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Development of an efficient and expedited cotyledon protoplast-based transient transformation system in *Gossypium hirsutum* L

WANG Zhicheng¹, CAO Shuaiting¹, CAI Qiuyan¹, GUAN Zhenhui¹, CHENG Hailiang^{1,2}, PENG Fanjia³, LI Yujun³, ZUO Dongyun^{1*}  and SONG Guoli^{1,2*}

Abstract

Background Cotton is an important crop providing the most natural fibers all over the world. The cotton genomics community has utilized whole genome sequencing data to construct an elite gene pool in which functional genes are related to agronomic traits. However, the functional validation of these genes is hindered by time-consuming and inefficient genetic transformation methods. Thus, establishing a transient transformation system of high efficiency is necessary for cotton genomics.

Results To improve the efficiency of transient transformation, we used the protoplasts isolated from the etiolated cotyledon as recipient. The enzymatic digestion buffer comprised 1.5% (w/v) cellulase, 0.75% (w/v) macerozyme, and 1% hemicellulase, osmotically buffered with 0.4 mol·L⁻¹ mannitol. After 5 h of dark incubation at 25 °C, uniform cotton protoplasts were successfully isolated with a yield of 4.6 × 10⁶ protoplasts per gram (fresh weight) and 95% viability. We incubated 100 μL protoplasts (2.5 × 10⁵·mL⁻¹) with 15 μg plasmid in the solution of 0.4 mol·L⁻¹ mannitol and 40% PEG 4000 for 15 min, ultimately achieving an optimal transient transfection efficiency of 71.47%.

Conclusions This transient system demonstrated effective utility in cellular biology research through successful applications in subcellular localization analyses, bimolecular fluorescence complementation (BiFC) verification, and prime editing vector validation. Through systematic optimization, we established an efficient and expedited protoplast-based transient transformation system and successfully applied this platform to cotton functional genomics studies.

Keywords Cotton, Cotyledon protoplast-based transient transformation, Prime editing, Protoplast isolation, Transient expression

*Correspondence:

Zuo Dongyun
zdy041@163.com
Song Guoli
sglzms@163.com

¹ State Key Laboratory of Cotton Bio-Breeding and Integrated Utilization, Institute of Cotton Research, Chinese Academy of Agricultural Sciences, Anyang, Henan 455000, China

² Zhengzhou Research Base, State Key Laboratory of Cotton Bio-Breeding and Integrated Utilization, School of Agricultural Sciences, Zhengzhou University, Zhengzhou 450001, China

³ Hunan Cotton Science Research Institute, Changde, Hunan 415000, China

Background

Cotton (*Gossypium hirsutum* L.) is the main source of natural fiber. Previous cotton genomic research has provided an elite gene pool in which genes are associated with agronomic traits (Liu et al. 2023). Although genomic selection could predict agronomic traits without dissecting causal genes, functional validation on target genes is still important in gene-driven breeding strategy (Liu et al. 2025). The first step for gene functional validation is the genetic transformation of target genes. Present genetic transformation pipelines are time-consuming and



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limit the progress of cotton functional genomic research. For the callus-based genetic transformation, the time is rather long (about 9–10 months) because of the dedifferentiation-redifferentiation process. Although the time of shoot apex-based transformation is shorter than callus-based one, the chimeras are inevitable which make the plant filtering complex and time-consuming (Ge et al. 2023). Thus, a new genetic transformation method overcoming the above two drawbacks should be developed.

The protoplast refers to a plant cell without cell wall. It has been widely used as the recipient of genetic transformation in many crops. In rice, protoplast transformation technology is extensively used for studies involving transient gene expression and photosynthesis processes. This includes analyses of subcellular localization and protein-protein interactions (Zhang et al. 2011). In peanut, the clustered regularly interspersed short palindromic repeats/CRISPR-associated protein 9 (CRISPR-Cas9) system has been employed to target allergen genes, demonstrating high efficiency in gene editing within protoplasts (Biswas et al. 2022). Furthermore, in jasmine, polyethylene glycol (PEG)-mediated protoplast transformation has been shown to be highly effective. This approach has led to the successful generation of transgenic plants, showcasing the potential of protoplast transformation in ornamental plants (Ahmed et al. 2021). Protoplasts, serving as transformation recipients, can be cultured in vitro to regenerate into whole plants (Jeong et al. 2021). In this case, only one cloned generation is necessary to pick up the desired homozygous results, accelerating the transformation process and avoiding chimeras. Although the transformation process necessitates cell wall regeneration and subsequent callus formation from protoplasts, which may involve longer timeframes or lower efficiencies compared with the conventional callus-based transformation systems, it still holds significant promise as a current transient transformation platform. Protoplast-based systems have already demonstrated their robust utility in functional gene validation and transient expression studies (Eeckhaut et al. 2013).

Recently, prime editing has been applied to plants. This technology is described as a “search-and-replace” genome editing method that mediates targeted insertions, deletions, and all 12 possible base-to-base conversions in living cells, it does not require double-stranded breaks (DSB) or donor DNA templates (Anzalone et al. 2019). Unlike base editing or Cas nuclease-mediated homology-directed repair (HDR), prime editing does not require a protospacer adjacent motif (PAM) sequence near the target sites, allowing for a wider range of target sites. The efficiency of prime editing varies significantly across different species and target selections (Lin et al. 2021; Sheri et al. 2025). Therefore, before genetic

transformation, it is crucial to utilize a time-efficient and effective protoplast transient transformation system for prime editor research. This approach allows for verifying the feasibility of applying prime editors in cotton, assessing differences in editing efficiency among various targets, predicting the editing efficiency for different targets, and optimizing prime editors accordingly.

In this study, we first explored a proper method to extract a number of protoplasts from cotton cotyledons. Based on enough protoplasts, we optimized the plasmid amount, PEG concentration, and incubation time to develop the cotton cotyledon protoplast-based transformation (CPT) system. In addition, the effectiveness of CPT was validated by subcellular localization, bimolecular fluorescence complementation (BiFC), and transformation of prime editing vectors. The CPT we developed in this research could be utilized widely in cotton genetic transformation, facilitating the cotton functional genomics research.

Materials and methods

Plant materials and growth conditions

The matured seeds of upland cotton (*G. hirsutum* L. acc. TM-1) were selected and sowed in pots (the volume ratio of vermiculite to soil is 2: 1) for 5–7 days (at 25 °C, and 50% relative humidity) to collect fully expanded cotyledons. Seedling cultivation is conducted under strictly controlled continuous darkness (0 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetically active radiation) to maintain etiolation. The collected explant materials were sterilized by 75% alcohol solution and washed for 3 times.

Vector construction and plasmid preparation

We used the prime editing system as described previously (Jin et al. 2023). The primary targets were *GhMYC2-like* and *GhPDS* genes, and the corresponding editing vectors were constructed. First, primers corresponding to the different targets and types of edits were designed (Table S1). After synthesis and purification by Sangon Biotech (Shanghai, China), these primers were used to clone the gRNA scaffold sequence from the gGTR empty vector. Subsequently, the inserted fragments were purified using the FastPure Gel DNA Extraction Mini Kit (Vazyme Biotech, Shanghai, China). The empty pRGB32-nCas9-H840A vector was double-digested using NEB restriction enzymes *Bsa* I and *Sbf* I-HF. The fragments obtained from cloning were inserted into the digested vector using the ClonExpress® Ultra One Step Cloning Kit (Vazyme Biotech, Shanghai, China) to obtain pRGB32-nCas9-H840A-PPE-GhMYC2-like&GhPDS, and likewise, obtained the paired prime editing vectors dual-pRGB32-nCas9-H840A-PPE-GhMYC2-like&GhPDS.

The panel of five engineered vectors [SG (4.0 kb), GFP-A (6.0 kb), GFP-B (8.0 kb), GFP-Amp (10.0 kb), and GFP-KanR (15.0 kb), derived from pre-existing constructs, encompasses a precisely controlled size gradient spanning from 4.0 to 15.0 kb, while universally retaining the green fluorescent protein (GFP) reporter gene as a functional expression marker. The pRI101-GFP and pRI101-GFP-GhMYB2 were used for subcellular localization; p326-104-GFP^N (GhSP2-GFP^N) and p326-106-GFP^C (GhFL-GFP^C) were used for BiFC assay (Cai 2024; Guan 2024).

All the constructed vectors (plasmid concentration > 1 000 ng·μL⁻¹) were transformed into *Agrobacterium tumefaciens* strain GV3101 via electroporation (voltage: 2.5 kV; pulse duration: 5 ms), following the standard protocols for transformation of plant-associated bacteria. The plasmids were extracted using the TIANprep Mini Plasmid Kit (TIANGEN biotech, Beijing, China).

Cotton cotyledon protoplast preparation and viability assessment

During preliminary optimization, four distinct concentration gradients were systematically evaluated for each enzymatic component, followed by temporal profiling of enzymatic hydrolysis spanning 1–7 h. Empirical results from these parameter screenings (enzyme ratios detailed in Table S2) guided the subsequent optimization process. An enzymatic digestion solution was formulated by combining 1.5% (weight per volume, w/v) Cellulase R10 (Yakult, Tokyo, Japan), 0.75% (w/v) Macerozyme R10 (Yakult, Tokyo, Japan), 1% (w/v) Hemicellulase (Sigma-Aldrich, Shanghai, China), 0.4 mol·L⁻¹ mannitol, 20 mmol·L⁻¹ KCl, 20 mmol·L⁻¹ 2-Morpholinoethanesulphonic acid (MES, pH 5.7), 10 mmol·L⁻¹ CaCl₂, and 0.1% (w/v) bovine serum albumin (BSA) in a 10 mL volume. The mixture was sterilized using a 0.22 μm pore-size Millipore Express® PES membrane filter and chilled to 4 °C. Healthy cotyledons of upland cotton were surface-sterilized, flattened on sterile Petri dishes, and sectioned into 0.5–1.0 mm transverse strips using a surgical scalpel under laminar flow conditions. Approximately 1.0 g of leaf strips were subsequently incubated in the pre-chilled enzyme solution with gentle orbital shaking (100 r·min⁻¹) at 25 °C in darkness for 5–6 h until complete tissue maceration was achieved, as evidenced by epidermal separation and a characteristic yellowish-brown supernatant. The enzymatic reaction was terminated through the addition of an equal volume of W5 buffer (2 mmol·L⁻¹ MES, 154 mmol·L⁻¹ NaCl, 125 mmol·L⁻¹ CaCl₂, 5 mmol·L⁻¹ KCl), followed by gentle agitation to neutralize residual enzymatic activity. The lysate was filtered through a 40 μm nylon mesh into 10 mL conical centrifuge tubes, after which protoplasts were pelleted

via horizontal centrifugation at 100 g for 2 min at 4 °C. The supernatant was aspirated carefully, and the pellet was resuspended in 2 mL ice-chilled W5 solution for 30 min on ice to promote further purification through gravitational sedimentation. Finally, purified protoplasts were reconstituted in MMG solution (4 mmol·L⁻¹ MES, 15 mmol·L⁻¹ MgCl₂, 0.4 mol·L⁻¹ mannitol) and maintained at 4 °C for downstream applications.

The protoplast yield was determined using a standard hemocytometer and light microscopy. Protoplast viability was assessed by incubating samples with fluorescein diacetate (FDA) at an optimal concentration (0.01% w/v) for 30 min in darkness. Fluorescent signals generated by esterase-catalyzed FDA hydrolysis in live cells were analyzed using epifluorescence microscopy (excitation/emission: 485/535 nm), with viability quantified as the percentage of fluorescein-positive protoplasts relative to the total counted cells ($n \geq 200$ per replicate). All reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

PEG-mediated protoplast transfection

PEG-mediated protoplast transfection was carried out as described previously with modifications (Mathur et al. 1998). For each transfection, 10 μL (8–19 μg) plasmid were gently mixed with 100 μL protoplasts ($2.5 \times 10^5 \cdot \text{mL}^{-1}$) in a 2 mL centrifuge tube. Then, 110 μL freshly prepared PEG solution [10%–60% (w/v) PEG 4000, 0.2 mol·L⁻¹ mannitol, 0.1 mol·L⁻¹ CaCl₂] were added and mixed gently. The transfection mixture was incubated in the dark for about 10 min. The transformation was stopped by adding 440 μL of W5 solution and mixing gently by inverting up and down 3–4 times. After centrifuging at 100 g for 4 min, the supernatant was removed, and the protoplasts were resuspended by adding 1 mL W5 solution. The transfected protoplast was incubated at 25 °C for 12 h in darkness. The incubation time can be appropriately extended based on the plasmid used in the transfection. All reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA; Table S3).

Verification of prime editor-mediated precise mutations in cotton protoplasts

PCR assay was used to assess the activity of the targeting vectors. The transformed protoplast collected and resuspended for more than 72 h were cultured in suspension. Then the samples were centrifuged at 100 g for 2 min to collect the transformed protoplasts. After the supernatant was removed, the collected protoplast samples were frozen in liquid nitrogen for 3–5 min. The frozen samples were thawed at room temperature, and then supplemented with 50 μL of sterile water. The

re-suspended protoplasts were heated on a heating block at 95 °C for 10 min and cooled at 4 °C for about 3 min. The protoplast suspension was used as template to perform PCR (forward primer: 5'-ggagtgagtacgggtgc-NNNNNNNNNNNNNN-3'; reverse primer: 5'-gagttg-gatgctggatggNNNNNNNNNNNNNN-3'). The PCR product was sent for next-generation sequencing (high-throughput tracking of mutations, Hi-TOM) to check for gene mutations at the target site (Liu et al. 2019).

Fluorescence microscopic examination

The plasmid pRI101-GFP was used to calculate the transformation efficiency of protoplasts and assess protein subcellular localization. The transformation efficiency (%) was calculated by dividing the protoplast number with bright green fluorescence by the total protoplast number. GFP fluorescence was observed under an OLYMPUS BX53 F Confocal Microscope for GFP (Olympus Corp, Tokyo, Japan). Images were acquired with a Leica TCS SP8 STED 3× Confocal Microscope (Leica Microsystems Inc, USA) using excitation wavelengths of 488 nm (GFP).

Results

Development of a method to extract protoplast from cotton cotyledons

The first step of cotton CPT is to obtain protoplasts of high yield. Previously reported researches indicated that explants with and without light exhibited different utility in protoplasts extraction (Lv et al. 2023). Here, we used cotyledons growing with and without light as the explants for cotton protoplast isolation. After the enzymatic digestion described in Methods part, we found

that explants without light underwent faster enzymatic breakdown than those with light. Moreover, the protoplasts extracted from etiolated explants showed higher purity than those extracted from normal explants (Fig. 1). This results showed that etiolated cotyledons (explants without light) were suitable for subsequent steps. After determining the best explant type, we need to furtherly improve the efficiency of enzymatic digestion.

According to the previous researches, cellulase and macerozyme played an important role in protoplast extraction (Aoyagi 2006). Therefore, we set different cellulase concentrations (0.5%, 0.75%, 1.5%, and 3%) for protoplast extraction, and found that 1.5% was the best considering both protoplast yield and viability (Fig. 2a, b; Table S2). We also explored that 0.75% was the most suitable concentration of macerozyme (Fig. 2c, d; Table S2). To address the toxic effects brought by macerozyme on protoplasts, we introduced hemicellulase into the digestion solution and found that 1% was the best concentration (Fig. 2e, f; Table S2). After thorough consideration of both yield and viability of protoplasts, we concluded that the optimal enzyme concentration composition for the protoplast enzymatic digestion solution is 1% cellulase, 0.75% macerozyme, and 1% hemicellulase. Although the concentrations of components (1.5% cellulase, 0.75% macerozyme, 1% hemicellulase) in solution have been determined, we need to ensure the best time for digestion. We set a different time intervals (from 1 to 7 h, 1 h as the interval) for enzymatic digestion, and found that 5 h enzymatic digestion had the highest protoplast yield of $4.6 \times 10^6 \cdot g^{-1}$ (Fig. 2g). The optimized enzymatic digestion regimen comprising 1.5% (w/v) cellulase, 0.75%

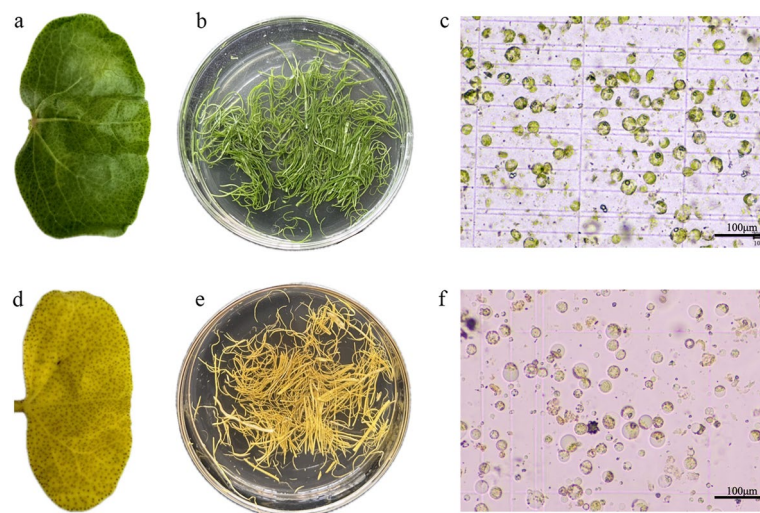


Fig. 1 Comparison of protoplast hydrolysis between green cotyledons and etiolated cotyledons. **a** green cotyledons; **b** green cotyledons in enzymatic hydrolysis; **c** green cotyledons after enzymatic hydrolysis observed under the microscope; **d** etiolated cotyledons; **e** etiolated cotyledons in enzymatic hydrolysis; **f** etiolated cotyledons after enzymatic hydrolysis observed under the microscope. Bars = 100 μm

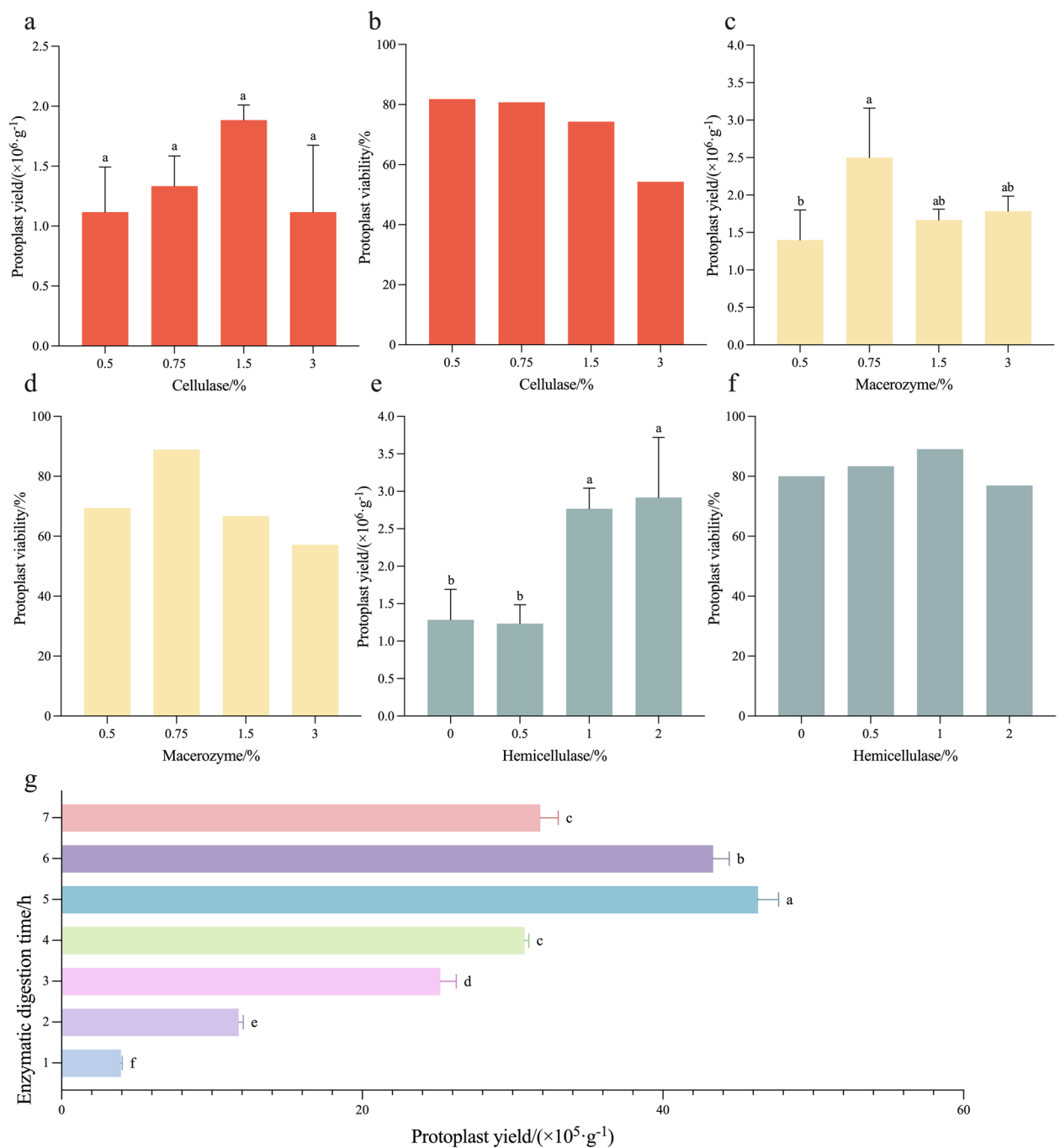


Fig. 2 Protoplast yield and viability at different enzyme concentrations and digestion times. The yield of protoplasts isolated with solutions containing different concentrations of (a) cellulase, (c) macerozyme, and (e) hemicellulase. The viability of protoplasts isolated with solutions containing different concentrations of (b) cellulase, (d) macerozyme, and (f) hemicellulase. g Protoplast yield at different digestion time. Different lowercase letters indicate significant difference ($P < 0.05$)

(w/v) macerozyme, and 1% (w/v) hemicellulase achieved maximal protoplast yield of $4.6 \times 10^6 \cdot g^{-1}$ (fresh weight) following 5 h enzymatic incubation. The viability of enzymatically isolated protoplasts was assessed via FDA staining. Fluorescence microscopic

analysis demonstrated that a single enzymatic digestion cycle routinely yields protoplast populations with an overall viability of approximately 95% (Fig. 3).

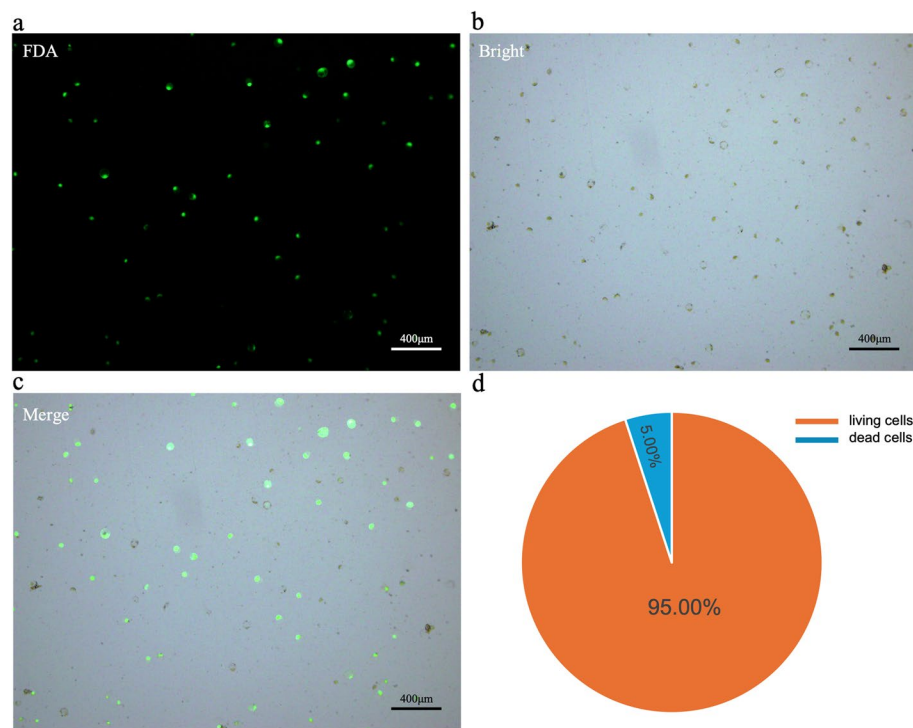


Fig. 3 Protoplast viability identification by FDA staining. **a** FDA staining results. **b** The results of brightfield which containing all the cells. **c** The merged results of **(a)** and **(b)**. **d** Cell viability assay result after FDA treatment. Bars = 400 μ m

Optimization on protoplast-based transient transformation

Since the suitable pipeline for protoplast extraction has been developed, optimization on transient transformation system is also necessary. Here, we used GFP as the reporter to construct pRI101-GFP vector for a PEG- Ca^{2+} -mediated genetic transformation on protoplast solution (2.5×10^5 protoplasts per mL) which is widely used in protoplast transient transformation. The previous optimization of the transformation included factors such as PEG concentration, Ca^{2+} concentration, the amount of plasmid, co-cultivation time, transformation time, and post-transformation culture reagents (Mathur et al. 1998). We conducted preliminary transient transformation experiments by referring to the published protoplast transient transformation system. However, the expression of GFP was observed in only 10% protoplasts, exhibiting low utility (Fig. 4). Therefore, it is necessary to optimize the transformation system for further applications.

In this research, we took 4 factors (plasmid size and amount, PEG concentration, and incubation time) into consideration for optimizing the genetic transformation system. We used 100 μ L protoplast solution in subsequent exploration. Plasmid is the most important cargo in genetic transformation implied that the amount and size of plasmid may influence the efficiency of genetic

transformation. We constructed 5 plasmid vectors with different sizes (SG, GFP-A, GFP-B, GFP-amp, and GFP-KanR, from short to long) to perform genetic transformation. The transforming results showed that longer plasmid vector had lower protoplast transformed efficiency, decreasing from 36.99% (transformed by SG) to 9.49% (transformed by GFP-KanR) (Fig. 5a). Although plasmid vectors with large size were difficult to be transformed, it could be overcome by more plasmids. As the amount of GFP-KanR increased, the transformation efficiency increased from 18.40% (8 μ g) to 36.85% (15 μ g). When the plasmid was over 15 μ g, the transformation efficiency started to decrease, indicating that the most suitable plasmid amount is 15 μ g (Fig. 5b).

PEG- Ca^{2+} mediated transformation method is diverse among different crops, yet the most suitable PEG-concentration is unknown for different plant materials, such as cotton cotyledon. Here, we used pRI101-GFP (11.1 kb) as plasmid vector, and set up 6 treatments [PEG 4000 concentration was from 10% (w/v) to 60%, with an interval of 10%] to perform genetic transformation. The results showed that transformation efficiency increased from 3.33% to 23.90% as the PEG concentration was increased from 10% to 40%, and the transformation efficiency decreased when PEG concentration was over 40%

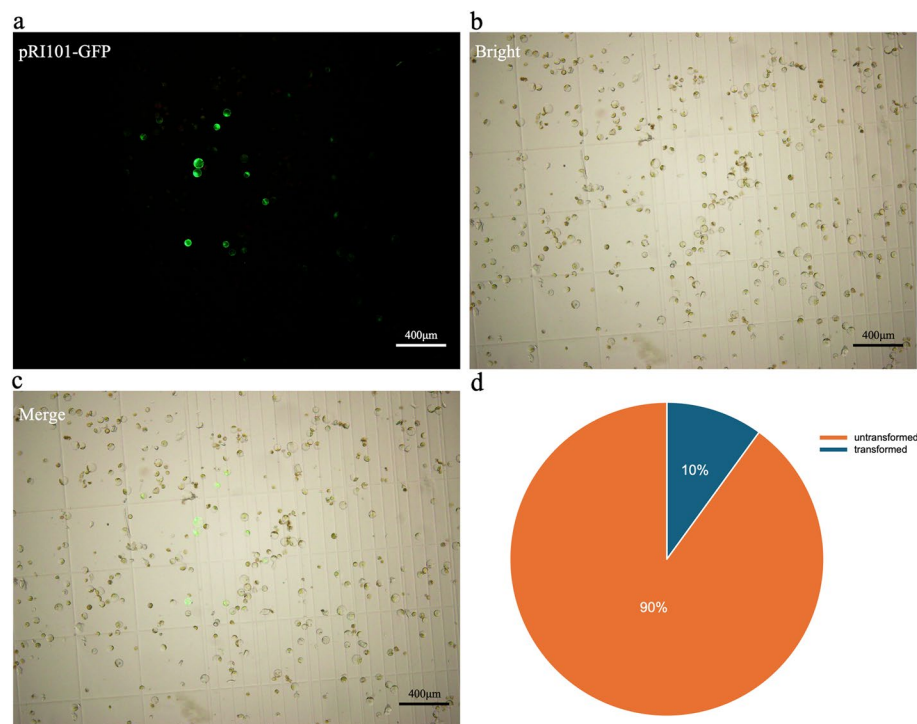


Fig. 4 Preliminary transformation results of pRI101-GFP. **a** The protoplast that was successfully transformed. **b** The results of brightfield which containing all the cells. **c** The merged results of (a) and (b). **d** The transformation efficiency of protoplasts using pRI101-GFP vector. Bars = 400 μ m

(Fig. 5c). We used 40% as the best PEG concentration in subsequent genetic transformation.

Incubation of protoplast and plasmid is the last step in genetic transformation system, and the importance of incubation time has been proved before (Ma et al. 2024). To explore the best incubation time, 4 incubation times (5 min, 10 min, 15 min, and 20 min) were set up to conduct protoplast-based transient transformation. We noticed that 15 min incubation had the highest transformation efficiency (50.98%), which is significantly higher than that of 5 min and 20 min (Fig. 5d).

The integration of the optimizations is the cotton CPT system. CPT could be summarized as follows: the sliced etiolated cotyledons were used to extract protoplast for 5 h with the solution of 1% cellulase, 0.75% macerozyme, and 1% hemicellulase. The plasmid is 15 μ g, PEG 4000 concentration is 40% (w/v), and incubation time is 15 min. The final transformation efficiency of cotton CPT reached at 71.47% (Fig. 6).

Utility of cotton CPT

Since the cotton CPT system was constructed, we need to evaluate its utility in functional genomics research. Protoplast-based subcellular localization analysis is a crucial method for preliminarily studying gene functions. Here, we used CPT system to conduct subcellular

localization experiment for a nuclei-located protein, GhMYB2 (Yang et al. 2013). The green fluorescence was observed in nuclei, indicating CPT was a convenient tool for subcellular localization experiment (Fig. 7).

BiFC is an important technology to validate protein-protein interaction in vivo. To evaluate the utility of CPT in BiFC experiment, we used an interacted vector pair (p326-104-GFP^N and p326-106-GFP^C, which contain *GhSP2* and *GhFL*, respectively) for the experiment. After incubation in darkness for 24 h, GFP fluorescence was observed under a laser scanning confocal microscope, while the negative control did not show GFP fluorescence (Fig. 8). This results indicated that CPT could be also applied in BiFC experiment.

Fast and efficient validation of prime editing vectors using cotton protoplasts

Based on the CPT system established, we were delighted to discover that using a prime editing vector containing a GFP reporter system resulted in a transformation efficiency of 30% to 50%. Therefore, we believe that this system can be utilized for the preliminary validation of prime editing feasibility in cotton. Following the completion of *Gossypium hirsutum* genome sequencing, *GhMYC2-like* and *GhPDS* were selected as target genes to assess the efficacy of

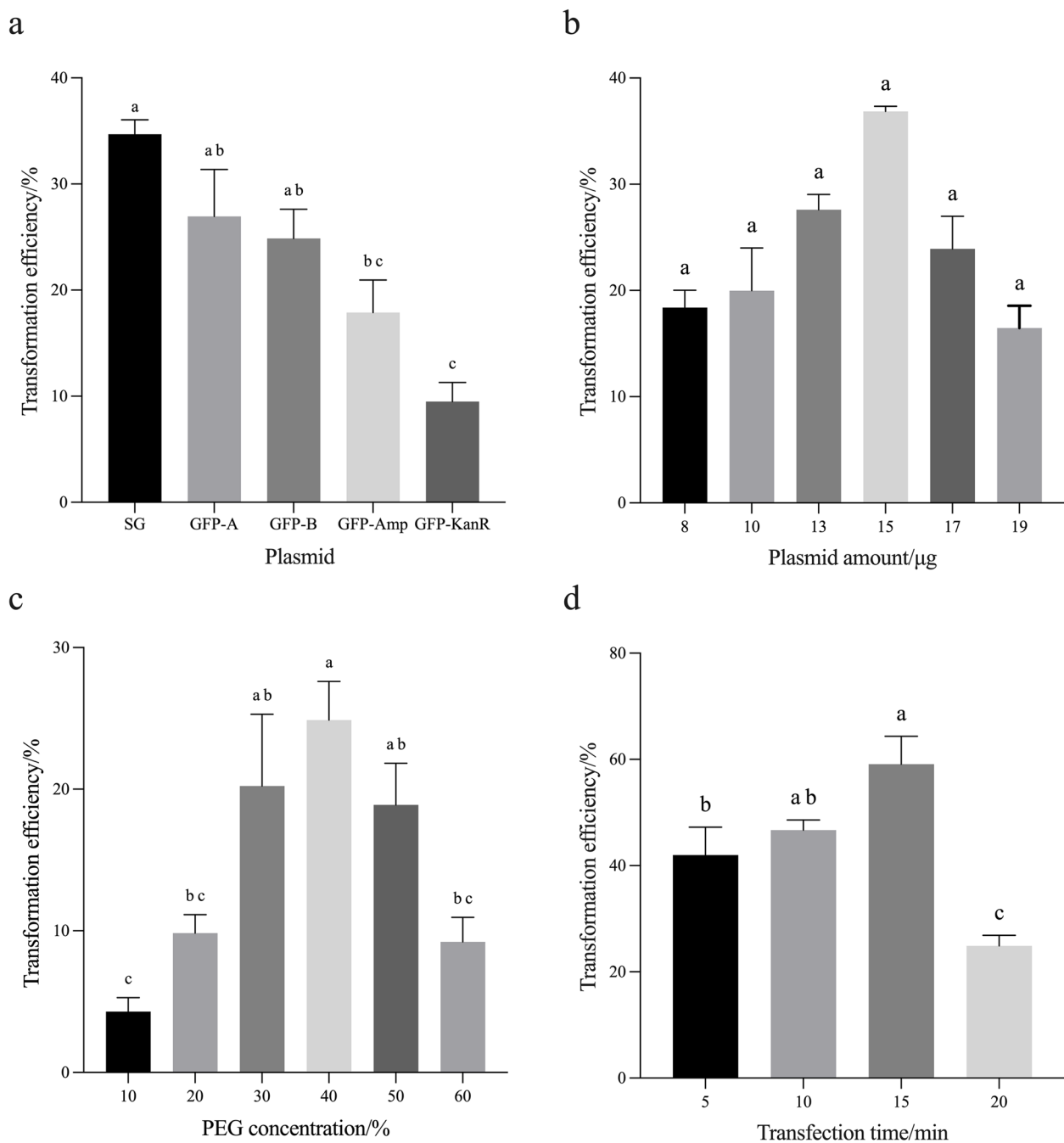


Fig. 5 Transformation efficiency under different vector sizes (a), different dosages of GFP-KanR plasmid (b), different PEG 4000 concentrations (c), and different incubation times (d). The vector size increases from the SG vector to the GFP-KanR vector. Different lowercase letters indicate significant difference ($P < 0.05$)

prime editing systems in cotton (Ma et al. 2018). We used paired prime editing vectors dual-pRGB32-nCas9-H840A-PPE-GhMYC2-like&GhPDS for the transient transformation of protoplasts. The protoplasts were gathered through centrifugation after incubation in the dark at room temperature for 72 h. We extracted

genomic DNA from the collected protoplasts, which were then used as the templates for PCR amplification with specific sequencing primers designed for the target regions, as shown in Table S1. The PCR products were subjected to agarose gel electrophoresis (Fig. S1), and bands of interest were selected for next-generation

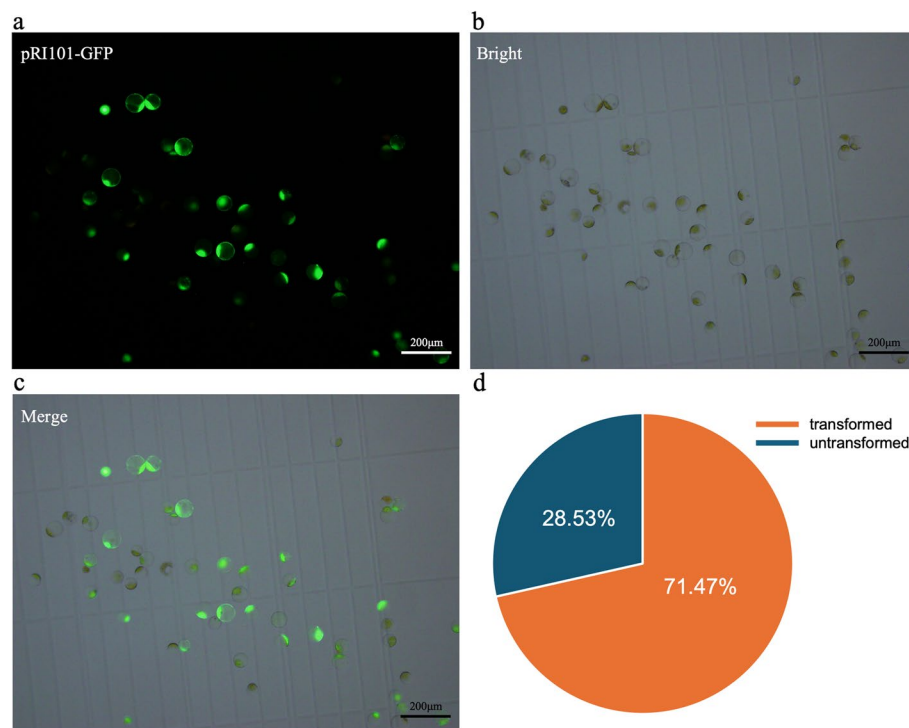


Fig. 6 Transformation results of pRI101-GFP by CPT system. **a** Protoplast that was successfully transformed. **b** Results of brightfield which containing all the cells. **c** Merged results of (**a**) and (**b**). **d** Transformation efficiency of protoplasts using pRI101-GFP vector. Bars = 200 μm

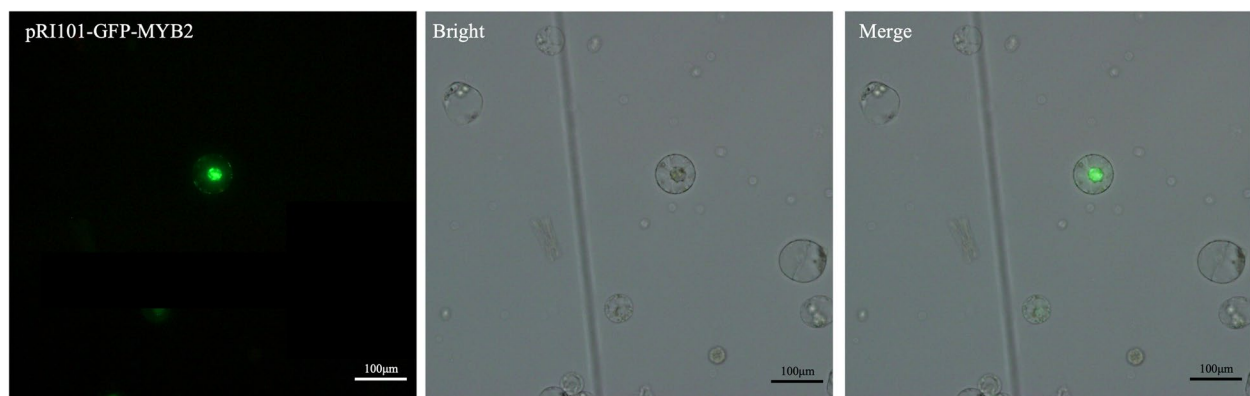


Fig. 7 Subcellular localization of GhMYB2. pRI101-GFP-MYB2 was transiently expressed in protoplast derived from cotton etiolated cotyledons. Bars = 100 μm

sequencing. Deep sequencing analysis of the *GhMYC2-like* target gene revealed that merely 0.28% of its locus exhibited prime editing proximal to the PAM site, while the remaining sites (99.72%) displayed complete maintenance of genomic integrity with no detectable indels or unintended modifications (Fig. 9).

Discussion

In this study, we developed a rapid and efficient protocol for cotton cotyledon protoplast isolation and transient transformation. The optimized protoplast isolation system and transient transformation system successfully achieved high viability, yield, and transient transformation efficiency. This protocol significantly reduces time and resources required for protoplast

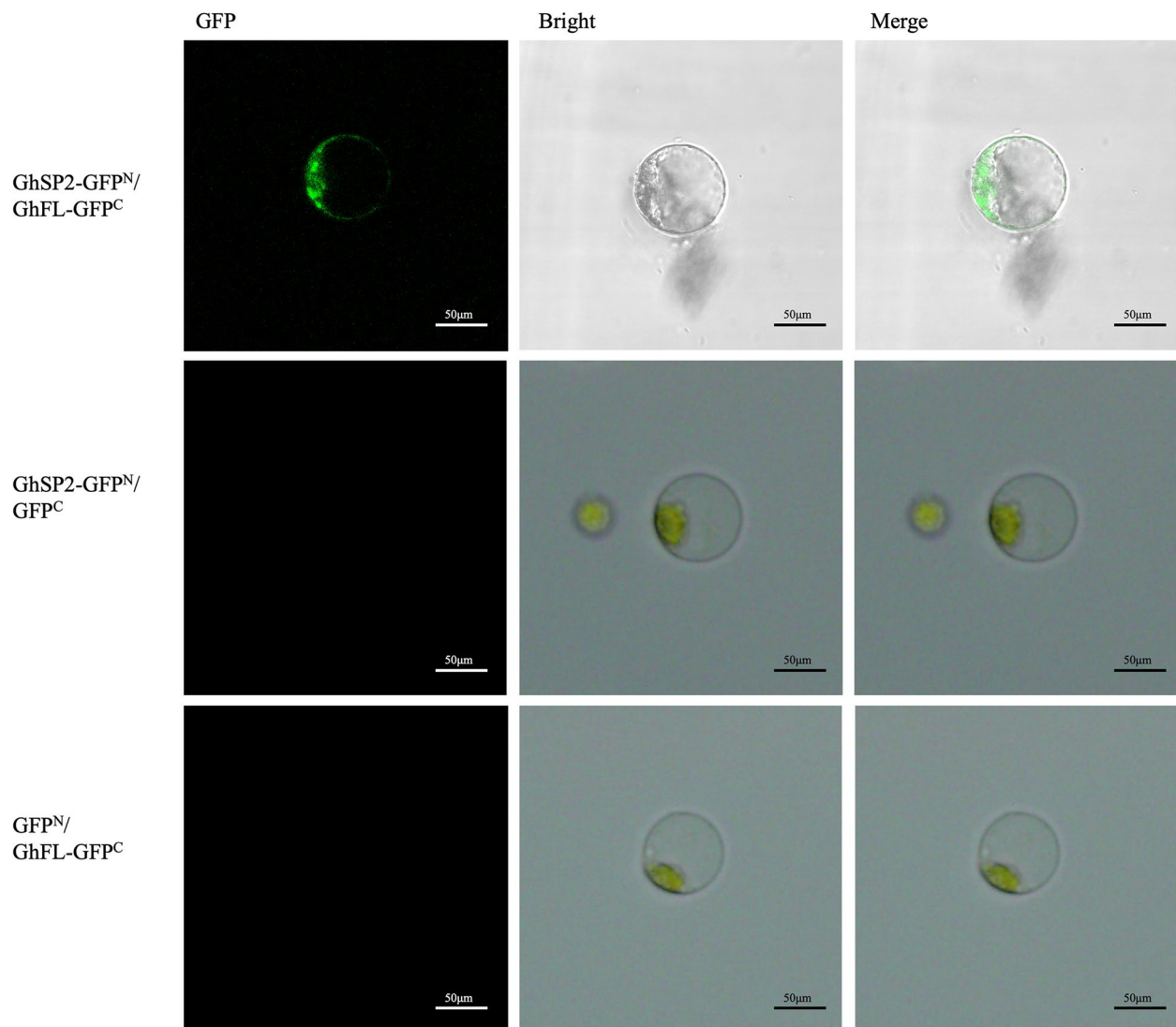


Fig. 8 Protein–protein interaction assays by BiFC in cotton etiolated cotyledons. The paired constructs p326-104-GFP^N(GhSP2-GFP^N) and p326-106-GFP^C (GhFL-GFP^C) were transiently co-expressed in protoplast. GhSP2-GFP^N or GhFL-GFP^C with the empty vector GFP^N or GFP^C were used as negative controls. Bars = 50 µm

1	7321	48.86%	SNP	T->A	GTCCAGTTTCGAGAGGAAAAGGTCGGGAAAAGATCAGGTGCAAGCTTATTTTAATGAAGTGATGGACGTGGACCGAATGGTAGATGGCGATGTGACTGATTATGAGTGGTACTATACCGTATC
2	6429	42.90%	WT	-	GTCCAGTTTCGAGAGGAAAAGGTCGGGAAAAGATCAGGTGCAAGCTTATTTTAATGAAGTGATGGACGTGGACCGAATGGTAGATGGCGATGTGACTGATTATGAGTGGTACTATACCGTATC
3	246	1.64%	SNP	T->A	GTCCAGTTTCGAGAGGAAAAGGTCGGGAAAAGATCAGGTGCAAGCTTATTTTAATGAAGTGATGGACGTGGACCGAATGGTAGATGGCGATGTGACTGATTATGAGTGGTACTATACCGTATC
4	96	0.64%	SNP	A->G	GTCCAGTTTCGAGAGGAAAAGGTCGGGAAAAGATCAGGTGCAAGCTTATTTTAATGAAGTGATGGACGTGGACCGAATGGTAGATGGCGATGTGACTGATTATGAGTGGTACTATACCGTATC
5	83	0.55%	SNP	G->A, T->A	GTCCAGTTTCGAGAGGAAAAGGTCGGGAAAAGATCAGGTGCAAGCTTATTTTAATGAAGTGATGGACGTGGACCGAATGGTAGATGGCGATGTGACTGATTATGAGTGGTACTATACCGTATC
6	55	0.37%	SNP	A->G, T->A	GTCCAGTTTCGAGAGGAAAAGGTCGGGAAAAGATCAGGTGCAAGCTTATTTTAATGAAGTGATGGACGTGGACCGAATGGTAGATGGCGATGTGACTGATTATGAGTGGTACTATACCGTATC
7	50	0.33%	SNP	G->A, T->A	GTCCAGTTTCGAGAGGAAAAGGTCGGGAAAAGATCAGGTGCAAGCTTATTTTAATGAAGTGATGGACGTGGACCGAATGGTAGATGGCGATGTGACTGATTATGAGTGGTACTATACCGTATC
8	44	0.29%	WT	-	GTCCAGTTTCGAGAGGAAAAGGTCGGGAAAAGATCAGGTGCAAGCTTATTTTAATGAAGTGATGGACGTGGACCGAATGGTAGATGGCGATGTGACTGATTATGAGTGGTACTATACCGTATC
9	42	0.28%	SNP	G->A	GTCCAGTTTCGAGAGGAAAAGGTCGGGAAAAGATCAGGTGCAAGCTTATTTTAATGAAGTGATGGACGTGGACCGAATGGTAGATGGCGATGTGACTGATTATGAGTGGTACTATACCGTATC
10	38	0.25%	SNP	T->C, T->A	GTCCAGTTTCGAGAGGAAAAGGTCGGGAAAAGATCAGGTGCAAGCTTATTTTAATGAAGTGACCGGACGTGGACCGAATGGTAGATGGCGATGTGACTGATTATGAGTGGTACTATACCGTATC
11	38	0.25%	SNP	A->G, T->A	GTCCAGTTTCGAGAGGAAAAGGTCGGGAAAAGATCAGGTGCAAGCTTATTTTAATGAAGTGATGGACGTGGACCGAATGGTAGATGGCGATGTGACTGATTATGAGTGGTACTATACCGTATC
12	23	0.15%	SNP	G->A	GTCCAGTTTCGAGAGGAAAAGATCAGGTGCAAGCTTATTTTAATGAAGTGATGGACGTGGACCGAATGGTAGATGGCGATGTGACTGATTATGAGTGGTACTATACCGTATC

Fig. 9 Next-generation sequencing results. PAM locus is in blue, and the non-specific mutation loci is in red. The columnar arrangement (from left to right) delineates sequencing analysis outcomes as follows: identified mutation types detected in sequencing, quantitative distribution of observed mutations, proportional representation (%) in total sequencing reads, taxonomic classification of mutational subtypes, specific mutational characteristics (base change patterns), systematized collation of sequencing data outputs

isolation and transformation compared with traditional methods.

Protoplasts are universally acknowledged as optimal targets for transient genetic modification, with their utilities in plant biology research, such as the preservation of genetic diversity, cellular hybridization, and the detection of mutants, thereby heralding expansive potential for plant breeding innovations (Reed et al. 2021). This investigation is predicated on devising a streamlined and efficient protocol for the isolation and transient genetic modification of protoplasts derived from the etiolated cotyledons of upland cotton. This initiative aspires to enrich the foundational research on cotton protoplasts, and to catalyze the refinement and application of prime editing methodologies in cotton.

In the selection of materials for protoplast isolation, etiolated cotyledons have been used in rice and maize (Lv et al. 2023; Jiang et al. 2018), but similar reports in cotton are absent. The findings indicate that etiolated cotyledons yield higher purity protoplasts compared with green cotyledons, making them superior for cotton protoplast isolation. The enzymatic method for isolating protoplasts requires precise proportions of enzyme components in the digestion solution. The types and concentrations of enzymes used for protoplast isolation vary depending on the plant species or the tissues within the same species. Specifically, for cotton, root cells yield high-quality and viable protoplasts in a digestion solution containing 1.5% cellulase and 0.75% macerozyme (Zhang et al. 2023b); for mesophyll cells, the optimal enzyme combination is 1.5% cellulase and 0.4% macerozyme (Li et al. 2014); while for cotton callus tissues, the best combination includes 1.5% cellulase, 0.5% macerozyme, and 0.5% hemicellulase (Wang et al. 2022). In this research, after exploring various types and concentrations of enzyme components, an enzyme solution containing 1.5% cellulase, 0.75% macerozyme, and 1% hemicellulase was found to successfully obtain protoplast yield and viability comparable to those achieved with an 8–9 h digestion, but within a significantly reduced digestion time of 5 h. This greatly shortens the enzymatic digestion period without compromising protoplast yield and viability.

Among the various factors that could affect the protoplasts efficiency of PEG-mediated transient transformation (Shen et al. 2014), we selected four primary factors (PEG concentration, plasmid amount and size, and transfection duration) for experimentation on isolated etiolated cotyledon protoplasts. The CPT system ultimately determined the optimal conditions to be 2.5×10^5 protoplasts·mL⁻¹, 40% PEG 4000, 15 µg plasmid, and 15 min of transfection time. In PEG-mediated transient transformation of protoplasts, the required PEG concentration varies among different plants. Studies have

shown that the optimal PEG concentration for PEG-mediated transformation in *Arabidopsis* mesophyll cells and cassava protoplasts were 20% and 25%, respectively (Yoo et al. 2007; Wu et al. 2017). In this study, the optimal PEG concentration for cotton cotyledon protoplast transformation was found to be 40%, which is consistent with the optimal PEG concentration for protoplasts isolated from other cotton tissues (Li et al. 2014; Wang et al. 2022; Zhang et al. 2023b). This indicates that the PEG concentration required for transient transformation of protoplasts does not significantly differ among different tissues of cotton. This study indicated that the optimal plasmid amount for transfecting cotton cotyledon protoplasts is 15 µg, which is consistent with the results previously observed (Li et al. 2014). However, the optimal plasmid amounts for protoplasts from cotton roots and callus tissues were 20 µg and 16 µg, respectively, indicating variability within the same plant (Zhang et al. 2023b; Wang et al. 2022). The optimal transfection time for cassava protoplasts was 10 min, while for cotton root cells it was 20 min, suggesting that transfection time varies across different plants and tissues (Zhang et al. 2023b; Wu et al. 2017). Future protoplast studies should determine the optimal transfection time based on the specific plants and tissues. Additionally, it was observed that plasmid size significantly impacts transformation efficiency. However, this is not an absolute rule. The transient transformation efficiency could be enhanced by increasing the amount of larger plasmids, whereas smaller plasmids are less demanding on the plasmid amount. It is generally believed that the plasmid concentration for transient transformation in protoplasts needs to exceed 1 000 ng·µL⁻¹ (Shan et al. 2014). This finding is particularly important for subsequent prime editing experiments.

The developed CPT system offers a rapid and effective means of investigating gene functions, including subcellular localization, protein-protein interaction verification, and prime editors. This system addresses the limitations of *Agrobacterium*-mediated transformation in model plants when dealing with genes exhibiting species-specific expression patterns (Azizi-Dargahlou et al. 2024). The application of prime editing has been successfully implemented in plant species such as wheat, rice, maize, and tobacco (Lin et al. 2020; Jiang et al. 2020; Zhang et al. 2023a; Ni et al. 2023), but related studies in cotton have not been reported. One of the challenges of using protoplasts for prime editing in cotton is the large size of the prime editing vectors, which typically hinders high-efficiency transient transformation in protoplasts. However, in this research, it was pleasantly surprising to find that using a GFP-reporter system with prime editing vectors achieved a high transformation efficiency of 30% to 50% in cotton cotyledon protoplasts, which

successfully reached the same level as the reported in rice protoplasts using prime editing vectors (28%–45%) (Jin et al. 2023). It can undoubtedly greatly reduce the interference of non-transformed protoplasts for our subsequent detection of editing efficiency, thus facilitating the detection of editing outcomes. This indicates that the CPT system can validate the feasibility of prime editing in cotton. Although the overall editing efficiency was still low and precise editing results were not detected, the paired prime editing vectors demonstrated that prime editing vectors can function in cotton. With further understanding of DNA repair mechanisms and optimization of the prime editing system, the CPT system could be used to construct a prime editing library (Yan et al. 2024). This would enable the prediction of editing efficiency for various editing types and sites in cotton, advancing the application of prime editing in cotton biotechnology.

Conclusion

An optimized protocol for high-efficiency isolation and transient transformation of etiolated cotyledon-derived protoplasts from upland cotton was established. The enzymatic digestion regimen employing 1.5% cellulase, 0.75% macerozyme, and 1% hemicellulase yielded 4.6×10^6 protoplasts per gram (fresh weight) via 5 h incubation. The maximal transient efficiency of 71.47% was obtained with the optimized conditions. The CPT system could be utilized in subcellular localization analyses, BiFC, and prime editing vector validation in cotton. Thus, the CPT system offers a valuable platform for accelerating the development and application of advanced genetic tools in cotton, ultimately contributing to the improvement of cotton varieties and the advancement of cotton biotechnology.

Abbreviations

BiFC	Bimolecular fluorescence complementation
CPT	Cotton cotyledon protoplast-based transformation
CRISPR-Cas9	Clustered regularly interspersed short palindromic repeats/CRISPR-associated protein 9
DSB	Double-stranded breaks
FDA	Fluorescein diacetate
GFP	Green fluorescent protein
HDR	Homology directed repair
PAM	Protospacer adjacent motif
PEG	Polyethylene glycol

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s42397-025-00227-z>.

Additional file: Table S1. Primer information. Table S2. The concentration of three enzymes used in different groups. Table S3. Kits and reagents information used in this research. Fig. S1. Agarose gel electrophoresis of protoplast PCR product

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Authors' contributions

Song GL, Zuo DY, and Wang ZC conceived and designed the research. Wang ZC, Cao ST, Cai QY, and Guan ZH performed the experiment. Wang ZC wrote the manuscript. Song GL, Cheng HL, Peng FJ, and Li YJ revised the manuscript.

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Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests. Author Song GL is an associate editor of *Journal of Cotton Research*, he was not involved in the journal's review of, or decision related to this manuscript.

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