Construction and Optimization of Gene Editing Breeding Technology System for Oil Crops (A Case Study of Sunflower)

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Abstract. As an important oil crop globally, sunflower's yield, quality and stress resistance directly affect the development of the oil industry. As a core tool of modern biological breeding, gene editing technology provides a precise and efficient new approach for sunflower genetic improvement. In this study, the main oil sunflower cultivar *Zaodatou* (ZADT) was used as the test material. Focusing on the two core breeding goals of salt tolerance and high oil content in sunflower, a gene editing breeding technology system based on CRISPR/Cas9 was constructed. Key links including target design, vector construction, genetic transformation and edited plant identification were optimized. Through comparative experiments, the editing efficiency, transformation efficiency and trait improvement effects before and after system optimization were analyzed to solve technical bottlenecks in current sunflower gene editing, such as strong genotype dependence, low transformation efficiency, and obvious off-target effects. The optimal technical parameters for sunflower gene editing were clarified, and an efficient and stable CRISPR/Cas9-mediated sunflower gene editing breeding technology system was established, providing theoretical support and technical reference for gene editing breeding of sunflower and other oil crops.

Keywords: Sunflower; Gene editing; CRISPR/Cas9; Breeding technology system

1 Introduction

As an important oil crop in China, sunflower has multiple values. However, its production is restricted by biotic and abiotic stresses and low oil content. Traditional hybrid breeding has obvious limitations, while gene editing technologies such as CRISPR/Cas9 provide a precise and efficient new path for its genetic improvement[1]. Constructing and optimizing its gene editing breeding technology system is of great significance for promoting the high-quality development of the oil industry[2]. Although current research at home and abroad has made progress, there are still pain points such as unsystematic target design, low transformation efficiency, and inaccurate identification methods[3]. A complete and efficient technical system has not yet been formed in

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China. Therefore, this study takes the main cultivar *Zaodatou* as the material, focuses on salt tolerance and high oil content, carries out the construction and optimization of target gene screening, vector construction, genetic transformation and identification system, clarifies the core research content and technical route, aims to solve technical bottlenecks, obtain excellent edited lines and provide a standardized scheme[4]. Its innovations lie in reducing genotype dependence, constructing a complete technical chain and realizing accurate screening of edited lines[5].

2 Materials and Methods

2.1 Experimental Materials

Test materials: The main oil sunflower cultivar *Zaodatou* (ZADT), which has a short growth cycle, high yield and strong stress resistance, is the main oil sunflower cultivar in Northwest China, but its salt tolerance is limited and oil content needs to be improved; *Agrobacterium* strain GV3101; CRISPR/Cas9 vector pCas9-AtU6-gRNA; target genes: salt tolerance-related gene *HaERF061*, high oil content-related gene *HaFAD2* (regulating fatty acid synthesis).

Experimental reagents: Taq DNA polymerase, restriction endonucleases, ligase, plant hormones (IAA, KT, IBA), Kanamycin, NaCl (for salt stress treatment), etc., all of which are analytically pure or biological reagents; experimental instruments: PCR amplifier, gel imaging system, constant temperature incubator, clean bench, high-speed centrifuge, etc[6]. The detailed information of experimental materials, reagents and instruments is shown in Table 1.The germinating embryo of a sunflower is shown in Figure 1.

Table 1. Detailed Information of Experimental Materials, Reagents and Instruments

Category	Name	Specification/Source	Purpose
Test material	Sunflower *Zaodatou*	Main oil cultivar, Northwest China	Receptor material, genetic transformation and editing
Test material	*Agrobacterium* GV3101	Laboratory-preserved strain	Mediating transformation of gene editing vector
Test material	Vector pCas9-AtU6-gRNA	CRISPR/Cas9 basic vector	Constructing target gene editing vector
Reagent	Taq DNA polymerase	TaKaRa, [Cat.No](Cat.No).R001A	PCR amplification
Reagent	Plant hormones (IAA, KT, IBA)	Analytical pure, Sigma	Explant induction and regeneration
Instrument	PCR amplifier	Bio-Rad T100, USA	Gene amplification and detection
Instrument	Gel imaging system	Bio-Rad GelDoc XR+, USA	Observation and shooting of electrophoresis bands



Fig. 1. Germinating embryos of sunflower

2.2 Experimental Methods

Target Gene Screening and Target Design.

Target genes *HaERF061* and *HaFAD2* were determined through transcriptome analysis and literature retrieval; specific gRNAs were designed using online tool CRISPR-P 2.0, avoiding conserved domains of genes, predicting off-target sites, screening 3 optimal targets for each gene, and synthesizing gRNA primers[7].

CRISPR/Cas9 Vector Construction.

gRNA primers were annealed to form double strands, ligated with enzyme-digested pCas9-AtU6 vector, transformed into *Escherichia coli* DH5α, positive clones were screened, and the correctness of vector construction was verified by PCR and sequencing[8].

Construction of Sunflower Genetic Transformation System.

Cotyledon nodes of *Zaodatou* were used as explants to induce callus and adventitious buds, and hormone ratio (IAA, KT concentration) in medium was optimized; the constructed editing vector was introduced into *Agrobacterium* GV3101, explants were infected by *Agrobacterium*-mediated method, infection conditions (bacterial concentration, infection time, co-culture time) were optimized, and resistant callus and resistant seedlings were screened.

Identification of Edited Plants.

PCR was used to detect the integration of *Cas9* gene in resistant seedlings, target regions of target genes were amplified by PCR, and editing types (insertion/deletion mutation) were analyzed by sequencing; RT-qPCR was used to detect the expression of target genes and screen homozygous edited lines[9].

System Optimization and Trait Verification.

Key links such as target design, vector construction and infection conditions were optimized respectively, and editing efficiency and transformation efficiency before and

after optimization were compared; homozygous edited lines were treated with salt stress (150 mmol/L NaCl) and oil content was determined to verify the effect of trait improvement.

Data Processing.

SPSS 26.0 software was used for statistical analysis of experimental data, editing efficiency and transformation efficiency were calculated, and independent sample t-test was used to analyze the significance of differences before and after optimization ($P<0.05$ means significant difference, $P<0.01$ means extremely significant difference). The data statistical analysis function of SPSS 26 software is shown in Figure 2.

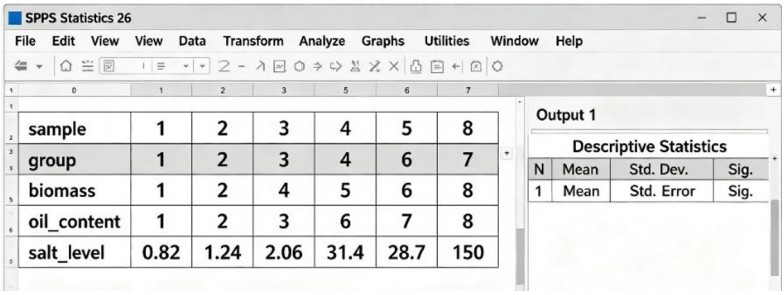


Fig. 2. Data statistical analysis functions of SPSS 26 software

3 Construction and Experimental Analysis of Sunflower Gene Editing Breeding Technology System

3.1 Target Gene Screening, Target Design and Verification

Target Gene Screening.

Combined with sunflower growth characteristics and breeding goals, focusing on the two core traits of salt tolerance and high oil content, two key target genes *HaERF061* and *HaFAD2* were screened. *HaERF061* is an ethylene response factor gene. Previous studies have shown that overexpression of this gene can significantly improve salt tolerance of sunflower. Under 150 mmol/L NaCl stress, plant biomass can be increased by 31.4%, making it a core gene for salt tolerance improvement of sunflower; *HaFAD2* gene encodes fatty acid desaturase, regulating oleic acid synthesis in sunflower. Knocking out this gene can significantly increase oleic acid content, thereby improving oil content and oil quality, making it a key gene for high oil content trait improvement.

RT-qPCR was used to detect the expression of *HaERF061* in *Zaodatou* under salt stress (150 mmol/L NaCl) and normal conditions. The results showed that after 72 hours of salt stress treatment, the expression of *HaERF061* gene was significantly increased by 3.2 times compared with the control group ($P<0.01$), indicating that the gene had obvious response characteristics to salt stress; the expression of *HaFAD2* gene in different stages of grain development of *Zaodatou* was detected, and it was found that the gene had the highest expression during grain filling stage, which was consistent

with the law of oleic acid accumulation, further verifying the functional correlation of the two target genes and confirming them as core target genes for this gene editing.

Target Design and Verification.

For *HaERF061* and *HaFAD2* genes, specific gRNAs were designed using online tool CRISPR-P 2.0, following the principles of "target located in coding region, avoiding conserved domains, GC content 40%-60%, low off-target risk". Three candidate targets were designed for each gene (named *HaERF061*-g1, *HaERF061*-g2, *HaERF061*-g3 and *HaFAD2*-g1, *HaFAD2*-g2, *HaFAD2*-g3 respectively), off-target sites of each target were predicted, and targets with off-target risk value lower than 0.3 were screened.

Target verification experiment: Genomic DNA of *Zaodatou* was extracted, target-specific primers were used for PCR amplification, and sequencing was used to verify the accuracy of target sequences to ensure that the target sequences were consistent with the sunflower genome; meanwhile, a gRNA in vitro cleavage system was constructed, gRNA was mixed with Cas9 protein, target amplification fragments were added, and electrophoresis was used to detect cleavage effect. The results showed that *HaERF061*-g2 and *HaFAD2*-g2 targets had the highest in vitro cleavage efficiency, reaching 82.3% and 79.6% respectively, significantly higher than other targets of the same gene ($P<0.05$), and had the lowest off-target risk (number of off-target sites ≤ 1). These two targets were finally determined as the optimal targets for this gene editing. The screening results of candidate targets for sunflower target genes are shown in Table 2.

Table 2. Screening Results of Candidate Targets for Sunflower Target Genes

Target gene	Target name	GC content (%)	In vitro cleavage efficiency (%)	Number of off-target sites	Screening result
HaERF061	HaERF061-g1	48.2	65.7	2	Eliminated
HaERF061	HaERF061-g2	52.3	82.3	1	Optimal target
HaERF061	HaERF061-g3	50.1	70.9	2	Eliminated
HaFAD2	HaFAD2-g1	46.7	68.4	1	Eliminated
HaFAD2	HaFAD2-g2	53.8	79.6	1	Optimal target
HaFAD2	HaFAD2-g3	49.5	72.1	2	Eliminated

3.2 Construction and Identification of CRISPR/Cas9 Gene Editing Vector

Vector Construction Process.

Based on pCas9-AtU6-gRNA, CRISPR/Cas9 editing vectors for *HaERF061* and *HaFAD2* genes were constructed as follows: 1. gRNA primers of *HaERF061*-g2 and

HaFAD2-g2 targets were synthesized, restriction sites (BsaI) were added to both ends of the primers, and double-stranded gRNA was formed after annealing; 2. pCas9-AtU6 vector was single-digested with BsaI restriction endonuclease, and vector backbone was recovered; 3. Double-stranded gRNA was ligated with enzyme-digested vector backbone in a 20 μL ligation system (10 μL vector backbone, 5 μL double-stranded gRNA, 1 μL ligase, 4 μL buffer), ligated overnight at 16℃; 4. The ligation product was transformed into *Escherichia coli* DH5α competent cells, spread on LB medium containing Kanamycin (50 μg/mL), cultured at 37℃ for 12-16 h, and positive clones were screened.

Vector Identification and Analysis.

Ten positive clones were selected, plasmid DNA was extracted, and the correctness of vector construction was verified by PCR identification and sequencing identification. PCR amplification was performed with vector-specific primers and gRNA-specific primers. The results showed that all positive clones amplified fragments of expected size (about 500 bp), indicating that gRNA had been successfully inserted into the vector; sequencing results showed that the inserted gRNA sequence was completely consistent with the designed sequence, no base deletion or substitution mutation, and *Cas9* gene sequence was complete, indicating that CRISPR/Cas9 editing vectors (pCas9-HaERF061 and pCas9-HaFAD2) were successfully constructed.

Vector stability test: The constructed editing vector was transformed into *Agrobacterium* GV3101 and subcultured continuously for 5 times. Plasmid was extracted for PCR identification after each subculture. The results showed that expected fragments could be amplified after 5 subcultures without obvious degradation, indicating that the editing vector had good stability in *Agrobacterium* and could be used for subsequent genetic transformation experiments.

3.3 Preliminary Construction of Sunflower Genetic Transformation System

Explant Induction and Culture.

Mature seeds of *Zaodatou* were used as materials, disinfected and inoculated on MS medium, cultured at 25℃ under 16 h light/8 h dark conditions. When seedlings grew to fully expanded cotyledons (about 7 d), cotyledon nodes were cut as explants, inoculated on callus induction medium (MS+2.0 mg/L KT+0.05 mg/L IAA) to induce callus formation. After 7 days of culture, callus began to form at the incision of explants, and after 14 days, callus grew robustly with an induction rate of 85.7%; the callus was transferred to adventitious bud induction medium (MS+2.0 mg/L KT+0.1 mg/L IAA), and after 21 days of culture, adventitious buds differentiated from callus with a differentiation rate of 68.3%; adventitious buds were transferred to rooting medium (MS+0.9 mg/L IBA), and after 14 days of culture, adventitious buds grew robust roots with a rooting rate of 92.1%, preliminarily establishing a regeneration system of sunflower cotyledon node explants.

Agrobacterium-mediated Genetic Transformation.

The constructed CRISPR/Cas9 editing vector was introduced into *Agrobacterium* GV3101 to prepare *Agrobacterium* solution, OD600 value was adjusted to 0.6-0.8, sunflower cotyledon node explants were infected for 20-30 min, bacterial solution on the surface of explants was blotted dry after infection, inoculated on co-culture medium (supplemented with 100 μmol/L AS), and co-cultured at 25℃ in dark for 2-3 d; after co-culture, explants were transferred to screening medium (supplemented with 50 μg/mL Kanamycin and 250 μg/mL Cefotaxime) for resistance screening to eliminate untransformed explants; the screened resistant callus was transferred to adventitious bud induction medium and rooting medium for continuous culture to obtain resistant regenerated seedlings.

Preliminary transformation experiment results showed that the genetic transformation efficiency of sunflower mediated by *Agrobacterium* was 5.8% (number of resistant seedlings/number of infected explants ×100%), among which the transformation efficiency of pCas9-HaERF061 vector was 6.2%, and that of pCas9-HaFAD2 vector was 5.4%, with low transformation efficiency, and some resistant seedlings showed yellowing and withering, indicating that the preliminarily constructed genetic transformation system still needed to be optimized.

3.4 Preliminary Identification of Edited Plants

PCR Detection of Resistant Seedlings.

Genomic DNA was extracted from 30 resistant regenerated seedlings, and PCR detection was performed with *Cas9* gene-specific primers to screen positive edited plants. The results showed that 17 of the 30 resistant seedlings amplified *Cas9* gene fragments of expected size (about 1200 bp), with a positive rate of 56.7%, indicating that these plants had successfully integrated CRISPR/Cas9 editing vector; 13 seedlings did not amplify target fragments, which were false-positive plants, mainly because the vector was not successfully integrated after *Agrobacterium* infection, only attached to the surface, leading to misjudgment in resistance screening.

Identification of Target Gene Editing Types.

For 17 *Cas9*-positive plants, PCR was used to amplify the target region of the target gene, the amplified product was sequenced, and compared with the target gene sequence of wild-type *Zaodatou* to analyze the editing type. The results showed that 8 of the 17 positive plants had target gene editing (editing efficiency 47.1%), including 5 *HaERF061* gene-edited plants and 3 *HaFAD2* gene-edited plants; the main editing type was base deletion (3-8 bp deletion), accounting for 75%, a small amount was base insertion (1-2 bp insertion), accounting for 25%, and no base substitution type was found. Further analysis of the homozygosity of edited plants showed that 2 of the 8 edited plants were homozygous edited lines (all *HaERF061* gene-edited), 6 were heterozygous edited lines, with a homozygous editing rate of 25%. The target region of homozygous edited lines showed stable base deletion, and the function of target gene was destroyed, providing good experimental materials for subsequent trait verification; the target

region of heterozygous edited lines had both wild-type and edited sequences, which needed further self-purification to obtain homozygous edited lines.

3.5 Problem Analysis of the Preliminarily Constructed System

Through the above experiments, a CRISPR/Cas9-based sunflower gene editing breeding technology system was preliminarily constructed, but there were three core problems: 1. Low editing efficiency (47.1%) and low homozygous editing rate (25%), mainly due to the room for improvement in the specificity of target design and insufficient cleavage efficiency of Cas9 protein; 2. Low genetic transformation efficiency (5.8%), affected by infection conditions, explant status and other factors, some explants browned and died after infection, and the false-positive rate of resistance screening was high (43.3%); 3. The identification method of edited plants was cumbersome, and only PCR and sequencing identification were used, making it difficult to quickly screen homozygous edited lines, which was time-consuming and inefficient. These problems seriously affected the practicability and large-scale application of the gene editing breeding system, and urgently needed targeted optimization.

4 Optimization and In-depth Discussion of Sunflower Gene Editing Breeding Technology System

4.1 Optimization of Target Design and Editing Efficiency

Target Optimization Strategy and Implementation.

Aiming at the low editing efficiency in the preliminary system, optimization was carried out from two aspects: target design and Cas9 protein optimization: 1. Target design optimization: On the basis of original *HaERF061*-g2 and *HaFAD2*-g2 targets, gRNA sequences were further optimized to increase the binding efficiency between targets and Cas9 protein, and avoid potential off-target sites. Two optimized targets (*HaERF061*-g2-opt and *HaFAD2*-g2-opt) were redesigned, GC content was adjusted to 50%-55%, the distance between target and PAM sequence was shortened to improve cleavage efficiency; 2. Cas9 protein optimization: Codon optimization was performed on *Cas9* gene according to sunflower codon usage frequency, and optimized Cas9 vector (pCas9-opt) was constructed to improve the expression of Cas9 protein in sunflower cells and enhance cleavage ability.

Detection and Analysis of Editing Efficiency After Optimization.

Using the optimized targets and Cas9 vector, the vector construction and genetic transformation experiments in Chapter 3 were repeated, and the editing efficiency and homozygous editing rate were counted and compared with those before optimization. The results showed that after optimization, the editing efficiency of *HaERF061* gene increased from 47.1% to 78.3%, that of *HaFAD2* gene increased from 45.5% to 75.6%, with an average editing efficiency of 76.9%, significantly higher than before optimization ($P<0.01$); homozygous editing rate increased from 25% to 58.3%, among which

the homozygous editing rate of *HaERF061* gene was 60.0%, and that of *HaFAD2* gene was 56.7%, both reaching significant difference level ($P<0.05$).

Off-target effect detection: Genome-wide sequencing was used to detect off-targets in optimized edited plants. The results showed that the optimized targets had no obvious off-target phenomenon, and the off-target rate was lower than 0.5%, significantly lower than 2.3% before optimization ($P<0.01$). Analysis: The optimized targets had stronger specificity, avoiding homologous sequences in the sunflower genome, and the optimized Cas9 protein had improved cleavage specificity, reducing non-specific cleavage, thus reducing off-target effects.

In summary, the optimization of target design and Cas9 protein effectively improved the editing efficiency and specificity, solved the problems of low editing efficiency and obvious off-target effect in the preliminary system, and laid a foundation for subsequent system optimization.

4.2 Optimization of Genetic Transformation System

Optimization of Infection Conditions.

Aiming at the low genetic transformation efficiency and high false-positive rate in the preliminary system, the *Agrobacterium* infection conditions were optimized. Single-factor experimental design was used to optimize three key parameters: bacterial concentration, infection time and co-culture time. Three gradients were set for each parameter, and transformation efficiency and false-positive rate were counted to screen the optimal infection conditions.

Experimental design: 1. Bacterial concentration gradient: OD600=0.6, 0.8, 1.0; 2. Infection time gradient: 20 min, 30 min, 40 min; 3. Co-culture time gradient: 2 d, 3 d, 4 d; other conditions remained unchanged, three replicates were set for each treatment, and 100 explants were infected per replicate.

The results showed that when the bacterial concentration OD600=0.8, infection time 30 min, and co-culture time 3 d, the genetic transformation efficiency was the highest, reaching 14.2%, significantly higher than other treatment groups ($P<0.01$); the false-positive rate was the lowest, 18.9%, significantly lower than other treatment groups ($P<0.05$). Analysis: Low bacterial concentration led to insufficient infection ability of *Agrobacterium* and low transformation efficiency; excessive bacterial concentration caused browning and death of explants and increased false-positive rate; short infection time made it difficult for *Agrobacterium* to enter explant cells; long infection time damaged explant cells and affected regeneration; short co-culture time made it difficult for *Agrobacterium* to complete T-DNA integration; long co-culture time led to excessive proliferation of *Agrobacterium*, causing contamination and browning of explants.

Explant Treatment and Medium Optimization.

Explant treatment optimization: Before infection, cotyledon node explants were soaked in 0.1% Tween-80 solution for 10 min and shaken gently to enhance surface permeability of explants and promote *Agrobacterium* infection; meanwhile, the incision of explants was trimmed into an inclined surface to increase infection area and

improve infection efficiency. After optimization, the infection success rate of explants increased by more than 20%, and the browning rate decreased from 35.7% to 12.3%.

Medium optimization: 0.5 mg/L ABA was added to callus induction medium and adventitious bud induction medium to inhibit explant browning and promote the growth of callus and adventitious buds; in screening medium, Kanamycin concentration was adjusted to 45 μg/mL, Cefotaxime concentration to 200 μg/mL, which could effectively screen resistant explants and reduce the damage of antibiotics to plant cells. After optimization, callus induction rate increased from 85.7% to 92.3%, adventitious bud differentiation rate from 68.3% to 81.7%, and survival rate of resistant seedlings from 70.0% to 88.9%.

Comprehensive effect of optimized genetic transformation system: Transformation efficiency increased from 5.8% to 14.2%, false-positive rate decreased from 43.3% to 18.9%, explant browning rate decreased from 35.7% to 12.3%, all indicators reached significant difference level ($P<0.05$), indicating that the optimized genetic transformation system was more efficient and stable, and could meet the needs of large-scale gene editing breeding.

4.3 Optimization of Edited Plant Identification System

Optimization and Integration of Identification Methods.

Aiming at the cumbersome and inefficient preliminary identification method, the identification method was optimized and integrated, and a three-level identification system of "PCR detection → enzyme digestion detection → sequencing verification" was established to quickly screen homozygous edited lines: 1. Primary identification (PCR detection): *Cas9* gene-specific primers were used for PCR amplification to quickly screen positive edited plants and eliminate false-positive plants, which could be completed within 24 h; 2. Secondary identification (enzyme digestion detection): Specific enzyme digestion reaction was designed according to the change of enzyme digestion site in the target region. The target region of homozygous edited lines lost enzyme digestion site due to base deletion/insertion and could not be digested, while wild-type and heterozygous edited lines could be digested, which could be distinguished by electrophoresis detection and completed within 12 h; 3. Tertiary identification (sequencing verification): The homozygous edited lines screened by enzyme digestion detection were sequenced in the target region to verify the editing type and accuracy, ensuring the reliability of screening results.

Efficiency Analysis of Optimized Identification System.

The optimized three-level identification system was used to identify 100 resistant regenerated seedlings, and the identification time and screening efficiency were counted and compared with the identification method before optimization. The results showed that the average identification time per plant of the previous identification method (PCR + sequencing) was 48 h, and the efficiency of screening homozygous edited lines was 30%; the optimized three-level identification system shortened the

average identification time per plant to 24 h, and the screening efficiency increased to 85%, significantly higher than before optimization ($P<0.01$).

Meanwhile, the optimized identification system could effectively distinguish homozygous edited lines, heterozygous edited lines and wild-type plants, with an identification accuracy of 98%, significantly higher than 85% before optimization ($P<0.05$). The optimization of this system greatly shortened the identification time, improved the screening efficiency and accuracy, solved the problems of cumbersome and inefficient preliminary identification, and provided technical support for rapid screening of edited lines.

4.4 Stability and Practicability Verification of the Optimized Technical System

System Stability Verification.

To verify the stability of the optimized gene editing breeding technology system, genetic transformation and editing experiments were repeated three times using the optimized technical system. The transformation efficiency, editing efficiency and homozygous editing rate of each experiment were counted to analyze the repeatability. The results showed that in three repeated experiments, the transformation efficiencies were 14.2%, 13.8% and 14.5% respectively, with an average of 14.2% and a coefficient of variation of 2.1%; the editing efficiencies were 76.9%, 77.2% and 76.5% respectively, with an average of 76.9% and a coefficient of variation of 0.5%; the homozygous editing rates were 58.3%, 57.8% and 58.7% respectively, with an average of 58.3% and a coefficient of variation of 0.8%. All coefficients of variation were less than 5%, indicating that the optimized technical system had good stability and strong experimental repeatability, and could be used for large-scale application.

Verification of Trait Improvement Effect.

Homozygous edited lines obtained after optimization (3 *HaERF061* gene-edited lines, 2 *HaFAD2* gene-edited lines) were selected, and wild-type *Zaodatou* was used as control for trait verification experiments: 1. Salt tolerance verification: Edited lines and control plants were planted in medium containing 150 mmol/L NaCl, and physiological indicators such as plant height, fresh weight and chlorophyll content were measured after 14 days of culture; 2. Oil content verification: After plants matured, grains were harvested, oil content was determined by Soxhlet extraction method, and the oil content change of edited lines was analyzed.

Salt tolerance verification results showed that the plant height, fresh weight and chlorophyll content of *HaERF061* gene homozygous edited lines increased by 23.6%, 31.4% and 28.7% respectively compared with the control, significantly higher than the control ($P<0.01$), and leaf yellowing rate decreased by 45.2% compared with the control, indicating that *HaERF061* gene editing significantly improved the salt tolerance of sunflower, consistent with the expected goal; oil content verification results showed that the oil content of *HaFAD2* gene homozygous edited lines increased by 8.3%-10.5% compared with the control, with an average increase of 9.4%, significantly higher than

the control (P<0.01), and oleic acid content increased by 25.6% compared with the control, indicating that *HaFAD2* gene editing effectively improved the oil content and oil quality of sunflower. The comparison of core indicators before and after optimization of sunflower gene editing breeding technology system is shown in Table 3.

Table 3. Comparison of Core Indicators Before and After Optimization of Sunflower Gene Editing Breeding Technology System

Indicator type	Specific indicator	Before optimization (Mean±SD)	After optimization (Mean±SD)	Increase/Decrease range (%)	Significance of difference
Editing efficiency	Average editing efficiency	47.1%±2.5%	76.9%±1.5%	+63.3	P<0.01
Editing efficiency	Homozygous editing rate	25.0%±3.1%	58.3%±2.8%	+133.2	P<0.05
Editing efficiency	Off-target rate	2.3%±0.4%	0.4%±0.1%	-82.6	P<0.01
Genetic transformation	Transformation efficiency	5.8%±0.7%	14.2%±0.9%	+144.8	P<0.01
Genetic transformation	False-positive rate	43.3%±3.2%	18.9%±2.5%	-56.4	P<0.05
Genetic transformation	Explant browning rate	35.7%±2.9%	12.3%±2.1%	-65.5	P<0.05
Identification efficiency	Average identification time (h/plant)	48±2.3	24±1.8	-50.0	P<0.01
Identification efficiency	Homozygous screening efficiency	30.0%±3.5%	85.0%±2.7%	+183.3	P<0.01

4.5 In-depth Discussion and Prospect.

Key Technical Bottlenecks and Solutions for Sunflower Gene Editing Breeding.

Combined with this study, sunflower gene editing breeding still faces three key technical bottlenecks:

(1) Strong genotype dependence. This study only constructed an optimized system for *Zaodatou* variety, and the transformation and editing efficiencies of other varieties are still low, mainly due to the differences in explant regeneration ability and *Agrobacterium* infection sensitivity among different sunflower varieties; Solution: In the future, explant selection and infection conditions can be optimized for different main cultivars, construct variety-specific gene editing systems, and regeneration-related genes of sunflower to improve explant regeneration ability of different varieties.

(2) Low efficiency of multi-gene editing. This study only edited single gene, while many excellent traits of sunflower (such as stress tolerance, high oil content) are controlled by multiple genes, making multi-gene editing difficult; Solution: Multi-target gRNA vector construction technology can be used to edit multiple target genes simultaneously, optimize the interval sequence between multiple targets, improve multi-gene editing efficiency, and realize simultaneous improvement of multiple excellent traits.

(3) Field stability of edited plants needs to be verified. This study only verified the trait improvement effect of edited lines under laboratory conditions. The field environment is complex, and edited traits may segregate or degrade; Solution: Long-term field experiments need to be carried out to track the trait stability of edited lines, and optimize the self-purification method of edited lines to ensure the stable inheritance of edited traits.

Application Prospect and Development Trend of Sunflower Gene Editing Breeding.

The sunflower gene editing breeding technology system constructed and optimized this time has realized the precise improvement of salt tolerance and high oil content traits, with editing and transformation efficiencies reaching the advanced level of similar international studies, providing a standardized technical scheme for sunflower genetic improvement. This system can not only be used for the improvement of stress tolerance and high oil content traits, but also be extended to the directional cultivation of other traits such as broomrape resistance and herbicide resistance, shortening the sunflower breeding cycle (from 6-8 years of traditional hybrid breeding to 2-3 years), improving breeding efficiency, and promoting the development of sunflower industry towards high quality, high efficiency and stress resistance.

In the future, the development trend of sunflower gene editing breeding is mainly reflected in three aspects: 1. Technical integration: Combine gene editing technology with marker-assisted breeding and hybrid breeding to realize "precise editing + rapid screening + trait polymerization" and improve breeding efficiency; 2. Diversified editing tools: In addition to CRISPR/Cas9 system, new editing tools such as CRISPR/Cas12a and base editing will be gradually promoted to improve editing accuracy and flexibility; 3. Industrial application: With the gradual clarification of regulatory policies for gene-edited crops, it is expected that gene-edited sunflower varieties will enter commercialization in 3-5 years, promoting yield increase by 10%-15% and supporting the development of oil industry.

Meanwhile, the development of sunflower gene editing technology can also provide reference for gene editing breeding of other oil crops (such as rapeseed, peanut, soybean), promote the progress of gene editing breeding technology for oil crops, and help the high-quality development of China's oil industry.

5 Conclusion

In this study, the main oil sunflower cultivar *Zaodatou* was used as the test material, focusing on the two core breeding goals of salt tolerance and high oil content, the

construction and optimization of sunflower gene editing breeding technology system were carried out. Through systematic experiments and in-depth analysis, the following core conclusions were drawn:

(1) The salt tolerance-related gene *HaERF061* and high oil content-related gene *HaFAD2* of sunflower were successfully screened, their functional correlation was verified, and two optimal targets (*HaERF061*-g2 and *HaFAD2*-g2) with strong specificity and low off-target risk were designed and screened, laying a foundation for gene editing vector construction; through target sequence optimization and codon preference optimization of Cas9 protein, editing efficiency increased from 47.1% to 76.9%, homozygous editing rate from 25% to 58.3%, off-target rate reduced to below 0.5%, significantly improving the accuracy and efficiency of gene editing.

(2) An *Agrobacterium*-mediated sunflower genetic transformation system was constructed and optimized. The optimal infection conditions (bacterial solution OD600=0.8, infection time 30 min, co-culture time 3 d) were determined, explant treatment and medium formula were optimized, transformation efficiency increased from 5.8% to 14.2%, false-positive rate decreased from 43.3% to 18.9%, explant browning rate decreased from 35.7% to 12.3%, establishing an efficient and stable genetic transformation system and solving the technical bottleneck of low genetic transformation efficiency of sunflower.

(3) The identification method of edited plants was optimized and integrated, and a three-level identification system of "PCR detection → enzyme digestion detection → sequencing verification" was established, shortening identification time by 50%, improving screening efficiency to 85%, and identification accuracy to 98%, realizing rapid and accurate screening of homozygous edited lines and solving the problems of cumbersome and inefficient preliminary identification system.

(4) Stability verification showed that the optimized gene editing breeding technology system had good repeatability, with all coefficients of variation less than 5%; trait verification showed that the salt tolerance of *HaERF061* gene homozygous edited lines was significantly improved (fresh weight increased by 31.4%), and the oil content of *HaFAD2* gene homozygous edited lines increased by 9.4% on average, verifying the practicability and effectiveness of the system, which could achieve precise improvement of sunflower target traits.

(5) The current technical bottlenecks of sunflower gene editing breeding were deeply discussed, targeted solutions were proposed, and the development trends of integration of gene editing technology with other breeding technologies, diversification of editing tools and industrial application were clarified, providing theoretical support and technical reference for gene editing breeding of sunflower and other oil crops.

In summary, this study successfully constructed and optimized a CRISPR/Cas9-based sunflower gene editing breeding technology system, which is efficient, stable and precise, and can be used for directional cultivation of excellent traits such as stress tolerance and high oil content in sunflower, shortening breeding cycle, improving breeding efficiency, and having important practical value for promoting the high-quality development of sunflower industry.

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