Development of SCAR Markers for Korean Wheat Cultivars Identification

Son Jae-Han¹, Kyeong-Hoon Kim¹, Sanghyun Shin¹, Induk Choi¹, Hag-Sin Kim¹, Young-Keun Cheong¹, Choon-Ki Lee¹, Sung-Il Lee², Ji-Yeong Choi², Kwang-Geun Park¹, Chon-Sik Kang^{1*}

¹Department of Rice and Winter Cereal Crop, National Institute of Crop Science, RDA, Iksan, 570-080, Korea

²Department of Molecular Bioscience, Kangwon National University, Chuncheon 200-701, Korea

ABSTRACT Amplified fragment length polymorphism (AFLP) is a molecular marker technique based on DNA and is extremely useful in detection of high polymorphism between closely related genotypes like Korean wheat cultivars. Six sequence characterized amplified regions (SCARs) have been developed from inter simple sequence repeat (ISSR) analysis which enabled the identification and differentiation of 13 Korean wheat cultivars from the other cultivars. We used six combinations of primer sets in our AFLP analysis for developing additional cultivar-specific markers in Korean wheat. Fifty-eight of the AFLP bands were isolated from EA-ACG/MA-CAC, EA-AGC/MA-CTG and EA-AGG/MA-CTA primer combinations. Of which 40 bands were selected to design SCAR primer pairs for Korean wheat cultivar identification. Three of 58 amplified primer pairs, KWSM006, KWSM007 and JkSP, enabled wheat cultivar identification. Consequently, 23 of 32 Korean wheat cultivars were classified by eight SCAR marker sets.

Keywords AFLP, Molecular marker, SCARs, Wheat

INTRODUCTION

Various molecular marker techniques have been introduced and are constantly being modified and developed to enhance their utility in the process of genome analysis (Swati *et al.* 1999). DNA-based molecular marker techniques are very useful in various fields like genetic engineering, plant molecular biology, physiology as well as plant breeding. In plant breeding program like wheat breeding, various molecular markers have been used for marker-assisted selection (MAS). MAS has been widely practiced in many crop breeding programs across the globe. This technique reduces the effort and time, while improving the efficiency of the selection in the plant breeding process.

AFLP is a PCR-based technique which is useful in the analysis of relationship between closely related genotypes. It is a complex technique that is coupled with convenience of random amplification polymorphic DNA (RAPD) based on PCR and random fragment length polymorphisms

(RFLP) having high reproducibility (Vos *et al.* 1995). AFLP comprises two different two steps. The first step is restriction of DNA using combination of restriction enzyme such as *MseI* and *EcoRI*. The second step is ligation of oligonucleotide adapters that are defined sequences including the respective restriction enzyme sites. AFLP has been used for DNA fingerprinting, cloning and also mapping of species-specific DNA sequences (Yong *et al.* 1996; Paglia *et al.* 1998). In addition, AFLP technique was used for mutation analysis caused by transposition, insertion and deletion of some nucleotide in genome as well as it has been used for the analysis of genetic map (Mackill *et al.* 1996; Voorrips *et al.* 1997), evolution (Heun *et al.* 1997) and research of bio-diversity (Barrett and Kidwell 1998). Similar to RAPDs or inter simple sequence repeats (ISSRs), bands of the species-specific obtained by AFLP can be converted into SCARs (Son *et al.* 2013). Thus, AFLP technique is an important tool with variety of applications.

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*Corresponding author Chon-Sik Kang, kcs1209@korea.kr, Tel: +82-63-840-2156, Fax: +82-63-840-2116

Genetic diversity among landraces of bread wheat between different geographic regions and phylogenetic relationships of durum wheat cultivars were analyzed by using AFLPs (Stodart *et al.* 2005; Martos *et al.* 2005). This technique is a useful tool for Korean wheat research. In this study, we developed specific DNA markers for Korean wheat cultivar identification using AFLP technique. We selected this technique among various molecular marker techniques because AFLP has been shown to generate many polymorphic DNA bands.

MATERIALS AND METHODS

Plant materials and DNA extraction

Genomic DNAs were extracted from young leaves of 32 Korean wheat cultivars using HiGene™ Genomic DNA Prep Kit (SolGent, Korea). They were chosen as major cultivars in Korea and representative species in the classification of wheat cultivars (Table 1).

AFLP analysis

Information of adaptor and primer sets for AFLP analysis is indicated in Table 2. Genomic DNA was digested with *MseI* and *EcoRI* restriction enzymes. Adapter ligation step was performed according to AFLP analysis system I manual (Invitrogen, USA). A total of 25 µl pre-amplification mixture containing 40 ng of adapter ligated DNA, 0.5 µM pre-amp primer mix, 1X PCR buffer plus Mg and 5U Platinum High Fidelity Taq polymerase (Invitrogen, USA) were amplified through PCR which was performed in 20 cycles at 94°C for 30 s, 56°C for 60 s and 72°C for 60 s. This protocol was described in AFLP Starter Primer Kit (Invitrogen, USA). Selective amplification reactions were performed with 20 ng diluted pre-amplified products, 5 µM primer sets, 0.2 mM dNTPs and 0.5 U Platinum High Fidelity Taq polymerase (Invitrogen, USA) in a total volume of 25 µl. Selective amplification was carried out for one cycle at 94°C for 30 s, 65°C for 30 s, 68°C for 60 s, and the annealing temperature was then repeatedly lowered by 0.7 °C for each cycle during 12 cycles, followed by 23 cycles at 94°C for 30 s, 56°C for 30 s, 68°C for 60 s and the

Table 1. 32 Korean wheat cultivars used in this study and their expression patterns for novel markers.

No.	Cultivar	Amplified pattern	No.	Cultivar	Amplified pattern
1	Ol	N	17	<i>Joeun</i>	KWSM007
2	Geuru	N	18	<i>Anbaek</i>	N
3	Dahong	N	19	Jopoom	N
4	Chungkye	N	20	Sinmichal	KWSM006
5	<i>Eunpa</i>	KWSM006	21	Jonong	N
6	Tapdong	N	22	Jokyoung	KWSM006/ KWSM007/JkSP
7	Namhae	N	23	Younbaek	N
8	Uri	N	24	Shinmichal1	N
9	Olgeuru	KWSM006	25	<i>Baekjung</i>	N
10	Alchan	N	26	Jeokjoong	N
11	Gobun	KWSM006	27	<i>Sugang</i>	KWSM006/KWSM007
12	<i>Keumkang</i>	KWSM007	28	Hanbaek	KWSM007
13	<i>Seodun</i>	N	29	Suan	N
14	Saeol	KWSM006	30	Dajoong	N
15	Jinpoom	N	31	<i>Goso</i>	KWSM006
16	<i>Milsung</i>	KWSM006	32	<i>Joah</i>	KWSM006

* Classified wheat cultivars by novel markers are in Italics and bold type. N indicates no-amplification.

final extension of 68°C for 5 min. The amplified DNA fragments were loaded on 6% polyacrylamide gel and detected by silver stain method.

Cloning and sequencing

Specific bands (300 - 1,000 bp) were eluted from the 6% polyacrylamide gel. The eluted DNA fragments were ligated into pGEM-T vector (Promega, USA), and the clones were transferred into DH5 α competent *E. coli* cells. Nucleotide sequencing was processed at genome analysis company Xenotech Inc., Korea. The sequences were determined by primer pairs M13 and SP6 using a PRISM3700 automatic DNA sequencer (ABI Inc., USA).

Sequence analysis and re-amplification

The sequence analyzed DNA fragment length was determined from minimum 300 to maximum 1,000 bp. These fragments were included in wheat species. The sequence information was confirmed in GenBank (NCBI, <http://www.ncbi.nlm.nih.gov>). Using the sequences, we designed the SCAR primers using primer3 (version 0.4.0, <http://frodo.wi.mit.edu/>). Designed primers were tested for classification of Korean wheat cultivars. PCR reaction was performed with 40 ng of genomic DNA, 200 μ M of each dNTP, 1X reaction buffer and 0.5 U Platinum High Fidelity Taq polymerase (Invitrogen, USA) in a total volume of 25 μ l. PCR condition was the same with selective amplification

reaction. The amplified DNA fragments were confirmed on 1.2% agarose gel electrophoresis. Using SCAR primers, the PCR reaction was performed in 35 cycles at 94°C for 30 s, 55–58°C for 30 s, 72°C for 1 min and the final extension at 72°C for 5 min. Using Jokyoung specific primer (JkSP), PCR reaction was performed by touch-down phase system. In the first seven cycles the annealing temperature was lowered at 1°C interval from 62°C to 55°C, while the second step was performed in 35 cycles at 94°C for 30 s, 58°C for 30 s, 72°C for 1 min and the final extension at 72°C for 5 min. The amplified DNA fragments were confirmed by loading on 1.2% agarose gel.

RESULTS

AFLP finger printing of Korean wheat cultivars

We used six combinations of primer sets for AFLP analysis (Table 2) to develop cultivar-specific markers in Korean wheat. Each primer set produced 70 to 100 detectable amplified bands with size ranging from 200 to 500 bp, depending on the primers and Korean wheat cultivars. Fifty-eight of the total amplified bands that are potential useful markers were selected from the three primer sets (Fig. 1). Twenty-one of the AFLP bands were isolated from EA-ACG/MA-CAC primer combination, 19 bands were isolated from AGC/CTG primer combination

Table 2. Nucleotide sequences of the AFLP primers.

Primer name	Sequences (5'→3')
<i>Eco</i> RI-adaptor	EA1: GACTGCGTACC EA2: AATTGGTACGCAGTC
<i>Mse</i> I-adaptor	MA1: TACTCAGGACTCATC MA2: GATGAGTCCTGAG
Anchored <i>Eco</i> RI-adaptor primers	CTGCGTACCAATTCA
Anchored <i>Mse</i> I-adaptor primers	GATGAGTCCTGAGTAAC
<i>Eco</i> RI- selective primers	
EA-ACG	CTGCGTACCAATTACG
EA-AGC	CTGCGTACCAATTACG
<i>Mse</i> I- selective primers	
MA-CAC	GATGAGTCCTGAGTAACAC
MA-CTG	GATGAGTCCTGAGTAAC
MA-CTC	GATGAGTCCTGAGTAAC

and 18 bands were isolated from EA-AGG/MA-CTA. These bands were unique to a specific or few cultivars. We eluted the cultivar-specific bands from the silver stained acrylamide gel and performed cloning and sequencing.

Analysis of three SCAR markers

We analyzed the sequences of 58 amplified DNA fragments and designed SCAR primer pairs for Korean wheat cultivar identification. Three of 58 amplified primer pairs KWSM007, KWSM008 and JkSP were able to differentiate the set of wheat cultivars used in the test. KWSM007 and JkSP primers were designed from each amplified DNA sequences (C2 and C17) of EA-ACG/MA-

CAC primer set and KWSM008 primer was designed from amplified DNA sequence (AGG10) of EA-AGG/MA-CTA primer sets (Fig. 1). C2 and AGG10 DNA fragments were 368 bp and 268 bp, respectively, but they did not match any gene. Result of BLAST search using a part of C2 DNA sequence revealed that AGG10 sequence was not annotated but the search identified matches to some region of *Triticum aestivum* chromosome 3B-specific BAC library (contig ctg0954b) sequences. Also, C17 DNA sequence was not annotated in wheat but it has a characteristic of retrotransposon that was *Ty3-gypsy* subclass in rice (LOC_OS11g28870).

Fig. 1. AFLP analysis of 32 Korean wheat cultivars. Sizes of the amplified products range from 200 to 500 bp. The red arrows indicate the eluted cultivar-specific DNA fragments. A; EA-ACG/MA-CAC selective-primer combination, B; EA-AGG/MA-CTA selective-primer combination. M; 1kb plus ladder and 10 bp size marker.

Table 3. The information of three SCAR markers for 32 Korean wheat cultivars identification.

Marker name	Forward sequences	Reverse sequences	Annealing temperature (°C)	Product size (bp)	Type of polymorphism
KWSM007	TAAGGTGGACTCTTCTGGTTGT	ACGACATTGTCATATCGTAAAG	56	215	Presence / absence
KWSM008	GAATTCAGGCTTCAGTTGATGG	AAGGATACAATTGAAGGAACAG	58	212	Presence / absence
JkSP	CAGAGTATACGAACGAGAACTAAC	TGACACGCGATCTCTGATCCTG	58*	468/150	Single or double

Application of three SCAR markers to Korean wheat cultivars

A total of 13 Korean cultivars were classified using the six SCAR markers sets developed in Son *et al.* (2013). Using this study as reference two SCAR markers KWSM007 and KWSM008 were added to the set of markers we developed.

The DNA band of 215 bp length was amplified by KWSM007 primer pairs in ten cultivars including Eunpa, Olgeuru, Gobun, Saeol, Milsung, Sinmichal, Jokyung, Sugang, Goso, and Joah. Then KWSM007 primer was applied to the six ISSR-derived SCAR primer sets (Son *et al.* 2013) as newly SCAR marker which enabled the identification of seven other cultivars (Table 1; Fig 2). In other words, 20 out of 32 Korean wheat cultivars were classified by combination of seven SCAR primer sets containing KWSM007 primer. These seven cultivars were Eunpa, Seodun, Milsung, Joeun, Anbaek, Goso, and Joah (Table 1).

The KWSM008 primer pairs amplified the DNA band of

212 bp length in five cultivars, Keumkang, Joeun, Jokyoung, Sugang and Hanbaek (Fig. 2). Hence, three more additional cultivars Keumkang, Baekjung and Sugang were distinguished from 32 cultivars.

However, the Jokyoung-specific/derived primer, amplified two distinct bands but only in Jokyoung, while the rest only showed single band (Fig. 2).

Consequently, 23 of 32 Korean wheat cultivars were classified by six ISSR-derived and two AFLP-derived SCAR markers combination (Fig 2). This molecular screening technique uses only few number of SCAR marker combination yet it efficiently distinguished some cultivars from numerous cultivars evaluated.

DISCUSSION

Various molecular marker techniques have been successfully demonstrated in plant research or genomics (containing genetics and cytology) and breeding for cultivar DNA

Fig. 2. The profiles of PCR amplified DNA fragments using developed SCAR markers in 32 Korean wheat cultivars and amplification patterns of SCAR markers. KWSM001 to KWSM006 indicate ISSR derived SCARs, KWSM007 and KWSM008 indicate AFLP-derived SCARs. A; KWSM006, B; KWSM007, C; JkSP, M; 1kb plus ladder, D; The + indicates amplified bands and - is absence. The red and blue letters indicate wheat cultivars that were identified by ISSR-derived SCARs and AFLP-derived SCARs, respectively.

fingerprinting, marker-assisted selection (MAS), and quantitative trait loci (QTLs) analysis (Lee *et al.* 2012). AFLP is a powerful molecular-marker technique because it produces multiple amplified fragments in each PCR reaction (Vos *et al.* 1995). The multiple steps in the AFLP process, however, make it worthwhile to convert the AFLP markers to SCAR markers that are easily produced by a single PCR reaction. AFLP analysis detects restriction-site polymorphisms by PCR analysis and it was difficult to detect a single cultivar-specific polymorphic fragment (De Jong *et al.* 1997; Negi *et al.* 2000). There are useful RAPD or SSR-derived SCAR markers for drought tolerance or other abiotic stresses in wheat (Deshmukh *et al.* 2012). AFLP markers have been used for genetic, species relationship, and simple DNA fingerprinting such as genetic erosion in durum wheat cultivars and between common wheat cultivars (Martos *et al.* 2005).

Through sequences analysis, we expected that C17 is related to wheat retrotransposon. Retrotransposons were derived from RNA intermediate (Class I transposon). They can amplify themselves through the replication in a genome and particularly abundant in plants. Many retrotransposons consisted of repeated DNA and they have been found in the plants having enormous genome like maize (49-78%) and wheat (~ 90%). Their genome size was increased by retrotransposons (San miguel *et al.* 1998; Li *et al.* 2004).

Six Korean wheat cultivar-specific marker sets have been developed from previous ISSR analysis (Son *et al.* 2013). These markers can identify specifically the 13 Korean wheat cultivars from other cultivars (Son *et al.* 2013). But using KWSM007 and KWSM008 in addition to six ISSR-derived SCAR primer sets, we were able to distinguish 23 out of 32 Korean wheat cultivars (Table 1). Likewise, we were able to develop a single cultivar-specific marker, called JkSP that was derived from Jokyoung.

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