Two-step chemical mutagen treatment to convert Fuhua 12, a normal oleic Spanish peanut cultivar, into its high oleic version

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Abstract. High oleic peanut food products have proved to have better keeping quality and several health benefits. Most of the high oleic acid peanut cultivars thus far released were bred through hybridization. Breeding through mutagenesis directly provides a possibility of keeping the main agronomic characteristics from the parental cultivars/lines unchanged in the new cultivars, which is of significance to their use in commercial production. The aim of the present study was to convert Fuhua 12, a normal oleic Spanish peanut cultivar, into a high oleic version through EMS (ethyl methane sulfonate) mutagenesis. The effect of the first round of EMS treatment of Fuhua 12 on oleic acid content was limited. However, after the second round of EMS treatment, we were able to identity a high oleic plant through near infrared spectroscopy (NIRS) and further studies by gas chromatography and sequence comparison of the mutated type and wild type FAD2A/FAD2B genes. The mutant had the same mutations in FAD2A and FAD2B as UF435. A G448A substitution in FAD2A and an A insertion (441_442insA) in FAD2B together contribute to the high oleic phenotype of the mutant. In this study, Fuhua 12 was successfully converted into a high oleic version without changing most of the agronomic
characters, demonstrating the utility of a two-step chemical mutagen treatment protocol in inducing useful mutations in the cultivated peanut. The novel method may expedite genetics and breeding studies in the cultivated peanut, and is of reference to other polyploid plant species.

1. Introduction

Peanut (*Arachis hypogaea* L.) is cultivated as an important oilseed crop globally. Fatty acid composition is of relevance to its quality as seed oil content of peanut may be as high as 50% or even more. Oleic (C18:1) and linoleic (C18:2) acids together account for about 80% of the total fatty acids in peanut [1-2]. High oleic peanut generally has more than 72% oleic acid and less than 7.7% linoleic acid [3]. In contrast, normal oleic peanut contains less oleic acid and more linoleic acid. Linoleic acid is prone to oxidization, whereas oleic acid is more stable. With increased oxidative stability, high oleic peanut has an extended shelf life [3-4]. As shown by previous studies, consumers benefit from intake of high oleic peanut food, resulting in lowered blood low-density cholesterol levels and ease of control of body weight and blood glucose [3-5]. In addition, alkyl oleate esters are useful as lubricant, surfactant, emulsifying agent, emollient, fuels additive and biodiesel [6]. High oleic peanut has been well recognized by leading food and oil processors and health-conscious consumers. Therefore, high oleic acid trait has become a must-have in peanut varietal releases [3].

Of the high oleic peanut cultivars thus far released worldwide for commercial production, most were developed through hybridization. C458 and M2-225, two high oleic peanut cultivars released in the USA, however, were directly selected from EMS and DES (diethyl sulfate) mutagenized populations, respectively [3].

Hybridization may cause a large scale of genetic recombination. To overcome the uncertainty frequently encountered in hybrid populations, backcrossing is often used to incorporate desirable trait(s) into a cultivar with wide use in food processing. Mutagenesis is another way to the same goal worthwhile trying. Breeding high oleic peanut cultivars through mutagenesis directly provides a possibility of keeping the main agronomic characteristics from the parental cultivars/lines unchanged in the new cultivars, which is of significance to their use in commercial production. Choice of appropriate parents is therefore of vital importance to the success of this breeding method, however, as the parents used should still be viable in productivity and adaptability even several years afterwards in a world full of competitions.

Widely cultivated in Liaoning province, Fuhua 12 is a Spanish-type normal oleic peanut cultivar bred for export at Sandy Land Amelioration and Utilization Research Institute of Liaoning, China [7]. The aim of the present study was to obtain its high oleic acid version through EMS mutagenesis.

2. Materials and methods
2.1. Materials

Fuhua 12, a high-yielding Spanish-type peanut cultivar with wide adaptability [7], was used. Maturing within 125-128 days, it is suitable for cultivation in northeast provinces of China (125°-135°E, 38°-56°N) [7], a major peanut production region with low risk of aflatoxin contamination possibly due to its cool climate.

2.2. Methods

2.2.1. Chemical mutagen treatment and identification of mutants with elevated oleic acid content.

1,000 Fuhua 12 seeds were soaked in 0.4% EMS (Solarbio, Beijing). Only the M3 single plants with morphology like the parent were chosen. M4 seeds from the selected single M3 plants were then analyzed for oleic acid content in bulk seed samples using near infrared spectroscopy (NIRS) on a NIR machine (Matrix I, Bruker Optics) as described earlier[3]. Of these, seeds with elevated oleic acid were mixed, and 200 well-filled seeds were randomly selected for further EMS (0.4%) treatment. Seeds from the M3 single plants of the second round of EMS treatment were screened for high oleic acid using NIRS [3]. Bulk seed samples from single plants were first used in search of high oleic plants; once identified, the high oleic single plant(s) were then analyzed for oleic and linoleic acid in individual single seeds [3]. High oleic acid phenotype was further confirmed by gas chromatography on an Agilent 7820A system. Injection volume was 2 μl. A silica capillary column (DB-wax, 30m×0.25mm×0.25μm) was programmed for an initial temperature of 210°C (for 9 min), then increasing at a heating rate of 20°C/min, to a final temperature of 230 °C (held for 5 min). A split ratio of 15:1 was employed. Temperature of detector was set at 300°C. Gas flow rates were 40ml/min for hydrogen, 400ml/min for air, and 66.5ml/min for helium (carrier gas), respectively.

2.2.2. Cloning and sequence analysis of FAD2A/FAD2B

2.2.2.1. Peanut DNA extraction

Genomic DNA was extracted from peanut cotyledonary slices distal to embryo ends, without affecting seed germinability, following the procedure described by Yu et al. [8].

PCR amplification of FAD2A/FAD2B from peanut genome

PCR amplification was performed on a Veriti™ 96-Well PCR machine using peanut genomic DNA. Gene-specific PCR primer pairs, aF19/R1 and bF19/R1[8], were utilized to amplify the FAD2A and FAD2B from the cultivated peanut genome, respectively (Table 1). The reactions were performed in a 50μl volume containing 2μl of DNA, 25μl of Gflext buffer, 1μl of each primer, 1μl of TKS Gflext polymerase (Tiangen, Beijing), and 20μl of sterile double distilled water. The thermal cycling profile was as follows: 94 °C for 6 min; 30 cycles consisted of 94 °C for 30 s, 53°C for 1 min, 72°C for 2 min; and a final extension of 72 °C for 4 min. The PCR product was resolved on a 1% agarose gel.
| Primer name | Sequence (5’ to 3’ ) |
|-------------|---------------------|
| aF19        | GATTACTGATTATGGACTT |
| bF19        | CAGAACCATTAGCTTTG   |
| R1          | CTCTGACTATGCATCAG   |

2.2.2.2. Ligation, E. coli transformation and identification of PCR positive colonies

PCR products of expected size from the genomic DNA amplifications were ligated into the pEASY-Blunt Zero vector (Tiangen, Beijing), and the ligation products were then used to transform the Trans-T1 Phage Resistant Chemically Competent Cell (Tiangen, Beijing) using the heat shock method following the manufacturer’s instructions. Putative transformants were identified through blue white screen, and the right recombinants were confirmed by PCR. The reaction (20μl) consists E. coli, 1μl of each primer, 10μl of 2×Bench Top Tap MasterMix (Biomiga, San Diego) and 8μl of sterile double distilled water. Primer pairs and PCR program were the same as aforementioned for peanut genomic DNA.

2.2.2.3. DNA Sequencing and sequence analysis

PCR positive colonies were sent to Shanghai Sunny Biotechnology Co., Ltd (Shanghai) for sequencing the inserts on ABI 3730XL. Bio-XM 2.6 and DNAMAN were used to analyze the DNA sequences.

2.2.3. Characterization of the high oleic mutant and Fuhua 12 for main agronomic traits

At harvest, 10 plants of high oleic mutant and 10 plants of Fuhua 12 were sampled randomly, and plant height, branch length, no. of pods per plant, 100-pod weight and 100-seed mass were measured and recorded following the methods of Yu et al [9].

3. Results and discussion

3.1. Identification of a high oleic mutant plant of Fuhua 12

The first round EMS treatment of Fuhua 12 had some effects on oleic acid content. Even though no high oleic plants were found, single plants with elevated oleic acid, but still lower than 75%, were identified in M1 generation. These were named 12M. Selected seeds from 12M were subjected to the second round EMS treatment. A high oleic peanut plant of the M3 generation of the second round EMS treatment, WC-103 (Figure 1), was initially identified as with over 75% oleic acid by NIRS, and its high oleic phenotype was later confirmed by GC analysis of its 44 single seeds (Table 2). Seeds of WC-103 proved to contain 76.91%–83.94% oleic acid in their total fatty acids. For example, oleic acid in WC12 was as high as 80.7%, in contrast to only 35.9% in Fuhua 12 (Figure 2, Table 2)
Figure 1. Breeding of “WC-103” with 2 rounds of EMS treatment and NIRS-aided selection.

Table 2. Oleic, linoleic acid in seeds of the high oleic peanut mutant plant, WC-103, revealed by GC.

| Seed serial No. | Oleic acid (O) (%) | Linoleic acid (L) (%) | O/L       |
|-----------------|--------------------|----------------------|-----------|
| WC1             | 83.94              | 2.40                 | 34.98     |
| WC2             | 82.31              | 2.99                 | 27.53     |
| WC3             | 82.86              | 3.03                 | 27.35     |
| WC4             | 82.66              | 2.56                 | 32.29     |
| WC5             | 83.05              | 2.83                 | 29.35     |
| WC6             | 79.21              | 4.81                 | 16.47     |
| WC7             | 78.95              | 2.92                 | 27.04     |
| WC8  | 77.23 | 5.44  | 14.20 |
| WC9  | 80.18 | 4.13  | 19.41 |
| WC10 | 81.75 | 3.08  | 26.54 |
| WC11 | 79.53 | 4.76  | 16.71 |
| WC12 | 80.70 | 3.47  | 23.26 |
| WC13 | 82.34 | 2.89  | 28.49 |
| WC14 | 82.71 | 2.83  | 29.23 |
| WC15 | 83.00 | 3.02  | 27.48 |
| WC16 | 83.30 | 2.91  | 28.63 |
| WC17 | 83.06 | 3.21  | 25.88 |
| WC18 | 82.53 | 2.68  | 30.79 |
| WC19 | 81.42 | 3.33  | 24.45 |
| WC20 | 81.39 | 3.41  | 23.87 |
| WC21 | 83.83 | 2.41  | 34.78 |
| WC22 | 82.61 | 2.74  | 30.15 |
| WC23 | 77.06 | 7.19  | 10.72 |
| WC24 | 83.20 | 2.38  | 34.96 |
| WC25 | 82.74 | 3.09  | 26.78 |
| WC26 | 81.97 | 3.38  | 24.25 |
| WC27 | 79.74 | 4.33  | 18.42 |
| WC28 | 81.12 | 3.31  | 24.51 |
| WC29 | 82.93 | 2.77  | 29.94 |
| WC30 | 80.34 | 3.79  | 21.20 |
| WC31 | 82.67 | 2.90  | 28.51 |
| WC32 | 79.36 | 4.52  | 17.56 |
| WC33 | 81.01 | 3.98  | 20.35 |
| WC34 | 79.68 | 4.54  | 17.55 |
| WC35 | 83.10 | 2.76  | 30.11 |
| WC36 | 83.17 | 2.74  | 30.35 |
| WC37 | 79.86 | 4.32  | 18.49 |
| WC38 | 82.52 | 3.20  | 25.79 |
| WC39 | 77.49 | 5.44  | 14.24 |
| WC40 | 76.91 | 5.58  | 13.78 |
| WC41 | 77.16 | 6.15  | 12.55 |
| WC42 | 78.94 | 4.61  | 17.12 |
| WC43 | 81.02 | 4.05  | 20.00 |
| WC44 | 81.55 | 3.92  | 20.80 |

Notes:  

- Oleic acid and linoleic acid reported as percentage of total fatty acids.  
- Peanuts with an O/L ratio no less than 9 can be classified as high oleic.
Figure 2. GC Chromatograms showing increased oleic acid and reduced linoleic acid in the high oleic mutant WC12 (a) as compared with the wild type Fuhua12 (b).

3.2. Comparison of FAD2A/FAD2B sequences of WC12, a high oleic mutant and Fuhua 12

The FAD2A and FAD2B from the mutated seeds (WC1, WC 2, WC3, WC4) and their parental cultivar (Fuhua 12) were cloned and sequenced. Multiple sequence alignment showed that the differences were the same as previously reported in UF435. A G448A substitution in FAD2A and an A insertion (441_442insA) in FAD2B were found in all the mutated seeds assayed, but absent in the wild type parent, Fuhua 12 (Figure 3).
Figure 3. Comparison of FAD2A and FAD2B partial DNA sequences from normal oleic Fuhua 12 and its high oleic mutant WC12, showing a G448A substitution in FAD2A and an A insertion (441_442insA) in FAD2B in WC12.

3.3. Main agronomic characters of WC-103 and Fuhua 12

As shown in Table 3, WC-103 was quite similar to Fuhua 12 in the main agronomic characters investigated. Of the 6 traits listed, only 1 (branch length) differed at 0.05 level. Indeed, the plant morphology of the mutant line and its parental cultivar was nearly identical (Figure 4).

Table 3. Main agronomic characters of the high oleic mutant (WC-103) and its parent, Fuhua 12.

| Material   | Plant height (cm) | Branch length (cm) | No. of branches per plant | No. of pods per plant | Pod weight per plant (g) | 100-pod weight (g) | 100-seed mass (g) |
|------------|------------------|--------------------|--------------------------|-----------------------|--------------------------|-------------------|------------------|
| WC-103     | 39.2±1.3         | 43.8±1.9*          | 6.8±0.8                  | 19.4±2.1              | 15.9±1.6                 | 206.6±3.2         | 74.3±1.8         |
| Fuhua 12   | 38.6±2.1         | 41.2±2.3           | 6.5±1.5                  | 18.2±2.5              | 14.6±2.1                 | 201.1±5.4         | 75.1±1.3         |

Note: Figure with an asterisk (*) indicated significant difference at 0.05 probability level according to LSD (Least Significant Difference) test.

Figure 4. Plant morphology of WC-103 (left) and its parental cultivar, Fuhua 12 (right).

4. Discussion and conclusions

In this study, we obtained a high oleic acid peanut mutant derived from the high-yielding, normal oleic food use peanut cultivar, Fuhua 12. This new version was almost identical to the original version in plant morphology and the main agronomic characters investigated, except for oleic and linoleic acid and branch length. The productivity and adaptability of the new line still need further evaluation.

Previously reported peanut induced mutants mostly, if not all, resulted from a single treatment with chemical/physical mutagen(s), and composite treatment was seldom studied. The present study,
however, demonstrated, for the first time as far as we know, the utility of a two-step chemical mutagen treatment protocol in inducing useful mutations in the cultivated peanut. As a tetraploid species with two sub-genomes and duplications in its genome [10], the cultivated peanut sometimes was considered not amenable to induced mutagenesis. A large number of mutations may be difficult to be identified due to gene redundancy. \( FAD2A \) from the A sub-genome and \( FAD2B \) from the B sub-genome are genes conditioning oleic acid content in the cultivated peanut [3,10]. Expression of the high oleic phenotype requires both genes to be dysfunctional [3,10]. In this study, though not confirmed, it is likely that the first round of EMS treatment mutated one of the \( FAD2A/FAD2B \) gene, and the second round mutated the other. Actually, we obtained more mutations in the mutagenized populations after the second round of EMS treatment as compared with those in one round of EMS treatment (data not shown). The two-step mutagenesis may expedite genetics and breeding studies in the cultivated peanut, and is of reference to other polyploid plant species. The present study suggests that, to get better results, more than one round of mutagen treatment or composite treatment may be necessary for a crop species with sub-genomes, if the outcome of a single round of mutagen treatment is not satisfactory.

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