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Research article

Development and application of KASP marker for high throughput detection of *AhFAD2* mutation in peanut

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ABSTRACT

Background: Cultivated peanut (*Arachis hypogaea* L.) is a major oilseed crop worldwide. Fatty acid composition of peanut oil may affect the flavor and shelf life of the resulting food products. Oleic acid and linoleic acid are the major fatty acids of peanut oil. The conversion from oleic acid to linoleic acid is controlled by the Δ12 fatty acid desaturase (FAD) encoded by *AhFAD2A* and *AhFAD2B*, two homoeologous genes from A and B subgenomes, respectively. One nucleotide substitution (G:C → A:T) of *AhFAD2A* and an "A" insertion of *AhFAD2B* resulted in high-oleic acid phenotype. Detection of *AhFAD2* mutation had been achieved by cleaved amplified polymorphic sequence (CAPS), real-time polymerase chain reaction (qRT-PCR) and allele-specific PCR (AS-PCR). However, a low cost, high throughput and high specific method is still required to detect *AhFAD2* genotype of large number of seeds. Kompetitive allele specific PCR (KASP) can detect both alleles in a single reaction. The aim of this work is to develop KASP for detection *AhFAD2* genotype of large number of breeding materials.

Results: Here, we developed a KASP method to detect the genotypes of progenies between high oleic acid peanut and common peanut. Validation was carried out by CAPS analysis. The results from KASP assay and CAPS analysis were consistent. The genotype of 18 out of 179 BC_4F_2 seeds was aabb.

Conclusions: Due to high accuracy, time saving, high throughput feature and low cost, KASP is more suitable for determining *AhFAD2* genotype than other methods.

1. Introduction

Cultivated peanut (*Arachis hypogaea* L.) is one of the most important oil crops worldwide. Oleic acid and linoleic acid are the major fatty acids of peanut oil, accounting for approximately 80% of the oil composition [1]. Linoleic acid contains two double bonds at the Δ9 and Δ12 positions of the hydrocarbon chain. Oleic acid with only one double bond at the Δ9 position is more stable to oxidation than linoleic acid [2]. As a result, the oleic acid and linoleic acid (O/L) ratio is an important parameter for oil quality determination. By determining fatty acid composition the first two natural high-oleate peanut mutants (F435-2-1 and F435-2-2) were identified. The O/L ratio of the mutants reached nearly 40:1 [3]. Numerous high O/L peanut cultivars such as 'SunOleic 95R', 'SunOleic 97R', 'Florida-07' and some Chinese peanut varieties including Kainong176, and K17-15 were developed from F435 via traditional breeding efforts [4,5,6]. High oleic acid content in these mutants was controlled by two major recessive genes [7]. Using Arabidopsis FAD2 cDNA as a probe, two homologous and non-allelic genes encoding Δ12 fatty acid desaturase were isolated and characterized in peanut [8,9]. Δ12 fatty acid desaturase is responsible for the conversion of oleic acid to linoleic acid in peanut [8,10]. One of these two genes was from A subgenome and designated as *AhFAD2A*. The mutant allele *fad2a* had G → A transition at position 448 after the start codon. This caused a nonsynonymous amino acid substitution from aspartic acid to asparagine (D150N). This mutation resulted in a dysfunctional AhFAD2A desaturase. FAD2 gene from B subgenome was designated as *AhFAD2B*. The mutant allele *fad2b* had an "A" insertion at position 442 after the start codon. This insertion resulted in a frame shift and led to an inactive *AhFAD2B* desaturase [9,10]. There are only 11 base pair difference between *AhFAD2A* and *AhFAD2B*, resulting in a difference of 4 amino acids.

Based on the gene character of *AhFAD2A* and *AhFAD2B*, DNA markers were developed for marker-assisted selection (MAS) to enhance the efficiency of high oleic acid peanut breeding program. Cleaved amplified polymorphic sequence (CAPS) markers were first developed for *AhFAD2A* [11] and *AhFAD2B* detection [12]. Subsequently, allele-specific polymerase chain reaction (AS-PCR) method was developed [13] and further optimized [14]. PCR product sequencing

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method was also used to distinguish *AhFAD2B* gene in the hybrids from crosses between normal cultivars and high oleate cultivars [15]. Real-time PCR amplification of *AhFAD2B* and *AhFAD2A* was used to discriminate wild type plants from the mutant alleles [16,17]. These various markers for *AhFAD2A* and *AhFAD2B* detection allow breeders and researchers to determine the genotypes of peanut lines with AABB, aabb and AaBb. There are nine genotypes in F_2 hybrids. It is difficult to distinguish all these genotypes within one experiment using the above-mentioned methods. Furthermore, utilization of restriction enzymes in CAPS and fluorescence-labeled probes in real-time PCR resulted in relatively high cost of these methods. Therefore, a simple, high throughput and low cost assay with high resolution is required.

KASP (Kompetitive Allele Specific PCR) is a novel competitive allele specific PCR for SNP genotyping assay based on dual FRET (Fluorescent Resonance Energy Transfer). In this method, the sample DNA is amplified with thermal cycler using allele specific primers. The allele specific primers are conjugated to fluorometric dye HEX and FAM at their 5′ end, respectively. When FRET cassette primer is hybridized with DNA, fluorometric dye and quencher are separated, leading to emitting of the corresponding fluorescence. The genotype is easily detected by reading fluorescent signals. Owing to low cost, high throughput, and high specificity and sensitivity, KASP has been extensively used in massive SNP genotyping studies such as genome-wide SNP assay for rice genotyping [18], molecular markers-assisted breeding of wheat leaf rust resistance [19], and soybean cyst nematode resistance [20]. In peanut, KASP assay was firstly applied to screen on 94 genotypes using 96 SNPs, [21]. In this study, the KASP method was developed to detect all genotypes of *AhFAD2A* and *AhFAD2B* in different cross progenies.

2. Materials and methods

2.1. Plant materials

To develop peanut with high oleate and high yield for domestic consumption, cross was made between high oleate donors, Kainong176 (developed by Kaifeng Academy of Agriculture and Forestry), and normal oleate and high yield peanut cultivars, Huayu 31 (developed by Shandong Peanut Research Institute).

2.2. DNA extraction

About 0.1 g of a slice of cotyledonary tissue from parents, F_1 and F_2 mature seeds was obtained and placed with a 3.97 mm steel ball in a 1.5 mL tube. Tubes were immersed in liquid nitrogen and tissue was finely ground by intensely shaking the tube for 1 min. DNA extraction was performed according to the protocol of TIANGEN plant genomic DNA extraction kit (category No. DP-305).

2.3. KASP method

The KASP method was used to detect crossing parents, F_1 and BC_4F_2 seeds. The KASP reaction and its components are described at http://www.lgcgenomics.com/genotyping/kasp-genotyping-reagents/how-does-kasp-work. Sequences of allele-specific and common primers are listed in Table 1. 1.5 μL 2× KASP PCR mix, 35–45 ng of genomic DNA template, 0.16 μM allele-specific forward primers, and 0.41 μM reverse primers were incorporated into 3 μL of KASP reaction. Amplification was performed in a Hydrocycler, water bath thermal cyclers, starting with 15 min at 94°C, a touchdown phase of 10 cycles at 94°C for 20 s and at 65°C for 60 s with a 1°C decrease in temperature per cycle, followed by 35 cycles of 94°C for 20 s and 55°C for 60 s. Once the thermal cycle is complete, BMG Omega F plate reader was used to read fluorescence signal. Fluorescence signal was acquired at 520 nm (green) and 556 nm (yellow) for 2 min at 25°C.

Table 1
Primers for KASP and CAPS.

Primer name	Sequence
AhFAD2A-Allele-l	5′-GTTTTGGGACAAACACTTCGTT-3′
AhFAD2A-Allele-2	5′-GTTTTGGGACAAACACTTCGTC-3′
AhFAD2A-common	5′-CGCCACCACTCCAACACC-3′
AhFAD2B-Allele-l	5′-CAAACACTTCGTCGCGGTCT-3′
AhFAD2B-Allele-2	5′-CAAACACTTCGTCGCGGTCG-3′
AhFAD2B-common	5′-CCGCCACCACTCCAACACA-3′
Allele-l Tail (FAM tail)	5′-GAAGGTGACCAAGTTCATGCT-3′
Allele-2 Tail (HEX tail)	5′ GAAGGTCGGAGTCAACGGATT-3′
aF19	5′-GATTACTGATTATTGACTT-3′
1056	5′-CCAACCCAAACCTTTCAGAG-3′
bF19	5′-CAGAACCATTAGCTTTG-3′
R1/FAD	5′-CTCTGACTATGCATCAG-3′

2.4. Validation of KASP using CAPS method

CAPS method [11,12] was used to validate the KASP results of *AhFAD2A* and *AhFAD2B*. Briefly, to detect *AhFAD2A* genotype, the genomic DNA was amplified with primers aF19 and 1056 (Table 1). Each reaction contained 1 μL of DNA, 2 μL of 10× PCR buffer (Takara), 0.2 μL of ExTaq DNA polymerase (Takara), 1.6 μL of 2.5 mM dNTPs (Takara), 0.5 μL of forward and reverse primers in 20 μL reaction system. The PCR condition was 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 51°C for 30 s, and 72°C for 1 min. The final extension step was at 72°C for 7 min. PCR products were digested with 1 U of *Hpy*99I restriction enzyme (New England Biolabs, Ipswich, MA) at 37°C for 4 h. The digested products were separated on a 2% (w/v) agarose gel and stained with ethidium bromide. To detect the *AhFAD2B* genotype, primer pairs bF19/R1(FAD) (Table 1) amplified the coding region of *AhFAD2B* genes using a total PCR reaction volume of 20 μL containing 0.5 μL DNA, 0.5 μL FastPfu DNA polymerase (Transgene Biotech), 4.0 μL of 5× Pfu buffer, 0.4 μL of 10 mM dNTPs (Transgene Biotech), and 0.5 μL forward and reverse primers R1(FAD), respectively. Amplification conditions were initiated at 94°C for 5 min for denaturation; 35 cycles of 94°C for 45 s, 52°C for 45 s, 72°C for 60 s; final extension at 72°C for 7 min. Eight microliters of the amplification products was digested by 0.2 μL or 2 U of *Hpy*188I (New England Biolabs, Ipswich, MA, USA). The digestion was performed at 37°C overnight. Digested products were separated on a 2% (w/v) agarose gel.

3. Results and discussion

3.1. Genotyping AhFAD2A and AhFAD2B by KASP

The genotypes of *AhFAD2A* and *AhFAD2B* genes in normal oleic peanut and high oleate peanut were designated as AABB and aabb, respectively. To develop the KASP method allele-specific primers were designed based on the sequences of wild type and mutant *AhFAD2A* and *AhFAD2B* genes (Table 1). The KASP assay mix contains three assay-specific non-labeled oligos: two allele-specific forward primers and one common reverse primer. The allele-specific forward primers differentiated the mutant allele from wild type allele. Due to the high sequence identity of coding region of *AhFAD2A* and *AhFAD2B* genes, the primers are the key to successfully distinguish these two genes. Fortunately, there is a SNP between these two genes at position 432 from the 5′ end of the open reading frame (C in *FAD2A* and A in *FAD2B*) [10]. Therefore, according to the competitive allele specific PCR theory of KASP, 3′ end of the common reverse primer was designed at this SNP position to discriminate A and B subgenomes (Fig. 1).

The allele-specific primers each harbored a unique tail sequence that corresponds with a universal FRET (fluorescence resonant energy transfer) cassette; *Ahfad2a*/*Ahfad2b*-Allele-1 Tail primer, the mutant