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A highly efficient protocol for tomato protoplast isolation and transfection via seedling etiolation

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Abstract

Protoplast-based transient expression systems serve as indispensable platforms for functional genomics, genome editing, and synthetic biology. While tomato (*Solanum lycopersicum*) is a premier model crop for genetic research, its utility in protoplast-based assays has been constrained by suboptimal isolation yields and low transfection efficiencies. In this study, we report that etiolation treatment of tomato seedlings (cv. Micro-Tom) significantly enhances both protoplast recovery and PEG-mediated transfection. Cotyledons subjected to an 8-day dark treatment yielded an average of 1.47×10^7 protoplasts/g FW, representing a 12-fold increase compared with non-etiolated controls (1.22×10^6 protoplasts/g FW). Furthermore, etiolated protoplasts exhibited superior viability at 90.8%, compared to 53.4% in light-grown counterparts. Most notably, the transfection efficiency using a GFP-reporter plasmid reached 58.2% in etiolated protoplasts, a 2.2-fold improvement over the 25.8% observed in non-etiolated cotyledons. Our results suggest that etiolation optimizes cellular physiology and enhances structural stability for exogenous DNA delivery. This study establishes a robust and streamlined protocol for high-efficiency protoplast isolation and transfection, providing a powerful tool to accelerate precision breeding and functional genomics in tomato.

Keywords Tomato, Protoplast, Etiolation, PEG-mediated transfection, Transient expression

Introduction

Tomato (*Solanum lycopersicum*) is one of the most widely produced and consumed vegetable crops worldwide, holding significant economic value as a global food resource. Characterized by a relatively small diploid genome (~ 900 Mb), a short life cycle, and well-established reference genome data, tomato serves as a key model crop for functional genomics and trait studies in the Solanaceae family [1, 2]. Recently, the escalation of abiotic stresses driven by climate change and shifting pest and disease patterns has posed a serious threat to food security. To address these challenges, there is an increasing demand for precision breeding technologies that can introduce and validate beneficial traits more rapidly than traditional cross-breeding [3, 4]. In particular, CRISPR/

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Cas-based genome editing has emerged as an essential tool for next-generation breeding, with the potential to accelerate the domestication of wild tomatoes and the integration of stress-resistance traits [5–8]. Therefore, to effectively apply these genome editing technologies, it is crucial to establish a platform that enables the rapid and precise validation of target gene functions.

While *Agrobacterium*-mediated T-DNA delivery is the most common method for plant transformation, it is often limited by its narrow host range, the lengthy regeneration process, and regulatory concerns regarding genetically modified organisms (GMOs) due to the risk of transgene integration [9, 10]. In cases where sgRNAs with low editing efficiency are used, there is a substantial risk of expending excessive time and resources before obtaining stable transformants [11, 12]. To address these limitations, protoplasts have emerged as a versatile alternative [12]. Protoplasts provide an optimized platform for transient expression analysis, as they can directly take up various macromolecules, including DNA, RNA, and proteins (ribonucleoproteins, RNPs) [7, 13, 14]. Utilizing this system enables the rapid screening of sgRNA cleavage efficiency prior to stable transformation. Furthermore, the direct delivery of Cas9-sgRNA RNP complexes into protoplasts allows for DNA-free genome editing, effectively avoiding transgene integration and minimizing off-target effects, which may serve as a strategic approach to bypass current GMO regulations.

Despite these advantages, the tomato protoplast system remains a significant challenge for widespread use due to low isolation yields, poor viability during incubation, and consequently low transfection efficiency [15]. These efficiency issues often lead to low reproducibility and complicate the processing of large sample volumes. Although protoplast-based CRISPR validation has been reported in various crops, the isolation and transfection of protoplasts in tomato still serve as a major technical bottleneck [16, 17]. Therefore, establishing a high-efficiency isolation and transfection protocol is essential to enhance the practicality of protoplast-based genome editing [12].

The yield and viability of protoplasts are heavily dependent on the physiological state of the plant tissue. Previous studies have reported that using etiolated tissues grown in the dark significantly improves protoplast yield and transfection efficiency not only in monocots, such as rice and maize, but also in dicots, including lettuce and Chinese cabbage [12, 18–20]. Etiolated tissues are characterized by excessive elongation growth and the absence of chlorophyll, which leads to structural and biochemical changes in cell wall composition [21]. These modifications facilitate enzymatic isolation and reduce mechanical stress on the plasma membrane by minimizing the physical presence of intracellular organelles [22–24]. Thus, etiolation treatment serves as an effective strategy

to create a highly competent physiological state for protoplast isolation and transfection.

Despite these benefits, the impact of etiolation on the yield, viability, and transfection efficiency of tomato protoplasts has not yet been reported. In this study, we quantitatively evaluate the effects of etiolation treatment on tomato seedlings regarding protoplast isolation efficiency, cell viability, and PEG-mediated transfection efficiency. The ultimate goal of this research is to establish a high-efficiency protoplast platform that can be readily utilized for functional genomics and the validation of CRISPR/Cas systems in tomato.

Materials & methods

Plant materials and growth conditions

Tomato (*Solanum lycopersicum* cv. Micro-Tom) was used as the experimental material in this study. Seeds were sown in commercial horticultural soil (Bunong, Republic of Korea) and germinated for 7 days at 24 °C under a photoperiod of 16 h light and 8 h dark. Non-etiolated seedlings were obtained by cultivating the plants for an additional 11 days under the same light and temperature conditions. Etiolated seedlings were germinated and grown under light conditions for the first 3 days at 24 °C to promote uniform germination and early seedling vigor. Subsequently, the seedlings were transferred to continuous darkness for an additional 8 days at 24 °C to induce etiolation. Relative humidity was maintained at 60–70% throughout the cultivation period, and irrigation was performed 2–3 times per week.

Protoplast isolation

Protoplast isolation was conducted with modifications from the protocols described previously [17, 25, 26]. Cotyledons grown in soil were sliced into 1 mm strips and immersed in 25 mL of digestion solution (1/4 MS liquid medium containing 4% Viscozyme and 3% Pectinex, 2% Celluclast, 3% [v/v] sucrose, and 0.4 M mannitol, pH 5.7). The enzymatic reaction was carried out at 24 °C in the dark for 2.5 h with gentle rotation at 15 rpm. The digested mixture was filtered through a 40 µm nylon mesh and diluted with an equal volume of W5 solution (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, 5 mM glucose, and 2 mM MES, pH 5.7). The protoplasts were collected by centrifugation at 360 g for 3 min and purified with a 20% (w/v) sucrose solution. The purified protoplasts were washed with W5 solution and resuspended in MMG solution (0.4 M mannitol, 15 mM MgCl₂, 4 mM MES, pH 5.7). The final concentration was adjusted to 2.5×10^5 cells/mL. Protoplast yield and viability were assessed using 0.05% fluorescein diacetate (FDA) staining under a light microscope and a hemocytometer (Marienfeld, Germany).

Protoplast transfection

Protoplast transfection was performed using the PEG- Ca^{2+} mediated method as previously described [14, 15, 27]. Specifically, 20 μL of plasmid DNA (20–40 μg) was added to 200 μL of the protoplast suspension (2.5×10^5 cells/mL) and mixed gently. An equal volume (220 μL) of PEG- Ca^{2+} solution was then added, and the mixture was incubated at room temperature for 5 min. To terminate the reaction, 880 μL of W5 solution was added, and the protoplasts were collected by centrifugation at 200 g for 2 min. The collected protoplasts were resuspended in 1 mL of KM solution (KM medium 3.62 g/L, 3% sucrose, 0.5 M mannitol, 2 mg/L 2,4-D, 0.5 mg/L BAP, 10% KM vitamin solution, pH 5.8) and incubated in the dark at 24 °C for 48 h. Transfection efficiency was calculated as

the percentage of GFP-expressing cells among the total number of protoplasts using a fluorescence microscope (Olympus BX51; GFP filter 400–650 nm).

Results

Protoplast isolation yield and viability of non-etiolated and etiolated seedlings

Non-etiolated plants were cultivated for 11 days after germination under a 16 h light/8 h dark photoperiod. etiolated plants were grown under the same light conditions for 3 days post-germination, followed by 8 days of continuous darkness to induce skotomorphogenesis (Fig. 1A). While the non-etiolated cotyledons exhibited normal development with green pigmentation, etiolated cotyledons showed distinct yellowing due to chlorophyll

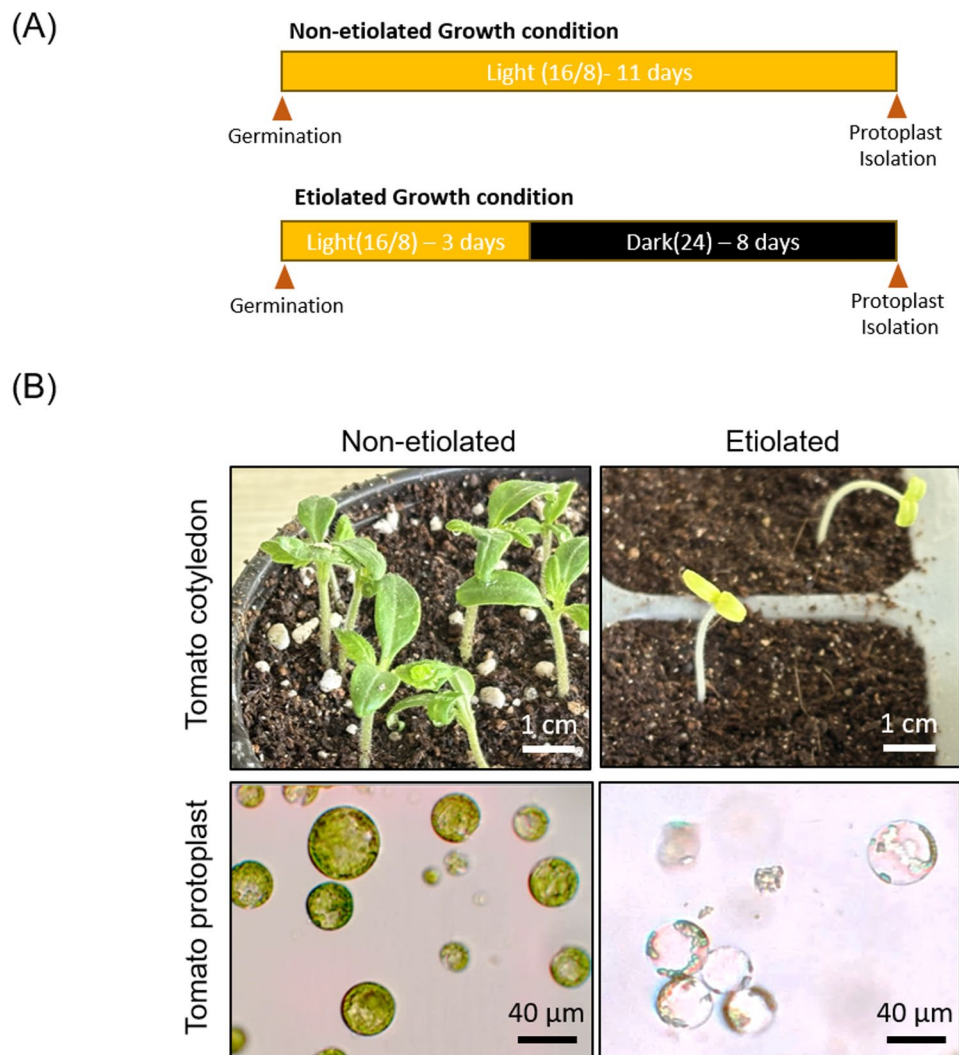


Fig. 1 Morphological comparison of tomato cotyledon and isolated protoplast under non-etiolated and etiolated growth conditions. **(A)** Schematic representation of the experimental timeline and growth conditions for protoplast isolation. Non-etiolated plants were grown under a long-day photoperiod (16 h light/8 h dark) for 11 days. For etiolation, seedlings were grown under the same light conditions for 3 days, followed by incubation in continuous darkness (24 h dark) for 8 days. **(B)** Representative images of tomato cotyledons (upper panels) and isolated protoplasts (lower panels) from each condition. Scale bars: 1 cm (upper panel), 40 μm (bottom panel)

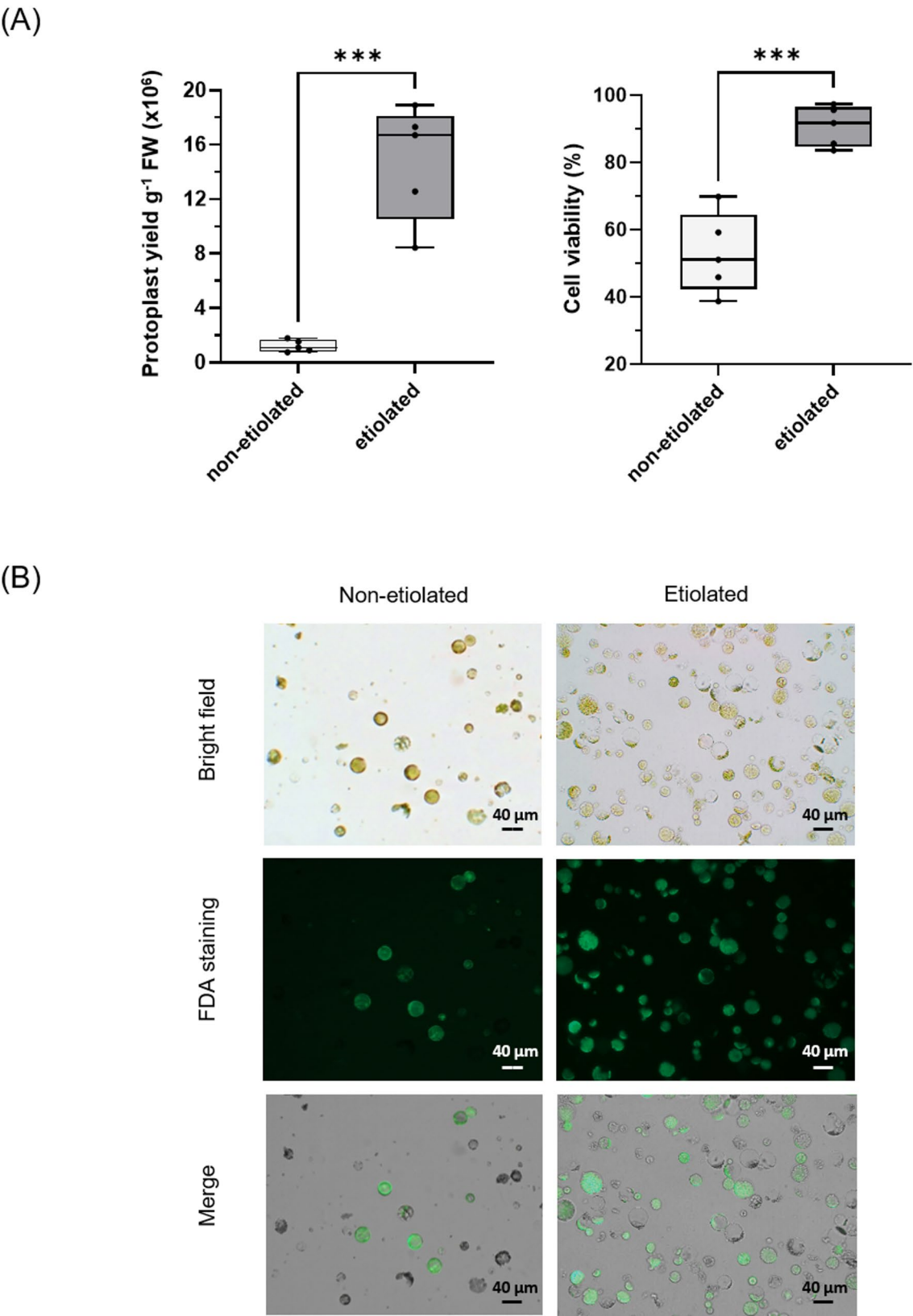


Fig. 2 (See legend on next page.)

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Fig. 2 Comparison of yield and viability of protoplasts isolated under non-etiolated and etiolated conditions. The yield and viability of isolated protoplasts were measured from non-etiolated and etiolated tomato cotyledons using a hemocytometer and FDA staining. **(A)** The quantification of protoplast yield (left) and cell viability (right). **(B)** Representative microscopy images of isolated protoplasts (top), live protoplasts stained green with FDA (middle), and merged images (bottom) visualized by fluorescence microscopy. Scale bars: 40 μm . Data are presented as mean $\pm$ s.e.m. for each group from 5 independent experiments ($n=5$). P-values were obtained using the Student's t-test analysis: *** $P<0.001$

deficiency and marked hypocotyl elongation. Consistent with these morphological changes, isolated non-etiolated protoplasts were enriched with chloroplasts, whereas etiolated protoplasts appeared transparent and devoid of chlorophyll (Fig. 1B).

Protoplast yield per fresh weight (FW) was quantified for each condition using a hemocytometer (Fig. 2A). The results showed that the isolation yield from non-etiolated seedlings averaged 1.22×10^6 cells/g FW, whereas etiolated seedlings yielded a significantly higher count of 1.47×10^7 cells/g FW, indicating a 12-fold increase in yield following etiolation treatment. To assess the integrity of the isolated protoplasts, FDA (Fluorescein Diacetate) staining was performed. FDA is a non-fluorescent compound that penetrates intact cell membranes and is hydrolyzed by intracellular esterases to produce fluorescein, which emits strong green fluorescence. Since this conversion only occurs in metabolically active cells, it is a reliable indicator of protoplast viability (Fig. 2B). The viability of protoplasts from non-etiolated cotyledons was 53.4%, while those from etiolated cotyledons showed a significantly higher viability of 90.8% (Fig. 2A). These findings suggest that the reduction in chlorophyll and tissue softening associated with growth in the dark not only facilitates enzymatic isolation but also helps maintain high viability by reducing mechanical stress during isolation.

Transfection efficiency of non-etiolated and etiolated protoplasts

To evaluate the effectiveness of the protoplast system for transient gene expression, PEG- Ca^{2+} mediated transfection was performed using the pMDC32_eGFP vector. Protoplast suspensions were adjusted to a final concentration of 2×10^5 cells/mL before transfection. Analysis of GFP expression (Fig. 3B) showed that non-etiolated protoplasts achieved a transfection efficiency of 25.8%, whereas etiolated protoplasts yielded a high efficiency of 58.2% (Fig. 3A). This represents an approximately 2.2-fold improvement over the control, suggesting that dark cultivation creates a highly competent physiological state that significantly enhances exogenous DNA delivery. In conclusion, etiolation treatment is an effective strategy for obtaining a high concentration of healthy protoplasts and ensuring high-efficiency transfection. These results demonstrate that etiolation is a valuable method for maximizing the yield and transfection potential of tomato protoplasts.

Discussion

Protoplasts represent a powerful tool for DNA-free genome editing; however, further improvements are required to enhance their efficiency in plants. Previous studies have primarily focused on chemical and physical methods to optimize protoplast protocols. In this study, we demonstrated that etiolation treatment is a strategy to improve protoplast isolation yield, viability, and transfection efficiency in tomato seedlings. The average protoplast yield obtained was 1.47×10^7 cells/g FW, which is substantially higher than the typical yields of $3\text{--}4 \times 10^6$ cells/g FW reported in existing protocols using green tomato cotyledons [11, 25]. While previous studies have reported the positive effects of etiolation in various crops such as lettuce, Chinese cabbage, and rice [12, 18–20], this study is significant as it provides a quantitative assessment of these improvements specifically optimized for the tomato system.

While etiolation is essential for significantly enhancing protoplast yield, our preliminary observations indicated that 11 days of continuous darkness markedly compromised seedling vigor, resulting in reduced biomass and underdeveloped cotyledons. By implementing an initial three-day photoperiod, we secured the metabolic resources and structural integrity necessary for robust seedling development, ultimately facilitating more stable protoplast isolation. In this study, soil-grown seedlings were utilized to ensure sufficient yield and viability of etiolated protoplasts. The seedlings cultured on MS medium may offer easier enzymatic digestion due to their tender and soft tissues.

Furthermore, the enzyme combination and incubation time were optimized to strike a balance between efficient tissue dissociation and the preservation of protoplast integrity. Given that prolonged enzymatic exposure can adversely affect yield and viability [26] and that a sufficient enzyme concentration is essential, we increased the enzyme concentration and shortened the incubation time.

It is important to note that etiolated protoplasts exhibited not only higher yields but also significantly improved viability compared to the non-etiolated control. This aligns with reports suggesting that etiolated seedlings maintain a highly healthy physiological state without signs of senescence or oxidative stress [28]. The structural flexibility characteristic of etiolated tissues likely enhances the efficiency of enzymatic cell wall degradation while simultaneously reducing mechanical stress

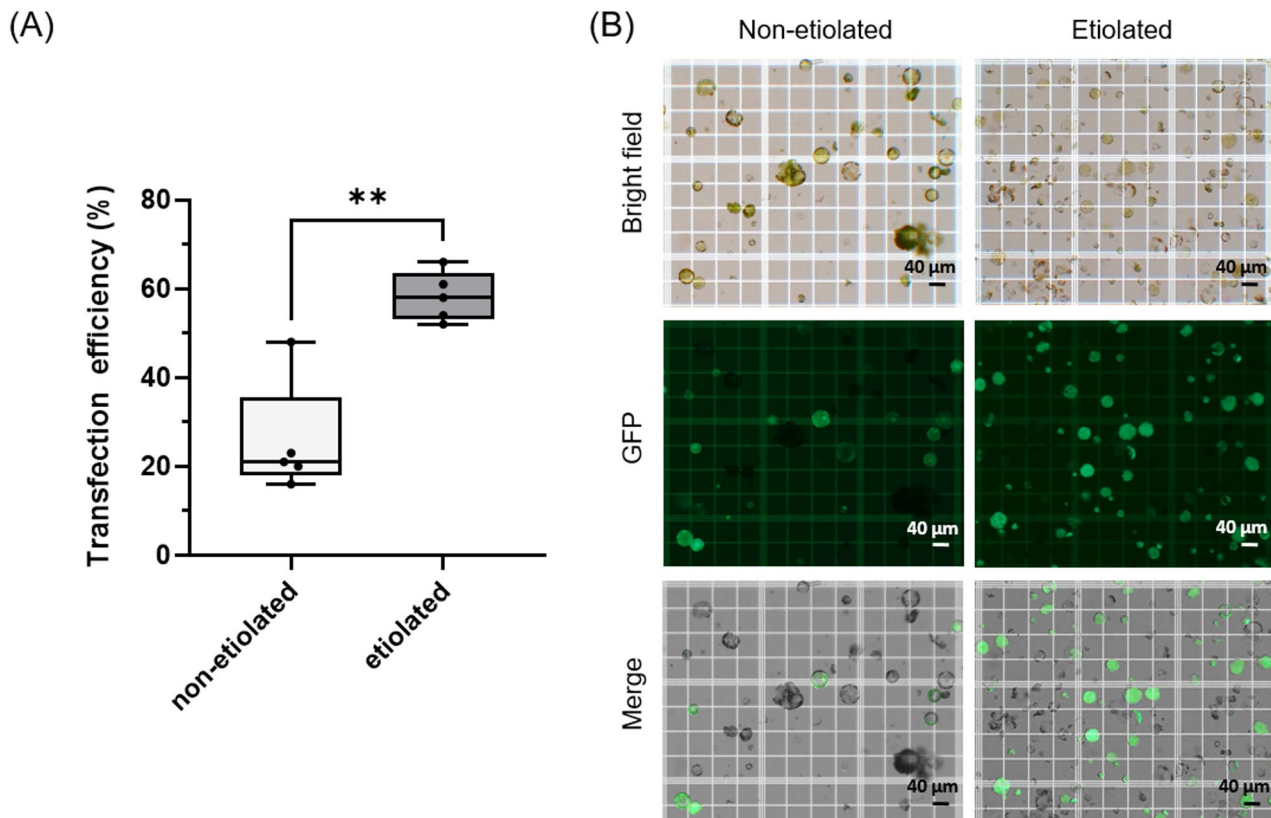


Fig. 3 Evaluation of transfection efficiency in non-etiolated and etiolated protoplasts. **(A)** Quantification of protoplast transfection efficiency using GFP-expression in non-etiolated and etiolated protoplasts. Transfection efficiency was determined by counting the number of GFP-expressing cells among a total of at least 200 protoplasts per replicate from randomly selected microscopic fields. **(B)** Representative images of isolated protoplasts (top), GFP-expressing protoplasts (middle) visualized by fluorescence microscopy. Scale bars: 40 μm. Data (mean ± s.e.m.) were calculated for each group from $n = 5$ independent experiments. P-values were obtained using the Student's t-test analysis: ** $P < 0.01$

on the cells during isolation, thereby ensuring high initial viability. Furthermore, the fact that etiolated protoplasts maintain intact plasma membrane active transport and signaling systems after isolation [29] suggests that they provide an optimal physiological state for the delivery of exogenous materials. This increased viability of etiolated tissues is consistent with studies reporting that hypocotyl-derived protoplasts exhibit higher viability than those derived from leaves, reflecting a similar trend observed in hypocotyl protoplast systems [26–31].

This high viability consequently led to increased transfection efficiency. Previous studies characterizing the rapid physical responses and single-cell signal transduction mechanisms in etiolated hypocotyl-derived protoplasts [32] suggest that the plasma membranes of etiolated tissues reside in a highly dynamic primed state, characterized by heightened sensitivity to external stimuli. Such enhanced membrane flexibility and elevated metabolic activity are considered pivotal factors that facilitated the efficient penetration and stable intracellular uptake of exogenous DNA during the transformation process. Furthermore, the superior transfection efficiency observed in etiolated protoplasts is thought to be closely

related to changes in the intracellular environment following the inhibition of chloroplast development. Etiolation-induced elongation growth and the absence of chlorophyll cause a reduction in organelle density and promote structural changes [21], which can act as factors that enhance plasma membrane stability against osmotic stress [22, 24]. In other words, as the physical space constraints imposed by chloroplasts are reduced and membrane fluidity is maintained, the entry of exogenous DNA into the cell likely becomes more efficient.

The high activity of etiolated protoplasts identified in this study suggests potential for enhancing future regeneration efficiency beyond simple transient expression analysis. As previously reported in sunflowers, where etiolated hypocotyl-derived protoplasts showed superior cell division and micro-callus formation compared to those derived from green mesophyll [33], the etiolated tomato protoplasts in this study are also expected to possess excellent physiological potential to overcome cultivation stress and sustain tissue development.

However, the primary focus of this study was to establish a highly efficient and practical protoplast-based platform for tomato research. While direct molecular-level

verification of changes in cell wall composition, ROS levels, or osmotic regulation mechanisms induced by etiolation was not performed here, the 12-fold increase in yield and 2.2-fold improvement in transfection efficiency clearly demonstrate the functional superiority of this etiolation-based system. Future follow-up studies, such as transcriptome or cell wall biochemical analysis, will further clarify the underlying mechanisms.

Given the physiological and morphological similarities within the genus *Solanum*, the protocol optimized in this study is expected to be broadly applicable to various tomato cultivars and related species [11, 34]. However, given the genotypic variations in cell wall composition and regeneration capacity, further cultivar-specific fine-tuning - such as adjustments to enzyme concentrations or incubation times - may be required to achieve maximum efficiency across different genetic backgrounds. Nevertheless, the streamlined protocol established in this study provides a robust and immediate solution for high-throughput sgRNA screening and precision breeding in tomatoes.

Acknowledgments

Not applicable.

Author contributions

YL was involved in the experiment, methodology, and writing the original draft; SHY provided resources and contributed to the methodology; SKH designed the experiment, edited the manuscript, and YK contributed to conceptualization, project administration, and supervision.

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Data availability

All data generated or analyzed in this study are included in this published article and its supplementary information files.

Declarations

Competing interests

YK is an Editor of *Applied Biological Chemistry*, yet Editor status has no bearing on the editorial consideration of this article. The authors declare that they have no competing interests.

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