

Establishment of a Transient Expression System in Jute Protoplasts

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Abstract

Jute is a stem fiber crop with important economic value, but functional analysis of candidate genes is constrained by inefficient and genotype-dependent transformation. Here, we established a rapid polyethylene glycol (PEG)-mediated transient expression system using cotyledon-derived protoplasts from 7- 12-day-old aseptic seedlings of cultivar Meifeng No. 4 (MF4). Cotyledons were digested for 5 h with 1.5% Cellulase R-10 and 1.0% Macerozyme R-10 in 0.5 mol L⁻¹ mannitol yielded up to 1.34×10^7 viable protoplasts g⁻¹ fresh weight, with > 90% viability. Transformation was maximized with 0.3 mol L⁻¹ CaCl₂, 0.5 mol L⁻¹ mannitol, 50% PEG 4000, 30 µg plasmid DNA, and a 75-min incubation, producing 59.52% GFP-positive cells. The platform accurately confirmed the nuclear localization of CcWRKY70 and successfully visualized/confirmed the HcMYB50-HcTT8a interaction in a bimolecular fluorescence complementation assay. This simple and reproducible jute-native system enables rapid subcellular-localization and protein-interaction analyses and provides a practical tool for gene-function studies in jute and related bast-fiber crops.

Introduction

Jute (*Corchorus capsularis* L.), widely known as the “golden fiber,” is a major renewable bast-fiber crop cultivated principally in Asia(Zhang et al., 2015). In addition to paper and textiles, jute is used in cellulose and stem-development research(Li et al., 2024), serves as a feedstock for nanocellulose and other value-added materials(Farooq et al., 2024). Beyond the fiber itself, jute sticks and processing residues also support additional industrial uses, including gas-purification adsorbents(Dutta et al., 2024). Protoplast-mediated transient transformation verification system has been applied in a variety of model crops, such as *Arabidopsis*(Lopez-Gomez and de Folter, 2025), rice(Zhong and Zhang, 2022), wheat(Luo et al., 2022) and sugarcane(Wang et al., 2021). However, no such system has been reported for jute. Although we have previously reported an *Agrobacterium*-mediated stable transformation system, it is still limited by genotype and regeneration ability (Jiang et al., 2024). Therefore, a jute-native protoplast platform is needed to enable rapid functional assays of genomic resources.

Most crop protoplast protocols involve enzymatic digestion with cellulase and macerozyme, followed by PEG-mediated DNA delivery(Wang et al., 2021). Direct transient assays in intact leaves are restricted to a small number of amenable species. Jute is particularly challenging because true leaves contain abundant mucilage and compact mesophyll that compromise digestion and imaging, while stable transformation remains inefficient(Jiang et al., 2024). Strategies in which *Agrobacterium*-infiltrated leaves are digested before microscopy can improve signal detection in pepper, but jute cotyledons wilt rapidly after infiltration and mature leaves release excessive mucilage(Chu et al., 2025). We therefore optimized a conventional PEG-mediated workflow using young and aseptic cotyledons as the starting materials.

Here, we define the key parameters governing jute protoplast isolation and transient transformation. The optimized workflow yielded up to 1.34×10^7 protoplasts g⁻¹ fresh weight with > 90% viability and 59.52%

transformation efficiency. Its utility was demonstrated by CcWRKY70 subcellular localization and BiFC analysis of the HcMYB50-HcTT8a interaction.

Results

Efficient isolation of viable jute protoplasts

To establish an efficient protoplast isolation system, we compared young cotyledons and true leaves as donor tissues. Cotyledons with lower mucilage yielded cleaner, more uniform protoplasts, whereas true leaves generated excessive debris that compromised protoplast yield (Fig. 1). We then optimized enzyme composition, digestion time, and osmoticum concentration. Enzyme optimization showed that 2.5% Cellulase R-10 plus 0.5% Macerozyme R-10 gave the highest total yield (12.92×10^6 protoplasts g^{-1} fresh weight), but 1.5% Cellulase R-10 plus 1.0% Macerozyme R-10 produced the greatest number of viable cells ($11.15 \times 10^6 \text{ g}^{-1}$) (Fig. 2A). Yield increased with digestion time and peaked at 5 h ($13.45 \times 10^6 \text{ g}^{-1}$), after which both total and viable cell numbers declined (Fig. 2B). At 0.5 mol L^{-1} mannitol, FDA-positive cells exceeded 90%, indicating optimal osmotic stabilization (Fig. 2C and D). Based on these results, all subsequent experiments were performed using cotyledons digested for 5 h in enzyme solution containing 1.5% Cellulase R-10, 1.0% Macerozyme R-10, and 0.5 mol L^{-1} mannitol.

Optimization of transient transformation

To establish an efficient PEG-mediated transformation system for jute protoplasts, we examined the effects of CaCl_2 concentration, osmoticum, and incubation time on transformation efficiency (GFP-positive cell rate). Increasing CaCl_2 from 0.1 to 0.3 mol L^{-1} progressively enhanced efficiency from 4.08% to 20.14%, but further elevation proved detrimental (Fig. 3A). Osmotic optimization revealed a sharp peak at 0.5 mol L^{-1} mannitol (37.58%), with efficiency dropping markedly at 0.6 mol L^{-1} (Fig. 3B). Time-course analysis showed that while 90-min incubation yielded the highest efficiency (54.92%), it caused extensive membrane damage; shortening to 75 min preserved cell integrity with a still-acceptable efficiency of 40.14% (Fig. 3C). These optimized conditions were adopted for subsequent transfection assays.

Further optimization of PEG concentration and plasmid DNA dosage revealed peak efficiencies of 54.45% at 50% PEG and 59.52% at 30 μg DNA (Fig. 3D, E). Combining these with the previously established conditions (0.3 mol L^{-1} CaCl_2 , 0.5 mol L^{-1} mannitol, and 75-min incubation), we defined the optimal transformation mixture as 50% PEG 4000 and 30 μg plasmid DNA. Under these conditions, strong GFP fluorescence was readily detectable 12–16 h post-transformation (Fig. 3F).

Subcellular localization of jute proteins

In order to verify the practicability and reliability of our protoplast system in subcellular localization analysis, we co-expressed CcWRKY70-GFP with the nuclear marker H2B-mCherry. WRKY70 proteins are generally nuclear-localized transcription factors in Arabidopsis, wheat, and tobacco (Guo et al., 2024; Song and Wu, 2024; Yang et al., 2025). The empty GFP was distributed in the cytoplasm and nucleus, while CcWRKY70-GFP overlapped with H2B-mCherry signal and localized in the nucleus. (Fig. 4). These results confirm that our jute protoplast system has the ability to reliably identify protein subcellular localization.

BiFC analysis of protein-protein interactions

To assess the feasibility of BiFC assays in our protoplast system, we fused the known interacting partners HcMYB50 and HcTT8a to the N- and C-terminal halves of YFP, respectively. Co-expression of HcMYB50-YN and HcTT8a-YC reconstituted a clear YFP signal, whereas the three negative-control combinations showed no detectable fluorescence (Fig. 5). These results confirm that jute protoplasts support BiFC analysis.

Discussion

The heritable transformation of jute remains a major bottleneck despite the availability of high-quality reference genomes (Zhang et al., 2021). Existing *Agrobacterium*-mediated approaches are time-consuming, genotype-dependent, and constrained by regeneration (Jiang et al., 2024). *The Crop Journal* reported the application of *Agrobacterium*-mediated CRISPR/Cas9 gene editing in jute. Our protoplast-based transient expression system enables researchers to rapidly screen genome editing efficiency in protoplasts before proceeding to stable transformation experiments. This system will significantly accelerate functional genomics and molecular breeding research in jute and other bast-fiber crops.

Donor tissue quality was decisive. Young leaves are commonly used in cotton (Wang et al., 2025) and hemp (Matchett-Oates et al., 2021), but jute true leaves release abundant mucilage and debris. Cotyledons from 7-12-day-old seedlings were less lignified, digested uniformly, and produced cleaner suspensions. The resulting cells also supported the expected nuclear localization of CcWRKY70, consistent with previous WRKY70 studies (Sen et al., 2017). The requirement for 0.5 mol L⁻¹ mannitol likely reflects species-specific osmotic tolerance and membrane properties (Zhang et al., 2023).

Transformation conditions for jute protoplasts were more stringent than in several other crops. While 30–50% PEG and short incubations are effective in many species (Yu et al., 2024), jute required 50% PEG for 75 min. Cotton and sugarcane protoplasts, by comparison, can be efficiently transformed with 40% PEG for about 15 min (Wang et al., 2021). Extending the jute treatment to 90 min increased transformation efficiency but caused severe cell deformation, indicating a narrow balance between transformation time and cell survival. The response to DNA dose also approached saturation, as reported in sugarcane (Wang et al., 2021), with the maximum efficiency at 30 µg.

Compared with hairy root transient expression, the platform completes the entire experiment within two weeks. It yields sufficient viable cells from minimal cotyledon tissue using routine reagents, and is applicable to both subcellular protein localization and dual-construct BiFC assays. The main limitation is the relatively long PEG exposure, which requires precise control, since extended incubation rapidly compromises membrane integrity.

Conclusions

In summary, we established a simple and reproducible jute protoplast system with > 90% viability and 59.52% transient transformation efficiency. The reliable nuclear localization of CcWRKY70 and reconstitution of the HcMYB50-HcTT8a interaction demonstrate that the platform is suitable for rapid functional genomics, protein-interaction screening, and future genome-editing validation in jute.

Materials and methods

Plant materials and growth conditions

Seeds of the pure-line jute cultivar Meifeng No. 4 were surface sterilized with 75% ethanol for 30 s and a chlorine-containing disinfectant (2.5% available chlorine) for 20 min, rinsed three times with sterile water, and germinated on MS basal medium. Seedlings were maintained at 25°C under a 14 h/10 h light/dark cycle. Cotyledons were excised from 7-12-day-old aseptic seedlings before full expansion of the first true leaf.

Plasmid constructs

The 35S::GFP reporter was used for transformation optimization. pUbi::CcWRKY70-GFP and p35S:H2B-mCherry were used for subcellular localization. pCAMBIA1300S-HcMYB50-YN, pCAMBIA2300S-HcTT8a-YC, pCAMBIA1300S-YN, and pCAMBIA2300S-YC were used for BiFC and negative-control assays. All plasmids were stored in the Laboratory of Bast Fiber Genetics, Breeding and Comprehensive Utilization, Fujian Agriculture and Forestry University.

Protoplast isolation

Approximately 1 g of cotyledons was cut into 3–5 mm pieces and immediately immersed in 10 mL enzyme solution containing 1.5% (w/v) Cellulase R-10, 1.0% (w/v) Macerozyme R-10, 0.5 mol L⁻¹ mannitol, 10 mmol L⁻¹ CaCl₂, 20 mmol L⁻¹ KCl, 20 mmol L⁻¹ MES, and 0.1% (w/v) BSA (pH 5.7). Samples were vacuum infiltrated at 50 kPa for 30 min and incubated at 45 rpm for 5 h in the dark. The digest was filtered through a 75-μm cell strainer and washed with W5 solution (154 mmol L⁻¹ NaCl, 125 mmol L⁻¹ CaCl₂, 5 mmol L⁻¹ KCl, and 2 mmol L⁻¹ MES-KOH, pH 5.7). Protoplasts were collected at 150 ×g for 5 min, washed once at 150 ×g for 3 min, resuspended in W5, and incubated on ice in the dark for 30 min. Before transformation, cells were transferred to MMG solution (0.5 mol L⁻¹ mannitol, 15 mmol L⁻¹ MgCl₂, and 4 mmol L⁻¹ MES-KOH, pH 5.7).

Cellulase R-10 and Macerozyme R-10 were purchased from Yakult (Japan), PEG 4000 was from Sigma-Aldrich (USA), and the remaining chemicals were of analytical grade.

Protoplast yield and viability

Protoplast concentration was determined with a hemocytometer and expressed as cells g⁻¹ fresh weight (Elias, 2022; Tian et al., 2023; Wang et al., 2021). Viability was quantified by FDA staining (1% stock; 1 µL per 100 µL suspension) after 5–10 min in the dark. Cells showing green fluorescence were scored as viable, and viability was calculated as the percentage of fluorescent cells (Chatukuta and Rey, 2020; Liu et al., 2019; Lu et al., 2025). Each treatment included three biological replicates.

PEG-mediated transformation

Fresh protoplasts in MMG solution (100 µL) were mixed with 30 µg plasmid DNA and an equal volume of transformation solution containing 50% (w/v) PEG 4000, 0.5 mol L⁻¹ mannitol, and 0.3 mol L⁻¹ CaCl₂. After 75 min at room temperature in the dark, transformation was stopped with 1 mL W5. Protoplasts were washed three times at 150 ×g for 2 min, resuspended in 1 mL W5, and incubated at 22°C in the dark for 12–16 h before fluorescence imaging. Three biological replicates were analyzed.

Subcellular localization

CcWRKY70-GFP and H2B-mCherry were co-transformed into jute protoplasts. After 12 h in the dark, GFP and mCherry signals were recorded by confocal microscopy.

Bimolecular fluorescence complementation

HcMYB50-YN and HcTT8a-YC were co-transformed into jute protoplasts. Empty YN plus empty YC, empty YN plus HcTT8a-YC, and HcMYB50-YN plus empty YC were used as negative controls. YFP fluorescence was examined after 16 h in the dark. Each combination included three biological replicates.

Declarations

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contribution

Huifan Kang: Methodology, Investigation, Data Curation, Formal analysis, Writing - Original Draft. Siyan Wu: Investigation, Visualization. Xin Tang: Methodology, Investigation. Shaolian Jiang: Writing - Review & Editing. Pingping Fang, Aifen Tao, Jiantang Xu, Jianmin Qi: Plant materials, Investigation. Qin Li:

Methodology. Liwu Zhang: Methodology, Supervision, Conceptualization, Funding acquisition, Writing - Review & Editing. All authors reviewed the manuscript.

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Figures

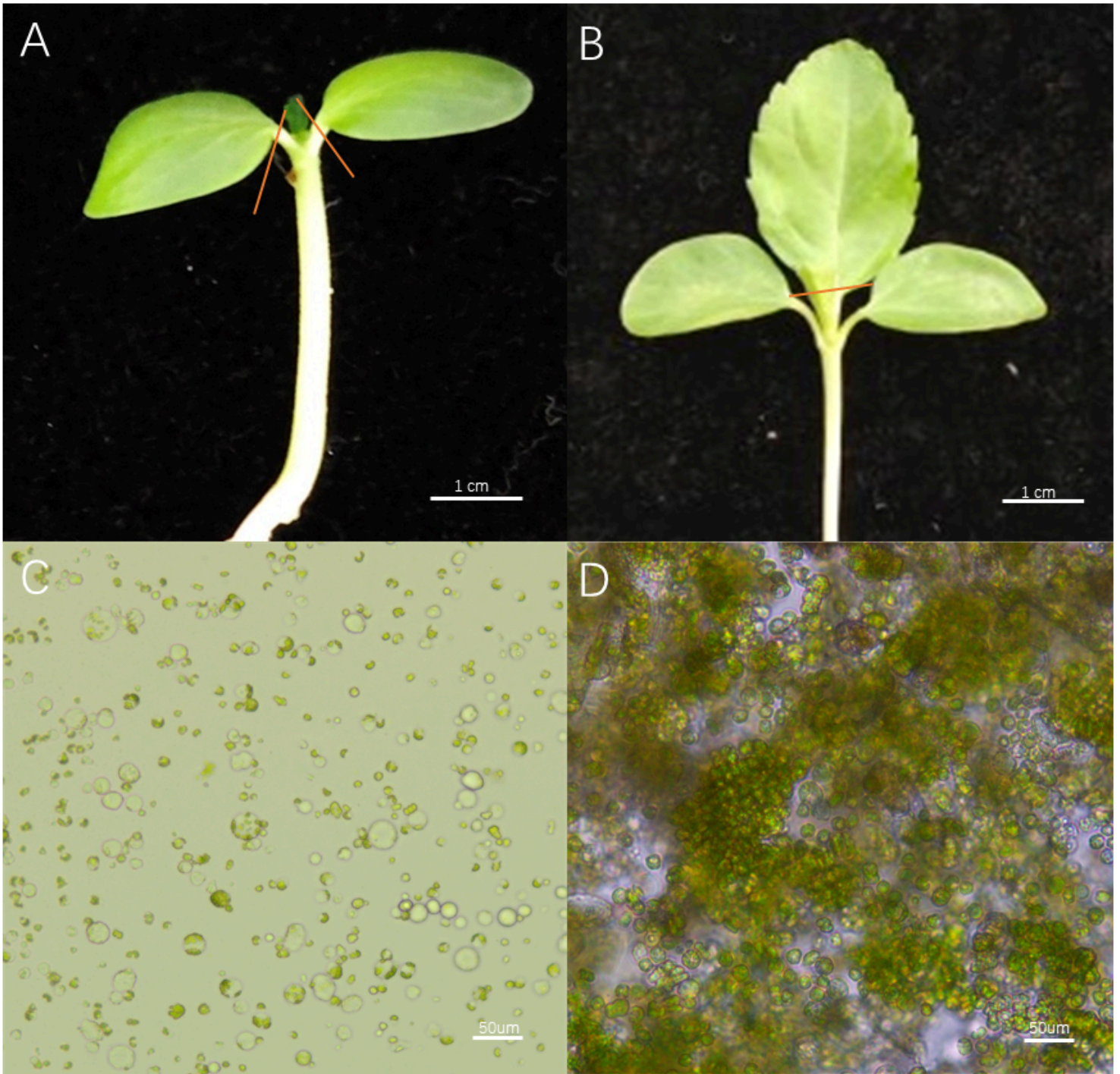


Figure 1

Comparison of cotyledon and true leaf tissues for jute protoplast isolation. (A, B) Young cotyledon and true leaf, respectively. (C, D) Protoplast suspensions obtained from cotyledons and true leaves, respectively. Scale bars, 50 μm .

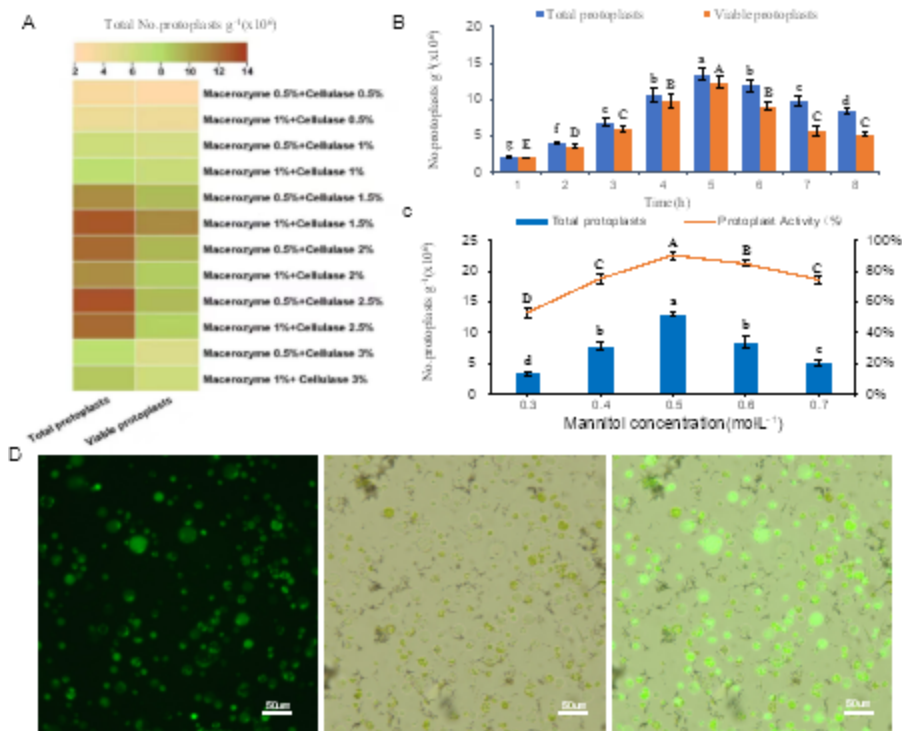


Figure 2

Optimization of jute protoplast isolation. (A) Effects of Cellulase R-10 and Macerozyme R-10 combinations on yield and viability. (B) Effects of digestion time. (C) Effects of mannitol concentration. (D) FDA fluorescence, bright-field, and merged images of isolated protoplasts. Scale bars, 50 μm .

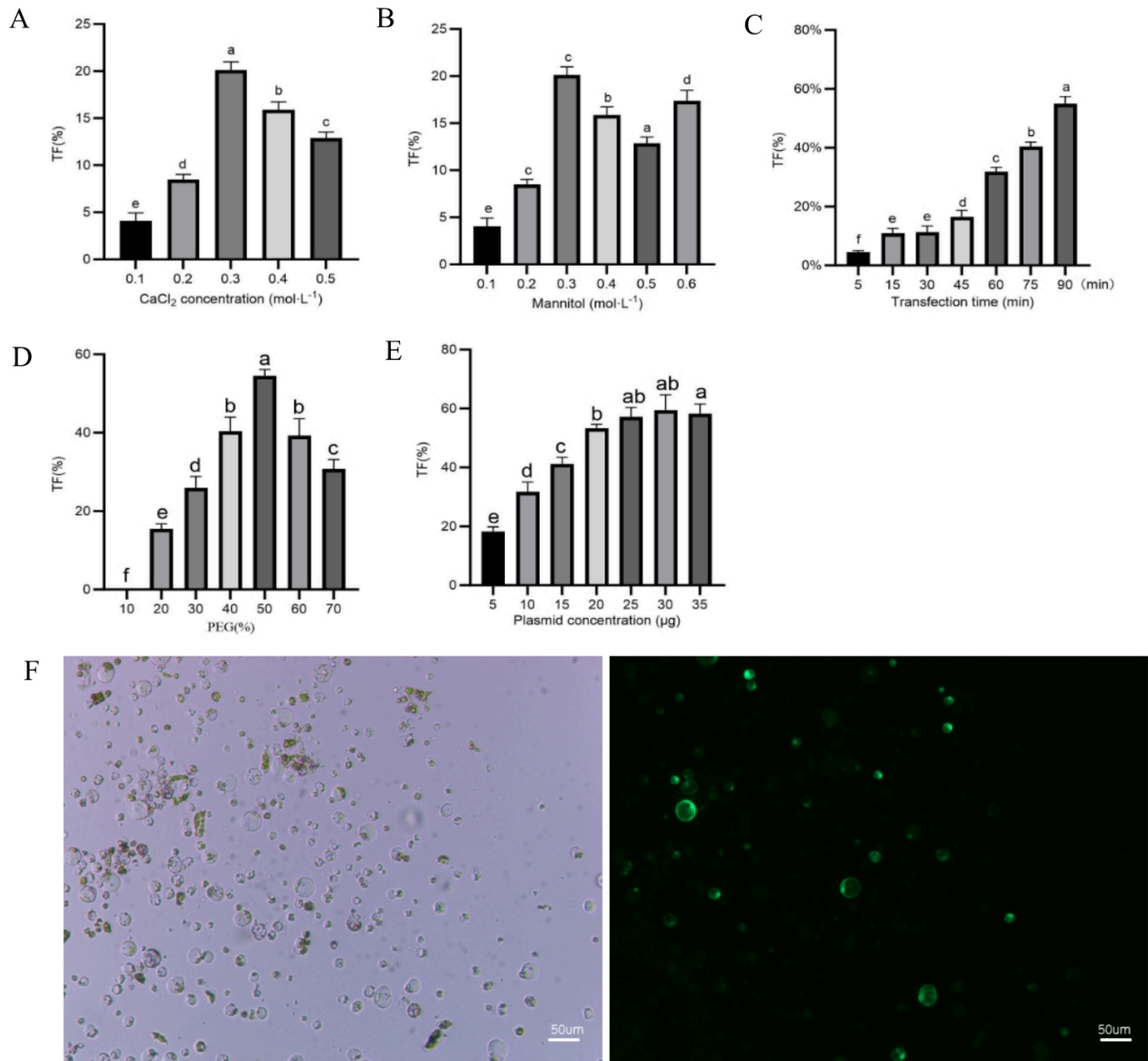


Figure 3

Optimization of jute protoplast transformation. (A-E) Effects of CaCl_2 concentration, mannitol concentration, transformation time, PEG concentration, and plasmid DNA amount, respectively. (F) Bright-field and GFP fluorescence images of transformed protoplasts. Scale bars, 50 μm .

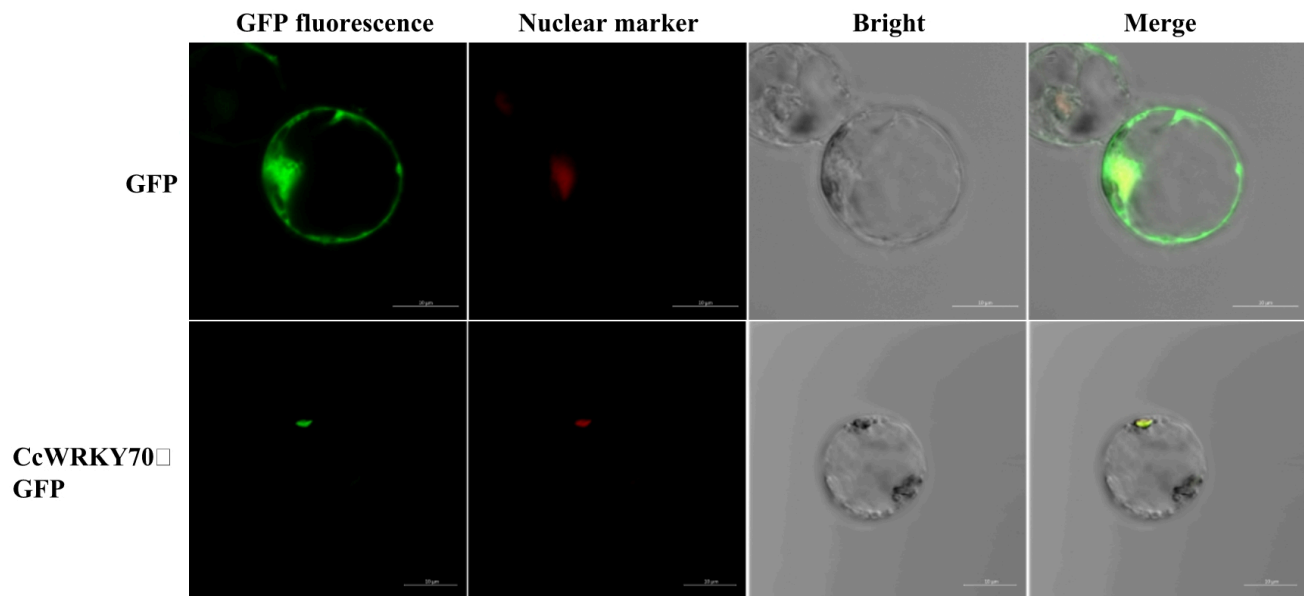


Figure 4

Subcellular localization of CcWRKY70 in jute protoplasts. GFP and CcWRKY70-GFP signals were compared with the H2B-mCherry nuclear marker under fluorescence, bright-field, and merged channels. Scale bars, 10 μ m.

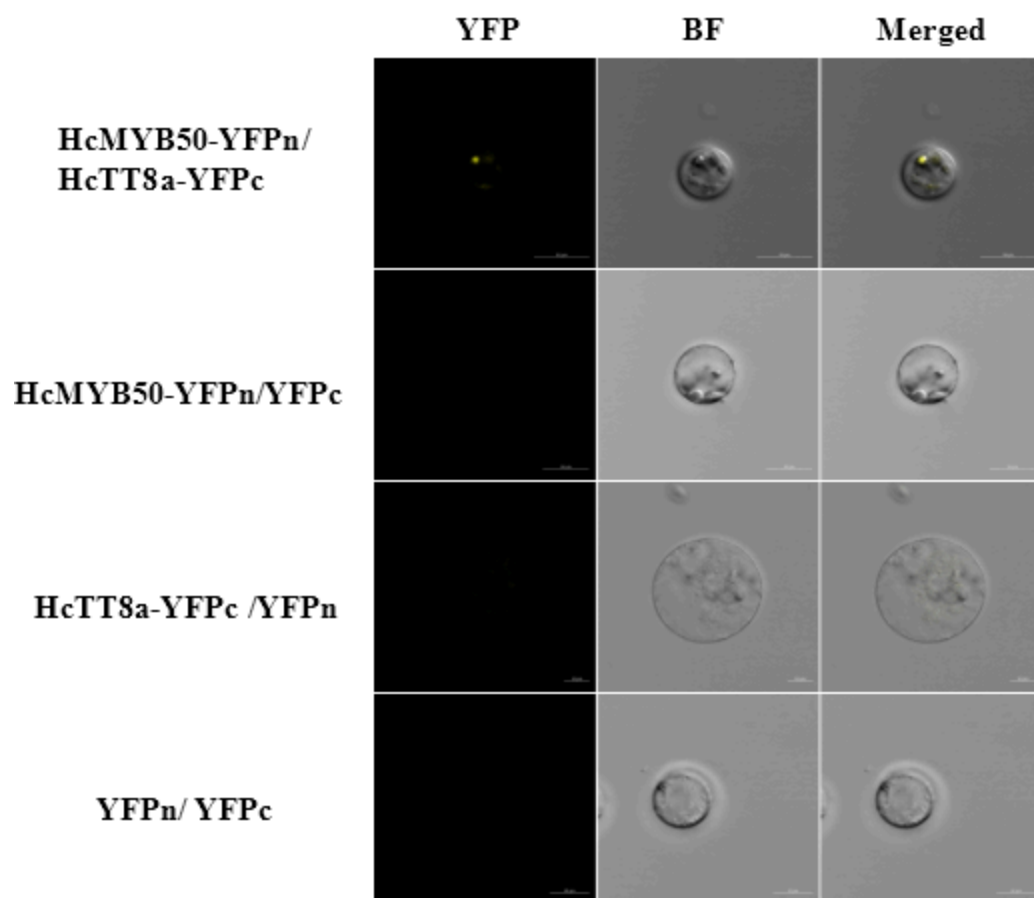


Figure 5

BiFC verification of the protein–protein interaction between HcMYB50 and HcTT8a.

Jute protoplasts were co-transformed with the following construct combinations: HcMYB50-YFP_n and HcTT8a-YFP_c; HcTT8a-YFP_c and YFP_n (empty vector, negative control); YFP_c (empty vector) and HcMYB50-YFP_n (negative control); YFP_n and YFP_c (empty vectors, negative control). BiFC fluorescence is represented by the YFP signal (FITC channel, shown in yellow). YFP, bright-field, and merged images are displayed. Scale bar = 10 μm.