Identification of a Novel Mutation in *FAD2B* from a Peanut EMS Mutant with Elevated Oleate Content

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Abstract: A Virginia type peanut mutant with more than 60% oleate content (E2-4-83-12) was selected from an EMS mutagenized population of LF2 (an export type peanut cultivar with 44.2% oleate) by near infrared reflectance spectroscopy. Cloning and sequencing of *FAD2B* from LF2 and E2-4-83-12 identified a novel mutation (C313T in the coding region) causing an H105Y substitution in the first histidine box of the *FAD2B* protein. GC-MS analysis of fatty acids in yeast cells harboring pYES2 with the mutated *FAD2B* detected no linoleate, confirming that *FAD2B* from E2-4-83-12 was dysfunctional. Loss-of-function *FAD2A* and *FAD2B* together contributed to elevated oleate phenotype of the peanut EMS mutant.

Key words: peanut, high oleate, *FAD2B*, yeast expression, novel mutation

1 INTRODUCTION

Peanut (*Arachis hypogaea* L.) is an important cash crop widely grown in warm areas. High oleate (c18:1) peanut is valued by oil/food processors and consumers, as it not only has better keeping quality but also provides multiple health benefits, i.e., reduced risk of cardiovascular disease, increased sensitivity to insulin, preventative effects on tumorigenesis, and amelioration of some inflammatory diseases¹⁻⁴. High oleate has become a major breeding objective of peanut.

F435, a spontaneous peanut mutant with 80% oleate, was first identified by Norden *et al.* (1987)⁵. Thereafter several high oleate peanut mutants induced by chemical mutagens or gamma ray irradiation were reported⁶⁻⁸. Thus far, at least 24 high oleate peanut cultivars have been released, most of which were from USA⁸. However, their availability for exploitation is restricted⁹. Moreover, the mutants and releases were of spreading or semi-spreading growth habit or had low yield potential, excluding their direct use in commercial production in countries like China, where only high yielding erect peanut cultivars are generally acceptable. In this situation, inducing high oleate mutants of high yielding adapted peanut cultivars seems to be a logical option, since agronomic characters in mutated populations tend to stabilize easily, and therefore less time and labor are needed in mutation breeding as compared with cultivar development through hybridization⁷.

The high oleate trait was believed to be controlled by 2 recessive genes, *FAD2A* and *FAD2B*¹⁰⁻¹³. In most cases, a point mutation from guanine to adenine in *FAD2A* and a 1-bp insertion (A:T) at position 442 after the start codon in *FAD2B* together cause a high oleate phenotype^{12, 14, 15}. Different from these results, in the report of Patel *et al.* (2004), insertion of miniature inverted-repeat transposable element (MITE) into *FAD2B* was found to be responsible for the high oleate trait in a peanut chemical mutant¹⁶.

In the present communication, we identified a peanut mutant with elevated oleate content by near infrared reflectance spectroscopy (NIRS), whose *FAD2B* had a novel point mutation that led to an amino acid substitution in one of the histidine boxes of the fatty acid desaturase.

2 EXPERIMENTAL PROCEDURES

2.1 Peanut cultivar and EMS mutagenesis

LF2, a high yielding Virginia type peanut cultivar with erect growth habit, was used in ethyl methanesulfonate (EMS) mutagenesis. Pretreatment, EMS treatment, post-treatment and cultivation were essentially the same as previously described by Wang *et al.* (2011) for sodium azide⁷, except that the 1.0% EMS (Sigma-Aldrich, Milwaukee, WI, USA) solution was prepared in 0.1 M phosphate buffered saline (pH 7.0). Mutagenized seeds along with the untreated

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Accepted October 23, 2011 (received for review August 23, 2011)

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Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online

<http://www.jstage.jst.go.jp/browse/jos/> <http://mc.manuscriptcentral.com/jjocs>

ed control (LF2) were sown on May 5, 2010 at the experimental plots, Nianzhitou, Laixi, China. Individual single peanut plants were harvested separately on September 12, 2010.

2.2 Screening of M_2 seeds for high oleate materials with NIRS

Single plants that produced adequate seeds for the sampling cup for bulk seeds were scanned with a NIRS machine (Matrix-I, Bruker Optics, Ettlingen, Germany) once. Of them, only the plants with more than 58% oleate (in total fatty acids) predicted by NIRS were further selected for single seeds NIRS analysis. Individual seeds were scanned 3 times, and oleate content was predicted by NIRS calibration equation for single peanut seeds. For NIRS analysis, the procedure depicted by Wang et al. (2011) was followed⁷.

2.3 GC analysis of fatty acids in single peanut seeds

Selected peanut single seeds with more than 59% oleate were sent to Food Supervising and Testing Center (Wuhan, China) to analyze fatty acid content by gas chromatography (GC). To avoid reduction in germination, a small seed portions (about 50 mg) distal to embryo end were sampled from each seed for analysis. Procedure for fatty acid determination by GC was the same as that described by Zeile et al. (1993)¹⁷.

2.4 Cloning and sequence analysis of *FAD2A* and *FAD2B* gene

DNA template was prepared according to the protocol of Yu et al. (2010)¹⁸. Gene-specific PCR primers, aF19/R1 and bF19/R1¹⁶, were used to amplify the *FAD2A* and *FAD2B* genes, respectively. PCR mixture (50 μ L) was comprised of 25 μ L of Tiangen 2 x *Taq* platinum Master Mix (Tiangen, Beijing, China), 5 μ L of DNA template, and 2 μ L of PCR primers (10 μ M) each. PCR program, run on a Tgradient thermal cycler (Biometra, Göttingen, Germany), was 95°C for 6 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. Purified PCR products were ligated into a pBS-T vector (Tiangen, Beijing, China) and transferred by heat shock method into chemically competent TOP10 *E. coli* cells (Tiangen, Beijing, China). White colonies harboring plasmids with desired inserts were identified by colony PCR and sequencing of the inserts by Genscript, Inc., Nanjing, China. Multiple alignments of sequences and translation were performed using the Lasergene™ DNASTar package (DNASTAR, Madison, USA).

2.5 Construction of recombinant yeast expression vector and yeast transformation

The auxotrophic *Saccharomyces cerevisiae* strain INVSc1 (*MATa his3-Δ1 leu2 trp1-289 ura3-52*) and the

shuttle vector pYES2 (Invitrogen, Carlsbad, CA, USA) were used to analyze the function of *FAD2B* from LF2 and E2-4-83-12. The coding sequences (cgs) of *FAD2B* were amplified using gene-specific primers with restriction enzyme sites, Jbf19b (5'-CCGGATCCCAGAAC-CATTAGCTTTG-3', *Bam*H I site underlined) and JR1 (5'-CCCTCGAGTCTCTGACTATGCATCAG-3', *Xho* I site underlined). *FAD2B* PCR products and pYES2 were both digested with *Bam*H I and *Xho* I, and were ligated to insert *FAD2B* cgs after the inducible yeast *GAL1* promoter and before the yeast *CYC1* terminator. The pYES-*FAD2B* constructs and the pYES2 vector (control) were transferred into the INVSc1 cells following the lithium acetate method described by Gietz et al. (1995)¹⁹. Selection was carried out on a synthetic complete agar medium without uracil (SC-U).

Well-isolated single colonies were picked and cultured separately in liquid SC-U medium at 30°C with agitation till OD_{600} was 0.4. Yeast vector DNA was extracted with TIAn-prep Yeast Plasmid DNA Kit (Tiangen, Beijing, China) and double digested with *Bam*H I and *Xho* I. RNA was extracted from the enzyme digestion identified yeast cultures grown in induction medium (SC-U medium supplemented with 2% galactose and 2% raffinose) using Tiangen RNApure Kit (Tiangen, Beijing, China). RT-PCR with gene specific primers (bf19/R1) was performed to ensure normal transcription of *FAD2B*.

2.6 Analysis of fatty acids in yeast by gas chromatography-mass spectroscopy (GC-MS)

Single colonies of yeast transformants capable of producing *FAD2B* transcripts along with those harboring pYES without insert were cultured individually in a 20 mL liquid SC-U medium at 30°C with agitation (200 r/min) overnight. Cultures (about 2 mL) were then transferred into induction medium (50 mL) to reach a final OD_{600} of 0.4. Cell pellets were collected by centrifugation at 1,500 g at 4°C for 5 min, re-suspended in 50 mL of inducing medium and then cultured at 30°C with agitation (200 r/min) for 48 h. Yeast cells were collected by centrifugation at 748 g at 4°C for 8 min, vacuum-dried, and then ground to a fine powder in liquid nitrogen²⁰. Twenty micrograms of yeast powder samples were placed into a crew cap test tube, to which 1 mL of saturated KOH CH₃OH solution was added. Ammonia was used as protectant. Saponification was done in a 75°C water bath for 10 min. Two milliliters of HCl CH₃OH solution (1 M) was added to the cooled reaction mixture. After incubated in a 75°C water bath for 10 min, the mixture was cooled down to room temperature, and 0.5 mL of hexane and 2 mL of distilled water were then added, thoroughly mixed, and kept undisturbed until hexane layer clearly formed. Hexane layer was collected for fatty acid determination by GC-MS (7890N/5975N) with ChemStation software (Agilent, Palo, Slto, CA, USA)²¹. For GC analysis, a

HP-5MS (30 m × 250 μm × 0.25 μm) capillary column was utilized. Column temperature was programmed from 50°C (held for 1min) to 190°C (20°C/min), to 240°C (4°C/min), and to 280°C (10°C/min, held for 2 min). High purity helium was used as the carrier gas. Splitless injection volume was 1 μL. Inlet temperature was kept at 280°C. Mass spectrometer (MS) was operated in full scan mode with a mass range of 35–400 amu at 70 eV electron ionization (EI). Temperature of ionization source, quadrupole and transfer line was 230, 150 and 300°C, respectively. A 3 min solvent delay was used to avoid acquiring unnecessary data.

3 RESULTS

3.1 NIRS-aided selection for high oleate content in the EMS mutagenized peanut population

Totally 1,040 peanut seeds soaked with 1.0% EMS were sown in the field in the spring. Only 588 (56.5%) peanut plants produced seeds and were harvested in the fall, of which 488 plants with seeds enough for the NIRS sampling cup for bulk seed samples were analyzed. Seven plants with more than 58% oleate predicted by NIRS were selected. Further quality evaluation with NIRS equation for single seed resulted in 6 seeds with higher than 59% oleate content. Among them E2-4-83-12, E2-4-178-18 and E2-4-256-24 were analyzed by GC, showing that they contained 64.3%, 66.4% and 62.9% oleate, much higher than the untreated control LF2 (44.2%). The linoleate content of the 3 seeds was 12.8%, 13.4% and 13.7%, respectively, lower than that in LF2 (34.4%).

3.2 Molecular characterization of *FAD2A* and *FAD2B*

With the primers af19/R1 and bf19/R1, both the high oleate mutant E2-4-83-12 and the untreated control (LF2) produced a unique band of expected size (1140 bp) on a 1.0% agarose gel. The bands were recovered and cloned, and PCR-positive colonies were sent for sequencing.

Sequence alignment of the *FAD2A* gene from LF2 (wild type) and from E2-4-83-12 (mutant type) revealed a G448A transition in the coding region (Fig. 1) causing a D150N substitution in the desaturase, as previously reported by Lopez *et al.* (2002)¹³, Chu *et al.* (2007)²³ and Mondal *et al.* (2011)⁶. An A634C transversion in the coding region of *FAD2A*, however, was a same sense mutation. Comparison of the *FAD2B* sequence from LF2 and from E2-4-83-12 showed a C to T transition in 313 position of the coding region (Fig. 2), which caused an H105Y (histidine to tyrosine) alteration in the *FAD2B* protein (Fig. 3). The amino acid substitution was located in the first histidine box (Fig. 3).

3.3 Identification of yeast transformants with *FAD2B* tran-

Fig. 1 Alignment of partial *FAD2A* sequences from LF2 (CK) and E2-4-83-12 (M).

Fig. 2 Alignment of partial *FAD2B* sequences from LF2 (CK) and E2-4-83-12 (M).

Fig. 3 Comparison of deduced amino acid sequences of *FAD2B* between the untreated control LF2 (CK) and mutant with elevated oleate content, E2-4-83-12 (M), note an H105Y substitution (shaded) in the first histidine box.

scripts and analysis of fatty acids in yeast by GC-MS

Yeast transformants harboring pYES2 with *FAD2B* were confirmed by presence of a band of 1140 bp in double digestion products of the vectors by *Bam*H I and *Xho* I. Yeast strains that produced *FAD2B* transcripts were further identified by RT-PCR (Data not shown). Yeast transformants with mutated or wild type *FAD2B* from E2-4-83-12 or LF2, respectively, which were capable of producing *FAD2B* transcripts, along with those harboring pYES2 without insert, were cultured in induction medium contain-

Fig. 4 GC-MS analysis of total fatty acids in *Saccharomyces cerevisiae* cells.

ing 2% galactose. Cell pellets collected by centrifugation were treated with saturated KOH CH₃OH solution. GC-MS analysis showed that the yeast strain with wild type *FAD2B* accumulated C18:2^{Δ9,12} (linoleate) (15.62% of total fatty acids), whereas yeast strains harboring pYES2 without insert or with mutant type *FAD2B* did not produce this fatty acid (Fig. 4).

3.4 Main agronomic characters in subsequent generation

Plant characters of the M₂ derivative of E2-4-83-12 including plant growth habit (erect), and size and shape of pods and seeds were identical to those of LF2. Seed weight of the M₂ plant was 30.9 g, comparable to that of the untreated control LF2 (31.3 g). Two of the randomly selected seeds (M₃) were analyzed for their fatty acid content by GC. Oleate content was 64.1% and 61.2%, and linoleate content was 16.0% and 18.4%, respectively.

4 CONCLUSION AND DISCUSSION

The present study demonstrated that selecting peanut seed with heritable elevated oleate and lowered linoleate from a large mutagenized population by NIRS was feasible. The productivity of the M₂ derivative of E2-4-83-12 was comparable to that of LF2 (though further evaluation is still needed), and no difference was detected by visual inspection in external characters of seeds, pods and plant between the mutant and LF2, showing that it was possible to induce desirable elevated oleate chemical mutants without compromise in yield and significant alterations in

external traits.

A novel mutation in *FAD2B* (C313T in coding region), which caused an H105Y substitution in the amino acid sequences of the *FAD2B* protein, was identified from the mutant with elevated oleate content, E2-4-83-12. Notably, the amino acid change was located in the first histidine box. It is well recognized that the 3 conserved histidine boxes of *FAD2* are vital to its activity and that any mutations in these regions may lead to reduction or loss of activity and specificity^{12, 13, 15}. GC-MS analysis of fatty acids detected no linoleate in yeast cells harboring pYES2 with the mutated *FAD2B*, verified that the *FAD2B* from E2-4-83-12 was dysfunctional. In this study, loss-of-function *FAD2A* and the mutated *FAD2B* together contributed to the elevated oleate phenotype of the peanut EMS mutant, E2-4-83-12.

According to Chen *et al.* (2010), oleate content in genotypes with wild type of both A and B genome, with wild type in A genome and mutant type in B genome (441_442insA), with mutant type in A genome (448 G>A) and wild type in B genome, and with mutant type in both A (448G>A) and B genomes (441_442insA) ranged from 36.0% to 48.2%, 54.4% to 58.6%, 51.9% to 61.6%, and 74.8% to 81.3%, respectively²². In the present study, LF2, the Virginia peanut cultivar with wild type *FAD2A* and *FAD2B*, only contained 44.2% oleate, falling in the variation range of oleate content in corresponding genotypes from the report of Chen *et al.* (2010)²²; despite that the cloned *FAD2* genes (*FAD2A* and *FAD2B*) from the EMS mutant E2-4-83-12 lost their function, the oleate content in the mutant was not as high as in previous reports of Chen and others^{5, 8, 22}, where 72%-80% oleate was not uncommon. This could be caused by heterozygous *FAD2A*/*FAD2B*, or other unidentified genes as indicated by Lopez *et al.*¹³ and Isleib *et al.* (2006)²⁴, which deserve further studies.

In peanut, oleate and linoleate together constitute about 80% of total fatty acids, and increase in oleate content is generally accompanied by decrease in linoleate level. Just as in other crop plants linoleate was reported to play an important role in cold tolerance, pest and disease resistance⁶, so in peanut, cultivar releases with >72% oleate were found to encounter germination/emergence problems at low temperature²⁵, and high oleate peanut lines also exhibited increased ability to support aflatoxin production during storage²⁶. Maintaining a good balance between oleate and linoleate in peanut is therefore considered judicious⁶, but how much oleate (linoleate) is most appropriate in peanut is still unknown. It is interesting to find out if higher than 60% but lower than 72% oleate content in the mutant identified in the present study can meet the demand.

ACKNOWLEDGEMENT

The authors sincerely thank the financial support from 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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