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Research Article

Molecular identification of sweet potato accessions using ARMS-PCR based on SNPs

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Abstract The sweet potato (*Ipomoea batatas* [L.] Lam.) is the sixth-most important crop in the world following rice, wheat, potato, maize, and cassava. Four varieties ('Beniharuka', 'Annobeni', 'Pungwonmi', 'Hogammi') and their Japanese cultivars are broadly distributed in South Korea. In the Korean marketplace, sweet potatoes are classified by color and shape, not by variety, making it necessary to differentiate varieties for uniform production and consumption. In this study, molecular markers were developed to distinguish the four varieties of sweet potato using SNPs and genotyping-by-sequencing (GBS) analysis via a tetra-primer amplification refractory mutation system (ARMS)-PCR. The results revealed that three variety-specific fragments (164 bp and 241 bp of SNP 04-27457768 and 292 bp of SNP 03-16195623) were amplified in the 'Beniharuka', 'Pungwonmi', and 'Annobeni' sweet potato varieties. There were instances where some varieties produced three bands within the gel electrophoresis, indicating heterozygosity at the given SNPs loci. DNA sequencing analysis also confirmed the results of electrophoresis at the SNPs loci. Overall, these molecular markers would provide a useful, rapid, and, simple evaluation method for the Korean sweet potato marketplace, where the mixing of varieties is a serious issue.

Keywords Sweet potato, SNPs, ARMS-PCR, Molecular marker

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Introduction

Sweet potato, *Ipomoea batatas* (L.), is a dicotyledon plant belonging to the family of *Convolvulaceae* (morning glory). This important root crop is rich in proteins, starch, and many other nutritional substances such as β-carotene and anthocyanins (Bovell-Benjamin 2007). Therefore, sweet potatoes are used as food, feed, and raw materials for industrial purposes.

Although the sweet potato is an important crop, its genome has not been completely sequenced yet. This is because sweet potato has complex genomes that are difficult to assemble. Sweet potato is hexaploid and highly polymorphic with a very large genome size (Arumuganathan and Earle 1991; Ozias-Akins and Jarret 1994).

The closest probable relative of sweet potato is known to be wild diploid species *Ipomoea trifida* (Roullier et al. 2013). Recently, genome research of *Ipomoea trifida* has been conducted and reported (Hirakawa et al. 2015). However, the origin of polyploidy (autopolyploidy, allopolyploidy) in sweet potato is still obscure (Wu et al. 2018).

Molecular markers are extensively used in plant research and breeding projects (Ganal et al. 2009). Restriction fragment length polymorphism (RFLP) was first known as molecular marker reported by Botstein et al (1980) to make human genetic linkage map. After the introduction of polymerase chain reaction (PCR) technology (Mullis and Faloona 1987), many molecular markers have emerged, including random amplified polymorphism DNA (RAPD) (Williams et al. 1990), amplified fragment length polymorphism (AFLP) (Vos et al. 1995), simple sequence repeat (SSR) (Zietkiewicz et al. 1994), and single molecular polymorphism (SNP). SNPs are allelic variations. They have ultra-high-throughput outcome. They are amenable to automation technology (Mammadov et al. 2012). For this reason, SNPs are commonly used molecular marker in many studies recently.

Genetic characteristics of sweet potato have not been elucidated yet. RAPD and AFLP markers have been used for genetic linkage of sweet potato (Kreigner et al. 2003; Ukoskit and Thompson 1997). SSR markers have been used to evaluate sweet potato diversity and characterize sweet potato (Karuri et al. 2010; Wang et al. 2011). Recently, genome sequencing and assembly have been used for sweet potato genome research (Yang et al. 2017; Wu et al. 2018). Thus, sweet potato has been studied in many ways, ranging from molecular markers to sequencing.

Four sweet potato varieties ('Beniharuka', 'Annobeni', 'Pungwonmi', and 'Hogammi') were selected in this study. They are known to have high yields in the Korea. In Korea, sweet potatoes are not distributed by variety, but are classified and distributed according to the color and taste of sweet potatoes. 'Beniharuka' is known as honey sweet potato on the Korean market, and this variety has been distributed in with 'Beniazuma' and 'Benisatsuma'. In this way, similar varieties are cultivated as the same varieties, and mixing of varieties occurs. Moreover, varieties cultivated mainly in East Asia show similarities in starch and shape, making it difficult to distinguish. The mix of varieties is difficult to prevent pests, which leads to lower yields and poor quality. Although several studies of molecular maker for various purposes were reported (Kou et al. 2017; Wang et al. 2011; Zhang et al. 2016), there are insufficient markers for existing cultivars. Thus, developing a marker for sweet potatoes is essential.

In this study, tetra-primer ARMS-PCR (Ye et al. 2001) was utilized to perform molecular identifications for four varieties of sweet potato based on our SNPs data sets. Candidate SNPs for these four varieties were identified from GBS data, and optimal annealing temperature of designed primers was then determined. DNA sequencing was performed to confirm their suitability for distinguishing the four varieties. Finally, two functional SNP markers were developed for distinguishing sweet potato varieties

Materials and Methods

Plant materials and Genomic DNA extraction

Sweet potato plants (*Ipomoea batatas* (L.) Lam.) were cultivated *in vitro* and grown at 28 ± 2°C with 16 hours of light and 8 hours of dark. Total genomic DNAs were extracted from leaf tissues of sweet potato cultured *in vitro* using the method reported previously (Kim and Hamada 2005). Briefly, fresh young leaves at seedling stage were ground to a fine powder in liquid nitrogen and extracted with pre-warmed CTAB buffer containing 2-mercaptoethanol. Contaminating substances were removed with chloroform : isoamyl alcohol (24:1). DNAs were precipitated with cold 2-propanol and washed with 70% ethanol. Air-dried DNA pellet was dissolved in water. DNA samples were treated with RNase A to deplete contaminating RNA. DNA quality and quantity were confirmed by spectrophotometry (DS-11 Series Spectrophotometer / Fluorometer, DeNovix Inc., Wilmington, DE, USA) and electrophoresis (Mupid-One, ADVANCE, Chuo-ku, Japan) using 1.5% agarose gel stained with ethidium bromide (MP Biomedicals Korea, Seoul, Korea).

Primer design and Tetra-primer ARMS-PCR

Each outer and inner primers were designed based on two SNPs (SNP 04-27457768, SNP 03-16195623) to identify four varieties of sweet potatoes. The program developed by Ye et al (2001) was used to design primers following their specifications and limiting fragment sizes to the range of 100 ~ 300 bp. Default settings were used for other parameters. Computer software to design primer for tetra-primers ARMS-PCR is available at: http://primer1.soton.ac.uk/primer1.html (Collins and Ke 2012).

Tetra-primer ARMS-PCR was conducted using four primers for molecular authentication of sweet potato varieties. PCR reactions were run in a 20 μl volume containing 50 ng of template DNA, 0.5 U of r-Taq DNA polymerase (TaKaRa, Japan), 2 μl of 10× reaction buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$), and 0.25 mM dNTPs. Concentrations of outer and inner primers were 0.25 μM and 0.5 μM, respectively. PCR amplification program for SNP 04-27457768 was: 5 min at 94°C; 35 cycles of 30 sec at 94°C, 30 sec at 55°C, and 30 sec at 72°C; and an additional 5-min extension step at 72°C. PCR amplification for SNP 03-16195623 was performed with the following program: 5 min at 94°C; 35 cycles of 30 sec at 94°C, 30 sec at 66°C, and 30 sec at 72°C; and an additional 5-min extension at 72°C. After PCR, all PCR products were analyzed by electrophoresis (Mupid-One, ADVANCE, Japan) using 20 μl aliquot and 1.5% (w/v) agarose gel (Agarose A; Bio Basic Inc., Canada) stained with ethidium bromide (MP Biomedicals Korea) and visualized via UV transilluminators (Gel documentation system ATTO, Japan). Fragment lengths were estimated by comparison with standard size markers (1-kb DNA ladder; iNtRON Biotechnology, Korea).

Sequencing and alignment

PCR products using outer primers were subjected to DNA sequencing analysis. Subsequently, dideoxy cycle sequencing PCR was performed using Bigdye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Warrington, UK) according to the manufacturer's protocols. After sequencing PCR, bead-based PCR clean up method was used. Sequencing PCR products were then run on an ABI 3730XL DNA Analyzer (Applied Biosystems). Sequence editing and assembly of contigs were performed using Sequence Scanner Software 2 (v2.0) (Life Technologies, Carlsbad, CA, USA). For sequence comparison among varieties, CLC Sequence Viewer 8 (CLC Bio A/S, Aarhus, Denmark, www.clcbio.com) was used and then manually adjusted by authors.

Results

SNPs marker selection

Molecular markers were selected to distinguish the four varieties ('Beniharuka', 'Annobeni', 'Pungwonmi', and 'Hogammi'), which are used in Korea without discrimination (Fig. 1). SNPs were filtered based on GBS data which was perform for 66 accession of sweet potato (unpublished data of our own lab). GBS is a technology for discovering SNPs in organism through multiplex sequencing. It can also decrease genome complexity by using restriction enzymes. Therefore, it is widely used for many SNP analyses (Elshire et al. 2011).

In the GBS data filtered by four cultivars, 2,543 SNPs showed different sequence. We randomly selected markers among them for the tetra-primer ARMS-PCR of the four cultivars. As a result, two SNPs (SNP 04-27457768, SNP 03-16195623) were selected.

Tetra-primer ARMS-PCR

Primers for ARMS-PCR targeting two SNP loci were designed to distinct four varieties of sweet potatoes (Table 1). Tetra-primer ARMS-PCR system is one of simple and

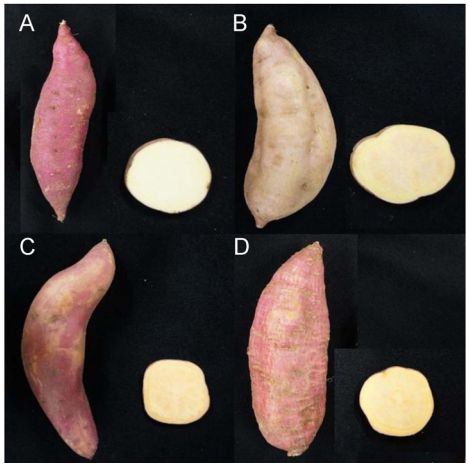

Fig. 1 The four varieties of sweet potato: (A) Beniharuka, (B) Annobeni, (C) Pungwonmi, and (D) Hogammi. Each box represents a cross section from the four varieties

Table 1 Primer designs for identifying the four varieties of sweet potato

SNP	System	Primer sequence (5'→3')[z]	Allele	Tm[y] (°C)	Amplicon (bp)
04-27457768	Forward inner primer	CTTTTGCTGTGGAAATGTAAATAA**G**A**G**	G	52.7	164
	Reverse inner primer	AATCTTCAGTGCTAGCTCATTTGTAA**T**G	C	55.4	241
	Forward outer primer	CCTCCTAAACTTTTGCAGCTAAGAGTATT		56.9	349
	Reverse outer primer	TCCAGAGTATATAACAAACCTTTGTGG		54.7	
03-16195623	Forward inner primer	CTTCCAACGTCTGACCGTGTAACCTA**T**C**C**	C	63.2	205
	Reverse inner primer	TTGACGGACGAAGTGGTGCTAGCC**A**T**A**	T	64.6	292
	Forward outer primer	AAACCGAGCAGACTCTTTCCCAGTTTCC		63.5	440
	Reverse outer primer	CTAGTTTGGCTATGGTGGGCAAGCTTTG		62.9	

[z]The mismatches for allele-specific primers are emphasized in bold
[y]Tm: Melting point temperature

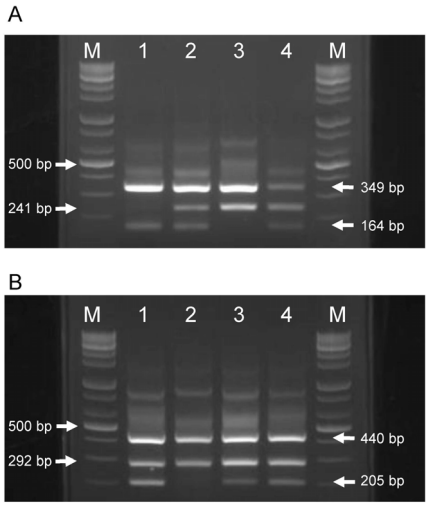

Fig. 2 The production of Tetra-primer ARMS-PCR using primers for (A) SNP 04-27457768 and (B) SNP 03-16195623. M, 1-kb DNA ladder: 1, Beniharuka; 2, Annobeni; 3, Pungwonmi; 4, Hogammi

economical methods for genotyping SNPs (Ye et al. 2001). Methods for genotyping SNPs have been applied to molecular identifications of varieties and species (Jeong et al. 2010; Kim et al. 2012; Park et al. 2006). Inner primers were designed based on SNP locus while modifying the 3' termini and the second nucleotide from the 3'-end of the original sequence. This is because inconsistency of the 3' termini only is insufficient (Ye et al. 2001). Outer primers were designed to be sequenced at both termini of the region including SNPs.

Figure 2A showed differences among the four varieties regarding SNP 04-27457768. Heterozygote was shown in 'Annobeni' and 'Hogammi' while homozygote was shown in 'Beniharuka' and 'Pungwonmi'. 'Beniharuka' and 'Pungwonmi' had a guanine and cytosine homo shape in this locus by showing only a band at 164 and 241 bp, respectively. The other two varieties had guanine and cytosine hetero shape in this locus by showing two bands at 241 bp and 164 bp.

In SNP 03-16195623, homozygote was shown in 'Annobeni' while heterozygote was shown in the other three varieties (Fig. 2B). 'Annobeni' showed a thymine homo shape in this locus by showing only a band at 292 bp. However, the other three varieties of sweet potato had thymine and cytosine hetero shape in this locus by showing two bands at 292 bp and 205 bp.

Alignment of DNA sequences

DNA sequences of PCR products were used to evaluate electrophoresis results of ARMS-PCR. Although Figure 3 looks like a homozygote in SNPs loci, heterozygote varieties could identify two allele based at the wavelength of the loci (Fig. 4). PCR product was amplified by using only the outer primers. The sequencing results coincide with tetra-primer ARMS-PCR experiments at the target region.

Discussion

Sweet potato is one of the most significant crops with abundant nutrients including starch and protein. Sweet potatoes have been as edible food, biomass, and so on. Many countries have bred varieties of sweet potato for various purposes and preferences. It is difficult to distinguish many sweet potato varieties only based on their morphological characteristics. Therefore, many previous studies have tried to develop molecular markers for sweet potato (Kou et al. 2017; Wang et al. 2011; Zhang et al. 2016).

It is important to develop markers for varieties in countries such as Korea, Japan, and China that cultivate sweet potatoes. In order to understand cross-country influx and the mix of varieties, markers for each cultivar should be developed. Since the early 2000s, sweet potatoes have been recognized as high-class foods. Its growing areas are expanding in Korea. However, due to cultivation of Japanese varieties rather than Korean varieties, it is urgent to develop markers for varieties classification in Korea. Especially, there is a problem of mixing varieties due to similarities of sweet potato varieties. In Korea, the market share of four varieties, including 'Beniharuka', 'Annobeni' (Japanese varieties), 'Pungwonmi', and 'Hogammi' (Korean varieties), accounts for more than 90%. However, sweet potato is not planted according to its varieties in Korea. These four kinds of varieties are mixed and planted because farmers plant sweet potatoes with apparent similarity. It will be important to establish molecular identification for systematic variety protection and breeding. In addition, specific markers are needed for different varieties in order to improve the quality of sweet potato and control pest insects.

GBS analysis have been used for SNPs development in various plants (Poland et al. 2012; Pootakham et al. 2015). In the present study, ARMS-PCR based on SNPs selected from GBS could identify the four most cultivated varieties of sweet potato in Korea (Fig. 1). However, the genome of sweet potato is complex due to polyploidy and heterozygote. We could confirmed through tetra-primer ARMS-PCR data that two SNPs represent three band of two or three in the four varieties (Fig. 2). The DNA sequencing also showed that some cultivars had two allele bases in the

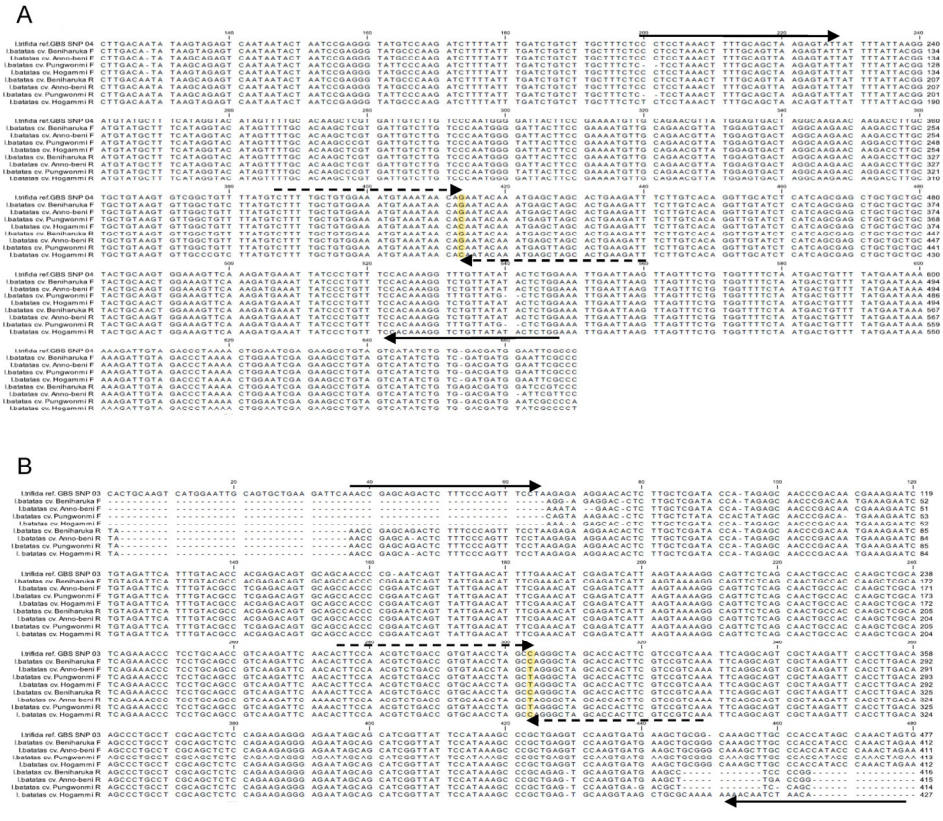

Fig. 3 DNA sequences of the four varieties and GBS data annotated from *I. trifida* in the (A) SNP 04-27457768 and (B) SNP 03-16195623 loci. The solid arrows and dashed arrows indicate outer primers and specific inner primers sites, respectively. Yellow boxes indicate the SNP sites

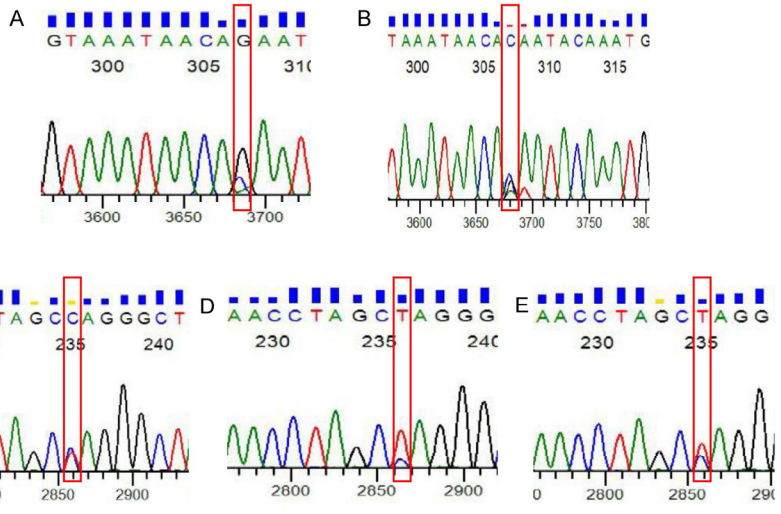

Fig. 4 Mixed sequence content following a heterozygote in (A-B) SNP 04-27457768 and (C-E) SNP 03-16195623. Red boxes indicate SNP sites showing two wavelengths of each allele. (A) SNP 04-27457768 in Annobeni, (B) SNP 04-27457768 in Hogammi, (C) SNP 03-16195623 in Beniharuka, (D) SNP 03-16195623 in Pungwonmi, (E) SNP 03-16195623 in Hogammi

SNPs loci (Fig. 4). A previous study using sweet potato has also shown that many PCR products have two or three bands after electrophoresis (Kou et al. 2017). Sugarcane, an allopolyploid plant, also coexists with ancestral ITS sequences in some hybrid generations, resulting in three bands (Yang et al. 2018).

Moreover, a previous study has performed phylogenetic analysis to test the hypothesis of hybridization (allopolyploids between *I. trifida* and *I. triloba* or/and in an autopolyploid by *I. trifida)* in sweet potato complex (Wu et al.

2018). However, the origin of sweet potato remains unclear. Unresolved sweet potato origin might be due to complexity of sweet potato genome and heterozygote of SNPs.

In this study, we performed tetra-primer ARMS-PCR and DNA-seq analysis using GBS-based SNPs to develop molecular markers for four varieties including Korean and Japanese cultivars. These varieties could be distinguished by electrophoresis using two SNPs developed in this study. Although some varieties showed three bands, they indicated heterozygote status of sweet potato. Although marker study of sweet potato is difficult due to their complicated genome, the results of this study can be one of the markers that can distinguish other varieties including 'Beniharuka', 'Annobeni', 'Pungwonmi', and 'Hogammi'. These molecular markers of sweet potato can be used for marker-assisted breeding and identification among many varieties bred for various purposes.

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