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Development of Gene Based STS Markers in Wheat

Sang-Kyu Lee*, Hwa-Young Heo**, Young-Up Kwon***, and Byung-Moo Lee*†

*Department of Plant Biotechnology, Dongguk University-Seoul, Seoul 100-715, Korea
**Department of Plant Sciences and Plant Pathology, Montana State University-Bozeman, MT 59717 USA
***National Institute of Crop Science, RDA, Suwon 441-857, Korea

ABSTRACT The objective of this study is to develop the gene based sequence tagged site (STS) markers in wheat. The euchromatin enriched genomic library was constructed and the STS primer sets were designed using gene based DNA sequence. The euchromatin enriched genomic (EEG) DNA library in wheat was constructed using the *Mcr*A and *Mcr*BC system in DH5α cell. The 2,166 EEG colonies have been constructed by methylated DNA exclusion. Among the colonies, 606 colonies with the size between 400 and 1200 bp of PCR products were selected for sequencing. In order to develop the gene based STS primers, blast analysis comparing between wheat genetic information and rice genome sequence was employed. The 227 STS primers mainly matched on *Triticum aestivum* (hexaploid), *Triticum turgidum* (tetraploid), *Aegilops* (diploid), and other plants. The polymorphisms were detected in PCR products after digestion with restriction enzymes. The eight STS markers that showed 32 polymorphisms in twelve wheat genotypes were developed using 227 STS primers. The STS primers analysis will be useful for generation of informative molecular markers in wheat. Development of gene based STS marker is to identify the genetic function through cloning of target gene and find the new allele of target trait.

Keywords : wheat, STS marker, gene based marker, PCR based marker

There are several molecular marker types available for identifying germplasm and for analyzing genetic mapping. The restriction fragment length polymorphism (RAPD), inter simple sequence repeat (ISSR) and amplified fragment length polymorphism (AFLP) markers are mainly made from undefined elements but simple sequence repeat (SSR) and STS markers are mainly made from defined elements. Molecular markers which derived from euchromatin enriched genomic DNA should be improved for specific allele tagging. The agarose gel based markers such as STS markers would be labor and cost effective and allele descriptions could give the information of band size. The EEG DNA library is constructed to use DNA probes for germplasm discrimination, linkage map construction that shown ready to use polymorphisms, and association analysis between marker allele types and phenotypic data sets.

An STS is a short unique genomic sequence that is amplified by using allele specific oligonucleotides as PCR primers. The DNA clones mapped by RFLPs can be used to design PCR-based STS primers (Mullis and Faloona, 1987). Many RFLP markers developed by the North American Barley Genome Mapping Project (NABGMP) (Kleinhofs *et al*., 1993) have been converted to STSs (Blake *et al*., 1996). These STSs could be useful in developing allele specific PCR-RFLPs at any locus on the barley-wheat genome in desirable populations without the need for sequencing or for designing primers.

The discovery of a large number of single nucleotide polymorphisms (SNPs) in crop has revealed the power of this technology to generate high resolution genetic maps. The large amount of plant sequencing data available in public databases represents a rich resource for SNP discovery using bioinformatics approaches. In rice, for example, thousands of candidate polymorphisms are identified by comparing the draft genome sequences from indica and japonica subspecies (Feltus *et al*., 2004). For other plant species, extensive expressed sequence tag (EST) collections provide an alternative source for the *in silico* detection of SNPs.

The objective of this study was to develop gene tagging marker by using euchromatin enriched genomic DNA library. In order to develop the gene based STS marker, the effective

†Corresponding author: (Phone) +82-2-2260-3307 (E-mail) bmlee@dongguk.edu
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genome specific primers were designed from genetic information of genomic DNA library. The STS primers analysis will be useful for generation of informative molecular markers in wheat. Development of gene based STS marker is to identify the genetic function through cloning of target gene and find the new allele of target trait.

MATERIALS AND METHODS

Construction of EEG library

The reference DNA of wheat in this study was 'Keumkang', a Korean variety. The DNA was extracted by the CTAB method (Saghai-Maroof *et al*., 1984). The DNA was digested with restriction enzyme, *Spe* I and was ligated with pGEM7zf(+) vector treated with *Xba* I. The restriction enzymes, *Xba* I and *Spe* I have compatible ends and the restriction site of *Xba* I is located on MCS of pGEM7zf(+) vector. The ligated vector was transformed into a *mcr*A and *mcr*BC active *E.coli* cell line which recognizes cytosine modifications. In order to increase the efficiency of transformation, the electroporation mediated transformation method was conducted.

Colony PCR amplification and design of STS primers

Colony PCR amplification was done in volumes of 25 ㎕, each containing 0.2 units of *Taq* polymerase; 20 pmole of primers; 250 μM of dNTPs; 1.0, 1.5, 2.0, or 2.5 mM of $MgCl_2$; 1X PCR buffer. Each reaction mixture was overlaid with mineral oil. The typical PCR amplification condition was 5 min at 95℃, followed by 40 cycles of 20 sec DNA denaturation at 95℃, 30 sec annealing at 55℃, and 2 min extension at 72℃, and a final 10 min incubation at 72℃. The PCR was done with a PTC-100TM thermal cycler (MJ Research, Inc.). Selected colonies which had 400-1200 bp of PCR products were selected and sequenced. And the sequence data was searched using the NCBI and GRAMENE web site to get additional genetic information. With the sequencing data of individual colonies, specific primers were designed using the Primer 3 program. The primers were designed with melting temperatures between 55 and 61℃, GC contents between 40 and 60, and lengths between 18 and 24 bases.

Detection of polymorphisms

The plant materials used for detecting polymorphisms with developed STS primers were common bread wheat (*Triticum aestivum* L.). Twelve wheat genotypes are distributed over Korea, China, Japan and North America (Table 1). These genotypes were not closely related plants by genetic side.

Template DNA used for PCR analysis was extracted from fresh leaf tissues by the CTAB method (Saghai-Maroof *et al*., 1984). After STS primers were designed, PCR was undertaken with twelve wheat genotypes. PCR amplification was done in

Table 1. Twelve wheat genotypes used for detecting polymorphism with developed STS primers.

No.	Variety Name	Country of origin	Cross combination
1	Keumgang	Korea	Geuru/Kanto 75//Eunpa
2	Chungnam Jaerae	Korea	Traditional cultivar or Local variety
3	Youngkwang	Korea	Yuksung 3/12SE
4	Sinmi Chal	Korea	Olgeuru//Kanto 107/Baihou
5	黑粒小麥 76	China	-
6	Sumai3	China	Funo/Taiwan Xiaomai
7	Chugoku 118	Japan	Chugoku 74///Milyang 23//Chugoku 69/Asominori
8	Kanto 107	Japan	Kanto 79/Kanto 82
9	Haru-yutaka	Japan	-
10	Leader	Canada	Fortuna/Chris
11	Clark	USA	Beau//65256A1-8-1/67137B5-16/4/Sullivan/3/Beau//5517B8-5-3-3/Logan; 65256A1-8-1=Caldwell sib
12	Early blackhull	USA	Blackhull selection

Table 2. Restriction enzymes used for detecting polymorphisms in 12 wheat genotypes.

No.	Enzyme	Recognition site	No.	Enzyme	Recognition site
1	*Alu* I	AG/CT	7	*Aci* I	C/CGC
2	*Hae* III	GG/CC	8	*Hpa* II	CC/GG
3	*Hinf* I	G/ANTC	9	*HpyCh4* IV	A/CGT
4	*Rsa* I	GT/AC	10	*HinP* I	G/ANTC
5	*Tsp509a* I	/AATT	11	*Mbo* I	/GATC
6	*Taq* I	T/CGA	12	*Mse* I	T/TAA

No. 1-4, 7-8 enzymes: Incubation at 37℃ for 3hr-overnight
No. 5, 6 enzymes: Incubation at 65℃ for 1-3hr

volumes of 50 ㎕, each containing 0.2 units of *Taq* polymerase; 20 pmole of primers; 250 μM of dNTPs; 1.0, 1.5, 2.0, or 2.5 mM of $MgCl_2$; 1X PCR buffer and 50 ng genomic DNA template. The PCR amplification condition was 5 min at 95℃, followed by 40 cycles of 30 sec DNA denaturation at 95℃, 2 min annealing and extension at 65℃ (two steps PCR). The PCR was done with a PTC-100TM thermal cycler (MJ Research, Inc.).

The monomorphic PCR products were subsequently digested with 12 different four- or five-base-cutter endonucleases (*Alu* I, *Hae* III, *Hinf* I, *Rsa* I, *Tsp*509*a* I, *Taq* I, *Aci* I, *Hpa* II, *HpyCh4* IV, *Hin*P I, *Mbo* I, and *Mse* I) (Table 2). One unit of each restriction endonuclease was added to 1 ul PCR products. Digested fragments were electrophoresed in 1.5% agarose gels.

RESULTS

Analysis of EEG library and design of STS primers

A total of 2166 colonies were observed from EEG genomic DNA library. The colony PCR products between 400-1200 base pairs were selected for high frequency of polymorphism in wheat. Among these colonies, 606 colonies were selected to be sequencing. The data of missing, duplication and abnormal GC content in sequencing data were excluded. The 27.95% of selected clones were used for design of STS primers. As a result, the 227 STS primers were designed from sequence data of 606 colonies. STS primers were designed using Primer 3 Software. The 227 STS primers were analyzed data such as accession information and match on cultivars using NCBI database. The genetic information matched on developed 227 STS primers was classified in Table 3. The

Table 3. Classification of genetic information matched on developed 227 STS primers.

Classification	Number of primer
Triticum aestivum (hexaploid)	104
Triticum turgidum (tetraploid)	43
Aegilops (diploid)	14
Other (rice, barely etc)	49
No match	17
Total	227

227 STS primers mainly matched on *Triticum aestivum* (hexaploid), *Triticum turgidum* (tetraploid), *Aegilops* (diploid), and other plants.

Detection of polymorphism with developed STS primers

The polymorphic bands of 227 STS primers were evaluated with the twelve wheat genotypes by two steps PCR. Two steps PCR products showed clearly single band for specific allele tagging. Twelve wheat genotypes represented the different origins, spike characteristics, and growth habit. Though amplification of STS primers showed no polymorphisms in wheat genotypes, the polymorphisms were shown at PCR products that treated with twelve restriction enzymes. The 32 polymorphisms were detected in PCR products after digestion with restriction enzymes. Table 4 showed the procedures for development of STS marker from the 2166 colonies of EEG library.

Polymorphisms were analyzed by amplification with STS primer and enzyme digestion in 12 wheat genotypes. The polymorphisms in KG 25 primer treated with enzyme *Alu* I, KG 30 primer treated with enzyme *Rsa* I, and KG 171

primer treated with enzyme *Rsa* I were shown Fig. 1. For example, the 480 bp PCR products derived from primer KG 30 were digested 360 bp and 120 bp with Rsa I (Fig. 1B). When the concentration of endonucleases was increased from 1 unit per reaction mixture to 2 units, the same results were observed.

Polymorphisms were detected in the PCR products after digestion with eight restriction endonucleases such as *Alu* I, *Hae* III, *Rsa* I, *Tsp*509*a* I, *Aci* I, *Hpa* II, *HpyCh4* IV, and *Mse* I. The STS primers, KG 7, KG 25, KG 30, KG 75 and KG 171 showed polymorphism by one restriction enzyme; KG 54 primer showed polymorphism by two restriction enzymes; KG 92 and KG 203 primers showed polymorphism by three restriction enzymes. In conclusion, the eight STS markers that showed 32 polymorphisms in twelve wheat genotypes were developed using 227 STS primers (Table 5).

Table 4. Procedures used for development of STS marker.

Procedures	Number
No. of colonies in EEG library	2166
Sequencing	606
Primer design	227
No. of wheat genotypes	12
Restriction enzyme	12
Combinations	144
Total no. of bands	2825
No. of polymorphism	32

Table 5. Primer sequence and enzyme used in developed STS markers.

Primer	Primer sequence	Expected product length (bp)	Enzyme
7	F:CATGGAAGAACAGGAGTTCG R:AAGGTGGACAGGATAATAGCA	530	*Hae* III
		340, 190	
25	F:CTCCACCCACTCACTCAGTT R:CAGGTGTAACTACAGGCCAAG	410, 250, 50	*Alu* I
		300, 250, 110, 50	
30	F:GGGCAAATCTGCAACACTAT R:TTCCCATTGTGACTGACCTT	480	*Rsa* I
		360,120	
54	F:GCCTTGCGAAGAGCTTTACT R:AAACTAGCGCCCTCTTCCTC	480,100	*Rsa* I
		250, 230, 100	
		580	*Tsp509a* I
		290, 100, 60	
75	F:CACAAATCACACAGGACCACA R:GCCATCATTGCCTAATGTGC	610, 80	*Rsa* I
		510, 100, 80	
92	F:AGAGAGAGAATGGCGACAGG R:AGCATGGGTAAGGCTAGCAA	300, 290	*Hpa* II
		290, 190, 110	
		590	*HpyCH4* IV
		500, 90	
		250, 210, 130	*Mse* I
		235, 210, 130	
171	F:ACCGACGGTTCTGGAGTTTG R:TAGGTGTATGGTGCCGGTGA	510	*Rsa* I
		400, 110	
203	F:ATGCTCCTGTTGTATGGAACG R:GACCAACTGCGTGACAGCAT	310, 240	*Aci* I
		300, 240	
		400, 160	*HpyCH4* IV
		390, 160	
		310, 250	*Mse* I
		300, 250	

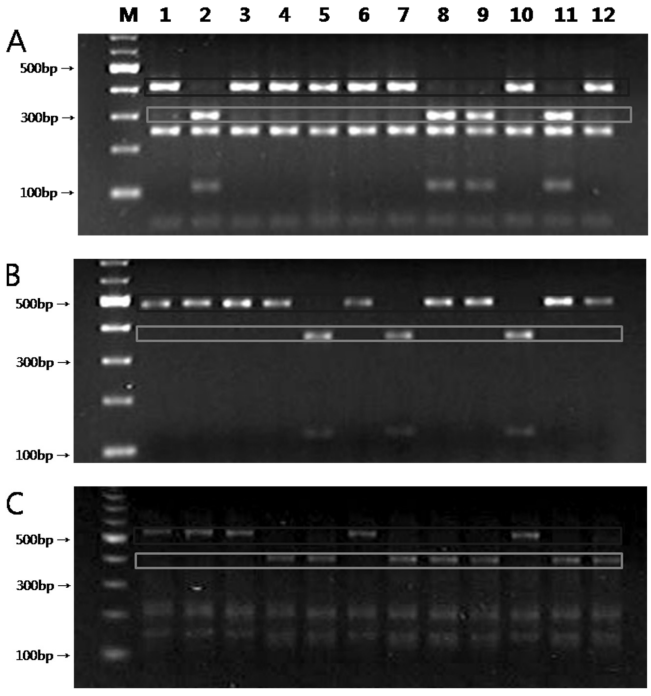

Fig. 1. Polymorphism analyzed by amplification with STS primer and enzyme digestion in 12 wheat genotypes. A: KG 25 primer treated with enzyme *Alu* I. B: KG 30 primer treated with enzyme *Rsa* I. C: KG 171 primer treated with enzyme *Rsa* I. M: size ladder marker and lane 1~12: wheat cultivars.

DISCUSSION

The development of STS markers from euchromatin enriched genomic DNA library could be used as a tool for genome analysis in wheat. The gene based STS marker has the advantages of safety and efficiency over traditional RFLP analysis and the elimination of confounding results due to amplification of repetitive DNA sequence. Additionally, once primers are developed and tested, the published sequences can be easily shared with other researchers without the trouble and expense of handling and shipping recombinant RFLP clones (Olson *et al*., 1989).

The wheat genotypes were distinguished by STS markers and restriction enzyme treatments (Table 6). The genetic analysis and germplasm evaluation of wheat genotypes could be assessed through genome specific SNP primer. The STS and SSR markers have been applied in variety identification, parentage assessment, genetic diversity analysis, molecular map construction, and in the assessment of property rights for plant varieties in major crops (Bredemeijer *et al*., 2002; UPOV-BMT, 2002; Esselink *et al*., 2003; Tommasini *et al*., 2003; Kwon *et al*., 2005; Weising *et al*., 2005).

The sequences of STS primers in barley were published by Sayed-Tabatabaei *et al*. (1998). Talbert *et al*. (1994) designed 37 primers from mapped RFLP clones in wheat. The twenty three primers amplified products that mapped to the expected homoeologous chromosome group. In general, primer sets designed from RFLP clones result in effective amplification in wheat. Additionally, 25 primers revealed polymorphisms among 20 wheat genotypes when PCR products were digested with restriction enzymes. These results suggest that the STS based PCR analysis will be useful for generation of informative molecular markers in wheat.

Integration of several linkage maps is necessary to detect

Table 6. Distinction of wheat genotypes by STS markers and restriction enzyme treatment.

Variety	Marker	Enzyme	Band size and pattern		Origin
Chungnam Jaerae	KG 54	*Rsa* I	280	O	Korea traditional cultivar
黑粒小麥 76	KG 92	*Hpa* II	300	O	China
	KG 92	*HpyCH4* IV	590	O	
	KG 7	*Hae* III	340, 190	O	
	KG 92	*Mse* I	250	X	
Sumai3	KG 75	*Rsa* I	510, 100	O	China
Kanto 107	KG 7	*Hae* III	340, 190	O	Japan
	KG 25	*Alu* I	410	X	
Leader	KG 203	*Mse* I	300	O	Canada
	KG 75	*Rsa* I	510, 100	X	

the common functional QTLs controlling agronomically important traits across different genetic backgrounds. Easy to use STS markers will allow different linkage maps to be integrated effortlessly. For example, QTLs for shoot differentiation in barley have been mapped on the long arm of chromosome 2H in three different mapping populations: Azumamugi × Kanto Nakate Gold (Komatsuda *et al*., 1993; 1995), Steptoe × Morex (Mano *et al*., 1996), and Harrington × TR306 (Takahashi *et al*., 1997).

A short term disadvantage for the STS PCR primers as compared to RAPDs primers is the need for sequence analysis before primers can be designed. However, this is accomplished only once. The STS primer sequences may find general utility for wheat genetic mapping and applied plant breeding studies. Empirical investigation will certainly be required to assess possible applications. Additionally, the eight STS marker pairs that developed in this study will be useful as landmark STSs for different wheat population.

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