Soybean Seed Extracts Preferentially Express Genomic Loci of *Bradyrhizobium japonicum* in the Initial Interaction with Soybean, *Glycine max* (L.) Merr

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Abstract

   Initial interaction between rhizobia and legumes actually starts 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 synthesis, 1-aminocyclopropane-1-carboxylate 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.
Key words: soybean seed extracts; *Bradyrhizobium japonicum*; expression clusters; genistein; symbiosis

1.   Introduction

   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

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in rhizosphere and were reported to correlate with the competition for the nodulation between rhizobia, e.g. non-motile and -chemotactic mutants required 10- to 30-fold number of cells to form nodules at the same rate as the wild strain.[1] 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.[2] Amino acids such as glutamate and dicarboxylic acids such as succinate are reported to have a strong attractive action to *Bradyrhizobium japonicum* cells.[3,4]

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 that specifically trigger various plant responses and initiation of cell division to form the nitrogen-fixing root nodules.[5,6] 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.[7–11] Unlike the exudates, however, these isoflavones were not chemoattractant of *B. japonicum* cells, although *Rhizobium* cells exhibited chemotaxis to their *nod*-genes inducer.[12,13] 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 2- to 3-fold induction in the presence of soybean seed extracts (SSE).[14] These reports suggest that the signal exchange in the early stages of *B. japonicum*-soybean symbiosis cannot be explained only by a simple paradigm of flavonoid-Nod factors.

The whole genomic sequence of *B. japonicum* USDA 110 (9.10 Mb) has been recently completed, and as in *Mesorhizobium loti* MAFF303099, *B. japonicum* also carries a symbiosis island of 681 kb on genome, in which most of the key genes relevant to the symbiotic nitrogen fixation (*nod, nif,* and *fix*) are concentrated.[15,16] A oligonucleotide microarray for genome-wide transcript analysis in *B. japonicum* under a variety of conditions, such as minimal versus rich medium, free-living versus bacteroids, aerobic versus microaerobic, and desiccation versus osmotic stress, has also been reported, recently.[17–19] 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, we 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. Materials and methods

2.1. Bacterial strains and culture conditions

Bradyrhizobium japonicum USDA 110 was used as a wild-type strain for global expression analysis throughout the experiment. *Bradyrhizobium japonicum* ZB977 harboring a plasmid (pZB32) with a *lacZ* fusion in *nodY* based on the background of *B. japonicum* USDA 110[8] was used as an indicator strain to determine the induction conditions (Supplementary Fig. S1). *Bradyrhizobium japonicum* USDA 110 and ZB977 were maintained on the yeast extract-mannitol agar (YMA) with chloramphenicol (30 μg/mL) and tetracycline (50 μg/mL), respectively, and cultured in yeast extract-mannitol broth (YMB) without antibiotics for the induction experiments in the presence of SSE or genistein.[20,21]

2.2. RNA preparation, hybridization, and image capture

Bradyrhizobium japonicum USDA 110 cells were pre-cultured in 50 mL of YMB without antibiotics for 3 days at 30°C and then scaled up to 200 mL of YMB for growing to mid- 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 (5.0 μM) under the conditions determined by *B. japonicum* ZB977. 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.[22] 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 MICROBExpress[TM] Kit (Ambion, Austin, TX, USA), cDNA labeling was carried out as described previously.[23]

We used a DNA macroarray of *B. japonicum* USDA 110, and hybridization, image acquisition, and data analyses were done as described previously.[23,24] 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% SDS for 10 min, and twice at 55°C in 0.3 × SSC

containing 0.1% 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.

2.3. 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 on the basis of 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 3960 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).[25]

2.4. 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://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Three hundred nanograms of total RNA was used as a template and real-time RT−PCRs were performed with MiniOpticon[TM] 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,[18,23] since the expression levels of *sigA* gene, which was regularly spotted on the array membranes, were not significantly changed with the SSE/genistein-treatment in this experimental condition.

3. Results

### 3.1. One giant expression region corresponds to the symbiosis island

The SSE-induced *nodY−lacZ* expression at the different cell densities was monitored by β-galactosidase activity (Supplementary Fig. S1), and the comparative genome-wide expression profiles of the cells in response to SSE and genistein were captured under the following conditions: initial cell density, $OD_{600} = 0.1$; SSE and genistein concentration, 20 μL/mL and 5 μM; induction periods, 0.5, 6 and 12 h post-induction (hpi). As shown in Fig. 1, 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 as compared with 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) (Fig. 1), 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 Fig. 5B, 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) were 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 five (6%) were significantly down-regulated in this region. The results here show that symbiosis island is induced by both SSE and genistein, but the expression levels were much more intensive for SSE-treatment.

3.2. Symbiosis island functions as great expression clusters

Fig. 2 shows the expression profiles of symbiosis island in a smaller window size. In consequence, four predominant expression clusters (ECs) were identified and designated as EC-I, -II, -III, and -IV considering more than 10 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 was 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 of it was also found in
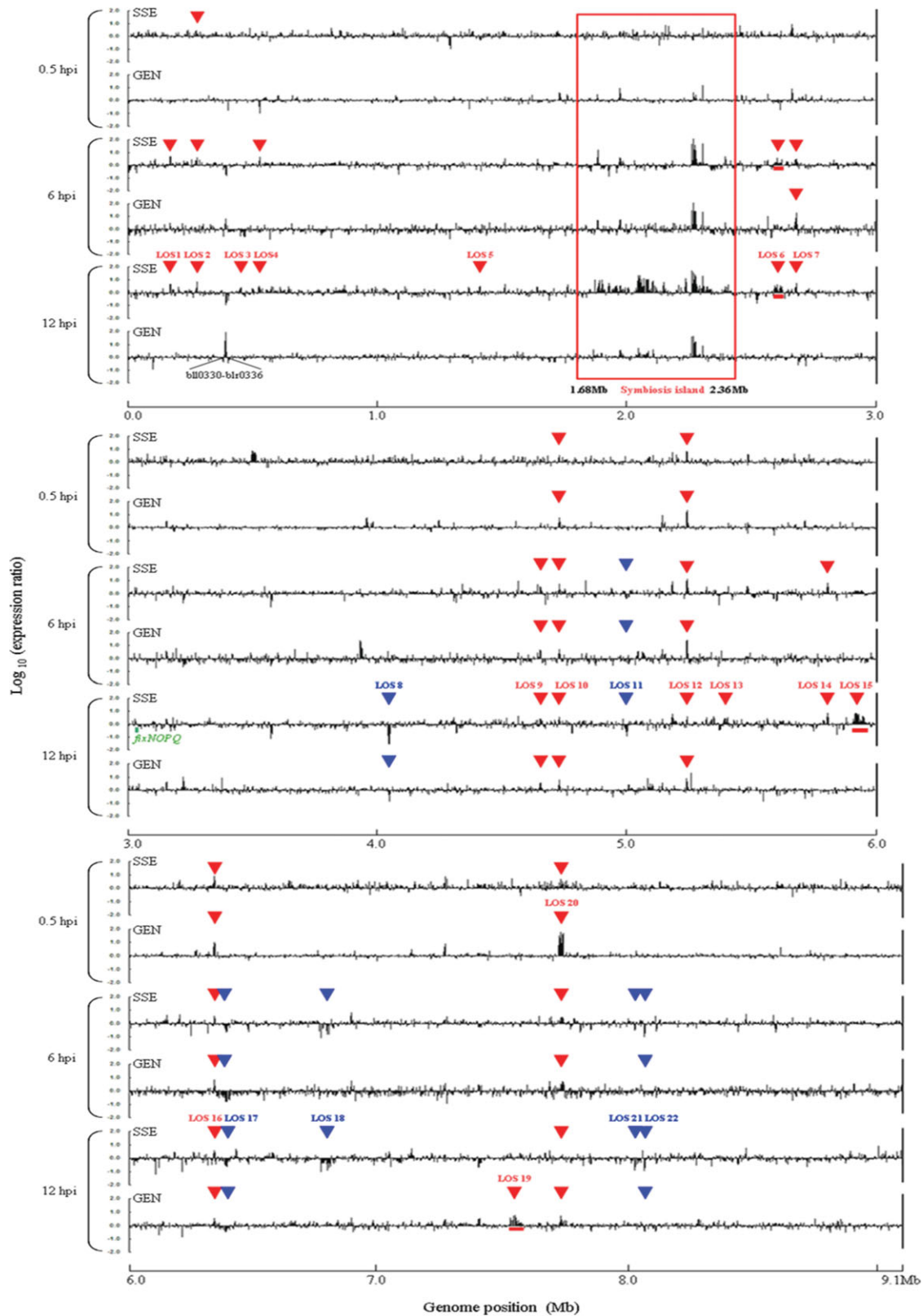
Figure 1. Genome-wide expression profiling of *B. japonicum* USDA 110 in response to SSE and genistein (GEN) at 0.5, 6, and 12 hpi, respectively. Expression profiles were constructed as the value of $\log_{10}$ (expression ratio) of 3960 clones covering the whole genome as described in Section 2. Expression 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 1.68–2.36 Mb.[16] 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 Supplementary Tables S3 and S4, respectively.
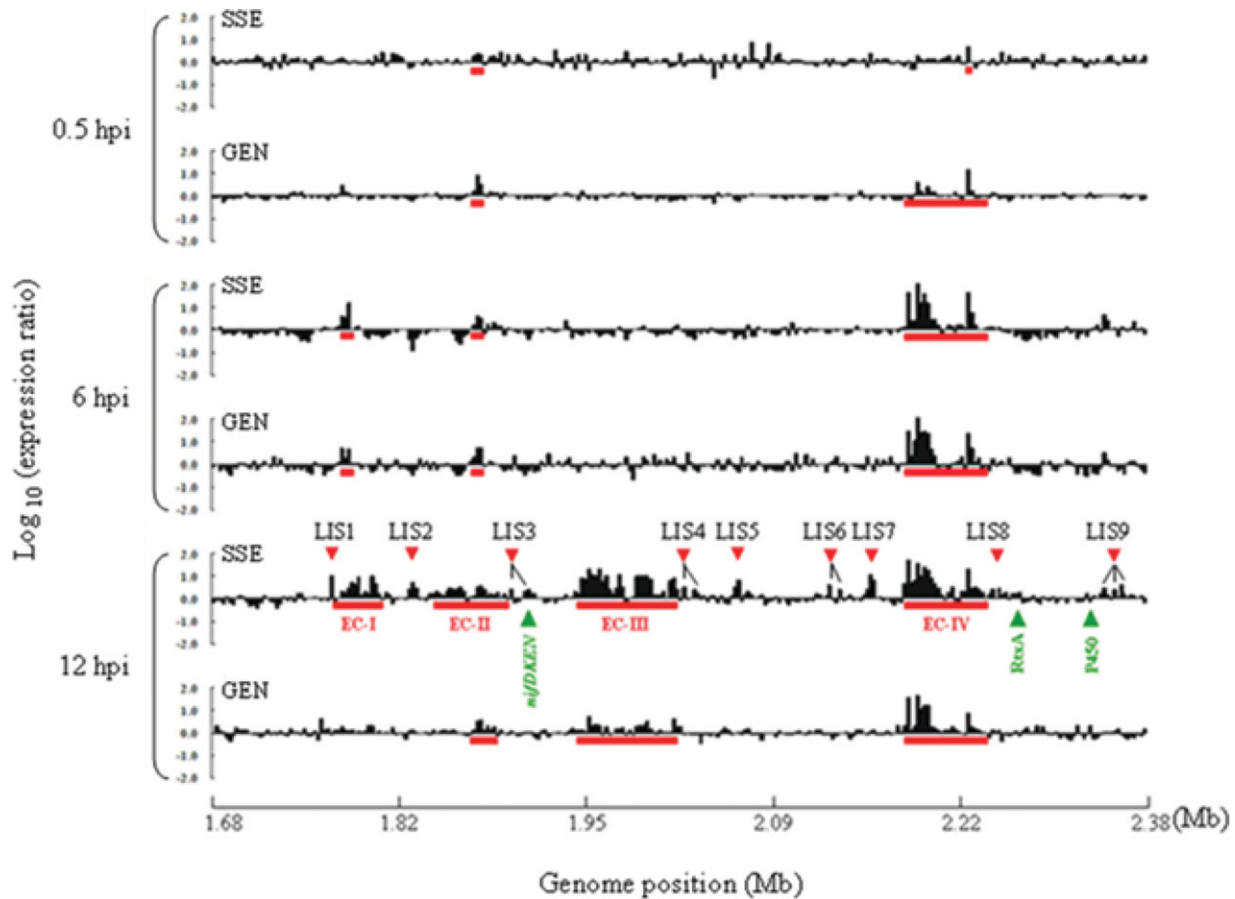
Figure 2. Expression profiles of the symbiosis island of *B. japonicum* USDA 110 induced by SSE and genistein (GEN) at 0.5, 6, and 12 hpi, respectively. Expression profiles were constructed as the value of $\log_{10}$ (expression ratio) of 276 clones covering symbiosis island as described in Section 2. Expression levels of each clone were sorted according to the genome coordinate and represented 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 arrows.

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 Figs 2 and 3 and Supplementary Table S1.

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 five genes that encode multi-drug 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 23 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 three genes that encode hypothetical (bll1703, blr1704) or unknown proteins (blr1705) was only induced by SSE at 12 hpi. Whereas, the latter locus covering four genes that encode two-component response regulator (bsl1713), NodW (bll1714), NodV (bll1715), and putative transposase (blr1716) was induced 2.1- to 8.6-fold by both SSE and genistein during 12 h. NodWV was identified as a two-component system that is unique to *B. japonicum* and directly induces *nodYABC* operon in response to genistein.[14,26,27] 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 20 clones among them (69.0%) were induced 2.4- to 20.7-fold by SSE at 12 hpi. On the other hand, most of the clones (20 among
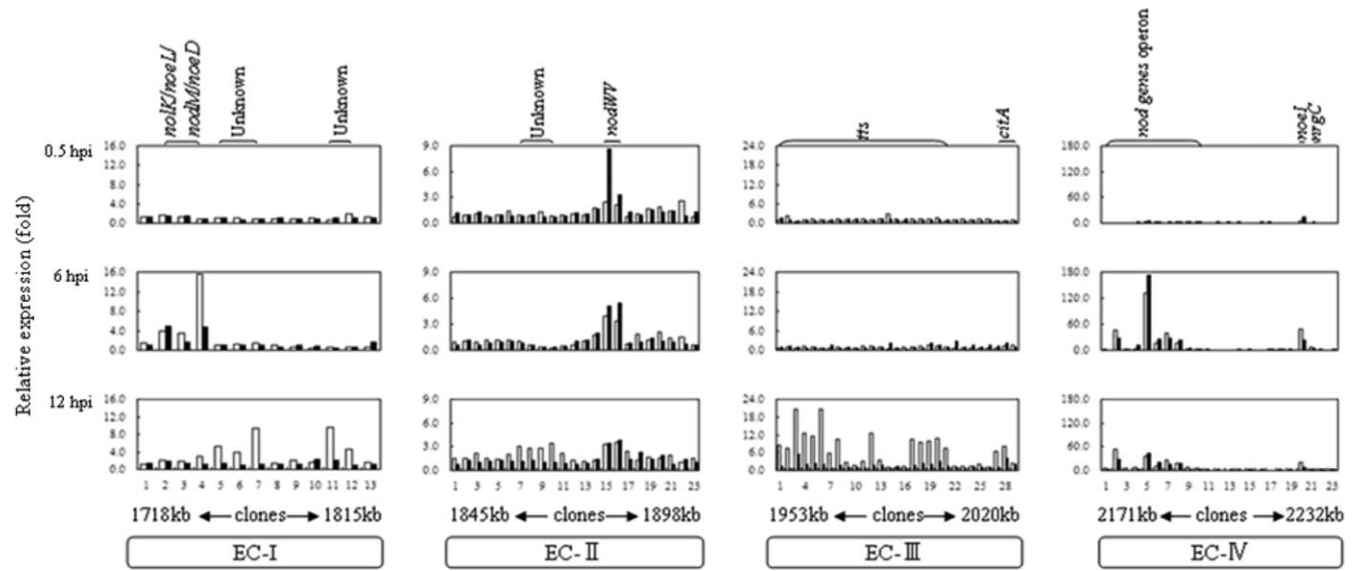
Figure 3. Comparative expression profiles of clones and their covered genes within ECs after the treatment of SSE or genistein at 0.5, 6, and 12 hpi, respectively. *tts* and *nod* genes operon correspond to *rhcC1C2JNQRSTUV* for the type III secretion system and *nolZY-nolA-nodD2-nodD1YABCSUIJ-nolMNO-nodZ* for the nodulation, respectively. The detailed expression profiles of EC-III and -IV are shown in Fig. 4 and Supplementary Table S1. White and black bars indicate the expression levels of clones in response to SSE and genistein, respectively.

29 clones) also tended to be up-regulated by genistein, however, only five clones (clone 3, 5, 18, 20, and 28) exhibited the significant expression levels, indicating that this cluster was significantly and preferentially induced by SSE (Figs 3 and 4A). 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, respectively. A genes cluster (*tts*) encoding recently identified type III secretion system (TTSS) of *B. japonicum*, *rhcC1C2JNQRSTUV* (bll1811−bll1842−blr1813−blr1816−blr1818−blr1819−bsr1820−blr1821−blr1822−bll1800),[16,28] 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.[29] 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, respectively.[30] This experiment showed that clones covering these genes were induced by SSE (Supplementary Table S1). Among six genes covered by the third locus, only two genes have putative functions, i.e. putative transposase (bll1861) and citrate−proton symporter (bll1864, *citA*), respectively. However, the presence of a *nod* box located between bll1862 and bsr1863, as well as a σ^{54} consensus between bll1864 and

blr1865,[28] suggests that the third locus might 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) (Figs 2 and 3). Among the 23 clones covered in EC-IV, 16 clones (clone 1−11, 15, and 20−23) (69.6%) were strongly induced by SSE at 12 hpi (2.8- to 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 (Fig. 4B). Clone 1−11 covering 31 genes from bsr2010 to bll2040 completely involved a great genes cluster, *nolZY*-[2genes]-*nolA*-[1gene]-*nodD2*-[1gene]-*nodD1YABCSUIJ-nolMNO-nodZ-fixR-nifA-fixA*(bsl2015-blr2038), which was reported previously.[16,31] The *nolZY-nolA-nodD2-nodD1YABCSUIJ-nolMNO-nodZ* has been identified as a common *nod* genes operon involved in the biosynthesis, modification and transport of Nod-factor and reported to be essential for the process of nodulation.[32,33] Downstream of this *nod* genes operon, clone 20 was induced at least 4.5-fold by both SSE and genistein during 12 h, suggesting that either or both of its covered two adjacent genes (bsr2061 and blr2062) were induced strongly. The blr2062 has been estimated to encode a nodulation protein, NoeI, although the function of bsr2061 is still unknown. These results support that *nod* genes 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.
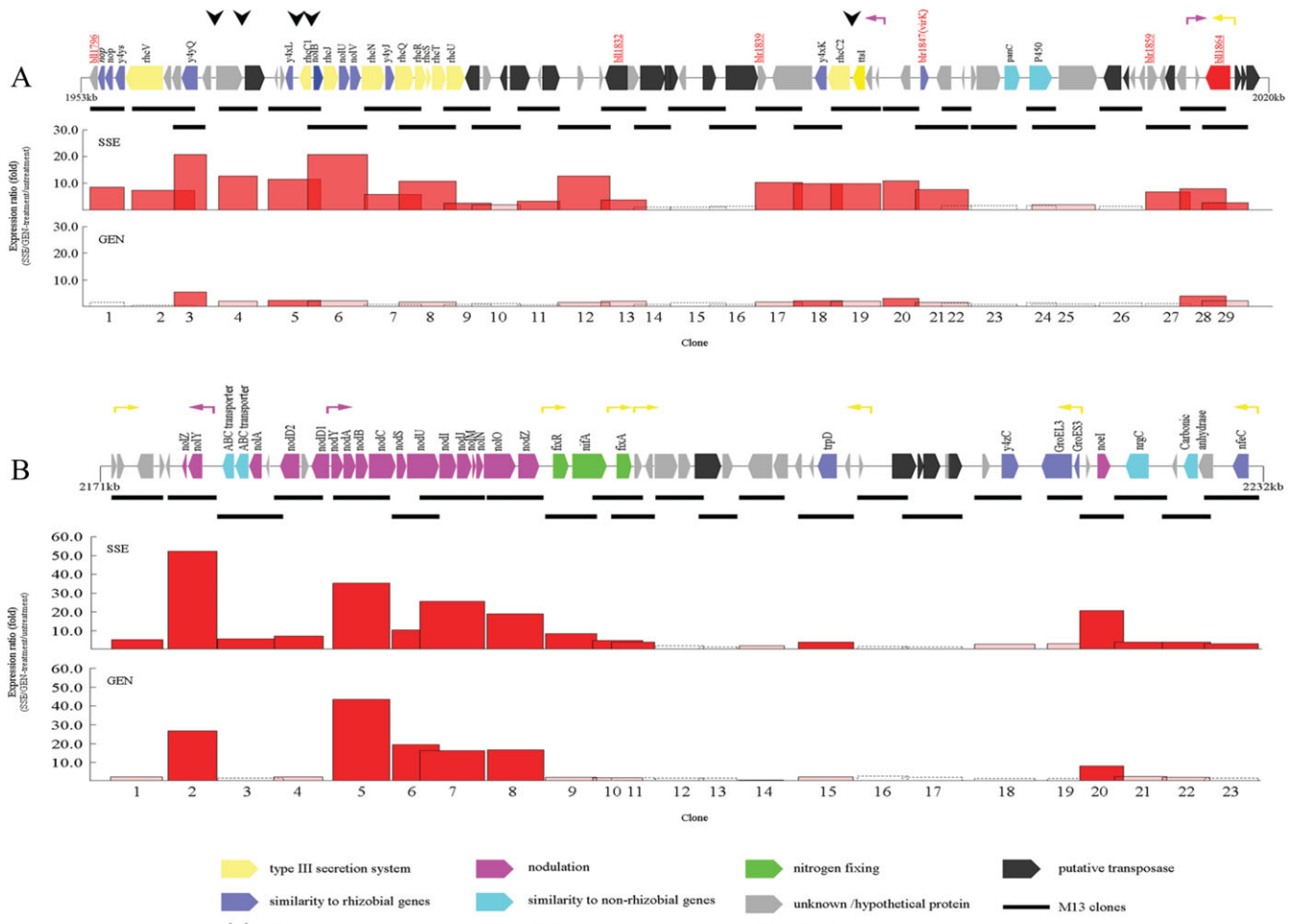
Figure 4. Expression profiles of clones and their covered genes in (**A**) EC-III and (**B**) EC-IV at 12 hpi after the treatment of SSE and genistein (GEN). Bars below the genes map indicate M13 clone inserts for macroarray construction and the box charts under M13 clone indicate differential expression of M13 clones in SSE/GEN-treated cells to untreated cells. The solid boxes indicate clones with strong (expression ratio $\geq$ 2.2-fold, red) and mediate (1.5-fold $\leq$ 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-ATCCAAACAATCGATTTTACCAATC), and δ^{54} consensus (TGGCAC-N5-TTGCT/A) are described previously.[28,30]

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.[34] The loci covered by clone 15 and 22−23 were also induced beyond 2.8-fold by SSE at 12 hpi, but only three genes bll2049, bll2065, and bll2067 have functions as anthranilate phosphoribosyltransferase (*trpD*), carbonic anhydrase, and nodulate formation efficiency C protein (*nfeC*), respectively.

Clones covering a large genes cluster, *nifDKENX-fer3-nifS-fixU-nifB-frxA-nifZ-nifH- fixBCX* (blr1743− bsr1775), which are located between EC-II and -III and likely to be required for nitrogen fixation under microaerobic conditions,[16] showed no potential changes of the expression in the presence of either SSE or genistein except for one clone

covering genes from bsr1749 to blr1752 (Fig. 2). One operon composed of eight ORFs (blr2077− bsl2084), which was reported to be likely involved in the biosynthesis of rhizobitoxine-like (RtxA) molecule,[28] 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 report that rhizobitoxine production is confined exclusively to genotype II strains of *B. japonicum* and cytochrome P450 are well expressed in bacteroids.[35,36]

3.3. Exclusively expressed loci by SSE

Nine loci covered by 19 clones inside symbiosis island but outside ECs (LISs: LIS 1−9) were exclusively
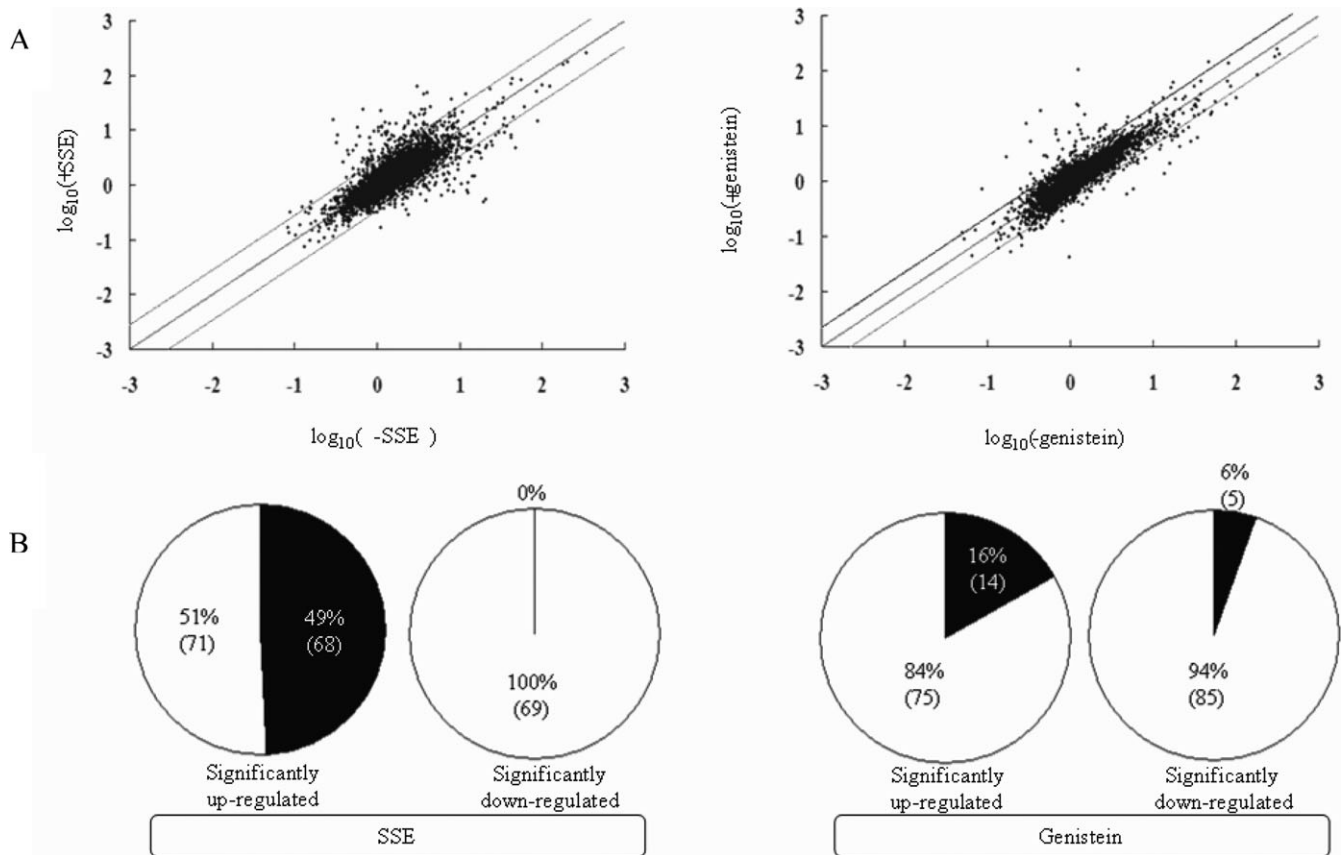
Figure 5. Scatter plots for the genome-wide expression of *B. japonicum* USDA 110 in response to SSE and genistein at 12 hpi, respectively. (**A**) Log plots of the hybridization signals for macroarray obtained in SSE/genistein-treated cells against those in untreated cells. The *x* and *y* axes indicate the $\log_{10}$ values of the spot signal normalized on the basis of 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 >2.2- and <0.4-fold, respectively.

induced by SSE at 12 hpi (Fig. 2 and Supplementary Table S2). The LIS 7 covered by two clones (brb19974, brb02802) was induced 2.8- to 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,[37] 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),[30] 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 (Fig. 1 and Supplementary Table S3). 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 genes cluster (*exo*) for exopolysaccharide (EPS) synthesis and deletion mutants within *exo* caused a delayed nodule initiation.[16,38] LOS 15 (from bll5380 to bll5420 at the coordinate 5,925,788−5,964,027) 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 not potentially induced by SSE but by genistein (Fig. 1 and Supplementary Table S3). Among 15 putatively induced genes, 12 genes (80.0%) 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.[30,39]

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- to 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.[23] 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.

3.4. Expression 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) (Fig. 1 and Supplementary Table S4). Two adjacent clones (brb11706, brb23885) in LOS 12 were induced 2.2- to 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.[40,41] Two adjacent clones (brb17502, brb07789) in LOS 10 were also induced strongly during 12 h, suggesting that three 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- to 57.3-fold). Two adjacent clones (brb07276, brb06980) in this locus and two adjacent clones (brb06269, brb14218) covering LOS 9 contained 10 putatively induced genes during 12 h, and five among them (50.0%) encode transcriptional regulatory protein. Six adjacent genes (blr2437−blr2442) covered by LOS 7 encode ABC transporter-related protein, whereas five of six 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 (Fig. 1 and Supplementary Table S4). Four adjacent genes encoding ABC transporter-related proteins (blr4553, blr4555−blr4557 in LOS 11), two adjacent genes encoding RhtB family transporter and two-component response regulator (bll7341 and bll7342 in LOS 22) and putative mono-oxygenase component (blr3679 in LOS 8) seemed to be repressed. However, all of six putatively repressed genes (blr5841−bll5846) in LOS17 encode hypothetical proteins.

3.5. 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 >2.2- and <0.4-fold, respectively. The candidates were selected according to the distribution profiles of scatter plots and the area corresponding to them is shown in Fig. 5A (Scatter plots for SSE- or genistein-treated cells at 0.5 and 6 hpi are shown in Supplementary Fig. S5). (ii) Selection of putatively regulated genes from the clones as described before,[23] and their validation by quantitative real-time RT−PCR.

As a result, 12 representative genes from some substantially regulated genomic loci (five genes in ECs, one gene in LISs, and six genes in LOSs) were selected and their relative amounts of transcripts were quantified by using real-time RT−PCR (Table 1). The primers of these selected genes were designed and confirmed as described in Section 2 (Supplementary Table S5). Among these 12 genes, genes encoding unknown protein (blr1649 in EC-I), RhcN (blr1816 in EC-III),

Table 1. Validation of putatively up- and down-regulated genes by real-time RT–PCR

Gene	Gene annotation	Location[a] (Clone[b])	Relative expression (fold)					
			SSE (h)			Genistein (h)		
			0.5	6	12	0.5	6	12
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 1681−2362 kb, respectively. EC-I, -II, -III, -IV indicate great expression clusters inside symbiosis island. LIS indicates loci inside symbiosis island but outside ECs and LOS indicates loci outside symbiosis island as shown in Figs 1 and 2.

[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 Supplementary Tables S1−S4.

and probable polygalacturonase (blr1993 in LIS 7) were exclusively up-regulated by SSE at 12 hpi (5.3- to 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- to 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 were maximal at 6 hpi (218.1- to 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. However, the high correlation of the expression ratio ($R^2 = 0.90$) between real-time RT–PCR and macroarray analyses showed the reliability of macroarray analyses with the validity of selection method for putatively induced genes.

4. Discussion

In this experiment, the symbiosis island of *B. japonicum* was collectively expressed as four predominant ECs (EC I–IV) in SSE-treated cells (Figs 1 and 2). The symbiosis island of *M. loti* MAFF303099 was also reported to be collectively expressed in bacteroids and function as clustered expression island (EI).[23] However, each symbiosis island functions as different great ECs, such as *nod* and *tts* induced by SSE in this experiment, whereas *nif, fix and fdx* induced in bacteroids of *M. loti* MAFF303099. 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,[42] was not potentially expressed in this experiment (Fig. 1). Genome-wide expression analyses on these two rhizobia clearly demonstrate that the rhizobia genome is a vehicle for the symbiosis island,

which function in distinctive gene clusters in the infection and differentiation stages of symbiosis.

Despite approximately the same final concentration of genistein between SSE- and genistein-induction medium (4.7 and 5.0 μM) (Supplementary Fig. S4), SSE-treated cells exhibited a distinctive genome-wide expression profiling since 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),[16] 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-pretreated cells was significantly enhanced (up to 12), compared with those formed with genistein-pretreated (six of mature nodules) as well as untreated cells (four of mature nodules) at 13 DAI (Supplementary Fig. S2). 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 (Supplementary Fig. S3). 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.[43,44] 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 are induced later than common *nod* genes operon (in EC-IV) and *nodW* (in EC-II) (Figs 2 and 3). 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.[45,46] 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 (Fig. 4), 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.[29] In this

experiment, the expression profile of *nodW* was similar between SSE- and genistein-treated cells (Table 1). 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 (Fig. 4B and Table 1), suggesting that higher expression of *tts* 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* in some pathogenic bacteria is controlled by environmental factors, such as temperature, nutrition, osmolarity, and quorum sensing.[47] These results suggest that the stronger expression of *tts* 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.[37] 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* genes 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 EPS in *Ralstonia solanacearum* was reported to be regulated in concert with type III secretion system.[48] 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.[23,49] Recently, blr0241 was also found to be well-induced in bacteroids,[18] implying that this gene plays an important role during symbiotic process. Higher expression of genomic loci relevant to ribosomal proteins 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,[11] 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- to 28.8-fold during 12 h (Table 1). 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.[50] In LOS 20, gene blr7023 encoding transcriptional regulatory TetR family was mainly induced by genistein during 12 h (3.6- to 8.0-fold). Besides, one clone (BJ7162) covering a single gene for a AcrB/AcrD/AcrF family protein (bll7019) was induced 2.6- to 33.3-fold by both SSE and genistein at 0.5 hpi (Supplementary Table S4), 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 (∼6.8 μM), the genome-wide expression of *B. japonicum* in response to daidzein (5.0 μM) at 12 hpi was also monitored. 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 (data not shown). These results indicate that daidzein is also a natural inducer of *nod* genes. It was reported that daidzein induced *nod* genes in *B. japonicum*.[7] However, no potential regulation of LISs and LOSs except for the LOS 8−10, 12, 16, 19 was observed for daidzein-treated cells (data not shown), 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.[11] In conclusion, the genome-wide expression analyses in this study reveal that symbiosis island functions as distinctive 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/array02/.

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