forests

MDPI

Article

Establishment of a Transient Transformation Protocol in *Cinnamomum camphora*

Siyi Zhang [1,2], Rong Hu [1,2], Lin Yang [3] and Zhaojiang Zuo [1,2,*]

[1] Zhejiang Provincial Key Laboratory of Forest Aromatic Plants-Based Healthcare Functions, Zhejiang A&F University, Hangzhou 311300, China
[2] State Key Laboratory of Subtropical Silviculture, Zhejiang A&F University, Hangzhou 311300, China
[3] Tianjin Key Laboratory of Animal and Plant Resistance, College of Life Sciences, Tianjin Normal University, Tianjin 300387, China; skyyl@tjnu.edu.cn
* Correspondence: zuozhaojiang@126.com

Abstract: *Cinnamomum camphora* is an excellent evergreen broad-leaved tree species with strong stress tolerance, but its molecular character revelation as well as ecological and economic value improvement were limited due to the lack of a genetic transformation system. To establish a simple and efficient transient transformation system for uncovering the molecular mechanism of plant tolerating stresses and promoting the selective breeding of good varieties, the infection method, co-cultivation time, infection solution concentration, and growth density of *Agrobacterium tumefaciens* containing green fluorescent protein (GFP)-based calmodulin protein 3 gene (*GCaMP3*) were identified by monitoring the fluorescence emitted from GCaMP3 bound to Ca^{2+}. Meanwhile, the transient transformation effects were evaluated via cytoplasmic Ca^{2+} concentration variations at high temperatures of 35 °C and 40 °C. When *C. camphora* leaves were infected with *A. tumefaciens* containing *GCaMP3* via injection and soaking, no significant difference was detected in the fluorescence intensity over 48 h, indicating that the two infection methods had the same transient transformation efficiency. By prolonging the co-cultivation time, the fluorescence intensity gradually increased, reached its strongest at the 48th h, and then gradually declined. For the infection solution concentration, an OD_{600} of 0.7 led to the strongest fluorescence intensity, with an increase of 42.2%, 13.7%, 4.2%, and 14.2%, respectively, compared to that at OD_{600} of 0.5, 0.6, 0.8, and 0.9. When *A. tumefaciens* growth density OD_{600} was 0.5–0.7, the strongest fluorescence intensity was detected after transient transformation. Combining these optimum conditions, *GCaMP3* was transferred into *C. camphora*, which indicated the variations in cytoplasmic Ca^{2+} concentration at high temperatures, with the fluorescence intensity at 35 °C and 40 °C increasing by 12.6% and 30.6%, respectively, in contrast to that at 28 °C. Therefore, it should be an efficient transient transformation system for *C. camphora*, with *A. tumefaciens* growth density OD_{600} of 0.5–0.7, infection solution concentration OD_{600} of 0.7, and co-cultivation time of 48 h by using both injection and soak infection methods, which is beneficial for uncovering the Ca^{2+} signal transduction in the plant tolerating stresses and promoting its molecular biology development and selective breeding of good varieties.

Keywords: *Agrobacterium tumefaciens*; cytoplasmic Ca^{2+}; *GCaMP3*; high temperature; infection method

Citation: Zhang, S.; Hu, R.; Yang, L.; Zuo, Z. Establishment of a Transient Transformation Protocol in *Cinnamomum camphora*. *Forests* **2023**, *14*, 1872. https://doi.org/10.3390/f14091872

Academic Editor: Jianjun Hu

Received: 25 August 2023
Revised: 11 September 2023
Accepted: 13 September 2023
Published: 14 September 2023

1. Introduction

Stable plant genetic transformation is a technology that can transfer exogenous genes into receptors via different methods to obtain transgenic plants, which plays an important role in gene function identification and genetic breeding. This technology mainly depends on the plant regeneration ability and gene introduction efficiency [1]. To date, it has been successfully applied to many herbs and crops, such as *Miscanthus sinensis*, wheat (*Triticum aestivum*), soybean (*Glycine max*), and cabbage (*Brassica oleracea*) [1–4]. In contrast to these plants, this technology is rarely applied to woody plants, except for *Populus*, due to its high heterozygosity and long breeding time [5,6].

Compared to stable genetic transformation, transient transformation exhibits a number of advantages, such as short periods, high efficiency, labor, and saving time [7]. There are many types of transient transformation, including *Agrobacterium*-mediated gene transformation, particle bombardment, electroporation, and poly-ethylene glycol-mediated transformation [8,9]. Particle bombardment can transfer exogenous genes into a broad range of plant species, but it requires expensive and special equipment [10]. For electroporation and poly-ethylene glycol-mediated transformation, high-vitality protoplasts are used as the receptors for exogenous genes. However, protoplast isolation is complicated, and it is hard to obtain a stable yield [11]. As an alternative approach, *Agrobacterium*-mediated gene transformation is widely used in transient assays with several advantages, such as straightforward operation, low cost, and easily obtaining transformation materials [12,13]. This technology was first developed in tobacco (*Nicotiana tabacum*) [14] and further applied to *Arabidopsis thaliana*, rice (*Oryza sativa*), tomato (*Solanum lycopersicum*), etc. [15–17]. However, its application in woody plants is slower than that in herbs. In recent years, many efforts were made to improve *Agrobacterium*-mediated gene transformation efficiency, which promoted its application in woody plants, such as *Populus*, apple (*Malus pumila*), tea plants (*Camellia sinensis*), and *Citrus* [18–21].

Cinnamomum camphora (L.) Presl. belongs to the genus *Cinnamomum* in Lauraceae, which is an excellent evergreen broad-leaved tree species with strong stress tolerance in subtropical regions. It not only exhibits ecological value by being used as landscaping tree species but also exhibits great economic value by extracting essential oil and using it as furniture material [22,23]. This species exhibits extensive genetic diversity, and simple sequence repeat (SSR) markers [24], expressed sequence tag-simple sequence repeat (EST-SSR) markers [25], and genome-wide single-nucleotide polymorphism (SNP) [26] have been developed for diversity analysis. However, research on other molecular biology aspects (especially stress tolerance) of the plant is very slow, which limits the improvement of the plant's ecological and economic value due to genetic breeding without transgenic assistance. Although a cold resistance gene is transferred to the embryogenic calluses of *C. camphora*, a large number of calluses are false positives, resulting in transgenic calluses not being screened out [27]. *C. camphora* contained lots of phenols and oxidases, which caused serious browning problems during the culturing of transgenic calluses [28]. Then, it is hard to identify gene functions by using a stable genetic transformation method in plants. Transient transformation is a powerful tool for analyzing the gene expression in the original species, but it has not been established in *C. camphora* until now. This is not beneficial for promoting the research on *C. camphora* molecular biology, although plant genome sequencing has already been completed [29].

Green fluorescent protein (GFP)-based calmodulin protein 3 (GCaMP3) contains a circularly permutated enhanced GFP flanked by calmodulin (CaM) and myosin light-chain kinase (M13), which shows green fluorescence in the absence of Ca^{2+} [30]. To date, this Ca^{2+} reporter gene *GCaMP3* has been used to uncover Ca^{2+} signal transduction in several herbs, such as *A. thaliana* and duckweed [31,32]. To establish a simple and efficient *Agrobacterium*-mediated transient transformation system in *C. camphora* by using *GCaMP3* as the reporter gene, we identified the infection method, co-cultivation time, infection solution concentration and growth density of *Agrobacterium tumefaciens* GV3101 containing *GCaMP3* during transient transformation process in this study. To the best of our knowledge, this is the first report of a transient transformation protocol in plants, which is not only beneficial for promoting the research on Ca^{2+} signal transduction in *C. camphora* tolerating stresses but also beneficial for promoting plant molecular biology development and selective breeding of good varieties.

2. Materials and Methods

2.1. Plant Material

Three-year-old *C. camphora* seedlings with a height of 80 cm were kept in an illumination incubator, and the regime was 16 h light at 28 °C and 8 h dark at 25 °C, with a

light intensity of 200 μmol·m^{-2}·s^{-1}. The relative humidity was 65 ± 5%. After adaptation for 15 days, the 3rd to 5th healthy leaves from the top were used for the transient transformation.

2.2. Preparation for Infection Solution

A. tumefaciens GV3101 containing *GCaMP3* was provided by Dr. Lin Yang at Tianjin Normal University, China, and the structure of *GCaMP3* plasmid is described in detail by Ren et al. [31]. The bacterium was activated 3 times on a Luria–Bertani (LB) solid plate containing 50 mg·L^{-1} kanamycin, 25 mg·L^{-1} rifampicin, and 25 mg·L^{-1} streptomycin at 28 °C. A single colony was randomly selected and kept in a 10 mL LB liquid medium with the same antibiotics at 28 °C for 18 h. Then, 1 mL of bacterial solution was transferred into a 20 mL of fresh LB liquid medium, and its OD value was recorded every 2 h to draw the growth curve. When the cell density reached a given concentration, they were harvested by centrifugation at 5000× *g* and used to prepare the infection solution by resuspending them in a solution containing 5 g·L^{-1} of sucrose, 0.04% of silwet-77, and 0.1 M of 2-morpholinoethanesulfonic acid (MES). After that, the infection solution was used for injection and soak infection (Figure 1), which are the two main methods used in *Agrobacterium*-mediated transient transformation.

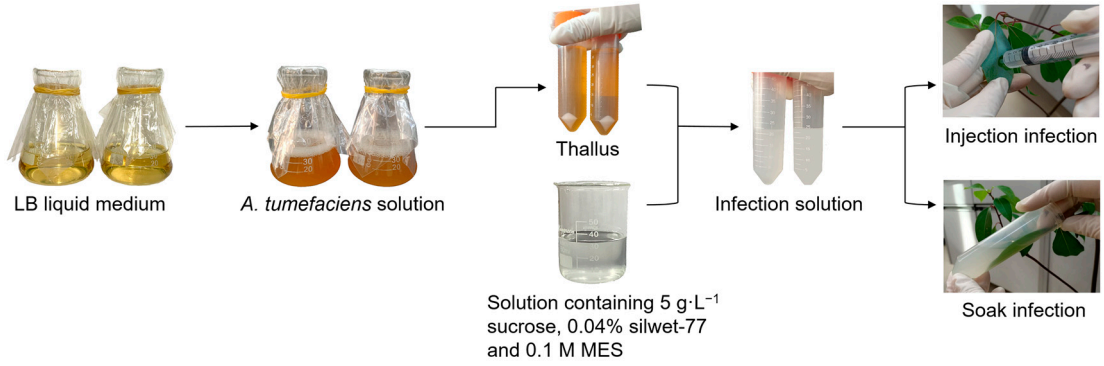

Figure 1. Experimental procedure of transient transformation in *C. camphora* leaves infected with *A. tumefaciens* via injection and soak infection.

2.3. Infection Methods

2.3.1. Injection Infection

C. camphora leaves were washed twice with sterile water, and the cuticle on the lower surfaces of the leaves was scraped off using a syringe needle without damaging the lower epidermis. The above *A. tumefaciens* in the logarithmic phase with an OD_{600} of 0.7 was used to prepare the infection solution, and the solution concentration was indicated by the bacterial cell concentration with an OD_{600} of 0.8. The infection solution was injected into the leaves through the scraped area by using a syringe without a needle, and the color around the injection site became dark. Then, the infected leaves were covered with a piece of silver paper to maintain darkness, and the plant was kept in an illumination incubator under normal culture conditions. After 12, 24, and 48 h, the fluorescence intensity and *GCaMP3* gene in the infected leaves were detected.

2.3.2. Soak Infection

C. camphora leaves were washed with sterile water and the cuticle was scraped off using a blade without damaging the lower epidermis. The infection solution was prepared following the injection method, and the scraped leaves were soaked in the solution for 20 min [31]. After being covered with a piece of silver paper, the plant was kept in an illumination incubator under normal culture conditions, and the fluorescence intensity and *GCaMP3* gene in the infected leaves were detected after 12, 24, and 48 h.

2.4. Determination of Optimum Co-Cultivation Time, Infection Solution Concentration, and A. tumefaciens Growth Density

To determine the optimum co-cultivation time, an OD_{600} infection solution concentration of 0.6 was prepared with the *A. tumefaciens* OD_{600} growth density of 0.7. After the soak infection step, *C. camphora* was kept in an illumination incubator under normal culture conditions, and the fluorescence intensity was measured after 12, 24, 48, 60, and 72 h.

To determine the optimum infection solution concentration, the *A. tumefaciens* OD_{600} growth density of 0.6 was used to prepare the infection solution, with the OD_{600} concentration of 0.5, 0.6, 0.7, 0.8, and 0.9, respectively. After the soak infection step, *C. camphora* was kept in an illumination incubator for 48 h, and the fluorescence intensity was measured.

To determine the optimum growth density of *A. tumefaciens*, the OD_{600} infection solution concentration of 0.7 was separately prepared using the bacterium at OD_{600} growth densities of 0.3, 0.5, 0.7, 0.9, and 1.1. After the soak infection step, *C. camphora* was kept in an illumination incubator for 48 h, and the fluorescence intensity was measured.

2.5. Assay of Cytoplasmic Ca^{2+} Concentration under High Temperature

To evaluate the actual effects of the transient transformation protocol, the OD_{600} *A. tumefaciens* with a growth density of 0.5 was used to prepare the infection solution, with an OD_{600} concentration of 0.7. This solution was used to infect *C. camphora* leaves via soaking, and then the plants were kept in three illumination incubators under normal culture conditions for 48 h, with 4 plants in each incubator. For high-temperature treatment, the temperature in the two illumination incubators was separately increased to 35 °C and 40 °C for 30 min, while the temperature in the other illumination incubator was maintained at 28 °C as the control. Then, the fluorescence intensity was detected, which indicated the cytoplasmic Ca^{2+} concentration.

2.6. PCR Analysis

The genomic DNA from *C. camphora* leaves was extracted following the cetyltrimethylammonium bromide (CTAB) method [33] using a DNA extraction kit (Beijing Kulaibo Technology Co., Ltd., Beijing, China). Polymerase chain reaction (PCR) was carried out using a PCR amplification kit (Takara Biotechnology Co., Ltd., Beijing, China). The cloning system contained 1.25 U Taq polymerase, 100 ng DNA, and 20 μM primers (forward primer: 5′-ATGGGTTCTCATCATCATCATCAT-3′, reverse primer: 5′-TCACTTCGCTGTCATCATTTGTA-3′). The amplification procedure was as follows: 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s. After 30 cycles, the amplified PCR fragments were analyzed via electrophoresis on a 1.5% agarose gel.

2.7. Fluorescence Intensity Measurement

In the successfully infected leaf cells, the expressed protein GCaMP3 bound to cytoplasmic Ca^{2+} and showed green fluorescence. This fluorescence was observed by using a fluorescence microscope (Olympus BX51, Olympus Corporation, Tokyo, Japan) at an excitation wavelength of 488 nm and an emission wavelength of 507 nm. The fluorescence intensity was analyzed using ImageJ (1.50i) software.

2.8. Statistical Analyses

At least 4 seedlings were used in each treatment, with each as a biological replicate, and 3 leaves were infected in a seedling, with each as a technological replicate. The statistical analyses among the treatments with significant differences at $p < 0.05$ were carried out by using Origin 8.0 according to the Tukey test in one-way analysis of variance (ANOVA).

3. Results and Discussion

3.1. Effects of Infection Methods on the Transformation Efficiency

In the cells, GCaMP3 is mainly located in the cytoplasm, which combines with Ca^{2+} to produce a green fluorescence [31,32]. When *A. thaliana* and duckweed were transformed

with *GCaMP3* using *A. tumefaciens* GV3101, green fluorescence was observed in the cells and veins [31,32]. Similar results were also observed in the present study. When *C. camphora* leaves were transformed with *GCaMP3* by injection and soak infection, green fluorescence was detected after 12 h, and the fluorescence intensity gradually increased by prolonging the co-cultivation time (Figure 2A). During the 48 h, there were no significant differences in the fluorescence intensity between the two infection methods (Figure 2B). However, no fluorescence was detected in the leaves infected with *A. tumefaciens* GV3101 with an empty vector using the two infection methods (Supplementary Figure S1). After PCR amplification using specific primers, the products of *GCaMP3* (1321 bp) showed bright electrophoretic bands during co-cultivation for 12, 24, and 48 h after injection and soak infection (Figure 2C). These results indicate that *GCaMP3* was successfully transferred into *C. camphora* leaf cells and the two infection methods had the same transformation efficiency.

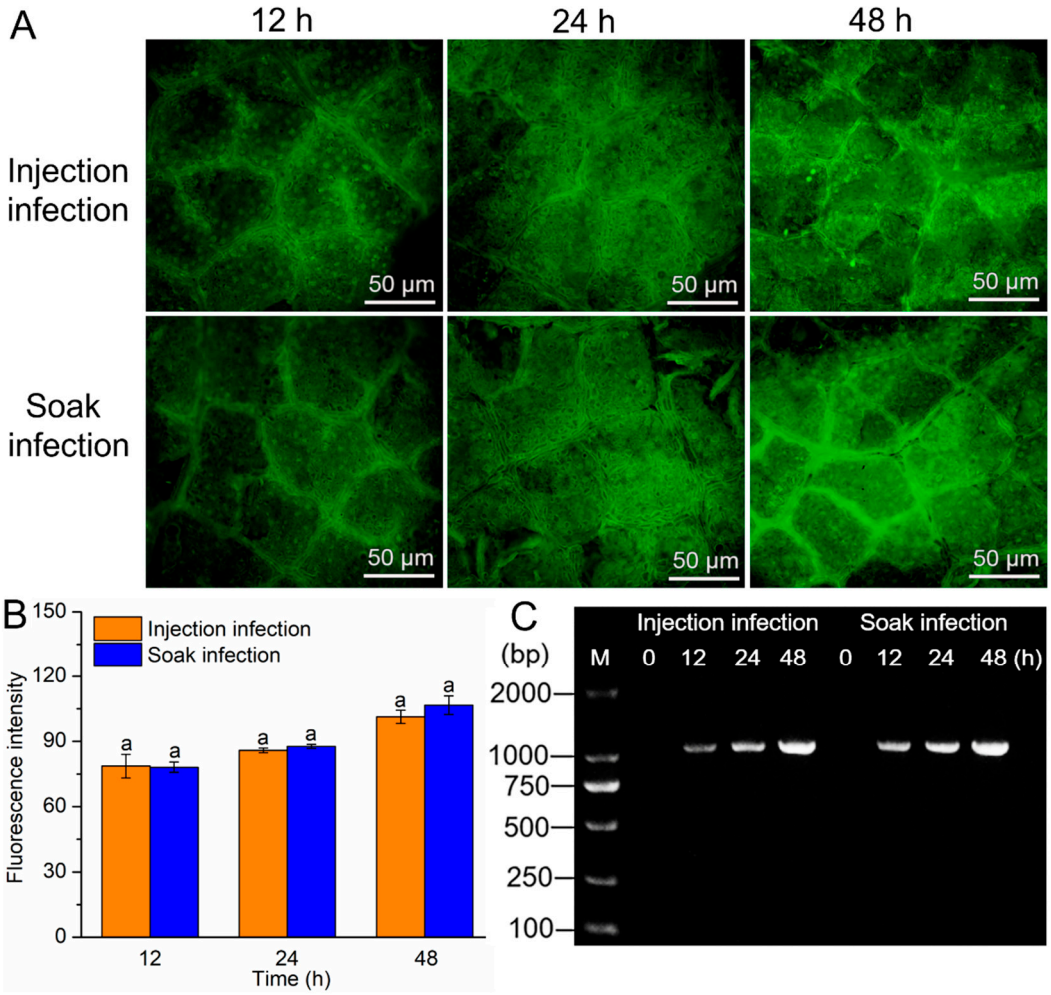

Figure 2. Transformation effects of injection and soak infection on *C. camphora* leaves. (**A**) Fluorescence images; (**B**) fluorescence intensity; (**C**) PCR results of *GCaMP3* gene in *C. camphora* leaves. M: DNA marker. 0 h: Before infection. At each time point, different lowercase letters indicate significant differences ($p < 0.05$) according to the Tukey test in one-way ANOVA. Means ± SE (n = 4).

Infiltration is the process of substances penetrating from the surface into the depth of plant tissues, which is specially applied in transient transformation mediated by *Agrobacterium* [34]. This process can be spontaneously performed under normal atmospheric pressure or forcedly performed by generating a pressure difference between the lamina surface and the inside of the leaves. In transient transformation, spontaneous infiltration is considered to be a less efficient manner than forced infiltration [35,36]. In this study, spon-

taneous infiltration and forced infiltration were carried out by soak infection and injection infection, respectively, but no difference was detected in the infection effects between the two methods (Figure 2). Compared to injection infection, there was a large leaf area in contact with *A. tumefaciens* during soak infection, which should result in many bacterial cells entering *C. camphora* leaves to improve infection efficiency. *C. camphora* produces an abundance of phenolic compounds [37,38], which can facilitate the expression of *vir* genes in *A. tumefaciens* to promote the transfer of T-DNA into the leaf cells [39,40]. During soak infection, the large contact area of *C. camphora* leaves might provide more phenolic compounds to promote the transfer of *GCaMP3* into leaf cells, resulting in a high transient transformation efficiency.

3.2. Effects of Co-Cultivation Time on the Transformation Efficiency

When *C. camphora* was infected with *A. tumefaciens* via soak infection and co-cultivated at 28 °C, green fluorescence was detected at the 12th h, and its intensity gradually increased by prolonging the co-cultivation time. The strongest fluorescence intensity was detected at the 48th h, after which it gradually declined with increasing co-cultivation time, and it disappeared at the 72nd h (Figure 3).

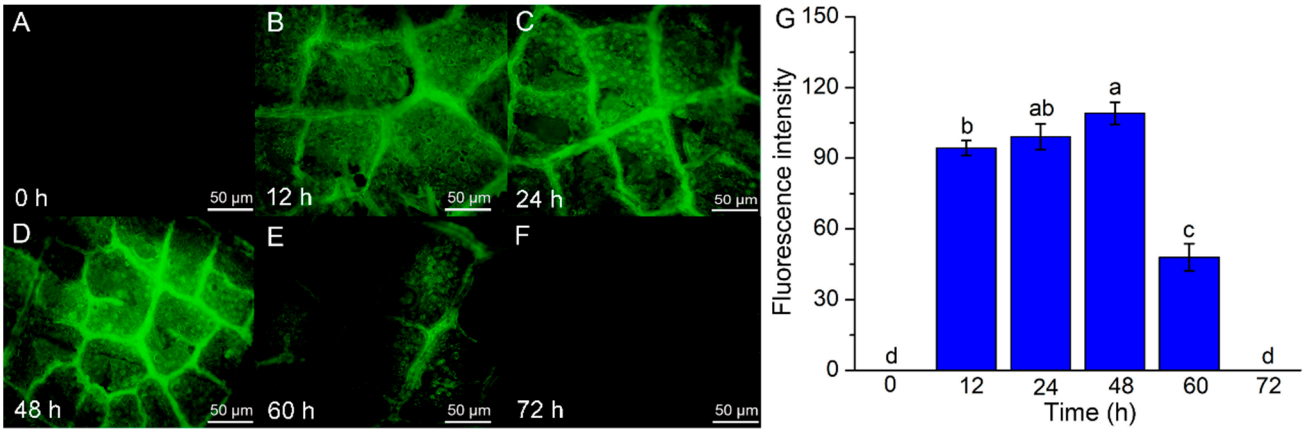

Figure 3. Effects of co-cultivation time on the transformation efficiency of *A. tumefaciens* containing *GCaMP3* in *C. camphora* leaves. (**A**–**F**) The fluorescence images after co-cultivation for 0, 12, 24, 48, 60 and 72 h, respectively. (**G**) Fluorescence intensity. Different lowercase letters indicate significant differences ($p < 0.05$) according to the Tukey test in one-way ANOVA. Means ± SE ($n \geq 4$).

The expression of exogenous genes is usually detected within 12 h after transient transformation, and the expression levels gradually increase by prolonging the co-cultivation time due to the gradual increase in exogenous genes entering the plant cells. However, the exogenous genes only exist for a short time, usually 3–5 days, in plant cells, as they are lost when the cells are divided [41,42]. In the present study, the fluorescence intensity in *C. camphora* disappeared after 72 h (Figure 3), which may have been caused by the loss of the *GCaMP3* gene during plant cell division.

The optimum expression time varies in different plants. When pineapple (*Ananas comosus*) calluses and Chinese birch (*Betula platyphylla*) leaves were transformed with *GFP* using *A. tumefaciens*, the strongest fluorescence intensity was detected on the third day of co-cultivation [43,44]. For apricot (*Armeniaca vulgaris*) calluses, the strongest fluorescence intensity was detected on the fourth day [45]. When avocado (*Persea americana*) callus was transformed with *EGFP* (enhanced green fluorescent gene) and *DsRed* (red fluorescent gene) using particle bombardment, the maximum fluorescence intensity was detected at the 24th and 72nd h, respectively, suggesting that the exogenous genes might also affect their expression in plant cells [46]. In this study, the strongest fluorescence intensity was detected at the 48th h, indicating that this should be the optimum co-cultivation time (Figure 3).

3.3. Effects of Infection Solution Concentration on the Transformation Efficiency

When *C. camphora* leaves were infected with *A. tumefaciens* at an OD_{600} infection solution concentration of 0.5, 0.6, 0.7, 0.8, and 0.9, the fluorescence intensity gradually increased with increasing concentration and reached the highest level at a concentration of 0.7. At this concentration, the fluorescence intensity increased by 42.2% ($p < 0.05$), 13.7% ($p < 0.05$), 4.2%, and 14.2% ($p < 0.05$), respectively, compared to that at OD_{600} of 0.5, 0.6, 0.8 and 0.9 (Figure 4).

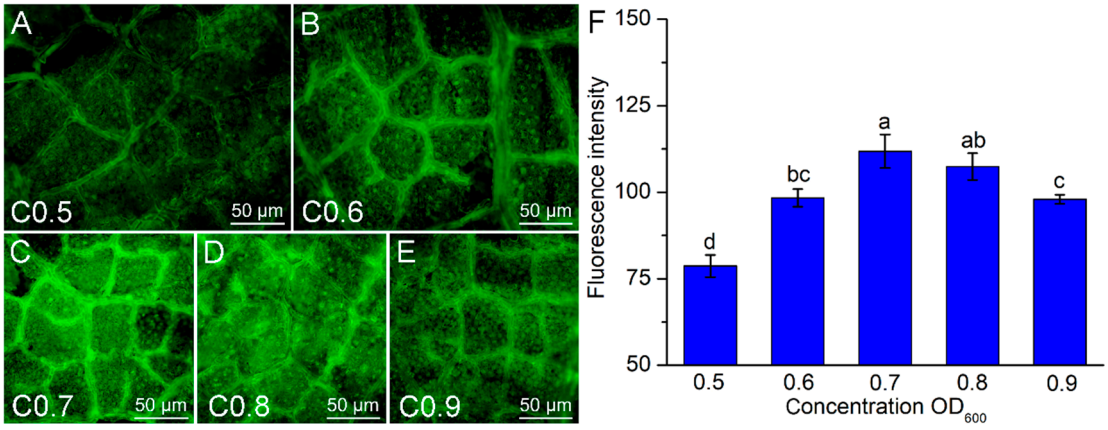

Figure 4. Effects of infection solution concentration on the transformation efficiency of *A. tumefaciens* containing *GCaMP3* in *C. camphora* leaves. (**A**–**E**) The fluorescence images using the infection solution concentration of 0.5 (C0.5), 0.6 (C0.6), 0.7 (C0.7), 0.8 (C0.8), and 0.9 (C0.9), respectively. (**F**) Fluorescence intensity. Different lowercase letters indicate significant differences ($p < 0.05$) according to the Tukey test in one-way ANOVA. Means ± SE (n = 4).

When OD_{600} infection concentration was 0.2 and 0.6, *A. tumefaciens* showed maximum transient transformation efficiency in sweetgum (*Liquidambar formosana*) and *Quercus robur*, respectively [47,48]. For *P. davidiana* × *P. bollena*, the maximum transient transformation efficiency was detected at *A. tumefaciens* OD_{600} concentration of 0.8 [49]. These results indicate that the receptor plants need specific *Agrobacterium* concentrations to obtain the maximum transformation efficiency due to their different sensitivity to the bacterium [50]. High concentrations of *Agrobacterium* always lower the transient transformation efficiency, as a high density of *Agrobacterium* may result in inhibition of the recipient cell respiration and even cause death of the bacterial cells for their competition [51,52]. This may be the reason for the low transformation efficiency of high infection concentration (especially OD_{600} of 0.9) in *C. camphora*, and OD_{600} of 0.7 may be the optimum infection concentration for high *A. tumefaciens* numbers and low adverse effects (Figure 4).

3.4. Effects of A. tumefaciens Growth Density on the Transformation Efficiency

Agrobacterium growth undergoes four growth stages, including lag phase, logarithmic phase, stationary phase, and decline phase [53]. The logarithmic phase is considered the most vigorous period for *Agrobacterium*, and the bacterium in this period is always used to transfer exogenous genes to plants [54]. For *A. rhizogenes* R1000, the logarithmic phase was at OD_{600} = 0.8–1.0 [55]. *A. tumefaciens* EHA105 and LBA4404 started to enter the logarithmic phase at 0.3 and 0.5, and ended at 2.4 and 2.7, respectively [56,57]. In the present study, *A. tumefaciens* GV3101 was in the logarithmic phase at an OD_{600} of 0.5–1.5 (Figure 5A). This indicates that different *Agrobacterium* species and strains have different growth rates.

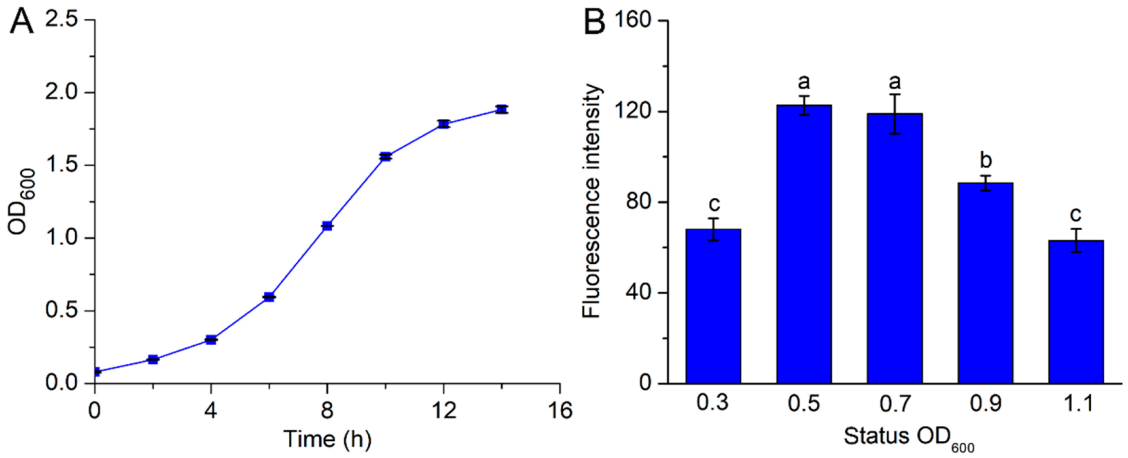

Figure 5. Effects of *A. tumefaciens* growth density on the transformation efficiency in *C. camphora* leaves. (**A**) *A. tumefaciens* growth curve; (**B**) fluorescence intensity. Different lowercase letters indicate significant differences ($p < 0.05$) according to the Tukey test in one-way ANOVA. Means ± SE (n = 4).

Agrobacterium is not suitable for gene transformation during the lag phase due to its low activity. However, it has very high activity in the middle and later logarithmic phases and is still not suitable for gene transformation, as the bacterium produces more toxins to disrupt the plant's physiological functions and even cause cell death [58,59]. When *C. camphora* was infected with the infection solution (OD_{600} of 0.7) separately prepared using *A. tumefaciens* GV3101 with OD_{600} growth densities of 0.3, 0.5, 0.7, 0.9, and 1.1, the strongest fluorescence intensity was detected at OD_{600} of 0.5 and 0.7 without significant differences between them (Figure 5B). At an OD_{600} growth density of 0.5–0.7, *A. tumefaciens* GV3101 was in the early logarithmic phase (Figure 5A) and exhibited the strongest infection activity to *C. camphora*, which might be caused by the bacterium's high activity and low toxicity to the plant.

In spinach (*Spinacia oleracea*), *A. tumefaciens* GV2260 with an OD_{600} concentration of 1.0 was the optimum growth density for transient transformation [60]. The highest transient transformation efficiency was found in *Paeonia lactiflor* callus and seedlings infected with *A. tumefaciens* EHA105 with OD_{600} of 0.6 and 1.2, respectively [61,62]. In the present study, the optimum OD_{600} growth density of *A. tumefaciens* GV3101 was 0.5–0.7 (Figure 5B). This might be caused by differences in the *Agrobacterium* strains and/or acceptor plants.

3.5. Effects of High Temperature on the Fluorescence Intensity

Ca^{2+} serves an important signaling function in plants that tolerate high temperatures, and it immediately accumulates in the cytoplasm when plants undergo heat shock; e.g., the cytoplasmic Ca^{2+} concentration in *A. thaliana* reached its first peak after heat shock for 10 s, and the second peak after 60 s [63]. Under high temperatures at 37 °C for 30 min, a considerable Ca^{2+} influx was detected in rice root cells, with a remarkable increase in cytoplasmic Ca^{2+} concentration [64]. In *Ganoderma lucidum*, a 2.5-fold increase in the cytoplasmic Ca^{2+} concentration was found when the plant was treated at a high temperature of 42 °C for 20 min [65].

Combining the optimum transient transformation conditions that were obtained, *C. camphor* leaves were infected with *A. tumefaciens* containing *GCaMP3* with a bacterial OD_{600} growth density of 0.5, an OD_{600} infection solution concentration of 0.7 and a co-cultivation time of 48 h using soak infection. Compared to the control at 28 °C, the fluorescence intensity increased by 12.6% ($p < 0.05$) and 30.6% ($p < 0.05$) under high temperatures of 35 °C and 40 °C, respectively, indicating that the quantities of Ca^{2+} that entered the cytoplasm gradually increased with increasing temperature (Figure 6). This was consistent with the cytoplasmic Ca^{2+} concentration increase under high temperatures in previous

studies [63–65], suggesting that this transient transformation system is efficient and can be applied to *C. camphor*.

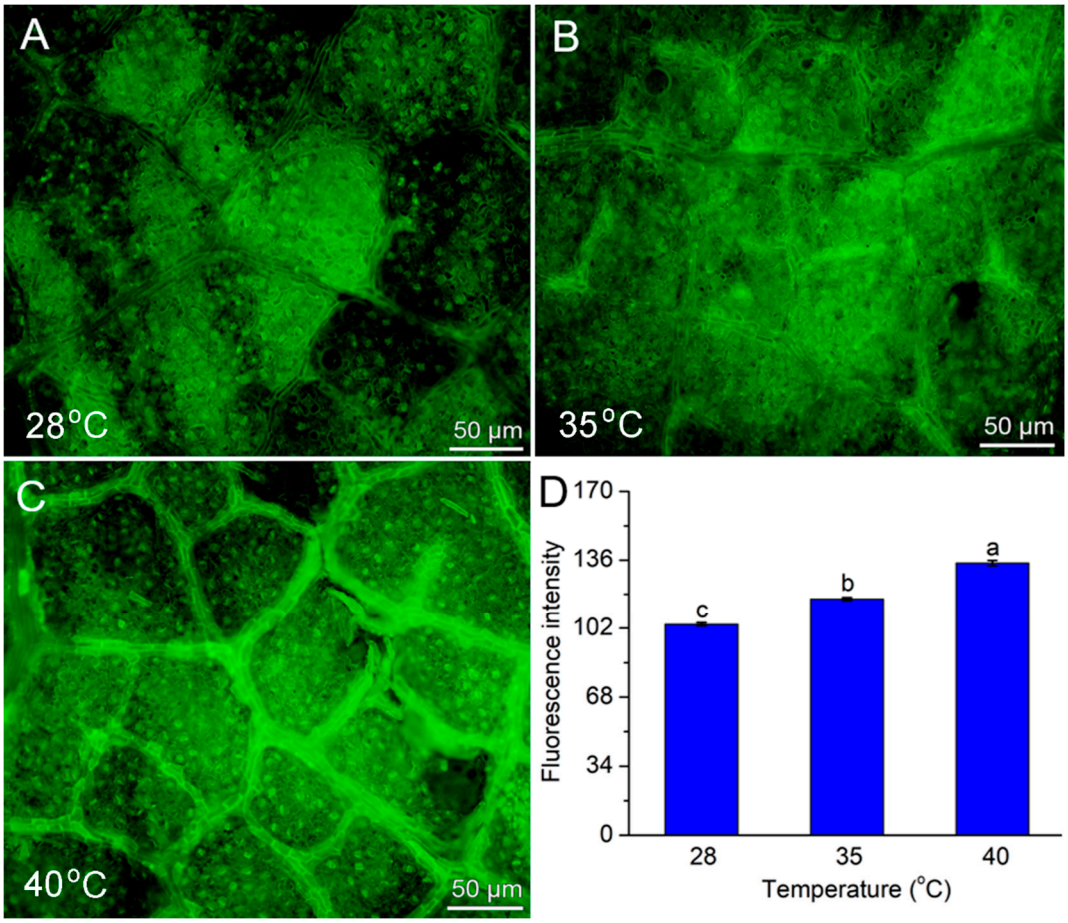

Figure 6. Effects of high temperatures on the fluorescence in *C. camphora* leaves transformed with *GCaMP3*. (**A**–**C**) Fluorescence images at 28 °C, 35 °C, and 40 °C, respectively; (**D**) fluorescence intensity. Different lowercase letters indicate significant differences ($p < 0.05$) according to the Tukey test in one-way ANOVA. Means ± SE (n = 4).

4. Conclusions

When *GCaMP3* was transferred to *C. camphora* leaves using *A. tumefaciens* through injection infection and soak infection, no significant differences were detected in the transient transformation efficiency between the two methods. During the transformation process, the optimum *A. tumefaciens* growth density, infection solution concentration and co-cultivation time were OD_{600} of 0.5–0.7, OD_{600} of 0.7, and 48 h, respectively. This transient transformation protocol combined with these optimum conditions exhibited effective effects that were indicated by the cytoplasmic Ca^{2+} variations in *C. camphora* under high temperatures, with a gradual increase in the fluorescence intensity with increasing temperature. This demonstrates that the protocol is suitable for the transient transformation of *C. camphora*, which is not only used to uncover the Ca^{2+} signal transduction in *C. camphora* tolerating stresses but also utilized in plant molecular biology research, promoting selective breeding of good varieties.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f14091872/s1, Figure S1: Transformation effects of *A. tumefaciens* with empty vector and *GCaMP3* on *C. camphora* during 48 h. When *C. camphora* was infected by *A. tumefaciens* GV3101 with empty vector (growth density OD_{600} of 0.5, infection concentration OD_{600} of 0.7) by using injection and soak infection methods, no fluorescence was detected during 48-h

co-cultivation. However, the fluorescence intensity in *C. camphora* infected by *A. tumefaciens* with *GCaMP3* gradually enhanced with prolonging the co-cultivation time.

Author Contributions: Methodology, L.Y. and Z.Z.; Investigation, S.Z. and R.H.; Writing—original draft, S.Z.; Writing—review & editing, Z.Z.; Supervision, Z.Z.; Funding acquisition, Z.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Natural Science Foundation of China (Grant No. 32371830, 31870585), and the Opening Project of Zhejiang Provincial Key Laboratory of Forest Aromatic Plants-based Healthcare Functions (Grant No. 2022E10008).

Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

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

References

1. Lian, Z.; Nguyen, C.D.; Liu, L.; Wang, G.; Chen, J.; Wang, S.; Yi, G.; Wilson, S.; Ozias-Akins, P.; Gong, H.; et al. Application of developmental regulators to improve in-planta or in vitro transformation in plants. *Plant Biotechnol. J.* **2022**, *20*, 1622. [CrossRef] [PubMed]
2. Wu, Y.F.; Zhou, N.; Ni, X.N.; Okoye, C.; Wang, Y.L.; Li, X.; Gao, L.; Zhou, G.K.; Jiang, J.X. Developing a long-term and powerful in vitro culture and *Agrobacterium*-mediated transformation system for *Miscanthus sinensis* (Poaceae). *Ind. Crop Prod.* **2021**, *161*, 113190. [CrossRef]
3. Janani, C.; Sundararajan, B.; Kumari, B.D.R. Construction and transformation of peroxisome proliferator activated receptor gamma (*RnPPARγ*) gene using *Agrobacterium tumefaciens* into *Glycine max* L. Merr. *Gene Rep.* **2019**, *16*, 100427. [CrossRef]
4. Li, X.N.; Li, H.Y.; Zhao, Y.Z.; Zong, P.X.; Zhan, Z.X.; Piao, Z.Y. Establishment of a simple and efficient *Agrobacterium*-mediated genetic transformation system to Chinese cabbage (*Brassica rapa* L. ssp. Pekinensis). *Hortic. Plant J.* **2021**, *7*, 117–128. [CrossRef]
5. Giorgio, G.; Ivana, G. Genetic transformation of fruit trees: Current status and remaining challenges. *Transgenic Res.* **2012**, *21*, 1163–1181.
6. Van Nocker, S.; Gardiner, S.E. Breeding better cultivars, faster: Applications of new technologies for the rapid deployment of superior horticultural tree crops. *Hortic. Res.* **2014**, *1*, 14022. [CrossRef]
7. Pushpa, K.; Rinku, C.; Narendra, T.; Prashant, K. A genotype-independent, simple, effective and efficient in planta *Agrobacterium*-mediated genetic transformation protocol. *Methods Psrotoc.* **2022**, *5*, 69.
8. Franz, L.; Anja, R.; Hannah, T.; Ralph, P. Gene gun-mediated transient gene expression for functional studies in plant immunity. *Methods Mol. Biol.* **2022**, *2523*, 63–77.
9. Wang, Y.Y.; Zhang, Y.A.; Dong, Y.X.; Li, D.L.; Shi, S.L.; Li, S.H.; Li, L.Z.; He, Y.J.; Li, J.Y.; Chen, H.Y.; et al. A highly efficient mesophyll protoplast isolation and PEG-mediated transient expression system in eggplant. *Sci. Hortic.* **2022**, *304*, 111303. [CrossRef]
10. Yang, Y.; Li, R.; Qi, M. In vivo analysis of plant promoters and transcription factors by agroinfiltration of tobacco leaves. *Plant J.* **2000**, *22*, 543–551. [CrossRef]
11. Chen, X.J.; He, S.T.; Jiang, L.; Li, X.Z.; Guo, W.L.; Chen, B.H.; Zhou, J.G.; Skliar, V. An efficient transient transformation system for gene function studies in pumpkin (*Cucurbita moschata* D.). *Sci. Hortic.* **2021**, *282*, 110028. [CrossRef]
12. Zottini, M.; Barizza, E.; Costa, A.; Formentin, E.; Ruberti, C.; Carimi, F.; Schiavo, F.L. Agroinfiltration of grapevine leaves for fast transient assays of gene expression and for long-term production of stable transformed cells. *Plant Cell Rep.* **2008**, *27*, 845–853. [CrossRef] [PubMed]
13. Zhong, S.W.; Dong, B.; Zhou, J.; Miao, Y.F.; Yang, L.Y.; Wang, Y.G.; Xiao, Z.; Fang, Q.; Wan, Q.Q.; Zhao, H.B. Highly efficient transient gene expression of three tissues in *Osmanthus fragrans* mediated by *Agrobacterium tumefaciens*. *Sci. Hortic.* **2023**, *310*, 111725. [CrossRef]
14. Fraley, R.T.; Rogers, S.G.; Horsch, R.B.; Sanders, P.R.; Flick, J.S.; Adams, S.P.; Bittner, M.L.; Brand, L.A.; Fink, C.L.; Fry, J.S.; et al. Expression of bacterial genes in plant cells. *Proc. Natl. Acad. Sci. USA* **1983**, *80*, 4803–4807. [CrossRef]
15. Nam, J.; Mysore, K.S.; Zheng, C.; Knue, M.K.; Matthysse, A.G.; Gelvin, S.B. Identification of T-DNA tagged *Arabidopsis* mutants that are resistant to transformation by *Agrobacterium*. *Mol. Gen. Genet.* **1999**, *261*, 429–438. [CrossRef]
16. Cho, J.H.; Lee, J.Y.; Kim, Y.W.; Lee, M.H.; Choi, S.H. Improvement of transformation efficiencies using *Agrobacterium*-mediated transformation of Korean rice. *Korean J. Crop Sci.* **2004**, *49*, 61–68.
17. Vinoth, S.; Gurusaravanan, P.; Jayabalan, N. Optimization of factors influencing microinjection method for *Agrobacterium tumefaciens*-mediated transformation of tomato. *Appl. Biochem. Biotechnol.* **2013**, *169*, 1173–1187. [CrossRef]
18. Lv, Q.R.; Chen, C.S.; Xu, Y.J.; Hu, S.K.; Wang, L.; Sun, K.; Chen, X.; Li, X.H. Optimization of *Agrobacterium tumefaciens*-mediated transformation systems in tea plant (*Camellia sinensis*). *Hortic. Plant J.* **2017**, *3*, 105–109. [CrossRef]
19. Lv, Y.D.; Zhang, M.L.; Wu, T.; Wu, T.L.; Zhong, Y. The infiltration efficiency of *Agrobacterium*-mediated transient transformation in four apple cultivars. *Sci. Hortic.* **2019**, *256*, 108597. [CrossRef]

20. Wen, S.S.; Ge, X.L.; Wang, R.; Yang, H.F.; Bai, Y.; Guo, Y.H.; Zhang, J.; Lu, M.Z.; Zhao, S.T.; Wang, L.Q. An efficient *Agrobacterium*-mediated transformation method for Hybrid Poplar 84K (*Populus alba* × *P. glandulosa*) using calli as explants. *Int. J. Mol. Sci.* **2022**, *23*, 2216. [CrossRef]
21. Yang, W.H.; Ren, J.Q.; Liu, W.R.; Liu, D.; Xie, K.D.; Zhang, F.; Wang, P.W.; Guo, W.W.; Wu, X.M. An efficient transient gene expression system for protein subcellular localization assay and genome editing in citrus protoplasts. *Hortic. Plant J.* **2022**, *9*, 425–436. [CrossRef]
22. Chen, C.H.; Zhong, Y.D.; Yu, F.X.; Xu, M. Deep sequencing identifies miRNAs and their target genes involved in the biosynthesis of terpenoids in *Cinnamomum camphora*. *Ind. Crop. Prod.* **2020**, *145*, 111853. [CrossRef]
23. Ma, R.; Su, P.; Jin, B.; Guo, J.; Tian, M.; Mao, L.Y.; Tang, J.F.; Chen, T.; Lai, C.J.S.; Zeng, W.; et al. Molecular cloning and functional identification of a high-efficiency (+)-borneol dehydrogenase from *Cinnamomum camphora* (L.) Presl. *Plant Physiol. Biochem.* **2021**, *158*, 363–371. [CrossRef] [PubMed]
24. Li, Z.; Zhong, Y.; Yu, F.; Xu, M. Novel SSR marker development and genetic diversity analysis of *Cinnamomum camphora* based on transcriptome sequencing. *Plant Genet. Resour.* **2018**, *16*, 568–571. [CrossRef]
25. Zhong, Y.; Yang, A.; Li, Z.; Zhang, H.; Liu, L.; Wu, Z.; Li, Y.; Liu, T.; Xu, M.; Yu, F. Genetic diversity and population genetic structure of *Cinnamomum camphora* in South China revealed by EST-SSR markers. *Forests* **2019**, *10*, 1019. [CrossRef]
26. Gong, X.; Yang, A.; Wu, Z.; Chen, C.; Li, H.; Liu, Q.; Yu, F.; Zhong, Y. Employing genome-wide SNP discovery to characterize the genetic diversity in *Cinnamomum camphora* using genotyping by sequencing. *Forests* **2021**, *12*, 1511. [CrossRef]
27. Shi, X.P.; Dai, X.G.; Liu, G.F.; Zhang, J.W.; Ning, G.G.; Bao, M.Z. Cyclic secondary somatic embryogenesis and efficient plant regeneration in camphor tree (*Cinnamomum camphora* L.). *In Vitro Cell. Dev. Biol.-Plant* **2010**, *46*, 117–125. [CrossRef]
28. El-Kader, E.M.A.; Serag, A.; Aref, M.S.; Ewais, E.E.A.; Farag, M.A. Metabolomics reveals ionones upregulation in MeJA elicited *Cinnamomum camphora* (camphor tree) cell culture. *Plant Cell Tissue Organ Cult.* **2019**, *137*, 309–318. [CrossRef]
29. Shen, T.F.; Qi, H.R.; Luan, X.Y.; Xu, W.L.; Yu, F.X.; Zhong, Y.D.; Xu, M. The chromosome-level genome sequence of the camphor tree provides insights into *Lauraceae* evolution and terpene biosynthesis. *Plant Biotechnol. J.* **2022**, *20*, 244–246. [CrossRef]
30. Krogman, W.; Sparks, J.A.; Blancaflor, E.B. Cell type-specific imaging of calcium signaling in *Arabidopsis thaliana* seedling roots using GCaMP3. *Int. J. Mol. Sci.* **2020**, *21*, 6385. [CrossRef]
31. Ren, Q.; Xu, Z.; Xue, Y.; Yang, R.; Ma, X.; Sun, J.; Wang, J.; Lin, S.; Wang, W.; Yang, L.; et al. Mechanism of calcium signal response to cadmium stress in duckweed. *Plant Signal. Behav.* **2022**, *17*, 2119340. [CrossRef] [PubMed]
32. Toyota, M.; Spencer, D.; Sawai-Toyota, S.; Wang, J.; Zhang, T.; Koo, A.J.; Howe, G.A.; Gilroy, S. Glutamate triggers long-distance, calcium-based plant defense signaling. *Science* **2018**, *361*, 1112–1115. [CrossRef] [PubMed]
33. Salehi, Z.; Amirahmadi, A.; Rezaei, A.; Farrokh, P.; Ghasemian, J. Comparison of five DNA extraction methods in three medicinal plants: *Peganum harmala* L., *Tamarix ramosissima* Ledeb., and *Potentilla reptans* L. *Mol. Biol. Res. Commun.* **2023**, *12*, 1–61.
34. Chincinska, I.A. Leaf infiltration in plant science: Old method, new possibilities. *Plant Methods* **2021**, *17*, 83. [CrossRef] [PubMed]
35. Vonasek, E.; Nitin, N. Influence of vacuum cooling on *Escherichia coli* O157:H7 infiltration in fresh leafy greens via a multiphoton-imaging approach. *Appl. Environ. Microb.* **2016**, *82*, 106–115. [CrossRef] [PubMed]
36. Ranjbaran, M.; Solhtalab, M.; Datta, A.K. Mechanistic modeling of light induced chemotactic infiltration of bacteria into leaf stomata. *PLoS Comput. Biol.* **2020**, *16*, e1007841. [CrossRef]
37. Suleiman, W.B. In vitro estimation of superfluid critical extracts of some plants for their antimicrobial potential, phytochemistry, and GC–MS analyses. *Ann. Clin. Microbiol. Antimicrob.* **2020**, *19*, 29. [CrossRef]
38. Yan, X.; Liang, S.; Peng, T.; Zhang, G.; Zeng, Z.; Yu, P.; Gong, D.; Deng, S. Influence of phenolic compounds on physicochemical and functional properties of protein isolate from *Cinnamomum camphora* seed kernel. *Food Hydrocolloid* **2020**, *102*, 105612. [CrossRef]
39. Zhao, H.M.; Jia, Y.Q.; Cao, Y.T.; Wang, Y.C. Improving T-DNA transfer to *Tamarix hispida* by adding chemical compounds during *Agrobacterium tumefaciens* culture. *Front. Plant Sci.* **2020**, *11*, 501358. [CrossRef]
40. Li, S.J.; Xu, B.L.; Niu, X.L.; Lu, X.; Cheng, J.P.; Zhou, M.L.; Hooykaas, P.J.J. JAZ8 interacts with *VirE3* attenuating *Agrobacterium* mediated root tumorigenesis. *Front. Plant Sci.* **2021**, *12*, 685533. [CrossRef]
41. Firoozabady, E.; Heckert, M.; Gutterson, N. Transformation and regeneration of pineapple. *Plant Cell Tissue Organ* **2006**, *84*, 1–16. [CrossRef]
42. Mo, R.L.; Huang, Y.M.; Yang, S.C.; Zhang, Q.L.; Luo, Z.R. Development of *Agrobacterium* -mediated transient transformation in persimmon (*Diospyros kaki* Thunb.). *Sci. Hortic.* **2015**, *192*, 29–37. [CrossRef]
43. Zhang, Y.; Wang, Y.C.; Wang, C. Gene overexpression and gene silencing in birch using an *Agrobacterium*-mediated transient expression system. *Mol. Biol. Rep.* **2012**, *39*, 5537–5541. [CrossRef]
44. Priyadarshani, S.V.G.N.; Cai, H.Y.; Zhou, Q.; Liu, Y.H.; Cheng, Y.; Xiong, J.J.; Patson, D.L.; Cao, S.; Zhao, H.; Qin, Y. An efficient *Agrobacterium* mediated transformation of pineapple with GFP-tagged protein allows easy, non-destructive screening of transgenic pineapple plants. *Biomolecules* **2019**, *9*, 617. [CrossRef] [PubMed]
45. Petri, C.; Alburquerque, A.; Garcéa-Castillo, S.; Egea, J.; Burgos, L. Factors affecting gene transfer efficiency to apricot leaves during early *Agrobacterium*-mediated transformation steps. *J. Hortic. Sci. Biotechnol.* **2004**, *79*, 704–712. [CrossRef]
46. Chaparro-Pulido, C.A.; Montiel, M.M.; Palomo-Ríos, E.; Mercado, J.A.; Pliego-Alfaro, F. Development of an efficient transient transformation protocol for avocado (*Persea americana* Mill.) embryogenic callus. *In Vitro Cell. Dev. Biol.-Plant* **2014**, *50*, 292–298. [CrossRef]

47. Mallón, R.; Vidal, A.M.V.N. High-efficiency *Agrobacterium*-mediated transformation in *Quercus robur*: Selection by use of a temporary immersion system and assessment by quantitative PCR. *Plant Cell Tissue Organ* **2013**, *114*, 171–185. [CrossRef]
48. Jiang, S.F.; Cui, Y.; Zhao, R.R.; Qi, S.Z.; Kong, L.S.; Zhao, J.; Li, S.S.; Zhang, J.F. *Agrobacterium tumefaciens*-mediated transformation of hybrid sweetgum embryogenic callus. *J. Beijing For. Univ.* **2021**, *43*, 9–17, (In Chinese with English abstract).
49. Han, X.; Han, B. Plant regeneration and *Agrobacterium*-mediated transformation of vacuolar H^+-ATPase c subunit gene in hybrid poplar *Populus davidiana* Dode × *P. bollena* Lauche. *BIO Web Conf.* **2017**, *8*, 03004. [CrossRef]
50. Miao, M.M.; Xu, R.; Zheng, L.J.; Zhou, H.L.; Zhang, Z.D.; Cheng, H. High-efficiency *Agrobacterium tumefaciens*-mediated transformation of cucumber (*Cucumis sativus* L.) using stem nodes as explants. *J. Hortic. Sci. Biotechnol.* **2009**, *84*, 199–203. [CrossRef]
51. An, D.D.; Danhorn, T.; Fuqua, C.; Parsek, M.R. Quorum sensing and motility mediate interactions between *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens* in biofilm cocultures. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 3828–3833. [CrossRef] [PubMed]
52. Sutradhar, M.; Mandal, N. Reasons and riddance of *Agrobacterium tumefaciens* overgrowth in plant transformation. *Transgenic Res.* **2023**, *32*, 33–52. [CrossRef] [PubMed]
53. Kasia, P.; Tanja, S.; Jürgen, T.; Karel, Š.; Karel, K.; Silke, K.; Dietmar, H.P.; Michal, K. Determining lineage-specific bacterial growth curves with a novel approach based on amplicon reads normalization using internal standard (ARNIS). *ISME J.* **2018**, *12*, 2640–2654.
54. Benny, A.; Alex, S.; Soni, K.B.; Anith, K.N.; Kiran, A.G.; Viji, M.M. Improved transformation of *Agrobacterium* assisted by silver nanoparticles. *BioTechnologia* **2022**, *103*, 311–317. [CrossRef] [PubMed]
55. Thilip, C.; Raju, C.S.; Varutharaju, K.; Aslam, A.; Shajahan, A. Improved *Agrobacterium rhizogenes*-mediated hairy root culture system of *Withania somnifera* (L.) Dunal using sonication and heat treatment. *3 Biotech* **2015**, *5*, 949–956. [CrossRef]
56. Iqbal, A.; Dave, N.; Kant, T. Comparative analysis of different nutrient media for growth of *Agrobacterium tumefaciens* under small volume cultures. *J. Appl. Life Sci. Int.* **2021**, *24*, 27–33. [CrossRef]
57. Irvin, L.; Ortiz, Y.Z.; Rivera, K.R.; Vaidya, B.N.; Sherman, S.H.; Batista, R.A.; Berríos, J.A.N.; Joshee, N.; Arun, A. Micropropagation of rare *Scutellaria havanensis* Jacq. and preliminary studies on antioxidant capacity and anti-cancer potential. *Molecules* **2021**, *26*, 5813. [CrossRef]
58. Ashraf-Dehkordi, E.; Alemzadeh, A.; Tanaka, N.; Razi, H. Effects of vacuum infiltration, *Agrobacterium* cell density and acetosyringone concentration on *Agrobacterium*-mediated transformation of bread wheat. *J. Consum. Prot. Food Saf.* **2021**, *16*, 59–69. [CrossRef]
59. Brown, P.J.B.; Chang, J.H.; Fuqua, C. *Agrobacterium tumefaciens*: A transformative agent for fundamental insights into host-microbe interactions, genome biology, chemical signaling, and cell biology. *J. Bacteriol.* **2023**, *205*, e00005-23. [CrossRef]
60. Cao, D.V.; Pamplona, R.S.; Kim, J.; Oh, Y.K.; Cho, S.K.; Ahn, J.; Yang, S.W.; Riu, K.Z.; Boo, K.H. Optimization of *Agrobacterium*-mediated transient expression of heterologous genes in spinach. *Plant Biotechnol. Rep.* **2017**, *11*, 397–405. [CrossRef]
61. Duan, S.Y.; Xin, R.J.; Guan, S.X.; Li, X.T.; Fei, R.W.; Cheng, W.; Pan, Q.; Sun, X.M. Optimization of callus induction and proliferation of *Paeonia lactiflora* Pall. and *Agrobacterium*-mediated genetic transformation. *Front. Plant Sci.* **2022**, *13*, 996690. [CrossRef] [PubMed]
62. Guan, S.; Kang, X.; Ge, J.; Fei, R.; Duan, S.; Sun, X. An efficient *Agrobacterium*-mediated transient transformation system and its application in gene function elucidation in *Paeonia lactiflora* Pall. *Front. Plant Sci.* **2022**, *13*, 999433. [CrossRef] [PubMed]
63. Li, Z.; Harper, J.F.; Weigand, C.; Hua, J. Resting cytosol Ca^{2+} level maintained by Ca^{2+} pumps affect environmental responses in *Arabidopsis*. *Plant Physiol.* **2023**, *191*, 2534–2550. [CrossRef] [PubMed]
64. Wu, H.C.; Luo, D.L.; Vignols, F.; Jinn, T.L. Heat shock-induced biphasic Ca^{2+} signature and OsCaM1-1 nuclear localization mediate downstream signalling in acquisition of thermotolerance in rice (*Oryza sativa* L.). *Plant Cell Environ.* **2012**, *35*, 1543–1557. [CrossRef] [PubMed]
65. Liu, Y.N.; Lu, X.X.; Ren, A.; Shi, L.; Zhu, J.; Jiang, A.L.; Yu, H.S.; Zhao, M.W. Conversion of phosphatidylinositol (PI) to PI4-phosphate (PI4P) and then to PI (4, 5) P_2 is essential for the cytosolic Ca^{2+} concentration under heat stress in *Ganoderma lucidum*. *Environ. Microbiol.* **2018**, *20*, 2456–2468. [CrossRef]