agronomy

MDPI

Article

Optimization of Protoplast Preparation System from Leaves and Establishment of a Transient Transformation System in *Apium graveolens*

Jiageng Du [1,†], Huitao Zhang [1,†], Weilong Li [1], Xiaoyan Li [1], Zhuo Wang [1], Ying Zhang [1], Aisheng Xiong [2,*] and Mengyao Li [1,*]

[1] College of Horticulture, Sichuan Agricultural University, Chengdu 611130, China; jiageng_du@163.com (J.D.); 15183923635@163.com (H.Z.); 15865899720@163.com (W.L.); lxy2324804342@163.com (X.L.); wangzhuo202208@163.com (Z.W.); 13086617252@163.com (Y.Z.)

[2] College of Horticulture, Nanjing Agricultural University, Nanjing 210095, China

* Correspondence: xiongaisheng@njau.edu.cn (A.X.); limy@sicau.edu.cn (M.L.)

† These authors contributed equally to this work.

Abstract: Protoplast culture and transformation technology offer a novel method for developing new plant varieties. Nonetheless, the effective preparation of protoplasts and transformation technology specific to celery has yet to be achieved. This study utilized celery seedling leaves as the primary materials to examine the key factors influencing protoplast isolation. The aim was to prepare leaf protoplasts with a high yield and of high quality and subsequently conduct transient gene transformation and expression. The findings indicated that the most effective procedure for isolating and purifying protoplasts was enzymatic digestion using an enzyme solution consisting of 2.0% cellulase, 0.1% pectolase, and 0.6 M mannitol for a duration of 8 h. Subsequently, the protoplasts were filtered through a 400-mesh sieve and purified through centrifugation at 200× *g*. Within this system, the overall protoplast yield was exceptionally high, reaching a viability rate of up to 95%. The transient transformation system yielded a maximum transformation efficiency of approximately 53%, as evaluated using the green fluorescent protein (GFP) as a reporter gene. The parameters of the transient transformation system were as follows: a protoplast concentration of 5×10^5 cells·mL^{-1}, exogenous DNA concentration of 500 μg·mL^{-1}, final concentration of PEG4000 at 40%, and transformation duration of 15 min. The transient transformation system was also utilized to further analyze the protein localization characteristics of the celery transcription factor AgMYB80. The findings indicated that AgMYB80 predominantly localizes in the nucleus, thereby confirming the reliability and effectiveness of the transient transformation system. This study successfully established an efficient system for isolating, purifying, and transforming celery protoplasts, and will serve as a basis for future studies on molecular biology and gene function.

Keywords: celery; protoplast preparation; transient transformation system; subcellular localization

Citation: Du, J.; Zhang, H.; Li, W.; Li, X.; Wang, Z.; Zhang, Y.; Xiong, A.; Li, M. Optimization of Protoplast Preparation System from Leaves and Establishment of a Transient Transformation System in *Apium graveolens*. *Agronomy* **2023**, *13*, 2154. https://doi.org/10.3390/agronomy13082154

Academic Editor: Moshe Reuveni

Received: 20 July 2023
Revised: 9 August 2023
Accepted: 14 August 2023
Published: 17 August 2023

1. Introduction

Protoplasts are plant cells devoid of cell walls, possessing both totipotency and vitality, enabling a range of metabolic activities within them [1]. Protoplasts are a distinctive single-cell system representing excellent fundamental research and crop enhancement materials. They offer valuable opportunities for investigating the physiology and genetics of plant cells, including cell wall formation and regeneration, somatic hybridization, and genetic transformation [2]. Plant protoplasts can be isolated and prepared from various plant tissues, including leaves, cotyledons, petals, roots, hypocotyls, cells in suspension culture, and callus tissues. Among these, leaves are the most frequently utilized tissue for protoplast isolation [3]. The methods employed for protoplast isolation primarily consist of mechanical, chemical, and enzymatic techniques [4,5]. Research has demonstrated that

enzymatic methods yield high protoplast yields with excellent integrity, which is why they are widely employed. However, the enzymatic method for protoplast isolation is influenced by several factors, including the type of explant, choice of enzyme, concentration of enzymes used, and duration of enzymatic digestion [6,7]. The optimal protoplast preparation system can vary depending on the specific plant tissue. For instance, in the case of tobacco (*Nicotiana tabacum* L.) leaf protoplasts, a digestion time as short as four hours is sufficient to achieve optimal results [8]. However, the yield of *Hibiscus cannabinus* hypocotyl protoplasts is relatively low after 9 h of digestion, whereas the maximum output is accomplished with a 12 h digestion period [9]. Furthermore, in the case of maize leaf protoplasts, the optimal result is achieved by employing 1.5% cellulase and 0.5% pectolase for a digestion period of 5 h [10]; for Arabidopsis leaf protoplast digestion, a highly effective combination consists of 1.5% cellulase and 0.4% pectolase [11].

After enzymatic or mechanical removal of their cell walls, protoplasts can readily incorporate exogenous organelles and nucleic acids under specific conditions. Transient gene expression is a rapid and effective cellular and genetic research technique. It proves particularly valuable for swiftly assessing gene function, including subcellular protein localization, protein–protein interactions, protein activity, and signal transduction [12,13]. Among the different methods for protoplast transformation, PEG-mediated transformation has been extensively researched and has seen widespread application. This method offers several advantages, including convenient operation, a shorter processing time, lower experimental costs, good reproducibility, and relatively high transformation efficiency. Additionally, PEG-mediated transformation allows the direct targeting of individual cells, enabling a more direct investigation of transient gene expression and study of exogenous substances. As a result, it is extensively utilized in plant molecular biology research [14,15]. At present, protoplast isolation and the preparation of a transient transformation system using protoplasts have been successfully conducted on various plant species. These include Arabidopsis [16], cucumber [17], maize [18], sweet cherry [19], and cassava [20]. These advancements provide a solid foundation for gene function analysis in these plants and offer valuable tools for further research in these species.

Celery (*Apium graveolens* L.) is a biennial vegetable from the Apiaceae family. It is renowned for its nutritional richness and diverse functional bioactive compounds, which make celery highly prized for its culinary applications and medicinal properties. It is popular among consumers both domestically and internationally [21]. The release of the whole genome data of celery has opened up valuable genetic resources for studying its evolution, gene function, functional analysis, and other related research areas [22]. A comprehensive and efficient method for protoplast preparation and transformation specifically tailored to celery is lacking. Existing techniques are time-consuming and challenging and often fail to produce protoplasts that meet the requirements of subsequent genetic transformation and gene functional studies. Subcellular localization studies primarily rely on tobacco or onion tissues as carriers. While heterologous expression systems can be employed for gene functional studies, including heterologous genetic backgrounds can potentially yield abnormal results and introduce confounding factors into the analysis. Arabidopsis gene proteins introduced into tobacco plants may undergo mislocalization, highlighting the limitations of using heterologous expression systems for subcellular localization analysis [8]. Establishing an efficient protoplast preparation and transformation system for celery is essential for studying its gene function and conducting gene editing. In the study, we report a simplified and efficient method for protoplast isolation and transient gene expression. Various factors affecting the efficiency of protoplast isolation including enzymatic composition, digestion time, mannitol concentration in the enzyme solution and centrifugal speed were evaluated to optimize protoplast isolation and purification procedures. Additionally, using green fluorescent protein (GFP) as a reporter gene, critical parameters that affect transient transformation efficiency were also investigated such as PEG concentration and transformation time. This transient gene expression system using leaf mesophyll protoplasts could be applied to analyze complex regulatory mechanisms and contribute to the study of protein

subcellular localization, protein–protein interactions and functional gene expression in celery and related species.

2. Materials and Methods

2.1. Plant Material

In this study, the celery variety 'Lvling Huangxinqin' was selected as the plant material. The seeds were placed into sterilized 50 mL centrifuge tubes and a disinfectant solution consisting of 20% sodium hypochlorite bleach and 80% sterile water was added. The tubes were subsequently shaken with 150 rotations per minute (rpm) using a shaker for 30 min at room temperature. The disinfectant solution was carefully placed in a laminar flow cabinet, and the sterilized seeds were thoroughly rinsed with sterile water five times to ensure cleanliness. After rinsing, the seeds were placed on clean filter paper and air-dried. Subsequently, the dried seeds were evenly distributed in a sealed bottle on a sterile MS (Murashige and Skoog) (3% (*w*/*v*) sucrose, 0.8% (*w*/*v*) agar, and pH 5.8) medium. The cultures were then incubated under sterile conditions at a temperature of 25 °C, with a humidity level of 40% and a light cycle of 16 h of light and 8 h of darkness.

2.2. Protoplast Isolation and Purification

A modified and optimized protoplast isolation protocol for celery has been adapted from Pua and Yoo's protocols [16,17]. To initiate the process, a stock solution was prepared, consisting of KCl, $CaCl_2$, MES, mercaptoethanol, BSA, and a separate solution containing mannitol. Subsequently, cellulase R-10 (at concentrations of 1.5%, 2.0%, and 2.5%) (Yakult Honsha Co., Ltd., Tokyo, Japan) and pectolase (at concentrations of 0.05%, 0.10%, and 0.20%) (Yakult Honsha Co., Ltd., Tokyo, Japan) were added to the enzyme solution. To ascertain the ideal concentration of mannitol (0.4, 0.5, 0.6, and 0.7 M) for the enzyme solution was used, we compared different concentrations of mannitol. The pH of the enzyme solution was adjusted to 5.8, and it was subsequently filter-sterilized using a 0.22 μm syringe filter. The solution was then stored at 4 °C for future use. For protoplast preparation, well-grown leaves from three-week-old plants were selected. A blade was used to carefully cut these leaves into 0.5–1 mm wide strips. The cut leaf strips were then immersed in the pre-prepared enzyme solution and incubated at 25 °C in the dark, with gentle shaking at 45 rpm. Protoplast yield and viability were assessed at different intervals to optimize the digestion time (6, 8, 10, and 12 h). The protoplasts were resuspended in a W5 salt solution (2 mM MES, 154 mM NaCl, 125 mM $CaCl_2$, and 5 mM KCl; pH 5.7) and gently mixed after isolation. The centrifugation speed was further optimized by testing different speeds (50, 100, 200, 300, and 400× *g*) to determine the most effective rate for separating and collecting the protoplasts.

The protoplast yield was quantified using a dual-chamber hemocytometer under an Olympus CX21 optical microscope (Olympus, Tokyo, Japan). The calculation of protoplast yield involved dividing the number of protoplasts obtained in the enzyme solution by the fresh weight of the plant leaves utilized in the enzyme solution. Protoplast viability was assessed using fluorescein diacetate (FDA) staining [23]. A solution of FDA (5 mg·mL^{-1}) was prepared by dissolving FDA in acetone. The isolated protoplasts were stained with FDA at 25 μL·mL^{-1} and incubated in the dark at room temperature for 20 min. Subsequently, the stained protoplasts were examined under a fluorescence microscope. Three random fields were selected for observation and photography, and each sampling was repeated three times. The number of fluorescent and total cells was counted to determine the protoplast viability rate. The protoplast viability rate was calculated by dividing the fluorescent protoplasts by the total number of protoplasts and multiplying the result by 100%.

2.3. Cloning of the AgMYB80 Gene

Total RNA was extracted from celery leaves using Total RNA Extraction Kit (Shenzhen Aweidy Biotechnology Co., Ltd., Shenzhen, China). Subsequently, cDNA was synthesized through reverse transcription using Reverse Transcription Kit (Beijing Ts-

ingke Biotech Co., Ltd., Beijing, China). The sequence of the *AgMYB80* gene was amplified using specific primers (forward: 5′-ATGAAGAACACGCCATTGT-3′; reverse: 5′-TAAATCATCTGAGGGTAGATCC-3′). The amplified product was then cloned into the pUCm-T vector and sent for sequencing at Sangon Biotech (Shanghai) Co., Ltd, Shanghai, China. After sequence verification, the specific primers (forward: 5′-ACGGGGGACTAGAG AGATCCATGCACATATATTTCA-3′; reverse: 5′-GCCCTTGCTCATGCATGCATGCATCCT CTCATTCCA-3′) were used to amplify the verified sequence. The amplified product was cloned into the pSPYE vector with a GFP tag using the *BamH* I site, resulting in the generation of recombinant plasmid 35S: AgMYB80-GFP. The recombinant plasmid was transformed into the Escherichia coli strain DH5α using the heat shock transformation method.

Plasmid extraction was conducted using SanPrep Column Plasmid DNA Mini Extraction Kit (Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China). The concentration of the extracted plasmid DNA was measured using a micro-nucleic acid protein quantifier and subsequently adjusted to a concentration of 1000 ng·μL^{-1}. The recovered plasmid DNA was then stored in a refrigerator at −20 °C for future use.

2.4. Protoplast Transformation

The protoplast transformation of celery involves utilizing a PEG-mediated transient protoplast transformation system, adapted from the established protocols used for Arabidopsis and tomato, with slight modifications to suit the specific requirements of celery [17,24]. The isolated protoplast solution was chilled on ice and precipitated for 30 min by gravity. The supernatant was discarded, and the protoplasts were resuspended in MMG solution (0.4 M mannitol, 15 mM $MgCl_2$, and 4 mM MES; pH 5.7). A mixture of 10 μL of the target plasmid and 100 μL of protoplasts was gently mixed, followed by 110 μL of PEG solution (PEG4000, 0.2 M mannitol, and 100 mM $CaCl_2$). The mixture was left at room temperature for 15 min. Different concentrations of PEG4000 (0%, 10%, 20%, 30%, 40%, and 50%) were tested to optimize the PEG concentration. To optimize the transformation duration, the mixture was incubated in the dark at room temperature for 5, 10, 15, 20, and 25 min. To stop the reaction, 420 μL of W5 solution was added. Transfected protoplasts were incubated in the dark for 18–24 h and resuspended in 100 μL of W1 solution (4 mM MES, 0.5 M mannitol, and 20 mM KCl).

In order to determine the efficiency of transformation, we counted the number of protoplasts expressing a GFP fusion protein. Transformation efficiency = (number of fluorescent protoplasts/total number of protoplasts) × 100%.

2.5. Subcellular Localization

The AgMYB80 fusion expression vector was transformed into celery protoplasts using the PEG-mediated method, and mCherry carrying a nuclear localization signal was used as a nuclear marker. The localization of AgMYB80 in celery protoplasts was observed using a laser confocal scanning microscope.

2.6. Data Analysis

All experiments were replicated three times. Statistical analysis of the data was performed using SPSS 18.0. The significant differences between treatments were determined using the least significant difference (LSD) test at $p \leq 0.05$. The heat map was generated using TBtools software (version 1.12).

3. Results

3.1. Effect of Enzyme Combination and Enzymatic Digestion Time on Protoplast Isolation from Celery Leaves

As depicted in Table 1 and Figure 1, 45 combinations were utilized for protoplast isolation from plant leaves to identify the most effective enzyme combination and optimal enzymatic digestion time. The results demonstrated significant variations in the number of

protoplasts obtained under different enzyme combinations and digestion time conditions. The enzymatic digestion was conducted throughout the period of 6 to 12 h, with a mannitol concentration of 0.6 M in the enzyme solution.

Table 1. The yield of protoplasts treated with different combinations of enzymatic hydrolysates.

No.	Enzyme-Liquid Combination	Enzymatic Hydrolysis Time (h)	Protoplast Number (10^7 Protoplasts·g^{-1} FW)	Active Quantity (%)
1	1.5% cellulase + 0.05% pectolase	4	2.75 t	78.89 p
2	1.5% cellulase + 0.10% pectolase	4	4.84 lm	80.11 op
3	1.5% cellulase + 0.20% pectolase	4	4.84 lm	83.22 ghijkl
4	2.0% cellulase + 0.05% pectolase	4	5.14 jk	82.37 klmn
5	2.0% cellulase + 0.10% pectolase	4	5.21 j	87.1 cd
6	2.0% cellulase + 0.20% pectolase	4	5.17 jk	84.49 fgh
7	2.5% cellulase + 0.05% pectolase	4	4.88 l	82.83 hijklm
8	2.5% cellulase + 0.10% pectolase	4	4.97 kl	82.52 ijklmn
9	2.5% cellulase + 0.20% pectolase	4	4.78l mn	85.44 def
10	1.5% cellulase + 0.05% pectolase	6	3.15 s	84.43 fghi
11	1.5% cellulase + 0.10% pectolase	6	6.55 fgh	83.44 ghijkl
12	1.5% cellulase + 0.20% pectolase	6	6.62 fgh	84.31 fghij
13	2.0% cellulase + 0.05% pectolase	6	7.03 e	85.48 b
14	2.0% cellulase + 0.10% pectolase	6	6.59 fgh	86.03 b
15	2.0% cellulase + 0.20% pectolase	6	6.39 hi	89.22 a
16	2.5% cellulase + 0.05% pectolase	6	6.28 i	84.29 fghij
17	2.5% cellulase + 0.10% pectolase	6	6.58 fgh	85.00 efg
18	2.5% cellulase + 0.20% pectolase	6	6.24 i	80.99 mno
19	1.5% cellulase + 0.05% pectolase	8	3.40 r	88.08 c
20	1.5% cellulase + 0.10% pectolase	8	7.37 d	87.18 cd
21	1.5% cellulase + 0.20% pectolase	8	7.62 c	90.67 a
22	2.0% cellulase + 0.05% pectolase	8	7.99 a	91.90 a
23	2.0% cellulase + 0.10% pectolase	8	8.01 a	93.00 a
24	2.0% cellulase + 0.20% pectolase	8	7.86 b	91.73 ab
25	2.5% cellulase + 0.05% pectolase	8	7.45 cd	83.00 hijkl
26	2.5% cellulase + 0.10% pectolase	8	7.58 cd	82.04 lmn
27	2.5% cellulase + 0.20% pectolase	8	7.10 e	80.23 op
28	1.5% cellulase + 0.05% pectolase	10	4.53 op	83.90 fghijkl
29	1.5% cellulase + 0.10% pectolase	10	6.53 fgh	86.62 cde
30	1.5% cellulase + 0.20% pectolase	10	6.76 f	84.10 fghijk
31	2.0% cellulase + 0.05% pectolase	10	6.67 fg	87.03 cd
32	2.0% cellulase + 0.10% pectolase	10	7.48 cd	85.09 efg
33	2.0% cellulase + 0.20% pectolase	10	7.10 e	82.48 jklmn
34	2.5% cellulase + 0.05% pectolase	10	6.66 fg	80.67 no
35	2.5% cellulase + 0.10% pectolase	10	6.5 gh	82.02 lmn
36	2.5% cellulase + 0.20% pectolase	10	6.53 fgh	71.64 s
37	1.5% cellulase + 0.05% pectolase	12	4.55 nop	75.05 r
38	1.5% cellulase + 0.10% pectolase	12	4.55 nop	76.84 q
39	1.5% cellulase + 0.20% pectolase	12	4.79 lm	74.43 r
40	2.0% cellulase + 0.05% pectolase	12	4.76l mno	77.09 q
41	2.0% cellulase + 0.10% pectolase	12	4.80 lm	75.04 r
42	2.0% cellulase + 0.20% pectolase	12	4.55 nop	71.89 s
43	2.5% cellulase + 0.05% pectolase	12	4.45 p	71.13 st
44	2.5% cellulase + 0.10% pectolase	12	4.63 mnop	69.87 t
45	2.5% cellulase + 0.20% pectolase	12	4.10 q	65.86 u

Notes: The letters represent statistically significant differences at $p \leq 0.05$.

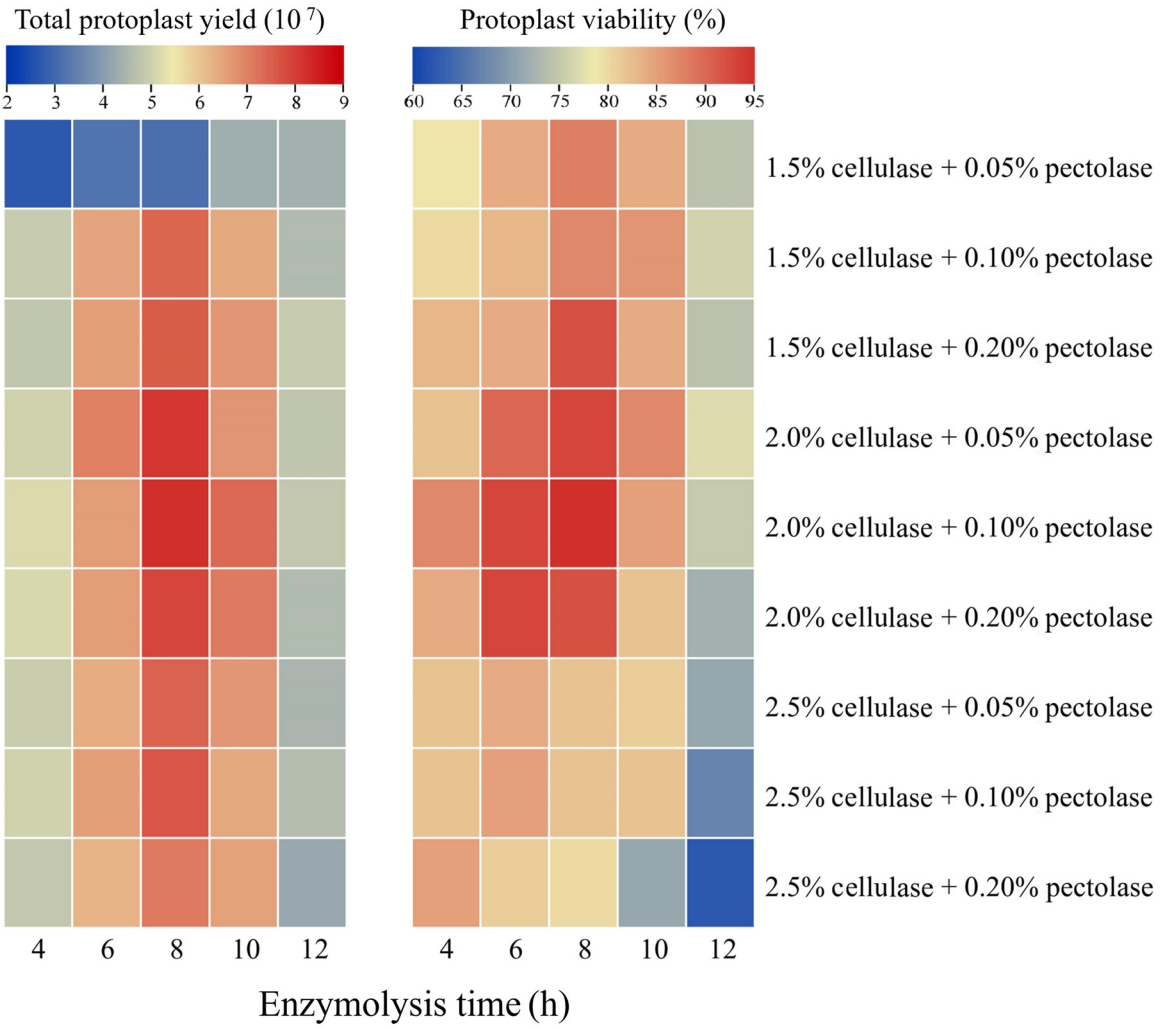

Figure 1. Effects of enzyme components on protoplast activity and yield of celery leaf protoplasts. Depending on the value, blue or red corresponds to a low or high value in the heatmap.

The findings from the cellulase concentration screening revealed that, with a constant pectolase concentration (0.2%) and digestion time (4 h), the protoplast yield and viability initially increased and subsequently decreased as the cellulase concentration increased. The highest yield of 5.17×10^7 protoplasts with a viability of 84.49% was achieved at a cellulase concentration of 2.0%. Similarly, in the pectolase concentration screening, with a constant cellulase concentration (2.0%) and digestion time (4 h), the protoplast yield and viability initially increased and then declined with higher pectolase concentrations. The highest yield of 5.21×10^7 protoplasts with a viability of 87.10% was attained at a pectolase concentration of 0.1%.

The results of the digestion time screening revealed that the protoplast yield exhibited an initial increase followed by a decrease as the digestion time was extended. The highest yield of 8.01×10^7 protoplasts with a maximum viability of 93% was achieved after eight hours of digestion. The protoplasts exhibited intact cell morphology, improved cell dispersal, and minimal fragmentation with this duration. In contrast, at 4 and 6 h of digestion, the cells remained clustered or only partially separated. Moreover, the yield was lower at 10 and 12 h of digestion, and the cell morphology was compromised with signs of cellular damage.

Based on the experimental findings, the optimal enzyme combination for protoplast isolation is a mixture of 2.0% cellulase and 0.1% pectolase, with an 8 h digestion time. This

combination has shown the highest protoplast yield and viability. Consequently, it will be employed for subsequent experiments in the study.

3.2. Effect of Mannitol Concentration on Protoplast Isolation

For optimum mannitol concentrations in enzyme solutions, a range of mannitol concentrations from 0.4 M to 0.8 M were tested. All treatments were performed using a combination of 2.0% cellulase and 0.1% pectolase for an 8 h digestion time. As the mannitol concentration in the enzyme solution increased, the yield and viability of protoplasts initially increased and then subsequently decreased (Figure 2A). The highest yield and viability of protoplasts were achieved with a mannitol concentration of 0.6 M, resulting in a total yield of 8.01 $\times$ 10^7 protoplasts with a viability of 93%. However, when the mannitol concentration was increased to 0.7 M and 0.8 M, the protoplast yield and viability decreased while the proportion of broken protoplasts increased. Conversely, when the mannitol concentration was reduced to 0.4 M and 0.5 M, the protoplast yield and viability were significantly lower compared to the optimal concentration of 0.6 M. Based on the results obtained, the optimal mannitol concentration in the enzyme solution for protoplast isolation is determined to be 0.6 M.

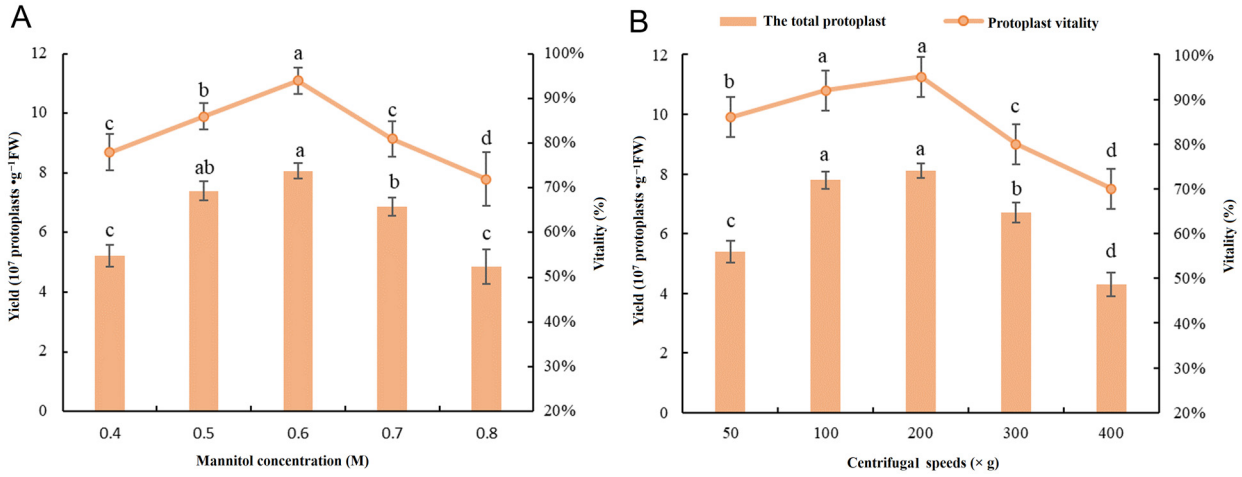

Figure 2. The yield of protoplasts from the protoplast isolation protocol under different mannitol concentrations (**A**) and centrifugation rates (**B**). The letters represent statistically significant differences at $p \leq 0.05$.

3.3. Effect of Centrifugation Speed on Protoplast Purification

Following enzymatic digestion using 2.0% cellulase and 0.1% pectolase in an enzyme solution containing 0.6 M mannitol for 8 h, the impact of different centrifugation speeds (50, 100, 200, 300, and 400$\times$ *g*) on protoplast purification was evaluated. Upon filtration through a 400-mesh sieve, the purified protoplasts were obtained. The outcomes of this purification process are presented in Figure 2B. The results indicate that as the centrifugation speed increased, the yield and viability of the lower layer protoplasts initially increased and then gradually decreased.

The optimal centrifugation speed for obtaining the highest viability and yield of protoplasts was 200$\times$ *g*, resulting in a yield of 8.22 $\times$ 10^7 protoplasts and viability of 95%. Further increases in centrifugation speed led to decreased protoplast yield and viability, accompanied by an increased proportion of broken protoplasts and fragments. At centrifugation speeds exceeding 300$\times$ *g*, some intact protoplasts were observed to be broken during the process, resulting in a lower yield of purified protoplasts. Hence, a centrifugation speed of 200$\times$ *g* was identified as the most suitable speed for the purification of protoplasts.

An optimized protocol for celery protoplast isolation involved an eighth round of enzymatic digestion using a mixture of 2.0% cellulase and 0.1% pectolase in an enzymatic

solution containing 0.6 M mannitol. Subsequent purification of the protoplasts was successfully performed through centrifugation at a speed of 200× *g*. This optimized protocol resulted in a high yield, viability, and purity of protoplasts. Following optimization, the total protoplast yield reached 8.22×10^7, with a viability of 95% being determined using FDA staining (Figure 3).

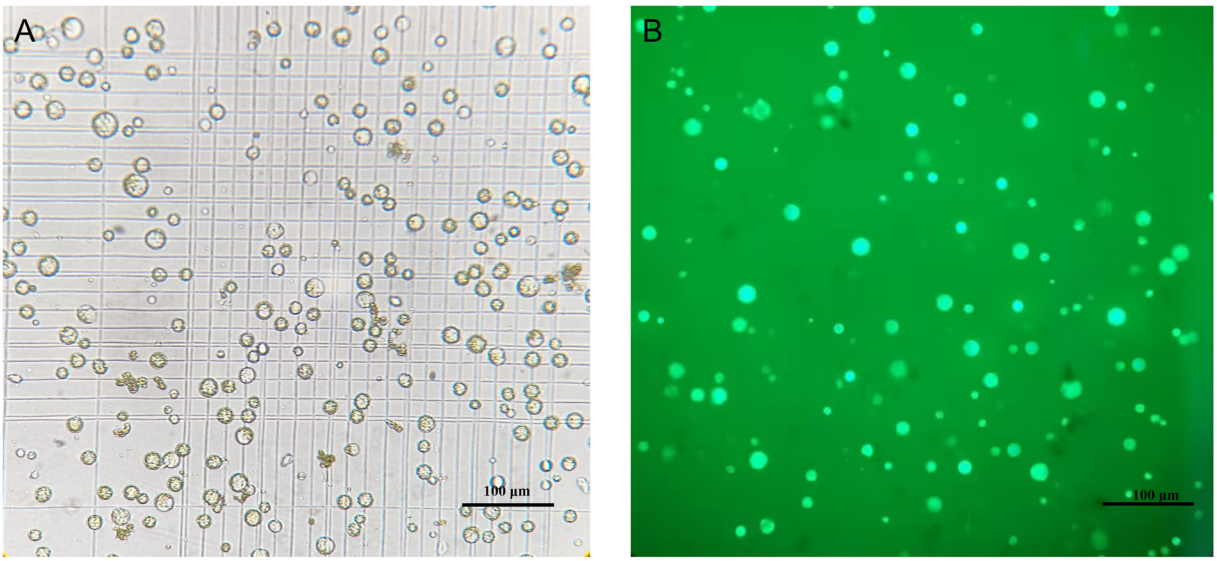

Figure 3. Yield and vitality detection of FDA-dyed protoplasts isolated from celery leaves under bright light (**A**) and ultraviolet light (**B**).

3.4. Protoplast Transformation of Celery Leaf Cells

This study utilized the PEG-mediated method to investigate the influence of protoplast concentration, exogenous DNA content, and PEG concentration on the efficiency of transient transformation in celery leaf protoplasts. The collected celery leaf protoplasts were diluted into cell suspensions of various concentrations and subjected to the same transformation method and incubation period. The efficiency of protoplast transformation was assessed using a fluorescence microscope, as depicted in Figure 4A,B. The optimal concentration of celery leaf protoplasts for transformation was determined to be 5×10^5 cells·mL^{-1}, with an exogenous DNA content of 50 μg·mL^{-1}, resulting in the highest transformation efficiency of approximately 53%. Deviating from this optimal concentration significantly decreased the transformation efficiency. For instance, at protoplast concentrations of 1×10^5 cells·mL^{-1} and 7×10^5 cells·mL^{-1}, the transformation efficiency was only around 23%. Similarly, higher or lower exogenous DNA concentrations had a negative impact on protoplast transformation efficiency. At an exogenous DNA content of 30 μg·mL^{-1}, the efficiency was less than 20%, while at a higher DNA content of 70 μg·mL^{-1}, the efficiency was less than 30%.

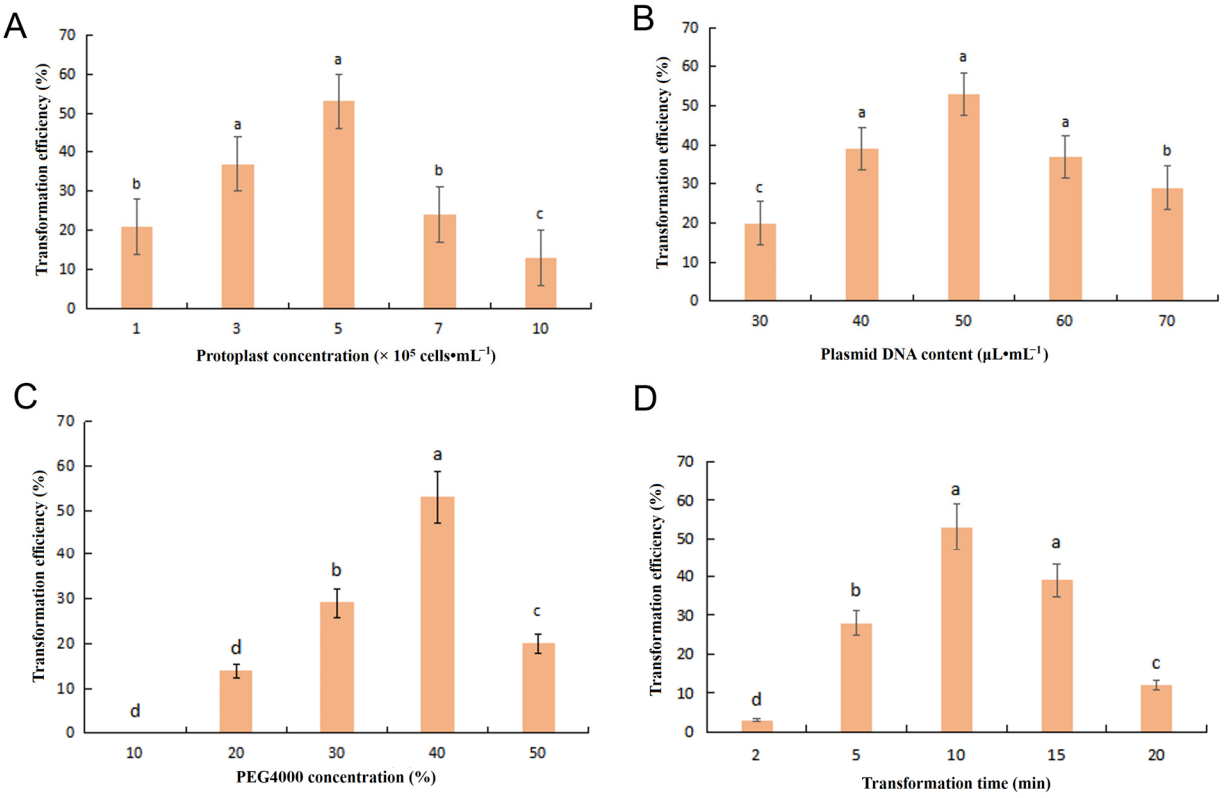

Figure 4. Transformation efficiency affected by protoplast concentration (**A**), plasmid DNA content (**B**), PEG4000 concentration (**C**) and transformation time (**D**). The letters represent statistically significant differences at $p \leq 0.05$.

Figure 4D illustrates that the transformation efficiency gradually increased with higher concentrations of PEG4000. Protoplasts displayed minimal successful transformation when the PEG4000 concentration was below 10%. The highest transformation efficiency was attained at PEG4000 concentrations of 30% and 40%, with no significant difference observed between the two concentrations. However, when the PEG4000 concentration exceeded 40%, a slight decrease in the transformation efficiency was observed. Notably, the transformation efficiency at a 50% PEG4000 concentration was lower than 30% and 40%.

Furthermore, the transformation efficiency demonstrated an initial increase with longer transformation durations (Figure 4C). The highest efficiency, reaching 40%, was achieved at 10 min of transformation, with no significant difference between 10 and 15 min. However, as the transformation time exceeded 20 min, a noticeable decline in the transformation efficiency was observed.

The optimized protocol for celery protoplast transformation involves using a protoplast concentration of 5×10^7 cells·mL^{-1} and a plasmid concentration of 50 μL·mL^{-1}. The transformation is performed by exposing the protoplasts to a 40% concentration of PEG4000 for 10 min. Utilizing this optimized system, the efficiency of celery leaf protoplast transformation can reach 53%.

3.5. Subcellular Localization of AgMYB80

Celery leaf protoplasts were used to validate the transient expression system by examining the subcellular localization of AgMYB80. The results confirmed the expected outcome, as the co-expression of the GFP-MYB80 fusion protein in celery leaf protoplasts resulted in a distinct GFP signal specifically localized in the nucleus (Figure 5). In contrast, the reporter gene GFP was observed in all organelles, serving as a control. This validation confirms that the expression of celery leaf protoplasts is a reliable method suitable for subcellular localization analysis.

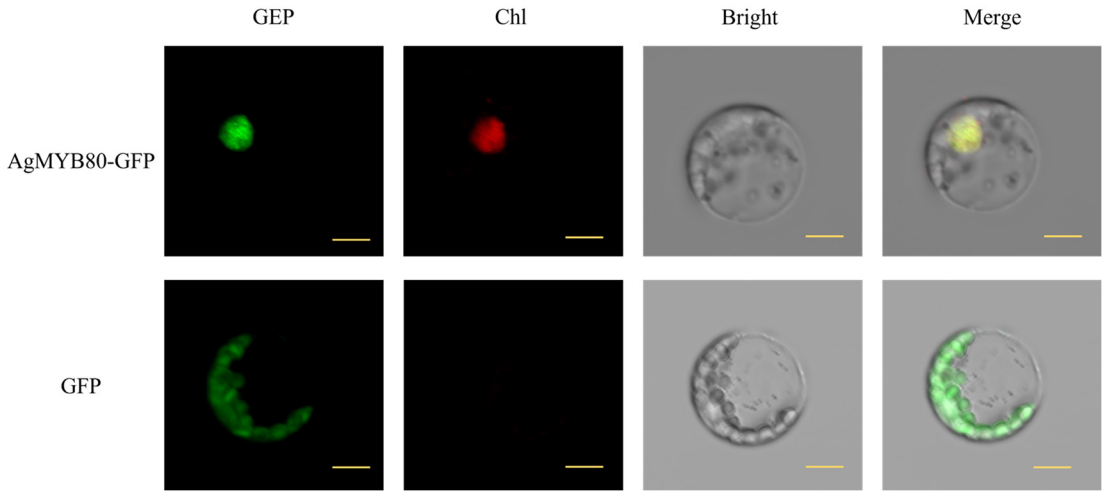

Figure 5. Subcellular localization analysis of AgMYB80 in celery leaf protoplasts under fluorescence microscopy. Scale bar = 15 μm.

4. Discussion

To establish an efficient transient transformation system, a stable protoplast isolation method must be employed to yield many highly viable protoplasts. Several factors play a crucial role in protoplast preparation, including the selection of plant tissues, enzyme concentration, and enzymatic digestion time. Careful consideration and optimization of these factors are necessary to ensure successful protoplast isolation and subsequent transformation experiments [6,7]. Healthy and young leaves are preferred for protoplast preparation. This study used 4-week-old celery tissue culture seedlings, improving protoplast practice and transient gene transformation efficiency. The enzyme composition and digestion time play a critical role in successful protoplast isolation, especially in the dissociation of leaf cells and the release of protoplasts. The specific enzymes and their optimal concentrations may vary depending on the plant species and tissue. For instance, previous studies in Arabidopsis demonstrated that a combination of 1.5% cellulase and 0.5% pectolase resulted in complete cell wall lysis and a reduced cell fragment presence [25]. The most favorable outcomes for maize leaf protoplast preparation were achieved using a combination of 1.5% cellulase and 0.5% macerozyme, as reported in previous research [10]. Digestion time and enzyme concentration have an interactive relationship, typically necessitating shorter digestion times with higher enzyme concentrations. In this experiment, various cellulase R-10 and pectolase Y-23 concentrations were employed to release protoplasts from leaf tissue at different digestion times. The findings revealed that a combination of 2.0% cellulase and 0.1% pectolase, with an 8 h digestion period, yielded the highest number of active and viable protoplasts. Furthermore, osmotic pressure plays a crucial role in protoplast isolation, and mannitol is commonly employed as an osmotic stabilizer in this process [26]. In the enzymatic function of protoplast isolation, the absence of cell walls leaves protoplasts vulnerable and in need of osmotic regulators to maintain the balance between their internal and external environments. Glucose, mannitol, and sorbitol are commonly added to adjust the enzymatic solution's osmotic pressure. In this study, different concentrations of mannitol were examined in the enzyme solution containing 2.0% cellulase and 0.1% pectolase. The results indicated that a mannitol concentration of 0.6 M resulted in an optimal protoplast yield and optimal viability, aligning with previous research findings [27].

Limited research has been conducted on the factors influencing protoplast purification, with only a few reports available in the current literature [28]. The efficient purification and isolation of protoplasts are essential to eliminate any undigested protoplast fragments and broken protoplasts [29]. Our experimental investigations assessed the factors influencing protoplast purification, particularly centrifugation speed. After subjecting the leaf samples

to enzymatic digestion using 2.0% cellulase and 0.1% pectolase in a solution containing 0.6 M mannitol for 8 h, the mixture was filtered through a nylon mesh to eliminate leaf fragments and cell clusters. To remove broken cell debris, centrifugation was employed. The purity of the obtained protoplasts was confirmed through optical microscopy. We observed that the highest yield of purified protoplasts was achieved at a centrifugation speed of 200× *g*. Similar observations have been reported, with lower centrifugation speeds being generally recommended to prevent protoplast rupture [28]. In our study, by optimizing various parameters affecting protoplast isolation and purification, we obtained high-yield and comparatively pure protoplasts satisfied for protoplast transformation. The yield and viability of protoplasts (8.22×10^7 protoplasts g^{-1} FW, 95%) were comparable to the values reported for perennial ryegrass (5.6×10^7 protoplasts g^{-1} FW, more than 80%) [30] and cassava (4.4×10^7 protoplasts g^{-1} FW, more than 90%) [31]. Notably, the system developed for celery protoplast isolation and purification in this investigation outperformed previous systems employed in other vegetable crops, such as Chinese cabbage [32], cabbage [33], and rape [34]. These discrepancies may be attributed to inherent characteristics of the plant itself. The results indicated that the system of protoplast isolation and purification using our protocol was highly efficient.

The PEG-mediated transformation of protoplasts is widely used in plant molecular biology studies. It has the advantages of convenience, a shorter processing time, and high transformation efficiency [35–39]. The success of transgenic expression systems depends on the choice of a suitable transformation method. Transformation efficiency is influenced by several factors, including the number of protoplasts, plasmid DNA concentration, PEG concentration, and transformation time [6,7]. Transformation efficiency can be hindered by an excessive or densely packed number of protoplasts or a low amount of plasmid DNA, leading to the limited entry of DNA into the protoplasts. Conversely, a low number of protoplasts and a high plasmid DNA concentration do not necessarily enhance transformation efficiency. This study achieved optimal transformation efficiency with a celery protoplast concentration of 8×10^7 per milliliter and a plasmid DNA concentration of 50 $\mu L \cdot mL^{-1}$. Previous research has also highlighted the significance of PEG4000 concentration and transformation time in maximizing transformation efficiency [20]. For the purpose of assessing the effect of PEG4000 concentration and transformation duration on the efficiency of transformation, this study optimized the transformation protocol for celery leaf protoplasts. The efficiency of protoplast transformation was evaluated using GFP as a detection marker. The findings revealed that both the PEG4000 concentration and transformation duration played a crucial role in determining the transformation efficiency of celery protoplasts. Elevated concentrations of PEG4000 and longer transformation durations positively affected the transformation efficiency. However, excessively high concentrations of PEG4000 or extended transformation durations had inhibitory effects on the transformation process. For instance, using PEG4000 at a concentration of 50% or duration of $\geq$20 min resulted in reduced transformation efficiency. For instance, using PEG4000 at a concentration of 50% or duration of $\geq$20 min resulted in reduced transformation efficiency. This is the same as what has been observed previous studies on Arabidopsis [16], rice [40] and other plants. In this study, the optimum combined conversion efficiency was found to be 53%. Although this efficiency value is comparable to that previously reported for sweet cherries [19], it is lower than the conversion efficiency value for the protoplast separation of Arabidopsis (60–90%) [16]. This difference in conversion efficiency can be attributed to intrinsic differences between plant species, with the higher conversion rates of Arabidopsis protoplasts. This observation is in accordance with the findings of earlier research, which underscores the existence of noteworthy inter-species disparities [41]. The disparities in transformation efficiency could be attributed to inherent differences between plant species, as Arabidopsis protoplasts exhibit higher transformation rates. In this study, we introduced a rapid transient transformation method as an alternative for investigating gene function in celery. By employing this system, we successfully examined the subcellular localization of

the AgMYB80 gene. The results confirmed the nuclear localization of AgMYB80 in celery protoplasts, validating the efficacy of this approach for subcellular localization studies.

5. Conclusions

The main findings of this study highlight the successful optimization of celery protoplast preparation and the establishment of an efficient, stable, and rapid transient expression system. By employing an enzymatic digestion process using 2.0% cellulase and 0.1% pectolase in an enzyme solution containing 0.6 M mannitol for 8 h, a substantial yield of 8.22×10^7 viable protoplasts was achieved using a centrifugation speed of $200\times$ *g*. Additionally, by adjusting the protoplast concentration to 5×10^7 cells·mL^{-1} and plasmid concentration to 50 μL·mL^{-1}, combined with 10 min of incubation using 40% PEG4000, a protoplast transformation efficiency value of approximately 53% was obtained.

These optimized methods and systems provide robust support for subcellular localization studies and protein interaction analysis. Through successful subcellular localization studies, the nuclear localization of the AgMYB80 protein was confirmed, offering key insights into the functional genomics, regulatory mechanisms, and gene positioning in the transcription factors of plants.

Furthermore, this study has significant implications for future research and potential applications in biotechnology. The efficient, stable, and rapid celery protoplast preparation and transformation system established here lays a solid foundation for functional genomic and gene editing studies on celery. It opens up new avenues for a deeper understanding of gene functions, metabolic pathways, the development of improved varieties, and proteomic research in celery. Moreover, this successful methodology holds the potential for adaptation and application in other areas of crop research, promising a wide-ranging impact in the field of plant science research and agricultural innovation.

In conclusion, the important findings and optimized methods of this study not only provide valuable tools for celery protoplast research but also present breakthroughs for biotechnological applications in related fields, offering vast prospects for future research and innovation in plant science.

Author Contributions: Conceptualization, M.L. and A.X.; methodology, H.Z., J.D., W.L. and X.L.; resources, J.D., Z.W. and Y.Z.; data curation and visualization, J.D.; writing—original draft preparation, H.Z. and J.D.; funding acquisition, M.L. All authors have read and agreed to the published version of the manuscript.

Funding: The research was supported by the National Natural Science Foundation of China (32002027), and the National Natural Science Foundation of Sichuan Province (2022NSFSC1647).

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Lei, R.; Qiao, W.J.; Hu, F.; Jiang, H.S.; Zhu, S.F. A simple and effective method to encapsulate tobacco mesophyll protoplasts to maintain cell viability. *Methods X* **2015**, *2*, 24–32. [CrossRef]
2. Davey, M.R.; Anthony, P.; Power, J.B.; Lowe, K.C. Plant protoplasts: Status and biotechnological perspectives. *Biotechnol. Adv.* **2005**, *23*, 131–171. [CrossRef] [PubMed]
3. Sheen, J. Signal transduction in maize and Arabidopsis mesophyll protoplasts. *Plant Physiol.* **2001**, *127*, 1466–1475. [CrossRef] [PubMed]
4. Gardossi, L.; Poulsen, P.B.; Ballesteros, A.; Hult, K.; Švedas, V.K.; Vasić-Rački, Đ.; Carrea, G.; Magnusson, A.; Schmid, A.; Wohlgemuth, R.; et al. Guidelines for reporting of biocatalytic reactions. *Trends Biotechnol.* **2010**, *28*, 171–180. [CrossRef] [PubMed]
5. Pinelo, M.; Arnous, A.; Meyer, A.S. Upgrading of grape skins: Significance of plant cell-wall structural components and extraction techniques for phenol release. *Trends Food Sci. Technol.* **2006**, *17*, 579–590. [CrossRef]
6. Chen, L.P.; Zhang, M.F.; Hirata, Y.T.; Cao, J.S.; Chen, Z.J. Efficient plant regeneration from cotyledon-derived protoplasts of cytoplasmic male-sterile tuber mustard (*Brassica juncea* Coss. var. tumida Tsen et Lee). *Acta Pharmacol. Sin.* **2001**, *27*, 437–440.
7. Zhang, Y.; Su, J.; Duan, S.; Ao, Y.; Dai, J.; Liu, J.; Wang, P.; Li, Y.; Liu, B.; Feng, D.; et al. A highly eficient rice green tissue protoplast system for transient gene expression and studying light/chloroplast-related processes. *Plant Methods* **2011**, *7*, 30. [CrossRef]

8. Wang, H.L.; Wang, W.; Zhan, J.C.; Huang, W.D.; Xu, H.Y. An efficient PEG-mediated transient gene expression system in grape protoplasts and its application in subcellular localization studies of flavonoids biosynthesis enzymes. *Sci. Hortic.* **2015**, *191*, 82–89. [CrossRef]
9. Thomas, M.R.; Rose, R.J. Enrichment for Nicotiana heterokaryons after protoplast fusion and subsequent growth in agarose microdrops. *Planta* **1988**, *175*, 396–402. [CrossRef]
10. Cocking, E.C. A method for the isolation of plant protoplast and vacuoles. *Nature* **1960**, *187*, 962–963. [CrossRef]
11. Reddy, C.R.K.; Dipakkore, S.; Kumar, G.R.; Jha, B.; Cheney, D.P.; Fujita, Y. An improved enzyme preparation for rapid mass production of protoplasts as seed stock for aquaculture of macrophytic marine green alga. *Aquaculture* **2006**, *260*, 290–297. [CrossRef]
12. He, P.; Shan, L.; Sheen, J. The use of protoplasts to study innate immune responses. *Methods Mol. Biol.* **2007**, *354*, 1–9. [PubMed]
13. Fujikawa, Y.; Nakanishi, T.; Kawakami, H.; Yamasaki, K.; Sato, M.H.; Tsuji, H.; Matsuoka, M.; Kato, N. Split luciferase complementation assay to detect regulated protein-protein interactions in rice protoplasts in a large-scale format. *Rice* **2014**, *7*, 11. [CrossRef]
14. Sheng, X.; Gu, H.; Yu, H.; Wang, J.; Zhao, Z.; Qi, Z. An efficient shoot regeneration system and Agrobacterium-mediated transformation with codA gene in a doubled haploid line of broccoli. *Can. J. Plant Sci.* **2016**, *96*, 1014–1020.
15. Jefferson, R.A.; Kavanagh, T.A.; Bevan, M.W. GUS fusions: Beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **1987**, *6*, 3901–3907. [CrossRef]
16. Yoo, S.D.; Cho, Y.H.; Sheen, J. Arabidopsis mesophyll protoplasts: A versatile cell system for transient gene expression analysis. *Nat. Protoc.* **2007**, *2*, 1565–1572. [CrossRef]
17. Huang, H.; Wang, Z.; Cheng, J.; Zhao, W.; Li, X.; Wang, H.; Zhang, Z.; Sui, X. An efficient cucumber (*Cucumis sativus* L.) protoplast isolation and transient expression system. *Sci. Hortic.* **2013**, *150*, 206–212. [CrossRef]
18. Chen, J.; Yi, Q.; Song, Q.; Gu, Y.; Zhang, J.; Hu, Y.; Liu, H.; Liu, Y.; Yu, G.; Huang, Y. A highly efficient maize nucellus protoplast system for transient gene expression and studying programmed cell death-related processes. *Plant Cell Rep.* **2015**, *34*, 1239–1251. [CrossRef]
19. Yao, L.P.; Liao, X.; Gan, Z.Z.; Peng, X.; Wang, P.; Li, S.J. Protoplast isolation and development of a transient expression system for sweet cherry (*Prunus avium* L.). *Sci. Hortic.* **2016**, *209*, 14–21. [CrossRef]
20. Wu, J.Z.; Liu, Q.; Geng, X.S.; Li, K.M.; Luo, L.J.; Liu, J.P. Highly efficient mesophyll protoplast isolation and PEG-mediated transient gene expression for rapid and large-scale gene characterization in cassava (*Manihot esculenta* Crantz). *BMC Biotechnol.* **2017**, *17*, 29. [CrossRef]
21. Li, M.; Zhou, J.; Du, J.; Li, X.; Sun, Y.; Wang, Z.; Lin, Y.; Zhang, Y.; Wang, Y.; He, W.; et al. Comparative Physiological and Transcriptomic Analyses of Improved Heat Stress Tolerance in Celery (*Apium graveolens* L.) Caused by Exogenous Melatonin. *Int. J. Mol. Sci.* **2022**, *23*, 11382. [CrossRef]
22. Li, M.Y.; Feng, K.; Hou, X.L.; Jiang, Q.; Xu, Z.S.; Wang, G.L.; Liu, J.X.; Wang, F.; Xiong, A.S. The genome sequence of celery (*Apium graveolens* L.), an important leaf vegetable crop rich in apigenin in the Apiaceae family. *Hortic. Res.* **2020**, *7*, 9. [CrossRef]
23. Pua, E.C. Plant regeneration from stem-derived protoplasts of Brassica alboglabra bailey. *Plant Sci.* **1987**, *50*, 153–160. [CrossRef]
24. Larkin, P.J. Purification and viability determinations of plant protoplasts. *Planta* **1976**, *128*, 213–216. [CrossRef] [PubMed]
25. Yu, G.H.; Cheng, Q.; Xie, Z.N.; Xu, B.; Huang, B.R.; Zhao, B.Y. An efficient protocol for perennial ryegrass mesophyll protoplast isolation and transformation, and its application on interaction study between LpNOL and LpNYC1. *Plant Methods* **2017**, *13*, 46. [CrossRef] [PubMed]
26. Iguti, S. Effects of mannitol on protoplasts of Saccharomyces cerevisiae. *Plant Cell Physiol.* **1968**, *9*, 573–576.
27. Hu, Y.; Huang, Y. Isolation and Transfection of Maize Endosperm Protoplasts. *Methods Mol. Biol.* **2022**, *2464*, 105–121.
28. Kanai, R.; Edwards, G.E. Purification of enzymatically isolated mesophyll protoplasts from c(3), c(4), and crassulacean Acid metabolism plants using an aqueous dextran-polyethylene glycol two-phase system. *Plant Physiol.* **1973**, *52*, 484–490. [CrossRef]
29. Navrátilová, B. Protoplast cultures and protoplast fusion focused on Brassicaceae: A review. *Hortic. Sci.* **2018**, *31*, 140–157. [CrossRef]
30. Zhang, J.; Li, H.; Jiang, Y.; Li, H.; Zhang, Z.; Xu, Z.; Xu, B.; Huang, B. Natural variation of physiological traits, molecular markers, and chlorophyll catabolic genes associated with heat tolerance in perennial ryegrass accessions. *BMC Plant Biol.* **2020**, *20*, 520. [CrossRef]
31. McMahon, J.; Sayre, R.; Zidenga, T. Cyanogenesis in cassava and its molecular manipulation for crop improvement. *J Exp Bot.* **2022**, *73*, 1853–1867. [CrossRef] [PubMed]
32. Sivanandhan, G.; Bae, S.; Sung, C.; Choi, S.R.; Lee, G.J.; Lim, Y.P. Optimization of Protoplast Isolation from Leaf Mesophylls of Chinese Cabbage (*Brassica rapa* ssp. *pekinensis*) and Subsequent Transfection with a Binary Vector. *Plants* **2021**, *10*, 2636. [CrossRef] [PubMed]
33. Kiełkowska, A.; Adamus, A. Peptide Growth Factor Phytosulfokine-α Stimulates Cell Divisions and Enhances Regeneration from *B. oleracea* var. *capitata* L. Protoplast Culture. *J. Plant Growth Regul.* **2019**, *38*, 931–944. [CrossRef]
34. Li, X.; Sandgrind, S.; Moss, O.; Guan, R.; Ivarson, E.; Wang, E.S.; Kanagarajan, S.; Zhu, L.H. Efficient Protoplast Regeneration Protocol and CRISPR/Cas9-Mediated Editing of Glucosinolate Transporter (*GTR*) Genes in Rapeseed (*Brassica napus* L.). *Front Plant Sci.* **2021**, *12*, 680859. [CrossRef] [PubMed]

35. Jeon, J.M.; Ahn, N.Y.; Son, B.H.; Kim, C.Y.; Han, C.D.; Kim, G.D.; Gal, S.W.; Lee, S.H. Efficient transient expression and transformation of PEG-mediated gene uptake into mesophyll protoplasts of pepper (*Capsicum annuum* L.). *Plant Cell Tissue Organ Cult.* **2007**, *88*, 225–232. [CrossRef]
36. Pitzschke, A.; Persak, H. Poinsettia protoplasts—A simple, robust and efficient system for transient gene expression studies. *Plant Methods* **2012**, *8*, 14. [CrossRef]
37. Burris, K.P.; Dlugosz, E.M.; Collins, A.G.; Stewart, C.N.; Lenaghan, S.C. Development of a rapid, low-cost protoplast transfection system for switchgrass (*Panicum virgatum* L.). *Plant Cell Rep.* **2016**, *35*, 693–704. [CrossRef]
38. Omar, A.A.; Dutt, M.; Gmitter, F.G.; Grosser, J.W. Somatic Embryogenesis: Still a Relevant Technique in Citrus Improvement. *Methods Mol. Biol.* **2016**, *1359*, 289–327.
39. Hong, S.Y.; Seo, P.J.; Cho, S.H.; Park, C.M. Preparation of leaf mesophyll protoplasts for transient gene expression in *Brachypodium distachyon*. *J. Plant Biol.* **2012**, *55*, 390–397. [CrossRef]
40. Burman, N.; Chandran, D.; Khurana, J.P. A Rapid and Highly Efficient Method for Transient Gene Expression in Rice Plants. *Front Plant Sci.* **2020**, *11*, 584011. [CrossRef]
41. Yang, D.; Zhao, Y.; Liu, Y.; Han, F.; Li, Z. A high-efficiency PEG-Ca^{2+}-mediated transient transformation system for broccoli protoplasts. *Front Plant Sci.* **2022**, *13*, 1081321. [CrossRef] [PubMed]