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Molecular Characterization of Hypernodulation in Soybean

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SS2-2, a hypernodulating soybean mutant, was isolated by EMS mutagenesis from Sinpaldalkong 2. This autoregulation mutant showed greater number of nodules and smaller plant size than its wild type Sinpaldalkong 2. SSR markers were used to identify DNA variation at SSR loci from different soybean LG. The only SSR marker that detected a length polymorphism between SS2-2 and its wild type ancestor was Satt294 on LG C1 instead of LG H, locating a hypernodulating gene. Sequencing data of flanking Satt294 indicated that the size variation was due to extra stretch of TTA repeats of the SSR motif in SS2-2, along with A→G transversion. In spite of phenotypic differences between the wild type and its hypernodulating mutants, genomic DNA polymorphisms at microsatellite loci could not control regulation of nodule formation. The cDNA-AFLP method was applied to compare differential display of cDNA between Sinpaldalkong 2 and SS2-2. After isolation and sequence comparison with many AFLP fragments, several interesting genes were identified. Northern blot analysis, immunolocalization and/or the yeast two-hybrid system with these genes might provide information on regulation of nodule development in SS2-2.

Keywords : cDNA-AFLP, EMS, hypernodulating mutant, root nodulation, SSR.

Nodule formation by the interaction between legumes and rhizobia is tightly regulated. This internal mechanism is termed 'autoregulation' (Carroll and Mathews, 1990; Caetano-Anollés & Gresshoff, 1991), controlling the amount of nodules developed on the root.

In soybean, supernodulating mutants were isolated from the cultivar Bragg by chemical mutagenesis using ethylmethane sulfonate (EMS) (Carroll et al., 1985a, b), and from cultivar Enrei by Akao & Kouchi (1992). Hypernodulating soybean mutants, characterized with nodulation which was more extensive than that of the wild type but smaller than that of a supernodulating mutant, were also developed independently by Carroll et al. (1985a, b) and by Gremaud and Harper (1989). More recently, Lee et al. (1997) isolated a hypernodulating mutant, SS2-2, from M_2 families of Sinpaldalkong 2 mutagenized by 30 mM EMS, showing no inhibition of nodule formation even in the presence of exogenous nitrate (Lee et al., 1997; Lee et al., 1998). Genetic mapping in a population segregating for hypernodulation, as well as hybridization between SS2-2 and *nts* mutants (Carroll et al., 1985a, b), revealed that hypernodulation character in SS2-2 was governed by the same locus controlling supernodulation in *nts* mutants (Ha and Lee, 2001).

Molecular markers have been frequently used to identify DNA variations in plant variants or mutants. Chemical mutagenesis is a random process that introduces changes at unspecified positions in a DNA molecule. Since point mutations occurred by EMS mutagenesis in these supernodulating/hypernodulating mutants, molecular markers, such as restriction fragment length polymorphisms (RFLPs) and amplified fragment length polymorphisms (AFLPs), have frequently been used to identify DNA variations in plant variants or mutants (Chowdari et al., 1998; Starman et al., 1999; Scott et al., 2000). These markers have been of great use for developing genetic linkage maps for a number of plant species because simple sequence repeats (SSRs) have several advantages over other molecular markers (Akagi et al., 1996; Cregan et al., 1999; Milbourne et al., 1998; Yu et al., 2000). The abundance of SSRs in the genome and their unusual high level of polymorphism make them an interesting tool for investigating the changes induced by mutagens (Ellegren, 2000).

The cDNA-AFLP method largely overcomes problems of repeatability and uncertainty in the identification of

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specific fragments associated with hypernodulation characters, and makes a simple and rapid verification of differentially expressed transcripts using PCR. This high throughput technique is an inexpensive gel-based method for analysis of gene expression patterns and can be used in any laboratory (Bachem et al., 1996; Qin et al., 2001).

This study aimed to identify the gene associated with hypernodulation using hypernodulating soybean mutant SS2-2, which was derived from EMS mutagenesis with its wild type Sinpaldalkong 2. The biological nitrogen fixation will become more interesting by molecular characterization of hypernodulation gene. Two major approaches, SSR marker analysis and cDNA-AFLP, were used for characterizing the gene associated with hypernodulation in soybean.

Materials and Methods

Plant materials. Seven soybean genotypes comprising three soybean mutants and their wild types were used (Table 1). Supernodulating mutant, nts382 (Carroll et al., 1985b) and hypernodulating mutant, nts1116 (Carroll et al., 1985a), were derived from cultivar Bragg. Bragg and its nodulating mutants, *nts382* and *nts1116*, were obtained from Dr. P.M. Gresshoff of the University of Queensland, Australia. Dr. J. E. Harper of the University of Illinois at Urbana-Champaign (now retired) kindly provided NOD1-3 (Gremaud and Harper, 1989) and its wild type, Williams 82. The other hypernodulating mutant, SS2-2, derived from Sinpaldalkong 2 was also used (Lee et al., 1997).

SSR marker analysis. Genomic DNA was isolated according to the procedure of Keim et al. (1988). After a total of 67 SSR markers were selected from the 20 soybean linkage groups (data not shown) based on the public soybean map (Cregan et al., 1999), amplicon length polymorphisms were screened. The 5'-end fluorescent-labeled forward primers (Applied Biosystems, Forster City, CA, USA) were labeled either with blue (6-Fam), green (Hex), or yellow (Ned) fluorescent tags (Ziegle et al., 1992).

Amplification using the polymerase chain reaction (PCR) was performed as described by Diwan and Cregan (1997), using a PTC-100 thermocycler (MJ Research, Inc., Watertown, MA, USA). Amplified products of a number of SSR markers with different fluorescent labels and/or allele sizes were pooled together, and pooled samples were loaded and separated on an ABI Prism 377 DNA sequencer (Applied Biosystems). GeneScan 672™ fragment analysis software and Genotyper™ software (Applied Biosystems) were used for gel image analysis and accurate characterization of the alleles.

Alleles of different sizes, obtained by amplification of soybean mutants and their wild types, were cloned and sequenced. The cloning process was based on the T/A cloning method (Clark, 1988; Mead et al., 1991).

Sequencing reactions were performed using a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) with a PTC-100 thermal cycler. Sequencing of the cloned DNA was performed using the ABI 377 sequencer (Applied Biosystems). The sequences of the amplified and cloned products were aligned using the CLUSTAL W multiple alignment package (Thompson et al., 1994).

cDNA-AFLP analysis. Using RNeasy kit (Qiagen Inc., Valencia, CA, USA), total RNA was isolated from the root nodule primordia of Sinpaldalkong 2 and SS2-2 soybean 7 days after inoculation of *Bradyrhizobium japonicum* (USDA110). Double-stranded cDNA was synthesized by cDNA synthesis kit (Roche Diagnostics Corporation, Indianapolis, IN, USA) with 10 μg of total RNA.

For cDNA-AFLP, 30 ng of cDNA was used. Digestion and ligation reaction mixtures were made in the same tube once with restriction enzymes (*Mse*I and *Eco*RI) and adaptors using plant AFLP kit, followed by preamplication of template for cDNA-AFLP (Applied Biosystem).

*Eco*RI+NNN primers, each with three selective nucleotides at the 3' end, were labeled with blue, green, and yellow fluorescent dye. Reactions were performed in 10 μL of a reaction mixture containing 0.2 mM dNTP, 3 mM $MgCl_2$, 76 pmol of labeled

Table 1. Summary of SSR maker analysis with seven different soybean genotypes. Number represents length of amplicon

Marker	LG[a]	Sinpal-dalkong 2	SS2-2	Bragg[b]	*nts382*[b]	*nts1116*[b]	Williams 82[c]	NOD1-3[c]
Satt415	B1	296	296	296	296	296	296	296
Satt063	B2	105	105	145	145	145	145	145
Satt294	C1	249	288	285	285	285	288	288
Sat_022	D2	220	220	228	228	228	220	220
Sat_039	F	112	112	112	112	112	112	112
Satt001	K	116	116	107	107	107	76	76
Satt143	L	272	272	272	272	272	247	247
Satt590	M	317	317	317	317	317	311	311
Sat_003	N	159	159	135	135	135	159	159
Satt243	O	202	202	211	211	211	202	202

[a] LG, linkage group.
[b] Carroll et al. (1985a, b).
[c] Gremaud and Harper (1989).

*Eco*RI (+AGG, ACC, ACT) primers, 125 pmol of *Mse*I (+CAA, CAT, CTT, CTG, CAC) primers, and 2.5 μL of diluted preamplification mixture. After amplification, 2 μL of the reaction mixture was combined with an equal volume of dye solution. The mixture was heated for 5 minutes at 95°C and then quickly cooled to 4°C. The samples were loaded on a 7% polyacrylamide/6 M urea gel and electrophoresed by ABI 377 (Applied Biosystems).

After the gel bands were excised, DNA was isolated (Sambrook et al., 1989). Reamplification was done using the same primer set and PCR conditions, except for the use of 20 μM of dNTP. Reamplified PCR samples were run on a 1.5% agarose gel and reamplified DNA fragment was extracted by the spin column (NucleoSpin Extract, Macherey-Nagel, Duren, Germany). Reamplified cDNA fragments were cloned into the pT-aDV vector system (Clontech Laboratories, Inc., Palo Alto, CA, USA). Preparation of sequencing reaction and sequencing analysis were performed as above.

Results and Discussions

SS2-2 is a hypernodulating soybean mutant generated by EMS mutagenesis with Sinpaldalkong 2. It produces greater nodules about five to ten times and smaller plant size than its wild type Sinpaldalkong 2 (Fig. 1).

Genomic DNAs from seven soybean genotypes were analyzed for polymorphisms at SSR loci. A total of 67 available primer pairs were tested and 10 SSR markers and their amplicons length were shown selectively in Table 1. These SSR markers, except Satt415 and Sat_039, revealed amplicon length polymorphisms among seven soybean genotypes. Satt294 was the only marker that showed an amplicon length polymorphism between Sinpaldalkong 2 and SS2-2 on LG C1 (Table 1). The larger fragment from the locus-specific amplification at Satt294 in SS2-2 compared with Sinpaldalkong 2 showed big stretch of TTA trinucleotide insertions along with A→G point mutation probably caused by EMS treatment (data not shown).

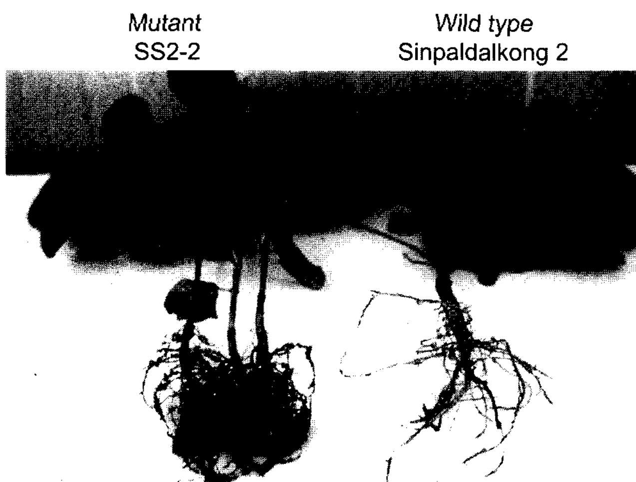

Fig. 1. Nodulation characters of Sinpaldalkong 2 and SS2-2. Greater numbers of nodules were shown in SS2-2.

The genomic length change at Satt294 is unlikely to be associated with hypernodulation characters. Previous study revealed that a hypernodulation gene was 18.9 cM away from Satt353 on LG H (Ha and Lee, 2001). This is near pA132, which is closely linked to the supernodulating *nts* gene, based on the public soybean map (Cregan et al., 1999). Also, Searle et al. (2003) recently reported that flanking markers, pUTG132a and UQC-IS1 on LG H, were used for isolation of the gene associated with super/hypernodulation characters in *nts* mutants. This is an indication that mapping population derived from the cross between Sinpaldalkong 2 and SS2-2 is not suitable for genetic mapping using SSR marker due to the lack of parental polymorphism.

Fig. 2 represents typical results of the AFLP-based fingerprinting of mRNA using fluorescence-labelled primers on an ABI-377. mRNAs extracted from root nodule primordia of Sinpaldalkong 2 and SS2-2 7 days after inoculation with *Bradyrhizobium japonicum* were used for cDNA-AFLP analysis. It was evident that the AFLP-based fingerprinting of mRNA resulted in the differences in the banding patterns, although that of SS2-2 was not different from that of its wild type Sinpaldalkong 2 at genomic DNA level. Hence, it can be concluded that cDNA-AFLP mapping could be useful with the segregating population derived from the cross between hypernodulating SS2-2 and its wild type Sinpaldalkong 2, whereas genomic DNA marker

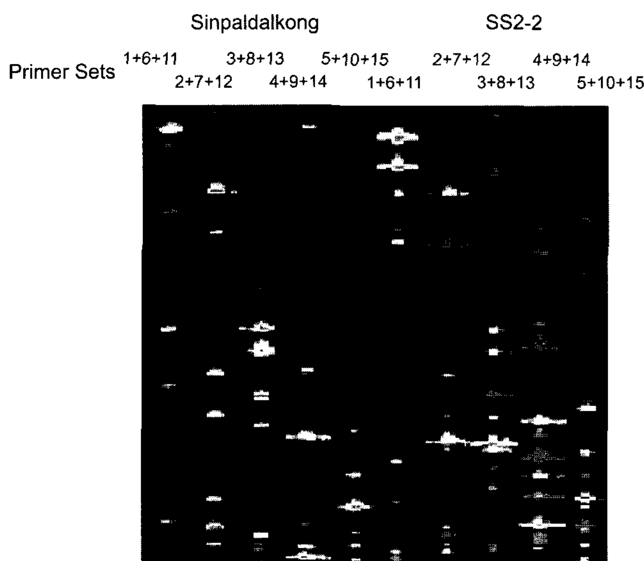

Fig. 2. cDNA-AFLP display of Sinpaldalkong 2 and SS2-2 with combinations of primers. Three different fluorescent-labeled primers were used. The number indicates different combinations of primer sets; for example, primer set 1+6+11 represents mixture of *Eco*RI+AAC and *Mse*I+CTT primers, *Eco*RI+AGG and *Mse*I +CTT primers, and *Eco*RI+ACC and *Mse*I+CAC primers, respectively.

mapping was not useful due to the lack of polymorphisms between two parents.

With each combination of primers, approximately 50 discrete bands were detected per lane (Fig. 2). Comparison of banding patterns using fluorescence-labelled primers on an automatic DNA sequencer ABI-377 revealed that a given combination of primers showed different patterns of cDNA prepared from nodules of Sinpaldalkong 2 and SS2-2, indicating the differential expression in two genotypes (Fig. 2). In this study, cDNA-AFLP analysis was performed with 33 combinations of primers and a total of 1,600 bands were detected.

A total of 712 bands were differentially amplified in the two DNA samples from Sinpaldalkong 2 and SS2-2, suggesting a high possibility of the identification of genes associated with hypernodulation in soybean by cDNA-AFLP mapping. For the identification of the differentially amplified DNA fragment, silver nitrate staining was used to clone the DNA fragment (data not shown). After a piece of the dried gel band of interest was cut out and cloned, about 650 bands were cut out of the differential amplified gels. Some of the reamplified PCR fragments were cloned into the T/A vector to allow sequence determination. With the differentially expressed fragments from Sinpaldalkong 2 and SS2-2, a total of 72 fragments were first analyzed based on homology comparison.

Table 2 shows a short list of expressed sequence tags (ESTs), showing high homology with Sinpaldalkong 2 specific fragments. Since many protein kinases were involved in the signal transduction pathway for pathogen attack and symbiosis, protein kinase isolog and receptor-like protein kinase precursor drew an attention (Table 2). Recently, it was reported that symbiosis receptor-like kinase (SYMRK) required for a symbiotic signal transduction pathway (Stracke et al., 2002) and a receptor kinase gene regulated symbiotic nodule development (Endre et al., 2002), supporting this studys data. Also, Searle et al. (2003) recently demonstrated that the receptor-like protein kinase GmNARK (*Glycine max* nodule autoregulation receptor kinase) controlled autoregulation of nodulation and was expressed in the leaf, involving a long-distance communication with nodule and lateral root primordia. Hence, this study particularly looked into these two fragments, #4 Sin-2 and #4 Sin-4-4. A short list of SS2-2 specific ESTs is shown in Table 3. Interestingly, it was found that nodulation regulating genes of #9 S-4 and #10 S-4-3 are similar to that of *Medicago truncatula*. These genes might have contributed to the control gene expression in Sinpaldalkong 2 and SS2-2 at transcriptional level.

cDNA-AFLP was used for identifying PCR fragments associated with differential gene expression in Sinpaldalkong 2 versus SS2-2. It was found that protein kinase/protein kinase precursor like ESTs from Sinpaldalkong 2 and partial genes were related to root nodulation from SS2-2. Northern blot analysis would be helpful for determining temporal and spatial gene expression in soybean, using total RNA extracted days after inoculation with *Bradyrhizobium japonicum* and interesting AFLP fragments as probes, along with immunolocalization and the yeast two-hybrid system for identifying interacting proteins. Full-length

Table 2. Homologies of sequences of cDNA-AFLP fragments expressed only in Sinpaldalkong 2 to sequences in the GenBank

EST	Size (bp)	Description	Species	Homology (%)
#4 Sin-2	42	GmTR:O04086 O04086 Ser/Thr protein kinase isolog. mRNA	*Glycine max*	100
#4 Sin-4-4	146	Q96387 Receptor-like protein kinase precursor	*Glycine max*	88
#9 Sin-2-3	102	Pathogen-infected compatible 1 (PIC1) Sorghum bicolor cDNA	*Glycine max*	67

Table 3. Homologies of sequences of cDNA-AFLP fragments expressed only in SS2-2 to sequences in the GenBank

EST	Size (bp)	Description	Species	Homology (%)
#9 s-7	75	ATPH_MARPO P06287 ATP synthase C chain (BI944542)	*Glycine max*	100
#8 s-8	107	Uncultured BVB19b 16S ribosomal RNA (AY013653)	Banisveld landfill bacterium	82
#9 s-7	347	Aldehyde dehydrogenase (BI498051)	*Glycine max*	51
#9 s-5-1	75	ATPH_MARPO P06287 ATP synthase C chain (BI944542)	*Glycine max*	89
#9 s-4	49	Enod8.3 (Enod8.3) gene, partial cds; and Enod8.2, Enod8.1 gene (AF463407)	*Medicago truncatula*	65
#4 s-2	148	RNA polymerase II largest subunit mRNA (AF241089)	*Pseudobiantes japonicus*	55
#10 S-7-1	75	mRNA for RD20 protein, complete cds (AB039924)	*Arabidopsis thaliana*	60
#10 S-4-1	32	Peptide synthetase-like protein (CPS1) (AF332878)	*Cochliobolus heterostrophus*	66
#10 S-2-1	35	BAC clone FW2.2, complete sequence (AF411809)	*Lycopersicon esculentum*	68
#10 S-4-3	50	Nodulated root cDNA clone	*Medicago truncatula*	81

cloning of these ESTs and getting sequence information from the rest of AFLP fragments will be the next stage of this research.

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