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


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 (C18:1) to linoleate (C18:2). 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 y 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 (Barkeley 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 F₁ 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 \(Ol₁Ol₂/Ol₁Ol₂\) or \(Ol₁O₂/Ol₁Ol₂\) and \(Ol₁Ol₂/Ol₁O₂\).

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 (\(F₁\)) 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 (\(Ol₁Ol₂/Ol₁Ol₂\)). CTWE and FB4 were used to prepare \(FAD2A/FAD2B\) mutant allele homozygote DNA templates (\(Ol₁Ol₂/Ol₁Ol₂\)), and the true hybrid seeds (\(F₁\)) from Huayu 40 pollinated with FB4 or CTWE were used to prepare wild-mutant heterozygote DNA templates (\(Ol₁Ol₂/Ol₁Ol₂\)).

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 \(F₁\) 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 (\(Ol₁\)), \(FAD2A\) mutant allele (\(ol₁\)), \(FAD2B\) wild allele (\(Ol₂\)), and \(FAD2B\) mutant allele (\(ol₂\)), 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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
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<tbody>
<tr>
<td>FAD2A-F</td>
<td>GATTACTGATTATTGACTTGCTTTG</td>
</tr>
<tr>
<td>FAD2A-G</td>
<td>GTTTTTGGGACAACACTCITT</td>
</tr>
<tr>
<td>FAD2A-A</td>
<td>AACACTTCGTCCGGCTT</td>
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<tr>
<td>FAD2B-F</td>
<td>CAGAACCATTAGCTTGTAGTGT</td>
</tr>
<tr>
<td>FAD2B-C</td>
<td>AACACTTCGTCGGCTT</td>
</tr>
<tr>
<td>FAD2B-A</td>
<td>CTCGACTATGCATCGAACTT</td>
</tr>
</tbody>
</table>

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.
for 30 sec, 54 °C for 30 s, and 72 °C for 1 min, and a final extension of 74 °C for 4 min.

2.4. Agarose gel electrophoresis of PCR products and band separation

PCR products were separated on a 1% agarose gel (MBBio, Qingdao, China) in 1 x 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. 1.2 kb 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 x 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 (x100) were used as templates in the second amplification, with the primer pairs abf19F (AAGCCTTTTCAAGGGTTCCA) and abf19R (GATGAGCCACATGCGTATCAGTT). The PCR mixture (50 µL) consisted of 25 µL of Tiangen 2 x Taq Platinum Master Mix (Tiangen, Beijing, China), 1 µL 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 30 s, 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 (Ol1Ol1Ol2Ol2) produced a 557 bp target band in Reaction I and a 539 bp target band in Reaction III, CTWE and FB4 (ol1ol1ol2ol2) produced target bands in Reaction II and IV (Figure 2a and Figure 2c), and the true F1 hybrids (Ol1ol1Ol2ol2) 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 F1:2 population

A total of 86 randomly selected single F1:2 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-PCR.
peanut varieties are used as female parents to cross with high oleate peanut materials with FAD2B
441_442insA; in such a case, true F1 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 F1:2 populations.
Genotyping medium oleate single F1:2 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 F2 generation, selection should be based on field performance. High
oleate single F2:3 seeds resulting from genotyped F1:2 seeds with both FAD2A and FAD2B mutant
alleles are to be retained as they may produce high oleate descendants in subsequent

table 3

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of seeds</th>
</tr>
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<tbody>
<tr>
<td>Ol1Ol1/Ol2Ol2</td>
<td>9</td>
</tr>
<tr>
<td>Ol1Ol1/Ol2ol2</td>
<td>7</td>
</tr>
<tr>
<td>Ol1ol1/Ol2Ol2</td>
<td>2</td>
</tr>
<tr>
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<td>ol1ol1/Ol2ol2</td>
<td>15</td>
</tr>
<tr>
<td>ol1ol1/ol2ol2</td>
<td>13</td>
</tr>
<tr>
<td>Total</td>
<td>86</td>
</tr>
</tbody>
</table>

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
alleles can be identified by NIRS. Continuous selection for productivity and other agronomic traits should be carried out in F2 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.

ACKNOWLEDGEMENT

The authors wish to thank support from the China Agricultural Research System (CARS-14), Qingdao Science & Technology Support Program (10-3-3-20-nsh, 09-1-3-67-jch), Shandong Natural Science Foundation (Y2008D11), and Shandong Key Project of Science & Technology (2009GG10009008).

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Recibido: 12/11/12

Aceptado: 11/1/13