In planta genetic transformation to produce CRISPRed high-oleic peanut

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Abstract
Compared to its normal-oleic counterpart, high-oleic peanut has better keeping quality and much more health benefits. Breeding high-oleic peanut through conventional means is a tedious process that typically takes several years. Genome editing, however, may shorten the duration. This study aimed to test the effectiveness of the node injection method coupled with CRISPR/Cas9 technology in inducing \textit{FAD2B} mutations and high-oleic phenotype in peanut. Huayu 23, a popular normal-oleic runner type peanut cultivar having dysfunctional \textit{FAD2A} and functional \textit{FAD2B}, was transformed with CRISPR/Cas9 construct targeting \textit{FAD2B}, resulting in two \textit{T1} seeds with over 80\% oleic acid and a 442 A insertion in \textit{FAD2B}. The high-oleic phenotype in \textit{T2} seeds was inheritable from the \textit{T1} generation. As a genotype-independent, simple and easy method for peanut genetic transformation, node injection has great potential in functional analysis of genes and peanut varietal improvement. This method is of reference value to other seed plant species.

Keywords Groundnut · \textit{Arachis} · High oleate · \textit{FAD2B} · Biallelic genome editing · Node injection transformation

Abbreviations

\begin{tabular}{ll}
AhCYP & \textit{Arachis hypogaea} cyclophilin \\
BW & Bacterial wilt \\
CRISPR & Clustered regularly interspaced short palindromic repeat \\
Cas & CRISPR-associated protein \\
FAD2 & Fatty acid desaturase 2 \\
FAD2A & Fatty acid desaturase 2A \\
FAD2B & Fatty acid desaturase 2B \\
FATB & Acyl-acyl carrier protein thioesterase B \\
GC & Gas-chromatography \\
MES & 2-(N-morpholino) ethanesulfonic acid sodium salt \\
nCas9 & Nicking Cas9 \\
NIRS & Near infra-red spectroscopy \\
OD & Optical density \\
RT-PCR & Reverse transcription polymerase chain reaction \\
CTF-1 & Soybean \textit{C2H2}-type transcription factor 1 \\
sgRNA & Single guide RNA \\
TALENs & Transcription activator-like effector nucleases \\
YEB & Yeast extract broth
\end{tabular}

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Introduction

Rich in culinary oil and highly digestible protein, the cultivated peanut (Arachis hypogaea L.) occupies an important position in human and animal nutrition, and rural economy (Li et al. 2023). Fatty acid profile is an indicator of its quality. Oleic and linoleic acids together constitute about 80% of total fatty acids in peanut seeds. As compared to linoleic acid, oleic acid is less prone to oxidation. Increase in oleic acid and decrease in linoleic acid in peanut seeds may result in extended shelf life of peanut produce and much more health benefits (Nkuna et al. 2021; Zhao et al. 2022). Hence, high oleate has become one of the main breeding objectives of peanut.

The cultivated peanut originated from polyploidization after hybridization of two diploid wild species (Bertioli et al. 2019). In the tetraploid cultivated peanut, FAD2A and FAD2B on the A and B sub-genome respectively, control the conversion of oleic acid to linoleic acid; expression of the high-oleic phenotype (at least 70% oleic acid content) in peanut cultivigen, therefore, requires inactivation of both genes (Nawade et al. 2018). Natural, chemical, and physical peanut mutants with high oleate have been reported and used in hybridization and backcross to develop high-oleic peanut cultivars (Wang et al. 2021, 2022; Han et al. 2022). In contrast to the lengthy process of conventional breeding, genome editing may be a much faster alternative. Using peanut germ with a cotyledon attached for transformation, Wen et al. (2018) demonstrated that TALENs-mediated targeted mutagenesis of FAD2 in peanut cv Yueyou 7 raised oleic acid content from 43–60%~80%, while decreasing linoleic acid content from 35.5% to lower than 20%. Yuan et al. (2019) induced FAD2B mutations in peanut protoplasts and hairy roots using CRISPR/Cas9 based genome editing, Zhang et al. (2021) bombarded the embryonic calli of peanut cv Luhua 11 with a CRISPR/ Cas9 gene editing vector targeting FAD2, and regenerated plants were obtained, but the fatty acid profiles of the descendants were not reported. Tang et al. (2022) produced genetically edited Huayu 23 lines with decreased saturated fatty acid and increased unsaturated fatty acid through knockout of FATB by CRISPR/Cas9 system. To minimize the negative effects on other plant tissues while improving the fatty acid profile of peanut seeds, Neelakandan et al. (2022a) performed CRISPR/Cas9 genome editing on FAD2 cis-regulatory motifs in peanut using the Agrobacterium-mediated calyx tube injection method, and obtained T1 seeds with elevated oleate content, two of which had 66.27% and 68.43% oleate, respectively. Neelakandan et al. (2022b) reported successful base editing in peanut FAD2 with CRISPR/nCas9 and hairy root transformation. The industry accepted standard for high oleic peanut is > 74% oleic acid (Davis et al. 2021). However, in the above mentioned genome editing studies, only Wen et al. (2018) obtained peanut seeds with an oleic acid content of 74% or more.

Suitable transformation procedures may facilitate the application of genome editing tools. Previously, the node injection method, an easy-to-follow in planta peanut transformation protocol, was developed at our laboratory (Wang et al. 2013). Using this method, SCTF-1, the soybean C2H2-type zinc finger protein gene (Song et al. 2012), was transferred to chill susceptible peanut. RT-PCR and Southern blot analysis confirmed the transgenic events. SCTF-1 transgenic peanut plants showed good tolerance to chill stress (V. M. Vacu, unpublished data). Likewise, transfer of AhCYP, a Ralstonia solanacearum infection responsive gene from Rihua 1, a Virginia type peanut cultivar with verified high resistance to BW both in field and at laboratory (Ding et al. 2012), to Huayu 40, a susceptible peanut cultivar, following the same protocol, enhanced BW resistance of the recipient (Wu et al. 2019).

The present study aimed to test the effectiveness of the node injection method in inducing FAD2B mutations and high-oleic phenotype in peanut using CRISPR/Cas9 technology.

Materials and methods

Plant material for genome editing and its cultivation

Huayu 23, a normal-oleic peanut cultivar of runner market type widely accepted by growers and food processors in China, was used in this study. As expected, Sanger sequencing of its FAD2A and FAD2B and subsequent sequence alignment revealed that this cultivar had a mutated FAD2A (486 G > A) and a wild type FAD2B. Peanut for genome editing was sown under polyethylene film mulching in an isolated region in SPRI Laixi Experimental Station on May 5, 2021. Agronomic practices were followed as routine (Wan 2003).

Vector construction and transformation of Escherichia coli

Target site of sgRNA, 501–520 position of the coding sequence (5’-catgaaataacccagggg-3’) of wild type FAD2B, was selected using CRISPR-GE (http://skl.scau.edu.cn/) (Fig. 1). To facilitate ligation in genome editing vector construction, two oligos which would produce overhangs after mixing, denaturation and annealing were generated with the online tool (http://biogle.cn/index/excrrspr) and synthesized (Tsingke, Qingdao) (FAD2B-1 F:...
5’-gggtgcatgaacaatccaccaggga-3’, FAD2B-1R: 5’-aaactccctggtggattgttcatgca-3’). CRISPR/Cas9 editing vector with target site incorporated was made with BGK41-Cas9 (Bio- gle Biotechnology Co. Ltd, Hangzhou) following manufacturer’s instructions. Briefly, BGK41-Cas9, oligo dimers and enzyme mix (Biogle CRISPR/Cas vector construction kit, Biogle Biotechnology Co. Ltd, Hangzhou) were mixed on ice, and incubated at 20 °C for 1 h. The ligation products were transformed into competent cells of *E. coli* strain DH5α. Positive clones identified using colony-PCR with the Cas9-F/Cas9-R primer pair (5’-tcgtgctgaccctgacactgtttga-3’, 5’-cttggcggtagccttgccgatttcc-3’) were sequenced with primer CXYW1 (5’-cccagtcacgacgttgtaaa-3’) (Tsingke, Qingdao) to confirm the inclusion of the target site in the vector. The newly constructed plasmid was named as BGK41-Cas9 recombinant vector FAD2B-1 (Fig. 1).

**Transformation of Agrobacterium and node injection transformation of peanut**

Genome editing construct was transformed into *Agrobacterium tumefaciens* strain GV3101 chemically competent cells (Veidi Biotech, Shanghai) according to the attached user’s guide. Preparation of *Agrobacterium* for node injection was based on Pan et al. (2020) with some modifications. Positive single clones were verified by bacterial suspension PCR using the Cas9-F/Cas9-R primer pair. 100 µl of freshly prepared bacterial suspension were cultured in 10 ml of YEB liquid medium at 28 °C with agitation (250 rpm) until OD600 reached 0.6–0.8 (about 12 h). The cultures were centrifuged at 6000 rpm for 1 min to collect the bacterial cells. Equal volume of infection solution containing 100 µmol/L acetosyringone (BBI Lifesciences, Hongkong), 10 mmol/L MES (Sangon Biotech, Shanghai) and 10 mmol/L MgCl2·6H2O was added to the pellets. freshly prepared resuspended bacterial pellets were used for injection. Node injection procedure was essentially the same as that in our previous report except for the plant age (Wang et al. 2013) (Fig. 2). Volume for each injection was approximately 5 µl (Wang et al. 2013). Injection was done between 6:00–8:00 a.m. on July 17, 2021, and the positions injected were marked with threads (Fig. 2). Inverted U-shaped metal wires were used to facilitate the entry of pegs into the soil from higher nodes (Fig. 2).
Fatty acid analysis of resultant T1 seeds

Pods were harvested when matured (Sept. 17, 2021). These pods were sun-dried and hand shelled. Oleic and linoleic acid contents of the individual single seeds were predicted with NIRS (Wang et al. 2014). T1 generation seeds of transgenic lines with at least 74% oleic acid along with the untreated control Huayu 23 were further analyzed for fatty acids by gas-chromatography using cotyledonary slices following the protocol of Yang et al. (2012).

Comparison of FAD2 sequences between the high-oleic T1 seeds and Huayu 23

FAD2A and FAD2B sequences of the high-oleic T1 seeds and Huayu 23 were amplified by PCR using primers aF19 (5’-gattactgtgattactg-3’)/R1 (5’-ctctgactatgcatcag-3’) and bF19 (5’-cagaaccattagctttg-3’)/R1, respectively (Patel et al. 2004), and DNA templates prepared from cotyledonary slices (Yu et al. 2010). PCR products were directly sequenced by Tsingke, Qingdao. Sequence comparison was done with the DNASTar Lasergene version 7.1.0.

Amplification of bar gene

PCR amplification of the bar gene was performed using DNA templates prepared from Cas9 empty vector (positive control), Huayu 23 (negative control) and 2 high-oleic T1 seeds respectively, with Bar-7F and Bar-6R primers (Bar-7F: 5’-caccatcgtcaaccactaca-3’, bar-6R: 5’-acccagcaggtgggttgta-3’). The PCR mixture (10 µl) consisted of 6.25 µl of 2×Taq Plus Master Mix II (Vazyme, Nanjing), 1 µl of DNA template, 0.5 µl of each primer (10 µM), and 4.25 µl of double distilled water. The PCR thermal profile was 95 °C 5 min, followed by 30 cycles of 95 °C for 40 s, 61 °C for 40 s, and 72 °C for 30 s, and a final extension of 72 °C for 10 min.

Cultivation of T1 plants

To obtain descendants as soon as possible, one T1 seed was sown in a pot in the winter of 2021, but unfortunately it died and did not set any pods. The remaining T1 seed along with a Huayu 23 seed was sown under a film mulch on May 23, 2022. Both grew to maturity and were harvested on September 20, 2022.

Fatty acid profiling of the T2 seeds harvested from T1 plant

Oleic and linoleic acid contents of the T2 seeds from the T1 plant both as bulk seed sample and as individual single seeds were determined by NIRS (Wang et al. 2014, 2021). Huayu 23 CK was also analyzed for main fatty acid content.

Results

Main fatty acids of the resultant T1 seeds and wild type Huayu 23

A total of 16 nodes were injected, which only resulted in 4 seeds. Among them, two seeds, Huayu 23-7-1 and Huayu 23-7-2, were classified as high-oleic by NIRS (Table 1; Fig. 3).

Fatty acid composition of the seeds of concern determined by GC was shown in Table 2. There were drastic changes in oleic, linoleic and palmitic acid contents in Huayu 23-7-1 and Huayu 23-7-2. Oleic acid increased from 52.15% in Huayu 23 to over 80% in Huayu 23-7-1 and Huayu 23-7-2, linoleic acid dropped from 25.72% in Huayu 23 to lower than 1.70% in Huayu 23-7-1 and Huayu 23-7-2. Accordingly, the oleic acid to linoleic acid ratio (O/L) rose from around 2.03 to more than 47. Palmitic acid content in Huayu 23-7-1 and Huayu 23-7-2 was 8.07% and 7.39%, respectively, much lower than that in Huayu 23 (15.55%).

FAD2A/FAD2B genotyping of the resultant T1 seeds and wild type Huayu 23

Multiple alignment of FAD2A/FAD2B sequences of different sources revealed that Huayu 23, Huayu 23-7-1 and Huayu 23-7-2, all had a mutated FAD2A (448 G > A), Huayu 23 possessed wild type FAD2B, whereas both the high-oleic T1 mutant seeds had a mutant type FAD2B (442 A insertion) (Fig. 4). However, the 442 A mutation in FAD2B was outside the expected target range, i.e. positions 501–520 of the wild-type FAD2B coding sequence, where no mutation was found.

Amplification of the bar gene

As expected, the 2 high-oleic peanut seeds produced a band with size equal to the positive control Cas9 empty vector.

Table 1 Chemical quality of single T1 peanut seeds and Huayu 23 predicted by near infra-red spectroscopy

| Identity     | Generation | Oleic acid (O) (%) | Linoleic acid (L) (%) | O/L |
|--------------|------------|--------------------|----------------------|-----|
| Huayu 23 (CK)| –          | 45.88              | 26.64                | 1.72|
| Huayu 23-7-1 | T1         | 74.33              | 5.39                 | 13.80|
| Huayu 23-7-2 | T1         | 78.96              | 3.84                 | 20.54|
Fig. 3  Two high oleic peanut seeds, Huayu 23-7-1 (left), Huayu 23-7-2 (middle) and wild type Huayu 23 (right)

Table 2  Fatty acids (%) in single peanut seeds determined by gas chromatography

| Identity       | Generation | Oleic acid    | Linoleic acid  | Palmitic acid | O/L |
|---------------|------------|---------------|----------------|---------------|-----|
| Huayu 23 (CK) | –          | 52.15 ± 0.24B | 25.72 ± 0.20A  | 15.55 ± 0.19A | 2.03|
| Huayu 23-7-1  | T₁         | 80.26 ± 0.32A | 1.68 ± 0.12B   | 8.07 ± 0.23B  | 47.69|
| Huayu 23-7-2  | T₁         | 80.83 ± 0.10A | 1.46 ± 0.06B   | 7.39 ± 0.38B  | 55.36|

In each column of the fatty acid content, figures followed by the same letter were not significantly different at 0.01 level. Fatty acid content was expressed as mean ± SE.

Fig. 4  Multiple sequence alignment of \(FAD2A\) and \(FAD2B\) from \(FAD2A\) V1.0 (peanutbase), Huayu 23 CK (wild type), Huayu 23-7-1 and Huayu 23-7-2
whereas the untransformed Huayu 23 (negative control) yielded no band (Fig. 5), verifying that the 2 high-oleic peanut seeds were transformants.

**T₁ plants grown from high-oleic T₁ seeds**

The two high-oleic T₁ seeds and the untransformed control were sown in soil in the isolated areas. Both T₁ seeds developed into plants, but one of them (Huayu 23-7-2) died prior to flowering. The remaining T₁ plant (Huayu 23-7-1) grew normally and set a total of 73 seeds.

**Oleic and linoleic acid contents and main agronomic characters of T₁ plant**

All the seeds from the T₁ plant Huayu 23-7-1 were firstly used as a bulk seed sample in NIRS. The T₁ plant had an oleic acid content of 79.07%, as against 44.52% in Huayu 23. Sixty-eight well-developed T₂ seeds were then analyzed with NIRS for individual single seeds, and all were found to be high-oleic (Table 3). Similarly, forty-five Huayu 23 seeds were also used as individual single seed samples in NIRS analysis and as expected, all were normal-oleic (Table 3). Compared to Huayu 23, the T₁ plant was shorter, set slightly bigger pods (Fig. 6; Table 4), and had much higher pod weight and seed weight (Table 4).

**Discussion**

Both the high-oleic mutant seeds had an F435 type FAD2B mutation (442 A insertion). It has been well documented that the 442 A insertion in FAD2B could cause the dysfunction in FAD2B due to the early appearance of a stop codon in the coding region caused by the frame shift mutation (Yu et al. 2008). The mutated FAD2B encoded a truncated oleate desaturase with the loss of a conserved histidine box, which was vital to enzyme activity. Yeast expression analysis also demonstrated that the 442 A insertion in FAD2B resulted in a loss-of-function FAD2B (Yu et al. 2008). Decreased transcription levels of FAD2B in F435 type high-oleic peanut were also observed (Jung et al. 2000). Since the genome-edited peanut seeds obtained in our study showed the same mutation in FAD2B as that reported by Yu et al. (2008) and Jung et al. (2000), it is inferable that the high-oleic phenotype is a consequence of the 442 A insertion in FAD2B. This was also consistent with the observation that FAD2B

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Table 3: Chemical quality of bulk and single T₂ peanut seeds and Huayu 23 predicted by NIRS

| Identity       | Generation | Bulk/Single | Oleic acid (%) | Linoleic acid (%) | O/L  |
|----------------|------------|-------------|----------------|-------------------|------|
| Huayu 23 (CK)  | –          | Bulk        | 44.52          | 35.99             | 1.24 |
| Huayu 23-7-1   | T₂         | Bulk        | 79.07          | 5.01              | 15.80|
| Huayu 23 (CK)  | –          | Single      | 37.82 ± 0.97   | 37.25 ± 0.61      | 1.04 ± 0.04 |
| Huayu 23-7-1   | T₂         | Single      | 75.60 ± 0.33   | 5.67 ± 0.26       | 22.91 ± 7.20 |

Fatty acid content and O/L were expressed as mean ± SE, where appropriate
transcript levels in high-oleic peanut were significantly lower than in its normal-oleic counterpart (Yu et al. 2008).

It seems that biallelic genome editing in this report is questionable. In fact, in a separate study, using a novel technology and with the help of NIRS for bulk seed samples, we were able to obtain high-oleic chemical mutant single peanut plants as early as in M1 from a popular normal-oleic Spanish market type cultivar with wild type FAD2A and wild type FAD2B (C. T. Wang, unpublished data). In other words, it appeared that in these high-oleic mutants, not only were both FAD2A and FAD2B mutated, but that these mutations were homozygous (C. T. Wang, unpublished data). This is very similar to the unexpectedly early appearance of the gene edited high-oleic mutants in this study. These may be ascribed to targeting earlier cells rather than postzygotic cells/tissues. In the present study, it is speculated that prior to the formation of the floral organs, the primordial cells that would differentiate to form male and female gametes were transformed. Even though the editing of the FAD2B gene was not biallelic at this stage, gamete fusion would give the chance to produce some zygotes with homozygous FAD2B mutation in T1 generation.

The possibility that the mutant FAD2B allele was in the background genotype of the cultivar used for transformation can be fully excluded. To be on the safe side, special care was taken to use breeder seeds, and the normal-oleic phenotype and FAD2A/FAD2B genotyping, and amplification of the bar gene all supported that genome editing of Huayu 23 was successful. Two peanut mutant T1 seeds with over 80% oleic acid were generated via CRISPR/Cas9 genome editing technology following the node injection method developed by Wang et al. (2013). The high-oleic phenotype was expressed in T2 seeds, clearly verifying the usefulness of the peanut transformation protocol. One of the advantages of the genome editing technology based on the present transformation scheme is that the high-oleic phenotype can be expressed as early as in T1 seeds, which will further shorten the breeding process (Wen et al. 2018, Neelakandan et al. 2022a).

It was noted that T1 plants and non-transgenic control differed in plant height, length of cotyledonary branches, number of effective branches, pod weight, and kernel weight. The T1 plant and its parent were cultivated apart to avoid possible mechanical and biological mixing. In addition to environmental factors, genetic factors such as the position effect of integration sites and/or the influence on non-target genes may bring about these changes. Observations on their descendants planted in close proximity will clarify whether these differences are caused by the environment.

The rationale behind the node injection method is that most of the peanut seeds set on the first (cotyledonary branches) and second pairs of branches, that the possibility of harvesting sound mature kernels from the lower nodes was high, and that peanut cells which will develop into reproductive cells, or “primordial” reproductive cells, can be transformed (Wang et al. 2013). Generally, only the first and second nodes counting from the intersection of the main stem and cotyledonary branches were injected at 30 days after sowing (Wang et al. 2013). However, in this study, when everything was ready, it was too late (63 days after sowing), and only higher nodes could be injected. That is the reason why only a small number of seeds were harvested. Failing to edit in the anticipated target site of FAD2B may be due to the small population and/or the unsuitable oligos designed for genome editing vector construction, as the website for oligo design had no peanut genome option. Anyway, two CRISPRRed high-oleic peanut seeds were identified from the 4 resultant seeds. Changes in oleic and linoleic acid contents in T1 and T2 seeds and the FAD2B sequences (442 A) of the two peanut T1 transformants demonstrated that the high-oleic phenotype was inheritable. In addition to high-oleic acid phenotype, high and stable productivity is a prerequisite for a peanut cultivar to be accepted by growers. If future commercialization is anticipated, it will still be necessary to assess the overall performance of the derived lines in due course. There is still a need to scale up the genome editing experiments and try out some new techniques in future studies. For example, as a more recent and more efficient technique, prime editing is more versatile than base editing in generating nearly all types of edits. It is

| Plant identity | Main stem height (cm) | Length of cotyledonary branches (cm) | Stem thickness (cm) | Rang of pod-bearing branches (cm) | Number of branches | Number of effective branches | Number of pods | Pod weight (g) | Seed weight (g) |
|----------------|----------------------|--------------------------------------|-------------------|---------------------------------|-------------------|-----------------------------|----------------|---------------|----------------|
| Huayu 23       | 37                   | 40                                    | 0.5               | 10                              | 9                 | 9                           | 53             | 44.18         | 27.92          |
| (CK)           |                      |                                      |                   |                                 |                   |                             |                |               |                |
| Huayu 23-7-1   | 25                   | 33                                    | 0.5               | 8                               | 19                | 17                          | 59             | 61.00         | 36.75          |

Table 4 Main agronomic characters of gene-edited Huayu 23 T1 plant and Huayu 23
a promising genome editing tool, as indicated by the study of Biswas et al. (2022).

In peanut, pods develop from pegs. One or multiple peg(s) is/are born at each node with peg(s), depending on cultivar (Nigam et al. 1990). In the case of multiple pegs, one injection may result in more than one pods, and in the meantime, the “primordial” reproductive cells at different developmental stages may increase the chances of being transformed. In this regard, the node injection method is advantageous over flower injection. A maximum of one pod can be harvested from a single flower. Our earlier study indicated that the node injection method was genotype independent (Wang et al. 2013), where no tissue culture procedure was needed, expanding its scope of use. Nevertheless, in-depth developmental studies on reproductive cells/tissues of the nodes may help optimize peanut transformation efficiency of this method.

Since the peanut node injection transformation method is easy to implement, peanut is an oilseed crop with oleosins, conducive to the separation and purification of expression products, and more importantly, peanut seeds can be eaten raw, the node injection method may thus facilitate peanut molecular pharming, for example, the production of edible vaccines. It is anticipated that the method, coupled with genome editing technology where necessary, will find wide utility in areas such as functional analysis of candidate peanut genes and breeding genome edited peanut cultivars for improved safety quality, ideotype, and other valuable traits, as long as they are controlled by oligenes. We believe that this node injection transformation method is not only useful to peanut, but also of some reference to other seed plant species.

Conclusions

Altogether, using the node injection transformation method and a CRISPR/Cas9 construct targeting FAD2B, two high-oleic T1 peanut mutant seeds with 442 A insertion in FAD2B were generated from normal-oleic cultivar Huayu 23 already having dysfunctional FAD2A. Amplification of the bar gene verified that the 2 high-oleic peanut seeds were true transformants. One of the T1 seed developed into a healthy plant. NIRS analysis demonstrated that the high-oleic phenotype was expressed in T2 seed generation. It is noteworthy that in this study the homozygous FAD2B mutation appeared in T1 seed generation. This may be explained by the transforming of cells that would generate male and female gametes, and the editing of FAD2B at very early developmental stages. Therefore, the outcome of this study is better than that reported in other studies.
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