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Optimized Regeneration of *Petunia* Protoplast and Its Association with Tissue Identity Regulators

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Abstract: The popular ornamental plant *Petunia* is also a valuable model plant in tissue culture. Cellular conversions during differentiation and regeneration have been investigated using various combinations of phytohormones; however, studies on genes for reprogramming toward desired tissue identities have been limited. In this study, we isolated *Petunia* protoplasts and cultured them in the callus, rooting, or shooting stages, which were used to establish the optimal protoplast culture conditions and to identify genes that epigenetically function as tissue identifiers. The optimal conditions for plasmolysis and enzyme digestion to obtain healthy protoplasts were compared, in which combinations of Viscozyme, Celluclast, and Pectinex (VCP) enzymes were more efficient in isolating protoplasts when followed by 21 to 25% sucrose purification and washing processes. The filtered and washed protoplasts started to divide at 1 day and developed into colonies after 3 weeks of culture, which showed higher efficiency in the Murashige and Skoog (MS) salt culture media compared to that in the Kao and Michayluk (KM) salt media. The pluripotent colonies formed calli on the solid medium supplemented with 3% sucrose after 4 weeks, and were destined to the same cell mass, rooting, or shooting on the regeneration medium. Three epigenetic controllers, *ATXR2*, *ATX4A*, and *ATX4B*, were highly expressed in calli, shoots, and organs of shoots and roots, respectively, confirming that dedifferentiation and regeneration of tissue identity is plastic.

Keywords: dedifferentiation; organogenesis; petunia; protoplast; regeneration

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1. Introduction

A protoplast is a plant cell lacking a cell wall, which is removed using fungal enzymes such as cellulase [1,2]. Under the influence of various hormones, almost all plant cells have the potential to regenerate through cell dedifferentiation and differentiation [1]. Leaf mesophyll is a common source of protoplasts and a universal system for the transient expression of plant genes and plant regeneration [2,3]; however, protoplast isolation depends on the explant and plant species [4,5]. Therefore, it is necessary to establish the conditions for protoplast culture and shoot regeneration when different plant cultivars or species are used. Initially, the protoplast was used for symmetric or asymmetric fusion of cells from the same or different species [6]. Recently, however, protoplast culture has drawn attention owing to its role in creating DNA-free plants using a CRISPR-related genome editing technology [7].

Research on *Petunia* protoplasts was started in the late 1970s to facilitate their isolation and culture. *Petunia* protoplasts were a suitable model system to characterize the effects of

explants, genotypes, and digestion enzymes [5,8,9]. Efficient protoplast isolation from leaf mesophyll cells and its transient gene expression protocols have already been determined for the cultivar Madness Midnight by our group [10]; the yields of mesophyll protoplasts have been found to be similar to those previously reported in *Petunia* genotypes [4]. This suggested that the cultivar and its protoplast system could be helpful in studying the genetics of phenotypic traits. With the development of technology, protoplast culture for the regeneration of various plants has become more efficient [2,11,12]. This efficient culture system can be the foundation for the research of other transient gene transformations, transgenic plants, or target-directed mutagenesis. Using the cultivar Madness Midnight and its protoplast system, the regeneration of genome-edited *Petunia* plants has also been successfully demonstrated [7].

Genes involved in callus, root, or shoot formation have been reported to play independent roles in corresponding differentiation processes. Generally, callus induction and proliferation are under the genetic control of a set of developmental regulators; the identification of such genes and regulatory elements will help in the development of in vitro systems in *Petunia*. According to recent studies by Lee et al. [13,14], *Arabidopsis trithorax-related 2* (*ATXR2*) and *Arabidopsis trithorax 4* (*ATX4*) were found to be dynamically upregulated during callus and shoot formation, respectively. Arabidopsis *atxr2*-defficient mutants showed defects in the formation of calli and adventitious roots, indicating their involvement in epigenetic regulation of cellular dedifferentiation [13]. The *ATX4* plays a vital role in regeneration by accumulating H3K4me3, which participates in shoot identity establishment. Arabidopsis *atx4*-defficient mutants exhibited enhanced callus and repressed shoot identity [15].

Although previously established protoplast isolation and regeneration protocols which are specific to the genome engineering of *Petunia* [7,10] are available, this study focused on developing simple and efficient protoplast isolation and protoplast-derived cell proliferation and regeneration methods, as well as investigating the molecular events involved in the dedifferentiation and regeneration phases. The results obtained from this study may be helpful in studying the functional analysis of developmental regulators involved in the cellular dedifferentiation of *Petunia* parental genomes.

2. Results and Discussion

2.1. Enzyme Combinations for Protoplast Isolation

P × *hybrida* cv. Madness Midnight leaf protoplast is known to have higher transformation capacity, which allows for efficient protoplast isolation and optimized culture conditions [7]. After 3 h of shaking incubation, protoplasts were released into the enzyme solution. Depending on the enzyme combinations, the yield of protoplasts varied between 1.82×10^6 and 6.9×10^6 cells per gram fresh weight (Figure 1A). For Macerozyme R and Cellulase R-1 (MC) enzyme combinations (EC 1, EC 2, and EC 3), the range of protoplast yield was 1.8×10^6–3.2×10^6 cells per gram fresh weight, while the range of protoplast was 3.8×10^6–6.9×10^6 for combinations of Viscozyme, Celluclast, and Pectinex (VCP) enzymes (EC 4, EC 5, EC 6, and EC 7). More protoplasts were obtained with the combinations of VCP enzymes than with those of MC enzymes, indicating that the VCP enzyme combinations were more efficient in isolating *Petunia* protoplasts. When the VCP enzyme concentration was increased, protoplast yield increased gradually; however, broken cells were detected at higher enzyme concentrations, as shown at EC 7. The lower number of cells at EC 7 is presumed to be due to damaged protoplasts resulting from the harsh removal of cell walls using higher enzyme concentrations.

Various factors affect the isolation of protoplasts, such as osmotic pressure, enzyme type, digestion time, incubation temperature, shaking, and pH value. To obtain high yields and highly viable protoplasts, a shorter enzyme digestion time was suggested. The currently established method will be applicable to the isolation of *Petunia* protoplasts, which was further verified by cell wall formation from the Fluorescein diacetate (FDA) test (Supplementary Figure S2) [4,10].

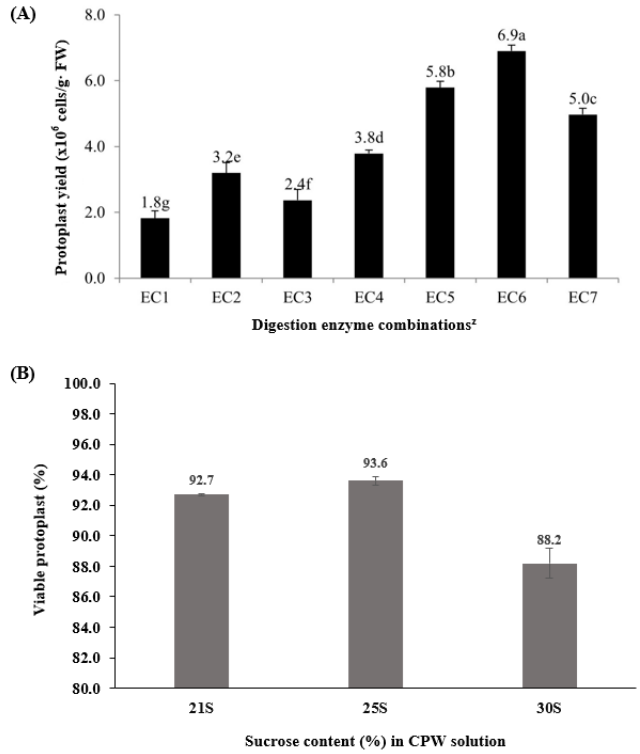

Figure 1. The calculated protoplast yield after enzyme digestion (**A**) and sucrose purification (**B**). The percentage of viable protoplasts was calculated by counting the green-dyed cells after reaction in the 0.01% FDA solution. Error bars indicate standard errors of the means and different letters indicate significant differences at $p \leq 0.05$ according to Duncan's multiple range test (DMRT). [z] Treatments with digestion enzymes comprised seven groups of enzyme combinations (EC). EC 1: 1.0% Cellulase R-10™ (CR) + 0.2% Macerozyme R-10™ (MR); EC 2: 1.5% CR + 0.5% MR; EC 3: 2.0% CR + 0.8% MR; EC 4: 0.2% Celluclast™ (Ce) + 0.2% Pectinex™ (PE) + 0.4% Viscozyme™ (Vz); EC 5: 0.4% Ce + 0.4% PE + 0.8% Vz; EC 6: 0.6% Ce + 0.6% PE + 1.2% Vz; EC 7: 0.8% Ce + 0.8% PE + 1.6% Vz.

2.2. Protoplast Purification and Protoplast Viability

Following centrifugation at 600 rpm for 5 min, the protoplasts were gathered in the middle of the two different solutions for purification. For the test with different concentrations of sucrose, the percentages of actively dividing protoplasts were determined after 1 day of culture. These were found to be 92.7%, 93.6%, and 88.2% from 21%, 25%, and 30% cell and protoplast washing (CPW) sucrose solutions for purification, respectively (Figure 1A). In addition, microscopic observation showed that the cells from the CPW 25% sucrose solution were round and intact.

This step determined the protoplast quality of the CPW solution containing round and healthy or broken protoplasts, as well as undigested cell debris. Separation of high-quality protoplasts was achieved with 21% to 25% sucrose solution. Previous studies have reported on feasible purification solutions for different plant species, such as 20% sucrose for *Petunia* [4], 25% sucrose for *Pinellia cordata* [16], and 30–44% sucrose for *Torreya nucifera* [17]. In the current work, determination of the optimized isolation enzyme and purification solution for healthy and viable protoplasts was further verified using the FDA method (Supplementary Figure S2).

2.3. Different Culture Media for Colony and Calli Induction

After culturing the protoplast cells for 24 h, the cells appeared to divide and form a cell mass 3 days after culture. After 4 weeks, the cell mass reached the microcolony phase (Figure 2(D1,E1)). The colonies were transferred to callus induction solid media consisting of Murashige and Skoog medium (MS), 2 mg/L 6-benzylaminopurine (6-BAP), 0.5 mg/L naphthalene acetic acid (NAA), 3% sucrose, and 0.6% agar (pH 5.8). In Kalanchoe species, the interaction between BAP and 2.4-D has been found to affect the formation of protoplast-

derived calli [18]. In this study, the calli were formed 3 weeks later (Figure 2(D3,E3)), indicating that BAP and NAA had a significant influence on the formation of calli as noted in previous reports on the plant species *Nigella damascene* [19] and *Chrysanthemum* [20]. After culturing in shallow-layered liquid media for 3 days, many cell masses formed which stuck to the bottom of the petri dish, and gentle shaking was necessary to separate the calli for subsequent shooting or rooting induction.

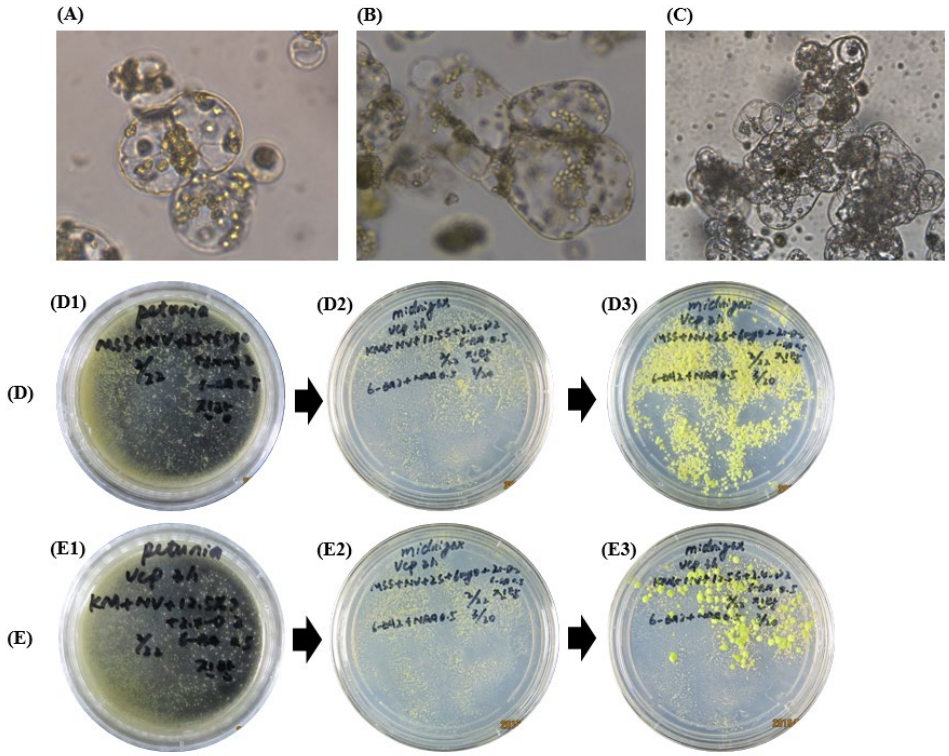

Figure 2. Protoplast-derived cell division for colony formation and proliferation in two callus induction media. A to C: Isolated protoplasts showing initiation of cell division on colony induction medium for 1 (**A**), 3 (**B**), and 7 (**C**) days after culture. The colonies were moved to callus induction media (**D** and **E**, respectively) as indicated. Medium D was composed of MS salt, 6% myo-inositol, 2% sucrose, 2 mg/L 2,4-D, and 0.5 mg/L 6-BAP. Medium E was composed of Kao and Michayluk (KM) salt, NLN vitamin, 12.5% sucrose, 2 mg/L 2,4-D, and 0.5 mg/L 6-BAP. The pictures in (**D1**,**E1**) were taken 4 weeks after colony formation. The pictures in (**D2**,**E2**) show the colony growing to form callus after transferring to callus induction media. The pictures (**D3**,**E3**) were taken 3 weeks after callus formation.

For callus growth, most plant protoplasts were cultured on MS salt [21,22], while protoplasts of Calibrachoa, sweet potato, and *Petunia* showed improved calli formation on KM salt [4,23]. Rather than the higher concentration of sucrose (medium E), the mixture with a lower concentration of myo-inositol and sucrose (medium D) was more effective for greater callus induction. Similarly, in previous studies, callus proliferation was preferred on MS salt medium with an energy source containing a mixture of myo-inositol and sucrose [17–19]. This result demonstrates the varying requirement of media sugar and salts according to plant genotypes and growth stages [19]. It also indicates that carbohydrate type and concentration are important factors in providing energy and adjusting the osmotic potential, which regulates nutrient absorption. Our study suggested that the MS salt medium with a relatively low level of sucrose was preferrable for *Petunia* callus proliferation, but further studies are needed to elucidate the detailed mechanisms.

2.4. Hormones for Shoot and Root Induction

Shoot regeneration was observed at different hormone combinations and concentrations, resulting in shoot induction efficiencies ranging from 3.7% to 77.8% (Table 1).

Treatments with zeatin 1 mg/L, thidiazuron (TDZ) 0.5 mg/L, or TDZ 1 mg/L similarly ensured higher shoot induction efficiency than treatments with NAA or indole acetic acid (IAA) combinations (Table 1 and Figure 3).

Table 1. Shoot induction efficiency using different hormones.

Hormones	Concentration (mg/L)	Shoot Induction Efficiency (%)
6-BAP + NAA	2.0 + 0.1	3.7 d [z]
TDZ + IAA	1.0 + 0.1	11.1 c
TDZ	0.5	74.0 a
TDZ	1.0	70.3 a
Zeatin	1.0	77.8 a
Zeatin	2.0	25.9 b

[z] Means within the column followed by different letters denote significant differences at $p \leq 0.05$ from the Duncan's multiple range test.

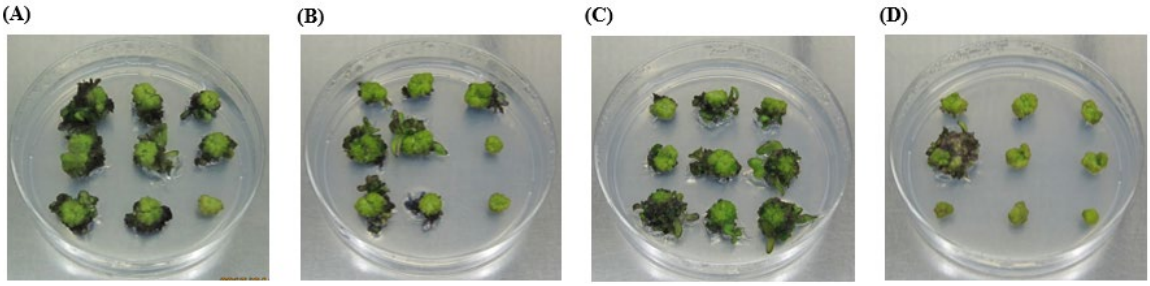

Figure 3. Shoot induction by cytokinin treatments in *Petunia* protoplast-derived regeneration, exhibiting relatively higher shooting rates of 74%, 70%, 78%, and 26% at thidiazuron (TDZ) 0.5 mg/L (**A**), TDZ 1.0 mg/L (**B**), zeatin 1.0 mg/L (**C**), and zeatin 2.0 mg/L (**D**), respectively.

Based on previous reports [4,24,25], various combinations of hormones were tested, but only TDZ or zeatin, as shown in Table 1, were found to be suitable for shoot regeneration in *Petunia*, demonstrating the species dependence for organogenesis. The direct induction of roots from zeatin supplementation in Kalanchoe species was quite different from our results [18]. Although a combination of 2 mg/L 6-BAP and 0.5 mg/L NAA was reported to show 41% regeneration in *Chrysanthemum* [20], more than 70% of shoot induction was achieved in *Petunia* supplemented with TDZ or zeatin alone. Interestingly, further growth of some *Petunia* calli was either arrested or continued to organogenesis depending on the level and type of phytohormones or protoplast-derived tissues, demonstrating that epigenic factors mediate the phase control of protoplast-based dedifferentiation or regeneration [19].

2.5. Epigenetic Regulation of Callus, Shoot, and Root Development in Petunia

ATX and its related ATXR proteins are generally methyltransferases; these are key regulators of chromatin state and gene expression that are necessary for the vegetative-to-reproductive phase transition [26]. To understand the molecular events surrounding developmental events such as callus, shoot, and root development, the gene expression patterns of *ATXR2*, *ATX4A*, and *ATX4B* were profiled (Figure 4). It was found that *PiATXR2* was significantly upregulated in callus and root tissues by 2.3 and 1.9 times, respectively, compared to the control (Figure 4A). The *ATXR2* has been found to be involved in cellular dedifferentiation during callus formation in *Arabidopsis* by inducing the expression of lateral organ boundaries domain (LBD) transcription factors, which is modulated by the histone methylation of H3K36me3 [13]. It has also been noted to play vital roles in de novo root organogenesis [14]. In contrast, both *PaATX4A* and *PiATX4B* were upregulated in shoot tissues (Figure 4A,B). The *PaATX4A* was highly repressed in both callus and root tissues (Figure 4A), while *PiATX4B* showed significant repression only in the callus (Figure 4B). This is consistent with the *Arabidopsis* expression study, where *ATX4*-deficient mutants showed enhanced callus formation. This suggested that the chromatin modifier ATX4

protein facilitates shoot formation during the plant regeneration process by activating shoot identity genes via H3K4me3 modification at these gene loci [15]. Analysis of the mRNA expression results demonstrated that *Petunia ATX* and *ATXR* genes could be involved in the epigenetic regulation of molecular events surrounding the acquisition of competence and early developmental events of callus, shoot, and root development.

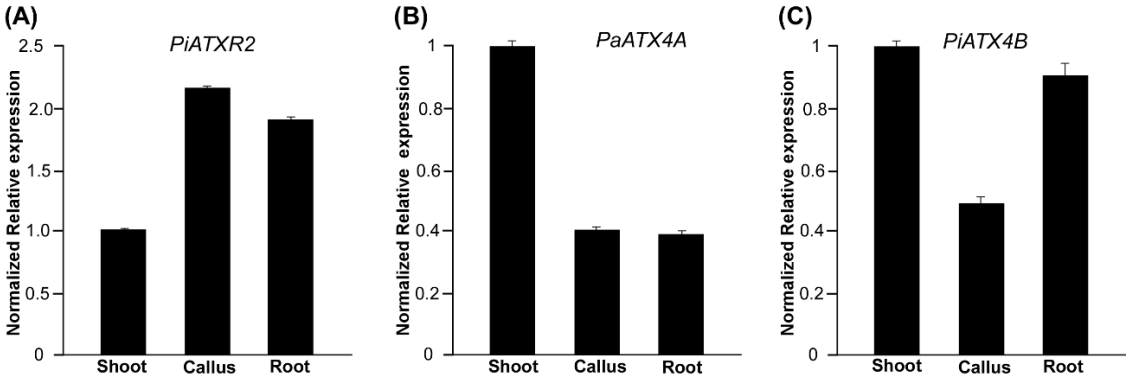

Figure 4. Transcriptional mRNA expression profiles of genes that act as molecular signatures of the different developmental processes of *P.* × *hybrida* cv. Madness Midnight. Expression of genes (**A**) *ATXR2*, (**B**) *PaATX4A* from *P. axillaris*, and (**C**) *PiATX4B* from *P. inflata* in developmental stages of callus, shoot, and root in *Petunia* protoplast-derived tissue culture. Expression levels of genes were normalized relative to the *elongation factor 1* gene. Transcriptional fold changes in shoot tissues (control) were set to level 1 for determining the fold changes in other tissues.

In summary, we obtained the optimized culture conditions by performing *Petunia* protoplast culture for regeneration. For protoplast isolation, we obtained the highest yield using the VCP enzyme combination of Viscozyme, Celluclast, and Pectinex at 1.2%, 0.6%, and 0.6%, respectively. Using a CPW 25% sucrose solution to purify protoplasts, we obtained more dynamic cells. After the addition of culture media, the protoplasts started to divide 1 d and 3 d later, and the cells became a cell mass. Approximately 3–4 weeks later, we observed cell colonies. After comparing two kinds of culture media, MS salt + 6% myo-inositol + 2% sucrose + 2 mg/L 2,4-D + 0.5 mg/L 6-BAP was chosen for colony induction to obtain more colonies. After 3–4 weeks, the produced colonies were transferred to callus induction media (MS + 2 mg/L 6-BAP + 0.5 mg/L NAA + 3% sucrose + 0.6% agar, pH 5.8), and small calli were formed 2 weeks later. Single green calli were picked up to MS + 1 mg/L zeatin + 3% sucrose + 0.7% Agar 1 mg/L for shoot induction. The small calli slowly started to grow larger about 2–3 weeks later, and small shoot tissue was produced around the callus. Shoot induction efficiency reached a maximum of 77.8%. After the regenerated shoots grew to 1–2 cm, the shoots were cut and cultured in MS + 3% sucrose + 0.7% agar media, and roots were formed one week later. Thus, the regenerated plant was successfully obtained. The duration of the entire process was less than three months. Some protoplasts proceeded to the dedifferentiation callus stage through successful cell proliferation, which was explained by the upregulation of *ATXR2*. *ATXR2* is a general regulator for callus and de novo root organogenesis, which was reported by stimulating the lateral organ boundaries domain (LBD) transcription factors [13]. Some of the pluripotent calli further transited to shoot organogenesis, which was confirmed by the enhanced *ATX4* gene that was reported to confer H3K4me3 deposition at shoot identity genes [15].

3. Materials and Methods

3.1. Plant Material

P. × *hybrida* cv. Madness Midnight seeds were sterilized in 2% NaClO solution for 20 min and washed 5 times with sterile distilled water for germination. The seedlings were further grown on MS basic medium containing 30 g/L sucrose and 0.8% agar, and the pH was adjusted to 5.8 [27]. In vitro plants were grown in culture room environments of 16/8 h

light (140 μmol m^{-2} s^{-1}) and dark photoperiod at 25 °C. Young, fully expanded leaves of the in vitro plants were selected and cut into 1–2 mm strips for protoplast isolation, as described in a previous report [10].

3.2. Enzyme Digestion and Purification of Protoplasts

Seven enzyme combinations (ECs) were prepared for enzyme digestion. Three treatments (MC enzyme) using Macerozyme R-10™ (MR; YAKULT Co. Ltd., Tokyo, Japan) and Cellulase R-10™ (CR; YAKULT Co. Ltd., Tokyo, Japan) were EC 1 (0.2% MR + 1.0% CR), EC 2 (0.5% MR + 1.5% CR), and EC 3 (0.8% MR + 2.0% CR). The other four treatments (VCP enzyme) using Celluclast™ (Ce; Novozyme Co. Ltd., Copenhagen, Denmark), Pectinex™ (PE; Novozyme Co. Ltd., Copenhagen, Denmark), and Viscozyme™ (Vz; Novozyme Co. Ltd., Copenhagen, Denmark) were EC 4 (0.2% Ce + 0.2% PE + 0.4% Vz), EC 5 (0.4% Ce + 0.4% PE + 0.8% Vz), EC 6 (0.6% Ce + 0.6% PE + 1.2% Vz), and EC 7 (0.8% Ce + 0.8% PE + 1.6% Vz). The combined enzymes were dissolved in CPW 9% solution containing 27.2 mg/L KH_2PO_4, 101.0 mg/L KNO_3, 1480.0 mg/L $CaCl_2 \cdot 2H_2O$, 246.0 mg/L $MgSO_4 \cdot 7H_2O$, 0.16 mg/L KI, and 0.025 mg/L $CuSO_4 \cdot 5H_2O$ [24], which was then combined with 9% mannitol (*W*/*V*) and 5 mM MES, and the pH was adjusted to 5.8. Leaf mesophyll cells were digested in each of the enzyme combinations with shaking at 40 rpm in three replicates at 25 °C under dark conditions for 3 h, and then the protoplast cells were isolated. The yield of protoplasts per gram of fresh weight leaves was calculated.

After 3 h of enzyme digestion, protoplasts in the enzyme solution were filtered through a 100 μm and 70 μm nylon mesh. The filtrate was centrifuged at 600 rpm for 5 min. The isolated protoplast pellets were washed once with CPW 9% mannitol solution. The obtained pellets were re-suspended in 5 mL CPW 9% mannitol solution and gently added to 5 mL of CPW with different concentrations of sucrose (21%, 25%, and 30%) for purification. The protoplasts were collected in the middle layer of the tube by centrifugation at 600 rpm for 5 min. The protoplasts were purified using different CPW sucrose solutions. The isolated cells in the middle layer were collected and diluted in a CPW 9% mannitol solution. The protoplasts (50 μL) were mixed with FDA solution to maintain a final concentration of 0.01%, which was kept in the dark for 5 min at room temperature. The cells were reacted with FDA solution and then observed using a green fluorescence microscope to count the number of green cells for protoplast viability.

3.3. Induction of Colony and Callus Formation

Finally, the purified protoplasts suspended in CPW 9% mannitol solution were collected by centrifugation at 600 rpm for 5 min. The collected cells were resuspended in the culture media, adjusted to a cell density of 2×10^5 cells mL^{-1} with a hemocytometer, and incubated at 25 °C in the dark. To compare the colony induction in different culture media, MS salt media consisting of MS salt, 6% myo-inositol, 2% sucrose, 2 mg/L 2,4-D, and 0.5 mg/L 6-BAP at pH 5.8 and KM salt media consisting of KM salt [28], NLN Vitamin [29], 2.5% sucrose, 2 mg/L 2,4-D, and 0.5 mg/L 6-BAP at pH 5.8 were used. After 3–4 weeks of culture, the colonies (micro-calli) were transferred to MS solid medium (pH 5.8) supplemented with 6-BAP (2 mg/L^{-1}), NAA 0.5 mg/L, 3% sucrose, and 0.7% agar at the same culture room conditions of 16 h light and 8 h dark cycles with 120 μmol m^{-2} s^{-1} intensity at 25 °C for callus induction.

3.4. Induction of Root and Shoot Generation

Two weeks after culturing in the solid medium, a small callus appeared and was transferred to a different hormone combination media on basic MS media with 3% sucrose in the same culture room conditions as callus induction. Different levels of cytokinins, including 6-BAP (2 mg/L), TDZ (0.5–1 mg/L), and zeatin (1–2 mg/L), a major phytohormone, were prepared with NAA (1 mg/L) or IAA (1 mg/L) as a minor.

3.5. In Silico Identification of Candidate Genes and Estimation of mRNA Expression Using qRT-PCR

To explore the molecular events during the in vitro *Petunia* plant regeneration, the candidate genes of *ATXR2* (AT3G21820.1) and *ATX4* (AT4G27910.1) were used to blast (BLASTP and BLASTN) against the parental genomes of *P. axillaris* (denoted as *PaATX4A*) and *P. inflata* (denoted as *PiATX4B*) (https://solgenomics.net/, accessed on 27 November 2019) and *Petunia*-specific *ATXR2* and *ATX4* genes were obtained (Supplementary Table S1), which were successfully amplified (Supplementary Figure S1).

Total RNA was extracted from *Petunia* tissues exhibiting callus, shooting, or rooting stages using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA synthesis was accomplished using PrimeScript® RT reagent with a gDNA eraser kit (Takara Korea Biomedical, Seoul, Republic of Korea). Primer pairs specific to target genes were designed using Primer Express (version 3.0, Applied Biosystems, Waltham, MA, USA), and are listed in Supplementary Table S1. A qRT-PCR analysis was carried out to estimate the relative mRNA expression level using the SYBR Green-based (iQ™ SYBR® Green Supermix) qRT-PCR method in a CFX Connect™ Real-Time PCR Detection System thermocycler (Bio-Rad, Daejeon, Korea). The qRT-PCR reactions and other parameters were performed according to a previous study [30,31].

3.6. Statistical Analysis

Statistical analyses were conducted using Statistical Product and Service Solutions for Windows (SPSS; version 12.1, IBM, New York, NY, USA). The data were analyzed using analysis of variance (ANOVA), and differences among the means were tested using Duncan's multiple range test ($p \leq 0.05$).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9020216/s1, Table S1: List of genes, designed primers, and amplicon characteristics. Figure S1: Agarose gel electrophoresis of qRT-PCR amplicons for reference and target genes from Petunia × hybrid. Arrows indicate the bands amplified by designed primers. EF reference gene of elongation factor 1; M DNA ladder; A_ATX4A, I_ATX4B, and I_ATXR2 target genes. Figure S2: Fluorescence microscope images collected from purified protoplast of CPW 25% sucrose solution mixed with 0.01% FDA solution. A: Protoplast stained by FDA in white light. B: Protoplast stained by FDA in green light. C: Combined A and B.

Author Contributions: Conceptualization, G.-J.L.; data curation, L.T., S.S., K.L., Y.J., F.Y. and J.Y.; formal analysis, L.T., Y.-S.K., S.S. and G.-J.L.; funding acquisition, O.-J.K. and G.-J.L.; investigation, L.T., S.S., K.L., Y.J., F.Y., J.Y. and Y.-S.K.; methodology, L.T., S.S., K.L., O.-J.K. and G.-J.L.; project administration, G.-J.L.; resources, G.-J.L.; software, L.T., S.S., K.L., Y.J. and Y.-S.K.; visualization, L.T., K.L. and S.S.; writing—original draft, L.T., S.S., K.L., Y.J., F.Y., J.Y. and Y.-S.K.; writing—review and editing, O.-J.K. and G.-J.L. All authors have read and agreed to the published version of the manuscript.

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References

1. Valente, P.; Tao, W.H.; Verbelen, J.P. Auxins and cytokinins control DNA endoreduplication and deduplication in single cells of tobacco. *Plant Sci.* **1998**, *134*, 207–215. [CrossRef]
2. 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]
3. Sheen, J. Signal transduction in maize and Arabidopsis mesophyll protoplasts. *Plant Physiol.* **2001**, *127*, 1466–1475. [CrossRef]
4. Meyer, L.; Serek, M.; Winkelmann, T. Protoplast isolation and plant regeneration of different genotypes of *Petunia* and *Calibrachoa*. *Plant Cell Tiss. Org. Cult.* **2009**, *99*, 27–34. [CrossRef]
5. Oh, M.H.; Kim, S.G. Plant-regeneration from petal protoplast culture of *Petunia hybrida*. *Plant Cell Tiss. Org. Cult.* **1994**, *36*, 275–283. [CrossRef]
6. Bona, C.; Gould, J.; Miller, J.C.; Stelly, D.M.; Louzada, E.S. In vitro regeneration of somatic symmetric and asymmetric hybrid citrus plantlets produced via protoplast fusion. *Hortscience* **2008**, *43*, 1206.

7. Yu, J.; Tu, L.; Subburaj, S.; Bae, S.; Lee, G.J. Simultaneous targeting of duplicated genes in Petunia protoplasts for flower color modification via CRISPR-Cas9 ribonucleoproteins. *Plant Cell Rep.* **2021**, *40*, 1037–1045. [CrossRef] [PubMed]
8. Fordlogan, J.; Sink, K.C. Plantlet Regeneration from Protoplasts of *Petunia alpicola*. *Hortscience* **1988**, *23*, 393–395. [CrossRef]
9. Sangthong, R.; Chin, D.P.; Hayashi, M.; Supaibulwatana, K.; Mii, M. Direct isolation of female germ units from ovules of *Petunia hybrida* by enzymatic treatment without releasing somatic protoplasts from ovular tissue. *Plant Biotechnol.* **2009**, *26*, 369–375. [CrossRef]
10. Subburaj, S.; Chung, S.J.; Lee, C.; Ryu, S.M.; Kim, D.H.; Kim, J.S.; Bae, S.; Lee, G.J. Site-directed mutagenesis in *Petunia* x *hybrida* protoplast system using direct delivery of purified recombinant Cas9 ribonucleoproteins. *Plant Cell Rep.* **2016**, *35*, 1535–1544. [CrossRef]
11. Oberwalder, B.; Schilde-Rentschler, L.; Ruoss, B.; Wittemann, S.; Ninnemann, H. Asymmetric protoplast fusions between wild species and breeding lines of potato—Effect of recipients and genome stability. *Theor. Appl. Genet.* **1998**, *97*, 1347–1354. [CrossRef]
12. Jeong, Y.Y.; Lee, H.Y.; Kim, S.W.; Noh, Y.S.; Seo, P.J. Optimization of protoplast regeneration in the model plant *Arabidopsis thaliana*. *Plant Methods* **2021**, *17*, 21. [CrossRef]
13. Lee, K.; Park, O.S.; Seo, P.J. Arabidopsis *ATXR2* deposits H3K36me3 at the promoters of LBD genes to facilitate cellular dedifferentiation. *Sci. Signal.* **2017**, *10*, eaan0316. [CrossRef] [PubMed]
14. Lee, K.; Park, O.S.; Seo, P.J. *ATXR2* as a core regulator of de novo root organogenesis. *Plant Signal. Behav.* **2018**, *13*, e1449543. [CrossRef]
15. Lee, K.; Park, O.S.; Choi, C.Y.; Seo, P.J. *ARABIDOPSIS TRITHORAX 4* Facilitates Shoot Identity Establishment during the Plant Regeneration Process. *Plant Cell Physiol.* **2019**, *60*, 826–834. [CrossRef]
16. Yang, X.; Ma, D.-D.; Jiang, F.-S.; Chen, N.-P.; Ding, B.; Jin, L.-X.; Qian, C.-D.; Ding, Z.-S. Protoplasts isolation, purification and plant regeneration of *Pinellia cordata*. *China J. Chin. Mater. Med.* **2014**, *39*, 4211–4215.
17. Zhang, Y.; Miao, J.; Xie, M.; Tian, S.; Dong, M.; Jiang, G. Isolation and purification of protoplast cell from the leaves of *Torreya nucifera*. *Biotechnol. Bulletin.* **2013**, *11*, 75.
18. Cui, J.; Mackenzie, K.K.; Eeckhaut, T.; Muller, R.; Lutken, H. Protoplast isolation and culture from *Kalanchoe* species: Optimization of plant growth regulator concentration for efficient callus production. *Plant Cell Tissue Org. Cult.* **2019**, *138*, 287–297. [CrossRef]
19. Klimek-Chodacka, M.; Kadluczka, D.; Lukasiewicz, A.; Malec-Pala, A.; Baranski, R.; Grzebelus, E. Effective callus induction and plant regeneration in callus and protoplast cultures of *Nigella damascena* L. *Plant Cell Tissue Org. Cult.* **2020**, *143*, 693–707. [CrossRef]
20. Adedeji, O.S.; Naing, A.H.; Kim, C.K. Protoplast isolation and shoot regeneration from protoplast-derived calli of Chrysanthemum cv. White ND. *Plant Cell Tissue Org. Plant Cell* **2020**, *141*, 571–581. [CrossRef]
21. Inokuma, C.; Sugiura, K.; Cho, C.; Okawara, R.; Kaneko, S. Plant regeneration from protoplasts of Japanese lawngrass. *Plant Cell Rep.* **1996**, *15*, 737–741. [CrossRef] [PubMed]
22. Mii, M.; Yuzawa, Y.; Suetomi, H.; Motegi, T.; Godo, T. Fertile Plant-Regeneration from Protoplasts of a seed-propagated cultivar of *Lilium X Formolongi* by utilizing meristematic nodular cell clumps. *Plant Sci.* **1994**, *100*, 221–226. [CrossRef]
23. Sihachakr, D.; Ducreux, G. Plant-regeneration from protoplast culture of sweet-potato (*Ipomoea-Batatas* Lam). *Plant Cell Rep.* **1987**, *6*, 326–328. [CrossRef] [PubMed]
24. Frearson, E.M.; Power, J.; Cocking, E. The isolation, culture and regeneration of *Petunia* leaf protoplasts. *Dev. Biol.* **1973**, *33*, 130–137. [CrossRef] [PubMed]
25. Vasil, V.; Vasil, I.K. Regeneration of tobacco and petunia plants from protoplasts an culture of corn protoplasts. *In Vitro* **1974**, *10*, 83–96. [CrossRef] [PubMed]
26. Chen, L.Q.; Luo, J.H.; Cui, Z.H.; Xue, M.; Wang, L.; Zhang, X.Y.; Pawlowski, W.P.; He, Y. ATX3, ATX4, and ATX5 encode putative H3K4 methyltransferases and are critical for plant development. *Plant Physiol.* **2017**, *174*, 1795–1806. [CrossRef] [PubMed]
27. Murashige, T.; Skoog, F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant* **1962**, *15*, 473–497. [CrossRef]
28. Kao, K. Chromosomal behaviour in somatic hybrids of soybean-*Nicotiana glauca*. *Mol. Gen. Genet.* **1977**, *150*, 225–230. [CrossRef]
29. Nitsch, C.; Nitsch, J. The induction of flowering in vitro in stem segments of *Plumbago indica* L. *Planta* **1967**, *72*, 355–370. [CrossRef]
30. Subburaj, S.; Ha, H.J.; Jin, Y.T.; Jeon, Y.; Tu, L.; Kim, J.B.; Kang, S.Y.; Lee, G.J. Identification of gamma-radiation-responsive microRNAs and their target genes in *Tradescantia* (BNL clone 4430). *J. Plant Biol.* **2017**, *60*, 116–128. [CrossRef]
31. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta Delta C(T)) method. *Methods* **2001**, *25*, 402–408. [CrossRef] [PubMed]