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Brief Communication

Enhancing peanut nutritional quality by editing *AhKCS* genes lacking natural variation

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Peanut (*Arachis hypogaea* L.) is a globally staple oilseed crop, extensively cultivated in tropical and subtropical regions. Due to its substantial oil (approximately 46%–58%) and protein (around 22%–32%) content, the peanut plays a pivotal role in addressing malnutrition and ensuring food security in many regions. The fatty acid profiles of vegetable oil and foods have recently garnered increased attention due to the potential impact on human health. Very long chain fatty acids (VLCFAs) are defined as fatty acids with a carbon chain length exceeding 18 atoms (Guyomarc'h *et al.*, 2021). Peanut kernels contain various VLCFAs, such as arachidic acid (C20:0), eicosenoic acid (C20:1), behenic acid (C22:0) and lignoceric acid (C24:0), but most of them are saturated fatty acids (SFAs). It is well understood that high levels of very long chain saturated fatty acid (VLCSFA) are associated with prevalence of atherosclerosis and cardiovascular disease (Bloise *et al.*, 2022). Therefore, reducing the VLCFA content in peanuts has gained more importance realizing its positive impact for improving the nutritional quality and health value.

The biosynthesis of VLCFAs in plants is known to be regulated by a key enzyme, *β*-ketoacyl-CoA synthase (KCS) (Wang *et al.*, 2017). In our previous study, a total of 30 *AhKCS* genes were identified in peanut genomes. After gene expression profiling and functional analysis, a pair of homologous gene *AhKCS1* and *AhKCS28* were identified as putative regulators of VLCFA contents in peanut kernels. The VLCFA content in available peanut germplasm accessions ranges from 4.3% to 9.8%, but no sequence variation was observed within or surrounding the *AhKCS1* and *AhKCS28* genes, suggesting the only possibility of further reduction of VLCFA content through gene editing (Huai *et al.*, 2020). Therefore, in this study, *AhKCS1* and *AhKCS28* were genetically disrupted using the CRISPR/Cas9 system to generate novel peanut mutants exhibiting significantly reduced levels of VLCFA content in kernels.

A CRISPR/Cas9 construct was designed to incorporate two single-guide RNAs (sgRNAs) that specifically target the homologous exon regions of *AhKCS1* and *AhKCS28* genes (Figure 1a,b). Firstly, this construct was introduced into normal oleate peanut cultivar Zhonghua 12 (ZH12) through *Agrobacterium tumefaciens*-mediated transformation (Huai *et al.*, 2023). A total of 66 independent positive T_0 transgenic ZH12 plants were successfully obtained. Among them, 61 exhibited mutations in both target genes, while two showed mutations in only one gene (Table S1). Three homozygous T_1 lines (A-2, A-3 and A-9) with mutations at both target sites for sgRNA1 and sgRNA2 in *AhKCS1* and *AhKCS28* genes, which caused translational frameshifts and premature stop codons, were selected for further study (Figures 1b and S1). None of the *AhKCS1*/*AhKCS28* double mutants exhibited any growth anomalies, and no apparent alteration in morphological and yield-related traits under both greenhouse and field conditions. Furthermore, resequencing of the three double mutants revealed no evidence of off-target mutations (Table S2).

The fatty acid composition of the harvested seeds from ZH12 and each double mutant was determined by gas chromatography (Figure 1c). The VLCFAs contents in the double mutants have been significantly decreased by 70.6%–100.0%. The VLCFA profiles of ZH12 showed four distinct peaks corresponding to C20:0, C20:1, C22:0 and C24:0. However, the peak of C20:1 and C24:0 was absent in all the three double mutants (Figure 1c). Although the peak of C20:0 was observed in both ZH12 and the double mutants, its content significantly decreased from 1.7% to 0.4%–0.5% in the double mutants. Similarly, while the content of C22:0 amounted to 2.8% in ZH12, it dramatically reduced to 0.3% in A-2 and was absent altogether in A-3 and A-9. Consequently, there was a substantial reduction from total VLCFA content of 6.9% observed within ZH12 down to merely 0.9%, 0.5% and 0.4% in A-2, A-3 and A-9, respectively, which were considerably lower than the value (4.3%) in naturally evolved germplasm materials (Figure 1d).

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The CRISPR/Cas9 construct was also introduced into a high oleate peanut breeding line JC30. In total, 63 independent positive T_0 transgenic JC30 plants were generated, out of which 60 exhibited mutations in both target genes (Table S1). Similarly, three homozygous T_1 lines (B-37, B-38 and B-59) harbouring truncated proteins of AhKCS1 and AhKCS28 were chosen to analyse the seed fatty acid composition (Figures 1b and S1). The double mutants of JC 30 exhibited only three peaks representing to C20:0, C20:1 and C22:0, while the peak of C24:0 was not detected (Figure 1c). The contents of C20:0 and C20:1 in double mutants of JC30 were reduced from 1.0% to 0.4%, while the C22:0 content was decreased from 1.4% to 0.2%. The VLCFA content in the double mutants of JC30 was reduced from 4.1% to 1.0%, which was slightly higher than that of double mutants of ZH12 (0.4%–0.9%). This relatively higher content can be attributed to the higher C20:1 content in the double mutants of JC30, which was absent in the double mutants of ZH12 (Figure 1d). The increase of C20:1 in double mutants of JC30 can be explained by an augment availability of substrate C18:1 in kernels. Interestingly, there was no significant difference in total

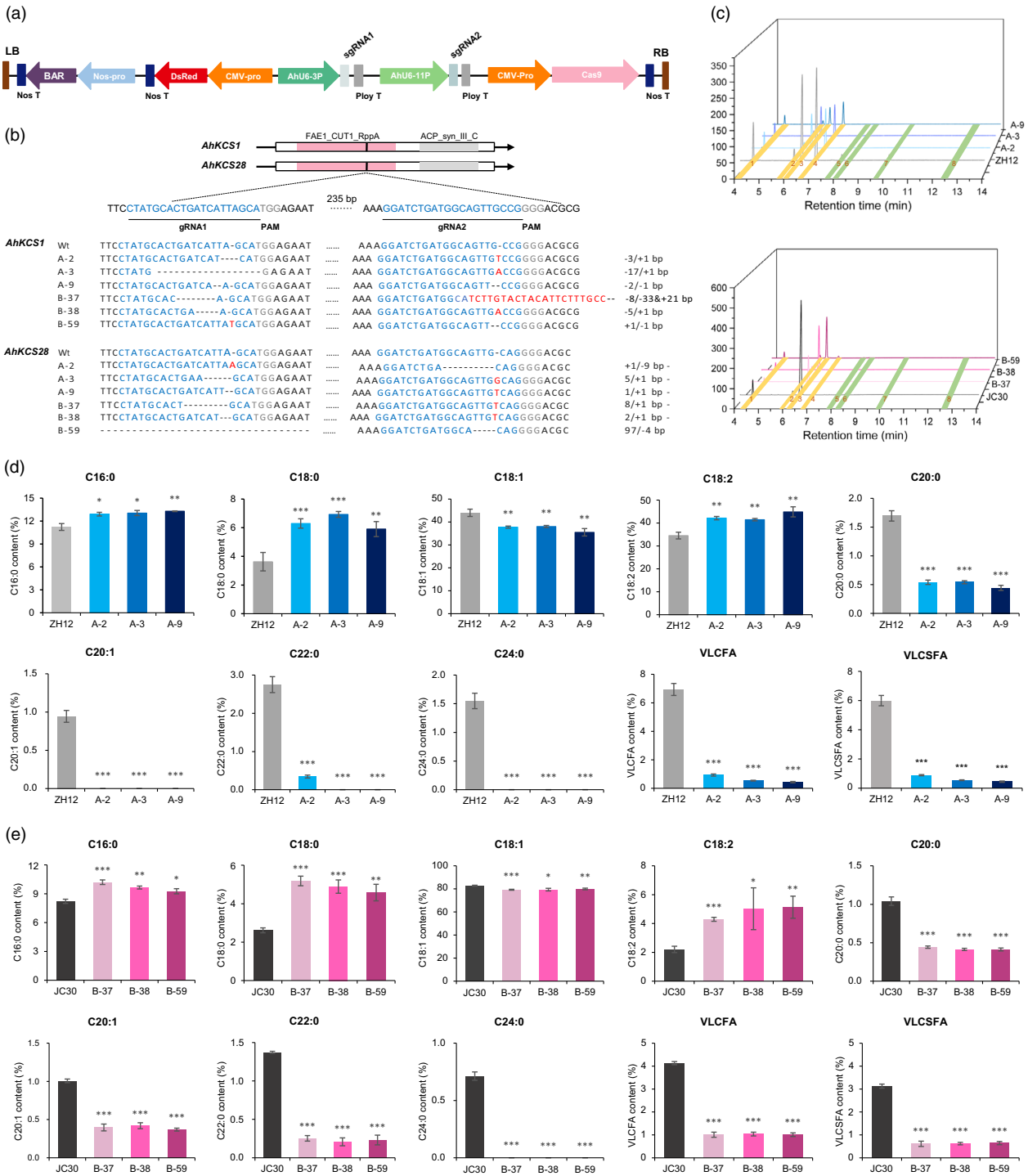

Figure 1 The depletion of *AhKCS1* and *AhKCS28* genes resulted in a significant reduction of the VLCFA content in peanut using the CRISPR/Cas9 system. (a) Schematic diagram of the T-DNA region of the CRISPR/Cas9 construct. (b) Characterization of edited *AhKCS1* and *AhKCS28* transgenic lines. The upper panel shows gene structure and CRISPR/Cas9 target sites in homologous exon regions of *AhKCS1* and *AhKCS28* genes. FAE1_CUT1_RppA, FAE1/Type III polyketide synthase-like protein domain; ACP_syn_III_C, 3-Oxoacyl-[acyl-carrier-protein (ACP)] synthase III C terminal domain. The lower panel shows the sequences of T_1 independent transgenic lines in the target sites. Wt, wild type (ZH12 and JC30); A-2, A-3 and A-9, transgenic lines derived from ZH12; B-37, B-38 and B-59, transgenic lines derived from JC30. PAM sites are indicated as grey letters; sgRNA sequences are indicated as blue letters; insertions are indicated as red letters; deletions are indicated as black dashes. The mutation types are shown on the right. (c) GC analysis of fatty acid methyl esters (FAMEs) from the harvested seeds of wild-type (ZH12 and JC30) and *AhKCS1* and *AhKCS28* double mutants. ZH12 is a normal oleate peanut cultivar, while A-2, A-3 and A-9 represent double mutants with normal oleate traits. JC30 is a high oleate peanut cultivar, while B-37, B-38 and B-59 represent double mutants with high oleate traits. The yellow belt indicates long chain fatty acids (LCFA), and the green belt indicates very long chain fatty acids (VLCFAs). Fatty acid peak identifies are: 1 = C16:0; 2 = C18:0; 3 = C18:1; 4 = C18:2; 5 = C20:0; 6 = C20:1; 7 = C22:0; 8 = C24:0. C16:0, palmitic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, linoleic acid; C20:0, arachidic acid; C20:1, eicosenoic acid; C22:0, behenic acid; C24:0, lignoceric acid. (d) Comparison of fatty acid composition between the normal oleate wild-type (ZH12) and double mutants of ZH12 (A-2, A-3 and A-9). (e) Comparison of fatty acid composition between the high oleate wild-type (JC30) and double mutants of JC30 (B-37, B-38 and B-59). VLCFA = C20:0 + C20:1 + C22:0 + C24:0; VLCSFA = C20:0 + C22:0 + C24:0. Student's t test was used for statistical analysis, single asterisk indicates significant differences at $P < 0.05$, double asterisk indicates significant differences at $P < 0.01$ and triple asterisk indicates significant differences at $P < 0.001$, all compared to the wild-type.

VLCSFA content between the double mutants derived from JC30 and ZH12 (0.6%–0.7% vs 0.4%–0.9%). Additionally, the levels of C16:0, C18:0 and C18:2 were found to be elevated, while the content of C18:1 was observed to be slightly reduced in both double mutants derived from JC30 and ZH12 (Figure 1c,d).

In summary, we demonstrated that *AhKCS1* and *AhKCS28* genes with no natural variation are the key genes for controlling the seed VLCFA content in peanut, and developed novel germplasm lines with low seed VLCFA content using genome-editing system. Furthermore, we also provided an efficient CRISPR/Cas9 genome editing platform for peanut, with great potential for expediting breeding programmes aimed at improving traits such as yield, quality and stress resistance.

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Author contributions

DH, RKV, BL and YL conceived and designed the experiments; HJ and LH supplied the peanut cultivars; XX, JW, NL, LY, YC, XW, QW, YK and ZW performed the experiments; DH, XX and MKP analysed the data; DH wrote the manuscript; DH, MKP, RKV, BL and YL contributed in data interpretation and revision of the manuscript. All authors have read and approved the final version of the manuscript.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

References

Bloise, A.M.N.d.L.G., Simões-Alves, A.C., Debora Santos, A., Morio, B. and Costa-Silva, J.H. (2022) Cardiometabolic impacts of saturated fatty acids: are they all comparable? *Int. J. Food Sci. Nutr.* **73**, 1–14.

Guyomarc'h, S., Boutté, Y. and Laplaze, L. (2021) AP2/ERF transcription factors orchestrate very long chain fatty acid biosynthesis during *Arabidopsis* lateral root development. *Mol. Plant* **14**, 205–207.

Huai, D., Xue, X., Li, Y., Wang, P., Li, J., Yan, L., Chen, Y. *et al*. (2020) Genome-wide identification of peanut KCS genes reveals that *AhKCS1* and *AhKCS28* are involved in regulating VLCFA contents in seeds. *Front. Plant Sci.* **11**, 406.

Huai, D., Wu, J., Xue, X., Hu, M., Zhi, C., Pandey, M.K., Liu, N. *et al*. (2023) Red fluorescence protein (DsRed2) promotes the screening efficiency in peanut genetic transformation. *Front. Plant Sci.* **14**, 1123644.

Wang, X., Guan, Y., Zhang, D., Dong, X., Tian, L. and Qu, L.Q. (2017) A β-ketoacyl-CoA synthase is involved in rice leaf cuticular wax synthesis and requires a CER2-LIKE protein as a cofactor. *Plant Physiol.* **173**, 944–955.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. The Sanger sequencing chromatograms of each target site in the homozygous T_1 lines.
Table S1. Summary of mutations at each target site in the T_0 generation.
Table S2. Detection of off-target mutation in A-2, A-3 and A-9 using genome resequencing.