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An AS-PCR assay for accurate genotyping of *FAD2A*/*FAD2B* genes in peanuts (*Arachis hypogaea* L.)

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RESUMEN

Ensayo AS-PCR para la genotipificación precisa de genes FAD2A/FAD2B en cacahuete (*Arachis hypogaea* L.)

FAD2A y FAD2B son genes homólogos de genomas A y B en cacahuete cultivado (Arachis hypogaea L.) que codifican las desaturasas de ácidos grasos que convierten oleato a linoleato. Para estudiar la genética de las variedades mejoradas de cacahuete alto oleato, fue desarrollado un protocolo sencillo del alelo específico PCR-(AS-PCR) para la determinación precisa del genotipo de FAD2A/FAD2B y discriminar el alelo silvestre y el mutante de ambos genes (FAD2A 448 G > A y FAD2B 441_442insA). Los resultados pueden servir para desarrollar un procedimiento viable para la producción de cultivares deseados de cacahuetes alto oleato a través de hibridación.

PALABRAS CLAVE: Alelo específico PCR (AS-PCR) – Cacahuete – FAD2A / FAD2B – Genotipificación.

SUMMARY

An AS-PCR assay for accurate genotyping of *FAD2A/FAD2B* gene in peanut (*Arachis hypogaea* L.)

FAD2A and *FAD2B* are homoeologous genes from A and B genomes in cultivated peanuts (*Arachis hypogaea* L.) encoding fatty acid desaturates which convert oleate to linoleate. To study the genetics of oleate and breed high oleate peanut cultivars, a simple allele specific-PCR (AS-PCR) protocol for the accurate genotyping of *FAD2A/FAD2B* was developed to discriminate the wild and mutant allele of both genes (*FAD2A* 448 G > A and *FAD2B* 441_442insA). The results may serve to develop a feasible procedure for producing highly desired high oleate peanut cultivars through hybridization.

KEY-WORDS: Allele specific PCR (AS-PCR) – FAD2A/ FAD2B – Genotyping – Peanut.

1. INTRODUCTION

The cultivated peanut, *Arachis hypogaea* L., is an important cash crop all over the world. Its seeds generally contain 44~56% oil. Oleate and linoleate together constitute about 80% of the total fatty acids in peanuts. Linoleate is prone to oxidation, producing noxious odors; moreover, it decreases both harmful low density lipoprotein (LDL) and beneficial high density (HDL) cholesterol levels. Oleate, however, is more stable; a high oleate peanut not only means prolonged shelf life (O'Byrne *et al*., 1997), but also helps to lower LDL cholesterol level, while maintaining HDL cholesterol level. The development of high oleate to linoleate ratio (O/L) varieties has therefore become a major breeding objective of the cultivated peanut.

The cultivated peanut is an allotetraploid (2n=4x=40). *FAD2A* and *FAD2B* are homoeologous genes from A and B genomes coding for 12-fatty acid desaturases, which convert oleate ($C_{18:1}$) to linoleate ($C_{18:2}$). So far, several high oleate peanut mutants have been reported (Norden *et al.* 1987, Ashri 1988, Wang *et al*., 2010). In most cases, a 1-bp substitution (G:C→A:T) at position 448 after the start codon (*FAD2A* 448 G > A) in *FAD2A*, caused a missense amino acid substitution from aspartic acid to asparagine (D150N), and a 1-bp insertion (A:T) at position 442 after the start codon in *FAD2B* (*FAD2B* 441_442insA), resulted in a frame shift (Jung *et al*., 2000, López *et al*., 2000). The activity of both desaturases in these high oleate mutants was confirmed to be significantly lower that in normal oleate peanut genotypes by enzyme assays, mutagenesis and gene expression in yeast systems (Ray *et al*., 1993, Bruner *et al*., 2001). These high oleate mutants can be used in hybridization programs to incorporate this valuable trait into high yielding adapted peanut cultivars.

For genetic studies and breeding applications, there is a need to identify peanut F1 and F2 hybrids with *FAD2A/FAD2B* mutant alleles or high oleate segregates in normal oleate × high oleate (*FAD2A* 448 G > A , *FAD2B* 441_442insA) crosses. Currently, some technologies have already been developed to partially meet the demand, which include, cleaved amplified polymorphisms (CAPS) (Chu *et al.,* 2007), real-time PCR genotyping assays (Barkley *et al*., 2010, 2011), near infrared reflectance spectroscopy (NIRS) (Wang *et al.,* 2010), direct sequencing of PCR products (Wang *et al.,* 2010) and allele specific-PCR (AS-PCR) (Chen *et al.* 2010). The first three methods require complicated operations or special instruments. Although direct sequencing can be used to identify

true F_1 hybrids, it is costly when handling large populations. NIRS cannot genotype the *FAD2A/FAD2B* alleles. As mentioned above, Chen *et al.* (2010) has already developed an AS-PCR assay for genotyping the *FAD2A*/*FAD2B* alleles in peanuts, but failed to differentiate between the genotypes of Ol_1ol_1/Ol_2Ol_2 and ol_1ol_1/Ol_2Ol_2, or Ol_1Ol_1/Ol_2ol_2 and Ol_1Ol_1/ol_2ol_2.

The objective of the present study was to develop an AS-PCR assay for the accurate genotyping each of the *FAD2A* and *FAD2B* allele in the cultivated peanut, as long as the mutant type *FAD2A* and *FAD2B* were *FAD2A* 448 G > A and *FAD2B* 441_442insA, respectively.

2. MATERIALS AND METHODS

2.1. Peanut material

Previously, FB4 and CTWE, 2 high-oleate peanut mutants developed by us, were identified as with *FAD2A* and *FAD2B* mutant alleles (*FAD2A* 448 G > A and *FAD2B* 441_442insA), and normal oleate Huayu 40 was verified to have wild type *FAD2A* and *FAD2B* (Wang *et al.,* 2010). True hybrids had been selected by the direct sequencing of *FAD2B* PCR products of the resultant seeds (F_1) harvested from female parents in the normal oleate Huayu 40 × high oleate (FB4 or CTWE) crosses (Wang *et al.*, 2010).

To test if the AS-PCR assay was accurate and reproducible, Huayu 40 was used to prepare an *FAD2A/FAD2B* wild allele homozygote DNA template ($Ol_1Ol_1Ol_2Ol_2$), CTWE and FB4 were used to prepare *FAD2A/FAD2B* mutant allele homozygote DNA templates ($ol_1ol_1ol_2ol_2$), and the true hybrid seeds (F_1) from Huayu 40 pollinated with FB4 or CTWE were used to prepare wild-mutant heterozygote DNA templates ($Ol_1ol_1Ol_2ol_2$).

To test the accuracy of the AS-PCR protocol in a segregation population (derived from a normal oleate × high oleate cross) with various genotypes, randomly selected single $F_{1:2}$ seeds with known *FAD2A/FAD2B* "genotypes" identified by the AS-PCR protocol described below were further tested for their real genotypes by direct sequencing.

2.2. AS-PCR primer design

Seven AS-PCR primers were designed according to the base difference between *FAD2A/FAD2B* alleles. To ensure specificity in detection, a mismatched base was placed in the second or third base from the 3' end of 4 primers (Table 1).

2.3. PCR mixture components and thermal cycling profile

Four PCR reactions were established to detect the wild and mutant alleles of *FAD2A/FAD2B,* respectively. As illustrated in Figure 1, Reaction I, Reaction II, Reaction III and Reaction IV were aimed to detect the *FAD2A* wild allele (Ol_1), *FAD2A* mutant allele (ol_1), *FAD2B* wild allele (Ol_2), and *FAD2B* mutant allele (ol_2), respectively. Each reaction (25 μL total volume) contained 12.5 μL of 2 × Taq PCR Mix (Tiangen, Beijing, China), 3 primers (volume listed in Table 2), and 1.5 μL of DNA template prepared from a slice of cotyledonary tissue (weight 3-5 mg) of a single seed following the method of *Yu et al*., (2010).

Table 1
Sequences of AS-PCR primers for *FAD2A*/*FAD2B* genotyping, with mismatched bases underlined

Primer	Sequence (5' to 3')
FAD2A-F	GATTACTGATTATTGACTTGCTTTG
FAD2A-G	GTTTTGGGACAAACACTTC<u>I</u>TC
FAD2A-A	GTTTTGGGACAAACACTTC<u>I</u>TT
FAD2B-F	CAGAACCATTAGCTTTGTAGTAGTG
FAD2B-C	AACACTTCGTCGCGGT<u>I</u>G
FAD2B-A	AACACTTCGTCGCGGT<u>I</u>T
FAD2-R	CTCTGACTATGCATCAGAACTTGT

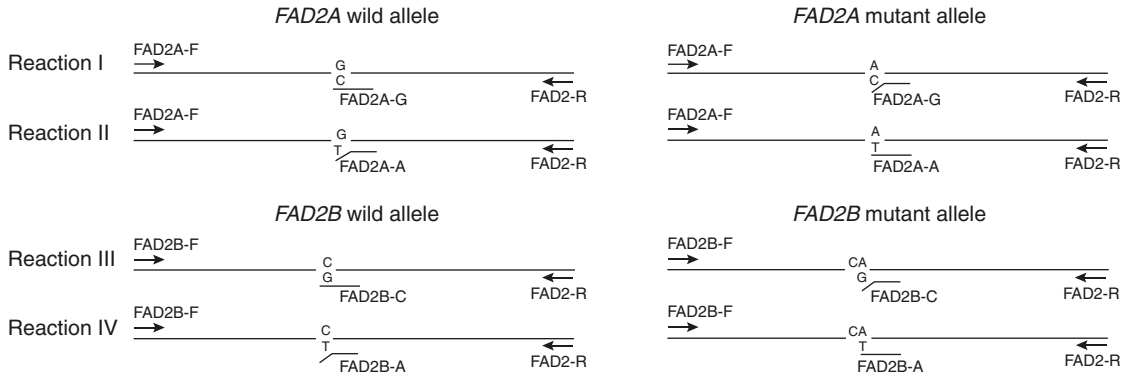

Figure 1
Illustration of the 4 reactions used in AS-PCR for *FAD2A/FAD2B* genotyping in peanut. Wild and mutant alleles of *FAD2A/FAD2B*, primers in each reaction and their annealing sites on the genes were shown.

Table 2
Primer combinations in the 4 PCR reactions for *FAD2A*/*FAD2B* genotyping

PCR reaction	PCR primer						
	FAD2A-F	FAD2A-G	FAD2A-A	FAD2B-F	FAD2B-C	FAD2B-A	FAD2-R
Reaction I	1μL	1μL					0.25 μL
Reaction II	1μL		1μL				0.25 μL
Reaction III				1μL	1μL		0.25 μL
Reaction IV				1μL		1μl	0.25 μL

[a] Primer concentration was 10 μM each.

The 4 reactions used the same PCR program: 94 °C for 1min, followed by 30 cycles of 94 °C for 30 s, 53 °C for 30 s and 72 °C for 90 s, and a final extension of 72 °C for 5 min.

2.4. Agarose gel electrophoresis of PCR products and band separation

PCR products were separated on a 1% agarose gel (MDBio, Qingdao, China) in 1 × TAE buffer by electrophoresis at 120 V for 24 min. The agarose gels were stained with GelRed dye (Biotium, CA, USA). The resulting bands were visualized under UV light.

All of the 4 reactions were supposed to produce a *ca* 1.2kb internal reference band amplified by the forward primer (FAD2A-F or FAD2B-F) and the reverse primer (FAD2-R), which was an indication of PCR success.

2.5. *FAD2A*/*FAD2B* genotyping by direct sequencing

The PCR primer pairs, aF19/R1 and bF19/R1, were used to amplify the *FAD2A* and *FAD2B* genes, respectively (Patel *et al.,* 2004). The PCR mixture (50 μL) consisted of 25 μL of Tiangen 2 × Taq Master Mix (Tiangen, Beijing, China), 2 μL of DNA template, and 2 μL of primers (10 μM each). The thermal cycling profile was 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 53 °C for 1 min, and 72 °C for 2 min, and a final extension of 74 °C for 4 min.

The diluted PCR products (×100) were used as templates in the second amplification, with the primer pairs abf19F (AAGCCTCTTTCAAGGGTTCCA) and abf19R (GATGAGCCACATGCGTATCAGTT). The PCR mixture (50 μL) consisted of 25 μL of Tiangen 2 × Taq Platinum Master Mix (Tiangen, Beijing, China), 1μl of DNA template, and 2 μL of primers (10 μM) each. The thermal cycling profile was 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 sec, 54 °C for 30s, and 72 °C for 1 min, and a final extension of 72 °C for 4 min.

The PCR products were then recovered and purified, and were sent to Genscript Inc, Nanjing, China, for direct sequencing. Heterozygotes were identified by overlapped peaks (Wang *et al.*, 2010).

3. RESULTS

3.1. *FAD2A/FAD2B* genotypes of Huayu 40, FB4, CTWE, (Huayu 40 × FB4)F1 and (Huayu 40 × CTWE)F1 revealed by AS-PCR

The banding pattern of the AS-PCR products was satisfactory (Figure 2). As expected, Huayu 40 ($Ol_1Ol_1Ol_2Ol_2$) produced a 557 bp target band in Reaction I and a 539 bp target band in Reaction III, CTWE and FB4 ($ol_1ol_1ol_2ol_2$) produced target bands in Reaction II and IV (Figure 2a and Figure 2c), and the true F_1 hybrids ($Ol_1ol_1Ol_2ol_2$) produced target bands in all 4 reactions (Figure 2b and Figure 2d), demonstrating that this method had potential to accurately detect each *FAD2A/FAD2B* allele from individual peanut seeds.

3.2. *FAD2A/FAD2B* genotyping in a normal oleate × high oleate $F_{1:2}$ population

A total of 86 randomly selected single $F_{1:2}$ seeds were genotyped by the AS-PCR assay. All 9 genotypes were detected in the population (Figure 3). Each of them appeared at least 2 times (Table 3). In all cases without exception, the trace files from the direct sequencing of *FAD2A/FAD2B* products amplified with these templates (Figure 4) were in full agreement with the results from AS-

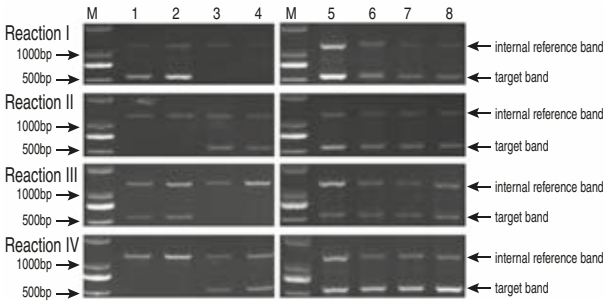

Figure 2
Banding pattern in the AS-PCR assay for *FAD2A/FAD2B* genotyping. Reaction I: Detection of *FAD2A* wild allele (Ol_1). Reaction II: Detection of *FAD2A* mutant allele(ol_1). Reaction III: Detection of *FAD2B* wild allele(Ol_2). Reaction IV: Detection of *FAD2B* mutant allele(ol_2). 1-2: wild homozygote(Huayu 40). 3-4: mutant homozygote(CTWE and FB4). 5-8: heterozygote (5-6: True F_1 hybrids of Huayu 40 × CTWE. 7-8: True F_1 hybrids of Huayu 40 × FB4). M: DL2000 marker (TaKaRa, Dalian, China).

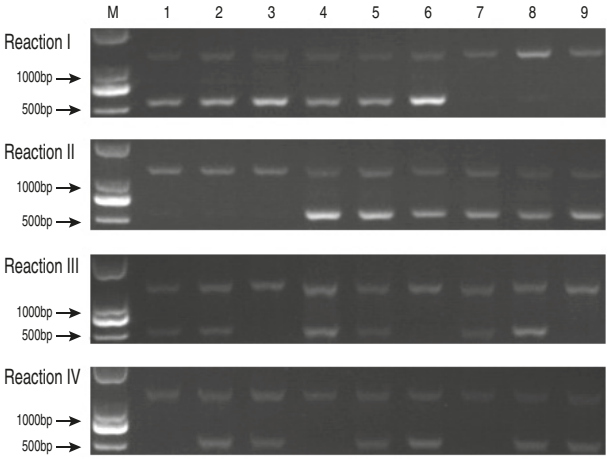

Figure 3
Banding pattern of 9 genotypes in the normal oleate × high oleate F1:2 populations. M: DL2000 Marker (TaKaRa, Dalian, China). 1: Ol_1Ol_1/Ol_2Ol_2. 2: Ol_1Ol_1/Ol_2ol_2. 3: Ol_1Ol_1/ol_2ol_2. 4: Ol_1ol_1/Ol_2Ol_2. 5: Ol_1ol_1/Ol_2ol_2. 6: Ol_1ol_1/ol_2ol_2. 7: ol_1ol_1/Ol_2Ol_2. 8: ol_1ol_1/Ol_2ol_2. 9: ol_1ol_1/ol_2ol_2.

PCR, showing that the AS-PCR method would cater to the requirements for accurately genotyping each of the *FAD2A/FAD2B* alleles in peanuts.

4. DISCUSSION

The AS-PCR protocol for peanut *FAD2A/FAD2B* presented here was advantageous over the one developed by Chen *et al.* (2010). Genotyping with the current protocol was accurate and rapid. About 40 minutes were saved in electrophoresis, and less time was needed in thermal cycling.

Based on the present AS-PCR protocol and NIRS calibration models reported earlier by our research group (Wang *et al.*, 2010), a feasible selection procedure for high oleate peanuts through hybridization can then be proposed, as long as the *FAD2A/FAD2B* genotypes involved are the same as in this study. Generally, normal oleate adapted

Table 3
Number of seeds with each of the 9 *FAD2* genotypes in the $F_{1:2}$ population

Genotype	No. of seeds
Ol_1Ol_1/Ol_2Ol_2	9
Ol_1Ol_1/Ol_2ol_2	7
Ol_1Ol_1/ol_2ol_2	2
Ol_1ol_1/Ol_2Ol_2	7
Ol_1ol_1/Ol_2ol_2	21
Ol_1ol_1/ol_2ol_2	8
ol_1ol_1/Ol_2Ol_2	4
ol_1ol_1/Ol_2ol_2	15
ol_1ol_1/ol_2ol_2	13
Total	86

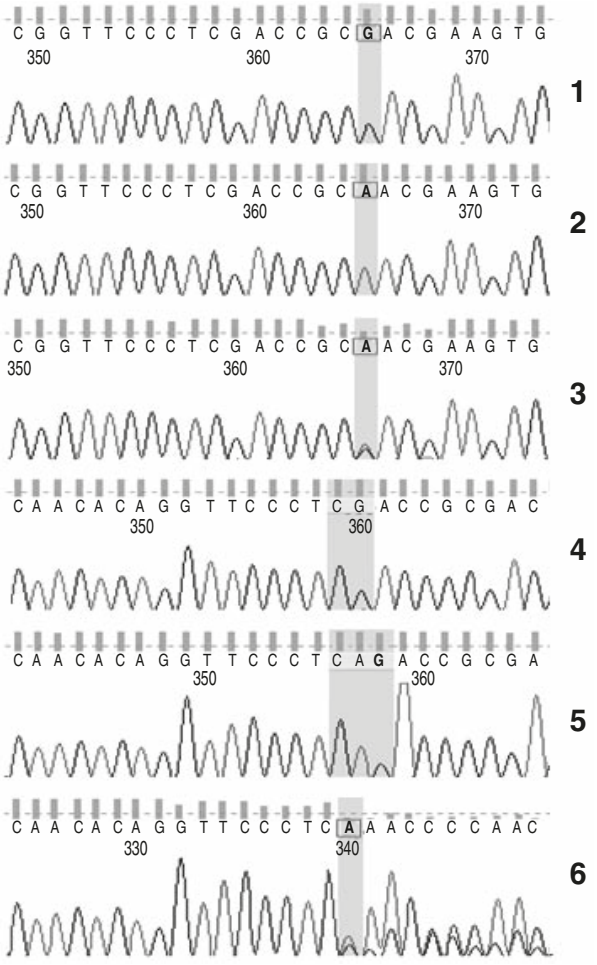

Figure 4
Accurate genotyping of *FAD2A/FAD2B* wild and mutant alleles by direct sequencing, note overlapped peak(s) in trace files of heterozygotes. 1: *FAD2A* wild allele homozygote (Ol_1Ol_1). 2: *FAD2A* mutant allele homozygote. (ol_1ol_1) 3: *FAD2A* wild and mutant allele heterozygote (Ol_1ol_1), note an overlapped peak of A/G at position 448 after the start codon. 4: *FAD2B* wild allele homozygote (Ol_2Ol_2). 5: *FAD2B* mutant allele homozygote (ol_2ol_2). 6: *FAD2B* wild and mutant allele heterozygote (Ol_2ol_2), note a series of overlapped peaks starting from the inserted A between the position 441 and 442 after the start codon.

peanut varieties are used as female parents to cross with high oleate peanut materials with *FAD2B* 441_442insA; in such a case, true F_1 hybrids with *FAD2B* 441_442insA can be easily identified by exploiting Reaction IV of the AS-PCR assay described here. With the help of NIRS, high oleate single seeds may be selected from $F_{1:2}$ populations. Genotyping medium oleate single $F_{1:2}$ seeds using our AS-PCR protocol, on the other hand, may find segregants with both *FAD2A* and *FAD2B* mutant alleles, which are also to be retained as they may produce high oleate descendants in subsequent generations. In contrast, phenotypic selection for high oleate, for example with NIRS or GC, will only keep high oleate seeds with the medium oleate ones discarded. In the F_2 generation, selection should be based on field performance. High oleate single $F_{2:3}$ seeds resulting from genotyped $F_{1:2}$ seeds with both *FAD2A* and *FAD2B* mutant

alleles can be identified by NIRS. Continuous selection for productivity and other agronomic traits should be carried out in F_3 and later generations. Backcrossing is generally recommended to cope with the low yield of high oleate genotypes, a common problem in high oleate crop breeding. Though proven useful, it is tedious and time-consuming. The proposed selection procedure increases the possibility for obtaining desirable segregates with high oleate; consequently, it may hasten the process for developing peanut cultivars with both high yields and high oleate.

The AS-PCR method presented here can also be used to study the genetics of the high oleate trait in peanuts; earlier reports indicated that there were other genes responsible for this trait in addition to *FAD2A* and *FAD2B* (Lopez *et al.*, 2000, Isleib *et al.,* 2006). It is interesting to know how great the contribution of *FAD2A/FAD2B* to the high oleate trait is in different cross combinations of the cultivated peanut.

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