



Creation of Early Flowering Germplasm of Soybean by CRISPR/Cas9 Technology

Jianan Han¹, Bingfu Guo², Yong Guo¹, Bo Zhang³, Xiaobo Wang^{4*} and Li-Juan Qiu^{1*}

¹ The National Key Facility for Crop Gene Resources and Genetic Improvement (NFCRI) and MOA Key Labs of Crop Germplasm and Soybean Biology (Beijing), Institute of Crop Science, Chinese Academy of Agricultural Sciences, Beijing, China, ² Crop Research Institute, Jiangxi Academy of Agricultural Science, Jiangxi, China, ³ School of Plant and Environmental Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA, United States, ⁴ College of Agronomy, Anhui Agricultural University, Hefei, China

OPEN ACCESS

Edited by:

Joachim Hermann Schiemann,
Julius Kühn-Institut,
Germany

Reviewed by:

Vinay Kumar,
Central University of Punjab,
India
Yuhai Cui,
Agriculture and Agri-Food Canada
(AAFC), Canada

*Correspondence:

Xiaobo Wang
wxbphd@163.com
Li-Juan Qiu
qjljuan@caas.cn

Specialty section:

This article was submitted to
Plant Biotechnology,
a section of the journal
Frontiers in Plant Science

Received: 25 June 2019

Accepted: 17 October 2019

Published: 22 November 2019

Citation:

Han J, Guo B, Guo Y, Zhang B,
Wang X and Qiu L-J (2019)
Creation of Early Flowering
Germplasm of Soybean by
CRISPR/Cas9 Technology.
Front. Plant Sci. 10:1446.
doi: 10.3389/fpls.2019.01446

Soybean is an important economic crop and a typical short-day crop, sensitive to photoperiod, and has narrow geographical adaptative region, which limit the creation of transgenic materials and reduce the breeding efficiency of new varieties. In addition, the genetic transformation efficiency of soybean is lower than that of many other crops, and the available receptor genotypes are limited. In this study, *Agrobacterium*-mediated transformation were used to introduce the CRISPR/Cas9 expression vector into soybean cultivar Jack and generated targeted mutants of *E1* gene controlling soybean flowering. We obtained two novel types of mutations, 11 bp and 40 bp deletion at *E1* coding region, respectively, and frameshift mutations produced premature translation termination codons and truncated E1 proteins, causing obvious early flowering under long day condition. In addition, no off-target effects were observed by predicting and analyzing the potential off-target sites of *E1* targets. Significant decreased *E1* gene expression of two novel mutants showed that the truncated E1 protein disinhibited *GmFT2a/5a* and increasing *GmFT2a/5a* gene expressions resulted obvious early flowering. Homozygous trans-clean mutants without T-DNA elements were also obtained and showed early flowering under long day condition. The photo-insensitive soybean transformation receptor we created laid a foundation for breeding excellent transgenic receptors suitable for high latitudes.

Keywords: soybean, CRISPR/Cas9, gene editing, *E1*, *Agrobacterium*-mediated transformation

INTRODUCTION

Soybean is rich of protein and oil and has high economic value. With the increasing demand for soybean globally, it is urgent to clarify gene function, and accelerate functional gene research and breeding speed for increasing yield and improving quality. In recent years, CRISPR (Clustered regular interspaced short palindromic repeat)/Cas9 (CRISPR-associated) provides an effective method for targeted genome editing and gene function research, and supplies a new idea for reverse genetics research. CRISPR/Cas9 system includes gene knock out, knock in, multiple genes and sites editing, large fragment deletion and replacement (Gratz et al., 2013; Shan et al., 2013; Feng et al., 2014; Gratz et al., 2014; Zhou et al., 2014). Feng et al. (2013) and Mao et al. (2013) firstly proved that CRISPR/Cas9 system could be used to target genome editing in crop by introducing site-directed mutations in specific genes in *Arabidopsis* and rice. Subsequently, CRISPR/Cas9 technology has

been widely used in several species including rice (Shan et al., 2013), wheat (Upadhyay et al., 2013), cotton (Gao et al., 2017), maize (Chen et al., 2018), *Arabidopsis* (Li et al., 2013), tobacco (Gao et al., 2015) and barley (Kapusi et al., 2017). Jacobs et al. (2015) first used CRISPR/Cas9 technology to knock out the green fluorescent protein gene (GFP), and produced targeted editing for nine soybean endogenous genes. Then CRISPR/Cas9 technology began to be widely applied in soybean (Cai et al., 2015; Jacobs et al., 2015; Li et al., 2015; Michno et al., 2015; Sun et al., 2015; Du et al., 2016; Tang et al., 2016; Cai et al., 2018; Li et al., 2019). Therefore, CRISPR/Cas9 is an effective tool for soybean targeted genome editing, providing a theoretical and technical basis for further research on soybean genome, as well as improving the breeding efficiency and accelerating the breeding process.

CRISPR/Cas9 system relies on transformation technology. However, soybean transformation efficiency is lower than other crops (Du et al., 2016), and receptor genotype dependence is a major limiting factor. The transformation efficiency of soybean is usually low, and the varieties suitable for soybean genetic transformation are very few (Donaldson and Simmonds, 2000; Guo et al., 2015). At the same time, soybean is a typical short-day crop and sensitive to photoperiod, which limits the geographical cultivated region (Xu et al., 2013; Wang et al., 2016), excellent varieties creation and breeding efficiency.

Soybean varieties are adapted to different latitudes and different photoperiods, so they need to have a series of photo-insensitivity. Photoperiod is the key meteorological factor that determines flower bud differentiation and adaptation to different ecological regions (Camara et al., 1997). The wide adaptability to different latitudes in soybean is controlled by some major genes and QTLs (Watanabe et al., 2012). At present, 11 genes have been identified to be related to soybean growth period (*E1-E10, J*) (Bernard, 1971; Buzzell, 1980; Buzzell and Voldeng, 1980; McBlain and Bernard, 1987; McBlain et al., 1987; Ray et al., 1995; Cober et al., 1996; Bonato and Antonio, 1999; Cober et al., 2010; Kong et al., 2014; Lu et al., 2017; Yue et al., 2017). Among these genes, *E1* has the greatest influence on soybean growth period with the most inhibitory of flowering and is considered as the most important gene controlling soybean growth period (Bernard, 1971; Cober et al., 1996; Watanabe et al., 2012; Xia et al., 2012) and is also a main selection locus in soybean breeding (Xia, 2017). *E1*, located at centromere of chromosome 6, is a unique transcription to legumes. It has bilateral nuclear location signal and DNA binding site, and is a flowering inhibitor related to the B3 domain (Xia et al., 2012). Seven alleles have been identified for *E1*. Its amino acids changes in the nuclear location signal region resulted in its protein distribution changing. *E1* protein distributes in the nucleus, while *e1* protein distributes in the nucleus and cytoplasm (Xia, 2013). *E1* gene expression is closely related to the length of day light. Under long day condition (LD), *E1* delays flowering by negative regulation of *GmFT2a/5a*, while its recessive alleles have early flowering time by disinhibiting *GmFT2a/5a* expression (Zhang et al., 2016). *E1* gene expression was significantly inhibited under short day condition which is the main factor of photoperiod sensitivity in soybean (Lü, 2015).

In this study, we used CRISPR/Cas9 system and *Agrobacterium*-mediated transformation to introduce the CRISPR/Cas9 expression vector into soybean cultivar Jack, knocked out *E1* gene and analyzed the effect of novel *E1* mutants to soybean flowering. It provides materials for breeding early flowering receptors, promotes the development of soybean genetic improvement, provides a basis for efficient soybean genetic transformation, and establishes an important guide for soybean gene function research, molecular breeding and variety layout.

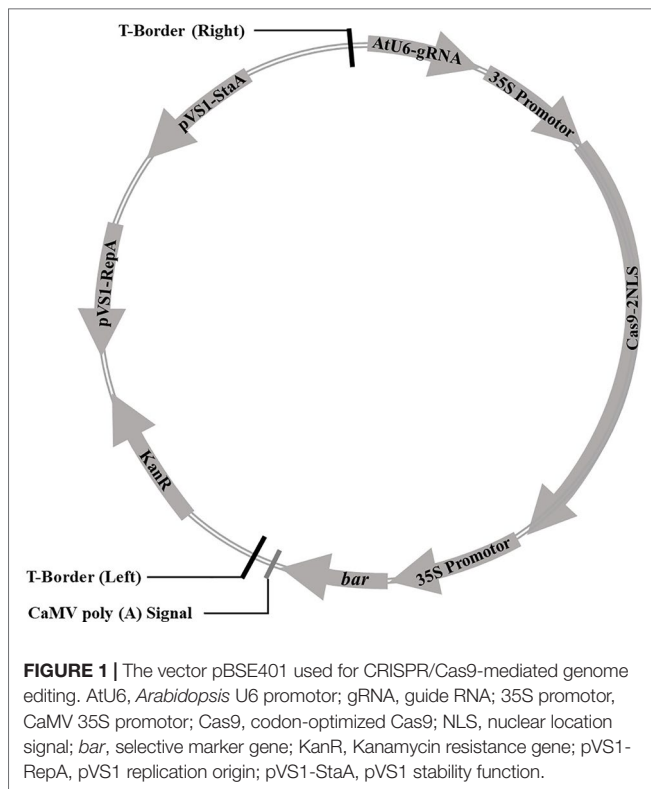
MATERIALS AND METHODS

Plant Materials and Growth Conditions

Soybean cultivar Jack was used for genetic transformation receptor. Jack (wild type as a control) and all seeds harvested from T₀ plants obtained through CRISPR/Cas9 and *Agrobacterium*-mediated transformation were planted on June 22, 2017 at the Shunyi Experimental Station of the Institute of Crop Sciences, Chinese Academy of Agricultural Sciences. T₂ homozygous mutants were planted in greenhouse under long day conditions (LD, 16 h/8 h, light/dark) and short-day conditions (SD, 12 h/12 h, light/dark) at 28, 70% relative humidity. The flowering time of each plant from seedling emergence to R1 stage (the first flower appeared on any node of the main stem) was recorded according to Fehr et al. (1971). Data analysis was performed by using Microsoft Excel, then adopted one-way analysis of variance. $P < 0.01$ was considered statistically significant.

CRISPR/Cas9 Expression Vector Construction

Cas9 sequence was optimized for the codon-optimized for dicotyledons and connected with CaMV 35S promoter at downstream, then assembled with sgRNA driven by *Arabidopsis* U6 promoter to construct a plasmid vector containing both sgRNA and Cas9. The bar gene as a selective marker was driven by CaMV 35S promoter. The CRISPR/Cas9 expression cassette was shown in **Figure 1**. The sequences were synthesized by Shanghai Sangon Biotech (Shanghai, China). The soybean endogenous gene *E1* (*Glyma.06G207800*) sequence and its information were downloaded from the Phytozome database (www.phytozome.net/). The optimal sgRNA sequences (20 bp) with the G as the first base were designed using online tool (<http://www.genome.arizona.edu/crispr/CRISPRsearch.html>). The base G was added at the 5' end artificially if the first base was not G (Ran et al., 2013). The online website (<http://www.rgenome.net/cas-offinder/>) was used to evaluate off-target effects. Two sgRNAs for the *E1* gene we selected were named as *E1*-SP1 (5'-CCCTTCAGATGAAAGGGAGCAG-3') and *E1*-SP2 (5'-CCACCATATGCGAAGCCTCTAA-3') respectively. Primers containing either of two sgRNAs were synthesized by Shanghai Sangon Biotech (**Supplementary Table 1**). Using overlapping polymerase chain reaction (PCR), two sgRNAs were cloned into pCBC plasmid. PCR products containing two sgRNAs were digested and inserted into the pHSE401 plasmid vector to construct the CRISPR/Cas9 expression vector containing Cas9



and sgRNA (**Supplementary Figure 1**). And then the CRISPR/Cas9 expression vector was transformed into *E. coli* Trans1 T1 (TransGen Biotech) used for soybean genetic transformation.

Agrobacterium-Mediated Soybean Transformation

The CRISPR/Cas9 expression vector was transferred into *Agrobacterium* strain EHA105 by electroporation. Soybean cultivar Jack was used for tissue culture and soybean transformation. Soybean genetic transformation procedure was referred to the described method in our laboratory (Guo et al., 2015) and made appropriate modification. The healthy soybean seeds were sterilized with chlorine for 16 h and germinated 5 d to prepare explants. Explants were submerged in agrobacterium suspension adding 0.02% surfactant (Silwet L-77) and wounded by ultrasonic treatment. After infection for 30–40 min, explants were co-cultured for 3 d. After the tissue culture (resistant bud induction, shoot elongation and rooting), transgenic plants were regenerated from explants. At the stage of resistant bud induction and shoot elongation, we added 10 mg L⁻¹ and 6 mg L⁻¹ glufosinate (Sigma-Aldrich, USA) respectively into culture medium to screen positive transformed cells.

Sequence Analysis of the Transgenic Plants

Total genomic DNA was extracted from every leaf sample following the modified cetyltrimethylammonium ammonium bromide (CTAB) protocol in the T₀, T₁ and T₂ generation (Saghai-Marooof

et al., 1984). To determine the types of mutation at target sites, we used specific primers (**Supplementary Table 2**) containing target sites in *E1* gene and genomic DNA as the template to amplify and analyze the target sites sequence. PCR products were detected by 1% agarose gel electrophoresis and then sequenced. Three types of mutations were identified by sequence peaks. Heterozygous mutations showed chaotic peaks after the target site, while wild types and homozygous mutations showed single peaks at the target. The sequences of homozygous mutations were aligned with wild types to further determine the variation of target. To screen and obtain *E1* targeted mutants without transgenic elements, PAT/Bar test strip was used to identify the selective marker *bar* gene. Two pairs of primers (**Supplementary Table 2**) were used to determine sgRNA/Cas9 on T-DNA elements by PCR.

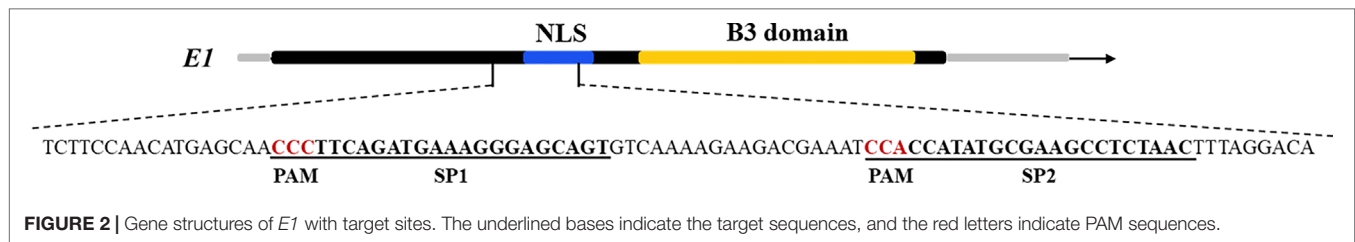
Real-Time Quantitative PCR Analysis of Gene Expression

Expression levels of *E1* and *GmFT2a/5a* in wild type plants and T₂ homozygous mutants were analyzed under LD and SD conditions, respectively. Every 5-day interval after 10 days after emergence (DAE), at 10 am (4 h after light), the trifoliolate leaves were sampled from plants with different genotypes under LD and SD conditions. Total RNAs were extracted using TransZol Up Plus RNA Kit (TransGen Biotech). For reverse transcription, the first-strand cDNA synthesis was performed using the TransScript First-strand cDNA Synthesis SuperMix Kit (TransGen, China). For qRT-PCR, gene expressions were examined using cDNA templates on an Applied Biosystems 7300 Real-Time PCR System. The relative gene expression levels followed the method (Pfaffl, 2001). The mRNA level of *GmActin* (*Glyma18g52780*) was used as a reference for normalization. Specific primers we used in this study were list in **Supplementary Table 2**. Three biological replicates were used for each gene.

RESULTS

CRISPR/Cas9-Mediated Mutations

Two targets for the *E1* gene (named *E1*-SP1 and *E1*-SP2, respectively) were designed (**Figure 2**), and the CRISPR/Cas9 expression vector were transferred into the soybean cultivar Jack by *Agrobacterium*-mediated genetic transformation to knock out soybean endogenous gene *E1*. The whole genome DNAs of transformed plants were used for PCR and sequence analysis. Combining with PAT/Bar test strip detection, 16 T-DNA positive plants were obtained (**Figure 3**), of which 12 plants had heterozygous mutations at target sites (**Supplementary Figure 2**). Then, all seeds derived from 9 heterozygous T₀ generation were planted in the Shunyi Station of the Chinese Academy of Agricultural Sciences under LD conditions (3 of 12 T₀ plants had no seed) and the types of mutation at target sites were determined. Six out of nine lines had three types of mutations: homozygous mutation, heterozygous mutation and non-mutation (**Table 1**). The rest three T₁ lines didn't show mutation, and site-directed mutagenesis of T₀ generation didn't inherit to its progeny.



Two types of homozygous mutations were detected at the target sites (named *e1-1* and *e1-2*, 11 bp deletion and 40 bp deletion, respectively) in three lines including L7 (*e1-1*), L9 (*e1-2*) and L16 (*e1-1*). All of them were frameshift mutations, resulting in premature translation termination codons (**Supplementary Figure 3**). The 11 bp deletion of *e1-1* created a truncated protein encoding 79 amino acids, and caused the absence of all the B3 domains while keeping part of nuclear location signal. The 40 bp deletion of *e1-2* created a truncated protein encoding 88 amino acids, and caused the absence of neither B3 domain or nuclear location signal (**Supplementary Figure 4**).

Potential Off-Target Analysis

In order to determine whether the CRISPR/Cas9 expression vector we used to have variations at potential off-targets and avoid the possibility of potential off-targets effecting on phenotype, by using online website (<http://cbi.hzau.edu.cn/crispr/>), four most potential off-target sites at the two targets (*E1*-SP1 and *E1*-SP2) were selected. Every potential off-target site mismatched 2-4 bases with the *E1* target sequences (**Table 2**). Using specific

primers of potential off-target sites (**Supplementary Table 2**) and genome DNAs of 35 T_1 mutants as templates for PCR, we didn't detect variation at four potential off-targets. The sequence comparison analysis shown in **Supplementary Figure 5** indicated that CRISPR/Cas9 expression vector had specific edits in *E1*-SP1 and *E1*-SP2 targets.

Inheritance Analysis and Phenotype Identification

To identify whether the mutations at the target of the homozygous T_1 mutants could inherited to T_2 generation stably, T_2 seeds derived from homozygous mutants of T_1 lines (L7, L9 and L16) were planted under LD and SD conditions, respectively (**Table 3**). Sequence analyses of 28 T_2 individuals indicated that CRISPR/Cas9-mediated mutagenesis of *E1* gene could be stably inherited from T_1 generation to T_2 generation and maintained the same type of variation. To analyze the flowering time accurately, R1 period of all above T_2 plants were recorded under LD and SD conditions respectively. Under LD condition, when T_2 homozygous mutants were flowering, the wild type plants had

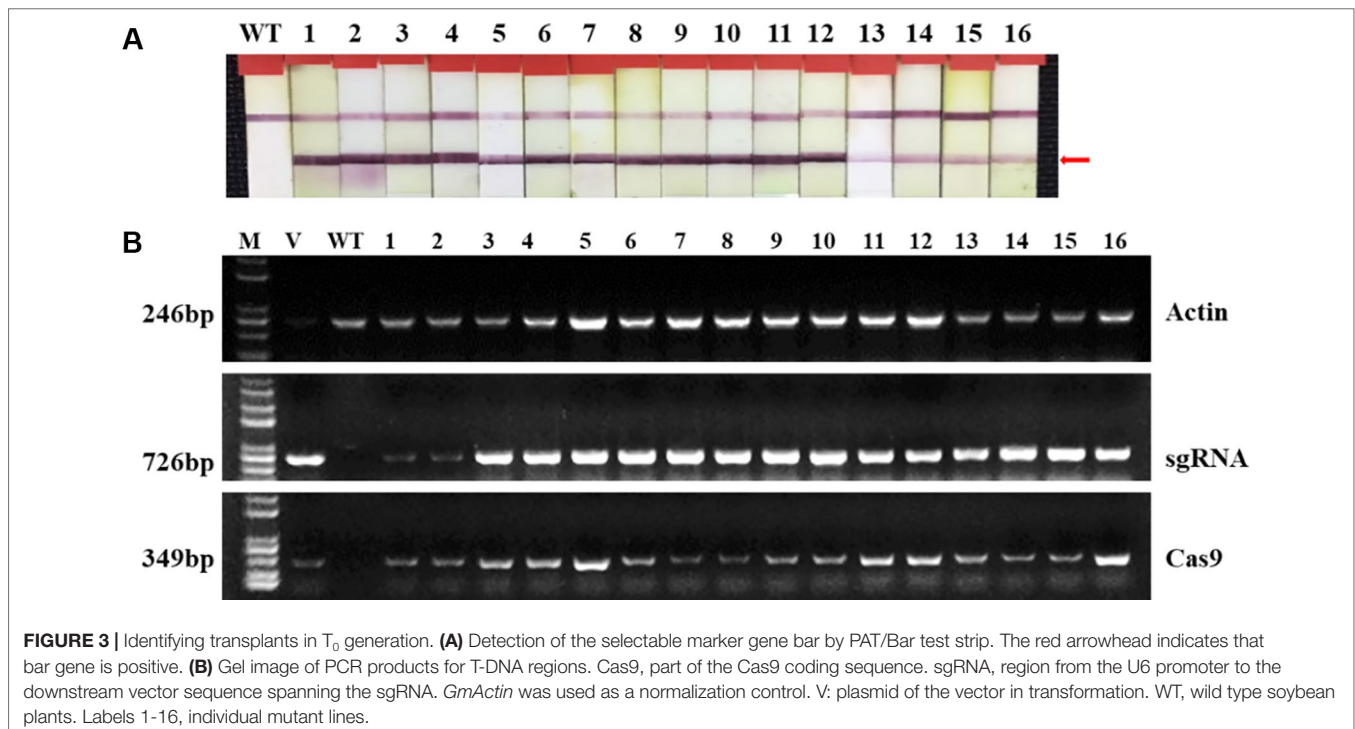


TABLE 1 | CRISPR/Cas9-mediated targeted mutants of *E1* in the T₁ generation.

T ₁ generation	No. of plants sequenced	No. of homozygous plants	No. of heterozygous plants	No. of no mutant plants
L1	4	0	3	1
L7	2	2	0	0
L9	4	2	2	0
L10	6	0	4	2
L11	10	0	8	2
L16	9	6	3	0
Total	35	10	20	5

not shown any flower buds. When wild type plants were flowering, T₂ homozygous mutants had obvious pods (**Figure 4A**). The recorded flowering dates showed that flowering time of mutants was significantly earlier than that of wild type plants ($P < 0.01$). The average flowering time of wild type plants were 57 d, while the average flowering time of T₂ homozygous mutants derived from three different lines (L7, L9 and L16) were 39 d, 38 d and 37 d, respectively (**Figure 4B**). However, under SD condition, when the T₂ homozygous mutants were flowering, the wild type plants were flowering (**Figure 4C**) and we didn't observe significant difference between wild type plants and mutants. The average flowering time of wild type plants was 25 d, while the average flowering time of T₂ homozygous mutants derived from L7, L9 and L16 were 24 d, 23 d, and 23 d, respectively (**Figure 4D**). Under natural condition, there were no significant differences in plant height, node number and branch number between mutants and wild type plants (**Supplementary Figure 6**).

Gene Expression Analysis of *E1/GmFT2a/GmFT5a*

Studies had shown that negative regulation model between *E1* and *GmFT2a/5a* was closely related to flowering time under LD and SD conditions (Kong et al., 2014; Xia et al., 2012). In order to clarify the correlation between the expression of *E1* and *GmFT2a/5a* with flowering time of mutants, RNA was extracted from trifoliolate leaves in two types of mutations of L7, L9 and L16 lines under LD and SD conditions at 10 am every fifth day after 10 DAE for five times (**Figure 5**). Wild type plants and mutants showed the similar expression patterns for each of three genes (*E1/GmFT2a/5a*) under LD or SD condition, but different expression levels. Under LD condition, *E1* had the highest expression level at 15 DAE both in mutants and wild type plants, but significantly lower *E1* expression level in mutants was observed at 15 and 20 DAE ($P < 0.01$). *GmFT2a/5a* exhibited two expression peaks at

15 DAE and 25 DAE both in mutants and wild type plants, but there were significantly higher expression levels in mutants at 15 and 25 DAE ($P < 0.01$). Under SD condition, all the three genes (*E1/GmFT2a/5a*) showed bimodal expression patterns and the expression peaks appeared at 15 DAE and 25 DAE, respectively. However, *E1* gene expression was significantly lower and almost no expression than that under LD condition.

Trans-Clean Mutants Without T-DNA Elements

To obtain novel soybean germplasm with homozygous mutation but without T-DNA elements, PAT/Bar test strip was used to identify the selective marker gene *bar* firstly and PCR strategy was used to exam sgRNA/Cas9 on T-DNA by using specific primers (**Supplementary Table 2**). Among three T₁ lines, only L7 didn't show T-DNA elements, and its 50 progenies were all free of T-DNA. Only 11 out of 211 homozygous mutants were free of T-DNA in T₂ generation derived from L9 and L16 (**Table 4, Figure 6**).

DISCUSSION

Soybean is an important source of protein and oil for human. Therefore, gene function exploration and utilization are of great significance to increase yield and improve seed quality. However, soybean is a short-day crop, sensitive to photoperiod and had narrow geographical cultivated regions. In *Agrobacterium*-mediated genetic transformation, soybean transformation efficiency is lower than many other crops, and depends on genotypes used for transformation. At present, the soybean receptors with higher transformation efficiency are limited. Therefore, its sensitivity to photoperiod and difficulty in developing transgenic offspring have significantly restricted the improvement of breeding efficiency of new varieties and germplasm. Therefore, it is important to study soybean growth-related genes, breed and create new soybean germplasm with wide adaptability to different latitudes. Among soybean growth-related genes, *E1* has the greatest impact on growth period and has the most obvious photoperiod response, so it is considered as a major gene for controlling soybean flowering (Bernard, 1971; Cober et al., 1996; Watanabe et al., 2012; Xia et al., 2012).

In this study, we screened and obtained early flowering homozygous mutants without transgenic elements of soybean endogenous *E1* gene by CRISPR/Cas9 system. The random integration of foreign genes in plant chromosome may lead

TABLE 2 | Potential off-target analysis at the two target sites of *E1* in the T₁ generation.

Target	Potential target	Physical position	Target sequence	No. of mismatch	Position
<i>E1</i> -SP2	OFF-1	<i>Glyma04g24640</i> 4: -28294190	CCACCATATGtGAAGCCTC c AAc	2	exon
	OFF-2	<i>Glyma10g33050</i> 10: -41421475	CCTCCATATG a GAAAGCC a CcAcC	4	exon
<i>E1</i> -SP1	OFF-3	<i>Glyma17g06520</i> 17: -4658911	CCATT a AaATGAAAGG a AGCAG c	4	intron
	OFF-4	<i>Glyma19g02555</i> 19: -2360418	CTTTTCAGATGAA gt GGAGCAG c	3	exon

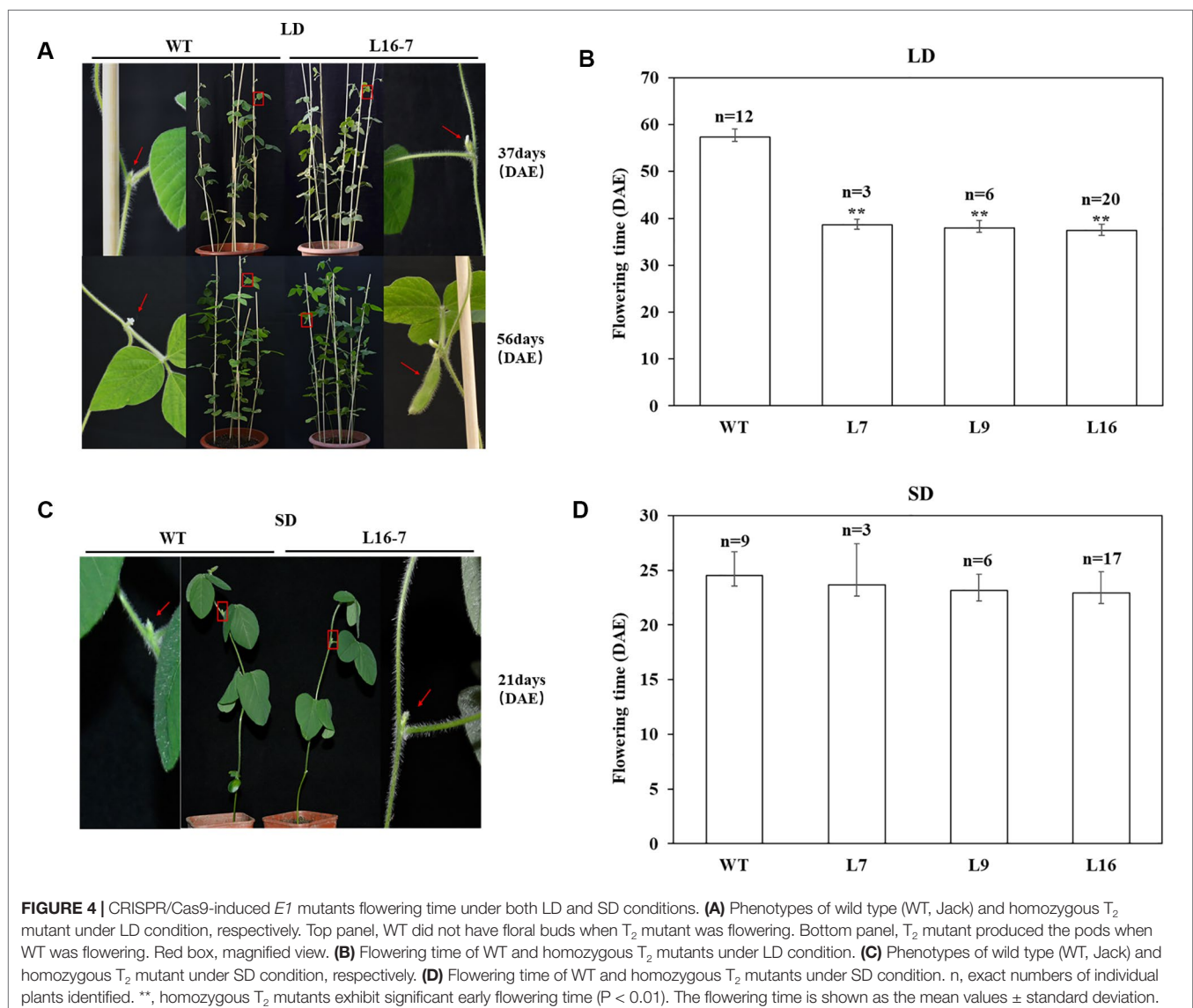
Mismatched bases are shown in lowercase red letters.

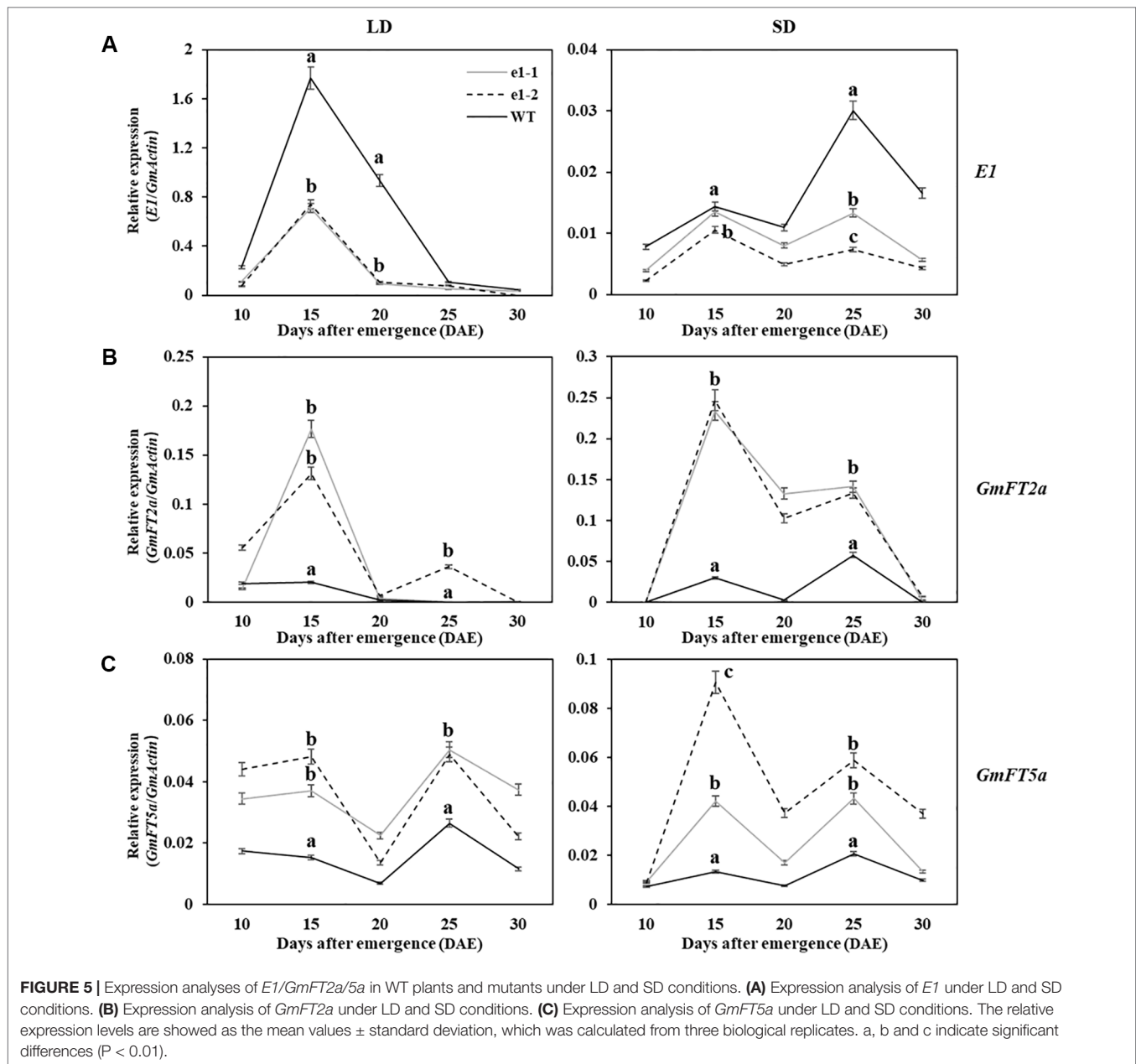
TABLE 3 | T_2 homozygous mutants under LD and SD conditions.

T_1 generation	Mutation type	T_2 generation	
		No. of plants in LD	No. of plants in SD
L7	40 bp delete	3	3
L9	11 bp delete	6	6
L16	40 bp delete	19	17

to destruction of plant endogenous gene, gene silencing and other undesirable phenomena, which caused a great controversy about the biosecurity of genetically modified organisms (Napoli et al., 1990). Therefore, the promotion and application of transgenic crops are greatly restricted (Zhang et al., 2011). CRISPR/Cas9 system affords an effective method to eliminate undesirable phenomena in the transformation by editing the target gene accurately. At the same time, the transgenic elements in CRISPR/Cas9 system such as Cas9 and

other selective markers can be lost by progeny separation or selfing and obtain trans-clean mutants (Cai et al., 2018; Chen et al., 2018). Besides, off-target effects could be reduced by selecting target sequences specifically (Xie et al., 2014; Xu et al., 2015). As an effective genome editing tool, CRISPR/Cas9 technology has been widely used in many crops and there are also many applications in soybean. Jacobs et al. (2015) reported the site-directed mutagenesis in soybean by using CRISPR/Cas9 technology firstly, which laid a good foundation for soybean genome editing. Many studies have also successfully obtained mutants without transgenic elements in many crops by CRISPR/Cas9. Haun et al. (2014) generated a high oleic acid content soybean variety without transgenic components and improved the quality of soybean. Cai et al. (2018) obtained trans-clean soybean homozygous mutants with late flowering time by using CRISPR/Cas9 technology to knock out *GmFT2a*.





E1 is a unique transcription factor to legumes and its B3 domain and nuclear location signal are important modules controlling soybean flowering (Xia et al., 2012). *E1* gene includes at least 7 allelic natural variations with *E1* and *e1-as* as the two basic genotypes. *e1-as* has a single missense mutation at the region of nuclear location signal. This one amino acid change led to the cell localization change and *e1* protein distribution in the nucleus and cytoplasm at the same time. However, *e1-as* is a leaky allele and has partially function of delaying flowering (Xia, 2013). The other three nonfunctional alleles are *e1-fs*, *e1-nl* and *e1-b3a*. *e1-fs* has 1 bp deletion in the B3 domain, and this frameshift mutation resulted in a truncated protein encoding 41 amino acids. *e1-nl* is a null allele and all the *E1* gene is deleted.

e1-b3a allele has 3 SNPs and 2 bp deletions in the B3 domain resulting in frameshift mutation (Zhai et al., 2015). *e1-re* and *e1-p* have variations at 5'UTR, but the flowering mechanism of these two alleles is unclear (Tsubokura et al., 2014). The two novel types of homozygous germplasm we obtained had 11 bp deletion and 40 bp deletion in the coding region, respectively, resulting in pre-terminate codons and truncated *E1* proteins. *e1-1* coded a 79-aa and deleted all B3 domain. *e1-2* coded an 88-aa and completely deleted the nuclear location signals and B3 domain.

In addition, we compared the flowering time (R1) of *E1* mutants and wild-type. Under LD condition, the two types of homozygous mutants showed about 20 days earlier flowering time than wild type plants. The flowering time of the wild type plants

TABLE 4 | Transgene-clean homozygous mutants from T₁ and T₂ generations.

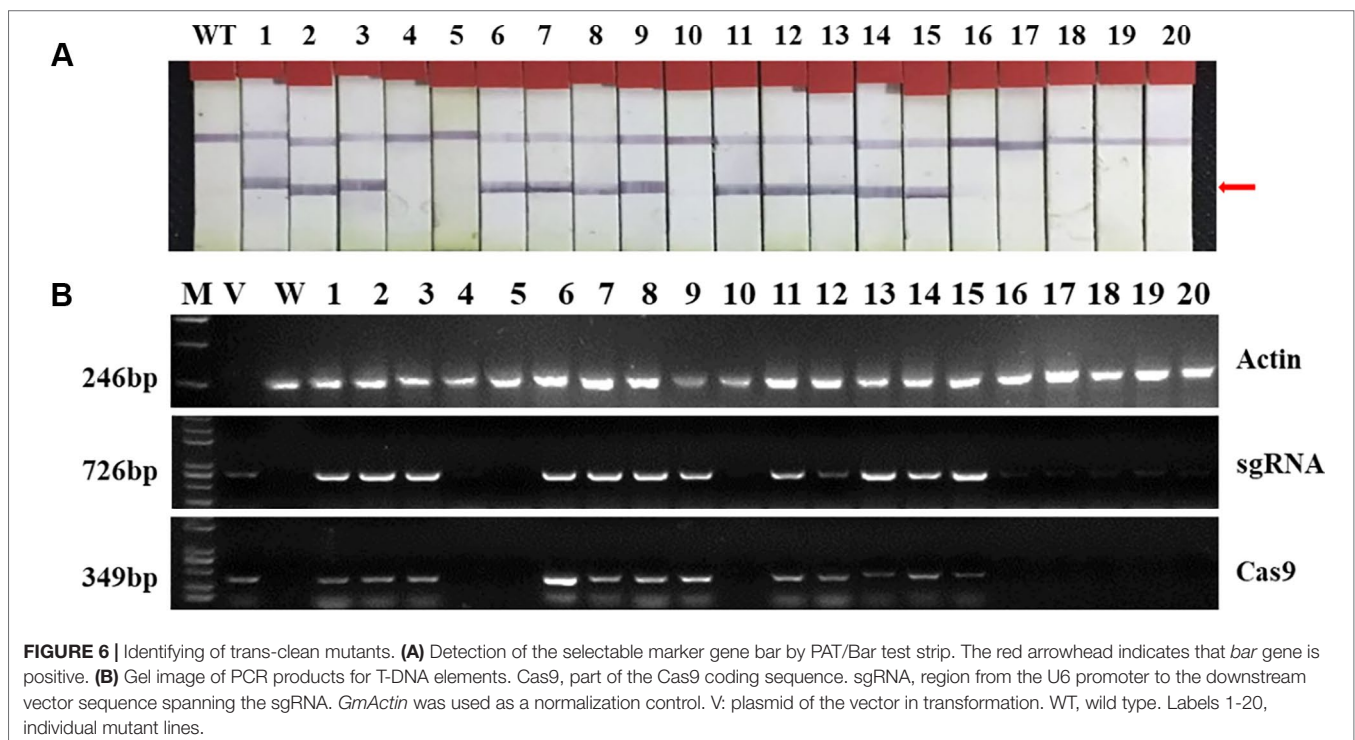
T ₁ generation	T-DNA	T ₂ generation	
		No. of plants identified	No. of trans-clean plants
L7	free	50	50
L9	positive	5	2
L16	positive	206	9
Total	–	261	61

was about 57 days and the average flowering time of mutants was only about 38 days. Studies have shown that the flowering time of cultivars carrying *E1*, *e1-as*, *e1-fs* and *e1-nl* were 70 d, 50 d, 30 d and 30 d under LD condition, respectively (Xia et al., 2012). In our study, the average flowering time of novel germplasm was about 38 days which was like the natural alleles *e1-fs* and *e1-nl*, indicating that the new *E1* mutants we obtained by CRISPR/Cas9 system may have the same mechanism for flowering as natural alleles. Studies have shown that nonsense mediated mRNA decay (NMD) reduces mRNAs with premature translation termination codons (PTCs) by down-regulating gene expression, and reduces its encoding truncated protein production (Baker and Parker, 2004; Maquat, 2004; Conti and Izaurralde, 2005; Maquat, 2005). The two mutants obtained in our study had bases deletion, leading to PTCs and truncated *E1* protein. We speculated that NMD resulted in decreased *E1* gene expression in mutants compared with wild type plants.

Soybean is a typical short-day crop and its flowering time is closely related to the length of day light. The expression of *E1* was

negatively correlated with *GmFT* (*GmFT2a/5a*) and controlled the *GmFT2a/5a* that functionally coordinated each other (Xia et al., 2012). In this study, compared with wild type plants, *E1* gene sequence of mutants caused not only truncated *E1* protein but also significantly decreased gene expressions. Meanwhile, *GmFT2a/5a* expressions of mutants were significantly increased due to decreased *E1* gene and appeared earlier flowering time. In order to identify whether the decreased *E1* gene expression had influence on its homologous gene *E1-L* (*Glyma04g24640.1/Glyma18g22670*) and avoid its potential effect on the flowering time, we analyzed the *E1-L* gene expression. Result showed that there was no obvious difference on *E1-L* gene expression between mutants and wild type plants (Supplementary Figure 7).

E1 is the most important gene controlling flowering and is also the major determinant to short-day crops. Photosensitivity in soybean reduced the flowering time, leading to early maturation and low yield in low latitude areas. Under SD condition, long juvenile trait can ensure enough growth period in soybean (Hartwig and Kiihl, 1979). *J* (*Glyma.04G050200*) is a transcriptional suppressor of *E1* gene and its recessive allele, *j*, delaying flowering time by disinhibit *E1*. The amplitude to delay flowering time is determined by different *E1* alleles (Lu et al., 2017). Furthermore, studies have shown that long-juvenile trait in soybean may be controlled by other genes (Ray et al., 1995, Valéria et al., 2000, Yue et al., 2017). Therefore, mutants we obtained not only provide soybean new receptor for high latitudes, and can also change the function of *J* at low latitudes, which may provide soybean high yield potential. In addition, it provides materials and theoretical basis for identifying other genes controlling long-juvenile trait and studying further the flowering regulation pathways. Compared with cross breeding,



using CRISPR/Cas9 technology to create early flowering soybean material has the advantages significantly short breeding period with high efficiency.

CONCLUSION

Our study provides materials support for breeding early-maturing transgenic receptors suitable for high latitudes and contributes to the soybean introduction. The photo-insensitive soybean transformed receptors could improve the soybean genetic development and contribute to efficient soybean genetic transformation. It offers important guidance for molecular breeding, soybean gene function research and variety development.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

REFERENCES

- Baker, K. E., and Parker, R. (2004). Nonsense-mediated mRNA decay: terminating erroneous gene expression. *Curr. Opin. Cell Biol.* 16 (3), 293–299. doi: 10.1016/j.ceb.2004.03.003
- Bernard, R. L. (1971). Two major genes for time of flowering and maturity in soybeans 1. *Crop Sci.* 11, 242–244. doi: 10.2135/cropsci1971.0011183X00110020022x
- Bonato, E. R., and Antonio, V. N. (1999). *E6*, a dominant gene conditioning early flowering and maturity in soybeans. *Genet. Mol. Biol.* 22, 229–232. doi: 10.1590/S1415-47571999000200016
- Buzzell, M. D. (1980). Adapting chronological stabilization to property risks. *Risk Manage.* 27 (2), 32–34.
- Buzzell, R. L., and Voldeng, H. D. (1980). Inheritance of insensitivity to long day length. *Soybean Genet. Newsletter* 7, 26–29.
- Cai, Y., Chen, L., Liu, X. J., Guo, C., Sun, S., Wu, C. X., et al. (2018). CRISPR/Cas9-mediated targeted mutagenesis of *GmFT2a* delays flowering time in soya bean. *Plant Biotechnol. J.* 16 (1), 176–185. doi: 10.1111/pbi.12758
- Cai, Y., Chen, L., Liu, X., Sun, S., Wu, C., Jiang, B., et al. (2015). CRISPR/Cas9-mediated genome editing in soybean hairy roots. *PLoS One* 10 (8), e0136064. doi: 10.1371/journal.pone.0136064
- Camara, G. M. S., Sedyama, T., Douradoneto, D., and Bernardes, M. S. (1997). Influence of photoperiod and air temperature on the growth, flowering and maturation of soybean (*Glycine max* (L.) Merrill). *Scientia Agricola* 54, 149–154. doi: 10.1590/S0103-90161997000300017
- Chen, R. R., Xu, Q. L., Liu, Y., Zhang, J. J., Ren, D. T., Wang, G. Y., et al. (2018). Generation of transgene-free maize male sterile lines using the CRISPR/Cas9 system. *Front. Plant Sci.* 9, 1180. doi: 10.3389/fpls.2018.01180
- Cober, E. R., Molnar, S. J., Charette, M., and Voldeng, H. D. (2010). A new locus for early maturity in soybean. *Crop Sci.* 50 (2), 524–527. doi: 10.2135/cropsci2009.04.0174
- Cober, E. R., Tanner, J. W., and Voldeng, H. D. (1996). Genetic control of photoperiod response in early-maturing, near-isogenic soybean lines. *Crop Sci.* 36 (6), 601–605. doi: 10.2135/cropsci1996.0011183X003600030013x
- Conti, E., and Izaurralde, E. (2005). Nonsense-mediated mRNA decay: molecular insights and mechanistic variations across species. *Curr. Opin. Cell Biol.* 17 (3), 316–325. doi: 10.1016/j.ceb.2005.04.005
- Donaldson, P. A., and Simmonds, D. H. (2000). Susceptibility to *Agrobacterium tumefaciens* and cotyledonary node transformation in short-season soybean. *Plant Cell Rep.* 19 (5), 478–484. doi: 10.1007/s002990050759
- Du, H., Zeng, X., Zhao, M., Cui, X., Wang, Q., Yang, H., et al. (2016). Efficient targeted mutagenesis in soybean by TALENs and CRISPR/Cas9. *J. Biotechnol.* 217, 90–97. doi: 10.1016/j.jbiotec.2015.11.005

AUTHOR CONTRIBUTIONS

JH performed the experiments, analyzed the data, and wrote the manuscript. BG, YG, and XW provided advice on experimental implementation. XW, BZ, and L-JQ supervised the project, reviewed and revised the manuscript.

ACKNOWLEDGMENTS

This work was supported by the National Science and Technological innovation Program of China.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2019.01446/full#supplementary-material>

- Fehr, W. R., Caviness, C. E., Burmood, D. T., and Pennington, J. S. (1971). Stage of development descriptions for soybeans, *Glycine max* (L.) Merrill. *Crop Sci.* 11 (6), 929–931. doi: 10.2135/cropsci1971.0011183X001100060051x
- Feng, Z. Y., Mao, Y. F., Xu, N. F., Zhang, B. T., Wei, P. L., Yang, D., et al. (2014). Multigeneration analysis reveals the inheritance, specificity, and patterns of CRISPR/Cas-induced gene modifications in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 111 (12), 4632. doi: 10.1073/pnas.1400822111
- Feng, Z. Y., Zhang, B. T., Ding, W. N., Liu, X. D., Yang, D. L., Wei, P. L., et al. (2013). Efficient genome editing in plants using a CRISPR/Cas system. *Cell Res.* 23 (10), 1229–1232. doi: 10.1038/cr.2013.114
- Gao, J. P., Wang, G. H., Ma, S. Y., Xie, X. D., Wu, X. W., Zhang, X. T., et al. (2015). CRISPR/Cas9-mediated targeted mutagenesis in *Nicotiana tabacum*. *Plant Mol. Biol.* 87 (2), 99–110. doi: 10.1007/s11103-014-0263-0
- Gao, W., Lu, L., Tian, X. Q., Xu, F. C., Liu, J., Singh, P. K., et al. (2017). Genome editing in cotton with the CRISPR/Cas9 system. *Front. Plant Sci.* 8, 1364. doi: 10.3389/fpls.2017.01364
- Gratz, S. J., Cummings, A. M., Nguyen, J. N., Hamm, D. C., Donohue, L. K., Harrison, M. M., et al. (2013). Genome engineering of *Drosophila* with the CRISPR RNA-guided Cas9 nuclease. *Genetics* 194 (4), 1029. doi: 10.1534/genetics.113.152710
- Gratz, S. J., Ukken, F. P., C Dustin, R., Gene, T., Donohue, L. K., Cummings, A. M., et al. (2014). Highly specific and efficient CRISPR/Cas9-catalyzed homology-directed repair in *Drosophila*. *Genetics* 196 (4), 961–971. doi: 10.1534/genetics.113.160713
- Guo, B. F., Guo, Y., Hong, H. L., Jin, L. G., Zhang, L. J., Chang, R. Z., et al. (2015). Co-treatment with surfactant and sonication significantly improves *Agrobacterium*-mediated resistant bud formation and transient expression efficiency in soybean. *J. Integr. Agric.* 14 (7), 1242–1250. doi: 10.1016/S2095-3119(14)60907-2
- Hartwig, E. E., and Kihl, R. A. S. (1979). Identification and utilization of a delayed flowering character in soybeans for short-day conditions. *Field Crops Res.* 2 (2), 145–151. doi: 10.1016/0378-4290(79)90017-0
- Haun, W., Coffman, A., and Clasen, B. M. (2014). Improved soybean oil quality by targeted mutagenesis of the fatty acid desaturase 2 gene family. *Plant Biotechnol. J.* 12 (7), 934–940. doi: 10.1111/pbi.12201
- Jacobs, T. B., Lafayette, P. R., Schmitz, R. J., and Parrott, W. A. (2015). Targeted genome modifications in soybean with CRISPR/Cas9. *BMC Biotechnol.* 15 (1), 1–10. doi: 10.1186/s12896-015-0131-2
- Kapus, E., Corcuera-Gómez, M., Melnik, S., and Stoger, E. (2017). Heritable genomic fragment deletions and small indels in the putative ENGase gene induced by CRISPR/Cas9 in Barley. *Front. Plant Sci.* 8, 540–. doi: 10.3389/fpls.2017.00540
- Kong, F. J., Nan, H. Y., Cao, D., Li, Y., Wu, F. F., Wang, J. L., et al. (2014). A new dominant gene conditions early flowering and maturity in soybean. *Crop Sci.* 54, 2529–2535. doi: 10.2135/cropsci2014.03.0228

- Li, C. L., Nguyen, V., Liu, J., Fu, W. J., Chen, C., Yu, K. F., et al. (2019). Mutagenesis of seed storage protein genes in soybean using CRISPR/Cas9. *BMC Res. Notes* 12 (1), 176. doi: 10.1186/s13104-019-4207-2
- Li, J. F., Norville, J. E., Aach, J., McCormack, M., Zhang, D., Bush, J., et al. (2013). Multiplex and homologous recombination-mediated genome editing in *Arabidopsis* and *Nicotiana benthamiana* using guide RNA and Cas9. *Nat. Biotechnol.* 31 (8), 688–691. doi: 10.1038/nbt.2654
- Li, Z., Liu, Z. B., Xing, A., Moon, B. P., Koellhoffer, J. P., Huang, L., et al. (2015). Cas9-guide RNA directed genome editing in soybean. *Plant Physiol.* 169 (2), 960–970. doi: 10.1104/pp.15.00783
- Lü, S. X. (2015). Studies on molecular mechanism regulating the expression of *E1* in Soybean [*Glycine Max* (L.) Merr.]. [dissertation]. northeast institute of geography and agroecology. *Chin. Acad. Sci.*
- Lu, S., Zhao, X., Hu, Y., Liu, S., Nan, H., Li, X., et al. (2017). Natural variation at the soybean *J* locus improves adaptation to the tropics and enhances yield. *Nat. Genet.* 49 (5), 773. doi: 10.1038/ng.3819
- Mao, Y. F., Hui, Z., Xu, N. F., Zhang, B. T., Feng, G., and Zhu, J. K. (2013). Application of the CRISPR-Cas system for efficient genome engineering in plants. *Mol. Plant* 6 (6), 2008–2011. doi: 10.1093/mp/sst121
- Maquat, L. E. (2004). Nonsense-mediated mRNA decay: a comparative analysis of different species. *Curr. Genomics* 5 (3), 75–190. doi: 10.2174/1389202043349453
- Maquat, L. E. (2005). Nonsense-mediated mRNA decay in mammals. *J. Cell Sci.* 118 (9), 1773–1776. doi: 10.1242/jcs.01701
- Mcblain, B. A., and Bernard, R. L. (1987). A new gene affecting the time of flowering and maturity in soybeans. *J. Heredity* 78 (3), 160–162. doi: 10.1093/oxfordjournals.jhered.a110349
- Mcblain, B. A., Bernard, R. L., Cremeens, C. R., and Korczak, J. F. (1987). A procedure to identify genes affecting maturity using soybean isoline testers. *Crop Sci.* 27 (6), 1127–1132. doi: 10.2135/cropsci1987.0011183X002700060008x
- Michno, J. M., Wang, X. B., Liu, J. Q., Curtin, S. J., Kono, T. J., and Stupar, R. M. (2015). CRISPR/Cas mutagenesis of soybean and *Medicago truncatula* using a new web-tool and a modified Cas9 enzyme. *GM Crops Food* 6 (4), 243–252. doi: 10.1080/21645698.2015.1106063
- Napoli, C., Lemieux, C., and Jorgensen, R. (1990). Introduction of a chimeric chalcone synthase gene into *Petunia* results in reversible co-suppression of homologous genes in trans. *Plant Cell* 2, 279–289. doi: 10.1105/tpc.2.4.279
- Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29 (9), e45. doi: 10.1093/nar/29.9.e45
- Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., and Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* 8, 2281–2308. doi: 10.1038/nprot.2013.143
- Ray, J. D., Hinson, K., Mankono, J. E. B., and Malo, M. F. (1995). Genetic control of a long-juvenile trait in soybean. *Crop Sci.* 35 (4), 1001–1006.
- Saghai-Marooof, M. A., Soliman, K. M., Jorgensen, R. A., and Allard, R. W. (1984). Ribosomal DNA spacer-length polymorphisms in barley: mendelian inheritance, chromosomal location, and population dynamics. *Proc. Natl. Acad. Sci. U.S.A.* 81 (24), 8014–8018. doi: 10.1073/pnas.81.24.8014
- Shan, Q., Wang, Y., Li, J., Zhang, Y., Chen, K., Liang, Z., et al. (2013). Targeted genome modification of crop plants using a CRISPR-Cas system. *Nat. Biotechnol.* 31 (8), 686–688. doi: 10.1038/nbt.2650
- Sun, X. J., Hu, Z., Chen, R., Jiang, Q. Y., Song, G. H., Zhang, H., et al. (2015). Targeted mutagenesis in soybean using the CRISPR-Cas9 system. *Sci. Rep.* 5, 10342. doi: 10.1038/srep10342
- Tang, F., Yang, S., Liu, J., and Zhu, H. (2016). *Rj4*, a gene controlling nodulation specificity in soybeans, encodes a thaumatin-like protein but not the one previously reported. *Plant Physiol.* 170 (1), 26–32. doi: 10.1104/pp.15.01661
- Tsubokura, Y., Watanabe, S., Xia, Z. J., Kanamori, H., Yamagata, H., Kaga, A., et al. (2014). Natural variation in the genes responsible for maturity loci *E1*, *E2*, *E3* and *E4* in soybean. *Ann. Bot.* 113 (3), 429–441. doi: 10.1093/aob/mct269
- Upadhyay, S. K., Kumar, J., Alok, A., and Tuli, R. (2013). RNA-guided genome editing for target gene mutations in wheat. *Genes Genomes Genet.* 3 (12), 2233–2238. doi: 10.1534/g3.113.008847 G3.
- Valéria, C. P., Almeida, L. A. D., Kiihl, R. A. D. S., and Rosolem, C. A. (2000). Inheritance of long juvenile period under short day conditions for the BR80-6778 soybean (*Glycine max* (L.) Merrill) line. *Euphytica* 112 (2), 203–209. doi: 10.1023/A:1003927817278
- Wang, Y., Gu, Y., Gao, H., Qiu, L., Chang, R., Chen, S., et al. (2016). Molecular and geographic evolutionary support for the essential role of *GIGANTEA* in soybean domestication of flowering time. *BMC Evolutionary Biol.* 16 (1), 79. doi: 10.1186/s12862-016-0653-9
- Watanabe, S., Harada, K., and Abe, J. (2012). Genetic and molecular bases of photoperiod responses of flowering in soybean. *Breed. Sci.* 61 (5), 531–543. doi: 10.1270/jsbbs.61.531
- Xia, Z. J. (2013). Research progresses on photoperiodic flowering and maturity genes in soybean (*Glycine Max* Merr.). *Acta Agron. Sin.* 39 (04), 571–579. doi: 10.3724/SP.J.1006.2013.00571
- Xia, Z. J. (2017). Research progress in whole-genome analysis and cloning of genes underlying important agronomic traits in soybean. *Chin. Bull. Bot.* 52 (2), 148–158. doi: 10.11983/CBB16087
- Xia, Z., Watanabe, S., Yamada, T., Tsubokura, Y., Nakashima, H., Zhai, H., et al. (2012). Positional cloning and characterization reveal the molecular basis for soybean maturity locus *E1* that regulates photoperiodic flowering. *Proc. Natl. Acad. Sci. U.S.A.* 109 (32), E2155. doi: 10.1073/pnas.1117982109
- Xie, K. B., Minkenberg, B., and Yang, Y. N. (2015). Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. *Proc. Natl. Acad. Sci.* 112 (11), 3570–3575. doi: 10.1073/pnas.1420294112
- Xie, K. B., Zhang, J. W., and Yang, Y. N. (2014). Genome-wide prediction of highly specific guide RNA spacers for CRISPR/Cas9-mediated genome editing in model plants and major crops. *Mol. Plant* 7 (5), 923–926. doi: 10.1093/mp/ssu009
- Xu, M. L., Xu, Z. H., Liu, B. H., Kong, F. J., Tsubokura, Y., Watanabe, S., et al. (2013). Genetic variation in four maturity genes affects photoperiod insensitivity and PHYA-regulated post-flowering responses of soybean. *BMC Plant Biol.* 13 (1), 91–91. doi: 10.1186/1471-2229-13-91
- Xu, R. F., Li, H., Qin, R. Y., Li, J., Qiu, C. H., Yang, Y. C., et al. (2015). Generation of inheritable and “transgene clean” targeted genome-modified rice in later generations using the CRISPR/Cas9 system. *Sci. Rep.* 5, 11491. doi: 10.1038/srep11491
- Yue, Y., Liu, N., Jiang, B., Li, M., Wang, H., Jiang, Z., et al. (2017). A single nucleotide deletion in *J* encoding *GmELF3* confers long juvenility and is associated with adaption of tropic soybean. *Mol. Plant* 10 (4), 656–658. doi: 10.1016/j.molp.2016.12.004
- Zhai, H., Lü, S. X., Wu, H. Y., Zhang, Y. P., Zhang, X. Z., Yang, J. Y., et al. (2015). Diurnal expression pattern, allelic variation, and association analysis reveal functional features of the *E1* gene in control of photoperiodic flowering in soybean. *PLoS One* 10 (8), e0135909. doi: 10.1371/journal.pone.0135909
- Zhang, X. Z., Zhai, H., Tian, X. J., Zhang, Y. P., Wu, H. Y., Lü, S. X., et al. (2016). Functional conservation and diversification of the soybean maturity gene *E1* and its homologs in legumes. *Sci. Rep.* 6, 29548. doi: 10.1038/srep29548
- Zhang, X., Zhang, J. F., Fu, W. F., Zhang, H. J., and Yuan, W. J. (2011). Advantages of transgene containment technologies. *Hereditas* 33 (5), 437–442. doi: 10.3724/SP.J.1005.2011.00437
- Zhou, H. B., Liu, B., Donald, P. W., Martin, H. S., and Yang, B. (2014). Large chromosomal deletions and heritable small genetic changes induced by CRISPR/Cas9 in rice. *Nucleic Acids Res.* 42 (17), 10903–10914. doi: 10.1093/nar/gku806

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Han, Guo, Guo, Zhang, Wang and Qiu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.