ISSR-derived Molecular Markers for Korean Wheat Cultivar Identification

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ABSTRACT Inter simple sequence repeat (ISSR) is a technique that leads to the development of novel specific molecular markers and relationship analysis between species. In this study, 54 ISSRs were used to develop cultivar-specific DNA sequence characterized amplified regions (SCARs) for Korean wheat cultivars (*Triticum aestivum*). Using nine of 54 UBC ISSR primers, 16 cultivar-specific fragments were detected among 32 wheat cultivars. Of the 16 primers, 10 successfully amplified targets in 32 cultivars and six novel cultivar-specific markers were finally selected. A total of 13 Korean wheat cultivars (Ol, Dahong, Namhae, Jinpum, Anbaek, Jopoom, Jokyung, Sinmichal 1, Jeokjoong, Dajung, Goso and Joah) were classified by newly developed six SCAR markers. Cultivar-specific markers can be used not only for cultivar fingerprinting but also for marker-assisted selection in wheat breeding program.

Keywords Molecular marker, ISSR, SCAR, Wheat

INTRODUCTION

Triticum aestivum, also known as common wheat, is an important food crop worldwide (Zhang *et al.* 2012), consumed by more than 40% of the world's population and is produced in more than 6,000,000 tons annually (Gupta *et al.* 2008). High quality elite cultivars that can adapt to climate change play a crucial role in wheat production (Zhu *et al.* 2011). Common wheat is a hexaploid ($2n = 6x = 42$) consisted of three distinct genomes A, B, and D which has seven pairs of chromosomes each genome (Gao *et al.* 2004). Although A, B and D genome are different, they are genetically related to each other. Both A and D genome are diploid in origin, but the origin of B genome is not yet clearly confirmed (Gill *et al.* 1991). Wheat has very large genome size which has more 80% of the entire genome consisted with repeat sequences like simple sequence repeat (SSR) or retrotransposon elements (Brenchley *et al.*

2012). Genes of each genome has tripled because common wheat germ cytological division is similar to that of normal diploid. These features facilitated the large genome research. Molecular markers have been used as a starter, genetic evolution research, and diagnosis in many crops including wheat. Using DNA-based molecular markers allows accurate, easy and rapid cultivar identification compare with phenotypic analysis and field growth test in wheat breeding program (Zhu *et al.* 2011). In addition, DNA-based molecular markers have been used in multi-purpose tools in various fields like taxonomy, physiology, embryology, and genetic engineering.

Simple sequence repeats (SSRs) or microsatellites were introduced by Litt and Luty (1989). SSRs are short repeated sequence motifs of 1 to 6 bp, present numerously in eukaryotic genomes including plant and animals (Tautz and Renz 1984). Although SSR markers have many advantages like easy to use and detect higher degree polymorphism,

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SSRs in coding regions are less abundant than in intergenic regions. Moreover, the process of developing SSR markers are difficult and complex such as in selecting and sequencing clones containing putative SSR motifs for designing and testing the flanking primers (Gao *et al.* 2004). Inter simple sequence repeat (ISSR) markers were reported by Zietkiewicz *et al.* (1994) which are developed by utilizing repeated microsatellites primers to amplify inter-SSR DNA sequences. Various microsatellites anchored at 5' end or 3' end are useful for amplifying genomic DNA which increase primer's specificity in PCR reaction (Godwin *et al.* 1997). Unlike other PCR-based techniques, ISSR does not require any information about target sequence of SSR regions (Tsumura *et al.* 1996). Advantages of ISSR include relatively low cost, high polymorphisms, and good reproducibility. ISSRs are highly rigid marker for scoring ISSR analysis because the random amplified markers are spurious or homoplasious bands (Bussell *et al.* 2005; Park *et al.* 2009). Kojima *et al.* (1998) showed that ISSR markers with Random amplified polymorphic DNA (RAPD) were used to construct genetic linkage map in Einkorn wheat. ISSR analysis is useful for detecting genetic diversity (Li and Ge 2001; Sica *et al.* 2005), and analyzing species relationship as well as marker-assisted selection (MAS) for molecular breeding in plant using a single primer composed of SSR (Hussain *et al.* 2000; Joshi *et al.* 2000; Pasakinskene *et al.* 2000). ISSR markers were also used for identification of abiotic stress like drought (Deshmukh *et al.* 2012). In addition, SSR and ISSRs markers are useful for cultivar identification (Crespel *et al.* 2009; Lu *et al.* 2009), specifically ISSR markers have been used for wheat cultivar identification (Nagaoka and Ogihara 1997) including others such as radish (Liu *et al.* 2008), lotus (Chen *et al.* 2008), and rose (Crespel *et al.* 2009).

In Korea, many kinds of wheat cultivars suitable for different baked products such as breads, cakes and cookies have been developed. Molecular markers are necessary tool to identify these Korean cultivars yet these remains to be investigated. In this study, we developed sequence characterization amplified region (SCAR) or sequence tagged site (STS) markers for identification of 32 Korean wheat cultivars based on specific alleles using ISSR technology.

MATERIALS AND METHODS

Plant materials and DNA extraction

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

PCR amplification and cloning

Information of nucleotide sequences of the primers for inter simple sequence repeat (ISSR) PCR amplification is indicated in Table 2. PCR amplification was performed with 40 ng of template genomic DNA, 5 μ M of primer, 2.5 mM of dNTP, and 1 unit of i-star maxII DNA polymerase (Intron, Korea) and Prime Taq DNA Polymerase (GENET Bio., Korea). The PCR annealing condition used consists 35 cycles of 94°C for 30 sec, 50°C for 30 sec and 72°C for 1 min. Amplified products were checked on 1.5% agarose gel electrophoresis where specific bands (300 - 1,000 bp) were eluted from the gel. The eluted DNA fragments were ligated into pGEM-T vector (Promega, USA), and cloned into DH5 α competent *Escherichia coli* cells. Plasmid DNA were sent to Xenotech Inc., Korea for sequencing with the primer pairs M13 forward and SP6 using a PRISM3700 automatic DNA sequencer (ABI Inc.).

Sequence analysis, primer design and re-amplification

The sequence-analyzed DNA fragment length was determined from 300 to 1,000bp. These fragments were included in wheat species (Fig. 1). The sequence information which was used to design the SCAR primers using primer3 (version 0.4.0, <http://frodo.wi.mit.edu/>) was confirmed in GenBank (NCBI, <http://www.ncbi.nlm.nih.gov>). Designed primers were tested for classification of Korean wheat varieties. PCR reaction was performed with 40 ng of genomic DNA, 200 μ M of each dNTP, 1X reaction buffer and 1 U i-star maxII DNA polymerase (Intron, Korea) in a total volume of 25 μ l. PCR conditions include initial denaturation at 94°C for 3 min followed by 35 cycles of 94°C for 30 sec, 50°C for 30, 72°C for 1 min and the final extension of 72°C for 5 min. The amplified DNA fragments were confirmed on 1.2% agarose gel electrophoresis.

Table 1. The 32 Korean wheat cultivars used in this study and information of PCR amplification patterns.

No.	Variety	SCAR marker name					
		812-1	812-4	816-1	816-2	808R	808A
1	Ol	+	+	-	+	-	-
2	Geuru	-	-	-	-	+	+
3	Dahong	+	-	-	+	-	-
4	Chungkye	-	-	+	-	+	+
5	Eunpa	-	-	-	+	+	+
6	Tapdong	-	-	-	+	-	-
7	Namhae	+	-	+	+	-	-
8	Uri	-	-	-	-	+	+
9	Olgeuru	-	+	-	-	+	+
10	Alchan	-	-	-	-	+	+
11	Gobun	-	+	+	+	+	+
12	Keumkang	-	-	-	-	-	+
13	Seodun	-	-	+	+	+	+
14	Saeol	-	-	-	-	+	+
15	Jinpoom	+	+	-	-	+	+
16	Milsung	-	-	+	+	+	+
17	Joeun	-	-	-	+	+	+
18	Anbaek	+	-	-	-	+	+
19	Jopoom	+	+	-	+	+	+
20	Sinmichal	-	-	-	-	+	+
21	Jonong	-	-	-	-	+	+
22	Jokyung	+	+	-	-	-	+
23	Younbaek	-	-	-	-	-	-
24	Sinmichal 1	+	+	-	-	-	-
25	Baekjung	-	-	-	-	-	+
26	Jeokjoong	+	-	-	-	-	+
27	Sugang	-	-	-	-	+	+
28	Hanbaek	-	-	+	+	-	-
29	Suan	-	-	-	-	+	+
30	Dajoon	+	+	-	-	+	+
31	Goso	+	-	-	-	+	+
32	Joa	+	+	-	-	+	+

* The + indicates amplified bands and - is absence

RESULTS AND DISCUSSION

ISSR amplifications

We have surveyed 54 ISSR primers for amplification using 32 Korean wheat cultivars, 14 of which showed consistent amplification and multiple polymorphism containing specific bands (Fig. 1). Other primers showing monomorphic banding pattern were not selected for further experiments in this study. ISSR primer UBC849 was profiled for identification of eight wheat cultivars in China wherein six different bands were amplified (Zhu *et al.*

2011), but when tested in Korean wheat cultivars there was no polymorphism detected UBC811 (Fig. 1). Although ISSR primer has been used for genetic linkage map in Einkorn wheat (Kojima *et al.* 1998), it was not used in this study because it failed to amplify the target allele. The 14 primers showing consistent and polymorphic amplification were consisted of all 3'-end anchoring (Fig. 1; Table 2). The number of amplified products varied from 1 to 8 with sizes that range from 200 to 1,000 bp depending on the primers and cultivars. The PCR reactions were carried out over three times in different thermocyclers (GeneAmp

PCR System 9700, ABI, USA; Biometra, Germany) using different DNA polymerase (Prime Taq DNA Polymerase, GENET Bio, Korea; i-star maxII, Intron, Korea) to obtain reproducible results. Since many bands were detected, the specific bands were eluted from the agarose gel for the sequencing. Polymorphisms were observed not only among different species like *Sumai3* (data not shown) but also among different 32 Korean wheat cultivars (Fig. 1).

Sequence analysis and designing the SCAR primers

We eluted 16 different DNA fragments from the agarose gel and performed the sequencing process. The number of eluted bands was one each in UBC808, UBC815, UBC816, UBC823 and UBC840, two in UBC807, and three each in UBC812, UBC813, and UBC819 (Fig. 1). BLAST analysis, querying the *Triticum* sequences deposited at NCBI, confirmed that the ISSR fragments were real DNA fragments from Wheat species, and further analysis of these primer pairs is therefore likely. However, all of the sequences are not coded with any functional genes because ISSR amplifies

genomic sequences between SSR loci or non-coding regions (Son *et al.* 2012). Hence, we designed the SCAR primers using primer3 input (Ver. 0.4.0) program from 16 analyzed DNA sequences based on ISSR amplicons. The primer length was fixed 20 to 24 bp to increase primer specificity and efficiency.

Application of SCAR markers to 32 Korean wheat cultivars

The newly developed markers were tested for reliability in classifying Korean wheat varieties. A total of ten cultivar-specific primer pairs which did not amplify or have generated non-specific band were discarded. With this, we used the remaining six primer pairs as SCARs (Table 3) which successfully amplified target sequences in Korean wheat cultivars with sizes of the amplicons corresponded well to expected length in sequence analysis (Fig. 2). Six SCAR primer pairs are generated from UBC808, UBC812 and UBC816. We tested these primer pairs over three times using different DNA polymerase and

Fig. 1. ISSR profiles of 32 Korean wheat cultivars (1-32, Table 1). The amplified products were represented by the sizes ranging from 200 to 1,000 bp. The yellow Arrows indicate the eluted DNA fragment. A; UBC807, B; UBC808, C; UBC812, D; UBC813, E; UBC816, F; UBC815, G; UBC819, H; UBC823, I; UBC836, J; 840, K; 844, L; 846, M; UBC849, N; UBC880.

PCR machines to validate the application of these SCARs for the identification of 32 Korean wheat cultivars. Although these markers have little disadvantage due to different annealing temperature in PCR condition, it did not affect the efficiency of the PCR assay. With these new marker

combinations, a total of 13 out of 32 Korean wheat cultivars were identified as unique from each other (Fig. 2). These include Ol, Dahong, Chungkye, Tapdong, Namhae, Olgeuru, Gobun, Jopoom, Jokyoung, Younbaek, Sinnichal 1, Jeokjoong and Hanbaek.

Table 2. Sequence information of ISSR primers used in this study.

No.	Primer	Sequence	No.	Primer	Sequence
1	UBC801	ATATATATATATATATT	28	UBC828	TGTGTGTGTGTGTGTGA
2	UBC802	ATATATATATATATATG	29	UBC829	TGTGTGTGTGTGTGTGG
3	UBC803	ATATATATATATATATC	30	UBC830	TGTGTGTGTGTGTGTGC
4	UBC804	TATATATATATATATAA	31	UBC831	ATATATATATATATATYA
5	UBC805	TATATATATATATATAC	32	UBC832	ATATATATATATATATYC
6	UBC806	TATATATATATATATAG	33	UBC831	ATATATATATATATATYA
7	UBC807	AGAGAGAGAGAGAGAGT	34	UBC832	ATATATATATATATATYC
8	UBC808	AGAGAGAGAGAGAGAGC	35	UBC833	ATATATATATATATATYG
9	UBC809	AGAGAGAGAGAGAGAGG	36	UBC834	AGAGAGAGAGAGAGAGYT
10	UBC810	GAGAGAGAGAGAGAGAT	37	UBC835	AGAGAGAGAGAGAGAGYC
11	UBC811	GAGAGAGAGAGAGAGAC	38	UBC836	AGAGAGAGAGAGAGAGYA
12	UBC812	GAGAGAGAGAGAGAGAA	39	UBC837	TATATATATATATATART
13	UBC813	CTCTCTCTCTCTCTT	40	UBC838	TATATATATATATATARC
14	UBC814	CTCTCTCTCTCTCTA	41	UBC839	TATATATATATATATARG
15	UBC815	CTCTCTCTCTCTCTG	42	UBC840	GAGAGAGAGAGAGAGAYT
16	UBC816	CACACACACACACACAT	43	UBC841	GAGAGAGAGAGAGAGAYC
17	UBC817	CACACACACACACACAA	44	UBC842	GAGAGAGAGAGAGAGAYG
18	UBC818	CACACACACACACACAG	45	UBC843	CTCTCTCTCTCTCTRA
19	UBC819	GTGTGTGTGTGTGTGTA	46	UBC844	CTCTCTCTCTCTCTRC
20	UBC820	GTGTGTGTGTGTGTGTC	47	UBC845	CTCTCTCTCTCTCTRG
21	UBC821	GTGTGTGTGTGTGTGTT	48	UBC846	CACACACACACACACART
22	UBC822	TCTCTCTCTCTCTCTCA	49	UBC847	CACACACACACACACARC
23	UBC823	TCTCTCTCTCTCTCTCC	50	UBC848	CACACACACACACACARG
24	UBC824	TCTCTCTCTCTCTCTCG	51	UBC849	GTGTGTGTGTGTGTGYA
25	UBC825	ACACACACACACACACT	52	UBC850	GTGTGTGTGTGTGTGYC
26	UBC826	ACACACACACACACACC	53	UBC876	GATAGATAGACAGACA
27	UBC827	ACACACACACACACACG	54	UBC880	GGAGAGGAGAGGAGA

* Y and R are single letter abbreviation for mixed base positions. Y is presenting C or T, R is presenting A or G. The red letters indicate the selected UBC primers for eluting specific bands.

Table 3. The PCR condition information of six novel SCAR markers for the identification of 32 Korean wheat cultivars.

Marker name	Forward sequences	Reverse sequences	Annealing temperature (°C)	Product size (bp)	Number of Polymorphic band
812-1	AAGAAGAAAAAGAAAGAGAGTTGAG	GTTGTGTTTTTATTTGTTTTTGAC	55	359	1
812-4	GCCGAGATTAAATTTGTTACTACT	AACATTTATGTTTGGTAAATTGAC	55	312	1
816-1	TGCTGAGGGCATATCTCAACA	GTGTTGCACGACGCTGTACT	58	682	1
816-2	TGCTGAGGGCATATCTCAACG	GTGTTGCACGACGCTGTACT	58	682	1
808R	GGCTTACTACCTACACTTGACC	GCAACAACAACCTTCTCCCAAT	58	392	1
808A	GGCTTACTACCTACACTTGACC	AACAACCTTCTCCCAATCCCT	58	392	1

Unlike the other 12 cultivars, Younbaek was identified distinctly from the others because of unamplified reactions from all primer sets. The 812-1 SCAR marker was amplified in 12 cultivars, Ol, Dahong, Namhae, Jinpum, Anbaek, Jopoom, Jokyung, Sinmichal 1, Jeokjoong, Dajung, Goso and Joah. Interestingly, no coded functions has been designated yet to this gene marker in *Triticum*. However, some of these cultivars have been reported to exhibit traits such as Jeokjoong which is known to have moderate resistance to *Fusarium* and is used for making noodle (Park *et al.* 2008), Baegjoong and Joah which are resistant to pre-harvest sprouting, and Goso which is very useful for making cookie as well as it has resistance to pre-harvest sprouting like Baegjoong, Joa and Sugang (Park *et al.* 2009). Similar to 812-1, the 812-4 is functionally non-coded gene marker which was specifically amplified in nine Korean wheat cultivars (Ol, Olgeuru, Gobun, Jopoom, Jokyung, Sinmichal 1, Dajung and Goso). Additionally,

the number of Korean wheat cultivars with amplifications were six for 816-1, 11 for 816-2, 21 for 808R, and 25 for 808A primer. The complete detail of the cultivar and primer pair combination with amplifications is shown in Fig. 2.

Both 816-1 and 816-2 are allele-specific primers. A nucleotide of 3'- end is different, 816-1 is A (T allele-specific primer) and 816-2 is T (A allele-specific primer). With this, Chungkye contains T allele because it showed amplification with 816-1 but not with 816-2 primer. On the otherhand, Ol, Dahong, Eunpa, Tapdong, Joeun and Jopoom have A allele while Namhae, Gobun, Seodun, Milseong, Jeokjoong, have both T and A alleles.

Since 19 Korean wheat cultivars were not clearly identified and classified by the six primer sets, we planned to proceed with both amplified fragment length polymorphism (AFLP) and transposon display (TD) techniques to detect more polymorphism. With this technique, we expect to classify

Fig. 2. The profiles of PCR amplified DNA fragments using developed SCAR markers in 32 Korean wheat cultivars. The + indicates amplified bands and - is absence. Red + indicated the distinguished 12 cultivars, 1; Ol, 3; Dahong, 4; Chungkye, 6; Tapdong, 7; Namhae, 9; Olgeuru, 11; Gobun, 19; Jopoom, 22; Jokyung, 23; Younbaek, 24; Sinmichal 1, 26; Jeokjoong, 28; Hanbaek, by six novel markers combination. A; 812-1, B; 812-4, C; 816-1, D; 816-2, E; 808R and F; 808A.

more cultivars using variable molecular markers in the future. Nonetheless in this study we were able to identify 13 out of 32 Korean wheat cultivars using the 6 primer pairs. The newly developed cultivar-specific SCAR markers can be used not only for cultivar fingerprinting but also for marker-assisted selection and inter-specific hybridization to combine important traits in the wheat breeding program (Burton 1992; Brilman 2001). This technique also saves time and money by performing only one PCR reaction.

CONCLUSION

Inter simple sequence repeat (ISSR) is a useful technique to analyze the relationship between species and to develop specific molecular markers without the need of target sequence information. In this study, we used ISSR primers to develop cultivar-specific DNA SCARs for Korean wheat cultivars. Six novel cultivar-specific markers (designated 812-1, 812-4, 816-1, 816-2, 808R, and 808A) were developed to identify 13 cultivars using 54 ISSR primers. A total of 13 cultivars were identified as unique based on specific alleles amplified which include Ol, Dahong, Namhae, Jinpum, Anbaek, Jopoom, Jokyoung, Sinmichal 1, Jeokjoong, Dajung, Goso and Joah. Of which, Jokyoung and Goso are few of the major cultivated wheat cultivars together with Keumkang in Korea. Cultivar-specific markers can be used not only for cultivar fingerprinting but also for marker-assisted selection in wheat breeding program. Furthermore, because ISSRs are based on universal repeat motifs in the genome, ISSRs can be used in molecular bio-techniques for development of novel marker and species relationship analysis in plants.

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