In Planta Genetic Transformation to Produce CRISPRed High-Oleic Peanut

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Research Article

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Abstract

In contrast to its normal-oleic counterpart, high-oleic peanut has better keeping quality and multiple health benefits. Breeding high-oleic peanut through conventional means is a tedious process generally requiring several years. Genome editing may shorten the duration. In this study, node injection method was used to transform normal-oleic Huayu 23, a popular peanut cultivar having loss-of-function *FAD2A*, with CRISPR/Cas9 construct targeting *FAD2B*, and two peanut mutants with over 80% oleic acid and 442A insertion in *FAD2B* were obtained. As a genotype-independent, simple and easy method for peanut genetic transformation, node injection has great potential in factional analysis of genes and in peanut varietal improvement.

Introduction

Rich in culinary oil and high digestible protein, the cultivated peanut (*Arachis hypogaea* L.) occupies an important position in human and animal nutrition. 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).

In the cultivated peanut, *FAD2A* (*fatty acid desaturase 2A*) and *FAD2B* (*fatty acid desaturase 2B*) control the conversion of oleic acid to linoleic acid, and expression of the high-oleic phenotype (at least 75% oleic acid content) in peanut cultigen 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. In contrast to the lengthy process of conventional breeding, the speed of genome editing may be much faster. With peanut germs with a cotyledon attached for transformation, Wen et al. (2018) demonstrated that TALENs (transcription activator-like effector nucleases)-mediated targeted mutagenesis of *FAD2* in peanut cv Yueyou 7 raised oleic acid content from 43–60%~80%, while decreased 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 (clustered regularly interspaced short palindromic repeat)/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.

Suitable transformation procedures may facilitate the application of genome editing tools. Previously, node injection method, an easy-to-follow *in planta* peanut transformation protocol, was developed at our laboratory (Wang et al. 2013). This study aimed to test its effectiveness in inducing high-oleic peanut mutations using CRISPR/Cas9 technology.

Material And Methods

Plant material and cultivation

Huayu 23, a popular peanut cultivar of runner market type widely accepted by growers and processors, was used in this study. Sanger sequencing of its *FAD2A* and *FAD2B* and subsequent sequence alignment revealed that this cultivar had a mutated *FAD2A* (448 G>A)and a wild type *FAD2B*. Peanut was sown under film mulching in an isolated region in Laixi Experimental Station on May 5, 2021. Agronomic practices were followed as routine.

Vector construction and transformation of *E. coli*

Target site of sgRNA, 501-520 position of the coding sequence, was selected based on wild type *FAD2B* using CRISPR-GE (http://skl.scau.edu.cn/). To facilitate ligation in genome editing vector construction, two oligos which would produce overhangs after mixing, denaturation and annealing were generated with online tool (http://biogle.cn/index/excrispr) and synthesized (Tsingke, Qingdao). CRISPR/Cas9 editing vector with target site incorporated was made with BGK41-Cas9 (Biogle Biotechnology Co. Ltd, Hangzhou) following manufacturer's instructions. Briefly, BGK41-Cas9, oligo dimers and enzyme mix (Biogle CRISPR/Cas vector construction kit) were mixed on ice bath, and then incubated at 20C for 1 h. The ligation products were transformed into competent cells of *E. coli* strain DH5α. Positive clones identified using colony-PCR with 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 injection was based on Pan et al. (2020) with some modifications. Positive single clones were verified by bacterial suspension PCR using Cas9-F/Cas9-R primer pair. 100 µl of freshly prepared bacterial suspension were cultured in 10 ml of YEB (Yeast Extract Beef) liquid medium at 28C with agitation (250 rpm) until OD_{600} reached 0.6-0.8 (about 12 h). The cultures were centrifuged at 6 000 rpm for 1 min to collect the bacterium. Equal volume of infection solution containing 100 µmol/L acetosyringone (AS), 10 mmol/L MES and 10 mmol/L $MgCl_2.6H_2O$ was added to the pellets. Resuspended bacterial pellets were used for injection. Node injection procedure was essentially the same as that in our previous report (Wang et al. 2013) (Fig. 2). 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 mental wires were used to facilitate the entry of pegs into the soil from higher nodes (Fig. 2).

Quality analysis of resultant seeds

Pods were harvested when matured (Sept. 17, 2021). These pods were sun-dried and hand shelled. The chemical quality of the individual single seeds was predicted with near infra-red spectroscopy (NIRS)

(Wang et al. 2014). Seeds with at least 74% oleic acid along with were further analyzed for fatty acids using gas-chromatography (GC) using cotyledonary slices follow the protocol of Yang et al. (2012).

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

FAD2A and *FAD2B* sequences of the high-oleic seeds and Huayu 23 were amplified by PCR using primers aF19 (5'-gattactgattattgactt-3')/R1 (5'- ctctgactatgcatcag-3') and bF19 (5'- cagaaccattagctttg-3')/R1 respectively (Patel et al. 2014), and 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.

Results

Quality of the resultant seeds and wild type Huayu 23 predicted by NIRS

A total of 16 nodes were injected, which only resulted in 4 seeds. Among them, 2 seeds, Huayu 23-7-1 and Huayu 23-7-2 were classified as high-oleic by NIRS (Table 1, Fig. 3). More than 5 percent points decrease in oil content and over 4.5 percent points increase in protein content were also noted (Table 1).

Table 1
Chemical quality of single peanut seeds predicted by NIRS

Identity	Oil	Protein	Oleic	Linoleic	O/L
Huayu 23 CK	54.72	22.27	45.88	26.64	1.72
Huayu 23-7-1	49.69	26.74	74.33	5.39	13.80
Huayu 23-7-2	46.92	30.83	78.96	3.84	20.54

Main fatty acids of the resultant seeds and wild type Huayu 23 by wet chemistry method

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.

Table 2
Fatty acids in single peanut seeds determined by GC

Identity	Oleic	Linoleic	Palmitic	O/L
Huayu 23 CK	52.15±0.24 B	25.72A±0.2	15.55±0.19 A	2.03
Huayu 23-7-1	80.26±0.32 A	1.68B±0.12	8.07±0.23 B	47.69
Huayu 23-7-2	80.83±0.1 A	1.46B±0.06	7.39±0.38 B	55.36
In each column, figures followed by the same letter were not significantly different at 0.01 level.				

FAD2A/FAD2B **Genotyping of the resultant 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 mutants had a mutant type *FAD2B* (442A insertion) (Fig. 4). The causal relationship between 442A in *FAD2B* and dysfunctional *FAD2B* has well been clarified (Yu et al. 2008). However, the 442A *FAD2B* mutation was out of the scope of the anticipated targeting site.

Discussion

In this study, two peanut mutant 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), clearly verified the usefulness of the peanut transformation protocol.

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, the possibility of harvesting sound mature kernels from the lower nodes was high, and that peanut cells that will develop into reproductive cells, or “primordial” reproductive cells, can be transformed (Wang et al. 2012). Generally, only the first and second nodes counting from the intersection of the main stem and cotyledonary branches were injected. However, in this study, when everything was ready, it was too late, only higher nodes could be injected, that’s why only a small number of seeds were harvested. Failing to edit in the anticipated target site may be ascribed to the small population. Anyway, 2 CRISPRed high-oleic peanut seeds were identified from the 4 resultant seeds.

Interestingly, in addition to oleic, linoleic and palmitic acid contents, the oil and protein contents were also altered in the 2 mutants. If the higher oil and lower protein phenotypes prove to be inheritable by further investigations, the mutants may be of some help to identify genes governing oil/protein contents in peanut.

In peanut, pods were developed from pegs. One or multiple peg(s) was/were born at each node, depending on cultivar. 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

chance of being transformed. In this regard, node injection method is advantageous over flower injection. 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.

Since the peanut node injection transformation method is easy to implement, and peanuts is an oilseed crop, its seeds may be eaten raw, the node injection method may facilitate peanut molecular pharming. 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 development of genome edited peanut cultivars with improved safety quality, ideotype, and high and stable productivity. This node injection transformation method is not only useful to peanut, but also of some reference to other seed plant species.

Declarations

Declaration of competing interest

The authors declare no conflicts of interest.

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Author contributions CTW designed and supervised the experiment. HWH and ZWW conducted the transformation experiment. ZY, GSS and XSS were responsible for peanut cultivation, NIR analysis, and regent supplies. STY performed GC analysis. CTW, HHW, and STY prepared the manuscript.

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Figures

Figure 1

Map of BGK41-Cas9 recombinant vector FAD2B-1 LB：T-DNA right border；RB：T-DNA left border； U6：Soybean U6 promoter；SG：sgRNA；e35S：enhanced 35S 35S promoter；Cas9：Optimized Cas9；NOS Ter：NOS terminator；35S：CaMV (cauliflower mosaic virus) 35S promoter；Bar：Barsta selection marker gene；PolyA Ter：PolyA terminator

Figure 2

Node injection of Agrobacterium suspension (upper) and use of inverted U-shaped mental wires to help soil penetration of pegs (lower)

Figure 3

Two high-oleic peanut seeds, Huayu 23-7-1(left), Huayu 23-7-2 (middle) and wild type Huayu 23 (right)

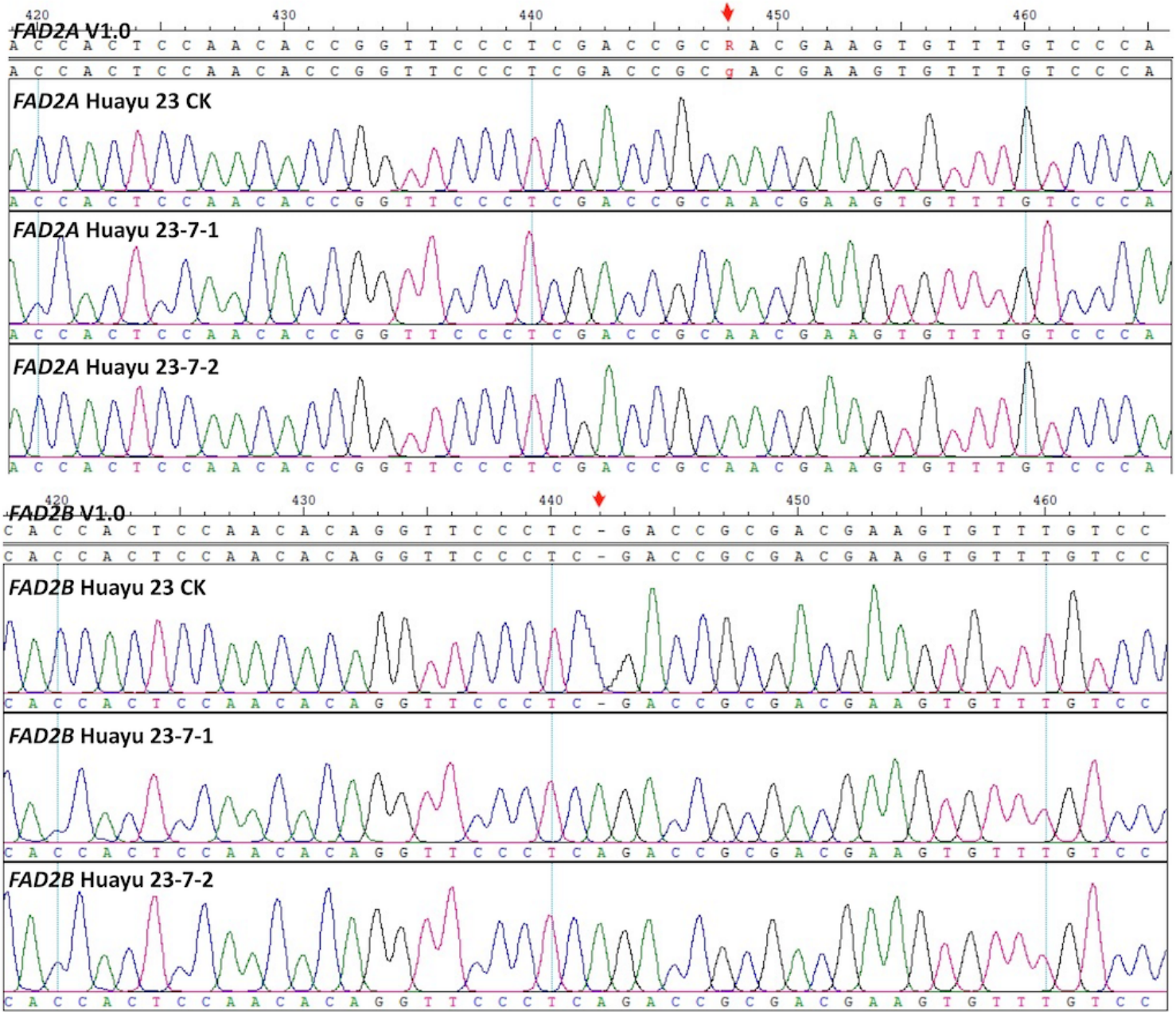

Figure 4

Fatty acids in single peanut seeds determined by GC