International Journal of Agricultural Sciences ISSN: 2167-0447 Vol. 1 (1), pp. 020- 024, December, 2011. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Vistra Green-Stained cDNA AFLP Technique for Identification of Transcript Profiles in Supernodulating Soybean

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Received December 5, 2011; Accepted December 22, 2011

To understand the molecular bases of symbiotic association, cDNA-AFLP technique was performed to identify differential transcripts between supernodulating soybean mutant, SS2-2, and its wild type, Sinpaldalkong 2. To determine optimum time point for identifying the transcripts, templates were prepared from soybean leaves depending on time after inoculation (TAI) of *Bradyrhizobium japonicum* on 3 soybean genotypes, SS2-2, Sinpaldalkong 2 and Jangyeobkong. While to identify the trasncripts associated in symbiotic interaction, cDNA-AFLP was carried out based on pre-determined time on 2 genotypes, SS2-2 and Sinpaldalkong 2. Gel-based vistra-green stained cDNA-AFLP system with non-denaturing polyacrylamide gel was established in this study. As the results, transcript-derived fragments (TDFs) showed the polymorphic banding patterns regardless of TAI. Using 22 primer combinations of *EcoR*I and *Mse*I, polymorphism levels of the TDFs among 3 genotypes were found to be the highest on one week after inoculation (WAI), determining the optimum time point for isolating differential transcripts profiles. A total of 4000 amplicons were recognized, leading 147 differential fragments to be specific in the SS2-2 (61 fragments) and 86 TDFs specific to the wild type. Approximately 80.3% of fragments were successfully reamplified, and used as candidates for differentially expressed genes in the symbiotic association specifically in supernodulating and normal soybeans.

Keywords: biological nitrogen fixation, *Bradyrhizobium japonicum*, cDNA AFLP, soybean, supernodulation, transcript derived fragmen (TDF)

INTRODUCTION

Several researchers have used chemical mutagenesis as an alternative approach to generate greater genetic variability in host nodulation response. Several hypernodulating and supernodulating soybean mutants with enhanced nodulation and partial tolerance in nodulation to the presence of high levels of NO_{-3} were isolated. Supernodulating soybean mutants were developed from cultivar 'Bragg' with EMS (ethyl methanesulfonate) mutagenesis by Carroll et al. (1985), and from cultivar 'Enrey' by Akao and Kouchi (1992), and more recently, SS2-2 (Lee et al., 1997).

The supernodulating mutant, SS2-2 shows pleiotropy, a gene influence several features of phenotypes. In which, pleiotropy, the ability of a single mutant gene to cause multiple mutant phenotypes, is a relative common but poorly understood phenomenon (Dudley et al., 2005). SS2-2 is characterized with greater nodule number, smaller root and plant size, than those of its wild type (Lestari et al., 2006). EMS treatment may causes point mutation on SS2-2 (Van et al., 2003). To differentiate supernodulating soybean and its wild type, transcript level is needed to be investigated.

The use of cDNA-AFLP has often been described as

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Figure 1. Nodulation types of supernodulating mutant, its wild type and the control genotype inoculated with *B. japonicum* at 7 week after inoculation

Table1. List of primers and adapters used to determine optimum time to identify differential transcripts using cDNA-AFLP technique

Primers/Adapters	Sequence
*Mse*I adapter	5'-GAC GAT GAG TCC TGA G-3' 3'-TA CTC AGG ACT CAT-5'
*Mse*I + 1 selective base	5'-GAT GAG TCC TGAG TAA C-3'
*Mse*I + 2 selective bases	+CA +CG
*Mse*I + 3 selective bases	+CGG +CGA +CTA +CGT +CAC +CTG +CGC +CAC +CAT
*EcoR*I adapter	5'-CTC GTA GAC TGC GTA CC-3' 5'-CAT CTG ACG CAT GGT -3'
*EcoR*I + 1 selective base	5'-GAC TGC GTA CCA ATT C A-3'
*EcoR*I + 2 selective bases	+AC
*EcoR*I + 3 selective bases	+ACC AAG

an extremely efficient method for isolation of differentially expressed genes (Simoes-Araujo et al., 2002). The cDNA-AFLP is also an inexpensive gel-based method for analysis of gene expression patterns and can be performed in any laboratory. cDNA-AFLP is more stringent and reproducible than other techniques with relatively high annealing temperatures can be used. In contrast to most hybridization-based technique (Qin et al., 2001), cDNA-AFLP is a reliable alternative to the often artifact-laden differential display method. With those advantages, cDNA-AFLP will allow to identify transcriptional levels of the interest transcripts in supernodulating mutants and the wild type. Thus, this study aimed to determine optimum time point after inoculation to identify differential trancripts as candidate for differentially expressed genes between supernodulating mutant, SS2-2 and its wild type using vistra green-stained cDNA-AFLP technique.

MATERIALS AND METHODS

Plant materials and growth conditions

Three soybean genotypes, supernodulating mutant, SS2-2 and its wild type, Sinpaldalkong 2, and a check genotype, Jangyeobkong were used in this study (Figure 1). Soybean seeds were surface-sterilized then germinate, and transferred into a plastic bag containing vermiculite. inoculated with suspensions of *B. japonicum* USDA 110 containing 10^8 CFU/ml (Delves et al., 1986).

The plants were inoculated with suspensions of *B. japonicum* USDA 110 containing 10^8 CFU/ml and kept in a greenhouse (Lestari et al., 2006). Young and healthy leaves were harvested with weekly intervals, collected for RNA extraction.

Total RNA extraction, poly $(A)^+$ mRNA and cDNA synthesis

Total RNA was isolated from frozen tissue using Trizol reagent (Molecular Research Center, Inc.), using glass wares and water treated with DEPC. Poly $(A)^+$ mRNA was isolated from total RNA using Oligotex mRNA Spin-Column (Qiagen, Valencia, CA, USA). Double-stranded cDNA was synthesized from poly $(A)^+$ mRNA (1 μg) according to cDNA Synthesis System Kit (Roche, Applied Science, Mannheim, Germany). Next step was purification of cDNA with phenol/chloroform/isoamyl alcohol, and precipitated with ethanol and resuspended in water. The cDNA was precipitated with NH_4OAc. The pellet was air-dried by evaporating residual ethanol and dissolved in appropriate volume with DEPC-treated water.

cDNA-AFLP analysis

cDNA-AFLP analysis was performed as described by Bachem et al. (1998) and Voz et al. (1995).

Restriction and ligation

Restriction fragments of the cDNA are produced by two different restriction enzymes; a frequent cutter (four-base restriction enzymes *Mse*I) and a rare cutter (six-base restriction enzymes *EcoR*I). Three types of restriction fragments are generated: one with *EcoR*I cuts at both ends, ones with *EcoR*I cuts at one end and *Mse*I cuts at the other and, and ones with *Mse*I cuts at both ends. The DNA fragments were then ligated with *EcoR*I and *Mse*I adapters. Adapters sequences are shown in Table 1.

Preselective amplification

Each preselective primer has a selective nucleotide that recognizes the subset of restriction fragment having the matching nucleotide downstream from the restriction site. The primary product of the preselective PCR are those fragments having one *Mse*I cut and one *EcoR*I cut, and also having the matching internal nucleotide.
Preselective amplification reaction was performed with two AFLP primers having a single nucleotide with two AFLP primers corresponding to *Mse*I and *EcoR*I, in a reaction containing the following components: 4 µl of resuspended cDNA sample, 0.3 µM of each preselective amplification primer, 1 U of AmpliTaq Polymerase (Applied Biosystem, Foster City, CA, USA), 1 x AmpliTaq buffer, 3 mM $MgCl_2$, 0.2 mM dNTPs-mix (Applied Biosystem, Foster City, CA, USA). PCR was performed with a MJ PTC-225-Peltier Thermal Cycler (MJ Research INC. Watertown, MA, USA). Determination of successful preselective amplification can be seen by smear band on 1% agarose gel. The PCR product was stored at –20 ^{o}C until required.

Selective amplification

Selective primers consist of an identical sequence to the preselective primers with one, two or three selective nucleotides at the 3'-end. This additional nucleotide was any of the possible combinations of the four nucleotides. Different primer combination generated different sets of fragments. To identify differentially expressed genes, all possible primer combination of *EcoR*I and *Mse*I with one, two and three selective bases were used.
Following the pre-amplification step, the product of preselective amplification PCR was diluted (10x) with TE. As much as 20 µl of PCR reaction was performed containing 5 µl of the diluted preselective reaction, 125 nM of *Mse*I and 76.5 nM of *EcoR*I, 0.5 U of AmpliTaq Polymerase (Applied Biosystem, Foster City, CA, USA), 10x AmpliTaq buffer, 3 mM $MgCl_2$, 0.2 mM of each dNTP. The selective amplification was performed for 36 cycles with following cycle profile; initial denaturation step at 94 ^{o}C for 2 min, denaturing step 94 ^{o}C for 30 sec, annealing step at 65 ^{o}C for 30 sec and extension step at 72 ^{o}C for 2 min. The annealing temperature was reduced by 0.7 ^{o}C each cycle from starting 65 ^{o}C to 56 ^{o}C and then remained at 56 ^{o}C for the remaining 23 cycles, and final extension step at 72 ^{o}C for 2 min.

Gel electrophoresis and vistra-green staining

Selective amplification products were separated by non-denaturing electrophoresis in 13% polyacrylamide running gel with 5% stacking gels. Electrophoresis was done using sequencing gel apparatus (Hoefer, SE 600 series, Pharmacia, Biotech Inc., San Francisco, California, USA) at 70 V constant power for 15 hr. Staining of DNA used vistra-green (Amersham Pharmacia, Biotech, Piscataway, NJ, USA) by incubating gel with 1:10,000 dilution of vistra green stock in water and allowed it to shake in dye solution for 10 - 60 min. Bands were then visualized on UV transilluminator.

Transcript-derived fragments (TDFs) isolation

The bands of interest showing polymorphism between SS2-2 and its wild type were selected, and removed from the gel by cutting slightly using a clean razor blade by holding a short wave UV light (approximately 240 nm). The excised band was placed in eppendorf 1.5 ml tube and then stored at liquid nitrogen or -80 ^{o}C. Extract DNA was recovered from gel by grinding and the gel was soaked in appropriate volume of TE buffer, then incubated on ice for 1 hr.

Clear supernatant on upper side was decanted and transferred to new tube and used for reamplification reaction.

Reamplification and purification of TDFs

Reamplification reaction was performed with the same primers in the selective amplification. Cycling conditions for consist of initial incubation at 72 ^{o}C for 30 sec, which was followed by 30 sec of denaturation at 94 ^{o}C, 30 sec, annealing at 68 ^{o}C for 1 min and 2 min of extension at 72 ^{o}C for 30 cycles, and 10 min of final extension at 72 ^{o}C. The reampilified cDNA-PCR products were purified by AccuPrep^{TM} PCR Purification Kit (Bioneer, Rockville, USA). The purified TDFs were separated in 1% agarose gel and stained with ethidium bromide. Single and clear band can be seen and visualized on UV transilluminator.

RESULTS

Technical aspects of vistra-green stained cDNA–AFLP

cDNA-AFLP technique was used with some revision to isolate genes associated with symbiotically growth in SS2-2 and its wild type. The use of vistra-green staining and UV transilluminator for detection greatly reduced the time necessary and was more affordable. Furthermore, pre-cast non-denaturing polyacrylamide ready gels were used instead of standard denaturing sequencing gels. Additionally, the recovery of DNA fragments from vistra-green stained polyacrylamide gels is less tedious than the original radioisotope and silver staining protocols.
The addition of selective nucleotides to the 3' end of primers enhanced the resolution of cDNA-AFLP. The lower complexity of the cDNA allows the use of one, two, and three selective nucleotides for both the *EcoR*I and *Mse*I primers, giving a lot of possible primer combinations. Briefly, we used more combination of primer with one and two selective bases on one side and three selective bases on another side instead of three selective bases on both primers (Durrant et al., 2000), expecting to enhance the polymorphism level of AFLP products. This step is critical for increasing polymorphism level of vistra-green stained-cDNA–AFLP. The primer combinations for selective amplification in this study were able to analyze considerable amounts of mRNA expressed in SS2-2 and its wild type.

In fact, more than 4,000 AFLP bands were recognized using these primer sets, which should be useful for discriminating the genes associated with symbiotic association between *B. japonicum* and SS2-2 compared to its wild type. Indeed, the resulting AFLP products ranged in length from 30 to 500 bp, and 10 - 40 bands were also observed for each primer combination. Even though the number of bands was lower than bands reported by either Bachem et al. (1996) or Durrant et al.

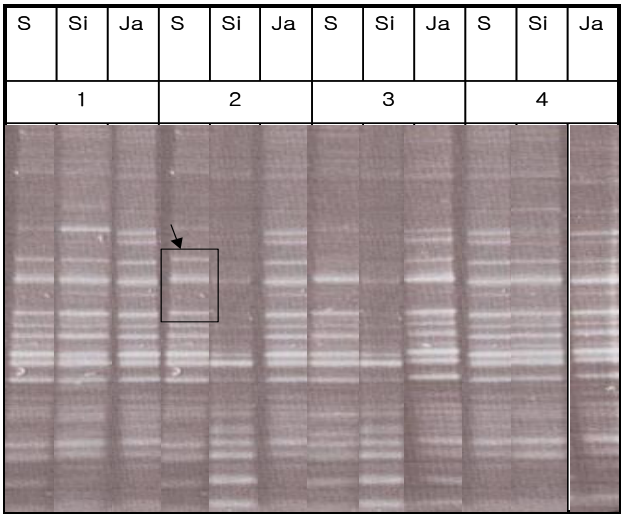

Figure 2. Vistra-green stained cDNA-AFLP showing the TDFs pattern from soybean leaves inoculated with *Bradyrhizobium japonicum.* The primer combination used (A: *Eco*RI-A and *Mse*I-CAT, B: *Eco*RI-AC and *Mse*I-CG). Each lane corresponds to amplified template from without inoculation (lane 1) or inoculation (1, 2 and 3 WAI, lanes 2, 3 and 4, respectively). Abbreviations: S, SS2-2; Si. Sinpaldalkong 2; Ja, Jangyeobkong. Arrow-labeled bands correspond to polymorphic band.

Table 2. Percentage of polymorphic cDNA-AFLP product generated with 22 different primer combinations of *Eco*RI and *Mse*I

No. total bands	*Eco*RI	*Mse*I	Percentage of polymorphic bands			
			0	1	2	3
			Weeks after inoculation			
19		CGG	10.5	15.8	10.5	0
18		CGA	11.1	11.1	11.1	11.1
15	ACC	CTA	0	13.3	20.0	6.7
14		CAC	7.1	7.1	7.1	7.1
14		CTG	7.1	14.3	7.1	7.1
16		CGC	6.2	12.5	6.2	6.2
11		CGG	0	18.2	9.1	9.1
17		CGA	11.8	0	11.8	0
12		CAC	0	0	0	0
10	AAG	CTG	10	10	0	10
13		CGT	0	15.4	7.9	0
18		CGC	5.6	5.6	5.6	0
21		CTA	9.5	9.5	14.3	4.8
33		CAT	3.0	9.1	6.1	6.1
25	A	CGG	8.0	12.0	12.0	8.0
23		CGA	4.3	13.0	8.7	8.7
27		CAC	7.4	14.8	11.1	7.4
20		CGC	5.0	10.0	15.0	10.0
12	AC	CA	8.3	25.0	8.3	8.3
20		CG	10.0	25.0	20.0	5.0
13	A	CA	0	7.7	7.7	7.7
16		CG	6.2	18.8	6.2	6.2
Mean			6.0	**12.2**	9.4	5.9

Table 3. Success of reamplification of extracted TDFs in SS2-2 and its wild type

Genotype	No. of extracted TDFs	No. of reamplified TDFs	Percentage (%)
SS2-2	61	48	78.7
Sinpaldalkong 2	86	70	81.4
Total	147	118	80.3

(2000), many differential fragments were detected (Figure 2).

Determination of optimum time to isolate differential transcripts

To determine optimum time to identify genes associated with symbiotic interaction in SS2-2 and its wild type, cDNA-AFLP was carried out using leaves of soybean plant inoculated with *B. japonicum* USDA 110 in different time point. Based on the results of cDNA-AFLP, transcript-derived fragments (TDFs) showed the polymorphic banding patterns independently of time after

For identifying genes involved in the symbiotic association, cDNA-AFLP was carried out on soybean leaves based on pre-determined time, one WAI. A total of 106 different primer combinations were used to two templates, and many TDFs displayed altered expression patterns in symbiotic association of supernodulating mutant and normal nodulating soybean. As a result, a total of 147 bands out of 4,000 amplicons were recognized as differentially expressed fragments, 61 TDFs in SS2-2 and 86 TDFs in the wild type. Among these, 48 TDFs (78.7% of excised bands) in SS2-2 and 70 TDFs (81.4% of excised bands) in Sinpaldalkong 2 were successfully reamplified (Table 3). TDFs shown a single band were purified then these were used for further study by cloning them into pGEM vector (and sequenced to identify their functions.

DISCUSSION

A point mutation detected in *GmNARK* between SS2-2 and Sinpaldalkong 2 indicated that the identified SNP in *GmNARK* (Acc. no. AY16665) could directly affect phenotypic variations for nodulation (Kim et al., 2005). Supernodulating mutant, SS2-2 shows pleiotropy, in which a gene influences several features of phenotypes. This single gene is inherited in Mendelian recessive (Mathews et al., 1990). SS2-2 shows quite different phenotypic characters compared to its wild type. SS2-2 is characterized with greater nodule number, smaller root inoculation (TAI) among SS2-2, Sinpaldalkong 2 and Jangyeobkong. Using 22 primer combinations of *EcoR*I and *Mse*I, 46 polymorphic bands out of 276 bands were observed in one WAI (Table 2) and in percentage, the polymorphic bands in one WAI was 12.2% (Table 3). The polymorphism level of the TDFs among three genotypes were found to be the highest in one WAI, determining the optimum time for isolation of differentially expressed genes related to symbiotic association in SS2-2 and its wild type.

Isolation of differential transcripts

growth, small plant size, higher seed protein level and lower carbohydrate content at early stage of growth. However, according to genotypic character, previous study by using SSR marker by Ha and Lee (2001) reported that there was slight difference in genomic level between SS2-2 and the wild type. So, cDNA-AFLP technique was chosen to detect the distinction in transcription level between SS2-2 and its wild type.

It showed that cDNA-AFLP successfully isolated 105 TDFs from mutant, SS2-2 and its wild type. cDNA-AFLP proved to be an efficient, powerful, highly accurate and high fidelity technology for identifying differential TDFs in SS2-2 and the wild type. cDNA-AFLP technique was also successful to discriminate differentially expressed genes in plants (Santaella et al. (2004); Kemp et al. (2005)) and for large scale analysis of gene expression.

Primer combination with less selective bases (one or two) used in this study produced a greater number of polymorphic bands than those with three selective bases on both primers. Previous study (Qin et al., 2001) reported that introducing selective bases in the 3' primer end reduces the complexity of the pattern and makes the amplification more specific. The result of this study also agreed with investigation by Santaella et al. (2004) that primer combination with two selective bases showed a higher number of bands than with three selective bases. So, the modified cDNA-AFLP performed in this study efficiently detects differential transcripts profiles in supernodulating mutant, SS2-2 and its wild type. TDFs identified in supernodulating and normal soybeans in this

study could be candidates for differentially expressed genes by sequencing analyses and homology study, followed by RT-PCR approach. So, we successfully surveyed transcriptional changes during symbiotic association, with no prior assumptions about the genes contributing in the symbiotic association between *Bradyrhizobium japonicum* and the supernodulating soybean compared to the normal soybean.

CONCLUSION

Detection in transcription level is needed to discriminate transcript or gene associated with symbiotic interaction between *B. japonicum* and supernodulating soybean mutant, SS2-2 compared with its wild type. Identification of differential transcripts between SS2-2 and its wild type showed the best time point on leaves, one week after inoculation (WAI) of *B. japonicum*. This determined time was used as the basis to carry out an identification of differentially expressed genes in the two genotypes for further study. cDNA-AFLP proved to be an efficient, powerful, highly accurate and high fidelity technology for identifying differentially TDFs in SS2-2 and the wild type.

ACKNOWLEDGEMENT

This study was supported by a grant from the International Atomic Energy Agency and the Crop Functional Genomics Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology (MOST) and the Rural Development Administration (RDA) of the Republic of Korea.

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