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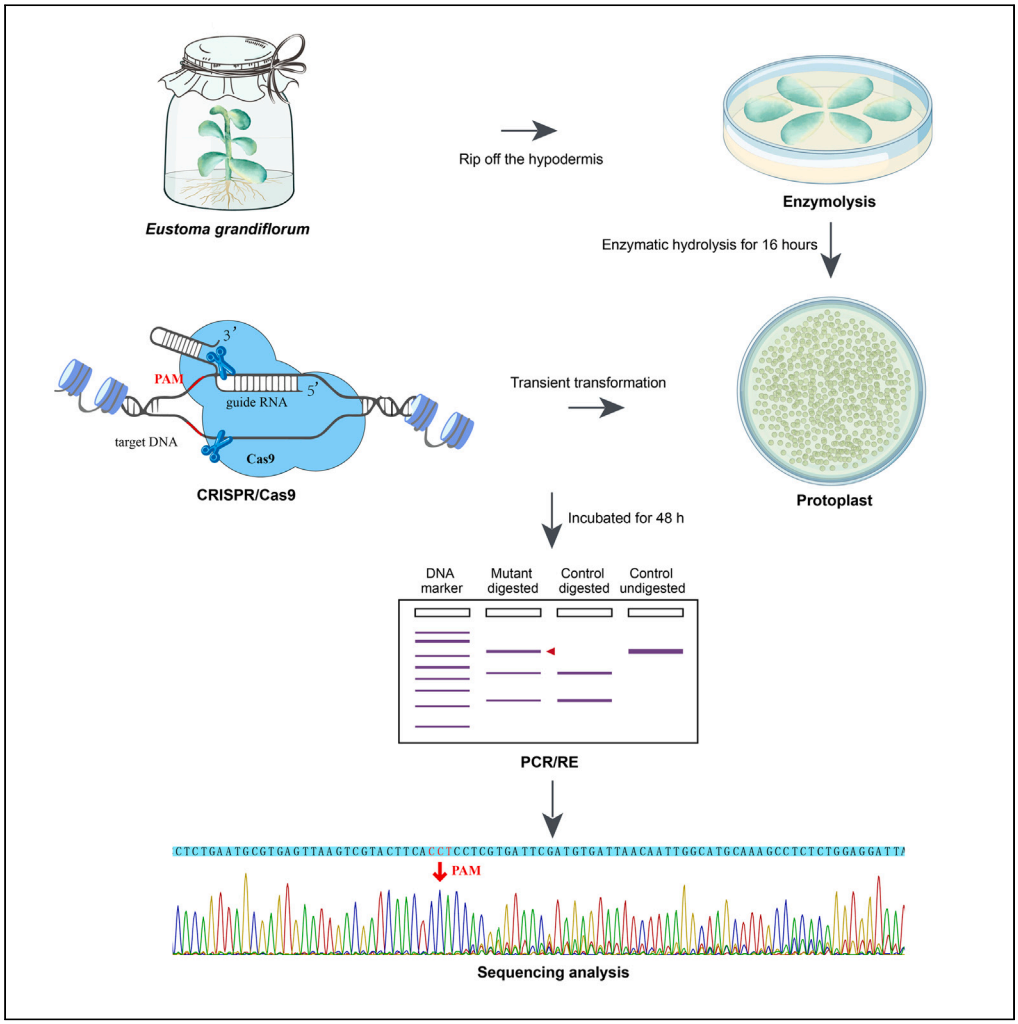

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Highlights

Established an efficient protoplast isolation and transient transformation system

Obtained natural active *U6* and *Cas9* promoters to enhance the expression levels

Acquired a highly efficient CRISPR-Cas9 vector and enhanced genome editing efficiency

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Optimization of CRISPR-Cas9 system in *Eustoma grandiflorum*

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SUMMARY

The optimization of the CRISPR-Cas9 system for enhancing editing efficiency holds significant value in scientific research. In this study, we optimized single guide RNA and *Cas9* promoters of the CRISPR-Cas9 vector and established an efficient protoplast isolation and transient transformation system in *Eustoma grandiflorum*, and we successfully applied the modified CRISPR-Cas9 system to detect editing efficiency of the *EgPDS* gene. The activity of the *EgU6-2* promoter in *E. grandiflorum* protoplasts was approximately three times higher than that of the *GmU6* promoter. This promoter, along with the *EgUBQ10* promoter, was applied in the CRISPR-Cas9 cassette, the modified CRISPR-Cas9 vectors that *pEgU6-2::sgRNA-2/pEgUBQ10::Cas9-2* editing efficiency was 37.7%, which was 30.3% higher than that of the control, and the types of mutation are base substitutions, small fragment deletions and insertions. Finally we obtained an efficient gene editing vector for *E. grandiflorum*. This project provides an important technical platform for the study of gene function in *E. grandiflorum*.

INTRODUCTION

The flowers of *Eustoma grandiflorum*, also known as *lisianthus*, are light and elegant. Both its scientific and common names are derived from Greek and roughly mean "large beautifully mouthed flower" and "dissolving flower," respectively. As the names imply, the plant is mostly used for fresh cut flowers, but also potted plants,[1,2] which has great ornamental and economic value. Flower color, floral, petal, and plant type are the main breeding traits. The development of a molecular breeding method for flowers would be highly significant in the creation of new varieties of horticultural flowers.

In the early stages, the investigation of gene function primarily relies on material derived from naturally occurring mutations, but useful natural mutations are scarce and acquiring the corresponding genetic material was very difficult indeed. Later, with the gradual maturity of genome sequencing technology, scientists applied RNA interference to the study of the biological functions of genes. However, mutations obtained by the method often have shortcomings, including that instability and low targeting efficiency, therefore a new generation of technologies for studying gene function came into being. In recent years, gene editing technology has played an important role in the study of gene function, genome editing technologies mainly include zinc finger nucleases (ZFNs),[3] transcription activator-like effector nucleases (TALENs),[4] and CRISPR-Cas9 technologies. The successful application of ZFN technology to gene editing solved the needs of gene editing to a certain extent, but ZFNs also have some non-specific recognition and cutting, and their construction is complicated, time-consuming and labor-intensive. Although TALENs are easier to assemble than ZFNs, and they are widely used in different species, their construction still remains complicated, which limits the deployment of TALEN technology. Instead, the CRISPR-Cas9 system is gaining popularity rapidly due to its simplicity and high specificity in targeting. It is considered to be a very useful method of genome engineering.[5–7]

CRISPR-Cas9 technology has rapidly become a very effective method for genome editing, and is thus widely used in animals and plants.[8–10] The system consists of Cas9, Cas1, Cas2, and Csn1 proteins as well as CRISPR RNA (crRNA) and *trans*-activating CRISPR RNA (tracrRNA).[11,12] Cas9 can form a complex with synthetic single guide RNA (sgRNA), the sgRNA is formed by fusion of crRNA and *trans*-activated tracrRNA, the sgRNA guides Cas9 to recognize and cleave the target DNA.[13] Cas9 protein is an endonuclease with two domains, one is an HNH nuclease domain and the other is a RuvC-like domain; the HNH nuclease domain cleaves the template strand, while the RuvC-like domain cleaves the coding strand.[14,15] This will produce DNA double-strand breaks (DSBs), and at the same time trigger the body's self-repair mechanism, either through non-homologous end-joining (NHEJ), or homology directed repair (HDR), so as to achieve site-specific modification of a genomic sequence.

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With the widespread application of CRISPR-Cas9 technology in plant gene editing, improving the gene editing efficiency of the CRISPR-Cas9 system has also become very important. The CRISPR-Cas9 system is mainly composed of a Cas9 endonuclease and sgRNA; to improve editing efficiency, it is necessary to express Cas9 and sgRNA efficiently.[16,17] The Cas9 endonuclease is mainly used to cut the target site to produce DNA DSBs in the endogenous target gene.[18] Recently, the plant *ubiquitin* (*UBQ*) promoter has been isolated as a strong promoter that can replace *the cauliflower mosaic virus 35S* (*35S*) promoter in the CRISPR-Cas9 system.[19,20] The sgRNA is mainly used to target and bind to endogenous target genes, cloning a promoter that can efficiently transcribe sgRNA is of great significance for improving the editing efficiency of the CRISPR-Cas9 system. The transcription of sgRNA is mainly driven by the *U3* or *U6* promoter, which are polymerase III (Pol III) promoters that facilitate the transcription of small RNAs, such as sgRNAs. This is because Pol III promoters can produce a precise 5′ terminal transcription start site, which is essential for sgRNA function. Additionally, RNA transcribed by Pol III promoters is usually not capped or polyadenylated, allowing the transcription product to function immediately in the cell.[21,22] The *U6* promoter uses G as the transcription initiation site. Many studies have shown that the efficiency of a *U6* promoter is substantially degraded when used in a distantly related species.[23] Moreover, there exist distinct *U6* promoters within the same species, and these *U6* promoters may exhibit significant variations in their transcriptional activities.[24–26] Nonetheless, statistical analysis revealed that endogenous *U6* promoters in plants are better able to drive transcription of sgRNA,[27–30] thereby improving the editing efficiency of the CRISPR-Cas9 system.

Traditionally, the agrobacterium-mediated transformation method has been employed to introduce its vector into a variety of plant cells, and genome edited plants distinguished from transgenic plants through DNA sequencing,[31,32] production of a stable genetic transformation system to evaluate the effect of CRISPR mutagenesis is time-consuming, while transient transfection of protoplasts is a very effective method for rapid detection of mutagenic effects. At present, protoplasts from many crops have been used to evaluate CRISPR-Cas9-based gene editing, such as *Solanum lycopersicum*, *Zea mays*, *Oryza sativa*, *Lactuca sativa*, and so on.[33–35] Although protoplasts can be used to determine the mutagenesis efficiency of target sites, their isolation conditions often need to be adapted to specific plant species, resulting in limitations when testing genome editing reagents in many non-model species.

Endogenous *U6* promoters have been successfully cloned in many species, and the editing efficiency of the resultant CRISPR-Cas9 system has been improved, the transcriptional activity of a *U6* promoter is not necessarily very high when used in distantly related species. To date, CRISPR-Cas9-mediated genome editing has not been explored in *E. grandiflorum*, so it stands to reason that no research has yet been done on any of its endogenous *EgU6* promoters. Bioinformatic analysis shows that there are at least seven *EgU6* promoters in *E. grandiflorum*, but the transcriptional activity of these promoters in germ cells is not clear. In this study, we optimized the *E. grandiflorum* CRISPR-Cas9 system by enhancing sgRNA expression using an endogenous promoter, and evaluated the editing efficiency of target sequences in CRISPR-Cas9-mediated target mutagenesis in transient protoplasts. Creating a highly efficient gene editing vector for *E. grandiflorum*, lays the foundation for studying of gene function in *E. grandiflorum*.

RESULTS

Establishment of the protoplast isolation and transient transformation system of *E.grandiflorum*

Based on the protocol used by Wu et al. on *Arabidopsis*,[36] we optimized the separation and purification of *E. grandiflorum* protoplast system (Figures 1A–1F). The optimal conditions for separating *E. grandiflorum* protoplasts are as follows: using 1.5% Cellulase R-10 and 1.0% Macerozyme R-10, maintaining a mannitol concentration of 0.9 M, and conducting enzymolysis for a duration of 16 h. The yield of *E. grandiflorum* protoplasts separated by the optimized protoplast separation process is above 0.5×10^7/g, and the survival rate was over 90%. The protoplasts obtained from this protocol were transfected with green fluorescent protein (GFP) using a polyethylene glycol (PEG)-mediated approach. Laser confocal microscopy was employed for visualizing the GFP-transfected protoplasts. (Figures 1G–1L). The protoplasts were quantified using a hemocytometer, and the transfection efficiency of these protoplasts exceeded 60%.

Identification of *U6*, *UBQ10*, and *actin* promoters in *E. grandiflorum*

In an effort to optimize the CRISPR-Cas9 system in *E. grandiflorum*, we conducted BLAST searches of the *E. grandiflorum* genome, using the *Arabidopsis AtU6-26* gene as the queries, identified and cloned seven *EgU6* small nuclear RNA (snRNA) genes, named *EgU6-1*, *EgU6-2*, *EgU6-3*, *EgU6-4*, *EgU6-8*, *EgU6-10*, and *EgU6-11* (Figure S1A). An analysis of multiple sequence alignments showed that seven *EgU6* genes exhibited relatively conserved snRNA transcript sequences compared with the corresponding *AtU6*, *TaU6* and *GmU6* genes; however, the upstream sequence element (USE) sequences and TATA-like box of *EgU6-1*, *EgU6-2*, and *EgU6-3* are relatively conserved, while other *U6* promoters are slightly different from those of *Arabidopsis*, wheat, and soybean (Figure 2A). The presence of the USE and TATA-like box in the promoters of *EgU6* genes suggests that these Pol III promoters might be productively involved in transcription. In addition, the aforementioned method was used to identify and clone one *EgUBQ10* and one *EgActin* (Figure S1B).

EgU6, *EgUBQ10*, and *EgActin* promoters can promote gene transcription and expression

The activity of the promoter can influence the expression of the target genes it regulates; we have developed a luciferin-based promoter reporting assay in *E. grandiflorum*. The method accurately identifies promoters with high activity and significant expression levels. First, we used a dual luciferase reporter assay to investigate the activity of amplified *EgU6* promoters,[37] and we obtained seven luciferase/renilla luciferase (Luc/Rluc) binary vectors with different truncated 350 bp *U6* promoters (Figure 2B). Based on the results of transient protoplast

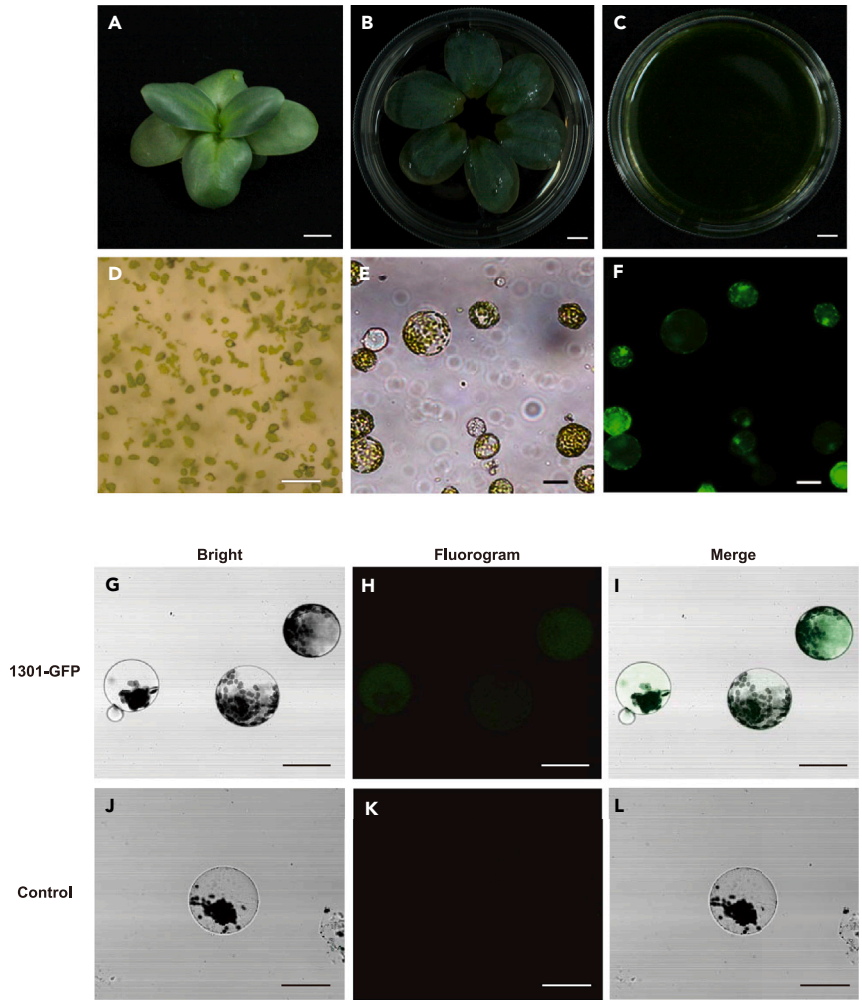

Figure 1. Establishment of the systems for protoplast isolation and transient transformation of *E. grandiflorum*

(A) Tissue cultured young plant of *E. grandiflorum*. Scale bar: 0.5 cm.

(B) The leaves without the hypodermis in the enzyme mixture. Scale bar: 0.5 cm.

(C) Protoplasts after enzymatic hydrolysis. Scale bar: 0.5 cm.

(D) Zoom-stereo microscope observation. Scale bar: 200 μm.

(E) Protoplasts imaging under natural light. Scale bar: 50 μm.

(F) Fluorescent microscope image taken after FDA (fluorescein diacetate) staining, the green fluorescence indicates active protoplasts. Scale bar: 50 μm.

(G–I) 1301-GFP plasmid was delivered to *E. grandiflorum* protoplasts using a PEG mediated method. Protoplasts were photographed after 21 h. Scale bar: 50 μm.

(G) Microscopic imaging of protoplasts under natural light. (H) Green color indicates GFP epifluorescence. (I) Overlay of epifluorescence and bright field images of transfected *E. grandiflorum* protoplasts.

(J–L) 1301-Empty plasmid was delivered to *E. grandiflorum* protoplasts using a PEG mediated method. Protoplasts were photographed after 21 h. Scale bar: 50 μm.

transformation, the values of Luc and Rluc were quantitatively analyzed, and the activities of different *EgU6* promoters were compared based on the ratio of luciferase activity between Luc and Rluc. The result showed that the seven *EgU6* promoters of *E. grandiflorum* all have transcriptional activity. Among *U6* promoters with conserved functional domains, *EgU6-2* exhibited the highest activity. Among *U6* promoters with unconserved functional domains, *EgU6-11* displayed the highest activity. Additionally, both promoters demonstrated higher activity compared to the *AtU6* and *GmU6* promoters. (Figure 2D). Based on our luciferase assay system, the activity of the *Cas9* promoters were also tested using this method (Figure 2C), the promoter activity of *EgUBQ10* is similar to that of *GmUBQ*, but higher than that of the *35S* promoter. However, compared to the *EgActin* promoter, *EgUBQ10* exhibits the strongest activity (Figure 2E).

Cloning and identification of the *EgPDS* gene of *E. grandiflorum*

Phytoene dehydrogenase (PDS) is one of the key enzymes in the process of carotenoid synthesis, the enzyme plays an important role in the synthesis of chlorophyll, and homozygous *PDS* gene mutants can produce albinism. We selected the *EgPDS* gene as the target endogenous

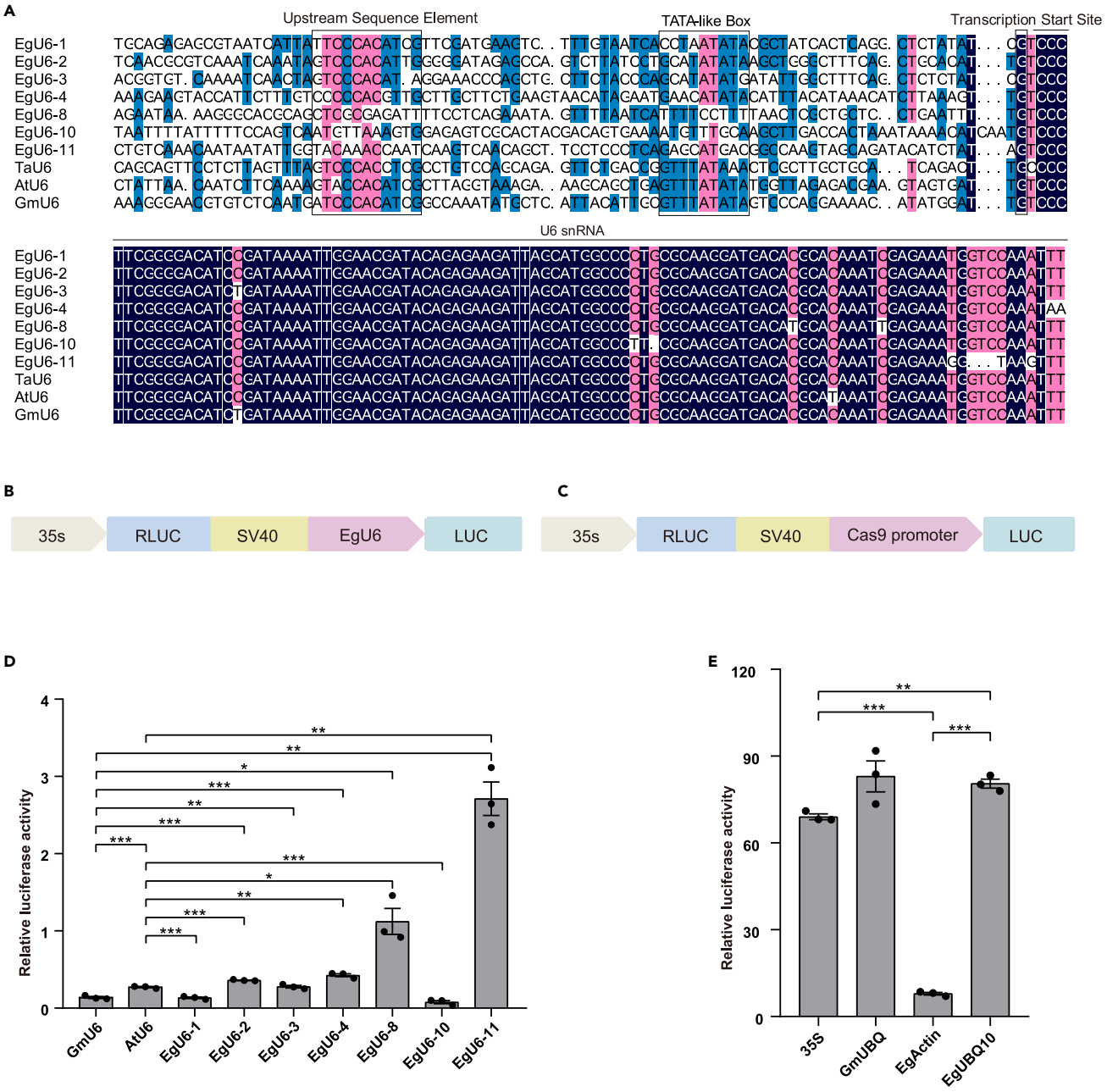

Figure 2. Identification and validation of *EgU6* promoters and *Cas9* promoters in *E. grandiflorum*

(A) Multi-sequence alignment of the *U6* promoter of *E. grandiflorum*, wheat, *Arabidopsis* and soybean. Black line denotes the *U6* snRNA transcript. USE (upstream sequence element), TATA-like box and the transcription start site are labeled with black boxes.

(B) Schematic diagram of the dual luciferase reporter vector for the *EgU6* promoter of *E. grandiflorum*.

(C) Schematic diagram of the dual luciferase reporter vector for the promoter of the *E. grandiflorum* internal reference gene.

(D) The result of dual luciferase reporter system detecting the *EgU6* promoter activity of *E. grandiflorum*. Data represent mean ± SEM of three biological replicates. Asterisks indicate significant differences (*p < 0.05, **p < 0.01, ***p < 0.001). Black dots indicate biological replicates. T-test was used for count of p value.

(E) The result of the dual luciferase reporter system detecting the promoter activity of the internal reference gene *UBQ10* and *Actin* of *E. grandiflorum*. Data represent mean ± SEM of three biological replicates. Asterisks indicate significant differences (*p < 0.05, **p < 0.01, ***p < 0.001). Black dots indicate biological replicates. t test was used for count of p value.

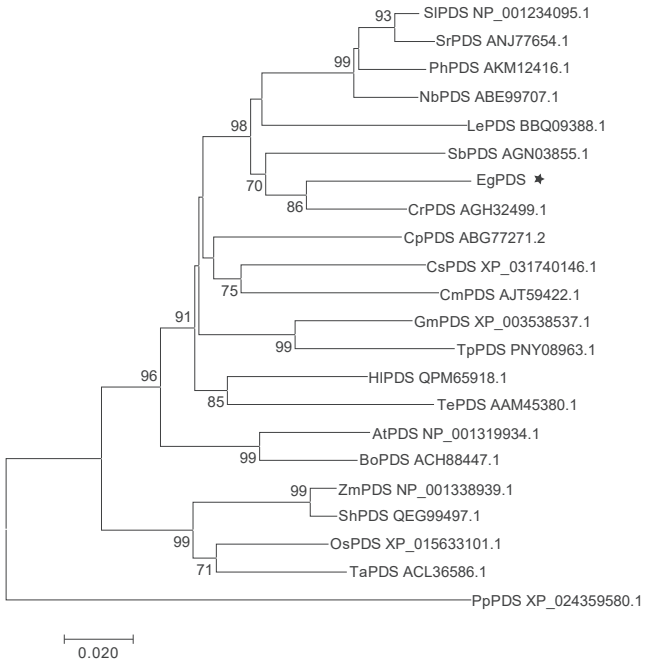

Figure 3. Phylogenetic analysis of the *PDS* gene of *E. grandiflorum* and that of other species

gene for editing. Using the sequence of the *Arabidopsis* PDS protein to screen out the *EgPDS* gene by local BLAST, only one copy of the gene was predicted. *EgPDS* has 14 exons, with 1,749 nucleotides of transcript sequence coding 582 amino acids. The evolutionary history was inferred using the neighbor joining method and using the amino acid sequences of *E. grandiflorum* and some known PDS proteins from other species; from the phylogenetic tree, it can be seen that of the *EgPDS* genes represented, that of *E. grandiflorum* is most closely related to the *PDS* gene of *Catharanthus roseus* (Figure 3).

Optimization of CRISPR-Cas9 vector for editing the *E. grandiflorum EgPDS* gene

To determine whether the use of *EgU6* and *UBQ10* promoters results in improved genome editing efficiency in *E. grandiflorum*, we constructed different CRISPR-Cas9 vectors. Recognition of the target site by sgRNA depends on the recognition of the protospacer adjacent motif (PAM) sequence. One target site was selected on the eighth exon, and there is a single restriction site *BssS* I at 3–4 base pairs upstream of the PAM. The other two sgRNA sites are located in the first and twelfth exons (Figure 4A). Using the CRISPR-Cas9 vectors of two model plants as the basic skeletons (Cas9-1 and Cas9-2), we successfully constructed CRISPR-Cas9 cassettes for editing the *EgPDS* gene of *E. grandiflorum* (Figure 4B).

The optimized CRISPR-Cas9 system enhances the editing efficiency in *E. grandiflorum*

The PEG-mediated transient transformation system of protoplasts is a very simple and effective method to verify the editing efficiency of CRISPR vectors. Protoplast DNA from the CRISPR-Cas9 vector, targeting sgRNA-2 at the editing site, was subjected to PCR/restriction enzyme (PCR-RE) analysis, then undigested bands were subcloned into a T/A cloning vector, the inserts of individual colonies are amplified by colony PCR and the mutation confirmed by digestion with *BssS* I (Figures 4C and 5A), and clones containing a putative mutagenized target gene are subjected to sequencing, we found the main types of endogenous gene mutation are base insertion, substitution and small fragment deletion (Figures 4D, 4E, 5B, and 5C). The result showed that the *pEgU6-11::sgRNA-2/pGmUBQ10::Cas9-2* editing efficiency was higher than *pEgU6-11::sgRNA-2/pCDC45::Cas9-1* (Table 1).

The editing efficiency of *pEgU6-11::sgRNA-2/pEgUBQ10::Cas9-2* was found to be 38.3%, which was 30.9% higher than that of the control; *pEgU6-2::sgRNA-2/pEgUBQ10::Cas9-2* editing efficiency was found to be 37.7%, which was 30.3% higher than that of the control. We found *pEgU6-2::sgRNA-2/pGmUBQ10::Cas9-2* and *pEgU6-11::sgRNA-2/pGmUBQ10::Cas9-2* have high editing efficiency (Table 1). However, their main types of endogenous gene mutation are substitution, on the contrary *pEgU6-2::sgRNA-2/pEgUBQ10::Cas9-2* has all types of editing. In conclusion, editing efficiencies are different in different vectors, and markedly higher efficiencies are obtained with the *EgU6* promoter and *EgUBQ10* promoter.

We also quantified the editing efficiency of CRISPR vectors targeting different sites. As there is no cleavage site at the target locus, PCR products were directly subcloned into a T/A cloning vector and sequenced. Our results indicate that sgRNA-1 and sgRNA-3 exhibited low mutation efficiencies (Table 1), the main types of mutation are base substitution (Figure S2). In order to rule out the possibility of single

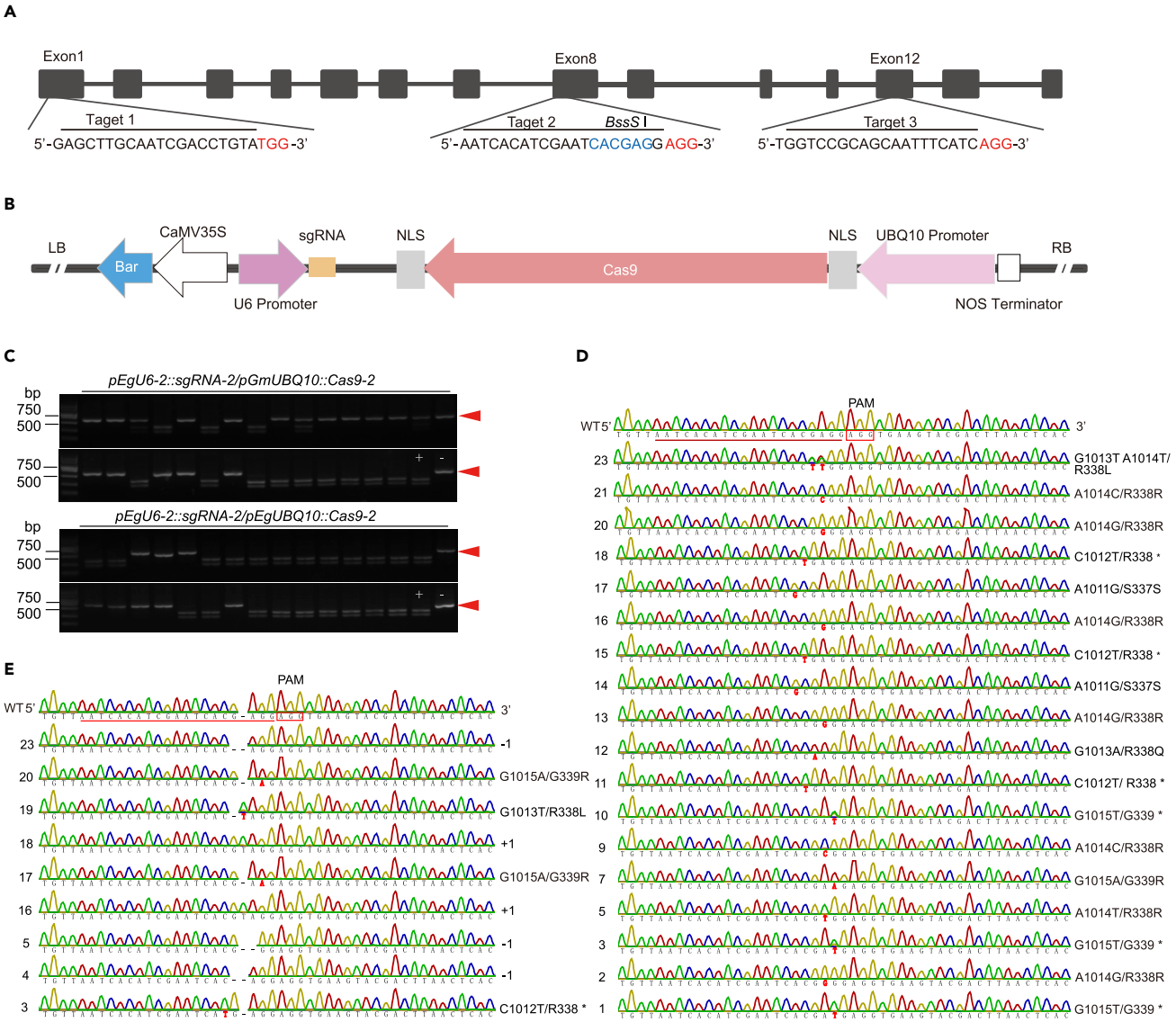

Figure 4. Construction of the *E. grandiflorum* CRISPR-Cas9 vector and targeted mutagenesis of the *EgPDS* gene in protoplasts

(A) Schematic diagram of target site selection of the *E. grandiflorum PDS* gene. Restriction enzyme sites are highlighted in blue letters; red letters indicate PAM sequences.

(B) *E. grandiflorum* CRISPR-Cas9 vector structure diagram.

(C) *pEgU6-2::sgRNA-2/pGmUBQ10::Cas9-2* and *pEgU6-2::sgRNA-2/pEgUBQ10::Cas9-2* mutagenized *EgPDS* gene target sites adopted the method of PCR-RE, undigested bands PCR products is subcloned into a T/A cloning vector. The inserts of individual colonies are amplified by colony PCR and the mutation confirmed by digestion with *BssS* I. Clones containing a putative mutagenized target gene (red arrowheads indicate mutated bands) are subjected to sequencing. "+" *BssS* I digested PCR products, "−" non-digested PCR products.

(D) *pEgU6-2::sgRNA-2/pGmUBQ10::Cas9-2* mutation types at the *PDS* gene target sites of *E. grandiflorum*. The red box is the PAM sequence, the red line is the sgRNA sequence.

(E) *pEgU6-2::sgRNA-2/pEgUBQ10::Cas9-2* mutation types at the *PDS* gene target sites of *E. grandiflorum*. The red box is the PAM sequence, the red line is the sgRNA sequence.

base substitutions arising from single nucleotide polymorphisms or random base pairing during PCR amplification, empty protoplasts (WT) were employed as templates for PCR amplification and subsequently ligated to TA/Blunt-Zero vector for sequencing of individual clones. The obtained sequencing results demonstrated a significantly lower mutation frequency at the target site compared to protoplast PCR amplification using *E. grandiflorum* CRISPR-Cas9 vector (Table 1). Thus, it can be inferred that the induction of single base substitutions at *EgPDS* target sites was attributed to the CRISPR-Cas9 system.

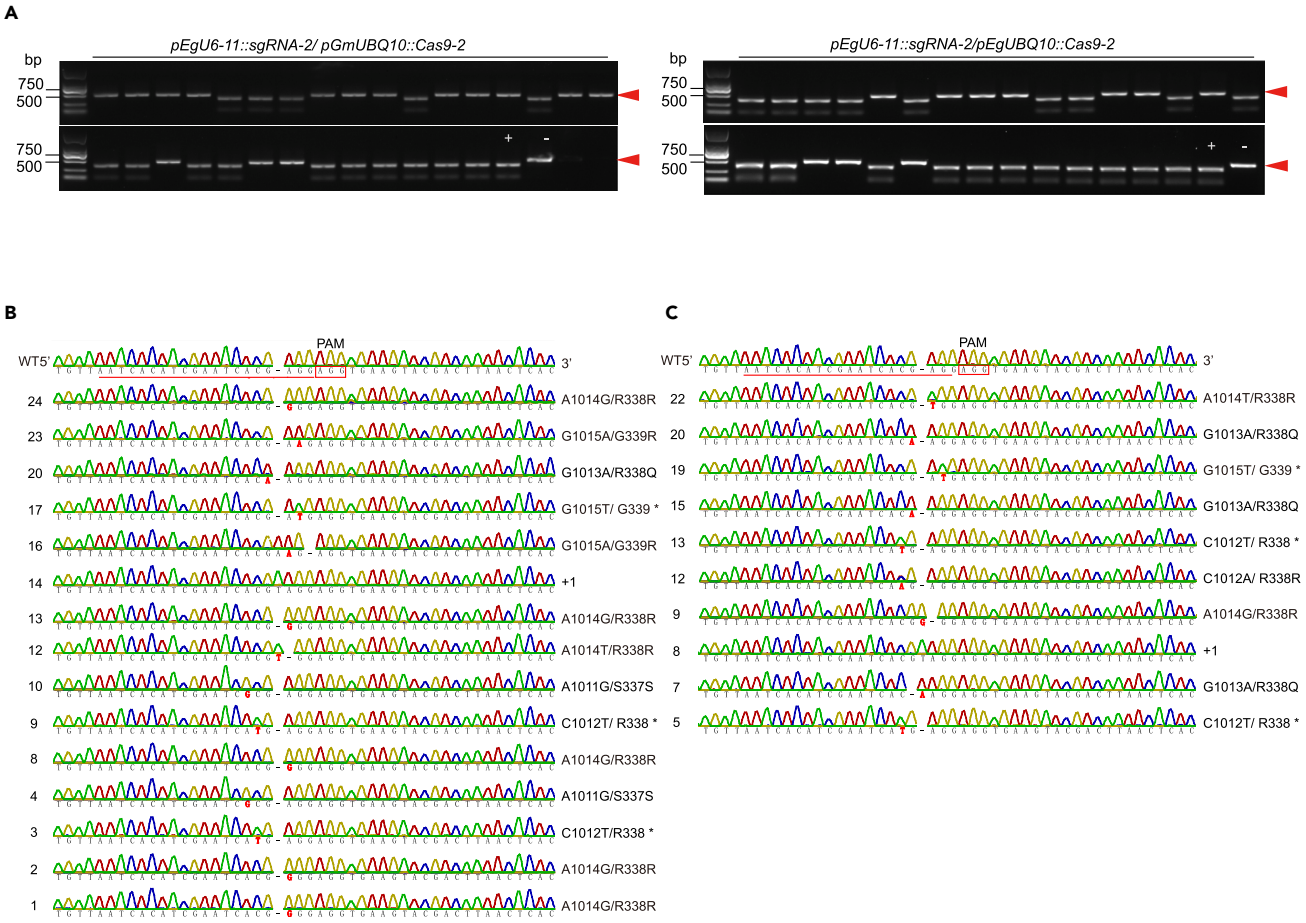

Figure 5. Targeted mutagenesis of the *EgPDS* gene in protoplasts

(A) *pEgU6-11::sgRNA-2/pGmUBQ10::Cas9-2* and *pEgU6-11::sgRNA-2/pEgUBQ10::Cas9-2* mutagenized *PDS* gene target sites adopted the method of PCR-RE, undigested bands PCR products is subcloned into a T/A cloning vector. The inserts of individual colonies are amplified by colony PCR and the mutation confirmed by digestion with *BssS* I. Clones containing a putative mutagenized target gene (indicated by triangle marks) are subjected to sequencing. "+" *BssS* I digested PCR products, "−" non-digested PCR products.

(B) *pEgU6-11::sgRNA-2/pGmUBQ10::Cas9-2* mutation types at the *PDS* gene target sites of *E. grandiflorum*. The red box is the PAM sequence, the red line is the sgRNA sequence.

(C) *pEgU6-11::sgRNA-2/pEgUBQ10::Cas9-2* mutation types at the *PDS* gene target sites of *E. grandiflorum*. The red box is the PAM sequence, the red line is the sgRNA sequence.

DISCUSSION

Assessing the stable transformation period of *E. grandiflorum* is not only time-consuming but also costly.[38] Therefore, it is crucial to meticulously select the most optimal CRISPR-Cas9 system before embarking on stable transformation, and the utilization of a protoplast transient transfection system could effectively address this predicament. The bottleneck is isolating high-quality protoplasts from *E. grandiflorum*. We developed protoplasts transient transformation system, which can simply and quickly verify the activity of *EgU6* promoters and the efficiency of CRISPR vectors. The yield and activity of protoplasts are affected by many factors, such as enzymatic hydrolysis conditions, enzymatic hydrolysis time, PEG induction time, and incubation time after plasmid transformation.[36,39] The optimal experimental method was obtained by testing different experimental treatments. We finally found that 1.5% Cellulase R-10, 1% the Macerozyme R-10, and 0.9M mannitol, produced the highest protoplasts. The transformation efficiency of protoplasts containing 30% PEG4000 was over 60%. Importantly, the transformation efficiency was not lower than that of protoplasts of model plants.[40] Protoplasts are not only useful for rapidly validating the mutagenesis efficiency of various RNA-guided endonucleases, promoters, sgRNA designs, or Cas proteins,[41,42] but can also be a platform for DNA-free gene editing, providing a method to generate mutants without foreign DNA. This approach circumvents the challenges and time-consuming process of offspring hybridization. However, the primary bottleneck of this technology remains protoplast regeneration.[43–45] On the other hand, there are currently no examples of protoplast fusion and its application in gene editing. If these technical hurdles can be overcome, CRISPR-mediated protoplast genome editing could usher in a new era in plant breeding.

Table 1. Editing efficiency statistics of the CRISPR to the *E. grandiflorum EgPDS* gene

Types	Transfection efficiency (%)	Target gene	Mutagenesis efficiency (%)	Sequenced mutations (bp)		
				Insertions	Deletions	Substitutions
WT	–	*EgPDS*	0.0%	None	None	None
pGmU6::sgRNA-2/pGmUBQ10::Cas9-2	90.2%	*EgPDS*	7.4%	None	None	2
pEgU6-2::sgRNA-2/pGmUBQ10::Cas9-2	82.7%	*EgPDS*	72.6%	None	None	18
pEgU6-11::sgRNA-2/pGmUBQ10::Cas9-2	86.6%	*EgPDS*	57.7%	1	None	14
pEgU6-2::sgRNA-1/pEgUBQ10::Cas9-2	61.3%	*EgPDS*	0.0%	None	None	None
pEgU6-2::sgRNA-2/pEgUBQ10::Cas9-2	79.6%	*EgPDS*	37.7%	2	3	4
pEgU6-2::sgRNA-3/pEgUBQ10::Cas9-2	69%	*EgPDS*	4.8%	None	None	1
pEgU6-11::sgRNA-1/pEgUBQ10::Cas9-2	62.7%	*EgPDS*	0.0%	None	None	None
pEgU6-11::sgRNA-2/pEgUBQ10::Cas9-2	87%	*EgPDS*	38.3%	1	None	9
pEgU6-11::sgRNA-3/pEgUBQ10::Cas9-2	70.2%	*EgPDS*	0.0%	None	None	None
pAtU6::sgRNA-2/pCDC45::Cas9-1	89.7%	*EgPDS*	3.7%	None	None	1
pEgU6-11-sgRNA-2/pCDC45-Cas9-1	88.3%	*EgPDS*	15.1%	None	None	4

U6 RNA is a small non-coding RNA, and its *U6* promoter can drive the transcription of sgRNA and is an important part of the CRISPR-Cas9 system, sgRNA activity and the expression of sgRNA/Cas9 greatly influence the efficiency of CRISPR-Cas9-mediated genome editing. The *U6* promoters of evolutionarily distantly related species cannot be used universally, even the activities of *U6* promoters from the same species are also very different.[23–25] Related articles report that application of plant species-specific promoters is an effective strategy for improving the efficiency of genome engineering in plants.[27,28] Therefore, the cloning and functional analysis of endogenous *U6* promoter is particularly important for the establishment of CRISPR-Cas9 genome editing system. Whether or not a CRISPR-Cas9 system driven by an *EgU6* promoter could edit endogenous genes had not been reported, so we mainly identify and clone *EgU6* promoters from *E. grandiflorum*. Achieving high sgRNA expression is essential for effective mutagenesis, so we used a dual luciferase detection system to test the activities of promoters. We showed that the 350 bp *EgU6* promoter was sufficient for driving *Luc* gene expression. However, the *EgU6-11* promoter achieves the highest expression of the *Luc* gene, but through analysis we found that its TATA-like Box and USE are not very conservative. Therefore, we also chose *EgU6-2* with TATA-like Box and USE conservative functional domain for research; it may serve as a suitable promoter for driving the expression of sgRNAs.

The results showed that the *pEgU6-11::sgRNA-2/pEgUBQ10::Cas9-2* vector had the higher gene editing ability compared with the *GmU6* promoter, the editing efficiency of the CRISPR-Cas9 vector increased by 30.9%; *pEgU6-2::sgRNA-2/pEgUBQ10::Cas9-2* editing efficiency was found to be 37.7%, which was 30.3% higher than that of the control; the editing efficiency of the CRISPR-Cas9 vector *pEgU6-11::sgRNA-2/pCDC45::Cas9-1* compared with the *AtU6* promoter increased 11.4%. Our results are consistent with previous reports suggesting that endogenous *U6* promoters produce higher editing efficiency than non-endogenous promoters.[28,30] We found the *pEgU6-2::sgRNA-2/pEgUBQ10::Cas9-2* main types of endogenous gene mutation are base insertion, substitution and small fragment deletion, *pEgU6-11::sgRNA-2/pGmUBQ10::Cas9-2* and *pEgU6-11::sgRNA-2/pEgUBQ10::Cas9-2* main types of endogenous gene mutation are substitution, it may be due to the fact that *EgU6-11* TATA-like Box and USE are not conservative. Additionally, we found the mutation efficiency of target site sgRNA-1 and sgRNA-3 was low, different targets producing different mutation efficiencies perhaps due to G/C content and the locations of the designed sgRNAs.[46] Previous studies have shown that *35S* and the *ubiquitin* promoters are typically used for the control of Cas9 expression, endogenic *ubiquitin* promoters can better drive Cas9 expression,[7,47] which is consistent with our results. We also found that CRISPR vectors constructed by Cas9-1 and Cas9-2 have different editing efficiencies, perhaps due to differences in codon preference between different species. However, whether the Cas9 protein we constructed for *E. grandiflorum* has obvious codon preference requires further study. In our study, we found that there are many single base substitutions at the target site, which may be related to the protoplasts state and/or the environment. After a DNA double strand is cut, possible activation of homologous repair mechanisms in protoplast cells is greatly activated. Another possible reason for the numerous single base substitutions is that Cas9 and sgRNA are transiently expressed, and the base substitutions produced at target sites cannot always be edited due to the lack of Cas9 and sgRNA.[48] Our study can thus facilitate the application of CRISPR-Cas9 technology to the future study of functional genes and mutant phenotypes in *E. grandiflorum*, thus providing an important technical platform for the development of new varieties of *E. grandiflorum*.

Conclusion

Firstly, we established a protoplast separation and transient transformation system using the leaves of *E. grandiflorum*. We evaluated and verified the editing efficiency of the CRISPR-Cas9 system by using transient transformation of protoplasts. Secondly, through dual luciferase assay, we identified the activity of endogenous *EgU6\UBQ\Actin* promoters and selected the best promoter to drive sgRNA and Cas9 for increased expression levels. We optimized the CRISPR-Cas9 system and improved editing efficiency of the *EgPDS* gene. Finally, we selected

a CRISPR-Cas9 vector with high editing efficiency. The evaluation of editing efficiency by transient protoplast transformation can serve as a basis for saving time and effort in stable genetic transformation. Additionally, an efficient CRISPR-Cas9 vector has laid a solid foundation for gene function research and breeding new varieties of *E. grandiflorum*.

Limitations of the study

The transient transfection system of protoplasts can quickly verify the mutagenic efficiency of promoters or Cas proteins, but it also has some limitations. It is an active naked plant cell surrounded by the plasma membrane, so its stability is poor. In order to establish a reliable protoplast evaluation system, we need to develop an optimal protoplast separation and instantaneous transformation system, which requires a significant amount of time and expense.

Additionally, due to the mixture nature of protoplasts, single-cell sequencing becomes challenging. When calculating the editing efficiency of different CRISPR vectors, T vectors are used and detected through Sanger sequencing with at least 30 monoclones per sample to determine their editing efficiency. Although this process involves a lot of work, we have confidence in the reliability of our results. Furthermore, there is untapped potential in the field of protoplast regeneration technology, which we anticipate will serve as a pivotal platform for enhancing novel cultivars and investigating gene functionality within *E. grandiflorum*.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.109053.

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AUTHOR CONTRIBUTIONS

X.L. designed the experiments, analyzed the data, and wrote the manuscript. F.B., C.K. W.X., and M.Z. performed the experiments. L.W. performed the bioinformatics analysis. K.S., Q.Z., Y.L., and Y.Z. guided the experiment. All authors read and approved of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
E. coli DH5α	2ND Lab	Cat# DL1001
Chemicals, peptides, and recombinant proteins		
2x Phanta Max Master Mix	Vazyme	Cat# P515-01
5 min TA/Blunt-Zero Cloning Kit	Vazyme	Cat# C601-01
ClonExpress II One Step Cloning Kit	Vazyme	Cat# C112-01
PrimeScript RT reagent Kit with gDNA Eraser	Takara Bio	Cat# RR047A
T4 DNA Ligase	Thermo Scientific	Cat# EL0013
Murashige and Skoog	PhytoTech	Cat# M153
Macerozyme R-10	Yakult	Cat# MX7351
Cellulase R-10	Yakult	Cat# MX7352
Xho I	New England Biolabs	Cat# R0146V
Sap I	New England Biolabs	Cat# R0569S
BssS I	New England Biolabs	Cat# R0681T
Asc I	New England Biolabs	Cat# R0558V
BstE II	New England Biolabs	Cat# R0162V
BamH I	New England Biolabs	Cat# R0136V
Hind III	New England Biolabs	Cat# R0104V
Bbs I	New England Biolabs	Cat# R0539V
Sal I	New England Biolabs	Cat# R1038V
Critical commercial assays		
Dual-Luciferase Reporter Assay System	Promega	Cat# E1910
Oligonucleotides		
Primers for cloning *EgU6-1* (See Table S1)	IDT	N/A
Primers for cloning *EgU6-2* (See Table S1)	IDT	N/A
Primers for cloning *EgU6-3* (See Table S1)	IDT	N/A
Primers for cloning *EgU6-4* (See Table S1)	IDT	N/A
Primers for cloning *EgU6-8* (See Table S1)	IDT	N/A
Primers for cloning *EgU6-10* (See Table S1)	IDT	N/A
Primers for cloning *EgU6-11* (See Table S1)	IDT	N/A
Primers for cloning *UBQ10-1* (See Table S1)	IDT	N/A
Primers for cloning *Actin-1* (See Table S1)	IDT	N/A
Primers for cloning *EgPDS* (See Table S1)	IDT	N/A
Primers for Luc/Rluc vector construction (See Table S2)	IDT	N/A
Primers for CRISPR vector construction (See Table S3)	IDT	N/A
Primers for target mutation validation (See Table S4)	IDT	N/A
Recombinant DNA		
pEgU6::Luc/35s::Rluc	This paper	N/A
pGmU6::sgRNA-2/pGmUBQ10::Cas9-2	This paper	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
pEgU6-2::sgRNA-2/pGmUBQ10::Cas9-2	This paper	N/A
pEgU6-11::sgRNA-2/pGmUBQ10::Cas9-2	This paper	N/A
pEgU6-2::sgRNA-1/pEgUBQ10::Cas9-2	This paper	N/A
pEgU6-2::sgRNA-2/pEgUBQ10::Cas9-2	This paper	N/A
pEgU6-2::sgRNA-3/pEgUBQ10::Cas9-2	This paper	N/A
pEgU6-11::sgRNA-1/pEgUBQ10::Cas9-2	This paper	N/A
pEgU6-11::sgRNA-2/pEgUBQ10::Cas9-2	This paper	N/A
pEgU6-11::sgRNA-3/pEgUBQ10::Cas9-2	This paper	N/A
pAtU6::sgRNA-2/pCDC45::Cas9-1	This paper	N/A
pEgU6-11::sgRNA-2/pCDC45::Cas9-1	This paper	N/A
pMD 18-T Vector- AtU6	This paper	N/A
pCAMBIA1301-GFP	This paper	N/A
Software and algorithms		
DNAman	LynnonBiosoft	https://www.lynnon.com/dnaman.html
Geneious	Biomatters	https://www.geneious.com

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yang Zhang (summerzhang@126.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

CRISPR vector backbones are available in our lab (College of Life Science, Northeast Forestry University, Harbin, 150040, China). Seeds of *E. grandiflorum* were used to generate the WT lines, the seeds were washed twice with 75% ethanol for 10 s each, surface sterilized with 2% (v/v) sodium hypochlorite for 5 min, and washed with sterilized water (autoclaved five times for 5 min) each before sowing on Murashige and Skoog M153 (PhytoTech; #M153) medium. Seeds were incubated under aseptic conditions at 24°C with a photoperiod of 16 h white light. Two weeks later, each plant was transplanted into sterile MS medium, and thereafter subcultured once a month.

METHOD DETAILS

Isolation and purification of *E. grandiflorum* protoplasts

E. grandiflorum protoplast isolation was optimized in accordance with the protoplast isolation method used by Yoo et al. with *Arabidopsis*.[39] The Macerozyme R-10 (Yakult; # MX7351) was set at 0.5%, 1.0% and 1.5%. The Onozuka R-10 cellulase (Yakult; # MX7352) was set at 1.0%, 1.5% and 2.0%. Mannitol was set at 0.2 M, 0.6 M and 0.9 M respectively, the enzymolysis time was set at 10 h, 16 h and 20 h and the centrifugation rate was set to 100 *g*, 200 *g* and 300 *g*, combination respectively. The mixture was then diluted to 10 mL with CPW solution (KH_2PO_4 27.2 mg, KNO_3 101 mg, $CaCl_2 \cdot 2H_2O$ 1.48 g, $MgSO_4 \cdot 7H_2O$ 246 mg, KI 0.16 mg, $CuSO_4 \cdot 5H_2O$ 0.025 mg, volume to 1 L with distilled water and filter sterilization), placed in a water bath at 55°C for 10 min, adjust the pH to between 5.75 and 5.8. After cooling, the epidermis-free leaves were added to the enzyme mixture and wrapped with tin foil, and stored for 16 h at room temperature. After enzymatic hydrolysis turned the mixture from brown to green, the solution was filtered with a 74 μm cell sieve to remove materials that were not completely enzymatically hydrolyzed. The filtrate was then centrifuged at 200 *g* for 2 min, to allow the protoplasts to settle. Then the upper layer of the enzyme solution was gently aspirated. The prepared CPW solution was added to wash the protoplasts. Protoplasts yield and survival rate were observed by

microscope after FDA staining, the protoplast suspension was 0.1 mL, placed in a 1.5 mL centrifuge tube, and the final concentration of the protoplast was 0.01%, which was mixed and placed at room temperature for 5 min. After observation by fluorescence microscope (Olympus, BX43, Japan), the fluorescing protoplasts were active, while those that did not produce fluorescence were inactive.

PEG-mediated transient transformation of *E. grandiflorum* protoplasts

Protoplasts were liberated by enzymatic hydrolysis and passed through a 74 μm membrane. The filtrate was centrifuged at 150 *g* for 2 min, and the supernatant discarded. The protoplast was diluted to a concentration of 2×10^5/mL by WS (500 μL MES [0.2 M], 125 μLKCl [2.0 M], 7.7 mL NaCl [1.0 M] and 6.25 mL $CaCl_2$ [1.0 M] in distilled water [final volume = 50 mL; pH 5.75–5.8]), and allowed to rest on ice for 30 min. The WS was drawn off without touching the protoplasts, which formed the bulk of the precipitate. The protoplasts were then resuspended in 2×10^5/mL by MMG (1 mL MES [0.2 M], 375 μL $MgCl_2$ [2.0 M] and 8.1975 g mannitol in distilled water [final volume = 50 mL; pH 5.75–5.8]). We took 2 mL centrifuge tubes, three replicates were performed for each sample, and each added into 100 μL of the protoplast-containing MMG solution, 10 μL of plasmid (1 μg/μL), and 110 μL of PEG (30% PEG 4000 has high induction efficiency. Mix 1.5 g PEG 4000, 500 μL $CaCl_2$, 0.4099 g mannitol, and water to 5 mL) to each tube, mixed its contents gently, and protected them from light at room temperature for 15 min. We then added 440 μL of WS, centrifuged at 100 *g* for 2 min, and discarded the supernatant. Finally, added 1 mL of WI (1 mL MES [0.2 M], 500 μL KCl [2.0 M] and 9.1085 g mannitol in distilled water [final volume = 50 mL; pH 5.75–5.8]) to each tube, shook the tube gently, transferred them to a Petri dish, and incubated in the dark for 21 h or longer. Refer to it for improvement.[49]

Identification of the *U6* and *ubiquitin* promoters in *E. grandiflorum*

The *AtU6-26* (AT3G13855) gene from *Arabidopsis* was used to identify the *E. grandiflorum U6* genes using the *E. grandiflorum* 10B-620 genome Database: https://www.ncbi.nlm.nih.gov/sra/DRX056155[accn]. 1.5 kb fragments upstream of the predicted *E. grandiflorum U6* genes were isolated and cloned into TA/Blunt-Zero (Vazyme; #C601-01) for sequencing. The seven identified *EgU6* genomic DNA sequences were amplified using specific primers (Table S1). In the same way, we searched for *UBQ10* and *Actin1* promoters sequences corresponding to the candidate *UBQ10* gene using the CDS of *Arabidopsis UBQ10* (GenBank: AT4G05320) and *Actin1* (GenBank: AT2G37620). We obtained one *UBQ10* promoter and one *Actin* promoter. The DNA sequences of the promoters were amplified using specific primers (Table S1).

Detection of the activities of the *EgU6* and *Cas9* promoters via transient expression in *E. grandiflorum* protoplasts

We used a dual luciferase detection system to compare the activity of the seven *EgU6* promoters, the *EgU6* promoters were truncated to 350 bp, to ensure that they contain two functional elements: the USE and TATA box. The Luc/Rluc vector was digested with *Xho* I, and the seven 350 bp *EgU6* promoters were appended to the front of *Luc* genes respectively, using the ClonExpress II One Step Cloning Kit (Vazyme; #C112-01). Similarly, 1500 bp *UBQ* and *Actin* promoters were appended to the front of *Luc* gene, respectively (Table S2). We transformed the plasmids of the dual luciferase reporter vectors of the promoter into the protoplasts of *E. grandiflorum* by PEG induction. After 21 h of incubation, we slowly aspirated the protoplasts from the Petri dish into a 2 mL tube, centrifuged it at 200 *g* for 1 min, and discarded the supernatant. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega; #E1910) in a Promega GloMax 20/20 luminometer (Promega; E5311), and compared the activity of different promoters by the ratio of Luc/Rluc.

CRISPR-Cas9 vector construction and guide RNA design

We used two CRISPR vector backbones for vector modification, the differences being in the *U6* promoters, the *Cas9* promoters and the Cas9 codons (Cas9-1 is a human code optimized *Cas9* gene, Cas9-2 is codon-optimized of soybean). One construction method is based on the model plant CRISPR skeleton as the original vector, where in the recognition of the target site by sgRNA depends on the recognition of the PAM sequence. This experimental approach is based on CRISPR-GE (Genome Editing), but also combines the principles of sgRNA design to select the target site. We generally used 20 bp upstream of the PAM as the target site, and designed three sgRNA sequences of the *PDS* gene of *E. grandiflorum*, named sgRNA-1, sgRNA-2 and sgRNA-3. The target sequence for sgRNA-2 contains a *BssS* I restriction site adjacent to the PAM. We used *Sap*I to digest the vector, and T4 ligase to connect sgRNA-2 to the vector to obtain the recombinant vector *pGmU6-sgRNA-2- pGmUBQ10-Cas9-2*. We used *Asc* I and *Sap* I to digest the recombinant vector. The promoters of *EgU6-2*, *EgU6-11* and sgRNA-1, sgRNA-2, sgRNA-3 were linked to the digested vector, six different CRISPR-Cas9 vectors were obtained, respectively named *pEgU6-2::sgRNA-1/pGmUBQ10::Cas9-2*, *pEgU6-2::sgRNA-2/pGmUBQ10::Cas9-2*, *pEgU6-2::sgRNA-3/pGmUBQ10::Cas9-2*, *pEgU6-11::sgRNA-1/pGmUBQ10::Cas9-2*, *pEgU6-11::sgRNA-2/pGmUBQ10::Cas9-2*, *pEgU6-11::sgRNA-3/pGmUBQ10::Cas9-2*. Then the six resultant CRISPR vectors were respectively digested with *BstE* II and *BamH* I, and the *pEgUBQ10* promoter was attached to the digested CRISPR vectors. The new recombinant vectors were named *pEgU6-2::sgRNA-1/pEgUBQ10::Cas9-2*, *pEgU6-2::sgRNA-2/pEgUBQ10::Cas9-2*, *pEgU6-2::sgRNA-3/pEgUBQ10::Cas9-2*, *pEgU6-11::sgRNA-1/pEgUBQ10::Cas9-2*, *pEgU6-11::sgRNA-2/pEgUBQ10::Cas9-2*, *pEgU6-11::sgRNA-3/pEgUBQ10::Cas9-2*.

In the other construction method, the original vector contains a CRISPR skeleton that uses the pCambia1300 Plant Expression Vector containing Cas9. In this approach, T4 ligase connects sgRNA-2 to the *AtU6-18T* vector, which has been digested with *Bbs* I. Then the *AtU6* promoter, sgRNA-2, and gRNA scaffold sequence recombinant fragments are joined to the expression vector 1300 containing Cas9, to obtain recombinant vector *pAtU6::sgRNA-2/pCDC45::Cas9-1*. The *AtU6-18T* cloning vector was digested with *Hind* III and *Bbs* I, *EgU6-11* as a template, the two restriction sites of *Bbs* I were affixed to the 3′ end of *EgU6-11* by PCR cloning. After the target fragment DNA was recovered and

purified by gel electrophoresis, the recombinant 18T vector was obtained using In-Fusion cloning. The restriction enzyme *Bbs* I was used to digest the recombinant 18T vector, T4 ligase was used to connect the sgRNA-2 of the *E. grandiflorum PDS* gene with 18T to obtain the recombinant the vector *EgU6-18T*. We then used *Hind* III and *Sal* I to digest the 1300 expression vector. Finally, the *EgU6-11* promoter, sgRNA-2 and the gRNA scaffold sequence were connected to the expression vector 1300 containing Cas9, to obtain the CRISPR vector named *pEgU6-11::sgRNA-2/pCDC45::Cas9-1*. All CRISPR vectors construction primers show in (Table S3).

Target mutation validation in the protoplasts

In the protoplast transient transformation experiment, to compare the editing efficiency of different CRISPR-Cas9 vectors, each constructed CRISPR-Cas9 vector and 35S-GFP-1301 expression vector was co-transformed into the *E. grandiflorum* protoplasts, and needs to undergo a minimum of 6 experimental repetitions (using 6 batches of protoplasts), and the protoplasts obtained from these 6 repetitions are subsequently pooled and collected after a 48-h incubation period, and genomic DNA was extracted using the CTAB method. To amplify the genomic region targeted by the sgRNA, three pairs of primers were designed. PCR conditions were 95°C for 3 min, 35 cycles of 95°C for 15 s, annealing (53°C–58°C) for 15 s, polymerization at 72°C for 30 s, followed by 72°C for 7 min. The target sequences were amplified by PCR using gene-specific primers (Table S4). The PCR product was digested by the *BssS* I restriction enzyme, target site sgRNA-2 all adopted the method of PCR-RE, and then undigested bands PCR products is subcloned into a T/A cloning vector, and clones containing a putative mutagenized target gene are subjected to sequencing. Others target sites sgRNA-1 and sgRNA-3 PCR products were cloned into TA/Blunt-Zero vectors (Vazyme, #C601-01), single colonies were sequenced. Monoclonal sequencing is guaranteed to be at least 30 successful, the total number of clones is N_t, and the number of clones with mutations is N_m. The sequencing results were compared using Geneious to analyze the mutation types of the target genes.

E. grandiflorum CRISPR-Cas9 vector editing efficiency statistics

This experiment was used to verify the editing efficiency of the *E. grandiflorum* CRISPR-Cas9 vector by means of transient expression, so we should first check the transfection efficiency of the plasmid under PEG induction. In this way, the editing capabilities of the CRISPR-Cas9 vector of *E. grandiflorum* can be assessed more accurately. By co-transforming with the 35S-GFP-1301 expression vector and the CRISPR vector, the GFP green fluorescent protein can emit green light under a fluorescent microscope. Then we can count the total number of protoplasts and the number of protoplasts transfected with GFP green fluorescent protein with a hemocytometer. We calculated the transient efficiency of the 35S-GFP-1301 expression vector co-transformed by different CRISPR-Cas9 vectors, and monoclonal sequencing calculates the likelihood of mutations. In the following equation, the transfection efficiency of *E. grandiflorum* protoplasts is W_1, the total number of clones is N_t, and the number of clones with mutations is N_m, the editing efficiency of the CRISPR-Cas9 vector for the *PDS* gene in *E. grandiflorum* was calculated to be $W=Nm/Nt/W1\times100\%$.

QUANTIFICATION AND STATISTICAL ANALYSIS

All experiments were independently repeated at least three times. Results are shown as means ± SEM. All statistical data analyses were performed using GraphPad PRISM version 8.0. the unpaired t-test was performed for two unpaired groups. *p value <0.05; **p value < 0.01; ***p value < 0.001.