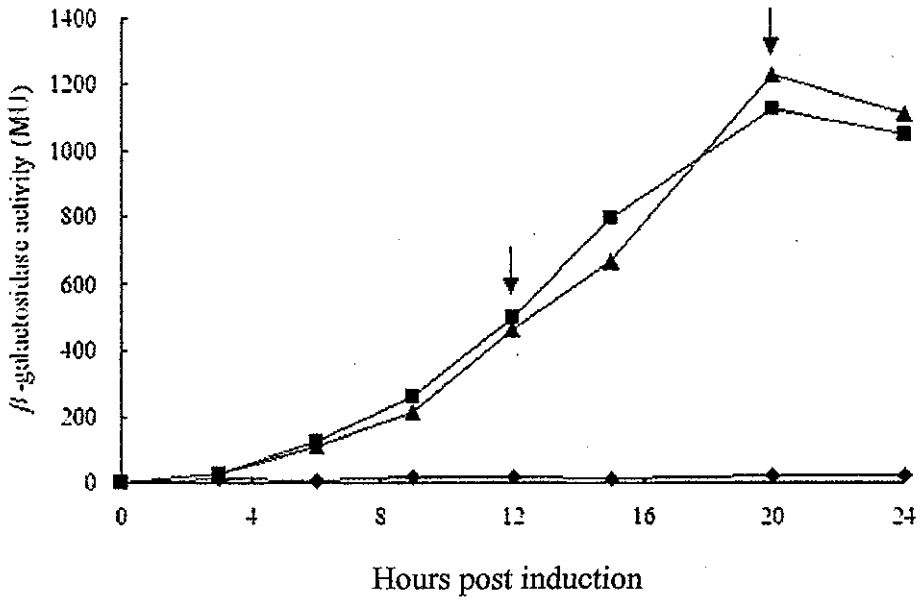

Figure 3.1 Influence of induction periods on the *nodY* expression at 15°C. *B. japonicum* ZB 977 cells grown to the log-phase were diluted to 0.1 of OD_{600} with fresh YMB culture medium containing no antibiotics and induced with SSE (■, 20 µl/ml culture), genistein (▲, 5 µM) or without inducers (◆) at 15°C for 24 h. A portion of the culture was withdrawn every 3 h during incubation and β-galactosidase activity was measured as described previously. Induction periods used for the macroarray analyses are shown by the arrows (12 and 20 hours post induction).

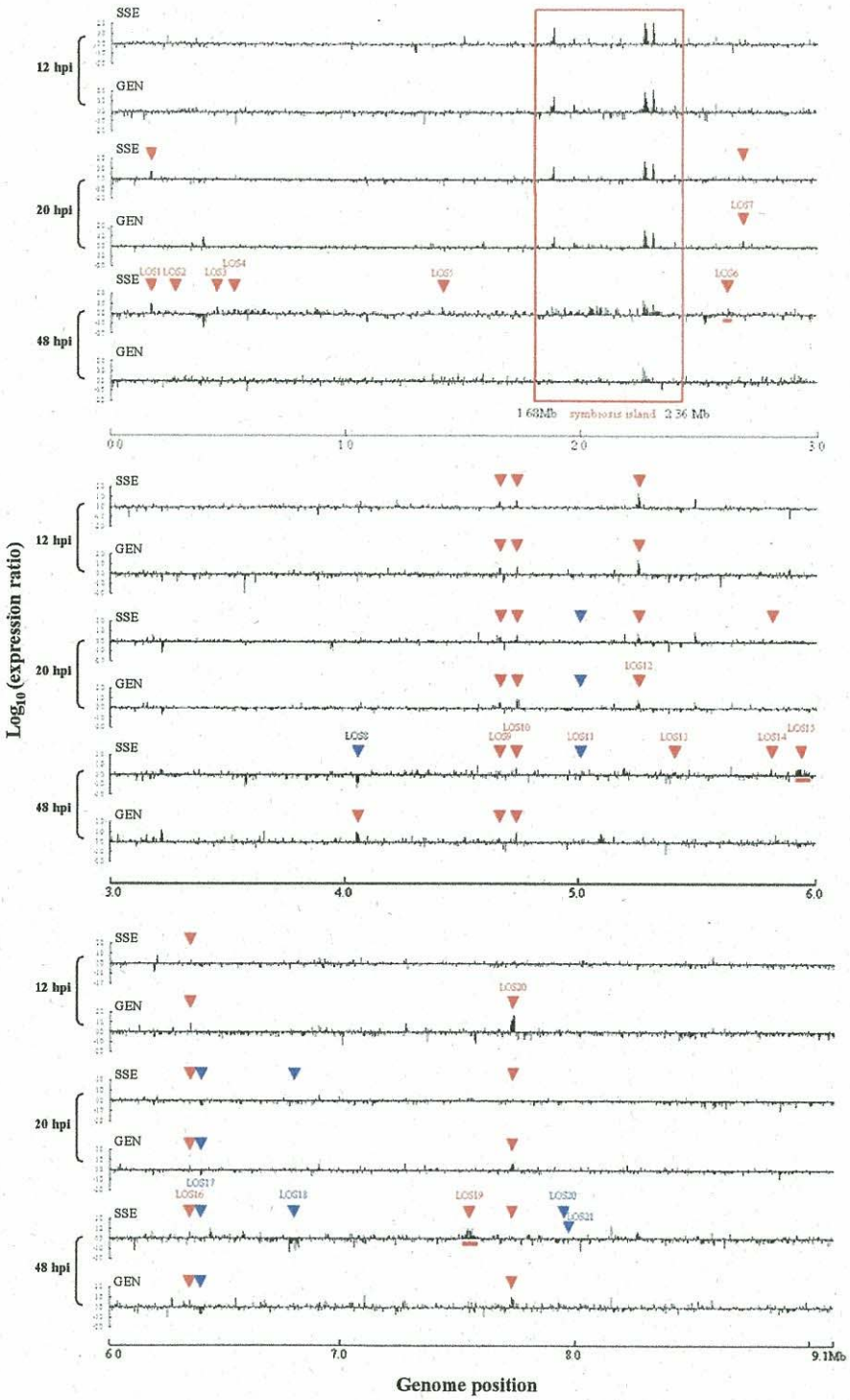

Figure 3.2 Genome-wide expression profiling of *B. japonicum* USDA 110 in response to SSE and genistein (GEN) at 12, 20 and 48 hpi at 15°C. Expression profiles were constructed as described in Materials and Methods. The symbiosis island is located on the chromosome at coordinates 1.68-2.36Mb. Red- and blue-colored arrowheads indicate positions of some strongly induced and reduced loci outside symbiosis island (LOSs: LOS1-22), which correspond to those in Figure 2.5 and cover genes as shown in Appendix 3 and 4, respectively.

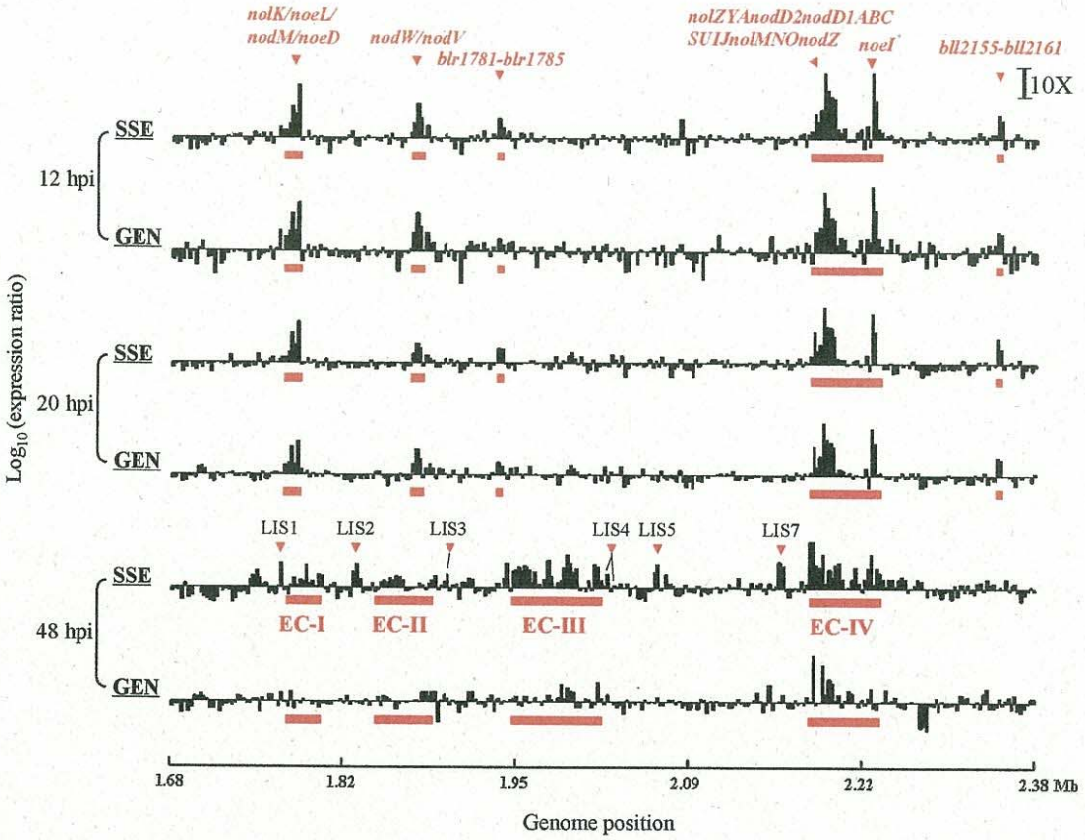

Figure 3.3 Expression profiles of the symbiosis island of *B. japonicum* USDA 110 induced by SSE and genistein (GEN) at 12, 20 and 48 hpi at 15°C. Expression profiles were constructed as described in Materials and Methods. Expression levels of each clone were sorted according to the genome coordinate and represented by a single bar. Four SSE-induced predominant expression clusters (EC-I to IV) at 12 hpi at 30°C are correspondingly underlined with red bars for SSE-and genistein-treatment at 48 hpi at 15°C. The loci inside or outside ECs.The SSE-induced loci inside symbiosis island but outside ECs (LISs) at 12 hpi at 30 °C are shown by the red arrows (LISs) and their covered genes can be seen in Appendix 2.

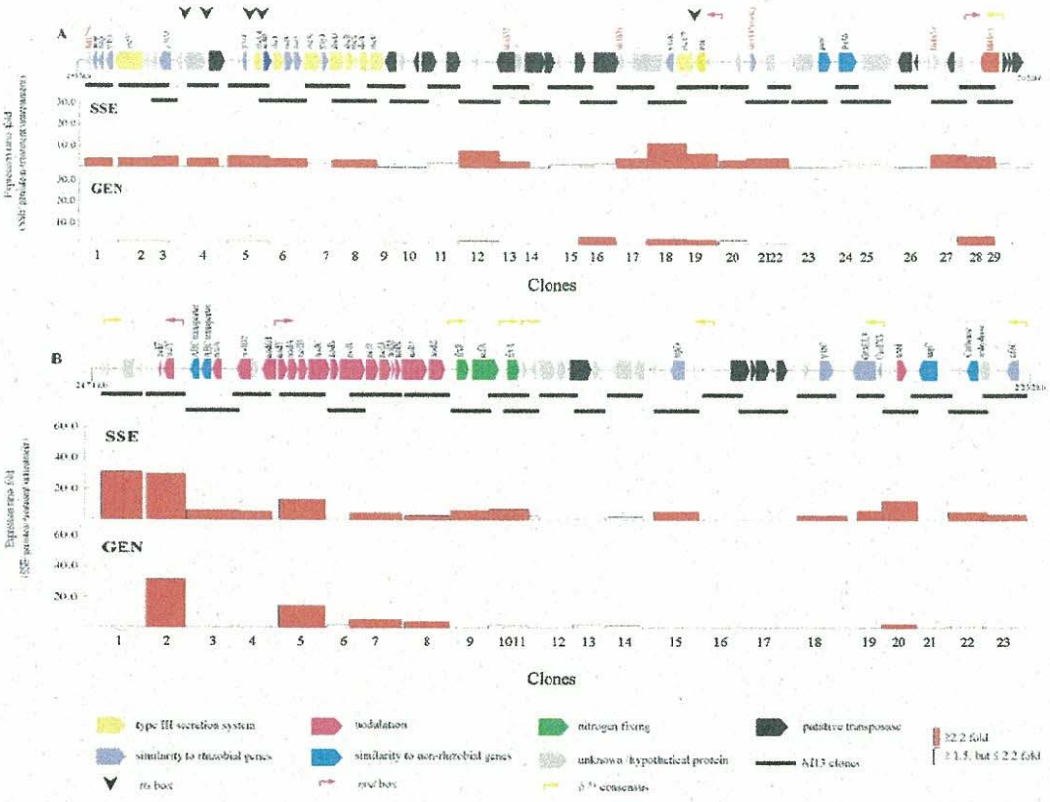

Figure 3.4 Expression profiles of clones and their covered genes in (**A**) EC-III and (**B**) EC-IV after the treatment of SSE and genistein (GEN) at 48 hpi at 15°C. Bars below the genes map indicate M13 clone inserts for macroarray construction and the box charts under M13 clone indicate differential expression of M13 clones in SSE / GEN-treated cells to untreated cells. The solid boxes indicate clones with strong (expression ratio≥2.2-fold, red) and mediate (1.5-fold ≤expression ratio<2.2-fold, pink) hybridization signal, while the dotted boxes indicate weak hybridization signal (expression ratio<1.5 fold). *tts* box (tcGTCAGctTNtcGaa AGct-N3-ccNcctA), *nod* box (ATCCA-N7-GATG-N6-ATCCAAACAATCGATTTTACCAAT C) and δ^{54} consensus (TGGCAC-N5-TTGCT/A) are described previously (Göttfert *et al.*, 2001; Süβ *et al.*, 2006).

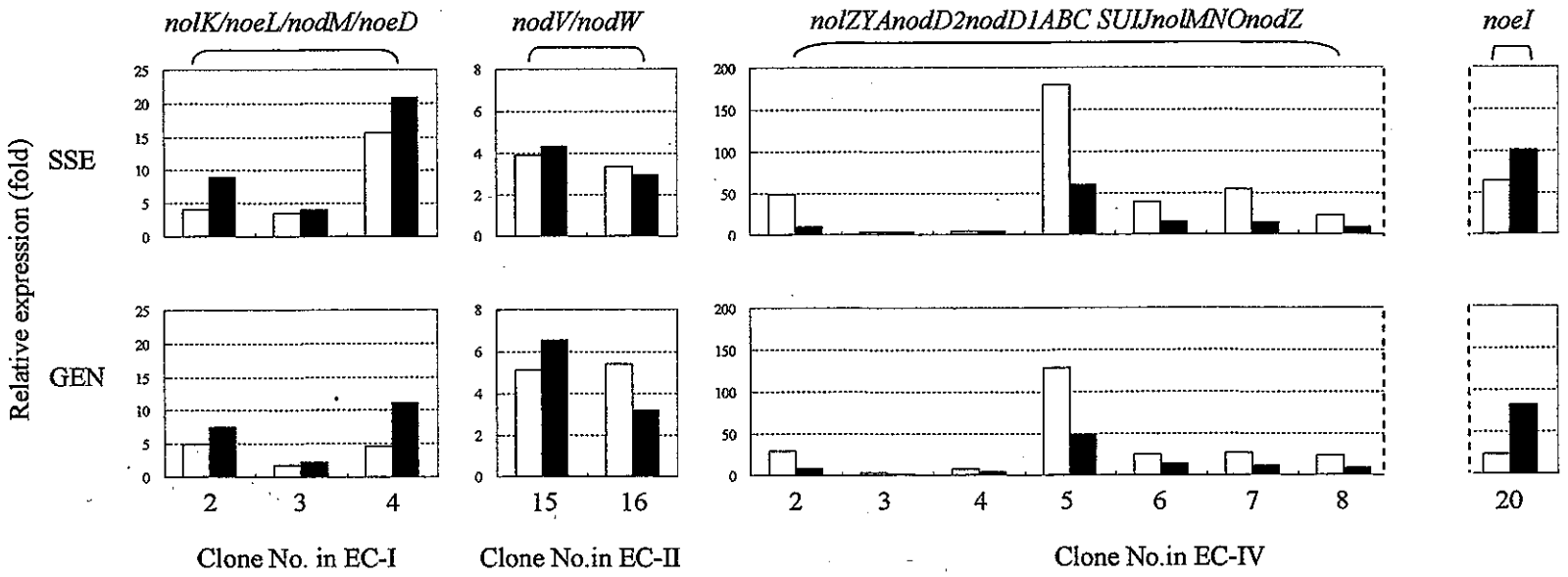

Figure 3.5 Comparative expression of nodulation loci in response to SSE and GEN at 6 hpi, 30°C(□) with those at 20 hpi, 15°C (■). Detailed information is shown in Appendix 1.

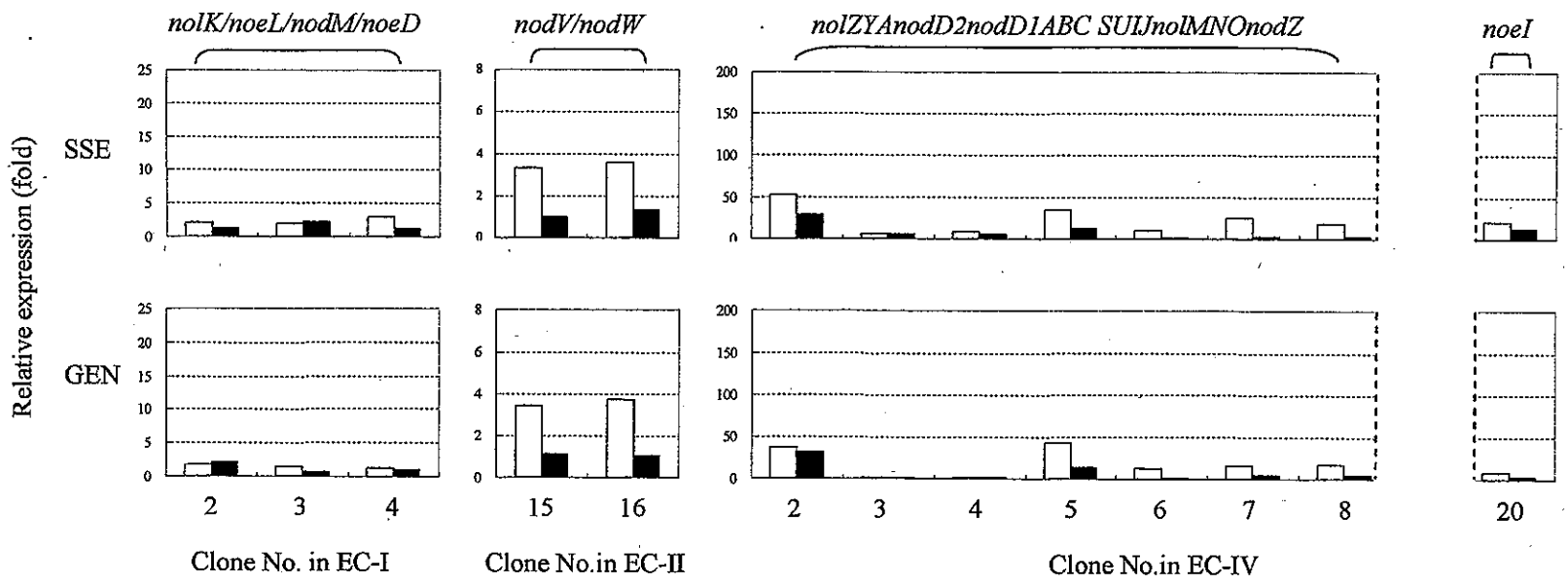

Figure 3.6 Comparative expression of nodulation loci in response to SSE and GEN at 12 hpi, 30°C (□) with those at 48 hpi, 15°C (■).Detailed information is shown in Appendix 1.

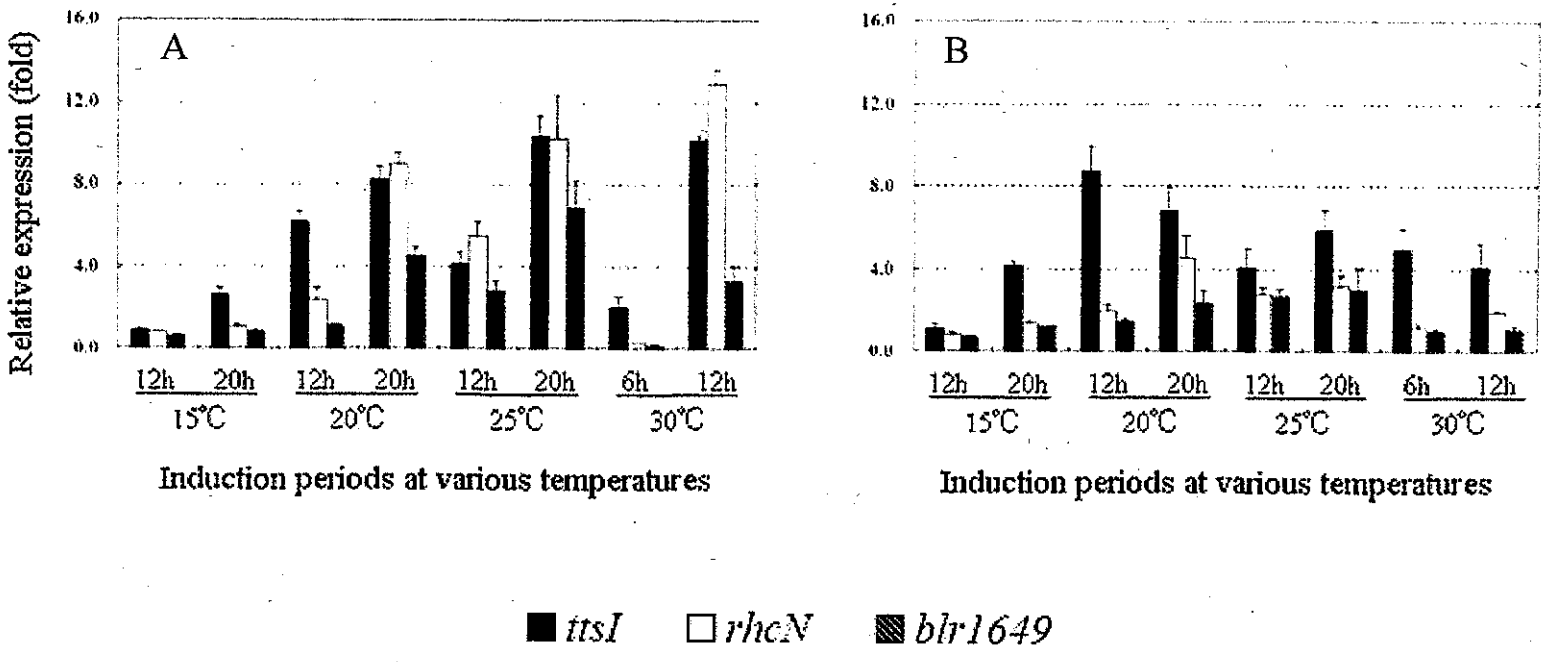

Figure 3.7 Effect of various temperatures on the expression of the *B. japonicum* TTSS genes in response to SSE (**A**) and genistein (**B**). The relative expression of three representative genes of TTSS genes, *ttsI*, *rhcN* and *blr1649* were quantified by using real-time PCR in normalization of the housekeeping gene, *sigA*.

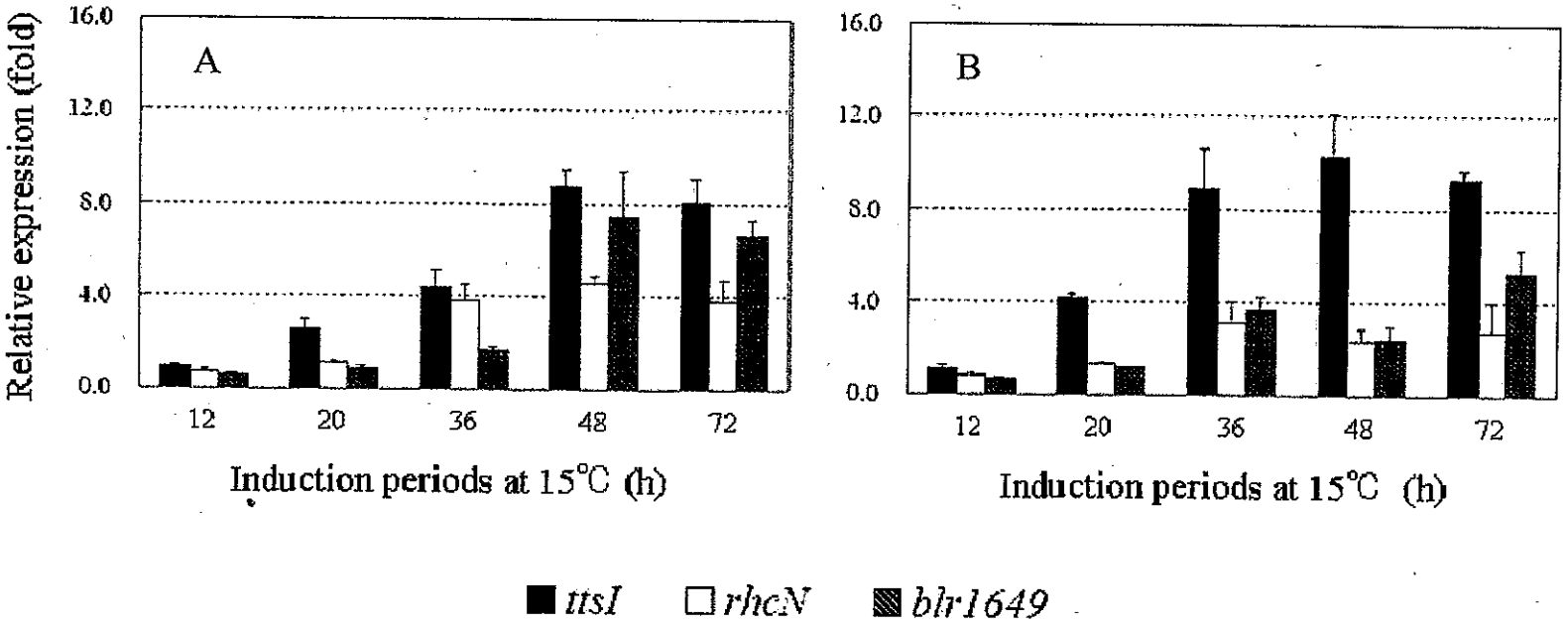

Figure 3.8 Effect of various induction periods at 15°C on the expression of the *B. japonicum* TTSS genes in response to SSE (**A**) and genistein (**B**). The relative expression of three representative genes of TTSS genes, *ttsI*, *rhcN* and *blr1649*, were quantified by using real-time RT-PCR in normalization of the housekeeping gene, *sigA*.

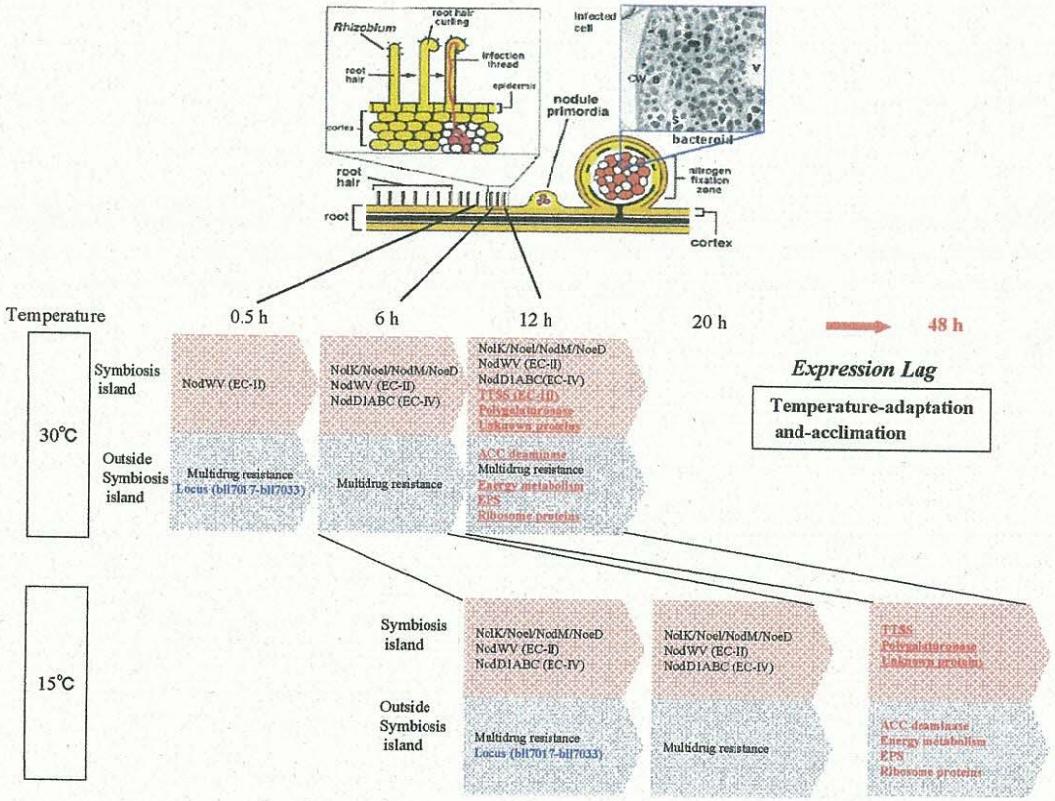

Figure 3.9 A dynamic comparisons of some potentially expressed genome loci of *B. japonicum* in response to SSE and genistein at 30°C with those at 15°C. Genome loci colored with black indicate a sharing of induction between SSE- and genistein-treatment. Genomic loci colored with red and blue indicate exclusive induction with either SSE- or genistein-treatment, respectively. The induction periods are illustrated with assumed corresponding stages of symbiotic development as described in picture above.

Table 3.1 Correlation analyses of genome-scale expression profiles of *B. japonicum* with either SSE- or GEN (genistein) -treatment between 30 and 15°C

			30°C					
			SSE			GEN		
			0.5 h	6 h	12 h	0.5 h	6 h	12 h
15°C	SSE	12 h	0.10	0.28	0.08			
		20 h	0.11	0.37	0.19			
		48 h	0.01	0.46	0.66			
	GEN	12 h				0.40	0.31	0.26
		20 h				0.31	0.46	0.24
		48 h				0.18	0.23	0.31

The number represents the correlation coefficient between the relative expressions of total 3,960 clones covering 98.5% of *B. japonicum* genome at 30 and 15°C for SSE or genistein treatment.

Table 3.2 The correlation analyses of genome-scale expression profiling of *B. japonicum* between SSE and GEN (genistein) treatment at either 30 or 15°C

			GEN					
			30°C			15°C		
			0.5 h	6 h	12 h	12 h	20 h	48 h
SSE	30°C	0.5 h	0.27	0.18	0.03			
		6 h	0.08	0.35	0.04			
		12 h	0.18	0.25	0.20			
	15°C	12 h				0.30	0.24	0.11
		20 h				0.29	0.38	0.20
		48 h				0.14	0.14	0.25

The number represents the correlation coefficient between the relative expressions of total 3,960 clones covering 98.5% *B. japonicum* genome under different conditions.

CHAPTER 4

Expression Analyses of *Bradyrhizobium japonicum* Type III Secretion System Genes

4.1 Introduction

Type III secretion system (TTSS) is a needle-like molecular machine highly conserved in Gram-negative bacteria to deliver bacterial effector proteins directly into host cells and then modulate host cellular functions (Cornelis, 2006). TTSS first attracted the attention of scientific community through its link between virulence in bacterial pathogens and animal and plant hosts (Hueck, 1998). Significant progresses have been made in understanding of its structure, assembly and mode of operation over the past few years. For example, the entire structure of *Yersinia enterocolitica* TTSS as well as ATPase, which is responsible for energizing the export process, was visualized at a resolution of approximately 1.6-1.7 nm (Cornelis, 2006). However, post-genomic explosion of knowledge has been revealing the wide distribution of bacterial TTSS and a rather broad role beyond universal virulence. For example, TTSS has been found in several opportunistic- and non-pathogenic organisms and to mediate pathogenic effects in a wide range of host (Pallen *et al.*, 2005). The expression of TTSS genes responds to environmental factors that usually correspond to the conditions encountered during the infection of a host, and is controlled by multicomponent regulatory networks which integrate a variety of environmental cues such as temperature, osmolarity, availability of

nutrients, divalent cations (in particular Ca^{2+}), pH, and growth phase (Hueck, 1998).

Since the first identification of genome region for TTSS in *Rhizobium* sp. NGR234 (Freiberg *et al.*, 1997), additional TTSS were found in a number of rhizobia, including *B. japonicum*, and depending on the host, symbiotic capacity of mutant strains is impaired, improved and unaltered (Marie *et al.*, 2001). In NGR234, the TTSS locus was reported to be expressed later than most *nod* genes, leading to a model that in the presence of plant-released flavonoids, NodD1, a positive transcriptional activator of *nod*-genes, activates Y4xI, which is a member of two components regulatory family and upstream of which a potential *nod*-box consensus sequence is present, and then the Y4xI in turn activates TTSS genes (Viprey *et al., 1998*).

In *B. japonicum*, besides NodD1 and TtsI (a homologue of Y4xI), another positive *nod*-genes regulator, NodW, is required for the expression of TTSS gene cluster and a model of regulatory cascade was proposed in the presence of genistein (Krause *et al., 2002*) (Figure 4.1). Recently, a conserved *tts* box upstream of TtsI-regulated genes has been found to be an essential promoter element of TTSS genes and the TTSS is active in early infection stages or in mature nitrogen-fixing nodules depending on the host plant (Zehner *et al., 2008*).

In the chapter 2 and 3, the genome-scale expression profiles of *B .japonicum* were monitored with time in the presence of SSE and genistein at common growth and low temperature, respectively. The results demonstrated that compared with genistein, TTSS genes were fully induced by SSE at common growth temperature, but showed a relatively weaker and a potentially delayed expression lag at low temperature, suggesting that besides flavonoids, other compounds within SSE are essential to coordinately induce TTSS genes and the expression of TTSS genes cluster is tightly

regulated in response to environmental clues. These captured genome-wide expression profiles with time allow a peer into the regulatory mechanism of *B. japonicum* TTSS genes. In the present chapter, it was found that two regulatory genes *nolA* and *nodD2* were exclusively and significantly induced by SSE and occurred simultaneously with the expression of TTSS genes. Furthermore, the expression analyses demonstrated that it is the mutation of *nodD2,* but not *nolA,* leading to the elimination of the expression of TTSS genes, suggesting that NodD2 is a critical component for the full induction of TTSS genes.

4. 2 Materials and Methods

Bacterial Strains and Culture Conditions

Bacterial strains and plasmids are listed in Table 2. 1. *B. japonicum* strains were maintained on the yeast extract-mannitol agar (YMA) medium supplemented with chloramphenicol (30 μg/ml), tetracycline (50 μg/ml), kanamycin (100 μg/ml), spectinomycin (100 μg/ml) and streptomycin (100 μg/ml). For pre-growth in the yeast extract-mannitol broth (YMB), appropriate antibiotics were used with following concentrations: chloramphenicol (30 μg/ml), tetracycline (25 μg/ml), kanamycin (50 μg/ml), spectinomycin (50 μg/ml) and streptomycin (50 μg/ml).

For RNA isolation, *B. japonicum* cells were pre-cultured in 20 ml of YMB with appropriate antibiotics for three days and scaled up to 200 ml of YMB without antibiotics for growing to log-phase (OD_{600}=0.3-0.5) at 30°C. The cell culture was then diluted with fresh YMB to OD_{600} of 0.1 and induced with SSE (20 μl/ml of culture) or genistein (5 μM).

β-Galactosidase Activity Assay

The induction assay of *nodY-lacZ* in the presence of SSE and genistein were described in the chapter 2 and 3. For the preparation of conditioned medium (CM), *B. japonicum* USDA 110 cells were cultured in 8500 ml of YMB and grown to an optical density at 600 nm (OD_{600}) of 1.6-1.8 at 30°C. The cells were then harvested by centrifugation (13,000 g). One half of the supernatant was 42.5-fold concentrated by rotary evaporation and used as CM for induction assays (Loh *et al., 2002*a). Another half was further 85-fold concentrated and extracted with ethyl acetate (1:1 v/v). After the ethyl acetate layer was dried by rotary evaporation, the residues were dissolved into 50% ethanol and used as CDF (cell density factor) described previously (Loh *et al., 2002b*). To avoid the effect of the stress of high saline caused by the concentrating process on the expression, 24 µl of CM and 12 µl of CDF per ml of induction medium were used.

RNA Isolation, cDNA Synthesis and Macroarray Analysis

DNA macroarray system of *B. japonicum* strain was described in the chapter 2. *B. japonicum* USDA 110 cells were pre-cultured in 30 ml of YMB without antibiotics for three days and then scaled up to 200 ml of YMB for growing to mid- log-phase (OD_{600}=0.3-0.5) at 30°C. The cells were then diluted with fresh YMB to OD_{600} of 0.1 and induced with SSE (20 µl/ml of culture) or genistein (5 µM) for periods determined by *B. japonicum* ZB977 at 15°C. RNA isolation, hybridization, image capture and data analyses were performed as described in the chapter 2. Briefly, the equal volume of ice-cold 5% (w/v) phenol/ethanol was added into the culture to

minimize the degradation of RNA. Total RNA was isolated with ISOGEN-LS (Nippon Gene, Tokyo, Japan) and then treated with DNase I. After removal of rRNA with the MICROB*Express*™ Kit (Ambion, Austin, TX, USA), the cDNAs were made by reverse transcriptase with the incorporation of [α-^{33}P] dCTP and hybridized with DNA array sheets at 55°C overnight. Hybridized signals were captured by using a BioImaging analyzer (BAS 5000, Fuji Film, Tokyo, Japan). The signal intensity of each spot was quantified by using ArrayVision software (GE Healthcare Bio-Sci, NJ, USA) and normalized on the basis of total sum of the signal intensities of spots on the array membrane and the data analysis was done as described in the chapter 2.

Quantitative Real-Time RT-PCR

Total RNA isolation was performed as described in the chapter 2 and the primers were designed by Primer 3 (http://fokker.wi.mit.edu/primer3/input.htm) (Rozen and Skaletsky, 2000). One to three hundred ng of total RNA were used as template and real-time RT-PCR reactions were performed with MiniOpticon™ Version 3.1 (Bio-Rad, Hercules, CA,USA) in combination with the QuantiTect SYBR Green RT-PCR (Qiagen GmbH, Hilden, Germany). Quantification was performed using the Pfaffl method according to the real-time RT-PCR application guide provided by Bio-Rad. A housekeeping gene of *B. japonicum, sigA,* which showed no significant change in expression level under the growth conditions, was used as an internal reference for quantitative real-time RT-PCR in the experiment.

4. 3 Results

Both nodD2 and nolA Are Strongly Co-induced with TTSS Genes by SSE, but not Genistein

As described in the chapter 3, the expression of clones covering *nod* genes showed a tendency to decrease with time from 6 to 12 hpi at 30°C and 12 to 48 hpi at 15 °C for both SSE and genistein treatments (Appendix 1). It is reasonable because the expression of *nodW* and *nodD1*, which was reported to encode two positive transcriptional activator of *nod* genes (Loh and Stacey, 2003), declined with time (Table 4.1). However, the induction of clone 3 and 4 within EC-IV occurred from 6 to 12 hpi at 30°C and 20 to 48 hpi at 15°C in the presence of SSE, and clone 4 showed a higher expression level than clone 3. On the contrary, the induction of these two clones decreased with time for the genistein-treatment (Figure 4.2). Although seven genes from *bll2017* to *bll2023* were covered by these two overlapped clones, only three genes, *bll2023*, *bll2021* and *bll2019*, were reported to encode regulatory proteins as NodD1, NodD2 and NolA, respectively. In *B. japonicum*, NodD1 responds to genistein and functions as a positive transcriptional activator of common *nodABCSUIJ* operon, whereas NodD2 and NolA form two key components in the feedback regulation as well as quorum regulation of the nodulation genes (Loh and Stacey, 2003). The unusual expression patterns of these two adjacent clones imply that the *nodD1nodD2nolA* gene region may involve in the regulation of some SSE-exclusively-expressed genome loci such as TTSS genes both at 12 hpi at 30°C and 48 hpi at 15°C. Thus, although the roles of other genes covered by these two clones might not be underestimated, here, a focus was made on the regulatory role of *nodD1nodD2nolA* gene region for the

induction of TTSS genes.

As shown in Figure 4.2, the induction of gene *nodD1*, which was individually PCR-amplified and regularly spotted as control clones on the array membrane, decreased with time for both SSE and genistein treatments and a significantly differential expression between *nodD1* and clone 4 was observed only for SSE-treatment at the condition of 30°C, 12 h and 15°C, 48 h. These results suggested that both or either of *nodD2* and *nolA* are strongly and exclusively induced by SSE because *nolA* was covered by only clone 3 but *nodD2* by both.

Consequently, the expression of *nodD2* and *nolA* was quantified by using real-time RT-PCR. As shown in Table 4.1, both of *nodD2* and *nolA* were strongly and exclusively induced by SSE at both 12 hpi at 30°C and 48 hpi at 15°C. A significantly higher induction (26.1-fold) was observed for *nodD2* at 12 hpi at 30°C, whereas the induction of *nolA* seemed not to be substantially influenced by low temperature. Interestingly, significant induction of *ttsI* was also observed for SSE-treatment at 12 hpi at 30°C (10.1-fold) and 48 hpi at 15°C (8.0-fold), suggesting that the induction of *ttsI* may accompany with those of *nodD2* and *nolA*.

On the other hand, the induction of *ttsI* by genistein seemed not to be substantially changed, being with a range of 4.1- to 5.3-fold with time at either 30 or 15°C. Besides, the genes *nodW* and *nodD1* were induced at a higher level by genistein at both 6 hpi at 30°C and 20 hpi at 15°C, compared at 12 hpi at 30°C and 48 hpi at 15°C, respectively. Taking into consideration that the TTSS genes were observed to be partially and weakly induced by genistein under the conditions of 30°C, 12h and 15°C, 48h, especially the former, suggesting that some undetermined growth phase-dependent components were required at the downstream of NodW and NodD1

in the regulatory pathway proposed by Krause *et al* (*2002*) for the induction of TTSS genes.

Induction of TTSS Genes Positively Responds to Culture Population Density

It has been established that the significant repression of *nod* genes of *B. japonicum* at high cell population density occurs via NolA-NodD2-mediated quorum control (Loh and Stacey, 2003) (Figure 4.3). Compared with genistein, SSE significantly stimulated the growth of *B. japonicum* cells at both 30 and 15°C, especially the former (Figure 4.4). In addition, the expression of three representative TTSS genes increased with time at certain temperature in the presence of SSE (Figure 3.7A), suggesting that TTSS genes are likely cell growth phase-dependent. Therefore, it is possible that the induction of TTSS genes is positively regulated via NolA-NodD2-mediated quorum.

Although supernatant from arabinose-gluconate (AG) and defined minimal medium were addressed to suppress *nod* genes expression in a density-dependent manner (Jitacksorn and Sadowsky, 2008; Loh *et al.*, 2001), there has been no report on YMB medium used throughout this study. Therefore, the effect of cell-density dependent factor (conditioned medium CM, see Materials and Methods) from YMB culture on the induction of *nodY-lacZ* in *B. japonicum* by SSE and genistein was firstly investigated. As shown in Figure 4.5, when conditioned medium (24 µl/ml) from culture (OD_{600}=1.6-1.8) was added to *B. japonicum* culture with various population densities (OD_{600}=0.1, 0.05 and 0.025), the level of *nodY* expression was reduced in the presence of SSE, but increased upon exposure of genistein. Besides, regardless of addition of CM, the induction of *nodY* was significantly increased when cell density decreased from

OD_{600} =0.1 to 0.025 for both treatments. Besides, addition of CDF also resulted in the repression of *nodY* induction (data not shown). These results suggest that supernatants from YMB culture grown to a high population density also contain a quorum-sensing factor that subsequently represses the expression of *nod* genes.

Subsequently, the effect of CM and CDF on the expression of TTSS genes in the presence of genistein, which was suggested to play a role in triggering the induction of TTSS genes thorough NodD1 and NodW as reported by Krause *et al* (2002) and shown in Figure 2.6, was investigated. As shown in Figure 4.6, addition of CM and CDF to the *B. japonicum* culture led to enhanced induction of two TTSS-related genes, *ttsI* and *rhcN*, but the induction of gene *blr1649* was not substantially changed. The result suggests that the genes for the regulatory activator and core structures of TTSS, not the secreted proteins, positively respond to culture population density (or quorum-activated). However, whether this activation is mediated by bradyoxetin, a recently reported unique quorum-signal accumulated in culture medium of *B. japonicum* cells at high population density (Loh *et al.*, 2002), more experimental evidences are needed.

Mutagenesis of nodD2, not nolA, Leads to the Elimination of TTSS *Expression*

Since both *nodD2* and *nolA* were exclusively induced by SSE at 12 hpi 30°C and 48 hpi at 15°C, meanwhile the full expression of TTSS genes cluster was detected. Thus, the expression of TTSS gene cluster in *nodD2* (strain Δ370) and *nolA* (strain BjB3) in response to SSE under these two conditions were examined by using real-time RT-PCR and macroarry. Firstly, using real-time RT-PCR, it was found that the induction of *ttsI* and *rhcN* were completely undetectable in strain Δ370, while a

higher expression level was observed in strain BjB3, compared with those in wild type (Figure 4.7). In addition, the expression of these three TTSS-related genes were also examined by addition of CM and CDF to the culture of the strain Δ370 in the presence of genistein at 12 hpi at 30°C. Unexpectedly, the induction of genes *ttsI* and *rhcN* were completely undetectable (Figure 4.6).

Subsequently, the array analyses was conducted to look at the expression profiling of EC-III which covered whole TTSS genes in response to SSE at 12 hpi at 30°C and 48 hpi at 15 °C. The results demonstrated that being consistent with the results of real-time RT-PCR above, the expression of EC-III was found in strain BjB3, but not in Δ370 (Figure 4.8). The result that mutagenesis of *nodD2*, not *nolA*, led to the elimination of the expression of TTSS gene cluster suggests that NodD2 is a critical component involved in the full induction of *B. japonicum* TTSS genes.

4. 4 Discussion

In the present study, both *nolA* and *nodD2* genes were found to be dramatically induced with time in the presence of SSE, but not genistein, and accompanied with the induction of TTSS gene cluster, leading to a hypothesis that the expression of TTSS genes is positively regulated by the both or either of NolA and NodD2. Although there have no report on the direct interaction between NolANodD2 and TTSS in the *B. japonicum*, the previous reports that i) TTSS genes as well as *nolA* and *nodD2* were maximally induced at the similar stage of symbiosis (e.g. bacteriods development) (Pessi *et al*, 2007), ii) *nolA* mutant showed a similar phenotype (e.g. a delayed nodule development) to TTSS mutants (Garcia *et al.*, 1996; Krause *et al.*, 2002) and iii) the similar involvement in the host plant genotype-specific dependence for both *nolA* and

TTSS (Sadowsky *et al.*, 1991; Krause *et al.*, 2002), strengthened the hypothesis that *nolA* and *nodD2* are involved the induction of TTSS genes. However, the following experimental evidences revealed that the NodD2, not NolA, is a novel compound required for the full induction of *B. japonicum* TTSS genes and that the induction of TTSS genes positively responds to culture population density.

Important pathogenicity determinants like EPS, degradative exoenzymes and TTSS were controlled in a cell-density-dependent manner in several plant pathogen (Soto *et al.*, 2006). The expression of TTSS genes was also reported to be positively controlled by quorum sensing in enterohemorrhagic and enteropathogenic *E. coli* (Sperandio *et al.*, 1999). Since the induction of *nod* genes in *B. japonicum* was negatively quorum-sensing controlled and the induction of TTSS genes occurred later those of *nod* genes, it is reasonable that quorum sensing positively control the expression of TTSS.

In the present chapter, the addition of CM and CDF enhanced the induction of TTSS genes in wild type *B. japonicum* in the presence of genistein, but unexpectedly, the induction of TTSS genes was completely undetected in *nodD2* mutant. Krause *et al.* (2002) proposed that in *B. japonicum*, the induction of TTSS genes cluster was dependent on the transcriptional activator protein NodW as well as the activation of *nodD1nodD2nolA* gene region, but in the same report, a part of TTSS genes having an upstream of *tts* box were found to be controlled by NodW and NodD1, not NolA NodD2 by using genistein as a inducer. As for genistein, the finding by Krause *et al.* (2002) was supported by my result that TTSS genes cluster was partially and weakly induced by genistein at 12 hpi at 30°C (Figure 2.6). It was considerably surprising, because according to this model, TTSS genes should be induced by genistein through NodD1 and NodW, but the level not influenced by the addition of CM and CDF in

nodD2 mutant. Thus, whether the genes *ttsI*, *rhcN* and *blr1649* were damaged in strain Δ370 was determined with the chromosome DNA as template by PCR amplification. The result showed that all three genes were detected in strain Δ370 (data not shown), raising the possibility that even a weaker induction of *nodD2* is essential for the activation of TTSS in genistein-treated wild type cells. Actually, *nodD2* showed an induction of 2.4-fold in the genistein-treated cells at 12 hpi at 30°C and 48 hpi at 15°C in this study (Table 4.1) and this was also addressed in the previous report that *nodD2* was expressed at a very low level in *B. japonicum* USDA 110 after incubation at 30°C for about 2 weeks (Göttfert *et al.*, 1992).

Although TTSS gene cluster was strongly induced in the SSE-treated cells, which showed a significantly rapid growth compared with genistein-treated cells, and the induction of TTSS positively responded in culture population density, it is still unknown whether the TTSS gene cluster is activated through the same pathway between these two physiological conditions due to the lack of experimental evidence showing that bradyoxetin, which is still not commercially available at the moment, accumulated in the SSE-treated cells. Nevertheless, the finding that the expression of a gene *blr1063* encoding a putative autoinducer synthase was strongly repressed by SSE at 12 hpi at 30°C and 48 hpi at 15°C (data not shown) and the result that it was the mutagenesis of *nodD2*, not *nolA*, leading to the elimination of TTSS expression, raise the possibility that the induction of TTSS in the SSE-treated cell is regulated in a different pathway with its induction in cells with high density population as proposed by Loh and Stacey (2003)(Figure 4.3).

Although a clear explanation can not be addressed why the expression of TTSS genes is not substantially influenced in SSE-treated strain BjB3, it was reported that soybean

inoculated with BjB3 showed a slight day in nodulation and the *B. japonicum nolA* encodes three functionally distinct proteins (Garcia *et al.*, 1996; Loh *et al.*, 1999), which suggest that the NolA play a more complex regulatory role in the *B. japonicum*-soybean interaction. On the other hand, not only TTSS but other genomic loci including those encoding polygalacturonase (*blr1993*) and pectinesterase (*blr1994*) which are preferentially induced by SSE in wild type was not detected in stain Δ370, suggesting that NodD2 may play a widely regulatory role in the nodulation efficiency, not just the repress of *nod* genes. Undoubtedly, a further genetic analysis in the gene region *nolAnodD2nodD1*, and probably other genes with unknown products as well, which were covered by clone 3 and 4 in the EC-IV, will provide a new insight into the *B. japonicum*-soybean interaction.

4. 5 Summary

In the present chapter, I provided experimental evidences that the induction of TTSS by SSE was accompanied with dramatic expression of both genes *nolA* and *nodD2*. Then, the role these two genes played in the induction of TTSS was initially investigated. The following experiment showed that the induction of TTSS genes was enhanced by the addition of either conditioned medium or cell density factor, suggesting expression of TTSS genes may be positively quorum-controlled. Finally, mutagenesis of *nodD2*, not *nolA*, led to the elimination of TTSS expression, indicating that NodD2 is a novel compound required for the full induction of TTSS.

4. 6 Figures and Tables

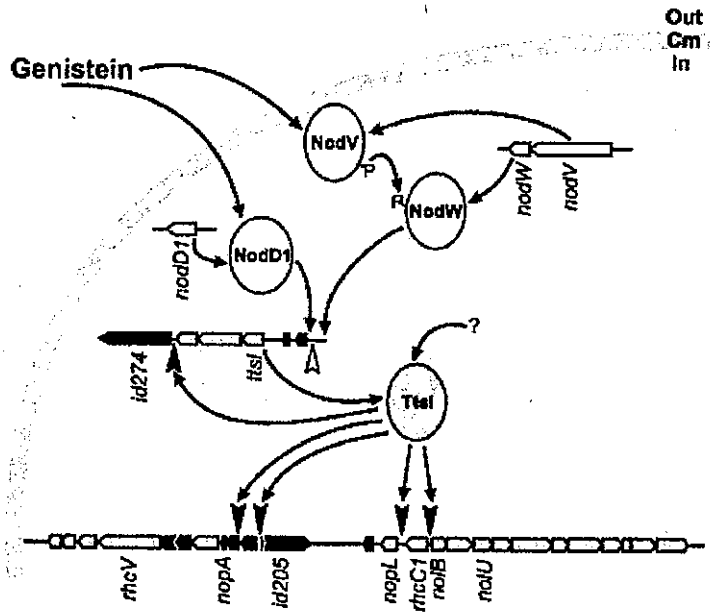

Figure 4.1 Model of the regulatory cascade controlling expression of the TTSS gene cluster. Open reading frames (ORFs) unique to *B. japonicum* are in black, and *nod* genes are shown in white. ORFs with homologues in tts gene clusters of other rhizobia are gray. ORFs related to IS elements are not shown. Gray arrowhead indicates the position of the nod box. Positions of tts boxes are marked by black arrowheads. Cm = cytoplasmic membrane (Krause *et al.*, 2002).

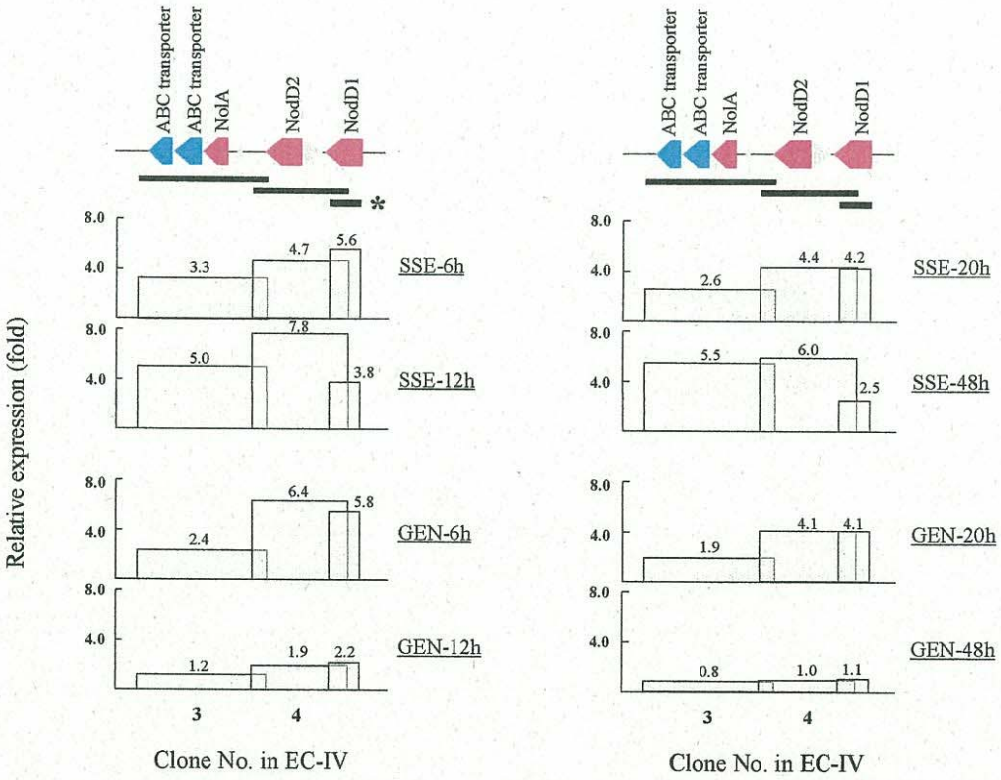

Figure 4.2 Differential expression of two adjacent clones, clone 3 (brb17558, at the coordinates 2,177,445-2,180,846) and 4 (brb00631, at the coordinates 2,180,444-2,182,985) which cover a *nodD1nodD2nolA* gene region, in the presence of SSE or GEN (genistein) with time at 30°C (A) and 15°C (B). Bars below the gene map indicate M13 clone inserts for macroarray construction and the box charts below them indicate differential expression of M13 clones in SSE/GEN-treated cells to untreated cells. Clone with a star indicate PCR-amplified *nodD1* fragment with the chromosome as template, which is spotted regularly on the array membrane. Genes encoding unknown or hypothetical proteins are colored gray

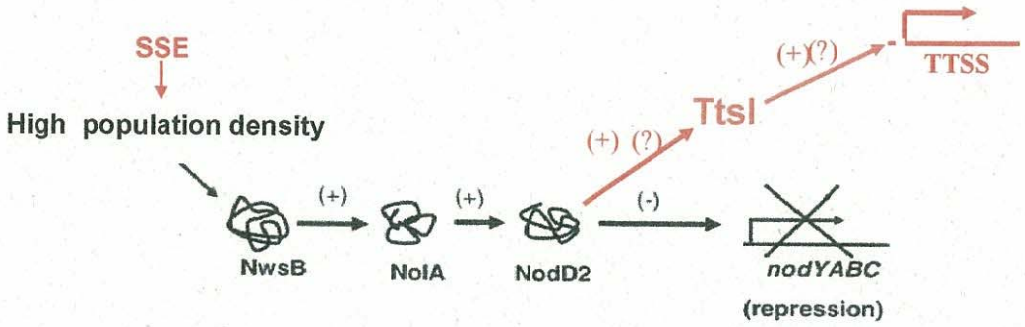

Figure 4.3 A proposed hypothesis that the induction of TTSS genes is positively regulated via NolA-NodD2-mediated quorum is illustrated as red pathway. The black pathway indicate a proposed pathway involved in the population density-dependent regulation of the *nod* genes by Loh and Stacey (2003). In response to bradyoxetin, a quorum signal accumulated with increasing population density, NwsB regulates the expression of NolA and NodD2. The expression of NodD2 leads to the repression of the *nodYABC* operon in *B. japonicum*.

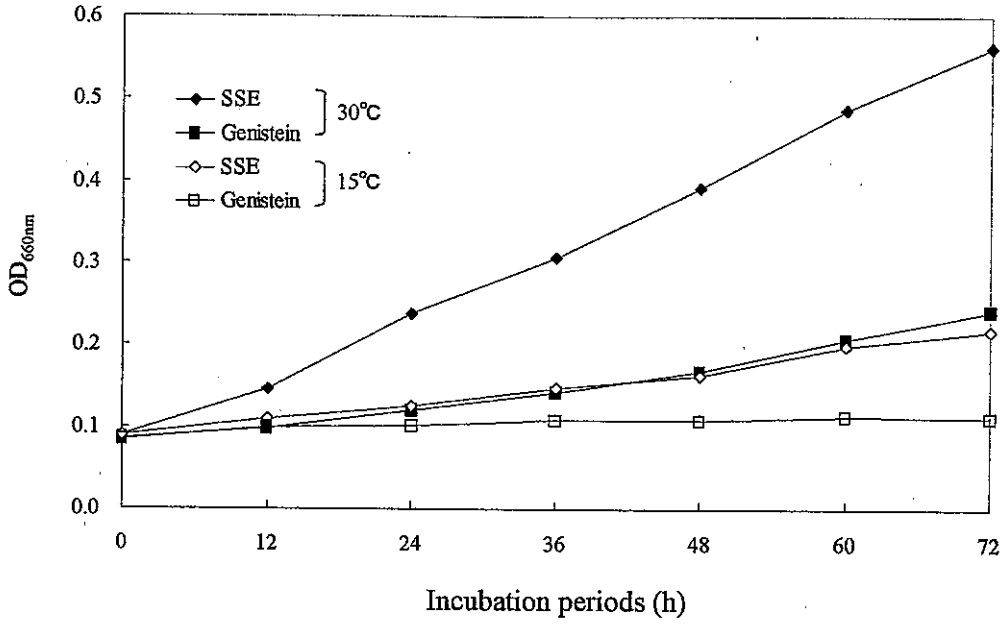

Figure 4.4 Growth of *B. japonicum* cells in the presence of SSE (20 µl/ml culture) and genistein (5 µM) at 30 and 15°C. The *B. japonicum* cells were pre-cultured at 30°C, then after the dilution of OD_{660nm} to 0.1, SSE or genistein was added to the culture and the cells were grown at 30 and 15°C for 72 hours.

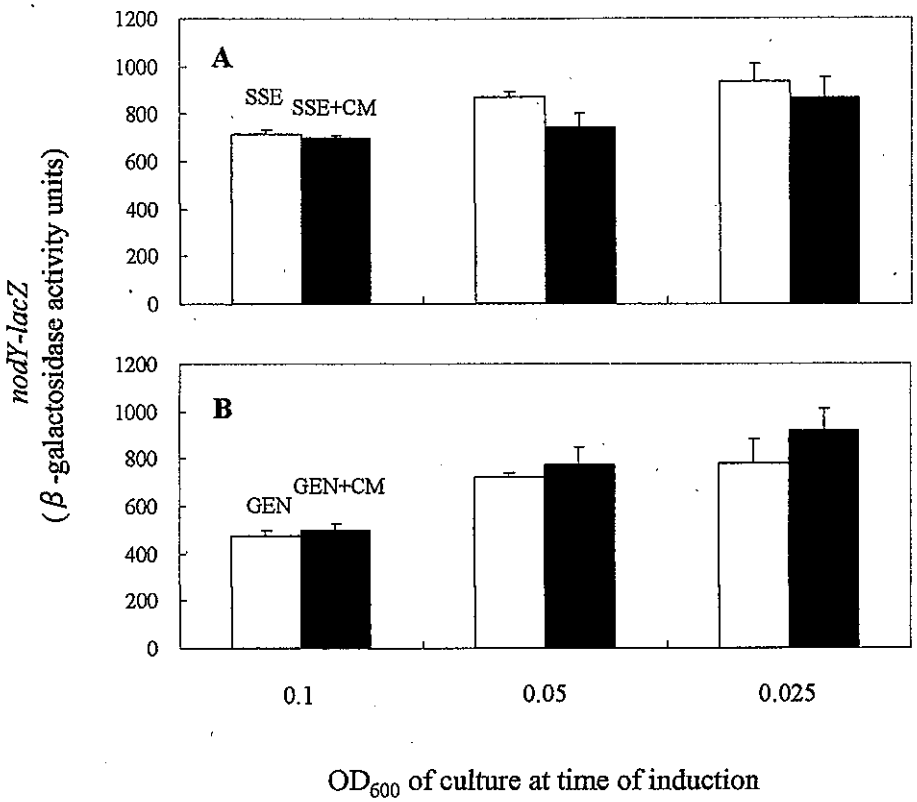

Figure 4.5 The effect of conditioned medium (CM) on the induction of *B. japonicum nodY* by the SSE (A) and genistein (B) at various initial cell densities. Conditioned medium was obtained from *B. japonicum* cultures grown to a high optical density (see Materials and Methods). The pre-grown *B. japonicum* culture was diluted to OD_{600}=0.1, 0.05 and 0.025 with fresh YMB, and then induced with SSE (20 µl/ml culture) or genistein (5 µM) at 30°C for 12 h.

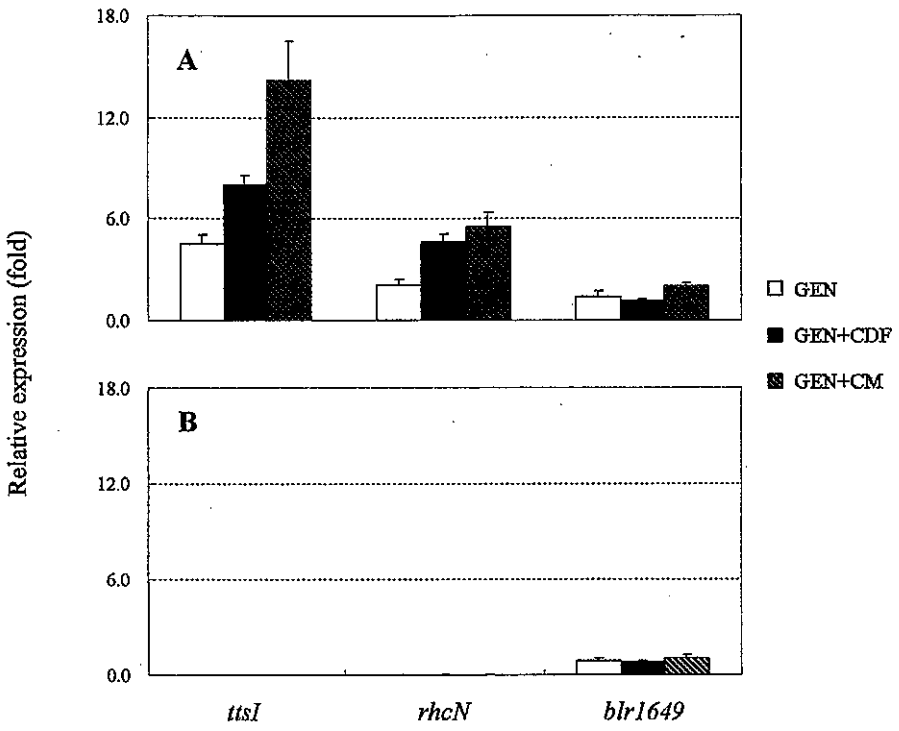

Figure 4.6 Effect of cell density-dependent factor on the induction of three TTSS genes, *ttsI*, *rhcN* and *blr1649*, in wild type (A) and *nodD2*-deleted strains Δ370 (B) by genistein (5 μM) at 30°C for 12 h. CM, conditioned medium from *B. japonicum* culture (OD_{600}=1.6~1.8). CDF, ethyl acetate-extracted CM described in Materials and Methods).

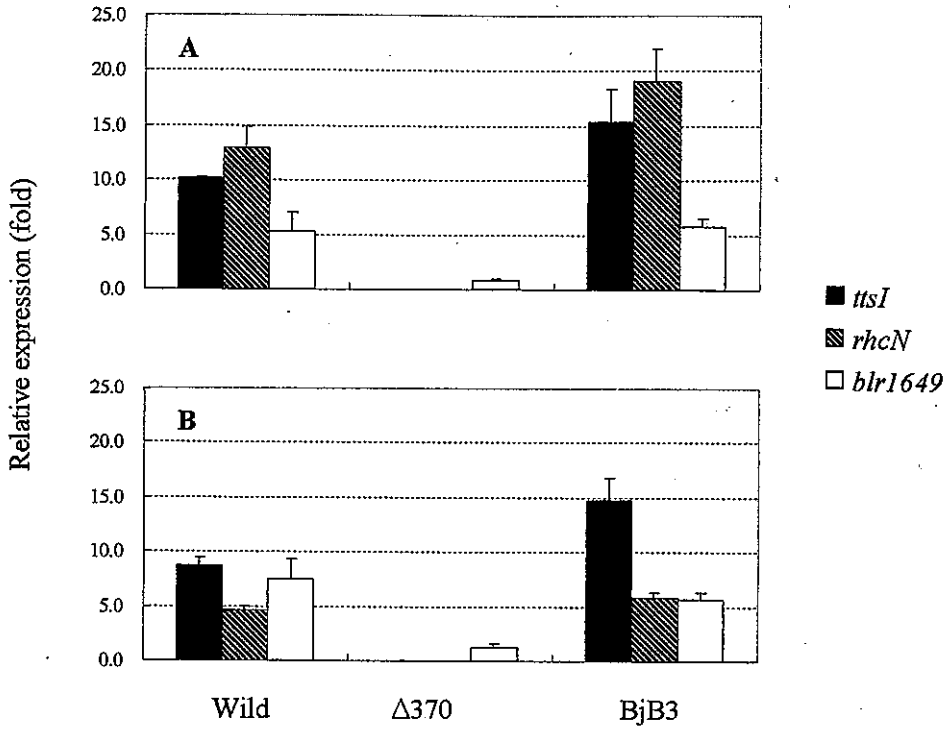

Figure 4.7 The expression analyses of three representative TTSS genes, *ttsI*, *rhcN* and *blr1649*, in the strains Δ370 and BjB3 upon exposure of SSE at 12 hpi at 30°C (A) and 48 hpi at 15°C (B), respectively. Expression levels were determined by real-time RT-PCR and normalized on the housekeeping gene of *B. japonicum*, *sigA*. *B .japonicum* USDA 110 was used as wild strain.

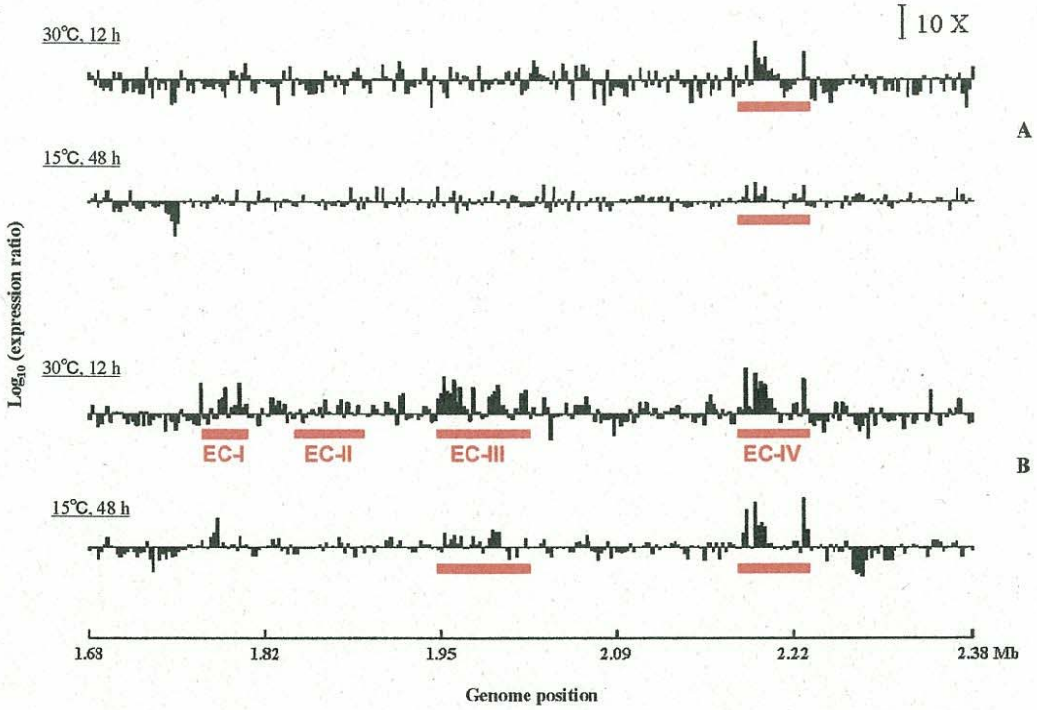

Figure 4.8 Expression profiles of symbiosis island in strains Δ370 (**A**) and BjB3 (**B**) in the presence of SSE at 12 hpi at 30°C and 48 hpi at 15°C, respectively. ECs (EC-I~IV) are underlined with red bars.

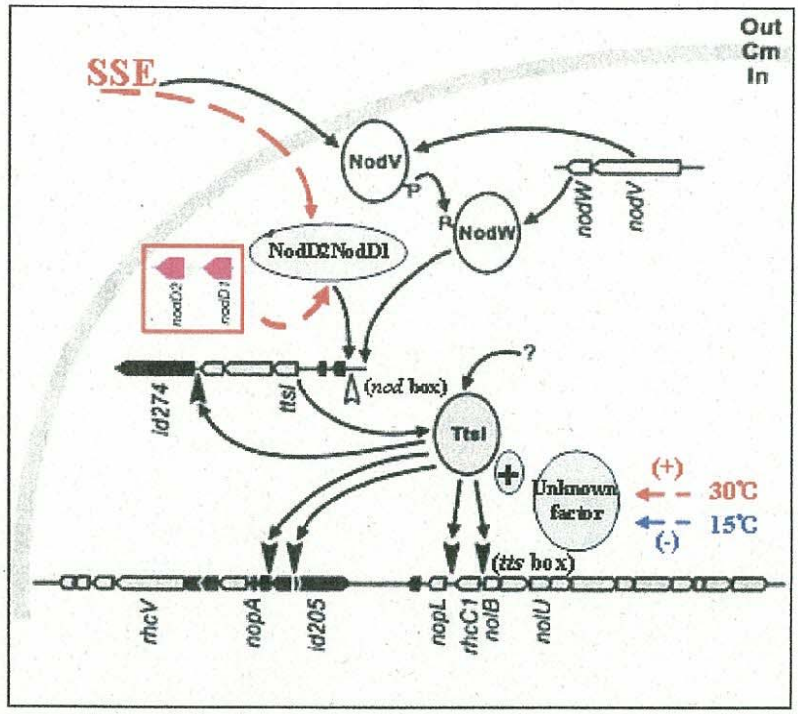

Figure 4.9 A modified regulatory circuit for the expression of TTSS genes cluster based on the model proposed by Krause *et al* (2002) with SSE as an inducer.

Table 4. 1 Quantification of several *nod*- and *tts*- regulatory genes in response to SSE and genistein at 30°C and 15°C by real-time RT-PCR

Inducer	Induction condition	*nodW*	*nodD1**	*nodD2*	*nolA*	*ttsI*	*tts* expression	Reference
SSE	30℃, 6h	2.3±0.2	5.6±0.9	3.8±0.9	3.2±0.3	2.1±0.5	undetectable	
GEN		3.2±0.5	5.5±0.6	4.1±1.3	2.7±0.2	4.9±1.0	undetectable	*Figure 2.6*
SSE	30℃, 12h	1.8±0.3	3.8±1.0	26.1±3.2	8.0±1.5	10.1±0.2	fully and strongly	
GEN		2.9±0.3	2.2±0.4	2.4±0.9	1.5±0.4	4.1±1.1	partially and weakly	
SSE	15℃, 20h	2.1±0.5	4.2±0.5	3.0±0.8	3.1±0.5	2.6±0.4	undetectable	
GEN		4.9±0.6	4.1±0.4	2.5±0.1	2.6±0.5	4.2±0.2	undetectable	*Figure 3.3*
SSE	15℃, 48h	0.6±0.0	2.5±0.3	5.8±0.7	7.6±1.9	8.0±1.5	fully but weakly	
GEN		1.0±0.3	1.1±0.3	2.4±0.3	2.2±0.3	5.3±1.4	partially and weakly	

* indicate relative expression from array analysis with a PCR-amplified fragment spotted on the membrane.

sigA, a housekeeping gene of *B. jaonicum*, was used as an internal control for quantitative real-time RT-PCR

CHAPTER 5

General Discussion

5. 1 Global Expression Profiles of *B. japonicum* in the Initial Interaction with Soybean, *Glycine max* (L.) merr, are Constructed

In present thesis, genome-wide expression profiles of *B. japonicum* in response to SSE and genistein at common growth and low temperature are captured by using a macroarray platform. These results provide a profound insight into the contribution of *B. japonicum* during early stage of *B. japonicum*-soybean symbiosis at the level of transcription and form a basic knowledge to screen the novel induced-genes, which are most probably involved in the symbiotic process. Nevertheless, it should be remembered that expression profiling only assays functionality in an indirect way, extensive efforts should be devoted to analyses of proteome and metabolome, which are the functional entities in *B. japonicum.* Furthermore, to understand the *B. japonicum*-soybean interaction during early stage of symbiosis, the molecular response of soybean partner to the inoculation of *B. japonicum* should be simultaneously analyzed by using functional tools. Recently, a dual-genome symbiotic chip was developed for coordinate study of signal exchange in *S. meliloti-Medicago truncatula* interaction (Barnett *et al.*, 2004), suggesting a unique tool to explore the rhizobia-legume symbiosis. Although the genome-wide expression portraits *B. japonicum* were captured with time and the expression of some novel genes were quantitatively confirmed, it is unknown whether these genes are essential for the

establishment of *B. japonicum*-soybean symbiosis, or when and where they function in the early stage of *B.japonicum*-soybean symbiosis. Analyses of mutant strains of these genes (e.g. knockout, overexpression and fusion with reporter genes) by using genetic method can provide important information on their functions in symbiotic association.

5. 2 Expression Response of *B. japonicum* to the SSE Should be Viewed as a " System", more than the Sum of Soybean-Released Compounds

This thesis revealed that compared with genistein, daidzein and mixture of both, SSE induced a distinctly differential genome-wide portrait of expression profiling of *B. japonicum*, suggesting that expression response of *B. japonicum* to the SSE should be viewed as a " system", more than the sum of soybean-released compounds. Most compounds in root exudates are traditionally viewed as simply energy substrates, building blocks for microbial protoplasm, or common growth cofactors (Philips and Streit, 1996). Although the compounds profiles of SSE are not determined in the present thesis, it may present the actual soybean-released compounds encountered by *B. japonicum* during initial infection process, leading to the coordinate expression of genes of *B. japonicum*. The present thesis also demonstrate that isoflavones (genistein and daidzein)-Nod factor relationship is important but not in full to explain the signal-interaction during the early stage of *B. japonicum*-soybean symbiosis. It is likely that the SSE-preferentially-induced genomic loci such as TTSS are involved in the nodulation efficiency other than capability because the mutant of these genes in the previous studies often resulted in the delayed nodule development, not the grossly defect in nodule formation.

Previously, it was shown that the actual amount of inducer present in SSE (ethanol

extract of the seeds), root and seed exudate, which obtained after 4-h as well as after 18-h imbibition of seeds in sterile water, differed significantly, but the chromatogram were very similar (Smit *et al.*, 1992). Soybean was chosen as one of the legume host plant *B. japonicum* in this study. Apparently, a further analyses of the genome-wide expression response of *B. japonicum* to its other plant partner such as cowpea will provide important information on genomic loci of *B. japonicum* involved in the specificity of symbiotic association.

5. 3 NolANodD2NodD1 May Play a "Turning Key" Role in Regulation of Induction of *nod* to TTSS, Probably other Gene Clusters

NolANodD2NodD1 has been reported to be involved in regulation of the fine-tuning expression of the nodulation genes (Loh and Stacey, 2003), yet, their regulatory functions have been mainly focused on the *nod* genes in response to soybean-released isoflavones (genistein and daidzein) by using classic genetic tools. By using macroarray technique in this study, it was found that although *n*od genes were induced early and did not show distinctly different expression pattern between SSE- and genistein-treated cells, clones covering *nolAnodD2nodD1* and TTSS as well as other genes loci were preferentially induced later by SSE, suggesting that NolANodD2NodD1 may play a "turning key" role in regulation of induction of *nod* to TTSS, probably other gene clusters on the whole genome of *B. japonicum*.

5. 4 Conclusions

B. japonicum-soybean symbiotic interaction involve continued and reciprocal signal exchanged, which is as complex on the plant side as for the bacterial partner. In this thesis, first, at the common growth temperature, SSE markedly induced four predominant genomic regions within a large symbiosis island (681kb), which includes type III secretion system (TTSS) and various nodulation genes. In addition, SSE-treated cells expressed many genomic loci containing genes for polygalacturonase (cell wall degradation), exopolysaccharide (EPS) synthesis, 1-aminocyclopropane-1- carboxylate (ACC) deaminase, ribosome proteins family and energy metabolism even outside symbiosis island. On the other hand, genistein-treated cells exclusively showed one expression cluster including common nod gene operon within symbiosis island and six expression loci including multidrug resistance, which were shared with SSE-treated cells. Second, low temperature led to a potentially delayed expression of those SSE-preferentially-induced genome loci including TTSS genes at 30°C compared with those genistein-induced loci relevant to nodulation and multidrug resistance. Finally, NodD2 was found to be a novel component required for the full induction of TTSS genes and then a modified regulatory circuit for the TTSS genes was proposed.

Taken together, this thesis originally captured genome-scale expression profiles of *B. japonicum* in the initial interaction with soybean. The results can provide a profound insight into *B. japonicum*-soybean symbiosis, and also a basic knowledge for screening novel genes relevant to this process.

References

Aguilar, M. M., Ashby, A. M., Richards, A. J. M., Loake, G. J., Watson, M. D., and Shaw, C. H. 1988, Chemotaxis of *Rhizobium leguminosarum* biovar *phaseoli* towards flavonoid inducers of the symbiotic nodulation genes, *J. Gen. Microbiol.*, **134**, 2741-2764.

Ampe, F., Kiss, E., Sabourdy, F., and Batut, J. 2003, Transcriptome analysis of *Sinorhizobium meliloti* during symbiosis, *Genome Biol.*, **4**, R15.

Banfalvi, Z., Nieuwkoop, A., Schell, M. G., Besl, L., and Stacey, G. 1988, Regulation of *nod* gene expression in *Bradyrhizobium japonicum*, *Mol. Gen. Genet.*, **214**, 420-424.

Barbour, W. M., Hattermann, D. R., and Stacey, G. 1991, Chemotaxis of *Bradyrhizobium japonicum* to soybean exudates, *Appl. Environ. Microbiol.*, **57**, 2635-2639.

Barnett, M. J., Toman, C. J., Fisher, R. F., and Long, S. R. 2004, A dual-genome symbiotic chip for coordinate study of signal exchange and development in a prokaryote-host interaction, *Proc. Natl. Acad. Sci. USA.*, **101**, 16636-16641.

Baumberger, I. C., Fraefel, N., Göttfert, M. M., and Hennecke, H. 2003, New NodW- or NifA-regulated *Bradyrhizobium japonicum* genes, *Mol. Plant-Microbe Interact.*, **16**, 342-351.

Becker, B. U., Kosch, K., Parniske, M., and Müller, P. 1998, Expolysaccharide (EPS) synthesis in *Bradyrhizobium japonicum*: sequence, operon structure and mutational analysis of an *exo* gene cluster, *Mol. Gen. Genet.*, **259**, 161-171.

Belkheir, A. M., Zhou, X.; and Smith, D. L. 2000, Response of soybean [*Glycine max* (L.) Merr.] cultivars to genistein-preincubated *Bradyrhizobium japonicum*: nodulation and dry matter accumulation under Canadian short-season conditions, *J. Agron. Crop Sci.*,

185, 167-175.

Broughton, W. J., Jabbouri, S., and Perret, X. 2000, Keys to symbiotic harmony, *J. Bacteriol.*, **182**, 5641-5642.

Caetano-Anolles, G., Wall, L. G., Micheli, A.T., Macchi, E. M., Bauer, W. D., and Favelukes, G. 1988, Role of motility and chemotaxis in efficiency of nodulation by *Rhizobium meliloti*, *Plant Physiol.*, **86**, 1228-1235.

Chang, W. S., Franck, W. L., Cytryn, E. J. et al. 2007, An oligonucleotide microarray resource for transcriptional profiling of *Bradyrhizobium japonicum*, *Mol. Plant-Microbe Interact.*, **20**, 1298-1307.

Cornelis, G. R. 2006, The type III secretion injectisome. *Nature Rev. Microbiol.*, **4**, 811-825.

Cornelis, G., Vanooteghem, J. C., and Sluiters, C. 1987, Transcription of the *yop* regulon from *Y. enterocolitica* requires trans-acting *pYV* and chromosomal genes, *Microb. Pathog.*, **2**, 367-379.

Cytryn, E. J., Sangurdekar, D. P., Streeter, J. G. et al. 2007, Transcriptional and physiological response of *Bradyrhizobium japonicum* to desiccation-induced stress, *J. Bacteriol.*, **189**, 6751-6762.

Dart, P and Day, J. 1971, Effects of incubation temperature and oxygen tension on nitrogenase activity of legume root nodules, *Plant Soil*, **35**, 167-184.

Denarie, J., Debeelle, F., and Prome, J. C. 1996, *Rhizobium* lipo-chitooligo-saccharide nodulation factors: signaling molecules mediating recognition and morphogenesis, *Annu. Rev. Biochem.*, **65**, 503-535.

Dobrindt, U., Hochhut, B., Hentschel, U., and Hacker, J. 2004. Genomic islands in pathogenic and environmental microorganisms, *Nature. Rev. Microbiol.*, **2**, 414-424.

Duzan, H. M., Mabood, F., Souleimanov, A., and Smith, D. L. 2006, Nod Bj-V ($C_{18:1}$, MeFuc) production by *Bradyrhizobium japonicum* (USDA110, 532C) at suboptimal growth

temperatures, *J. Plant. Physiol.*, **163**, 107-111.

Eggleston, G ., Huber, M. C., Liang, R, T., Karr, A. L., and Emerich, D. W. 1996, *Bradyrhizobium japonicum* mutants deficient in exo-and capsular polysaccharides cause delayed infection and nodule initiation, *Mol. Plant-Microbe Interact.*, **9**, 419-423.

Evans, J., Hochman, Z., O'Connor, G. E., and Osborne. G. J. 1988, Soil acidity and *Rhizobium*: their effects on nodulation of subterranean clover on the slopes of southern New South Wales, *Aust. J. Agric. Res.*, **38**, 605-618.

Fischer, H. M. 1994, Genetic regulation of nitrogen fixation in *rhizobia*, *Microbiol. Rev.*, **58**, 352-386.

Freiberg, C., Fellay, R., Bairoch, A., Broughton, W. J., Rosenthal, A., and Perret, X. 1997, Molecular basis of symbiosis between *Rhizobium* and legumes, *Nature*. **22**, 352-354.

Galibert, F., Finan, T. M., Long, S. R. et al. 2001, The composite genome of the legume symbiont *Sinorhizobium meliloti*, *Science*. **293**, 668-672.

Garcia, M., Dunlap, J., Loh, J., and Stacey, G. 1996, Phenotypic characterization and regulation of *nolA* gene of *Bradyrhizobium japonicum*, *Mol. Plant-Microbe Interact.*, **9**, 625-635.

Gaworzewska, E. T., and Carlile, M. J. 1982, Positive chemotaxis of *Rhizobium leguminosarum* and other bacteria towards root exudates from legumes and other plants, *J. Gen. Microbiol.*, **128**, 1179-1188.

Giraud, E., Moulin, L., Vallenet, D. et al., 2007, Legumes symbioses: absence of nod genes in photosynthetic *Bradyrhizobia*, *Science*. **316**, 1307-1312

González, V., Santamaria, R. I., Bustos, P. *et al.* 2006, The partitioned *Rhizobium etli* genome: genetic and metabolic redundancy in seven interacting replicons, *Proc. Natl. Acad. Sci. USA.*, **103**, 3834-3839.

Graham, T. L. 1991, Flavonoid and isoflavonoid distribution in developing soybean seeding tissues and in seed and root exudate, *Plant Physiol.*, **95**, 594-603.

Grob. P., Hennecke, H., and Göttfert, M. 1994, Cross-talk between the two-component regulatory system NodVW and NwsAB of *Bradyrhizobium japonicum*, *FEMS Microbiol. Lett.*, **120**, 349-353.

Göttfert, M., Groβ, P., and Hennecke, H. 1990, Proposed regulatory pathway encoded by the *nodV* and *nodW* genes, determinants of host specificity in *Bradyrhizobium japonicum*, *Proc. Natl. Acad. Sci. USA.*, **87**, 2680-2684.

Göttfert, M., Holzhäuser, D., Bäni, D., and Hennecke, H. 1992, Structural and functional analysis of two different *nodD* genes in *Bradyrhizobium japonicum* USDA 110, *Mol. Plant-Microbe Interact.*, **5**, 257-265.

Göttfert, M., Lamb, J.W., Gasser, R., Semenza, J., and Hennecke, H. 1989, Mutational analysis of the *Bradyrhizobium japonicum* common *nod* genes and further *nod* box-linked genomic DNA regions, *Mol. Gen. Genet.*, **215**, 407-415.

Göttfert, M., Röthlisberger, S., Kündig, C., Beck, C., Marty, R., and Hennecke, H. 2001, Potential symbiosis-specific genes uncovered by sequencing a 410-kilobase DNA region of the *Bradyrhizobium japonicum* chromosome, *J. Bacteriol.*, **183**, 1405-1412.

Halverson, L. J. and Stacey, G. 1984, Host recognition in the *rhizobium*-soybean symbiosis, *Plant Physiol.*, **74**, 84-89.

Han, T., Bausch, C., Richmond, C., Blattner, F. R., and Conway, T. 1999, Functional genomics: expression analysis of *Escherichia coli* growth on minimal and rich media, *J. Bacteriol.*, **181**, 6425-6440.

Hanin, M., Jabbouri, S., Broughton, W. J., Fellay, R., and Quesada-Vincens, D. 1999, In: Stacey, G., Keen, N.T (eds) Plant-Microbe Interactions, **4**. APS Press, Minnesota, pp.1-37.

Hirsch, A. M. 1999, Role of lectin (and rhizobial exopolysaccharides) in legume nodulation, *Curr. Opin. Plant Biol.*, **2**, 320-326.

Hueck, C. J. 1998, Type III protein secretion systems in bacterial pathogens of animals and plants, *Microbiol. Mol. Biol. Rev.*, **62**, 379-433.

Inaba, M., Suzuki, I., Szalontai, B. et al. 2003, Gene-engineered rigidification of membrane lipids enhances the cold inducibility of gene expression in *synechocystis*, *J. Biol. Chem.*, **278**, 12191-12198.

Ito, N., Itakura, M., Saeki, K. et al. 2006, Global genes expression in *Bradyrhizobium japonicum* cultured with vanillin, vanillate, 4-hydroxybenzoate, and protocatechuate, *Microbes Environ.*, **21**, 240-250.

Jitacksorn, S and Sadowsky, M. J. 2008, Nodulation gene regulation and quorum sensing control density-dependent suppression and restriction of nodulation in the *Bradyrhizobium japonium*-soybean symbiosis, *Appl. Environ. Microbiol.*, **74**, 3749-3756.

Jones, F. R. and Tisdale, W. B. 1921, Effect of soil temperature upon the development of nodules on the roots of certain legumes, *J. Agric. Res* (Cambridge), **22**, 17-37.

Jordan, D. C. 1984, In: Krieg, N.R., Holt, J.G. (eds) Bergey's Manual of Systematic Bacteriology, Vol.1, Williams and Wilkins, London, pp. 234-244.

Kanbe, M., Yagasaki, J., Zehner, S., Göttfert, M., and Aizawa, S. I. 2007, Characterization of two sets of subpolar flagella in *Bradyrhizobium japonicum*, *J. Bacteriol.*, **189**, 1083-1089.

Kaneko, T., Nakamura, Y., Sato, S. et al. 2000, Complete genome structure of the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti*, *DNA Res.*, **7**, 331-338.

Kaneko, T., Nakamura, Y., Sato, S. et al. 2002, Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA 110, *DNA Res.*, **9**, 189-197.

Kape, R., Parniske, M., and Werner, D. 1991, Chemotaxis and *nod* gene activity of *Bradyrhizobium japonicum* in response to hydroxycinnamic acids and isoflavonoids, *Appl. Environ. Microbiol.*, **57**, 316-319.

Kosslak, R. M., Bookland, R., Barkei, J., Paaren, H. E., and Appelbaum, E. R. 1987, Induction of *Bradyrhizobium japonicum* common *nod* genes by isoflavones isolated from *Glycine max*, *Proc. Natl. Acad. Sci. USA.*, **84**, 7428-7432.

Krause, A., Doerfel, A., and Göttfert, M. 2002, Mutational and transcriptional analysis of the

type III secretion system of *Bradyrhizobium japonicum, Mol. Plant-Microbe Interact.*, **15**, 1228-1235.

Kündig, C., Hennecke, H., and Göttfert, M. 1993, Correlated physical and genetic map of the *Bradyrhizobium japonicum* 110 genome, *J. Bacteriol.*, **175**, 613-622.

Lambert de Rouvroit, C., Sluiters, C., and Cornelis, G. R. 1992, Role of the transcriptional activator, VirF in the expression of the pVY plasmid genes of *Yersinia enterocolitica, Mol. Microbiol.*, **6**, 395-409.

Lang, K., Lindemann, A., Hauser, F., and Göttfert, M. 2008, The genistein stimulon of *Bradyrhizobium japonicum, Mol. Gen. Genom.*, **279**, 203-211.

Leibovitch, S., Migner, P., Zhang, F., and Smith, D. L. 2001, Evaluation of the effect of soyasignal technology on soybean yield [*Glycine max* (L.) Merr.] under field conditions over 6 years in eastern Canada and northern united states, *J. Agron. Crop Sci.*, **187**, 281-292.

Leveau, J. H. J., and Lindow, S. E. 2002, Bioreporters in microbial ecology, *Curr. Opin. Microbiol.*, **5**: 259-265.

Lodeiroa, A. R., Silvina, L. L., Triso, E. E. V., and Gabriel, F. 2000, Stimulation of adhesiveness, infectivity, and competitiveness for nodulation of *Bradyrhizobium japonicum* by its pretreatment with soybean seed lectin, *FEMS Microbiol. Lett.*, **188**, 177-184.

Loh, J., Lohar, D., Andersen, B., and Stacey, G. 2002a, A two-component regulator mediates population density dependent expression of the *Bradyrhizobium japonicum* nodulation genes, *J. Bacteriol.*, **184**, 1759-1766.

Loh, J., Carlson, R. W., York, W.S., and Stacey, G. 2002b, Bradyoxetin, a unique chemical signal involved in symbiotic gene regulation, *Proc. Natl. Acad. Sci. USA.*, **99**, 14446-14451.

Loh, J., Garcia, M., and Stacey, G. 1997, NodV and NodW, a second flavonoid recognition system regulating *nod* gene expression in *Bradyrhizobium japonicum, J. Bacteriol.*, **179**,

3013-3020.

Loh, J. and Stacey, G. 2001, Feedback regulation of the *Bradyrhizobium japonicum* nodulation genes, *Mol. Microbiol.*, **41**, 1357-1364.

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

Loh, J., Stacey, M. G., Sadowsky, M. J., and Stacey, G. 1999, The *Bradyrhizobium japonicum nolA* gene encodes three functionally distinct proteins, *J. Bacteriol.*, **181**: 1544-1554.

Loh, J., Yuen-Tsai, J. P. Y., Stacey, M. G., Lohar, D., Welborn, A and Stacey, G. 2001, Population density-dependent regulation of the *Bradyrhizobium japonicum* nodulation genes, *Mol. Microbiol.*, **42**, 37-46.

Lynch, D. H. and Smith, D. L. 1993, Soybean (*Glycine max*) nodulation and N_2-fixation as affected by exposure to a low-zone temperature, *Physiol. Plant.*, **88**, 212-220.

MacLean, A. M., Finan, T. M., and Sadowsky, M. J. 2007, Genome of the symbiotic nitrogen-fixing bacteria of legume, *Plant Physiol.*, **144**, 615-622.

Marie, C., Broughton, W. J., and Deakin, W. J. 2001, *Rhizobium* type III secretion systems: legume charmers or alarmers?. *Curr. Opin. Plant Biol.*, **4**, 336-342.

Mazurier, S., Lemunier, M., Hartmann, A., Siblot, S., and Lemanceau, P. 2006, Conservation of type III secretion system genes in *Bradyrhizobium* isolated from soybean. *FEMS Microbiol. Lett.*, **259,** 317-325.

Minamisawa, K., Seki, T., Onodera, S., Kubota, M., and Asami, T. 1992, Genetic relatedness of *Bradyrhizobium japonicum* field isolates as revealed by repeated sequences and various other characteristics, *Appl. Environ. Microbiol.*, **58**, 2832-2839.

Nellen-Anthamatten, D., Rossi, P., Preisig, O. et al. 1998, *Bradyrhizobium japonicum* FixK2, a crucial distributor in the FixLJ-dependent regulatory cascade for control of genes inducible by low oxygen level, *J. Bacteriol.*, **180**, 5251-5255.

Nienaber, A., Huber, A., Göttfert, M., Hennecke, H., and Fischer, H. M. 2000, Three new

NifA-regulated genes in the *Bradyrhizobium japonicum* symbiotic gene region discovered by competitive DNA-RNA hybridization, *J. Bacteriol.*, **182**, 1472-1480.

Norris, D. O and Date, R. A. 1976, In: Shaw, N. H., Bryan, W. W(eds) Legume Bacteriology, Tropical Pasture Principles and Methods, CAB Bulletin, **51**, 134-174.

Olsthoorn, M. M. A., Stokvis, E., Haverkamp, J., Spaink, H. P., and Thomas-Oates, J. E. 2000, Growth temperature regulation of host-specific modifications of Rhizobial lipo-chitin oligosaccharides: The function of *nodX* is temperature regulated, *Mol. Plant-Microbe Interact.*, **13**, 808-820.

Pallen, M. J., Beaston, S. A., and Bailey, C. M. 2005, Bioinfomatics, genomics and evolution of non-flagellar type-III secretion systems: a Darwinian perspective, *FEMS Microbiol. Rev.*, **29**, 201-229.

Parniske, M., Ahlborn, B., and Werner, D. 1991, Isoflavonoid-inducible resistance to phytoalexin glyceollin in soybean *rhizobia*, *J. Bacteriol.*, **173**, 3432-3439.

Penmetsa, R.V. and Cook, D. R. 1997, A legume ethylene-insensitive mutant hyperinfected by its *Rhizobial* symbiont, *Science*, **275**, 527-530.

Pessi, G., Ahrens, C. H., Rehrauer, H. et al. 2007, Genome-wide transcript analysis of *Bradyrhizobium japonicum* bacteroids in soybean root nodules, *Mol. Plant-Microbe Interact.*, **20**, 1353-1363.

Philips, D. A. and Streit, W. 1996, In: Stacey, G., Keen, N.T (eds) Plant-Microbe Interactions, **1**. Chapman and Hall, New York, pp. 236-271.

Rozen, S., and Skaletsky, H. J. 2000, In: Krawetz, S., and Misener, S (eds) Bioinformatics Methods and Protocols: Primer3 on the WWW for General Users and for Biologist Programmers, Humana Press, Totowa, New York, pp. 365-386.

Sadowsky, M. J., Cregan, P. B., Göttfert, M. et al. 1991, The *Bradyrhizobium japonicum nolA* gene and its involvement in the genotype-specific nodulation of soybeans, *Proc. Natl. Acad. Sci. USA.*, **88**, 637-641.

Sanjuan, J., Grob, P., Göttfert, M., Hennecke, H., and Stacey, G. 1994, NodW is essential for full expression of the common nodulation genes in *Bradyrhizobium japonicum, Mol. Plant-Microbe Interact.*, **7**, 71-77.

Sarma, A. D. and Emerich, D. W. 2005, Global protein expression pattern of *Bradyrhizobium japonicum* bacteroids: a prelude to functional proteomics, *Proteomics*, **5**, 4170-4184.

Schultze. M. and Kondorosi. A. 1998, Regulation of symbiotic root nodule development. *Annu. Rev. Genet.*, **32**, 33-57.

Smit, G., Puvanesarajah, V., Carlson, R.W., Barbour, W. M., and Stacey, G. 1992, *Bradyrhizobium japonicum nodD1* can be specifically induced by soybean flavonoids that do not induce the *nodYABCSUIJ* operon, *J. Biol. Chem.*, **267,** 310-318.

Soto, M. J., Sanjuan, J., and Olivares, J. 2006, Rhizobia and plant-pathogenic bacterial: common infection weapons, *Microbiol.*, **152**, 3167-3174.

Sperandio, V., Mellies, J. L., Nguyen, W., Shin, S., and Kaper, J. B. 1999, Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in enterohemorrhagic and enteropathogenic *Escherichia coli, Proc. Natl. Acad. Sci. USA.*, **96,** 15196-15201.

Süβ, C., Hempel, J., Zehner, S., Krause, A., Patschkowski, T., and Göttfert, M. 2006, Identification of genistein-inducible and type III-secreted proteins of *Bradyrhizobium japonicum, J. Biotechnology.* **126**, 69-77.

Tarrand, J. J., Krieg, N. R., and Döbereiner, J. 1978, A taxonomic study of the *spirillum lioferum* group, with description a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. *Can. J. Microbiol.*, **24**, 967-980.

Uchiumi, T., Ohwada, T., Itakura, I. et al. 2004, Expression islands clustered on the symbiosis island of the *Mesorhizobium loti* genome, *J. Bacteriol.*, **186**, 2439-2448.

Valls, M., Genin, S., and Boucher, C. 2006, Integrated regulation of the type III secretion

system and other virulence determinants in *Ralstonia solanacearum*, *PLoS Pathogens.*, Vol. 2, No. 8, e82 doi:10.1371/journal.ppat.0020082.

Viprey, V., Greco, A. D., Golinowski, W., Broughton, W. J., and Perret, X. 1998, Symbiotic implications of type III protein secretion machinery in *Rhizobium*, *Mol. Microbiol.*, **28,** 1381-1389.

Wright, S. F. and Weaver, R. W. 1981, Enumeration and identification of nitrogen-fixing bacteria from forage grass, *Appl. Environ. Microbiol.*, **54**, 1831-1837.

Young, J. P., Crossman, L. C., Johnston, A.W. et al. 2006, The genome of *Rhizobium leguminosarum* has recognizable core and accessory components, *Genome Biol.*, **7**, R34.

Zahram, H. H. 1999, *Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate, *Microbiol. Mol. Biol. Rev.*, **63**, 968-989.

Zehner, S., Schober, G., Wenzel, M., Lang, K and Göttfert, M. 2008. Expression of *Bradyrhizobium japonicum* type III secretion system in legume nodules and analysis of the associated tts box promoter, *Mol. Plant-Microbe Interact.*, **21**, 1087-1093.

Zhang, F. and Smith, D. L. 1994, Effects of low root zone temperatures on the early stages of symbiosis establishment between soybean [*Glycine max* (L.) Merr.] and *Bradyrhizobium japonicum*, *J. Exp. Bot.*, **45**, 1467-1473.

Zhang, F. and Smith, D. L. 1996, Genistein accumulation in soybean (*Glycine max* [L.] Merr.) root systems under suboptimal root zone temperatures, *J. Exp. Bot.*, **47**, 785-792.

Zhang, F. and Smith, D. L. 1997, Application of genistein to inocula and soil to overcome low spring soil temperature inhibition of soybean nodulation and nitrogen fixation, *Plant Soil.*, **192**, 141-151.

Zhang, H. Prithiviraj, B., Souleimanov, A., D'Aoust, F., Charles, T. C., Driscoll, B. T., and Smith, D. L. 2002, The effect of temperature and genistein concentration on lipo-chitooligosaccharide (LCO) production by wild-type and mutants of *Bradyrhizobium japonicum*, *Soil. Bio. Biochem.*, **34**, 1175-1180.

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Appendix

Appendix-1 Expression cluster (ECs: ECI–IV) within symbiosis island and their covered genes

ECs	Clone No.	30℃ SSE 0.5h	30℃ SSE 6h	30℃ SSE 12h	30℃ GEN 0.5h	30℃ GEN 6h	30℃ GEN 12h	15℃ SSE 12h	15℃ SSE 20h	15℃ SSE 48h	15℃ GEN 12h	15℃ GEN 20h	15℃ GEN 48h	Genome position start	Genome position end	Covered gene (entirely or partially) [a]	Annotated gene function
EC-I	1 (brb19696)	1.2	1.4	1.1	1.2	1.1	1.4	2.7	2.2	0.6	4.0	2.4	0.7	1781581	1784324	blr1625 blr1626 blr1627 bsr1628	blr1625(unknown protein), blr1626(ketopantoate hydroxymethyltransferase),
	2 (brb17883)	1.6	4.0	2.1	1.4	4.9	1.8	9.8	8.8	1.2	14	7.5	2.0	1784749	1787190	blr1629	blr1627(unknown protein),bsr1628(unknown protein),blr1629(multidrug resistance protein)
	3 (brb17576)	1.2	3.5	1.9	1.4	1.7	1.4	5.6	4.0	2.2	4.1	2.2	0.6	1788212	1790738	bll1630 bll1631	bll1630(*nolK*), bll1631(*noeL*)
	4 (brb15873)	0.9	16	2.9	0.9	4.6	1.2	47	21	1.2	29	11	1.0	1791307	1794382	blr1632 blr1633	blr1632(*nodM*), blr1633(*noeD*)
	5 (brb01552)	1.1	1.0	5.3	1.0	1.0	1.4	0.8	0.9	1.7	0.5	1.6	0.9	1794440	1797214	bll1634 blr1635 bll1636 bsl1637 blr1638	bll1634(unknown protein), blr1635(hypothetical protein), bll1636(unknown protein)
	6 (brb09807)	1.0	1.3	3.9	0.7	1.1	1.0	1.0	0.9	1.6	0.9	1.1	1.2	1795520	1798232	blr1635 bll1636 bsl1637 blr1638	bsl1637(unknown protein), blr1638(unknown protein)
	7 (brb00313)	0.8	1.5	9.4	0.9	1.0	1.1	1.6	1.4	5.3	1.4	1.3	1.0	1798668	1801069	bsl1639 blr1640	bsl1639(unknown protein),blr1640(unknown protein)
	8 (brb15892)	0.8	1.1	1.4	1.1	0.7	1.1	0.9	0.9	1.3	0.6	0.8	1.0	1800157	1804238	blr1640 bll1641 bll1642 bll1643	bll1641(putative transposase), bll1642(putative transposase), bll1643(unknown protein)
	9 (brb09855)	0.8	0.5	2.1	0.8	1.0	1.2	1.1	1.2	1.6	1.2	1.1	0.8	1804683	1807581	blr1644 blr1645 bsr1646 bll1647	blr1644(hypothetical protein), blr1645(putative transposase), bsr1646(putative transposase)
	10 (brb10847)	1.1	0.4	1.5	0.8	0.8	0.9	0.6	0.7	1.1	0.6	0.9	0.9	1806974	1809047	bll1647 bll1648	bll1647(putative transposase), bll1648(unknown protein)
	11 (brb02447)	0.7	0.6	9.6	1.1	0.5	2.1	1.1	0.9	2.7	1.4	1.0	0.6	1808585	1811215	bll1648 blr1649 blr1650	blr1649(unknown protein), blr1650(unknown protein)
	12 (brb09249)	1.8	0.6	4.7	1.1	0.6	0.9	0.7	0.8	2.6	1.2	0.9	0.8	1810567	1813243	blr1649 blr1650 bsl1651 bsl1652 blr1653 bsl1654 blr1	bsl1651(unknown protein), bsl1652(unknown protein), blr1653(unknown protein),
	13(brb08303)	1.2	0.6	1.5	1.1	1.7	1.1	0.7	1.3	0.9	1.1	1.3	0.9	1812932	1815640	blr1655 blr1656 blr1657	bsl1654(unknown protein), blr1655(putative transposase), blr1656(putative glycosyl hydrolase) blr1657(putative transposase)
EC-II	1 (brb24717)	0.7	0.9	1.5	1.1	0.6	0.8	1.0	0.8	1.3	1.1	0.8	1.0	1845670	1848572	blr1689 bsr1690 blr1691 blr1692	blr1689(putative transposase),bsr1690(putative transposase)
	2 (brb22433)	1.0	1.0	1.6	1.0	1.2	1.3	0.8	0.6	0.7	0.7	1.3	1.0	1849817	1852313	blr1693	blr1691(putative transposase), blr1692(putative transposase),blr1693(unknown protein)
	3 (brb12549)	1.1	0.9	2.1	1.3	0.6	1.0	0.8	0.9	0.9	0.8	1.3	1.4	1852782	1854434	blr1693 bll1694 bsr1695	bll1694(unknown protein), bsr1695(hypothetical protein)
	4 (brb03652)	0.8	1.1	1.5	0.7	0.9	1.2	1.0	0.9	1.4	1.3	0.9	0.7	1855217	1857673	bll1697 blr1698 blr1699 blr1700	bll1697(unknown protein), blr1698(putative transposase)
	5 (brb20145)	0.9	1.1	1.4	0.9	0.9	1.3	1.2	0.9	1.7	0.9	0.8	1.5	1855908	1858656	bll1697 blr1698 blr1699 blr1700	blr1699(hypothetical protein), blr1700(putative transposase)
	6 (brb06394)	1.4	1.1	2.0	0.8	1.1	1.1	0.6	0.7	1.5	0.6	0.5	1.3	1857567	1860881	blr1700 bll1701 blr1702	bll1701(conjugal transfer protein), blr1702(putative transposase)
	7 (brb12717)	1.0	1.1	3.0	0.9	0.8	1.1	1.3	0.8	1.9	0.8	0.9	1.2	1860071	1863482	bll1701 blr1702 bll1703	bll1703(hypothetical protein)
	8 (brb05517)	0.8	0.6	2.8	0.9	0.6	1.3	0.9	0.8	1.6	1.0	1.0	1.0	1861416	1864899	blr1702 bll1703 blr1704	blr1704(hypothetical protein)
	9 (brb20922)	1.3	0.3	2.8	0.7	0.3	1.1	0.9	1.0	2.4	0.4	0.7	0.6	1865058	1866909	blr1704 blr1705	blr1705(unknown protein)
	10 (brb14981)	0.8	0.2	3.4	0.7	0.3	1.0	0.5	0.9	2.4	0.6	1.1	0.9	1866481	1869128	blr1705	
	11 (brb12245)	0.9	0.5	2.1	0.8	0.5	0.8	0.7	0.6	1.7	0.9	0.9	1.0	1868893	1871661	blr1705 blr1706	blr1706(putative transposase)
	12 (brb08121)	1.0	0.6	1.2	1.2	1.0	0.7	1.0	0.8	0.8	0.9	0.7	0.9	1872717	1875419	blr1707 bsl1708 blr1709 bll1710 bll1711	blr1707(putative transposase), bsl1708(unknown protein)
	13 (brb09455)	1.0	1.0	1.2	1.1	1.2	0.9	1.4	0.8	0.8	1.2	0.8	1.0	1874319	1877132	blr1709 bll1710 bll1711 blr1712	blr1709(unknown protein), bll1710(putative transposase)
	14 (brb00851)	1.7	1.8	1.3	1.6	1.9	1.4	3.0	1.9	0.9	3.8	2.3	1.0	1875786	1878463	bll1711 blr1712	bll1711(putative transposase), blr1712(putative transposase)
	15 (brb16383)	2.4	3.9	3.3	8.6	5.1	3.5	13	4.3	1.0	15	6.6	1.1	1878444	1880835	bsl1713 bll1714 bll1715	bsl1713(two-component response regulator), bll1714(*nodW*)
	16 (brb09426)	2.1	3.4	3.6	3.3	5.4	3.7	4.6	3.0	1.3	5.8	3.2	1.0	1880238	1883701	bll1715 blr1716	bll1715(*nodV*), blr1716(putative transposase)
	17 (brb20961)	0.7	0.7	2.5	1.3	0.8	1.4	1.1	0.8	1.2	1.1	0.8	1.9	1884707	1886463	blr1717 bll1718	blr1717(putative transposase), bll1718(*dctA*)
	18 (brb00344)	1.0	1.9	1.3	0.9	0.9	2.2	2.4	1.8	0.9	3.7	1.7	2.0	1886228	1889034	bll1718 blr1719 blr1720	blr1719(*modB*), blr1720(*hupS*)
	19 (brb01205)	1.6	1.2	1.7	1.5	1.4	1.4	0.7	1.3	1.4	1.7	2.1	1.4	1888919	1891209	blr1720 blr1721	blr1721(*hupL*)
	20 (brb18679)	1.8	2.1	1.5	1.3	1.0	1.9	1.1	1.2	1.7	1.9	1.7	1.9	1890042	1892878	blr1721 blr1722 bsr1723 blr1724	blr1722(*hupC*), bsr1723(*hupD*)
	21 (brb04777)	1.4	1.4	1.9	1.4	1.0	0.7	0.9	0.7	2.5	0.5	0.8	1.9	1892130	1894733	blr1722 bsr1723 blr1724 bsr1725 blr1726 blr1727	blr1724(HupD protein homolog), bsr1725(*hupF*), blr1726(unknown protein)
	22 (brb01640)	2.6	1.5	1.1	0.8	0.7	1.3	0.8	0.7	0.8	0.5	1.6	0.2	1893677	1896325	blr1726 blr1727 blr1728 bsr1729 blr1730	blr1727(hupH), blr1728(*hupK*), bsr1729(*hupK*), blr1730(*hyp* A)
	23 (brb16443)	0.7	0.6	1.6	1.3	0.5	1.0	1.0	0.9	1.2	0.9	1.5	1.1	1895504	1898742	bsr1729 blr1730 blr1731 blr1732 blr1733 blr1734 blr1	blr1731(hypB)blr1732(HypB homolog), blr1733(putative transposase) blr1734(putative transposase), blr1735(hypF)
	1 (brb13387)	1.1	0.6	8.4	1.6	0.8	1.4	0.5	0.6	3.7	0.6	1.0	1.2	1953234	1955152	bll1796 bll1797 bll1798 bll1799	bll1796(unknown protein), bll1797(hypothetical protein), bll1798(hypothetical protein)
	2 (brb20482)	2.1	1.0	7.3	0.9	1.2	0.6	0.9	0.9	4.0	0.9	1.8	1.5	1955583	1959091	bll1800 bll1801 bll1802 bll1803	bll1799(hypothetical protein),bll1800(*rhcV*), bll1801(hypothetical protein), bll1802(unknown protein)

	3 (*brb22651*)	0.4	0.7	21	0.8	0.9	5.4	1.5	0.9	4.8	1.3	1.1	0.8	1957854	1960402	bll1802 bll1803 bll1804 bll1805	bll1803(hypothetical protein), bll1804(unknown protein), bll1805(unknown protein)
	4 (brb16958)	1.0	1.1	13	1.1	0.7	1.9	1.3	1.0	3.8	1.3	0.8	0.7	1960944	1963118	blr1806 blr1807	blr1806(unknown protein), blr1807(putative transposase)
	5 (brb19337)	1.2	0.8	11	0.7	0.9	2.4	0.8	1.0	5.2	1.1	1.2	1.5	1963709	1966632	bsl1808 bsr1809 bll1810 bll1811 blr1812	bsl1808(unknown protein), bsr1809(unknown protein), bll1810(hypothetical protein)
	6 (brb05339)	1.0	0.5	21	1.0	0.8	2.1	1.5	1.1	4.0	0.8	1.8	0.9	1965936	1969257	bll1811 blr1812 blr1813 blr1814 blr1815 blr1816	bll1811(*rhcC1*), blr1812(*nolB*), blr1813(*rhcJ*), blr1814(*nolU*), blr1815(*nolV*)
	7 (brb10404)	0.8	0.6	5.7	1.0	1.5	0.8	0.9	1.2	1.5	1.3	1.0	1.0	1969072	1972279	blr1816 blr1817 blr1818 blr1819	blr1816(*rhcN*), blr1817(hypothetical protein)
	8 (brb05528)	1.2	0.8	11	0.8	0.8	1.8	0.8	0.9	3.4	0.3	1.1	1.3	1971019	1974191	blr1818 blr1819 bsr1820 blr1821 blr1822	blr1818(rhcQ), blr1819(rhcR), bsr1820(*rhcS*)
	9 (brb12558)	0.8	0.7	2.6	1.2	0.8	0.9	0.9	1.0	1.3	1.4	1.1	1.0	1973473	1976118	blr1821 blr1822 bll1823 bll1824 blr1825	blr1821(rhcT), blr1822(rhcU),bll1823(putative transposase), bll1824(putative transposase)
	10 (brb10822)	0.8	0.5	1.8	1.2	0.7	1.0	1.2	0.7	1.0	1.1	0.9	0.8	1975080	1977819	bll1823 bll1824 blr1825 bll1826 blr1827	blr1825(hypothetical protein),bll1826(putative transposase),
	11 (brb16517)	1.3	1.1	3.1	1.0	0.7	0.6	1.0	0.8	1.8	0.6	0.8	1.1	1977635	1980145	blr1827 bsl1828 blr1829	blr1827(putative transposase),bsl1828(unknown protein)
	12 (brb18368)	1.1	1.3	12	0.9	1.1	1.6	0.8	0.6	7.6	0.7	0.9	1.6	1979895	1982954	blr1829 blr1830 bsr1831 bll1832	blr1829(putative transposase),blr1830(unknown protein)
	13 (brb02519)	1.3	0.9	3.3	1.4	1.0	1.7	0.7	0.8	2.5	0.7	0.9	1.2	1982455	1984938	bsr1831 bll1832 blr1833 blr1834	bsr1831(unknown protein), bll1832(putative transposase),blr1833(unknown protein)
	14 (brb10936)	2.8	0.4	1.0	1.2	2.1	0.8	1.0	1.6	1.1	1.5	1.1	1.0	1984263	1986905	blr1833 blr1834 blr1835 bll1836	blr1834(putative transposase)
EC-III	15 (brb24693)	1.1	0.8	1.0	1.0	0.5	1.4	0.9	0.7	1.5	1.3	0.5	1.3	1986166	1989515	blr1835 bll1836 blr1837	blr1835(putative transposase), bll1836(unknown protein)
	16 (brb08573)	0.6	1.1	1.2	1.4	0.2	0.8	1.1	1.2	1.8	2.0	0.9	3.2	1988599	1991306	blr1837 blr1838	blr1837(probable reverse transcriptase/maturase family protein)
	17 (brb21085)	1.4	1.0	10	0.8	0.8	1.7	1.0	1.1	4.5	0.9	1.0	1.3	1991265	1993957	blr1838 blr1839 bll1840	blr1838(DNA invertase),blr1839(hypothetical protein),bll1840(unknown protein)
	18 (brb24301)	1.2	1.2	9.6	0.8	0.8	2.2	0.7	1.7	11	0.7	1.5	2.7	1993458	1996234	bll1840 bll1841 bll1842	bll1841(hypothetical protein), bll1842(*rhcC2*)
	19 (brb00710)	1.3	1.5	9.7	0.9	2.0	1.9	0.7	2.2	6.5	0.9	2.0	2.3	1995604	1998476	bll1842 bll1843 bll1844 bsl1845	bll1843(*ttsI*), bll1844(unknown protein), bsl1845(unknown protein)
	20 (brb16150)	1.6	1.1	11	0.9	1.5	3.1	0.7	1.3	3.4	0.9	1.7	2.1	1998548	2001101	bll1846	bll1846(unknown protein)
	21 (brb22129)	0.8	0.9	7.5	0.8	1.0	1.5	1.0	0.9	4.3	1.2	1.3	0.7	2000922	2003936	blr1847 bll1848 bsl1849	blr1847(VirK protein homolog)
	22 (brb07973)	0.9	0.8	1.5	1.1	2.6	1.1	0.5	1.6	0.9	0.8	0.9	0.7	2002415	2004111	bll1848 bsl1849	bll1848(unknown protein),bsl1849(unknown protein)
	23 (brb02985)	1.1	0.5	1.4	0.6	0.8	0.8	0.9	0.9	1.0	0.2	0.9	1.0	2004132	2006781	blr1850 blr1851 blr1852	blr1850(unknown protein), blr1851(unknown protein),
	24 (brb13744)	0.9	0.7	1.5	0.8	1.5	1.3	0.7	0.5	0.8	0.6	0.8	1.3	2007332	2009049	blr1853	blr1852(similar to pantoate--beta-alanine ligase),blr1853(cytochrome P450 family protein)
	25 (brb17822)	1.3	0.7	2.1	0.8	0.9	1.1	1.1	0.6	1.7	0.9	1.1	1.2	2007672	2011287	blr1853 blr1854	blr1854(hypothetical protein),bll1855(putative transposase),bsl1856(putative transposase)
	26 (brb09811)	1.3	0.8	1.1	0.9	1.6	1.0	1.4	1.2	0.9	1.5	1.0	0.7	2011538	2014005	bll1855 bsl1856 bsl1857 bll1858	bsl1857(unknown protein),bll1858(hypothetical protein)
	27 (brb07356)	0.6	0.7	6.4	0.6	1.2	1.1	0.7	0.6	5.9	0.6	0.6	0.9	2014224	2016745	blr1859 bll1860 bll1861 bll1862	blr1859(unknown protein), bll1860(hypothetical protein)
	28 (brb11331)	0.7	1.3	8.0	0.6	2.0	4.0	1.0	0.8	5.0	0.5	1.0	3.9	2016186	2018818	bll1862 bsr1863 bll1864	bll1861(putative transposase), bll1862(unknown protein), bsr1863(unknown protein)
	29 (brb24554)	0.9	1.6	2.4	0.7	0.9	1.9	1.2	0.9	1.7	1.4	0.7	1.3	2017438	2020145	bll1864 blr1865 blr1866 blr1867	bll1864(citrate-proton symporter),blr1865(putative transposase)
	brb06125	1.4	2.4	1.4	1.3	1.2	0.8	4.2	2.8	1.4	2.4	2.6	1.2	1940649	1943229	blr1781 blr1782 blr1783 bsr1784	blr1781(putative transposase), blr1782(putative transposase), blr1783(putative transposase)
	brb08484	1.3	1.3	1.1	1.2	1.8	1.1	2.2	2.7	1.0	1.6	1.9	0.9	1941709	1945060	blr1783 bsr1784 blr1785 bll1786	bsr1784(unknown protein), blr1785(unknown protein), bll1786 (unknown protein).
																blr1866(putative transposase), blr1867(hypothetical protein)	
	1 (brb16501)	0.7	1.4	4.9	0.9	1.1	1.9	1.7	1.7	31	0.4	1.3	1.1	2171906	2174553	bsr2010 blr2011 bll2012 bsr2013	bsr2010(unknown protein), blr2011(unknown protein), bll2012(hypothetical protein)
	2 (brb03146)	1.1	49	52	0.8	29	38	6.1	10	30	4.1	7.9	32	2174833	2177390	bsl2014 bsl2015 bll2016	bsr2013(unknown protein),bsl2014(unknown protein), bsl2015(nolZ), bll2016(nolY)
	3 (brb17558)	0.5	3.3	5.0	0.8	2.4	1.2	3.3	2.6	5.5	1.4	1.9	0.8	2177445	2180846	bll2017 bll2018 bll2019 bsl2020 bll2021	bll2017(similar to ABC transporter, amino acid binding protein)
	4 (brb00631)	0.5	4.7	7.8	1.2	6.4	1.9	4.3	4.4	6.0	5.9	4.1	1.0	2180444	2182985	bll2021 blr2022 bll2023	bll2018(similar to ABC transporter, amino acid binding protein), bll2019(*nolA*)
	5 (brb16006)	1.8	180	35	4.0	127	44	138	60	13	80	49	15	2183559	2186529	blr2024 blr2025 blr2026 blr2027	bsl2020(hypothetical protein),bll2021(*nodD2*), blr2022(unknown protein), bll2023(*nodD1*)
	6 (brb12293)	1.3	40	10	1.8	25	13	33	16	1.4	24	14	1.8	2186683	2189145	blr2027 blr2028 blr2029	blr2024(*nodY*), blr2025(*nodA*), blr2026(*nodB*)
	7 (brb04139)	1.1	54	25	1.2	27	16	20	14	3.1	11	11	4.0	2188140	2191540	blr2029 blr2030 blr2031 bsr2032 blr2033 blr2034	blr2027(*nodC*), blr2028(*nodS*), blr2029(*nodU*)
	8 (brb02183)	1.4	22	19	2.5	24	17	18	7.7	2.3	9.9	7.9	3.9	2191696	2194646	blr2034 blr2035	blr2030(*nodI*), blr2031(*nodJ*), bsr2032(*nolM*), blr2033(*nolN*)
	9 (brb09684)	1.2	4.2	8.4	1.4	4.5	1.9	1.9	1.5	5.9	1.7	1.3	1.2	2194740	2197454	blr2036 blr2037	blr2034(*nolO*), blr2035(*nodZ*),blr2036(fixR)
	10 (brb13132)	1.4	3.9	4.6	1.4	2.3	1.6	1.6	1.4	6.7	3.0	1.7	1.8	2197266	2199877	blr2037 blr2038 blr2039	blr2037(*nifA*)
	11 (brb05758)	1.1	1.9	3.0	0.9	0.7	1.2	2.2	1.3	2.1	2.4	1.5	0.7	2198241	2200507	blr2038 blr2039 bll2040	blr2038(fixA), blr2039(unknown protein),bll2040(unknown protein)
EC-IV	12 (brb07250)	1.3	0.8	1.4	1.0	0.9	1.1	0.8	1.0	1.0	1.2	1.2	1.1	2200593	2203079	blr2041 blr2042 blr2043	blr2041(unknown protein), blr2042(hypothetical protein)
	13 (brb17339)	1.5	1.2	0.9	1.0	0.8	1.4	0.8	1.0	1.1	1.1	1.3	2.3	2202861	2204874	blr2043 blr2044	blr2043(putative transposase), blr2044(unknown protein)
	14 (brb23691)	1.7	2.1	1.9	0.9	0.6	0.3	0.9	1.3	1.9	0.8	0.9	2.0	2204996	2207367	bll2045 bll2046	bll2045(unknown protein),bll2046(unknown protein)
	15 (brb04111)	1.0	1.8	3.4	1.1	1.0	1.9	1.6	1.3	4.7	1.4	1.2	1.0	2208107	2210995	bll2047 bsl2048 bll2049 bsl2050	bll2047(unknown protein),bsl2048(unknown protein), bll2049(*trpD*), bsl2050(unknown protein)
	16 (brb13906)	1.1	0.7	0.9	1.4	1.1	1.5	1.9	0.9	1.5	2.6	1.1	0.7	2211248	2213852	bsr2051 blr2052	bsr2051(unknown protein), blr2052(probable maturase; reverse transcriptase)

17 (brb18612)	1.7	1.6	0.6	0.9	1.4	1.2	0.5	1.0	0.8	0.5	1.0	1.3	2213619	2216729	blr2052 blr2053 blr2054 blr2055 blr2056	blr2053(putative transposase), blr2054(putative transposase), blr2055(unknown protein)
18 (brb12393)	0.9	2.0	2.1	0.8	2.1	0.9	2.4	1.8	2.2	2.6	1.8	0.9	2217410	2219872	blr2057 blr2058	blr2056(putative transposase),blr2057(unknown protein), blr2058(hypothetical protein)
19 (brb14990)	0.9	1.2	2.1	1.1	0.4	0.9	0.6	0.6	4.5	2.2	0.5	1.4	2221313	2223106	bll2059 bll2060	bll2059(GroEL3 chaperonin), bll2060(GroES3 chaperonin)
20 (brb14561)	4.5	64	20	14	23	7.8	173	101	13	200	82	2.6	2223023	2225328	bsr2061 blr2062	bsr2061(unknown protein), blr2062(*noeI*)
21 (brb06007)	0.9	7.4	3.2	1.7	4.9	1.9	16	10	1.3	19	10	0.7	2224845	2227612	bll2063	bll2063(phenolhydroxylase homolog)
22 (brb02423)	0.6	1.7	3.2	0.9	1.1	1.5	2.0	1.0	4.5	2.7	1.2	0.6	2227362	2229876	bsl2064 bll2065 bll2066	bsl2064(unknown protein), bll2065(carbonic anhydrase)
23 (brb10454)	0.8	1.1	2.8	0.8	1.8	1.4	1.5	1.0	3.3	1.6	1.3	1.0	2229579	2232429	bll2066 bll2067	bll2066(hypothetical protein), bll2067(nodulate formation efficiency C protein)
brb18434	1.1	4.6	2.9	1.1	3.2	1.1	5.9	6.7	1.0	4.1	3.7	0.8	2328495	2331560	bll2154 bll2155 blr2156 blr2157	bll2154(unknown protein), bll2155(unknown protein), blr2156(hypothetical protein)
brb14204	1.7	2.9	1.4	1.1	1.4	1.0	3.7	3.0	1.1	3.8	3.3	1.4	2331774	2335384	bll2158 bll2159 bll2160 bll2161 bll2162	blr2157(hypothetical protein),bll2158(unknown protein), bll2159(putative transposase) bll2160(putative transposase), bll2161(putative transposase), bll2162(unknown protein).

Appendix 2 Exclusive expression of genetic loci inside symbiosis island (LISs:LIS 1-9) but outside ECs and their covered genes by SSE

LIS	Clone	30℃						15℃						Genome position		Covered gene (entirely or partially) [a]	Annotated gene function
		SSE			GEN			SSE			GEN			start	end		
		0.5h	6h	12h	0.5h	6h	12h	12h	20h	48h	12h	20h	48h				
LIS-1	brb03648	0.8	1.1	9.9	1.2	0.6	0.8	1.8	1.4	6.4	1.0	1.2	1.9	1778863	1781788	bll1623 bsl1624	bll1623(unknown protein),bsl1624(unknown protein)
LIS-2	brb22657	0.6	0.4	2.8	0.7	0.5	1.1	0.6	0.7	3.4	0.6	0.8	1.0	1833483	1835743	trnI-CAU bll1674 bll1675 blr1676	bll1674(unknown protein), bll1675(putative transposase)
	brb19858	0.6	0.2	5.2	0.6	0.4	1.3	0.6	0.7	5.7	0.5	0.9	0.9	1835228	1837697	bll1675 blr1676 bsr1677	blr1676(hypothetical protein)
	brb14354	1.0	0.5	3.1	0.9	0.6	1.1	1.0	0.8	1.9	1.1	0.8	0.9	1837512	1840709	bsr1677 bsl1678 blr1679 blr1680 blr1681 bsr1682 bsr1683	bsr1677(hypothetical protein), bsl1678(hypothetical protein)
																	blr1679(similar to ABC transporter permease protein)
																	blr1680(probable ABC transporter permease protein),blr1681(putative transposase)
																	bsr1682(putative transposase), bsr1683(hypothetical protein)
LIS-3	brb04656	1.0	1.0	2.5	1.1	0.6	1.2	1.1	0.9	2.9	0.7	0.8	1.3	1902907	1905914	blr1738 bsr1739 blr1740	blr1738(alanine dehydrogenase), bsr1739(ferredoxin), blr1740(putative transposase)
	brb09172	0.7	0.4	2.6	0.8	0.5	1.1	0.8	0.6	1.4	0.6	0.8	0.8	1915143	1918076	bsr1749 bsr1750 bll1751 blr1752 blr1753	bsr1749(hypothetical protein), bsr1750(ferredoxin), bll1751(unknown protein)
																	blr1752(host-inducible protein A homolog), blr1753(hypothetical protein)
LIS-4	brb03193	0.7	0.8	3.4	0.7	0.5	0.8	0.9	0.8	3.0	0.7	0.6	1.7	2021956	2024883	blr1869 bsl1870 bsl1871	blr1869(unknown protein),bsl1870(unknown protein), bsl1871(unknown protein)
	brb00593	1.4	0.4	2.5	1.0	0.6	0.8	0.8	0.8	1.1	0.5	1.1	0.7	2028816	2031868	bll1875 blr1876 bll1877 bsr1878	bll1875(hypothetical protein),blr1876(unknown protein)
																	bll1877(unknown protein), bsr1878(unknown protein)
LIS-5	brb00173	2.0	0.5	3.4	0.7	0.6	1.0	0.8	1.1	3.2	0.4	1.0	0.9	2056957	2060272	blr1901 blr1902 bsr1903 blr1904	blr1901(hypothetical protein), blr1902(putative bacA), bsr1903(hypothetical protein)
	brb12094	0.5	0.6	6.6	0.6	0.5	1.1	0.7	0.8	5.8	0.8	0.7	1.4	2059746	2061842	blr1904 blr1905	blr1904(hypothetical protein), blr1905(unknown protein)
LIS-6	brb06413	0.7	0.8	3.9	1.0	1.0	0.8	1.0	0.9	1.0	1.1	1.0	0.9	2122942	2126227	blr1961 bsr1962 bll1963 blr1964 bsl1965	blr1961(hypothetical protein), bsr1962(unknown protein), bll1963(hypothetical protein)
																	blr1964(putative sugar hydrolase), bsl1965(unknown protein)
	brb03735	0.8	1.0	2.4	1.0	0.8	0.9	0.9	0.7	1.5	0.9	0.9	1.0	2131749	2134607	bll1973 bll1974 blr1975 bsr1976 bll1977	bll1973(putative transposase), bll1974(hypothetical protein)
																	blr1975(unknown protein), bsr1976(hypothetical protein), bll1977(hypothetical protein)
LIS-7	brb19974	0.6	0.7	2.8	1.0	2.2	0.8	1.1	1.3	1.1	1.1	1.0	1.0	2149355	2152134	blr1992 blr1993	blr1992(unknown protein)
	brb02802	0.8	1.1	12	0.7	0.9	1.2	0.7	1.1	7.0	0.9	0.9	1.1	2150601	2152336	blr1993 blr1994	blr1993(probable polygalacturonase), blr1994(pectinesterase)
LIS-8	brb03958	1.4	1.3	2.7	1.3	1.6	1.4	0.8	0.5	2.0	1.0	1.1	1.2	2240603	2244199	blr2073 blr2074	blr2073(NoeE homolog), blr2074(NoeE homolog)
	brb14883	0.9	1.3	3.0	0.8	1.1	1.4	1.1	1.0	1.9	0.9	1.0	0.8	2243947	2246051	bll2075 blr2076	bll2075(unknown protein), blr2076(putative transposase)
LIS-9	brb18434	1.1	4.6	2.9	1.1	3.2	1.1	5.9	6.7	1.0	4.1	3.7	0.8	2328495	2331560	bll2154 bll2155 blr2156 blr2157	bll2154(unknown protein), bll2155(unknown protein),
																	blr2156(hypothetical protein), blr2157(hypothetical protein)
	brb01950	1.1	1.2	2.6	1.1	0.9	1.1	1.0	1.0	2.2	0.8	1.0	1.1	2336746	2339505	bsr2164 blr2165	bsr2164(unknown protein), blr2165(putative transposase)
	brb21447	1.0	1.3	4.2	0.8	0.7	0.9	1.1	0.8	1.3	1.7	0.8	0.6	2342251	2345238	blr2168 blr2169 blr2170 blr2171	blr2168(putative transketolase alpha subunit protein)
																	blr2169(putative transketolase beta subunit protein)
																	blr2170(probable ABC transporter substrate-binding protein)
																	blr2171 (probable ABC transporter permease protein)

Appendix 3 Exclusively regulated genomic loci outside symbiosis island (LOS 1-6,13-15, 18, 21) and their covered genes by SSE

Regulation	LOS	Clone	30℃ SSE 0.5 h	30℃ SSE 6h	30℃ SSE 12h	30℃ GEN 0.5h	30℃ GEN 6h	30℃ GEN 12h	15℃ SSE 12h	15℃ SSE 20h	15℃ SSE 48h	15℃ GEN 12h	15℃ GEN 20h	15℃ GEN 48h	Genome position start	Genome position end	Covered gene (entirely or partially) *	Annotated gene function
UP	LOS 1	brb04736	0.9	4.7	4.4	0.5	1.1	0.9	1.2	6.2	11	0.6	0.9	1.0	145257	147987	blr0149 blr0150	blr0149(cytochrome O ubiquinol oxidase subunit II),blr0150(cytochrome O ubiquinol oxidase subunit I),
		brb07092	0.8	4.9	4.6	0.6	1.7	1.0	1.5	5.1	7.6	0.4	1.1	0.6	148480	149993	blr0150 blr0151 blr0152 blr0153	blr0151((cytochrome O ubiquinol oxidase subunit III), blr0152 (cytochrome O ubiquinol oxidase subunit IV) blr0153(probable surfeit locus protein 1)
	LOS 2	brb01807	2.4	2.3	1.5	1.0	1.4	1.0	2.6	1.8	1.4	1.7	1.7	1.7	228013	230602	blr0232 bll0233 bll0234 bll0235 bll0236	blr0232(unknown protein), bll0233(hypothetical protein), bll0234(hypothetical protein),
		BJ7001	2.4	1.3	0.9	0.8	0.6	0.7	1.2	1.2	1.6	0.6	0.7	2.9	231441	232294	blr0237	bll0235(hypothetical protein),bll0236(cysteine synthase/cystathionine beta-synthase family protein)
		brb19376	2.8	2.1	2.3	0.8	0.5	1.3	1.3	1.8	2.2	0.7	1.1	1.7	233117	236090	blr0238 blr0239 bll0240 blr0241	blr0237(transcriptional regulatory protein LysR family)
		brb07072	1.4	3.9	6.9	0.6	1.0	0.7	1.6	2.7	6.7	0.4	1.1	0.8	235249	237522	bll0240 blr0241 bll0242	blr0238(oxidoreductase);blr0239(probable citrate-proton symporter) blr0240(transcriptional regulatory protein AsnC family) blr0241(1-aminocyclopropane-1-carboxylate deaminase (EC 4.1.99.4),bll0242 (ATP-dependent DNA helicase)
	LOS 3	brb02036	0.9	1.0	2.2	1.1	0.6	0.8	1.2	1.3	4.7	1.0	0.9	0.7	407220	409785	bll0378 bll0379	bll0378(dihydroxy-acid dehydratase); bll0379(ABC transporter permease protein)
		brb03641	2.0	0.8	2.5	0.8	0.5	0.9	1.2	1.5	5.5	1.1	1.0	0.9	408773	411543	bll0378 bll0379 bll0380 bll0381	bll0380(ABC transporter ATP-binding protein); bll0381(ABC transporter substrate-binding protein)
	LOS 4	brb09412	0.5	1.1	2.3	0.7	0.5	1.0	0.6	0.9	2.5	0.6	1.1	0.5	473160	476035	bll0438 bll0439 bll0440 bll0441	bll0438(unknown protein),bll0439(ATP synthase epsilon chain);bll0440(ATP synthase beta chain)
		brb07194	0.6	1.0	2.4	0.9	0.7	1.1	0.7	0.8	2.7	0.7	1.3	0.5	476331	478628	bll0442 bll0443	bll0441(ATP synthase gamma chain),bll0442(ATP synthase alpha chain)
		brb23090	0.7	2.3	3.2	0.3	0.6	1.1	1.1	1.7	2.5	0.1	0.8	0.5	477843	479857	bll0442 bll0443 blr0444	bll0443(ATP synthase delta chain)
		brb02885	0.8	4.3	2.3	0.1	0.5	1.0	0.9	3.0	3.0	0.0	0.5	0.8	479167	481398	blr0444 bll0445	blr0444(hypothetical protein) bll0445 (primosomal protein N')
	LOS 5	brb20438	0.6	0.9	2.7	0.6	0.5	1.0	0.8	1.1	2.3	0.3	0.9	0.4	1301866	1304423	blr1184 bll1185 bll1186 bsl1187 bll1188	blr1184(hypothetical protein),bll1185(FoF1 ATP synthase B chain);bll1186(FoF1 ATP synthase B' chain)
		brb13254	0.5	1.0	3.1	0.6	0.4	0.9	0.8	0.7	2.6	0.4	0.4	0.4	1303798	1306681	bll1186 bsl1187 bll1188 bsl1189 blr1190 blr1191 blr	bll1187(FoF1 ATP synthase C chain),bll1188(FoF1 ATP synthase A chain) bsl1189(FoF1 ATP synthase subunit I);blr1190(unknown protein) blr1191(hypothetical protein), blr1192(hypothetical protein)
	LOS 6	brb09645	1.4	1.2	1.9	0.8	2.2	0.9	1.4	1.3	0.9	1.5	0.8	0.9	2561229	2563595	blr2358 blr2359	blr2358(probable glycosyl transferase), blr2359(hypothetical protein)
		brb15520	1.5	1.6	3.5	0.7	1.8	1.1	1.1	0.9	2.2	0.9	0.9	0.5	2561938	2564514	blr2358 blr2359 blr2360	blr2360(hypothetical protein)
		brb02692	1.3	1.5	2.5	0.6	1.8	1.1	1.0	1.1	1.6	0.8	1.1	0.9	2564680	2567408	blr2360 bll2361 bll2362	bll2361(unknown protein), bll2362(*exoP*)
		brb02534	0.9	1.0	1.5	0.7	1.1	0.5	1.0	1.1	1.5	0.8	0.8	0.6	2566776	2569688	bll2362 bll2363 bll2364	bll2363(unknown protein), bll2364(hypothetical protein)
		brb02058	1.4	1.6	2.3	0.8	1.1	1.5	1.1	1.4	1.5	1.1	0.9	0.8	2570129	2572546	bll2365 bll2366 blr2367	bll2365(unknown protein), bll2366(hypothetical protein)
		brb01016	1.7	3.6	4.0	0.7	2.3	1.2	1.1	1.7	1.7	1.3	1.4	0.9	2571788	2574315	bll2366 blr2367 blr2368	blr2367(putative acetyltransferase), blr2368(unknown protein)
		brb22261	1.1	1.5	1.7	1.2	1.1	1.2	1.0	1.2	1.3	1.0	1.2	1.5	2574485	2577223	blr2368 blr2369 blr2370	blr2369(*exoZ*), blr2370(unknown protein)
		brb07090	0.9	1.5	1.6	0.6	1.7	1.7	1.1	1.1	1.2	1.2	1.2	0.8	2576309	2578515	blr2370 blr2371 blr2372	blr2371(*cysE*),blr2372(unknown protein)
		brb02867	0.8	1.3	1.1	0.5	0.8	1.1	1.6	1.3	0.5	1.4	1.8	0.2	2579729	2582127	bll2373	bll2373(unknown protein)
		brb15981	1.0	1.7	2.1	0.8	1.0	1.5	1.1	0.9	2.4	0.8	1.0	1.0	2584003	2585439	blr2374	blr2374(probable glycosyl transferase)
		brb08951	1.3	1.2	1.9	0.6	1.5	1.0	1.2	1.1	1.5	0.7	1.2	0.7	2585568	2588560	blr2374 bll2375 bll2376 bll2377	bll2375(unknown protein), bll2376(probable glycosyl transferase)
		brb02062	0.6	1.4	3.6	0.8	0.6	0.9	0.9	1.2	5.1	0.7	0.8	0.5	2588270	2591009	bll2376 bll2377 bll2378	bll2377(probable glycosyl transferase), bll2378(unknown protein)
		brb14589	2.5	1.5	2.2	0.7	1.4	1.1	1.1	1.2	1.3	0.7	1.4	0.6	2588909	2591337	bll2377 bll2378 bll2379	bll2379(putative polysaccharide export protein)
		brb21527	1.0	2.8	2.9	0.6	1.6	0.8	1.8	2.0	2.4	1.0	1.2	0.6	2593191	2595246	bll2380 bll2381	bll2380(probable glycosyl transferase), bll2381(probable glycosyl transferase)
		brb20591	0.7	1.4	1.9	1.2	0.8	1.0	1.0	0.7	1.3	0.8	0.9	1.3	5433214	5436736	bsl4900 bll4901 bll4902 bll4903 bll4904	bsl4900(hypothetical protein), bll4901(hypothetical protein), bll4902(hypothetical protein)
		brb17359	0.9	1.3	2.7	1.0	0.6	1.2	0.9	0.7	2.1	0.6	0.7	1.2	5436732	5439280	bll4904 bll4905	bll4903(birA bifunctional protein), bll4904(NADH ubiquinone oxidoreductase chain N)
		brb03104	0.8	1.3	2.7	0.8	0.5	1.4	0.9	0.8	2.0	0.5	0.8	0.8	5438397	5441499	bll4905 bll4906 bll4907	bll4905(NADH dehydrogenase I chain M), bll4906(NADH ubiquinone oxidoreductase chain L)
		brb10650	0.8	1.3	2.3	1.0	0.7	1.3	1.1	0.9	2.3	0.7	0.7	0.8	5440677	5443993	bll4906 bll4907 bll4908 bll4909 bll4910	bll4907(hypothetical protein), bll4908(NADH ubiquinone oxidoreductase chain J)
		brb14818	1.2	1.1	1.4	1.0	0.7	1.0	0.7	0.9	1.5	0.6	0.8	0.7	5444167	5447785	bll4911 bll4912 bsl4913 bll4914	bll4909(NADH ubiquinone oxidoreductase chain I) bll4910(NADH ubiquinone oxidoreductase I chain H)

UP	LOS 13	brb13576	0.9	1.3	2.5	1.1	0.7	1.1	0.7	0.7	2.3	0.5	1.0	0.9	5447480	5450163	bll4912 bsl4913 bll4914 bll4915 bll4916	bll4911(NADH ubiquinone oxidoreductase chain G)
		brb20641	0.7	1.3	3.4	0.9	0.6	1.3	0.5	1.2	3.0	0.5	1.1	0.9	5449226	5451988	bll4915 bll4916 bll4917 bll4918 bll4919	bll4912(NADH ubiquinone oxidoreductase chain F), bll4913(unknown protein)
																		bll4914(NADH-ubiquinone oxidoreductase chain E (EC 1.6.5.3))
																		bll4915(hypothetical protein);bll4916(NADH ubiquinone oxidoreductase chain D)
																		bll4917(NADH ubiquinone oxidoreductase chain C)
																		bll4918(NADH ubiqionone oxidoreductase chain B);bll4919(NADH ubiquinone oxidoreductase chain A)
	LOS14	brb01427	0.7	2.9	3.5	0.5	0.5	0.9	1.0	1.9	2.6	0.5	0.9	1.0	5823599	5826683	blr5272 bsr5273 bll5274 bll5275	blr5272(arsenical pump membrane protein), bsr5273(unknown protein), bll5274(hypothetical protein)
		brb07471	0.6	6.5	7.4	0.7	0.5	0.8	1.0	3.6	14	0.3	0.9	1.2	5825567	5828129	bll5274 bll5275 blr5276 blr5277	bll5275(transcriptional regulatory protein GntR family),blr5276(hypothetical protein)
		brb07919	0.5	3.1	3.3	0.9	0.5	0.9	1.0	1.7	5.1	0.6	0.8	1.0	5827915	5830702	blr5276 blr5277 blr5278 bll5279	blr5277(gluconate dehydrogenase),blr5278(IdnD L-idonate 5-dehydrogenase)
																		bll5279(unknown protein)
	LOS 15	brb00383	0.9	1.6	3.7	1.0	1.0	1.7	0.8	1.4	3.3	1.2	1.3	2.5	5925788	5927382	bll5380 bll5381	bll5380(preprotein translocase SECY subunit), bll5381(50S ribosomal protein L15), bsl5382(50S ribosomal protein
		brb20776	0.7	1.6	6.1	0.9	0.9	1.2	0.7	1.1	4.7	0.6	0.9	0.5	5927313	5929967	bll5381 bsl5382 bll5383 bll5384 bll5385 bll5386	bll5383(30s ribosomal protein S5),bll5384(50S ribosomal protein L18), bll5385(50S ribosomal protein L6)
		brb06422	0.8	1.6	7.2	0.5	0.7	1.4	0.7	1.0	4.1	0.5	0.7	0.3	5929796	5932172	bll5386 bll5387 bll5388 bll5389 bll5390 bsl5391 bsl5	bll5386(30S ribosomal protein S8),bll5387(30S ribosomal protein S14), bll5388(50S ribosomal protein L5)
		brb17247	0.6	1.5	6.5	0.7	0.8	1.3	0.6	1.3	4.1	0.6	1.1	0.3	5931838	5933984	bsl5391 bsl5392 bll5393 bll5394 bll5395 bsl5396	bll5389(50S ribosomal protein L24),bll5390(50S ribosomal protein L14),bsl5391(30S ribosomal protein S17)
		brb21087	0.7	1.5	5.4	0.7	0.8	1.3	0.8	1.2	4.3	0.8	1.2	0.4	5934251	5936958	bll5397 bll5398 bll5399 bll5400 bll5401	bsl5392(50S ribosomal protein L29),bll5393(50S ribosomal protein L16), bll5394(30S ribosomal protein S3)
		brb16061	0.7	1.6	6.1	0.9	0.8	1.2	0.8	1.3	4.7	0.6	1.0	0.4	5934973	5937684	bll5398 bll5399 bll5400 bll5401 bll5402	bll5395(50S ribosomal protein L22),bsl5396(30S ribosomal protein S19),bll5397(50S ribosomal protein L2)
		brb08165	0.7	1.5	4.7	0.8	0.7	1.0	1.1	1.2	3.4	0.6	1.0	0.6	5938579	5941638	bll5403 bll5404 bll5405	bll5398(50S ribosomal protein L23),bll5399(50S ribosomal protein L4), bll5400(50S ribosomal protein L3)
		brb04566	0.7	1.6	6.2	0.8	0.9	1.4	0.9	1.0	5.0	0.7	1.2	0.4	5938604	5940204	bll5403	bll5401(30S ribosomal protein S10),bll5402(elongation factor TU), bll5403(translation elongation factor G)
		brb11323	1.3	1.2	1.5	1.1	1.4	0.9	0.8	1.7	1.1	0.8	1.4	0.8	5941490	5945067	blr5406 bll5407	bll5404(30S ribosomal protein S7), bll5405(30S ribosomal protein S12), blr5406(hypothetical protein)
		brb02695	0.6	0.9	1.8	0.7	0.9	0.9	1.0	1.1	1.0	1.0	0.9	0.8	5943669	5946249	bll5407 bll5408	bll5407(ABC transporter), bll5408(hypothetical protein)
		brb03672	0.6	1.4	2.2	1.0	1.0	0.8	1.1	1.1	2.1	0.8	1.0	0.8	5944983	5947676	bll5407 bll5408 bll5409	bll5409(DNA-directed RNA polymerase beta' chain)
		brb15817	0.5	1.7	1.8	1.3	0.4	2.0	1.1	1.3	1.1	2.4	1.0	0.9	5949409	5950953	bll5409	bll5410(DNA-directed RNA polymerase beta chain)
		brb22887	1.0	1.7	1.7	1.1	0.6	0.7	1.1	1.2	1.5	1.8	1.1	0.8	5953134	5954342	bll5410	bll5411(50S ribosomal protein L7/L12)
		brb04775	0.7	1.4	4.2	0.9	0.8	1.0	1.0	1.0	3.1	0.7	0.8	0.4	5955545	5957982	bll5410 bll5411 bll5412 bll5413	bll5412(50S ribosomal Protein L10), bll5413(2-hydroxyacid dehydrogenase)
		brb13427	1.1	1.4	2.6	0.6	1.0	0.9	0.8	1.0	3.8	0.9	0.8	0.7	5958263	5960809	bll5413 bll5414 bll5415 bll5416 bsl5417	bll5414(50S ribosomal protein L1), bll5415(50S Ribosomal Protein L11)
		brb13863	0.8	1.4	3.6	0.8	1.0	0.9	1.1	0.9	4.0	0.8	0.9	0.8	5959260	5961351	bll5414 bll5415 bll5416 bsl5417 bll5418	bll5416(transcription antitermination protein), bsl5417(preprotein translocase)
		brb05295	0.9	1.0	2.9	0.9	0.9	0.8	1.3	1.0	1.8	1.0	1.0	0.8	5961020	5964027	bll5418 bll5419 bll5420	bll5418(putative D-mycarose 3-C-methyltransferase)
																		bll5419(hypothetical protein), bll5420(putative phosphoheptose isomerase)
	LOS 19	brb04738	1.1	1.2	2.4	1.0	1.6	3.9	0.7	2.0	7.0	0.6	2.0	1.6	7540433	7543339	blr6843 bll6844 bsl6845 blr6846	blr6843(transcriptional regulatory protein MarR family);bll6844(unknown protein);bsl6845(unknown protein)
		brb07062	1.0	1.3	1.9	0.7	0.8	2.6	1.4	1.6	8.3	1.4	1.4	2.0	7543325	7545942	blr6846 bll6847 bll6848 bll6849 bll6850	blr6846(two-component response regulator)
		brb04395	0.7	1.3	1.5	1.0	0.9	1.2	1.1	1.0	2.8	1.0	1.1	1.5	7544197	7546812	bll6847 bll6848 bll6849 bll6850 bll6851	bll6847(hypothetical protein); bll6848(hypothetical protein);bll6849(hypothetical protein);
		brb03335	1.2	1.3	1.1	1.2	0.9	0.9	0.9	1.5	2.9	1.3	1.2	1.0	7546023	7548546	bll6850 bll6851	bll6850(probable flagellar biosynthetic protein),bll6851(flagellar biosynthesis protein)
		brb05157	1.2	0.7	0.7	0.8	1.1	2.3	1.0	1.7	6.4	0.8	1.1	1.7	7548759	7550466	bsl6852 bll6853 bll6854 bll6855 bll6856	bsr6852(flagellar biosynthetic protein);bll6853(hook formation protein),bll6854(flagellin synthesis repressor protein
		brb01103	1.4	0.5	1.3	0.8	0.7	2.5	1.0	1.8	16	0.4	1.1	1.3	7550500	7552997	bll6856 bll6857 bll6858	bll6855(probable flagellar protein),bll6856(probable flagellar hook-associated protein)
		brb13004	1.4	0.7	1.0	0.8	0.9	5.3	0.7	1.8	12	0.4	1.0	0.7	7552161	7554667	bll6857 bll6858 bll6859 bll6860	bll6857(hook associated protein I homolog),bll6858(flagellar hook protein)
		brb13860	1.2	1.1	0.6	1.0	0.6	0.9	1.3	1.1	1.4	1.2	1.0	3.8	7554103	7556604	bll6859 bll6860 bll6861	bll6859(hypothetical protein); bll6860(unknown protein),bll6861(probable chemotaxis protein precursor)
		brb15303	1.4	1.3	0.9	1.1	0.7	1.6	1.1	1.0	3.7	1.7	0.8	0.5	7557990	7560507	bll6862 bll6863 bll6864	bll6862(probable flagellar motor protein); bll6863(unknown protein); bll6864(flagellar M-ring protein)
		brb19251	0.9	0.8	1.2	0.6	1.1	6.2	0.9	1.9	8.1	0.4	0.7	0.3	7561370	7564255	bll6865 bll6866 bll6867	bll6865(flagellin); bll6866(flagellin); bll6867(flagellar biosynthetic protein)
		brb07735	0.9	1.0	0.9	0.5	0.4	3.8	0.8	1.8	6.7	0.5	1.0	0.7	7562179	7565456	bll6866 bll6867 bll6868 bll6869	bll6868(flagellar protein); bll6869(flagellar L-ring protein precursor)
		BJ7169	0.9	1.0	1.3	1.0	0.8	0.9	0.8	1.3	4.0	0.8	1.0	3.3	7565515	7566267	bll6870	bll6870(hypothetical protein)
		brb02602	0.9	1.1	1.0	0.8	0.8	2.0	1.3	1.9	3.5	0.9	1.5	0.8	7566428	7569607	bll6871 bll6872 bll6873 bll6874 bll6875 bll6876	bll6871(flagellar P-ring protein precursor); bll6872(probable flagellar protein);
		brb14708	1.4	0.8	1.1	1.1	1.0	0.7	1.0	1.3	8.0	0.6	0.7	1.2	7569003	7572036	bll6874 bll6875 bll6876 bll6877 bll6878	bll6873(flagellar basal-body rod protein); bll6874(flagellar hook-basal body complex protein)
		brb00559	0.9	1.1	1.4	0.8	1.3	1.7	0.9	1.3	2.3	1.1	1.0	0.9	7570457	7573188	bll6877 bll6878 bll6879 bll6880 bll6881	bll6875(flagellar basal-body rod protein); bll6876(flagellar basal-body rod protein)
		brb16425	1.4	0.9	1.3	1.1	0.9	2.5	1.1	1.8	11	1.7	1.2	1.1	7572507	7575109	bll6880 bll6881 bll6882 blr6883	bll6877(flagellar biosynthetic protein); bll6878(probable flagellar motor switch protein)
																		bll6879(probable flagellar motor switch protein); bll6880(unknown protein)
																		bll6881(hypothetical protein), bll6882(chemotaxis protein),blr6883(hypothetical protein)
		brb17677	0.9	0.1	0.1	0.7	0.7	0.9	0.3	0.3	0.0	0.9	1.3	1.0	6803542	6805866	bll6183	bll6183(hypothetical protein)
		brb04919	1.6	0.9	0.4	0.9	0.6	1.1	0.9	0.8	0.4	0.7	1.0	0.9	6806056	6808159	bll6183 bll6184 blr6185	bll6184(two-component sensor histidine kinase); blr6185(two-component response regulator)

	brb21053	1.2	0.4	0.4	0.8	0.9	0.9	1.3	0.7	0.4	1.9	1.2	1.6	6808422	6810041	blr6186 bll6187	blr6186(esterase D); bll6187(hypothetical protein)
	brb19970	1.2	0.5	0.7	0.5	0.6	1.2	1.2	0.8	0.3	0.6	1.1	1.5	6810004	6812991	bll6187 blr6188 blr6189 blr6190	blr6188(hypothetical protein); blr6189(hypothetical protein);blr6190(ABC transporter ATP-binding protein)
	brb00577	1.2	0.5	0.4	0.9	0.6	1.1	1.0	0.6	0.3	0.5	0.5	0.9	6812628	6815116	blr6190 blr6191 blr6192 bsr6193 bll6194	blr6191(ABC transporter permease protein);blr6192(hypothetical protein)
	brb19372	1.0	1.2	0.4	0.7	0.6	1.0	0.7	0.9	0.4	0.7	1.0	1.3	6813822	6816494	blr6191 blr6192 bsr6193 bll6194 blr6195 bll6196	bsr6193(unknown protein); bll6194(hypothetical protein),blr6195(hypothetical protein)
	brb00336	1.3	0.7	0.6	1.2	0.8	1.2	1.2	0.8	0.4	1.4	1.2	1.5	6816563	6818731	blr6195 bll6196 blr6197 bll6198	bll6196(putative methanol oxidation protein),blr6197(hypothetical protein)
	brb00330	1.6	0.7	0.6	1.0	0.8	1.1	0.8	1.3	0.9	0.8	1.2	2.2	6818133	6820611	blr6197 bll6198 blr6199 blr6200	bll6198(bhypothetical protein),blr6199(ABC transporter substrate-binding protein)
	brb03633	1.5	0.8	0.7	1.1	0.7	1.0	0.7	1.0	1.3	0.7	0.8	3.1	6818971	6821755	bll6198 blr6199 blr6200 blr6201 blr6202	blr6200(ABC transporter permease protein),blr6201(ABC transporter ATP-binding protein)
LOS18	brb00301	2.0	1.0	0.7	1.0	1.0	1.6	0.9	0.8	0.4	0.8	1.0	0.4	6820107	6822540	blr6200 blr6201 blr6202 blr6203	blr6202(hypothetical protein),blr6203(unknown protein)
	brb18073	0.8	0.2	0.4	1.1	1.5	0.8	0.8	0.6	0.4	0.9	1.3	1.8	6822071	6824542	blr6202 blr6203 bsl6204 bll6205 bll6206	bsl6204(hypothetical protein); bll6205(hypothetical protein); bll6206(hypothetical protein)
	BJ7131	0.6	0.1	0.1	0.7	0.3	2.0	0.8	0.5	0.1	0.8	1.8	0.6	6825170	6826671	blr6207	blr6207(probable quinoprotein ethanol dehydrogenase precursor (EC 1.1.99.-))
	brb24507	0.6	0.6	0.4	0.9	0.5	1.1	1.5	0.8	0.6	1.2	0.6	0.6	6826718	6828345	blr6207 blr6208 blr6209	blr6208(putative cytochrome C55X precursor); blr6209(unknown protein)
	brb03580	1.0	0.1	0.2	1.2	0.6	1.2	1.1	0.3	0.2	0.9	0.8	0.7	6828411	6831030	blr6210 blr6211 bll6212	blr6210(hypothetical protein); blr6211(unknown protein)
	brb23581	1.0	0.3	0.2	1.0	0.8	1.2	1.5	0.5	0.2	0.7	0.9	1.1	6829824	6832439	blr6211 bll6212 blr6213	bll6212(transcriptional regulatory protein AraC family)
	brb15565	0.7	0.9	0.4	0.7	0.8	1.4	2.0	0.6	0.3	0.8	1.0	1.7	6832515	6835422	blr6213 blr6214 blr6215 blr6216	blr6213(methanol dehydrogenase large subunit-like prote);blr6214(putative cytochrome c protein)
	brb10395	0.9	0.8	0.4	1.3	1.2	0.9	1.3	0.7	0.3	2.4	1.4	1.6	6834420	6837301	blr6215 blr6216 bsr6217 blr6218 blr6219	blr6215(Icohol dehydrogenase class III), blr6216(hypothetical protein), bsr6217(unknown protein)
	brb11784	1.0	0.6	0.2	0.7	0.8	1.4	1.0	0.7	0.1	1.0	1.3	0.4	6840320	6842085	bll6220 bll6221	blr6218(putative oxidoreductase protein), blr6219(putative aldehyde dehydrogenase) bll6220(putative alcohol dehydrogenase precursor), bll6221(Rieske iron-sulfur protein)
	brb19414	1.4	0.3	0.1	1.0	1.0	1.5	0.6	0.9	0.1	1.1	2.2	2.8	8039932	8042674	bll7306 bll7307 bll7308	bll7306(two-component response regulator); bll7307(two-component response regulator)
LOS 21	brb00154	1.0	0.3	0.2	0.8	0.8	1.0	0.8	0.8	0.2	0.9	1.2	0.7	8043817	8046068	bll7309 bll7310 bll7311	bll7308(unknown protein),bll7309(unknown protein) bll7310(hypothetical protein), bll7311(hypothetical protein)

*: Genes supposed to be exclusively up-or down-regulated by SSE at 12 hpi were underlined.

Appendix 4: Regulation of genomic loci outside symbiosis island (LOS 7-12,16-17, 20, 22) and their covered genes by both SSE and genistein

Regulation	LOS	Clone	30℃ SSE 0.5h	30℃ SSE 6h	30℃ SSE 12h	30℃ GEN 0.5h	30℃ GEN 6h	30℃ GEN 12h	15℃ SSE 12h	15℃ SSE 20h	15℃ SSE 48h	15℃ GEN 12h	15℃ GEN 20h	15℃ GEN 48h	Genome position start	Genome position end	Covered gene (entirely or partially) *	Annotated gene function
UP	LOS7	brb21116	0.8	2.5	2.1	0.9	3.7	0.9	1.1	2.7	0.8	0.9	3.8	0.8	2653305	2656410	bsl2435 blr2436 blr2437 blr2438	bsl2435(hypothetical protein),blr2436(glycerol-3-phosphate dehydrogenase)
		brb03205	1.2	2.9	2.8	0.6	7.2	0.8	0.5	2.0	0.4	0.5	4.3	0.7	2655849	2658215	blr2437 blr2438 blr2439	blr2437(ABC transporter ATP-binding protein)
		brb14004	0.8	3.1	5.4	0.6	18	1.1	0.5	3.3	0.4	0.4	19	1.2	2657509	2660598	blr2439 blr2440 blr2441 blr2442	blr2438(ABC transporter ATP-binding protein) blr2439(ABC transporter permease protein) blr2440(ABC transporter permease protein),blr2441(hypothetical protein) blr2442(ABC transporter substrate-binding protein)
	LOS 9	brb06269	1.7	3.4	2.6	1.4	4.7	3.5	2.8	2.8	2.2	3.8	3.6	1.4	4683435	4686482	bll4251 bll4252 bll4253	bll4251(MFS permease); bll4252(putative hydrolase)
		brb14218	1.6	2.8	2.5	2.1	4.1	3.8	3.3	3.0	1.3	4.3	4.5	2.6	4685774	4689719	bll4253 bll4254 blr4255 bll4256	bll4253(transcriptional regulatory protein AraC family) bll4254(transcriptional regulatory protein AraC family) blr4255(transcriptional regulatory protein AraC family); bll4256(unknown protein)
	LOS10	brb01762	2.2	1.8	2.1	2.1	2.3	2.4	1.7	2.1	2.8	3.3	4.3	5.3	4768249	4770588	blr4318 bll4319	blr4318(hypothetical protein)
		brb17502	1.5	3.9	2.8	2.8	4.9	3.2	3.9	2.9	3.6	3.9	5.9	6.8	4769091	4771323	bll4319	bll4319(putative multidrug resistance protein)
		brb07789	3.5	5.5	3.6	5.7	2.9	6.1	4.8	4.9	5.7	7.3	9.9	12	4770696	4774431	bll4319 bll4320 bll4321	bll4320(probable RND efflux membrane fusion protein)
		brb18178	1.1	1.9	1.6	2.7	2.1	1.9	2.5	1.4	2.6	2.4	3.3	6.5	4773953	4776685	bll4321 blr4322 bll4323	bll4321(putative outer membrane channel lipoprotein) blr4322(transcriptional regulatory protein TetR family), bll4323(probable bacterioferritin)
	LOS 12	brb11706	6.9	9.2	2.2	14	22	7.8	27	7.5	0.8	32	7.9	1.0	5285628	5288526	bll4771 blr4772 blr4773	bll4771 (unknown protein), blr4772(unknown protein)
		brb23885	6.0	13	3.8	21	28	4.4	75	19	1.6	53	15	2.2	5286568	5289745	blr4772 blr4773 blr4774	blr4773(nwsA)
		brb04065	1.4	2.8	0.9	2.2	2.3	1.7	9.9	2.5	0.8	12	3.2	1.7	5288076	5290601	blr4773 blr4774 blr4775	blr4774(nwsB), blr4775(two-component response regulator)
	LOS 16	brb23266	7.9	3.6	3.6	11	6.9	3.6	4.4	2.0	3.6	19	6.1	15	6355896	6358532	blr5790 bsr5791 blr5792 blr5793	blr5790(putative lignostilbene-alpha,beta-dioxygenase),bsr5791(unknown protein),
		brb22037	3.7	2.4	2.6	7.7	2.5	2.2	2.1	1.3	4.4	6.8	2.6	5.2	6358247	6360634	blr5792 blr5793 bsr5794 blr5795 bll5796	blr5792(hypothetical protein),blr5793(hypothetical protein),bsr5794(hypothetical protein) blr5795(unknown protein), bll5796(Fumarate hydratase class I (EC 4.2.1.2))
	LOS 20	brb19679	1.1	0.9	0.7	6.1	1.6	1.1	0.7	0.7	1.2	5.5	1.2	2.0	7729680	7731626	bll7017 bll7018	bll7017(transcriptional regulatory protein LuxR family), bll7018(unknown protein)
		BJ7162	2.6	1.7	0.8	33	1.2	1.4	1.0	1.6	1.5	19	1.9	6.8	7732105	7735128	bll7019	bll7019(AcrB/AcrD/AcrF family protein)
		brb12742	1.3	1.4	1.1	21	1.6	2.0	1.6	1.2	0.6	15	2.4	1.4	7735902	7739183	bll7020 bll7021 bll7022 blr7023 bll7024	bll7020(efflux protein), bll7021(HlyD family secretion protein)
		brb14752	2.4	1.2	1.1	21	2.1	1.3	1.6	1.1	1.0	20	1.8	4.5	7736079	7738415	bll7020 bll7021 bll7022	bll7022(unknown protein),blr7023(transcriptional regulatory protein TetR family)
		brb07276	4.6	2.8	1.4	57	3.4	5.7	1.5	1.7	2.0	23	4.2	12	7738326	7740920	blr7023 bll7024 bll7025	bll7024(transcriptional regulatory protein TetR family)), bll7025(hypothetical protein)
		brb06980	1.8	2.8	1.3	8.7	2.8	2.8	1.4	0.7	2.1	13	1.3	7.9	7740752	7742620	blr7026	blr7026(unknown protein), blr7027(hypothetical protein)
		brb11559	3.2	3.0	1.9	37	5.4	1.5	2.1	1.1	1.4	49	4.6	7.2	7742522	7745552	blr7026 blr7027 blr7028 blr7029	blr7028(Acetoacetate decarboxylase (EC 4.1.1.4))
		brb08642	2.1	2.2	1.1	51	4.4	2.1	1.3	1.1	1.6	40	3.6	5.1	7744483	7747504	blr7028 blr7029 blr7030 blr7031 bll7032	blr7029(d-beta-hydroxybutyrate dehydrogenase),blr7030(unknown protein),
		brb00329	1.1	2.3	0.7	3.8	1.4	1.9	0.8	1.6	1.4	7.1	2.2	2.0	7746514	7748456	blr7031 bll7032 bll7033	blr7031(hypothetical protein),bll7032(putative cyclic NTP-blinding protein) bll7033(LuxA-like protein)
Down	LOS 11	brb24028	1.0	0.5	0.4	0.8	0.5	0.7	0.7	0.6	0.7	0.6	0.6	1.6	5043533	5046007	blr4550 bll4551 bll4552	blr4550(glucokinase (EC 2.7.1.2)),bll4551(two-component response regulator)
		brb06530	1.0	0.4	0.2	1.0	0.8	0.7	1.0	0.4	0.3	0.8	0.8	0.7	5045061	5047480	bll4551 bll4552 blr4553	bll4552(unknown protein),blr4553(ABC transporter substrate-binding protein)
		brb05409	1.1	0.4	0.1	0.7	0.5	0.9	0.6	0.4	0.1	0.5	0.7	0.5	5047069	5049615	blr4553 blr4554 blr4555 blr4556	blr4554(hypothetical protein),blr4555(similar to ABC transporter permease protein)
		brb03357	0.9	0.9	0.3	0.7	1.2	1.2	1.0	0.6	0.4	1.7	1.0	0.7	5051171	5053672	blr4557 blr4558 bsr4559 bll4560	blr4556(ABC transporter permease protein),blr4557(ABC transporter ATP-binding protein) blr4558(probable d-3-phosphoglycerate dehydrogenase) bsr4559(hypothetical protein), bll4560(hypothetical protein)
	LOS 8	brb13845	1.2	1.0	0.0	0.8	0.5	0.4	2.1	0.5	0.0	0.2	0.5	8.9	4066292	4069387	blr3677 blr3678 blr3679	blr3677(putative monooxygenase component),blr3678(putative oxidoreductase)
		brb16105	2.4	1.1	0.0	0.9	0.6	0.1	0.3	0.4	0.0	0.2	0.5	8.9	4069698	4072061	blr3679 blr3680 blr3681 blr3682	blr3679(putative monooxygenase component),blr3680 (hypothetical protein)

	brb08086	1.1	1.2	0.2	1.0	0.5	0.5	0.9	0.6	0.2	1.0	0.6	6.5	4072007	4075099	blr3682 blr3683 blr3684 blr3685	blr3681(hypothetical protein),blr3682 (hypothetical protein),blr3683(chaperonin GroEL) blr3684(unknown protein),blr3685 (transcriptional regulatory protein Fis family)
LOS 17	brb01031	0.8	0.4	0.3	0.7	0.3	0.4	0.5	0.5	0.6	0.7	1.1	0.3	6407174	6409725	blr5840 blr5841 bll5842 bll5843	blr5840(hypothetical protein),blr5841(hypothetical protein)
	brb02759	0.9	0.3	0.3	0.9	0.2	0.3	0.6	0.3	0.6	0.8	0.3	0.2	6410484	6412461	bll5843 bll5844	bll5842(hypothetical protein), bll5843(hypothetical protein)
	brb24974	0.5	0.2	0.3	0.7	0.2	0.4	0.4	0.3	0.5	0.6	0.3	0.2	6411464	6413904	bll5844	bll5844(hypothetical protein)
	brb12403	0.6	0.2	0.3	0.7	0.2	0.4	0.7	0.6	0.4	0.9	0.4	0.1	6413720	6416366	bll5845	bll5845(hypothetical protein)
	brb03026	0.6	0.2	0.2	0.7	0.1	0.2	0.6	0.5	0.5	0.7	0.4	0.2	6416373	6418971	bll5846	bll5846(hypothetical protein)
LOS 22	brb08591	0.6	0.1	0.1	0.7	0.4	0.4	0.5	0.7	0.3	0.3	0.7	0.4	8077590	8080244	bll7340 bll7341 bll7342	bll7340(hypothetical protein),bll7341(RhtB family transporter)
	brb09669	0.7	0.2	0.2	0.9	0.7	0.3	0.7	0.5	0.3	0.8	0.5	0.4	8078788	8081248	bll7341 bll7342 blr7343	bll7342(two-component response regulator) blr7343(probable short-chain dehydrogenase)

**: Genes supposed to be up-or down-regulated by both SSE and genistein were underlined.*