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Construction of a Genetic Transformation System for *Populus wulianensis*

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Abstract: Transgenic technology is a potent tool for verifying gene functions, and poplar serves as a model system for genetically transforming perennial woody plants. However, the current poplar genetic transformation system is limited to a few genotypes. In this study, we developed an efficient transformation system based on the *Agrobacterium*-mediated transformation of *Populus wulianensis*, a rare and endangered tree species endemic to Shandong Province. Aseptic seedlings of *P. wulianensis* were used as experimental materials, and the optimal medium for inducing adventitious buds was explored as 1/2(NH_4NO_3) MS + 0.05 mg/L naphthalene acetic acid (NAA) + 0.5 mg/L 6-benzylaminopurine (6-BA), resulting in up to 35 adventitious buds. The selection resistance critical pressure of 300 mg/L for timentin can effectively inhibit the growth of *Agrobacterium* while promoting the induction of adventitious buds in leaves. The critical screening pressure for kanamycin for producing resistant adventitious buds and inducing resistant rooting seedlings was 100 mg/L. We optimized several independent factors, which significantly enhanced the efficiency of genetic transformation. The leaves were infected with *Agrobacterium* suspension diluted twice by adding 100 µmol/L acetylsyringone (β-AS) (OD_{600} = 0.6) for 15 min, followed by co-culture in the dark for 3 d. Using this improved transformation system, we obtained transgenic *P. wulianensis* clones overexpressing the enhanced green fluorescent protein (*EGFP*) gene through direct organogenesis. Among the 112 resistant buds obtained, 17 developed resistant rooting in seedlings. Eight positive plants were identified through DNA, RNA, and protein level analyses, with a positivity rate of 47.06%. This study provides a foundation for developing and utilizing *P. wulianensis* germplasm resources and lays the groundwork for resource improvement.

Keywords: *Populus wulianensis*; leaf redifferentiation; genetic transformation

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Citation: Wang, Y.; Song, C.; Han, Y.; Wang, R.; Guan, L.; Mu, Y.; Sun, T.; Xie, X.; Zhao, Y.; Xu, J.; et al. Construction of a Genetic Transformation System for *Populus wulianensis*. *Forests* **2024**, *15*, 1474. https://doi.org/10.3390/f15081474

Academic Editor: Eduardo Notivol

Received: 7 May 2024
Revised: 16 August 2024
Accepted: 19 August 2024
Published: 22 August 2024

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1. Introduction

The reproduction of woody plants is characterized by long growth cycles and seasonal dormancy [1]. Tissue culture technology addresses the challenge of asexual reproduction and provides a basis for studying gene function and physiological characteristics.

Plant genetic transformation serves to explore gene function and to develop plant varieties with specific traits, particularly in long-generation trees. The establishment of this plant genetic improvement method has assisted the application of traditional breeding methods [2] and accelerates forest tree breeding [3]. *Agrobacterium tumefaciens*-mediated transformation is an effective technique for introducing exogenous DNA into plant genomes, facilitating gene function and breeding studies [4,5]. Since 1986, when Parsons et al. [6] first demonstrated the successful expression of exogenous genes in poplar

using transgenic technology, the field of poplar genetic engineering has rapidly advanced. The successful genome sequencing of *Populus trichocarpa* in 2006 marked the transition of genomic research in forest trees into the post-genomic era [7]. In recent years, various poplar species have developed genetic transformation systems, achieving significant breakthroughs in gene function research and other areas [8,9].

Agrobacterium-mediated genetic transformation is affected by numerous factors such as plant genotype, explant type, strain type, vector-receptor affinity, *Agrobacterium* infection mode, pre-culture time, bacterial fluid concentration, infection time, co-culture time, and antibiotic selection [10]. Therefore, to establish an efficient genetic transformation system, it is essential to coordinate these factors with the induction of resistant adventitious buds before conducting the experiments. Studies have indicated that various types of explants, including leaves, petioles, hypocotyls, stem internodes, stem segments, roots, cell suspension cultures, and buds [8,9,11], can enhance the efficiency of *Agrobacterium*-mediated transformation in *Populus* [7,12]. For instance, Yu et al. [13] achieved a transformation yield of 16.28% in male diploid *P. tomentosa* by screening transformed leaves with 30 mg/L kanamycin (Kan) after 12 h of pre-culture using an *Agrobacterium* concentration (OD_{600}) of 0.4, 20 min of infection time and 24 h of co-culture. Wen et al. [14] achieved a transformation frequency exceeding 50% in callus transformation by adding 100 μM acetylsyringone (β-AS) to an *Agrobacterium tumefaciens* suspension with OD_{600} of 0.6, infecting for 15 min and co-culturing for 2 d, with explants pre-cultured for 6 d. Ma et al. [15] obtained an average transformation efficiency of 23.60% for *P. alba* var. *pyramidalis* leaf explants by inducing leaf callus with 90 mg/L hygromycin, screening callus-induced adventitious buds twice for resistant transformants, and optimizing factors such as an *Agrobacterium* OD_{600} of 0.3–0.5, 10 min of infection, addition of 100 μM AS, and the use of different vectors.

P. wulianensis S. B. Liang & X. W. Li, a member of Salicaceae, is a dioecious, deciduous hardwood [16] and, similar to other poplar species, is valuable for timber. However, its small wild population, limited habitat, high risk of extinction, and sensitivity to human activities warrant its classification as a PSESP. The declining effective population size and increasing isolation of this species have enhanced the genetic drift and reduced gene flow, resulting in a loss of genetic diversity [17,18]. This loss has severely reduced the ability of species to withstand harsh environments, ultimately accelerating their extinction. The transgenic technology enables rapid and targeted genetic improvement in *P. wulianensis*, facilitating the development of new varieties that can adapt to climate change and meet evolving needs. However, establishing a culture medium for leaf redifferentiation to induce adventitious buds in *P. wulianensis* and breaking through its efficient genetic transformation system are currently unresolved. In this study, we selected the aseptic seedlings of *P. wulianensis*, an endemic and endangered tree species in Shandong, screened the orthogonal combinations of leaf-induced adventitious buds and the main experimental conditions of *Agrobacterium*-mediated genetic transformation, and established and optimized the regeneration system and the genetic transformation system of *P. wulianensis*, in order to provide reference for the study of genetic transformation of other woody plants and lay a foundation for molecular assisted breeding in the later stage.

2. Materials and Methods

2.1. Source of Materials

2.1.1. Plant Materials of *P. wulianensis*

The leaf redifferentiation test of *P. wulianensis* used 3–4 mature, healthy leaves below the apical bud of the aseptic seedlings stored in our laboratory as experimental materials, and 15–20 leaves were inoculated in each medium, with three replicates. After inoculation, the leaves were grouped for aseptic culture, maintaining a controlled temperature of (25 ± 5) °C during the day and (18 ± 5) °C at night, with a light cycle of 16 h per day and a light intensity of 3000–5000 LX. The medium was changed every 10 d, and after 35 d of culture, the number of regenerated buds was recorded, and the health state of leaf growth was evaluated.

The materials for antibiotic screening experiments were as above, and the leaves were divided into three parts (leaf base with petiole, leaf middle, and leaf tip) (Figure 1); 3–4 samples were placed in each petri dish with three replications for each gradient concentration. The optimal differentiation medium selected from the above tests was used, and the leaves were cultivated in the dark for 8 days; the culture conditions were unchanged.

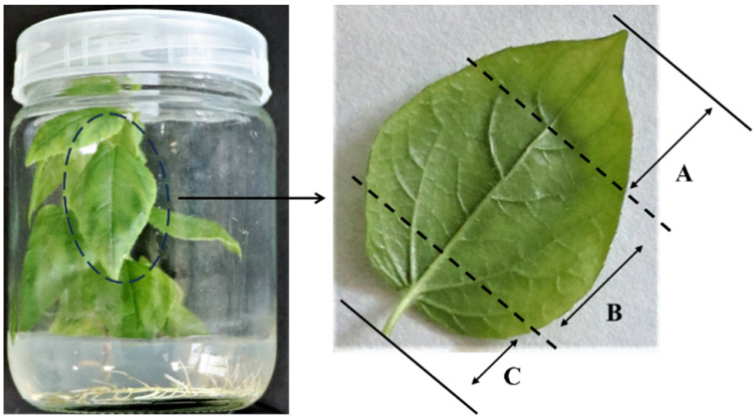

Figure 1. Diagram of the blade cutting area. A, B, and C represent the tip, middle, and base parts of the leaf, respectively.

2.1.2. Strain and Plasmid

The strain used in the experiment was *Agrobacterium tumefaciens* GV3101, and the plant expression vector was PEZR-(K)-LC, which is a binary expression vector carrying the Kan resistance gene and fluorescent protein gene (*EGFP*) (Figure 2).



Figure 2. Expression vector PEZR—(K)—LC map.

2.2. Methods

2.2.1. Leaf Adventitious Bud Induction

Four base media, Naphthalene acetic acid (NAA) (Sigma, Darmstadt, Germany), 6-Benzylaminopurine (6-BA) (Sigma, Darmstadt, Germany), and dark culture time, were selected as the influencing factors, with three levels for each factor. The design used an orthogonal table, $L_9(3^4)$ (Table 1). This study analyzed the effects of these different factors at various levels on leaf redifferentiation of *P. wulianensis*.

Table 1. Orthogonal factors for induction of regeneration of adventitious buds from sterile.

Level	Basal Medium (A)	NAA (mg/L) (B)	6-BA (mg/L) (C)	Dark Culture Time (d) (D)
1	(1/2NH_4NO_3) MS	0.01	0.1	0
2	1/2MS	0.05	0.5	4
3	MS	0.10	1.0	8

2.2.2. Antibiotic and Bacteriostatic Agent Concentration Screening Experiment

The effect of timentin (Coolaber, Beijing, China) on *Agrobacterium* GV3101 growth was tested at concentrations ranging from 0 mg/L (control) to 250 mg/L in 50 mg/L increments. The effect of timentin on the induction of adventitious buds in *P. wulianensis* leaves was tested on three parts of the treated leaves (Figure 1). The leaf middle was tested at concentrations ranging from 0 mg/L to 850 mg/L in 50 mg/L increments, while

the other two parts were tested at concentrations ranging from 0 mg/L to 900 mg/L in 100 mg/L increments. The effect of Kan (Sigma, Darmstadt, Germany) on the induction of adventitious buds in leaves was tested on three parts of the treated leaves at concentrations ranging from 0 mg/L to 200 mg/L in 25 mg/L increments. Additionally, the above adventitious buds were selected and separately inoculated with different concentrations of Kan (0, 25, 50, 75, 100, 125, 150, 175, and 200 mg/L) and timentin (0, 100, 200, 300, 400, 500, 600, 700, and 800 mg/L) on a rooting medium of 1/2 MS + 0.05 mg/L NAA + 15 g/L sucrose + 7.0 g/L agar powder [19], for adventitious buds induction and rooting critical pressure. The medium was changed every 7–10 d in the above experiments, and the differentiation of induced adventitious buds on leaves and their subsequent rooting were counted after 30 d.

To test the antibacterial effect of the bacteriostatic agent timentin, *Agrobacterium tumefaciens* GV3101 was activated and inoculated into 1 mL of YEB liquid medium (containing 50 mg/L Kan and 25 mg/L rifampicin) and shaken with reference to the method of Jia et al. [20]. The 20 μL liquid was spread on a solid medium (1/2(NH_4NO_3) MS + 0.05 mg/L NAA + 0.5 mg/L 6-BA) containing different concentrations of timentin for culture. After 4 d of inverted dark culture incubation at 28 °C, *Agrobacterium* growth was observed.

2.2.3. Genetic Transformation of *P. wulianensis* Leaf Discs

The plant expression vector PEZR-(K)-LC was transferred into *Agrobacterium* GV3101 using the electric shock transformation method, and the bacteria were preserved after Polymerase chain reaction (PCR) identification. The activation culture of *Agrobacterium* is shown in Section 2.2.2, and after activation, *Agrobacterium* was inoculated into 100 mL of medium for mass amplification. The OD_{600} = 0.6 bacterial solution was centrifuged and resuspended in 2 times the liquid medium of 1/2(NH_4NO_3) MS without hormones, antibiotics, or sucrose. We used the leaf disc transformation method for genetic transformation. The middle of 3 to 4 leaves under the apical leaf was taken, cut transversely, placed in the above *Agrobacterium* suspension with 100 μmol/L β-AS for 15 min, and shaken approximately 10 times every 2 min. After imbibition, the leaves were blotted dry with sterile filter paper and placed abaxially facing up into a solid co-culture medium without antibiotics (1/2(NH_4NO_3) MS + 0.05 mg/L NAA + 0.5 mg/L 6-BA + 100 μmol/L β-AS). They were incubated in the dark at 25 °C for 3 d, and then, the leaves were transferred to a solid medium containing antibiotic screening agents (1/2(NH_4NO_3) MS + 0.05 mg/L NAA + 0.5 mg/L 6-BA + 100 mg/L Kan + 300 mg/L timentin) for the resistant adventitious buds screening test. The medium was changed every 3 d, and after two changes, it was changed once every 7 d. The data on resistant adventitious buds were recorded after 50 d. The resistant buds were transferred to a solid medium (1/2 MS + 0.05 mg/L NAA + 100 mg/L Kan + 300 mg/L timentin) for further cultivation. After 35 d, the data on resistant rooted seedlings were recorded, and robust-rooted Kan-resistant plants were selected as the primary transformed plants.

2.2.4. Evaluation of Transformation Factors on Genetic Efficiency

A single-factor gradient experimental design was employed in the optimization test of the genetic transformation system. All factors, except the experimental ones, followed the genetic transformation methods outlined earlier. The single factor of bacterial concentration (OD_{600} = 0.6 bacterial solution was diluted 0, 2, 4 times with 1/2(NH_4NO_3) MS without any hormone and sucrose liquid medium), β-AS addition concentration (0, 100, and 200 μmol/L), infection time (5, 15 and 20 min), and dark culture (co-culture) time (1, 3, and 6 d) were used to investigate the genetic transformation efficiency. In this case, the average number of Kan-resistant adventitious buds cultured for 35 d was used as an indicator of transformation efficiency.

2.2.5. PCR and RT-PCR Analyses

The DNA from the resistant rooted seedling and wild-type (WT) plant strains was extracted using the CTAB method [21]. PCR detection was performed using the PEZR-(K)-LC vector primer sequence 35SF/EGFPR (5′-GACGCACAATCCCACTATC-3′ and 5′-GGTGCTCAGGTAGTGGTTGT-3′). The total PCR amplification system volume was 30 µL, including 15 µL of Takara^{TM} Premix Taq (Takara, Dalian, China), 0.6 µL each of the 10 µmol/L upstream and downstream primers, and 0.6 µL of DNA, reached 30 µL with ddH_2O. The gene amplification procedure included pre-denaturation at 95 °C for 3 min, followed by denaturation at 95 °C for 10 s, annealing at 56 °C for 30 s, and extension at 72 °C for 1 min, set up for 30 cycles, and finally, full extension at 72 °C for 10 min. The amplification products were stored at 4 °C and analyzed using 1.2% agarose gel electrophoresis. The total RNA was extracted from the leaves of resistant lines using a Plant RNA Extraction Kit (Vazyme, Nanjing, China) 1 month after transplantation. The RNA reverse transcription was performed using the PrimeScript™ IV 1st strand cDNA Synthesis Mix Kit (Takara, Dalian, China). Actin (5′-AAACTGTAATGGTCCTCCCTCCG-3′ and 5′-GCATCATCACAATCACTCTCCGA-3′) was used as the internal reference gene, following the TaKaRa^{TM} Premix Taq reaction system. Primer pairs for the *EGFP* gene (5′-GAAGAACGGCATCAAGGT-3′ and 5′-GCTCAGGTAGTGGTTGTC-3′) were designed to undergo semi-quantitative PCR to detect the relative expression levels of the integrated exogenous gene.

2.2.6. Observation of EGFP Expression

We removed the lower epidermis of the leaves of transgenic *EGFP* and WT lines for fluorescence expression. Leaf epidermis were observed with an Axio Imager 2 (Zeiss, Oberkochen, Germany) fluorescence microscope. EGFP fluorescence was observed with 473 nm laser excitation and emission between 487 nm and 521 nm, and 4′,6-Diamidino-2-phenylindole (DAPI) fluorescence was observed with 360 nm laser excitation and emission 460 nm.

2.2.7. Statistical Analysis

The efficiency of genetic transformation was calculated using the formula positive rate = identification of positive plants/total number of resistant rooted seedlings × 100%. Data processing and graphing were performed using Excel 2010 (Microsoft, Redmond, WA, America) and -Prism 9 (GraphPad, San Diego, CA, America). SPSS Statistics 26 software (IBM, Armonk, NY, America) was used for the analysis of variance (ANOVA) and multiple comparisons of the experimental data. Duncan's test was employed for comparison, with the least significant difference level (p) set at 0.01.

3. Results

3.1. Effects of Medium Components on the Induction of Adventitious Buds of P. wulianensis Leaves

The multifactorial ANOVA results for the number of adventitious buds induced by the leaves of *P. wulianensis* with different treatments. The F values for factors A, C, and D were 77.43, 15.39, and 49.29, respectively, reaching a highly significant level ($p < 0.01$). Conversely, the NAA concentration had no significant effect on the number of regenerated buds (Tables 2 and S1). This suggested that the base medium had the most significant influence on *P. wulianensis* leaf redifferentiation, followed by dark culture time and 6-BA hormone concentration, and the NAA hormone concentration had the least effect.

Further multiple comparative analyses of the three factors (base medium, dark culture time, and 6-BA hormone concentration) revealed (Table S2) that leaf inoculation into medium 1 induced the highest number of adventitious buds at 38, demonstrating a highly significant difference compared with media 2 and 3. Therefore, 1/2(NH_4NO_3) MS was selected as the optimal base medium in this study.

Table 2. Orthogonal test results of adventitious bud regeneration in detached leaves.

Code	A	B	C	D	Average Number of Regenerated Buds
1	3(MS)	2 (0.05)	3 (1.0)	1 (0 d)	1.67 ± 0.72^{e}
2	3(MS)	3 (0.10)	1 (0.1)	2 (4 d)	17.33 ± 1.70^{c}
3	2(1/2MS)	1 (0.01)	3 (1.0)	2 (4 d)	10.00 ± 0.82^{d}
4	2(1/2MS)	3 (0.10)	2 (0.5)	1 (0 d)	3.33 ± 2.05^{e}
5	2(1/2MS)	2 (0.05)	1 (0.1)	3 (8 d)	15.00 ± 3.27^{c}
6	1(1/2(NH_4NO_3) MS)	3 (0.10)	3 (1.0)	3 (8 d)	22.33 ± 2.05^{b}
7	1(1/2(NH_4NO_3) MS)	1 (0.01)	1 (0.1)	1 (0 d)	19.00 ± 2.94^{c}
8	3(MS)	1 (0.01)	2 (0.5)	3 (8 d)	17.67 ± 1.70^{c}
9	1(1/2(NH_4NO_3) MS)	2 (0.05)	2 (0.5)	2 (4 d)	34.67 ± 3.30^{a}

Influencing factors A, B, C, and D were basic medium, NAA, 6-BA, and dark culture time, respectively. The numbers in front of the brackets represent the numbers of different levels under the factor. Different lowercase letters indicate significant differences at the 0.05 level based on LSD.

The analysis of the effects of dark culture treatments on leaf adventitious bud regeneration (Table S2) revealed that 1 (0 d) of dark culture significantly differed in the number of adventitious buds compared to 2 (4 d) and 3 (8 d). However, the difference between days 2 (4 d) and 3 (8 d) was not significant. The number of induced adventitious buds was highest after 8 d of dark culturing (23 buds). Subsequently, the dark treatment time was set to 8 d.

The analysis of 6-BA addition concentration on leaf adventitious bud regeneration (Table S2) revealed a highly significant difference in the number of induced adventitious buds in treatment 3 (1.0) compared to treatments 1 (0.1) and 2 (0.5). However, the difference between treatments 1 (0.1) and 2 (0.5) was not statistically significant. Treatment 3 (1.0) had the lowest number of induced adventitious buds. Therefore, the subsequent addition of 6-BA concentration was 0.5 mg/L.

The optimal medium combination for inducing adventitious buds was $A_1B_2C_2D_3$, which corresponded to 1/2(NH_4NO_3) MS + 0.05 mg/L NAA + 0.5 mg/L 6-BA. Under these conditions, 35 adventitious buds per leaf were induced after 8 d of dark culture (Table 2 and Figure 3).

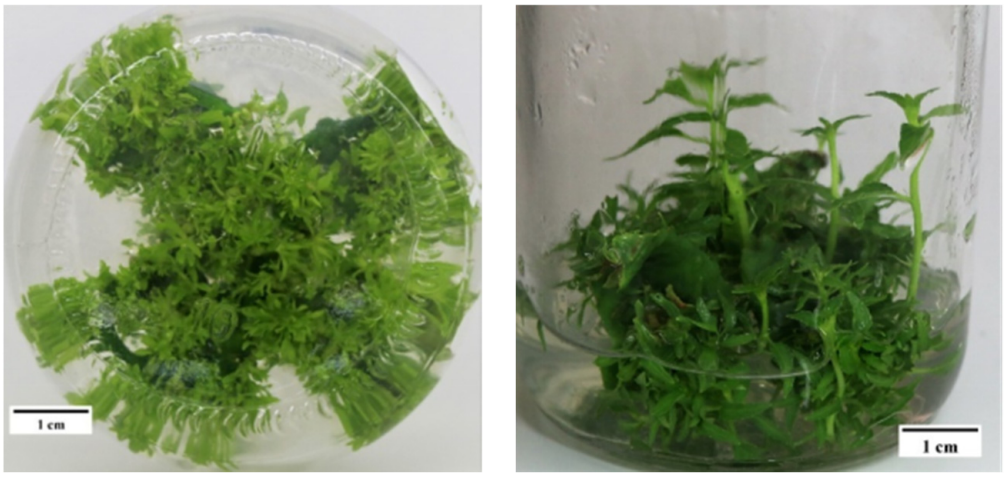

Figure 3. Adventitious bud induction. (**A**,**B**) were the best induction medium for leaf differentiation of adventitious buds and the optimal induction medium for 35 d elongation of adventitious buds, respectively.

3.2. Screening of Critical Antibiotic Concentrations

In plant genetic transformation systems, two types of antibiotics are commonly used: bacteriostatic agents (which inhibit *Agrobacterium* growth) and screeners (which identify

transformed agents). The selection of antibiotics and their critical concentrations are crucial for establishing a genetic transformation system. In this experiment, the vector used was PEZR-(K)-LC, with Kan as the selective antibiotic marker. Timentin was selected as the bacteriostatic agent for genetic transformation mediated by *Agrobacterium tumefaciens*.

3.2.1. Determination of Critical Screening Pressure for Timentin

The inhibitory effect of timentin on *Agrobacterium tumefaciens* GV3101 increased significantly with higher timentin concentrations (Figure 4). No bacterial spots were observed in the culture medium at concentrations $\geq$ 200 mg/L. Consequently, the timentin concentration for the subsequent genetic transformation test was set at $\geq$200 mg/L.

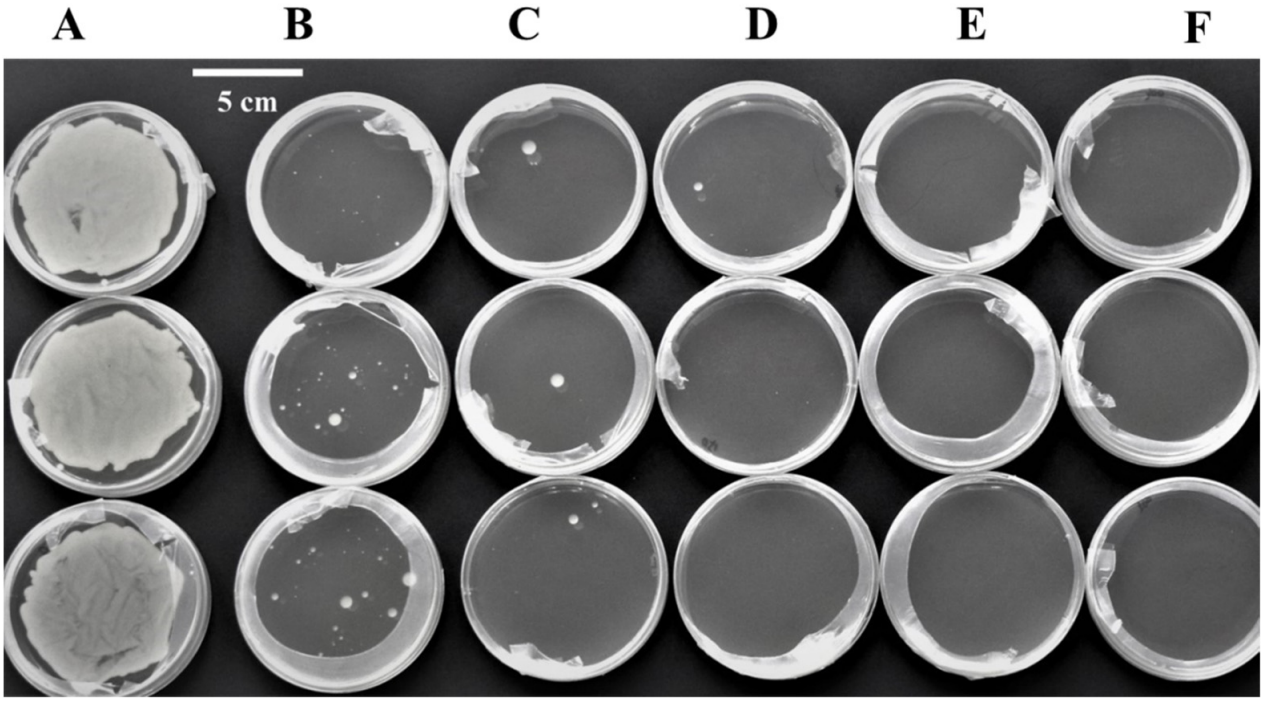

Figure 4. Effects of timentin concentrations on *Agrobacterium* Growth. (**A**–**F**) were 0, 50, 100, 150, 200, and 250 mg/L, respectively.

To study the effect of timentin on the induction of adventitious buds in leaves, various antibiotic concentrations were added to the optimal medium. The number of differentiated buds in different parts of the leaves (base, middle, and tip) initially increased and then decreased with increasing antibiotic concentrations until no more adventitious buds were induced (Figure 5).

When using the middle of the leaves as explants, the number of clustered buds was 32.3 at 0 mg/L bacteriostatic agent concentration, and it reached a maximum of 50.3 at 300 mg/L. However, at bacteriostatic agent concentrations of $\geq$450 mg/L, the number of differentiated adventitious buds significantly decreased (Figure 5i and Table 3). Therefore, the optimal timentin concentration for this purpose was 300 mg/L.

When using the leaf base as explants, the number of induced adventitious buds was 14.7 at 0 mg/L timentin concentration and peaked at 44 at 400 mg/L, the highest observed level. However, the number of differentiated adventitious buds significantly decreased at bacteriostatic agent concentrations $\geq$ 700 mg/L (Figure 5ii and Table 3). Therefore, the optimal timentin concentration for leaf-base explants was determined to be 400 mg/L.

When using the leaf tip as the explant, the number of induced adventitious buds was 16.0 at 0 mg/L and peaked at 24.3 at 300 mg/L, the highest timentin concentration. However, the number of differentiated adventitious buds significantly decreased when the

bacteriostatic agent concentration was ≥500mg/L (Figure 5ii and Table 3). Therefore, the optimal timentin concentration for leaf tip explants was determined to be 300 mg/L.

The analysis of timentin's effect on adventitious bud rooting revealed that although the rooting rate remained at 100% with increasing bacteriostatic agent concentrations, the growth of rooted seedlings was affected. Growth slowed notably at timentin concentrations ≥ 400 mg/L (Figure 6). Consequently, the timentin concentration for the subsequent induction of root formation in leaf adventitious buds was set at 300 mg/L.

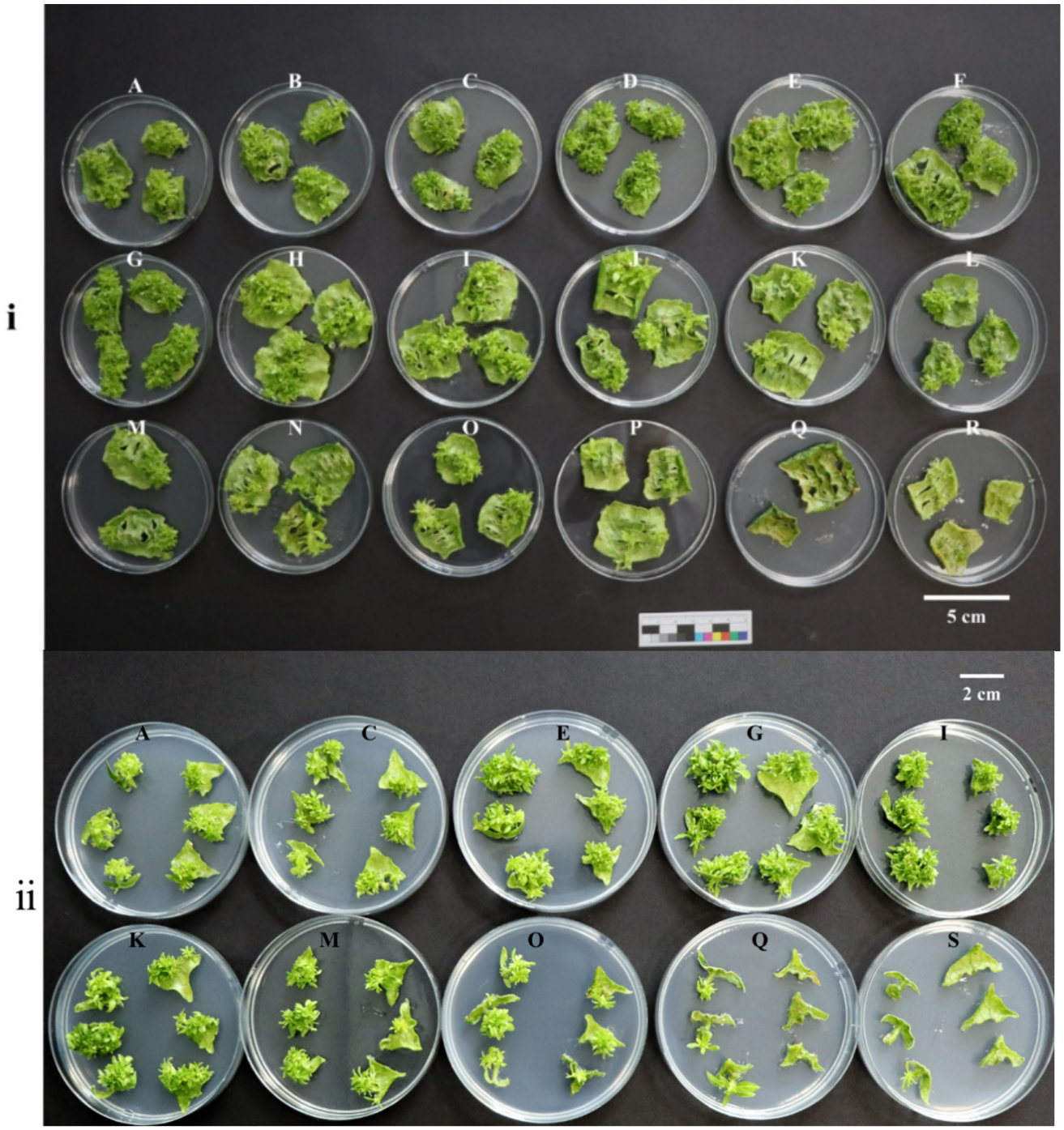

Figure 5. Effects of timentin concentrations on the induction of adventitious buds. (**i**,**ii**) indicate adventitious bud results in the tip part of the leaf and the middle/base part of the leaf differentiation after the addition of timentin, respectively. (A–S) timentin with concentrations of 0, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, and 850 mg/L was added to the medium.

Table 3. Statistics of adventitious buds under timentin treatment.

Code	Concentration mg/L	Middle of the Leaf	Top of the Leaf	Bottom of the Leaf
A	0	32.30 ± 1.15 ^{cd}	16.00 ± 0.66 ^{bc}	14.70 ± 0.18 ^{e}
B	50	37.70 ± 0.35 ^{bc}	/	/
C	100	39.30 ± 0.98 ^{bc}	17.70 ± 0.98 ^{bc}	15.30 ± 1.12 ^{e}
D	150	41.30 ± 1.52 ^{ab}	/	/
E	200	42.30 ± 1.34 ^{ab}	20.001.25 ^{ab}	24.00 ± 1.75 ^{cd}
F	250	49.30 ± 0.33 ^{a}	/	/
G	300	50.30 ± 1.71 ^{a}	24.30 ± 1.26 ^{a}	35.70 ± 1.27 ^{b}
H	350	40.00 ± 1.11 ^{bc}	/	/
I	400	34.00 ± 0.68 ^{bc}	20.70 ± 1.31 ^{ab}	44.00 ± 2.05 ^{a}
J	450	27.30 ± 1.28 ^{d}	/	/
K	500	16.00 ± 0.53 ^{e}	13.00 ± 1.09 ^{cd}	26.00 ±1.02 ^{c}
L	550	14.70 ± 1.14 ^{e}	/	/
M	600	11.70 ± 0.39 ^{ef}	7.70 ± 0.88 ^{d}	17.00 ± 0.72 ^{de}
N	650	8.00 ± 0.27 ^{ef}	/	/
O	700	7.70 ± 1.02 ^{ef}	5.70 ± 0.42 ^{e}	12.70 ± 0.93 ^{e}
P	750	7.00 ± 0.83 ^{ef}	/	/
Q	800	2.70 ± 0.57 ^{f}	0.00 ± 0.00	3.70 ± 0.14 ^{f}
R	850	1.70 ± 0.24 ^{f}	/	/
S	900	/	0.00 ± 0.00	1.70 ± 0.70 ^{f}

The data represent the mean ± standard deviation of three replicates. Different lowercase letters indicate significant differences at the 0.05 level based on LSD.

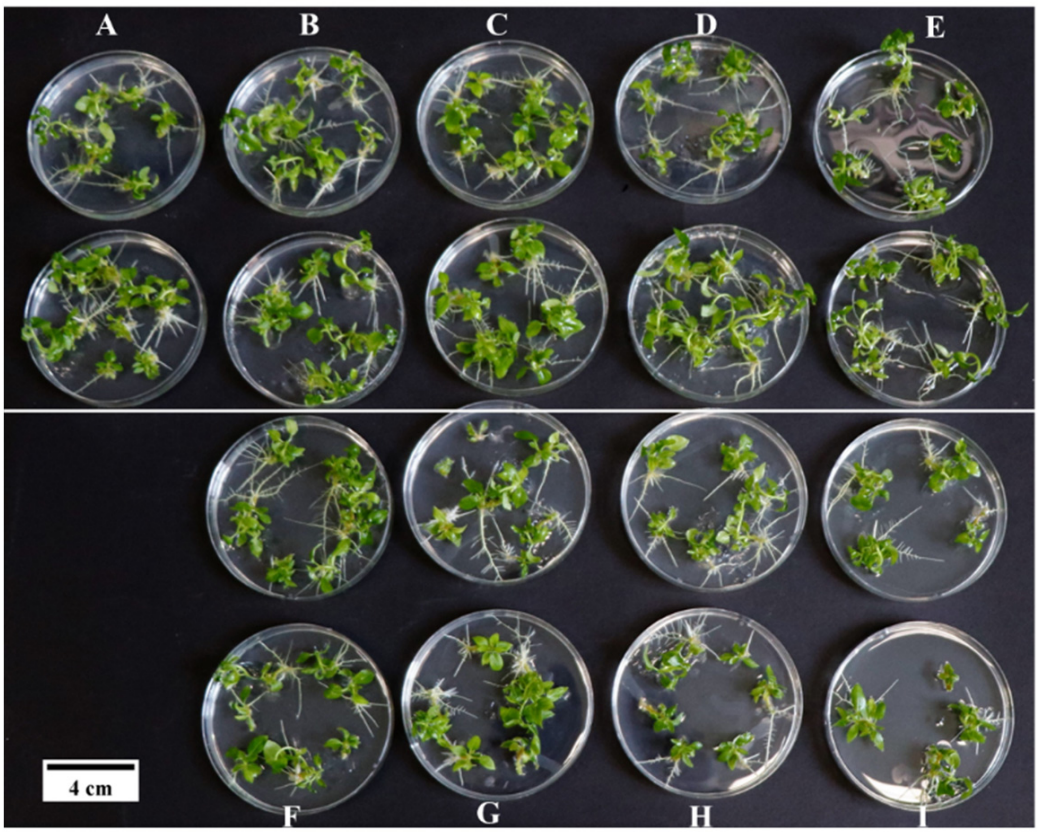

Figure 6. Effects of timentin concentrations on the rooting of adventitious buds. (A–I) timentin with concentrations of 0, 100, 200, 300, 400, 500, 600, 700, and 800 mg/L was added to the medium. Two replicates were used for each gradient in the figure.

3.2.2. Determination of Kan Critical Screening Pressure

To determine the critical pressure of *P. wulianensis* on Kan, the tests were conducted on Kan concentrations to induce adventitious buds and rooting in leaves (base, middle, and tip). The results revealed that Kan significantly inhibited adventitious bud induction in *P. wulianensis* leaves (Figure 7 and Table 4), with an inhibitory effect increasing at higher concentrations.

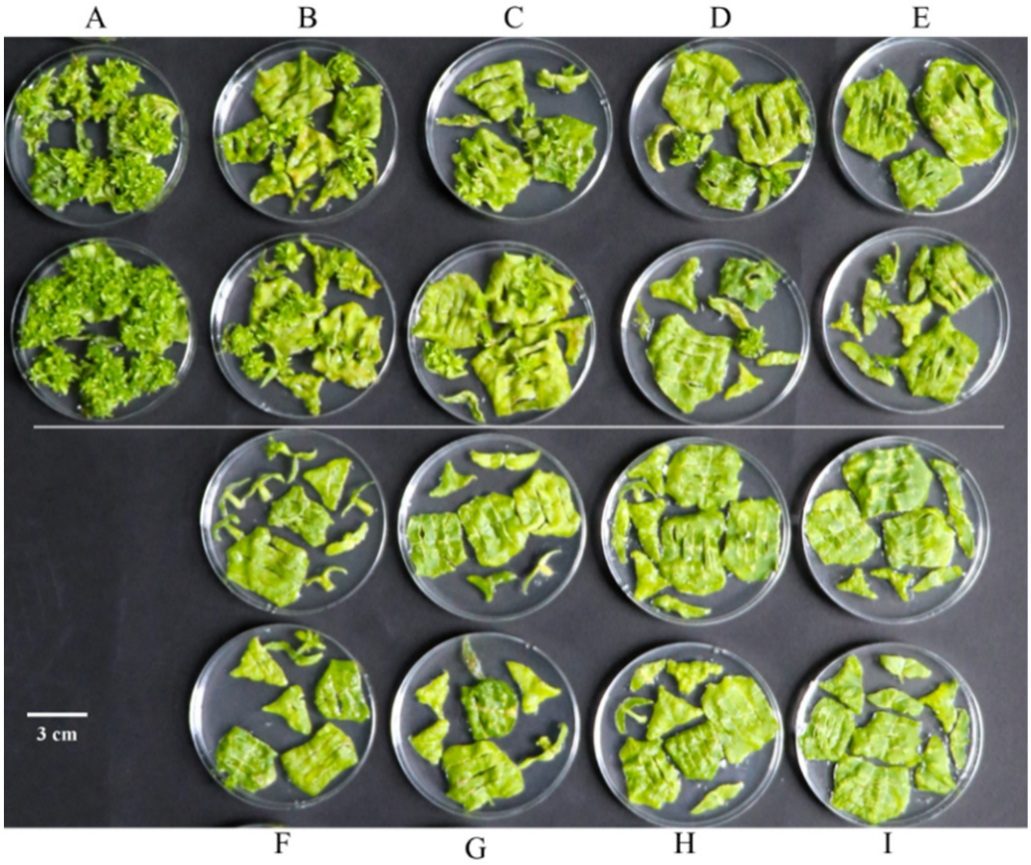

Figure 7. Effects of Kan concentrations on adventitious budding induction in the leaves. (A–I) Kan with concentrations of 0, 25, 50, 75, 100, 125, 150, 175, and 200 mg/L was added to the medium. Two replicates were used for each gradient in the figure.

When the leaf base was used as the explant, few adventitious buds formed at a Kan concentration of 125 mg/L, and the base only enlarged at 150 mg/L without differentiated buds. When the middle of the leaf was used as the explant, a small number of adventitious buds appeared at a Kan concentration of 75 mg/L, whereas at 100 mg/L, the leaf blades yellowed without differentiated buds. Using the leaf tip as the explant, 25 mg/L Kan produced a few adventitious buds, and 50 mg/L led to yellowing of leaves without buds. These results indicated that the critical Kan concentrations were 150 mg/L for the leaf base, 100 mg/L for the leaf middle, and 50 mg/L for the leaf tip as genetic transformation materials.

Kan also inhibited the adventitious rooting of *P. wulianensis*, with the inhibitory effect increasing with concentration (Figure 8). In the control group without Kan, the rooting rate was 100%, and the plants were healthy and green. At a Kan concentration of 25 mg/L, the rooting rate decreased to half that of the control group, and seedling growth was slightly slower than that of the control. At 75 mg/L, the rooting rate was 23.08%, with curled and vitrified leaves. At a concentration of 100 mg/L, the antibiotic completely inhibited adventitious bud rooting, resulting in dwarfed plants. At concentrations exceeding 100 mg/L, the adventitious buds ceased growth and extension, whereas the leaves exhibited yellowing,

drying, and death. The optimal Kan concentration for rooting *P. wulianensis* adventitious buds was 100 mg/L (Table 4).

Table 4. Adventitious bud differentiation and adventitious bud rooting of leaves under different concentrations of Kan.

Kan Concentration(mg/L)	Indeterminate Number of Buds	Leaf-Induced Growth Status	Rooting Rate (%)	Adventitious Buds Induce Rooting
0	33.30 ± 1.28 [a]	All three parts of the leaf blade were dark green and had the highest number of differentiated buds.	100.00	All rooted, green plants, healthy growth.
25	15.70 ± 1.09 [b]	All three parts of the leaf blade were green, and 50% of the B part of the leaf produced adventitious buds.	50.00	Half rooted, and the other leaves were light green.
50	5.00 ± 0.27 [c]	Leaf A was slightly yellow; the rest was green, and 30% of leaf B produced adventitious buds.	42.67	Some were rooted, whereas others were yellowed.
75	3.70 ± 0.33 [c]	Only the C part of the leaf produced adventitious buds, whereas the other parts of the leaf did not produce adventitious buds.	23.08	A small part of the leaves took root, and a small part of the leaves appeared vitrified.
100	2.00 ± 0.18 [c]	Only a small part of the leaf C part produced adventitious buds, and the leaf B part appeared yellow.	0.00	The base was swollen; none of them took root, and the adventitious buds grew slowly.
125	1.70 ± 0.15 [c]	Very few leaves had adventitious bud differentiation in the leaf C part, and no adventitious buds were produced in the others.	0.00	A few had swollen bases; none of them took root, and adventitious buds had stagnated growth.
150	0.00 ± 0.00	Dehydration began to appear in all three parts of the leaves, and there was no redifferentiation or adventitious buds.	0.00	The base was not enlarged and did not take root, and the adventitious bud leaves were partially yellowed.
175	0.00 ± 0.00	The three parts of the leaf showed different degrees of whitening, with no redifferentiation or adventitious buds.	0.00	The base was not swollen and did not take root, and the adventitious bud leaves were curled and yellowed.
200	0.00 ± 0.00	The three parts of the leaves were dehydrated and whitish, and there were no adventitious buds.	0.00	The base was not swollen and did not take root, and the leaves of adventitious buds were partially dry.

The data represent the mean ± standard deviation of three replicates. Different lowercase letters indicate significant differences at the 0.05 level based on LSD.

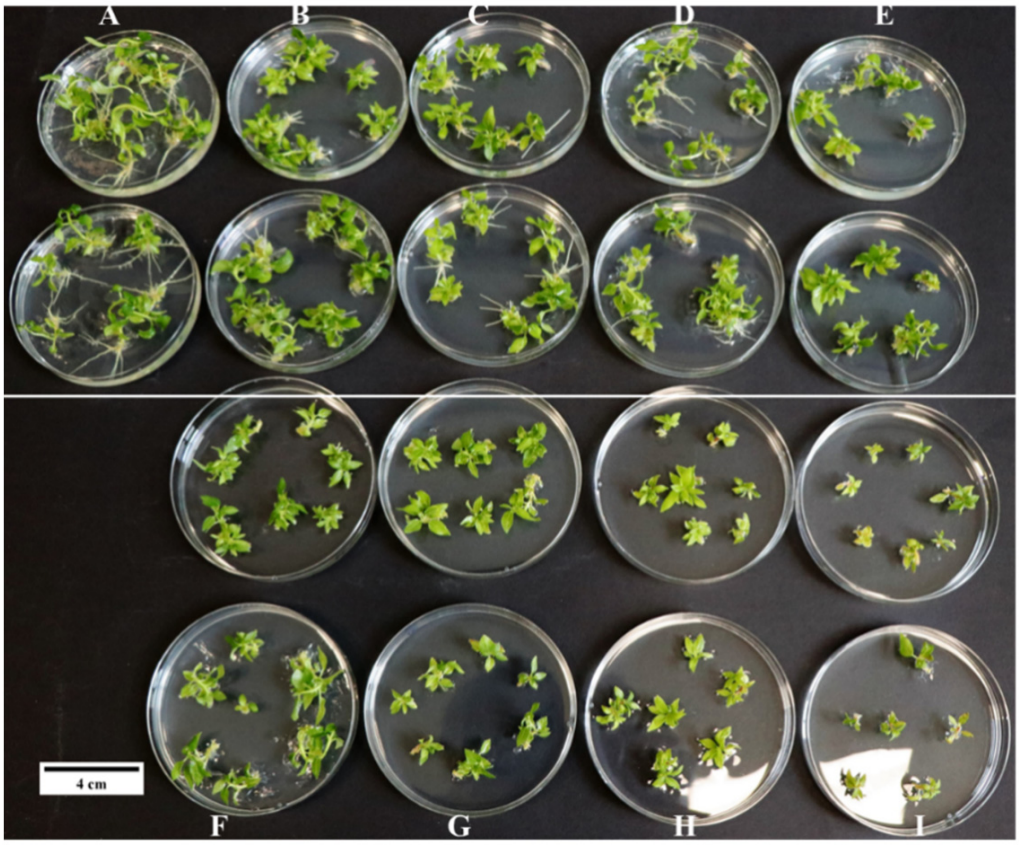

Figure 8. Effects of Kan concentrations on adventitious budding induction from rooting. (A–I) Kan with concentrations of 0, 25, 50, 75, 100, 125, 150, 175, and 200 mg/L was added to the medium. Each gradient was repeated twice.

3.3. Optimization of a Genetic Transformation System for P. wulianensis

The effect of *Agrobacterium* on the transformation efficiency of *P. wulianensis* was also studied. Using a high concentration (OD_{600} = 0.6) infected an average of one adventitious bud per leaf disc, with the discs fading green and becoming necrotic (Figure 9). The inhibitory efficiency of timentin against *Agrobacterium* was low, along with the substantial *Agrobacterium* propagation. When the concentration of *Agrobacterium* was OD_{600} = 0.3, an average of five adventitious buds were induced in each leaf disc. The regenerated buds exhibited good growth and produced a lower and controllable amount of *Agrobacterium*. However, at an *Agrobacterium* concentration of OD_{600} = 0.15, it was challenging to induce resistant buds in the leaf discs, which eventually dried and died (Table 5). Consequently, the optimal *Agrobacterium* concentration for infection was determined to be OD_{600} = 0.3.

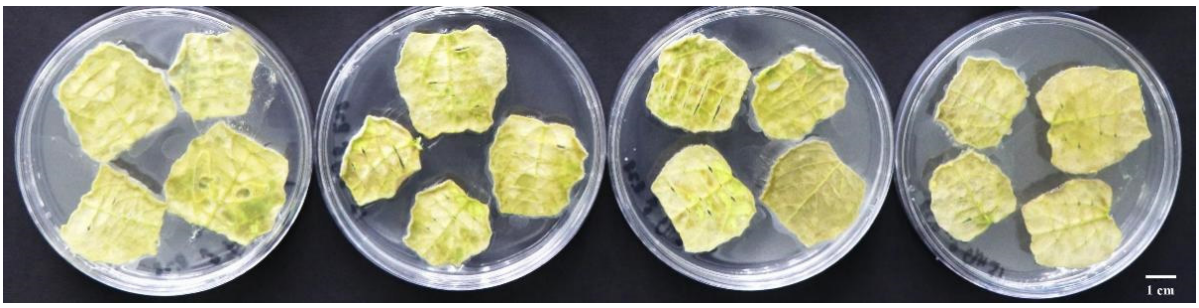

Figure 9. Leaf discs after infection with a high concentration of *Agrobacterium* (OD_{600} = 0.6) and culturing for 10 d.

Table 5. Effect of bacterial concentration on screening resistant buds of *P. wulianensis*.

OD_{600}	Number of Resistant Buds	Growth State
0.60	1.44 ± 0.51^{b}	The leaves gradually turned pale green and eventually withered, often displaying white plaques.
0.30	5.11 ± 0.92^{a}	A few leaf edges showed signs of fading with faintly visible colonies.
0.15	3.89 ± 0.87^{ab}	Several leaves exhibited fading with faintly visible colonies.

The results are expressed as mean ± standard error of three independent experiments. Different letters within each variable indicate statistically significant differences at $p < 0.05$. Same below.

The duration of *Agrobacterium* infection significantly affected *P. wulianensis* transformation (Table 6). A 15-min infection resulted in the highest differentiation efficiency, with an average of six resistant adventitious buds per leaf disc. Shorter infection times led to fewer regeneration buds, whereas longer times dried up leaf discs, reducing regeneration bud numbers. Hence, a 15-min infection was deemed optimal in this study.

Table 6. Effect of infection time on transformation of *P. wulianensis* resistant buds.

Infection Time/min	Number of Resistant Buds	Growth State
5	3.36 ± 0.45^{b}	Leaf disks remained green, with no colonies visible.
15	6.38 ± 1.28^{a}	Leaf blade margins displayed reddish brown, whereas wounds exhibited multiple buds and pale mycorrhizae.
20	2.92 ± 0.31^{b}	Leaf disks appeared dry with visible marginal mycorrhiza.

The results are presented as the means and standard errors from three independent experiments. Within each variable, values with different letters indicate statistically significant differences at the $p < 0.05$ level.

The addition of β-AS to both the infection solution and the co-culture solid medium significantly influenced the transformation rate of *P. wulianensis* leaves (Table 7). The number of resistant buds increased with increasing β-AS concentrations. However, at 200 μmol/L, regenerated resistant buds exhibited brown spots and poor growth. At 100 μmol/L, leaf discs remained green, and regenerated buds grew healthily and abundantly, making this the final selected concentration.

Table 7. Effect of β-AS on screening of resistant buds.

β-AS Concentration /μmol·L^{-1}	Number Of Resistant Buds	Growth State
0	2.94 ± 0.73^{b}	Dry spots appeared with yellowing of the leaf margins.
100	5.95 ± 1.12^{a}	Leaf disks remained green, with many buds forming around the wounds.
200	6.45 ± 1.34^{a}	Brown spots developed, with more buds forming around the wound.

The results are presented as the means and standard errors from three independent experiments. Within each variable, values with different letters indicate statistically significant differences at the $p < 0.05$ level.

In the study on the effect of co-culture (dark culture) on *P. wulianensis* transformation efficiency, we observed that after 1 d of dark culture, leaf discs did not produce adventitious buds, gradually withered, and died later (Figure 10). The highest transformation rate occurred after 3 d of co-culture, with an average of 10 resistant adventitious buds per leaf disc. Prolonged co-cultivation beyond three days led to flourishing *Agrobacterium* populations, causing leaf disc deterioration and a significant decrease in resistant adventitious bud production (Table 8). The optimal co-culture time was determined to be 3 d.

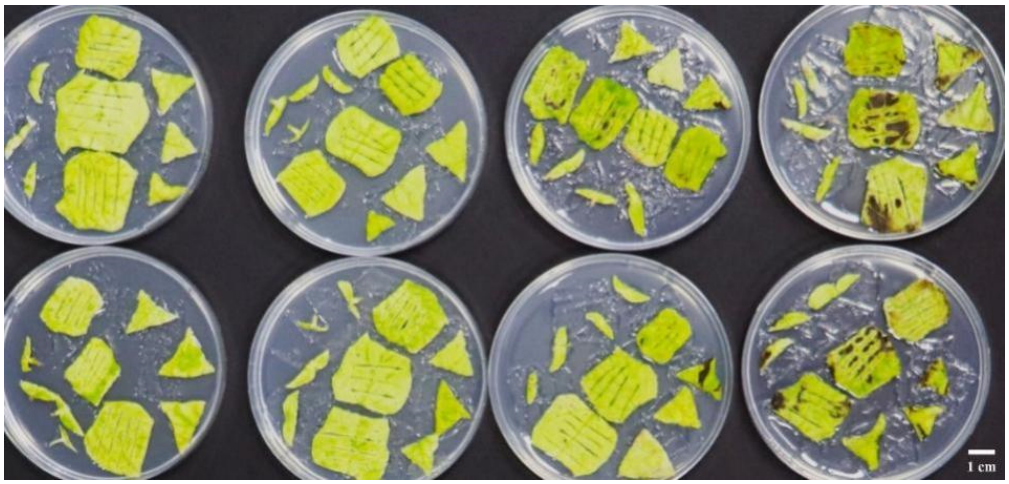

Figure 10. Leaf discs were cultured for 1 d, and adventitious buds were differentiated.

Table 8. Effect of dark culture (co-culture) time on transformation.

Co-Culture Time/d	Number of Resistant Buds	Growth State
1	0.00 ± 0.00^{c}	Leaf disks appeared greenish but dry without any visible adventitious buds.
3	6.11 ± 1.43^{a}	Yellowing was observed along the margins of the leaf disks, with green adventitious buds but no visible mycorrhizae.
6	2.95 ± 0.22^{b}	Late leaf discs appeared dry and exhibited white fungal spots.

The results are presented as the means and standard errors from three independent experiments. Within each variable, values with different letters indicate statistically significant differences at the $p < 0.05$ level.

3.4. Verification of a Genetic Transformation System for P. wulianensis

A genetic transformation system for *P. wulianensis* was established as follows: leaves were infected with MS liquid medium resuspended to an OD_{600} of 0.3 for 15 min and co-cultured on differentiation medium for 3 d, followed by transfer to adventitious bud screening medium containing Kan 100 mg/L and timentin 300 mg/L. The medium was changed every 3 d for the first week and every 7 d from the second week. After 35 d of culture, the resistant adventitious buds induced from the leaves were transferred to the resistant rooting medium containing Kan 100 mg/L and timentin 300 mg/L. After 30 d, resistant rooted seedlings were selected for refinement and transplantation, and DNA and RNA levels were examined after an additional 20 d of growth.

To validate the transformation system of *P. wulianensis*, we used a PEZR-(K)-LC plasmid carrying the *EGFP* gene. Following the established genetic transformation system, we obtained 112 Kan-resistant adventitious buds that grew on the rooting medium, resulting in 17 resistant rooted seedlings (Figure 11). After transplantation (Figure 12A), the genomic DNA was extracted from the plants for PCR detection. All eight rooted seedlings showed amplified bands of the expected sizes (729 bp), indicating successful integration of the *EGFP* gene into the *P. wulianensis* genome. The proportion of positive plants was 47.06% of the total number of root seedlings (Figure 12B). Semi-quantitative PCR confirmed the presence of target bands in all eight transgenic-positive plants, with no target bands found in WT plants (Figure 13). In order to detect whether EGFP protein was expressed, we tore off the epidermis of the leaves of overexpressed *EGFP* gene and WT strains for fluorescence microscopy. The results in Figure 14 show that fluorescent signals were found at the location of the nuclei of the DAPI-stained cells in the leaves of plants overexpressing the *EGFP* gene, which were not found in the WT strain. All of the above results suggested the stable expression of the exogenous *EGFP* gene in the *P. wulianensis* genome.

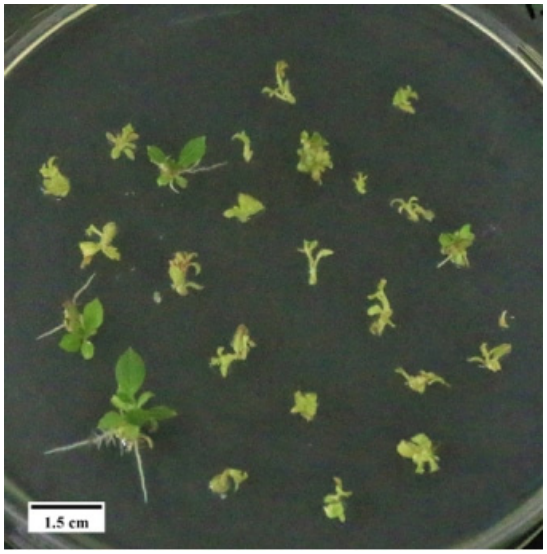

Figure 11. Rooted seedlings for Kan resistance screening.



Figure 12. Transgenic *P. wulianensis*. (**A**,**B**) represent the electropherograms of seedlings transplanted, resistant rooting seedlings, and DNA molecular identification, respectively. M, Marker. PC, Positive plants. WT, Wild type. H_2O, negative control. Numbers 1–17, transgenic lines L1–L17.

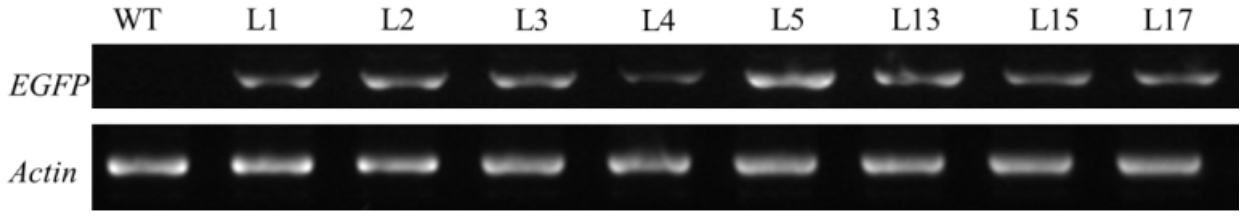

Figure 13. Semi-quantitative PCR detection of *EGFP* in transgenic *P. wulianensis*. L1–5, L13, L15, and L17 were identified as positive clonal clones at the DNA level. WT, wild type.

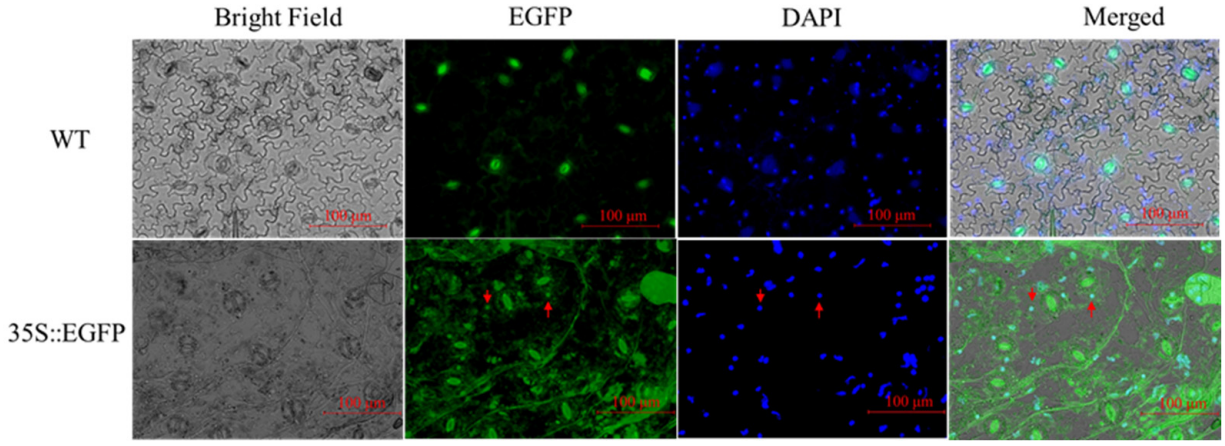

Figure 14. Monitoring of EGFP expression. The brightfield, EGFP fluorescence, DAPI fluorescence, and the combined field (merged EGFP fluorescence, DAPI fluorescence, and brightfield) were present for the fusion protein (EGFP) (**up**) and control (WT) (**down**), respectively. Arrows in the same direction indicate the expression of EGFP in the nucleus.

4. Discussion

4.1. Comparison of Adventitious Bud Regeneration System of Poplar

The organogenesis pathway has an impact on genetic transformation. The existing poplar transformation systems generally use two main methods for bud induction. One method is indirect induction: induce cell division and callus growth on the cut surfaces of the explants, followed by bud induction from the callus [15]. Yevtushenko et al. [12] inoculated infested explants into a healing-induction medium supplemented with 6-BA and NAA for cultivation and then transferred them to the bud differentiation medium for screening of resistant adventitious buds after 2 to 3 weeks. This indirect induction way can effectively reduce the false positive rate of *P. nigra* NM6 transgene but also the transformation efficiency of poplar. For example, the use of *Agrobacterium tumefaciens* to infect the 40–50 d seedling-aged stem segments of *P. trichocarpa* and then screening the resistant indeterminate buds after the induction of callus, with a maximum transformation efficiency of only 5.7% [22]. In addition, callus induction is time-consuming and complex, and methods involving the callus stage may result in plants that differ from the maternal plant owing to somatic clonal variation [23]. Another approach is direct induction: induce buds directly from *Agrobacterium*-infected poplar-sliced explants [2,24]. For example, Li et al. [25] improved the formulation of shoot regeneration medium (SRM) and inoculated *Agrobacterium*-infected tissue culture seedlings explants into SRM medium, which could directly differentiate into buds without going through the redifferentiation stage of callus. This not only effectively reduced the transgenic cycle of *P. trichocarpa* to 2 months but also increased the genetic transformation efficiency to 26.7%. In addition, the integration of exogenous genes based on the direct/indirect organogenesis pathway resulted in a high level of gene chimerism. In order to reduce the problem of this transformation, Zhan et al. [26] also proposed a " root first and then bud " method, in which *Agrobacterium tumefaciens* was used to infect the leaf discs of *P. kitakamiensis*, and the rooting hormone (1.5 mg/L IBA) was used to induce the differentiation of the leaf discs to produce adventitious roots, and then cells were induced to differentiation adventitious buds to form complete resistant plants. The proportion of rooted adventitious buds obtained in this way was as high as 55%, and the positive rate was as high as 76.5%. In this study, direct induction through orthogonal tests revealed that the average number of adventitious buds induced by leaves exceeded 35 when using medium (1/2(NH_4NO_3) MS + 0.05 mg/L NAA + 0.5 mg/L 6-BA) and dark culture for 8 d (Table 2), and the tissue culture seedlings could be successfully sub-cultured. The average number of induced adventitious buds in the leaves of the poplar and *P. tremuloides* hybrids ranged from 3 to 20 (Table 9). For example, the average numbers

of induced adventitious buds in leaf differentiation of *P. tomentosa* [27], *P. tremuloides* [28], *P. leucopyramidalis* [29], and *P. tremula* × *P. tremuloides* [30] were 3.4, 10, 8.2, and 2.73, respectively. Therefore, this study system is one of the best systems among the poplar tree species currently available.

Table 9. Comparison of the number of adventitious buds induced by Sect. *Populus* leaves.

Populus Species/Hybrid	Explants	Adventitious Buds	Reference
P. tomentosa (clone GM107)	Leaf	20.00	Huang et al., 2023 [31]
P. alba × *P. glandulosa*	Leaf	20.00	Li et al., 2007 [32]
P. adenopoda × poplar84K	Leaf	14.80	Zhang et al., 2010 [33]
P. dabidiana × *P. bolleana*	Leaf	12.10	Wang et al., 2010 [34]
P. hopeiensis	Leaf	11.40	Wu et al., 2021 [35]
P. tremuloides	Leaf	10.00	Huang et al., 2011 [28]
P. Leucopyramidalis	Leaf	8.20	Li et al., 2008 [32]
P. tomentosa (male diploid)	Leaf	6.63	Yu et al., 2022 [13]
P. tremula × *P. alba*	Leaf	4.27	Wang et al., 2009 [30]
P. tomentosa	Leaf	3.40	Li et al., 2016 [27]
P. tremula × *P. tremuloides*	Leaf	2.73	Wang et al., 2009 [30]

4.2. Comparison of Poplar Genetic Transformation Systems

The efficiency of poplar genetic transformation is closely related to plant genotypes. Numerous studies have shown significant differences in the transformation rates between genotypes, even within the same plant species. Table 10 demonstrates the varying positive yields in genetic transformation using leaf discs from both *P. tremuloides* and *P. tremuloides* hybrids. Long et al. [36] achieved an optimized genetic transformation efficiency of 9.38% using *P. tomentosa* as the test material. In contrast, Hu et al. [37] found that *P. tomentosa* transformed more easily, with a transformation efficiency as high as 36.32%. Yu et al. [13] obtained 86 resistant plants through genetic transformation using male diploid poplar as the test material, 14 of which were identified as positive single plants through molecular analyses. In addition, when using *P. dabidiana* × *P. bolleana* leaves as exosomes for genetic transformation, different studies reported significant differences in transformation efficiencies: 9.3% [2]; 30.0% [4]; and 93.3% [38]. Ma et al. [15] sequenced the genome of *P. alba* var. *pyramidalis* during genomic research, achieving an average positive transformation rate of 23.6% using leaf disc indirectly induced genetic transformation. For the same poplar, *P. tremuloides* [39], the positive transformation rate through leaf-induced indirect pathways was as high as 94.17%, possibly because of varying degrees of sensitivity to *Agrobacterium* among different genotypes.

Table 10. Statistics of the genetic transformation of *Populus* tree species.

Populus Species/Hybrid	Positive Rate	Selective Agent and Its Concentration(mg/L)	Bacterial Inhibitor and Its Concentration (mg/L)	Reference
P. dabidiana × *P. bolleana*	9.30%	25–50, Kan	500, Cef	Wang et al., 2011 [2]
P. tomentosa	9.38%	20–50, Kan	150–250, Cef	Long et al., 2010 [36]
Male diploid *P. tomentosa*	16.28%	30, Kan	200 Cef + 200 timentin	Yu et al., 2022 [13]
P. alba var. *pyramidalis*	23.60%	90, Hygromycin	400, Cef	Ma et al., 2019 [15]
P. dabidiana × *P. bolleana*	30.00%	50, Kan	Unmarked	Han et al., 2013 [4]
P. tomentosa	36.32%	30–50, Kan	300, Cef	Hu et al., 2005 [37]
P. alba × *P. glandulosa* cv. 84K	51.54%	50–60, Kan	300, Cef	Zhang et al., 2005 [40]
P. dabidiana × *P. bolleana*	93.33%	30–60, Kan	Unmarked	Zang et al., 2008 [38]
P. canescens × *P. grandidentata*	93.33%	60–80, Kan	250 Cef + 500 Car	Dai et al., 2003 [41]
P. tremuloides (clone 271)	94.17%	40–100, Kan	300, Cef	Tsai et al., 1994 [39]

This study successfully integrated the *EGFP* gene into the genomic DNA of *P. wulianensis* using leaf discs, achieving stable expression with a positive transformation rate of 47.06%. Establishing a genetic transformation system for *P. wulianensis* not only enriched

the germplasm resources of *P. tremuloides* but also provided transgenic "tools" for molecular plant breeding of poplar and other woody plants. This system can also serve as a technical and theoretical basis for genetic engineering.

4.3. Analysis of Factors Affecting Conversion Efficiency

The genetic transformation plays a crucial role in plant molecular biology and functional genomic research. Since the establishment of the first successful poplar transformation system [6], poplar has been transformed for genetic improvement and gene function studies more frequently than any other woody species [42,43]. Several factors influence transformation efficiency and regeneration during poplar genetic transformation, such as explants material, bacterial type, growth stage, the concentration of added β-AS, concentration of the bacteriostatic agent, co-culture time, and duration of *Agrobacterium* infection. The concentration of the added inducer is an important influencing factor. *Rhizobium* infects only the damaged parts of the plant, and the cells release some inducers similar to phenols or acidic polysaccharides when the plant undergoes damage and repair. Therefore, the cellular damage signals from the environment are transmitted to *Agrobacterium* cells through outer membrane protein when necessary, which ultimately leads to the expression of the *vir* genes and T-DNA transfer [41]. The most effective inducer is AS, which can activate the *vir* gene on the *Agrobacterium* plasmid and improve the transformation rate [15]. Li et al. [25] used the leaf blade of hybrid poplar (*P. nigra* var. *betulifolia* × *P. trichocarpa*) as the material, they found that AS and pH were the main factors affecting the efficiency of the genetic transformation with the help of *GUS* reporter genes, and the concentration of AS should be 25–30 μmol/L, while too high concentration would reduce the genetic transformation efficiency. In addition, the addition of 100 μmol/L AS to the medium of infiltration-transformed *P. alba* var. *pyramidalis* promoted the effective knockdown and efficient overexpression of the target gene [15]. Additionally, the bacteriostatic agent is also an important influencing factor. Bacteriostatic agents effectively kill or inhibit *Agrobacterium* in transformed explants; they can also affect the growth, isolation, and differentiation of plant cells. Higher concentrations of bacteriostatic agents generally result in better bacteriostatic effects but also increase the side effects of the explants [44]. In a study on tea tissue culture seedlings, bacteriostatic agents induced clumping of seedlings. Yang et al. [45] observed that Cefquinome and carbenicillin significantly reduced the survival rate of tea explants, the clumped bud induction rate of single explants, and the proliferation rate of clumped buds, showing a concentration-dependent inhibitory effect. Similar effects were also noted in *Nicotiana tabacum* [46] and *Fraxinus profunda* [47].

In this study, timentin was used as a bacteriostatic agent in the genetic transformation of *P. wulianensis* leaf discs. The results showed that timentin at concentrations $\geq$ 200 mg/L not only effectively inhibited the *Agrobacterium* growth but also increased the number of induced adventitious buds in all parts of the leaf blade (leaf base, middle, and tip). This positive effect is similar to timentin's promotion of tufted bud growth rate and seedling formation rate in tea tissue-cultured seedling explants [45], its effectiveness in inducing secondary embryogenesis in *Chrysanthemum* [48], and promotion of adventitious bud differentiation in *N. tabacum* leaf discs (127% increase after one month of culture) [46]. This effect may be attributed to the chemical structure of timentin, which resembles growth hormones, such as 2,4-D and NAA, thereby exhibiting hormonal effects in tissue culture [49].

4.4. False Positive Exclusion Analysis

In plant genetic transformation, chimeric buds comprising transgenic and non-transgenic parts form with a certain probability [50,51]. PCR is commonly used to identify transgenic plants because it is a powerful and direct tool for detecting target genes inserted into the genome [2]. In this study, we successfully detected transgenic poplar plants using PCR with an optimized vector and gene cross-specific primers, confirming the successful insertion of the *EGFP* gene into the poplar genome in eight transgenic poplar lines (Figure 12A). Direct identification of resistant tissue culture leaves can yield "false-positive" results due

to *Agrobacterium* residues. Using *Agrobacterium*-specific primers, such as the *tzs* gene [24,52], or sequences other than plasmid T-DNA, such as the *aadA* gene [14], for contamination identification may inadvertently exclude positive plants. In this study, resistant seedlings underwent transplantation for refinement and were grown under natural light for approximately 1 month. This approach ensured that the gradual dilution of *Agrobacterium* did not interfere with the identification results. Additionally, the DNA and RNA levels were verified to ensure the accuracy of the experimental results. In addition, based on the efficient genetic transformation system of *P. wulianensis* obtained in this experiment, it has been successfully applied to the genetic transformation of *PwuWRKY48* gene [53].

5. Conclusions

In this study, we established a genetic transformation system using *P. wulianensis* as a genotype for the first time. First, we established the optimal medium (1/2(NH_4NO_3) MS + 0.05 mg/L NAA + 0.5 mg/L 6-BA) for efficient leaf redifferentiation of adventitious buds and also explored the effects of Kan and timentin on the induction of adventitious buds and adventitious bud-induced rooting of *P. wulianensis* leaves. Meanwhile, we determined and optimized a number of factors affecting the efficiency of genetic transformation, including the concentration of a bacterial solution (OD_{600} = 0.3), infection time (15 min), co-culture time (3 d), and β-AS (100 μmol/L), etc. Finally, we established a genetic transformation system of *P. wulianensis* with high efficiency and stable expression of exogenous genes and verified its transformation of exogenous *EGFP* gene, resulting in a high positive transformation yield of 47.06%. These findings can facilitate genetic improvements in breeding programs and provide a foundation for future gene research.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/f15081474/s1, Table S1: Variance analysis of effects of different factors on the regeneration number of *P. wulianensis* adventitious buds; Table S2: Duncan multiple comparisons of the effects of the three factors on the induction of adventitious buds in *P. wulianensis*.

Author Contributions: Conceptualization, J.X. and Y.L.; methodology, Y.H., R.W., L.G., Y.M., T.S., X.X. and Y.Z.; investigation, Y.W.; writing—original draft preparation, Y.W. and C.S.; writing—review and editing, J.X.; supervision, J.X. and Y.L.; funding acquisition, Y.W. and Y.L. All authors have read and agreed to the published version of the manuscript.

Funding: We gratefully acknowledge financial support for this research from the Youth Project of Shandong Provincial Natural Science Foundation (ZR2020QC165), the Subject of Key R & D Plan of Shandong Province (2021LZGC02304), and the Innovation Team for Conservation and Utilization of Precious Tree Species Germplasm project of the Department of Natural Resources of Shandong Province (LZYZZ202398).

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

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

Abbreviations

MS	Murashige and Skoog medium
NAA	Naphthalene acetic acid
6-BA	6-Benzylaminopurine
EGFP	Enhanced green fluorescent protein
DAPI	4′,6-Diamidino-2-phenylindole
PCR	Polymerase chain reaction
β-AS	Acetylsyringone
WT	Wild type

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