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Identification of DNA Variations Using AFLP and SSR Markers in Soybean Somaclonal Variants

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ABSTRACT: Somaclonal variation, defined as phenotypic and genetic variations among regenerated plants from a parental plant, could be caused by changes in chromosome structure, single gene mutation, cytoplasm genetic mutation, insertion of transposable elements, and DNA methylation during plant regeneration. The objective of this study was to evaluate DNA variations among somaclonal variants from the cotyledonary node culture in soybean. A total of 61 soybean somaclones including seven R_1 lines and seven R_2 lines from Iksannamulkong as well as 27 R_1 lines and 20 R_2 lines from Jinju 1 were regenerated by organogenesis from the soybean cotyledonary node culture system. Field evaluation revealed no phenotypic difference in major agronomic traits between somaclonal variants and their wild types. AFLP and SSR analyses were performed to detect variations at the DNA level among somaclonal variants of two varieties. Based on AFLP analysis using 36 primer sets, 17 of 892 bands were polymorphic between Iksannamulkong and its somaclonal variants and 11 of 887 bands were polymorphic between Jinju 1 and its somaclonal variants, indicating the presence of DNA sequence change during plant regeneration. Using 36 SSR markers, two polymorphic SSR markers were detected between Iksannamulkong and its somaclonal variants. Sequence comparison amplified with the primers flanking Satt545 showed four additional stretches of ATT repeat in the variant. This suggests that variation at the DNA level between somaclonal variants and their wild types could provide basis for inducing mutation via plant regeneration and broadening crop genetic diversity.

Keywords: amplified fragment length polymorphism (AFLP), cotyledonary node culture, simple sequence repeat (SSR), somaclonal variation, soybean

Regeneration of plants from tissue culture often causes genetic change due to its severe stress exposed during plant regeneration. This phenomenon is termed somaclonal variation (Larkin and Scowcroft, 1981), which can be defined as genetic and phenotypic variations among regenerated plants from a single donor clone. This can provide basis for selection of new mutants among somaclonal variants through plant regeneration that can be used for broadening crop genetic diversities.

Somaclonal variation has been reported in soybean using organogenic and embryogenic callus cultures (Barwale *et al.*, 1986) and using cotyledonary node cultures (Freytag *et al.*, 1989). More recently, soybean mutants with high seed oil content were selected from regenerated plant via embryogenesis and organogenesis (Nguyen *et al.*, 2001).

Until recently, traditional mutation induction for crop improvement has been restricted to broadening the genetic base of germplasms (Stephens *et al.*, 1991). This was accomplished without explicit knowledge of the molecular bases underlying the mutation process. Nowadays, mutation techniques have expanded beyond direct use in breeding due to scientific advance in applied molecular genetics and genomics (Phillip *et al.*, 1994). Therefore, understanding the molecular basis for somaclonal variations will be beneficial for plant geneticists and breeders to broaden genetic bases by generating new genetic sources and to improve crop varieties. However, somaclonal variation does not always cause phenotypic change. As phenotypic variations are expected to occur at a much lower frequency due to the occurrence of mutation in the non-coding region, the absence of visible variation does not preclude the absence of all variation among the regenerated progenies (Evans *et al.*, 1984).

The objectives of this study were to determine the types, frequencies, rates and patterns of molecular change induced by regeneration from cotyledonary node culture commonly used for soybean crop tissue culture and to clarify DNA variation using amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) marker analyses.

MATERIALS AND METHODS

Plant materials

Somaclonal variants were regenerated from two soybean cultivars, Iksannamulkong and Jinju 1. The variants were obtained from transformation with GUS gene, according to the procedure of regeneration from the cotyledonary node (Freytag *et al.*, 1989). After successful regeneration, R_0 plants were transplanted to a greenhouse for self-pollination

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to obtain R_1 and R_2 plants. A total of seven R_1 and seven R_2 lines from Iksannamulkong and 27 R_1 lines and 20 R_2 lines from Jinju 1 were obtained and used in this study.

DNA isolation

To obtain DNA from somaclones and two wild type soybeans for AFLP and SSR analyses, leaves prior to full expansion were harvested from 5-day seedlings grown in the greenhouse. Genomic DNA was isolated using CTAB method according to the procedure of Keim *et al.* (1988).

AFLP marker analysis

The AFLP analysis was performed as described previously (Vos *et al.*, 1995). Briefly, digestion with *Mse*I (New England Biolabs, Beverly, MA, USA) and *Eco*RI (Promega, Madison, WI, USA) and ligation with *Eco*RI and *Mse*I adapters using T4 DNA ligase (New England Biolabs, Beverly, MA, USA) were performed in a tube and the reaction mixture was diluted and stored at −20°C.

Preselective amplification reaction was performed using two AFLP primers with a single selective nucleotide with AmpliTaq polymerase (Applied Biosystems, Foster City, CA, USA) in a PTC-100 Programmable Thermal Controller (MJ Research Inc. Watertown, MA, USA).

For selective amplification, selective *Eco*RI-primers were labeled with blue (6-Fam), green (Hex), or yellow (Ned) fluorescent tags (Ziegle *et al.*, 1992, Table 1). About 80 nM of the fluorescent labeled *Eco*RI selective primer was added into the diluted preselective reaction. After amplification, loading and separation of samples were followed by Van *et al.* (2003). GeneScan software (Applied Biosystems, Foster City, CA, USA) was used for gel image analysis and Genotyper software (Applied Biosystems, Foster City, CA, USA) was used for accurate characterization of the bands and automated data output.

SSR marker analysis and DNA sequence determination

A total of 36 SSR markers were selected (one to three markers per linkage groups) from the SoyBase (http:// soybase.org/) and were screened for amplicon length polymorphism against two parents and somaclonal variants. The SSR marker analysis was followed by Kim *et al.* (2004).

For sequence determination of polymorphic bands as revealed by SSR analysis, DNA fragment was excised and extracted using the NucleoSpin® Extract kit (MACHEREY-NAGEL Inc., Easton City, PA, USA). Sequencing reactions of polymorphic bands were mixed with SSR reverse primer (without fluorescent tag) and BigDye Terminator Cycle Sequencing Kit 2.0 (Applied Biosystems, Foster City, CA, USA). After the products were ethanol-precipitated, sequence analysis was performed with ABI 3700 DNA sequencer (Applied Biosystems, Foster City, CA, USA). ABI Prism SeqScape Software version 2.0 (Applied Biosystems, Foster City, CA, USA) was used for alignment and detection of mutation.

RESULTS AND DISCUSSION

In contrast to the agronomic potential of induced mutation through plant regeneration, the precise effects of plant regeneration on DNA sequence change in regenerated plants have not been described well. Table 1 indicated DNA variations detected by AFLP marker analysis for 14 somaclones

Table 1. Polymorphic bands detected by AFLP marker analysis for Iksannamulkong, Jinju 1, and their somaclonal variants.

		AFLP primer set																		
	*Mse*I	CTT						CTG						CGA						
	*Eco*RI	AAC	AAG	ACA	ACT	ACC	AGG	AAC	AAG	ACA	ACT	ACC	AGG	AAC	AAG	ACA	ACT	ACC	AGG	
Iksannamulkong and its somaclones	Total	33	19	11	43	15	30	38	23	20	26	18	11	20	17	20	16	25	19	
	Polymorphic	0	0	0	1	0	0	0	1	0	2	0	1	0	0	0	2	1	1	
Jinju 1 and its somaclones	Total	31	22	12	40	15	29	36	23	20	29	20	12	19	24	24	20	26	22	
	Polymorphic	0	1	0	0	0	0	0	0	0	0	1	0	0	2	0	0	0	1	
		AFLP primer set																		Total
	*Mse*I	CCC						CCT						CTA						
	*Eco*RI	AAC	AAG	ACA	ACT	ACC	AGG	AAC	AAG	ACA	ACT	ACC	AGG	AAC	AAG	ACA	ACT	ACC	AGG	
Iksannamulkong and its somaclones	Total	22	25	10	22	23	17	53	54	37	30	30	26	20	31	21	28	20	19	892
	Polymorphic	0	0	0	1	1	1	0	2	0	0	1	0	1	0	0	0	0	1	17
Jinju 1 and its somaclones	Total	23	22	13	20	19	18	47	55	40	27	29	22	24	28	20	21	20	15	887
	Polymorphic	0	0	1	1	0	0	0	1	0	1	0	0	0	0	0	2	0	0	11

from Iksannamulkong and 47 somaclones from Jinju 1. Using 36 primer combinations, *Mse*I as one primer and *Eco*RI with 3' extension of a single nucleotide, 892 bands from Iksannamulkong and 887 bands from Jinju 1 were generated ranging in size from 80 to 500 bp. Of 892 bands 17 polymorphic bands were detected between Iksannamulkong and its somaclonal variants, and 11 polymorphic bands of 887 bands were detected beween Jinju 1 and its somaclonal variants. This suggests the presence of changes in DNA sequences via plant regeneration.

Mutation rates vary tremendously depending on the species and the kind of gene. In nature, mutation rate of one gene after next generation is 10^{-6}~10^{-4} in higher organisms (Weaver and Hedrick, 1997). A total of 892 AFLP fragments were summed to be 178,400 bp and 177,400 bp for Iksannamulkong and Jinju 1, respectively. On the assumption of a base change per polymorphic AFLP band in this study, base change was estimated to occur at a frequency of 9.5×10^{-5} (17/178,400 bp) in Iksannamulkong and 6.2×10^{-5} (11/177,400 bp) in Jinju 1. Direct comparison cannot be made between mutation rate of one gene and base change frequency. Also, as the base change detected by AFLP marker analysis in this study was not scanned in the whole genome, there might be high possibility to extrapolate the estimation of the rate of base change. However, it may provide general sight for understanding the frequency of base change during plant regeneration.

Microsatellite regions were known to undergo mutation at several order rates of magnitude higher than those of unique eukaryotic DNA sequences (Ellegren, 2000). SSR marker analysis was performed to identify DNA variations near the microsatellite region. It is interesting to note that two (Satt545 and Satt147) of 36 SSR markers produced polymorphic bands in two somaclonal variants of Iksannamulkong. This suggests fairly high genomic change near microsatellite regions. However, no polymorphic bands were detected in somaclonal variants from Jinju 1. SSR fragments, amplified with Satt545 and Satt147 from Iksannamulkong and its somclonal variants, were excised from 3% agarose gel for sequence determination (Fig. 1). The polymorphic bands amplified with Satt545 were sequenced successfully, while the bands amplified with Satt147 were not. The fragment from the locus-specific amplification at Satt545 in Iksannamulkong compared to a somaclonal variant revealed four additional stretches of ATT repeats in the variants (Fig. 2). Probably, insertion of ATT repeats may be caused by plant regeneration. A model for microsatellite mutation was established based on replication slippage by Levinson and Gutman (1987). Based on this model, the direction of slippage from 3' to 5' or from 5' to 3' determines the insertion or deletion of SSR. Replication slippage from 3' to 5' direction may cause in the insertion of SSR in a somaclonal variant.

Field evaluation was performed to know the phenotypic differences in the qualitative (flower color and abnormal morphology) and quantitative traits (flowering, maturity, plant height, and pod number) between somaclonal variants and their wild types. However, in spite of some DNA variation, none of the variants exhibited any phenotypic variations compared to its parents (data not shown), indicating that the genomic change is unlikely associated with those characters measured in the field. However, if genomic

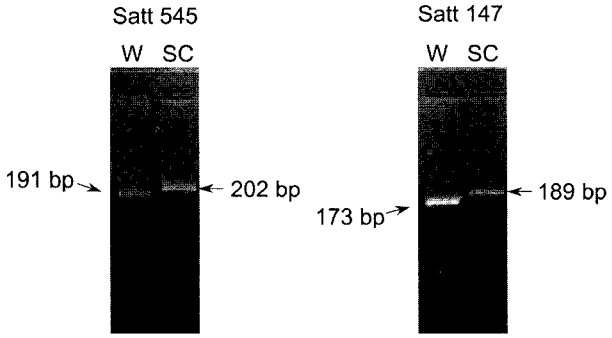

Fig. 1. Gel image showing the polymorphic amplicons for the somaclonal mutant and its wild type, Iksannamulkong. W: Iksannamulkong, SC: somaclone.

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1        10        20        30        40        50        60        70        80        90
|--------+---------+---------+---------+---------+---------+---------+---------+---------+-
W  CCCCCCCNCCCCATTCCATATTTGTTTAACCTTCCAATCCTATTCCCTT ATTATTATTATTATTATTATTATTATTATTATTATTATT
SC CCCCCCCNCCCCATTCCATATTTGTTTAACCTTCCAATCCTATTCCCTT ATTATTATTATTATTATTATTATTATTATTATTATTATT

92      100       110       120       130       140       150       160       170       180
|-------|---------+---------+---------+---------+---------+---------+---------+---------+-
W  ATTATTATTATTATTATT----------CCCTTAATCTTGATTGGGGATAATTACTTACTTTCAATCCCTTGTGTTNGCCCAATTGTA
SC ATTATTATTATTATTATTATTATTATTATTCCCTTAATCTTGATTGGGGATAATTACTTACTTTCAATCCCTTGTGTTNGCCCAATTGNA
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Fig. 2. Comparison of aligned amplicon sequences (5' → 3') from somaclonal mutant (SC) and its wild type (W), Iksannamulkong at the Satt 545 locus.

change occurred in a coding region during plant regeneration, it may provide basis for mutant induction affecting phenotypic traits.

In summary, this study suggests that mutation is induced at the DNA level such as single base change and SSR insertion via plant regeneration using tissue culture. Although plant breeders could not observe phenotypic differences in the field, variations at the DNA level from somaclonal variants could provide basis for broadening genetic diversity in crop.

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