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Direct sequencing of RAPD products provides a set of SCAR markers for discrimination of sweet potato cultivars

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Abstract

Sweet potato (*Ipomoea batatas* L. Lam) is one of the most commercially important crops worldwide and still the major food source in many developing countries. In order to accelerate the marker-assisted selection for sweet potato breeding, development and optimal use of easy-to-use and sequence-specific genetic markers, are required. Here, we analyzed 200 pairs of the random amplified polymorphic DNA (RAPD) markers to uncover the polymorphisms in sweet potato and subsequently developed 13 pairs of sequence-characterized amplified region (SCAR) markers. In contrast to the random amplification of RAPD markers, the newly-developed SCAR markers revealed lower ratio of polymorphisms, but showed higher repeatability, user-friendly and were sequence-specific. Furthermore, we employed these markers to exploit the genetic diversity of 27 Korean sweet potato cultivars, which could be distinguished and grouped. Overall, these results show that the newly-developed and sequence-specific SCAR markers could be used for marker-assisted selection (MAS) for breeding for elite sweet potato varieties in South Korea.

Keywords: Sweet potato, molecular markers, RAPD, SCAR, genetic diversity.
Abbreviations: RAPD_Random amplified polymorphic DNA; SCAR_Sequence characterized amplified region; ISSRs_inter-simple sequence repeats; AFLPs_amplified fragment length polymorphisms; Polymerase chain reaction (PCR).

Introduction

Sweet potato (*Ipomoea batatas* L. Lam) belongs to the Convolvulaceae family and is the seventh most important food crop worldwide after wheat, rice, maize, potato, barley, and cassava (Huaman, 1999). It has wide adaptability on marginal land and nowadays it is still considered as one of the most important food crops in many developing countries (Zhang et al., 2000). Sweet potato is asexually inherited and each cultivar is considered as a clone with identical genetic background. However, there are many variations in these clones depending on the environmental conditions (soil, climate, fertilizer, etc.) under which these are cultivated. These variations are minor and uneasily detectable since most of the sequence variations do not results in the visual changes of its architecture and development (for instance, biotic and abiotic stresses). The PCR-based genetic markers are derived from sequence variations and these are functional and detectable. Therefore, these are recognized as one of the most efficient tools to decipher sequence variations in many research fields, such as, genetic diversity, marker-assisted selection, map-based cloning (Poczai et al., 2013). Many molecular markers have been established which effectively can be used for cultivar discrimination. Random amplified polymorphic DNA (RAPD), inter-simple sequence repeats (ISSRs), and amplified fragment length polymorphisms (AFLPs) are representative of PCR-based methods, as these methods do not require genome sequence information (Vidal et al., 2000). These methods have been applied to barley (Russell et al., 2000), wheat (Christiansen et al., 2002), and maize (Lu and Bernardo, 2001) to investigate the genetic polypmorphism at inter- or/and intra-species level. Additionally, polymorphism in the sweet potato cultivars has been analyzed using SSR marker (Hwang et al., 2002), ISSR marker (Hu et al., 2003), and RAPD analyses (Dubreuil and Charcosset, 1999). Among these, RAPD analysis has widely been used for biomarker development, since it is simple, quick, and relatively inexpensive (Moon et al., 2010). RAPD is also an informative marker system which is particularly of interest for disserting the genetic diversity. However, RAPD markers are dominant and sequence-nonspecific (Paran and Michelmore, 1993). Therefore, to increase the reproducibility, RAPD markers have been converted into sequence characterized amplified region (SCAR) markers in which along with polymorphic bands that are needed, multiple bands are generated simultaneously. SCAR markers are co-dominant markers and generally reveal higher degree of polymorphism. However, development of sequence-specific and user-friendly SCAR markers is one of the most crucial steps in analyzing the genetic diversity. In this study, we have developed the RAPD-derived user-friendly SCAR

markers system which can be utilized for analyzing the genetic diversity and marker-assisted selection in sweet potato.

Results

RAPD pattern of 27 sweet potato cultivars

Sweet potato tubers are good source of carbohydrates in many tropical areas and are cultivated worldwide. However, analysis of biochemical properties of different sweet potato tubers has revealed that different cultivars showed different biochemical and nutritional properties (Ravindran et al., 1995). Since there are not much morphological differences among different sweet potato cultivars, development of an alternative rapid method for authentication of a particular sweet potato cultivar is very crucial. In this study, 200 random decamer primers were screened, of which 148 primers produced distinct, reproducible, and polymorphic amplification of all DNAs. As shown in Fig. 1, many polymorphic bands were observed in the agarose gels. A total of 815 polymorphic fragments were scored; 437 bands (53.6%) were monomorphic among the 27 cultivars, whereas 371 bands (46.4%) showing polymorphic bands were identified with an average of 5.5 bands per primer (Supplementary Table 1).

SCAR primer designing from sequenced RAPD fragments

To discriminate the 27 sweet potato cultivars, we performed SCAR analysis based on the RAPD. As shown in Fig. 2, a representatively polymorphic band amplified by the AA01 random primer, was cloned and sequenced (Fig. 2A). The nucleotides of the sequence matched completely with the corresponding RAPD primer used (data not shown). Based on the sequences of polymorphic bands obtained from RAPD, a total of 59 SCAR primers were designed which were used to amplify genomic DNA from the 27 cultivars. Among these, 13 successful polymorphic SCAR primers, listed in Table 2, showed polymorphisms in the 27 sweet potato cultivars, whereas 46 primer sets were found to be monomorphic. As shown in Fig. 2B, a single, distinct, and brightly resolved band was obtained. For the identification of genetic variability in the 27 sweet potato cultivars, we amplified genomic DNA using the 13 newly-developed SCAR primers. As shown in Fig. 3, most of the SCAR primers showed polymorphism in several sweet potato cultivars. However, A11-4 and B13_02 SCAR primer sets showed a specific band in "Whitestar" cultivar. Hae-pi-me specific band was amplified by E05-1 SCAR primer. Interestingly, G01_08 SCAR primer amplified two PCR bands with different molecular sizes (514 bp and 441 bp) (Fig. 3) which were cloned and sequenced. Analysis of these two amplicons showed that 73 bp were deleted from 441 bp amplicon (Fig. 4). This data suggested that the two PCR fragments were identified by InDel (insertion and deletion) marker in the region of sequences amplified by G01_08_700 SCAR primer (Fig. 3 and Fig. 4). Consequently, the 13 SCAR primer pairs developed in this study could be utilized as potential markers.

Genetic relationship among 27 sweet potato cultivars

The genetic relationship between the 27 sweet potato cultivars used, were determined by dendrogram analysis. A dendrogram is a tree-structured graph to visualize the distances or similarities among different genes or samples (Hu et al., 2013). The PCR products generated from SCAR analysis were used to calculate the genetic

Table1. The list of sweet potato cultivars used for this experiment.

No	Name	Origin	Flesh color
1	Ha-yan-mi	korea	White
2	Whitestar	USA	White
3	Bio-mi	korea	White
4	Sin-ja-mi	korea	Purple
5	Ja-mi	korea	Purple
6	Yeon-ja-mi	korea	Purple
7	Bo-ra-mi	korea	Purple
8	Ju-hwang-mi	korea	Orange
9	Sin-hwang-mi	korea	Orange
10	Saeng-mi	korea	Orange
11	Hae-pi-mi	korea	Orange
12	Yeon-hwang-mi	korea	Yellow
13	Dae-you-mi	korea	Yellow
14	Khu-syu	Japan	Yellow
15	Sin-cheon-mi	korea	Yellow
16	Mat-na-mi	korea	Yellow
17	Sin-nyul-mi	korea	Yellow
18	Jin-heung-mi	korea	Yellow
19	Geon-pung-mi	korea	Yellow
20	Jung-mi	korea	Yellow
21	Yeon-mi	korea	Yellow
22	Hel-ssi-mi	korea	Cream
23	Go-geon-mi	korea	Cream
24	Geon-mi	korea	Cream
25	Hong-mi	korea	Cream
26	Yul-mi	korea	Cream
27	Sin-geon-mi	korea	Cream

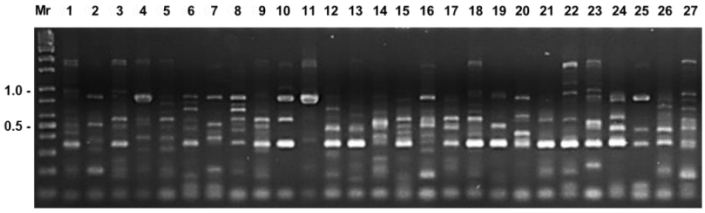

Fig 1. PCR amplification of RAPD marker using random primer E19 revealed informative polymorphic information across the 27 collected sweet potato cultivars. Number 1 to 27 refer to the sweet potato cultivars listed in Table 1. Fragments generated by amplification were separated according to size on 1.5% agarose gels run in 1X TBE buffer, stained with EtBr, and visualized by illumination with UV Gel doc (ATTO, Kyoto, Japan). On the left side, size of the standards (Mr: 1kb plus DNA ladder) is indicated.

distances between the 27 sweet potato accessions (Fig. 5). To analyze the genetic relationships between 27 sweet potato cultivars (25 Korean and two foreign sweet potato cultivars), genetic similarity using Dice's index was established among each possible accession pair (Supplementary Table 2). All varieties were scored for the presence (1) or absence (0) of band fragments of each of the amplified products. Only distinct and polymorphic bands were scored. Fragments showing the same gel mobility but different intensities were not distinguished from each other when cultivars were compared. Monomorphic fragments were not scored. Based on the infor-

Table 2. The sequences of 13 newly-developed SCAR markers.

No	Primer name	Forward sequence (5'-3')	Reverse sequence (5'-3')	Fragment (bp)
1	A11-4	CTTGGCTTCAAATGCTTTCC	AATCGCCGTTGTTTCTCTGA	806
2	A16-4	AACTTGAAGCTCAGCGACGA	CCAGCGAAGTATATAAATGATACGA	415
3	E05-1	GGGAGGTGATCTTAACAGCAAA	TCAGGGAGGTAACTGACCAT	550
4	E17-6	CTGCCGTATACTATTGTTAATAGCC	CCGTTGGTATGGAGATGGAG	574
5	E17-7	TCGACATGATATTGTAGCTGTGT	CGCTCCAACAACTCTAATGC	520
6	E17-8	CTGCCGTTATATACAAAGGAAAGAG	AGATCTGACCTCGGGATTACAA	555
7	F17-4	CCCGGGAATTTAAATATGGA	CCGGGAATCGGAAATCAT	455
8	F19-1-7	GGAGGCGATGCAGAGAGAT	TCTAGACCTCTCAAGTAACCCAAAA	408
9	G01-06-700	ACGGAGGAAAGGGAGGATTT	AGCCTCAGCACATAACGACG	514 & 441
10	G01-13	ACGGAGGAAAGGGAGGATTT	GTGAACCAATCCTAGGCTTG	473
11	AA08-18-750	TCCGCAGTAGGCAAGTCATA	TAGAGTGGGATATGCACTTGCA	703
12	B13-02-600B	AATCTGGCCCTGTCGCTTAT	ACATCGGCTCTCCCTAAAGC	582
13	AA01-24-700	CTCCTTGCATCCAGGCTTTC	CGCCGATGATATCCTTATGC	587

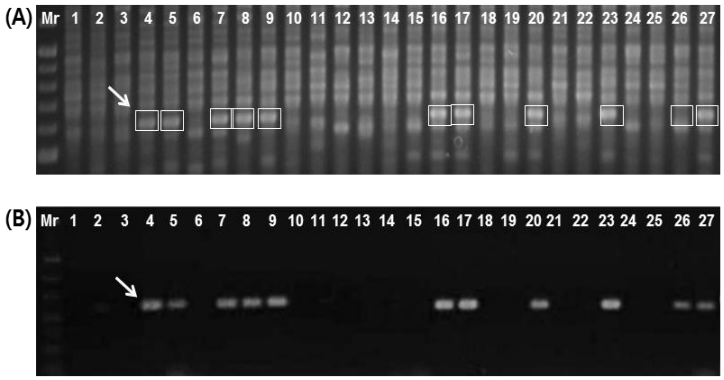

Fig 2. Comparison of RAPD and RAPD-derived SCAR marker. Test of markers were performed by analyzing 27 sweet potato cultivars. 1 kbp DNA ladder is indicated. (A) Gel picture of RAPD marker. (B) Gel picture of RAPD-derived SCAR marker. Arrow indicates the amplified polymorphic DNA with same molecular weight.

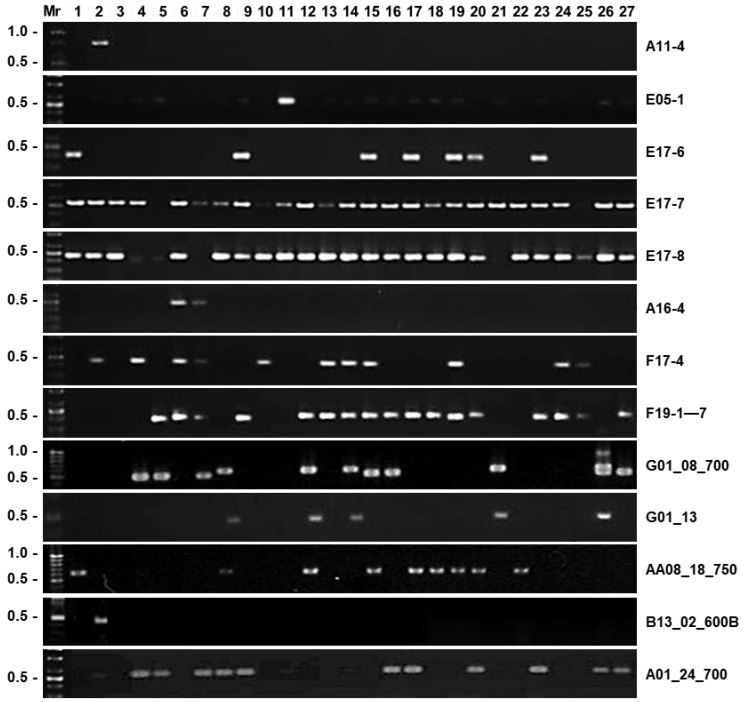

Fig 3. SCAR profiles of 27 sweet potato cultivars by using 13 different SCAR markers. 1 kbp DNA ladder is represented.

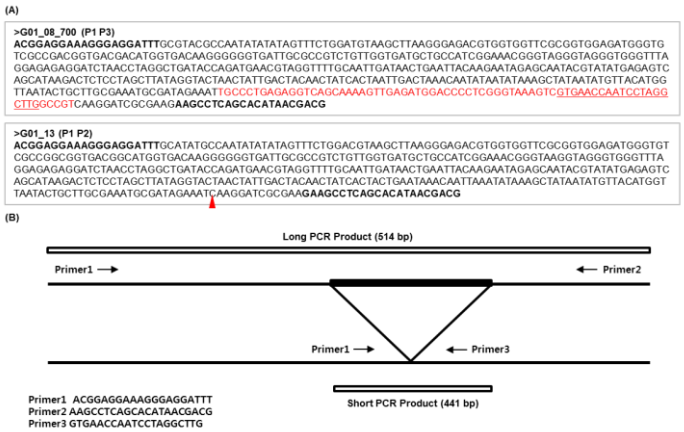

Fig 4. Sequence and schematic view of InDel marker among SCAR markers. (A) PCR products were sequenced for InDel detection. Red code indicates inserted fragment. Bold code indicates SCAR primer set. (B) Schematic view of InDel region. A given segment of DNA is present (black box).

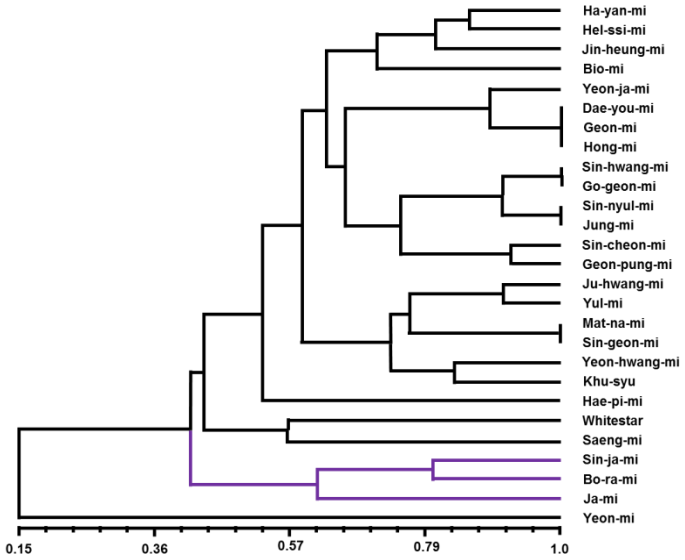

Fig 5. A dendrogram tree of 27 sweet potato cultivars using 13 SCAR markers. Dendrogram was generated using NTSYS 2.1 software.

mation generated by scoring polymorphic bands, genetic similarity using Dice's index was established between each possible accession pair. Dice's coefficient range was from 0.222~0.909 with a mean of 0.573. Four groups (cultivar no. 9 and 16, 9 and 20, 8 and 26, 17 and 23) showed the highest coefficient (0.909), whereas three groups (cultivar no. 5 and 19, 7 and 22, 15 and 21) showed the lowest similarity (0.222). According to sweet potato morphological flesh colors, the 27 accessions were clustered in two major groups. The first group was composed of white-fleshed sweet potatoes, which including Ha-yan-mi, Hel-ssi-mi, Jin-heung-mi, and Bio-mi cultivars while the second group comprised of only purple-fleshed sweet potatoes, including Sin-ja-mi, Bo-ra-mi, and Ja-mi cultivars (Fig 5. Purple line). However, most of the other cultivars were not clustered. Since morphological characteristics can vary considerably under different environmental conditions, classification of sweet potatoes remains very difficult. However, understanding of the genetic similarity between cultivars in Korea, as well as their genetic relationships is important for efficient conservation and utilization of germplasm resources.

Discussion

In this study, RAPD analysis was carried out to find out the genetic diversity in the sweet potato cultivars. Connolly et al., (1994) and Jarret and Austin (1994) reported that averages of 3.7 and 3.9 RAPD polymorphic bands per primer, respectively, in sweet potato, revealing that these values were less than our results. A similar pattern was demonstrated in a previous study on sweet potato (Lee et al., 1997; Lee et al., 1998; Lee et al., 2013). Based on the RAPD analysis, these results suggest that sweet potato varieties cultivated in Korea exhibit a high degree of genetic variability since it is an outcrossing hexaploid, and variations might be caused by somatic mutation fixed by vegetative propagation. Thus, these results led us to further discriminate sweet potato cultivars by using more specific approaches such as SCAR. RAPD analysis is a simple method to reveal high degree of polymorphism and does not require the information about the genome sequence of the species.

Therefore, it can easily be used to find out the degree of polymorphism in sweet potato cultivars. However, it also has its own limitations like poor reproducibility, and stability. Multiple polymorphic bands appeared in one sample some of which may have only slight size difference between the cultivars. This make it more difficult to distinguish different samples. SCAR markers are generated from RAPD markers based on the sequence analysis. This sequence based SCAR markers show high specificity and reproducibility than that of RAPD markers. The RAPD-SCAR method was already used for authentication
of *Dimocarpus longan* (Yang et al., 2013), *Phyllanthus emblica* (Dnyaneshwar et al., 2006), as well as distinguish susceptible and resistance race of *Elwusine indica* (Cha et al., 2014). However, SCAR analysis for sweet potato variety authentication is not carried out yet. In our study, an overall, single, distinct, and brightly resolved band was obtained using the SCAR markers, revealing that the specific SCAR markers could be utilized for assessment of the genetic diversity in cultivated sweet potato accessions. Dendrogram analysis indicated that those sweet potato accessions could successfully be used for investigate the genetic relationship between different accessions which may help us to apply this method for further applications such as searching of disease resistance accessions or quality related screenings. Furthermore, it was observed that specificity for SCAR markers was low in sweet potatoes due to the complexity of the polyploidy genome. However, we also found that some of those SCAR primers have specificity to some sweet potato cultivars, indicating that there is still a possibility of developing distinct markers. For this reason, cultivar-specific markers for each cultivar are expected to be used for screening plants at very early developmental stages to support breeding programs. Studies on discriminating various cultivars are needed for the development of such cultivar-specific markers.

Materials and Methods

Plant materials and DNA extraction

Twenty-seven Korean sweet potato cultivars were used in current study. All plants were cultivated under the climatic chamber condition (16 day/8 night, 22°C, 70% humidity) in the Bio-energy Crop Research Center, National Institute of Crop Science (NICS), Rural Development Administration (RDA), Suwon, South Korea. Young leaves were sampled and stored in -80°C. Extraction of genomic DNA was performed according to the previously described procedure (Doyle and Doyle, 1990). The quality and quantity of DNA samples were determined by a Nanodrop spectrophotometer (Thermo, Wilmington, USA) and gel electrophoresis by using lamda-DNA. The original DNA stocks were stored at -20°C while a few aliquots were diluted to the working concentration (10 ng/µL) for the PCR reactions.

Polymerase chain reaction (PCR) and gel electrophoresis

PCR amplification was performed as described previously with some modifications (Moon et al. 2010). A final reaction volume of 20 µL was prepared containing 20-30 ng of genomic DNA, 1 U of *Taq* DNA polymerase enzyme (Solis Biodyne, Tarty, Estonia), 100 µM of each dNTPs (Promega, Madison, Wi, USA), 1x *Taq* DNA polymerase buffer with 25 mM $MgCl_2$, and 5 µM of random primer. PCR was performed using following protocol: 5 min for initial denaturation, followed by 40 cycles of 30 sec at 95°C, 1 min annealing at 40°C (RAPD primers) or 55-65°C (SCAR markers), and 2 min extension at 72°C, and finally PCR extension by 5 min at 72°C. PCR products were resolved on 1% agarose gel (3h, 120V) and visualized using gel documentation (ATTO, Tokyo, Japan). The oligonucleotides were ordered from Bioneer, Daejeon, South Korea.

Gel extraction, cloning, digestion and Sanger sequencing

DNA bands showing polymorphisms among the cultivars were eluted using Agarose Gel extraction Ultra kit (Solgent, Daejeon, Korea). The purified fragments were ligated into pGEM-T easy vector (Promega, Madison, Wi, USA) according to manufacturer's protocol. The recombinant plasmids were transformed into *E. coli* strain DH 5α (Real Biotech Corporation, Taipei, China), which were subsequently cultured on the LB (Luria Bertani) solid medium containing 50 µg/mL ampicillin (37°C, 12-16 h). Positive colonies were picked up and trans-cultured in 4 mL of LB liquid medium containing 50 µg/mL ampicillin (37°C, 12-16 h). Bacterium solutions were subsequently sampled for plasmid mini-prep by applying a Plus Plasmid Mini kit (Nucleogen, Gyeonggi-do, Korea). The proposed recombinants were digested by *Eco*RI restriction enzyme (Promega, Madison, Wi, USA). The plasmids carrying inserts were subsequently subjected for sequencing on an ABI Prism 3700-Sequencer (Applied Biosystems, Foster City, CA, USA).

Primer design for SCAR markers and data analysis

SCAR markers were derived from the sequences of DNA fragments showing polymorphisms. Primer designing was performed by using the online software 'Primer3'. Oligonucleotides were ordered from Mbiotech, Seoul, Korea. These sequence-specific primers were tested as described above. Data of the polymorphisms were analyzed by the NTSYS-pc software ver. 2.1 (Rohlf, 2002). A dendrogram was constructed using the SAHN (Sequential, agglomerative, hierarchical, and nested clustering parameters) program based on the UPGMA (Unweighted Pair-Group Method with Arithmetical Averages) method.

Conclusion

A high degree of genetic polymorphism was observed among sweet potato cultivars which can be used to develop markers for cultivar identification. In this study, a total of 13 SCAR markers were developed by sequencing of RAPD polymorphic bands. These SCAR markers could be successfully used for the identification of sweet potato cultivars. Among these, three specific SCAR markers and one InDel marker were identified for discrimination of 27 sweet potatoes and the genetic diversity among different cultivars were further analyzed. Overall, SCAR markers developed in this study, have great potential for marker-assisted breeding and can be used to screen plants at very early developmental stages in sweet potato.

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Competing Interests

The authors have declared that no competing interest exists.

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