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Optimization of in vitro adventitious shoot regeneration and genetic transformation systems in blueberry (*Vaccinium corymbosum*) using a heterogeneous *Ruby1* gene

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Abstract

Blueberry is an economically important fruit crop, and demand for it is rising. Despite being essential for advancing precision breeding of blueberries, genetic transformation platforms remain limited for transgenic applications. In this study, we used the widely cultivated blueberry cultivar 'Legacy' (*Vaccinium corymbosum*) as the experimental material to establish an efficient adventitious shoot-regeneration system and an *Agrobacterium*-mediated transformation protocol. Adventitious bud development was promoted via a two-step protocol: induction on WPM medium with 2 mg/L TDZ and 0.5 mg/L NAA, followed by elongation on WPM medium with 3 mg/L ZT. Younger leaves from the shoot tips were selected as explants for the regeneration system. Wounding treatment, placing the abaxial (back) side in contact with the medium, and a 12-d dark treatment at the initial regeneration stage significantly improved the adventitious shoot regeneration rate. Building upon this regeneration system, an *Agrobacterium*-mediated genetic transformation protocol was developed using a vector carrying the reporter genes *enhanced green fluorescent protein (Egfp)* and *CgRuby1*, an anthocyanin-regulating transcription factor from purple pummelo (*Citrus grandis*). The optimal parameters included culturing *Agrobacterium*-mediated in liquid medium, preparing the *Agrobacterium* suspension with an OD₆₀₀ of 0.8, a 1-h vacuum infiltration, 6 d of co-cultivation, and selection on adventitious shoot regeneration medium containing 10 mg/L kanamycin, which enhanced the regeneration rates of resistant shoots. Polymerase chain reaction (PCR) analysis confirmed that transgenic plants carrying *CgRuby1* were achieved with a final transformation efficiency of 6.5%. Phenotypic analysis revealed that the transgenic plants accumulated significantly higher anthocyanin levels in their leaves than the wild-type plants. In addition, key blueberry anthocyanin biosynthetic genes, including *VcCHS*, *VcFHT*, *VcDFR*, *VcANS*, and *VcUGT*, were markedly upregulated in the transgenic lines. Taken together, we have successfully established an efficient transformation system that may hold great potential for functional characterization of genes involved in various processes.

Keywords Blueberry, Adventitious shoot regeneration, *Agrobacterium*-mediated transformation, Anthocyanin synthesis, *Ruby1*

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Introduction

Blueberry (*Vaccinium* spp.) is an economically important fruit crop worldwide, with demand for it rising. Commercial blueberry production relies heavily on conventional crossbreeding, which faces significant challenges owing to heterozygosity, polyploidy, and lengthy evaluation cycles. These constraints not only prolong breeding efforts, but also contribute to decreased genetic diversity

in cultivars, increasing their vulnerability to biotic and abiotic stresses (Brevis et al. 2008; Gupta et al. 2015). Genetic transformation has emerged as a promising tool for accelerating fruit tree breeding and enabling precise trait improvements (Hao et al. 2024). This approach can be implemented using methods mediated by *Agrobacterium tumefaciens* or *A. rhizogenes*, or vector-less transfer techniques (Hao et al. 2024). However, transgenic applications for blueberries are relatively underdeveloped. Therefore, the establishment of robust genetic transformation platforms is essential for advancing precision breeding strategies for high-value blueberries.

Efficient and stable adventitious shoot regeneration systems are fundamental for establishing robust genetic transformation systems (Shah et al. 2018). Explant selection significantly influences regeneration efficiency. Leaves from in vitro-grown shoots are commonly used, and leaf position affects regeneration potential (Debnath, 2009). In addition, the phytohormone composition of the culture medium plays a critical role in optimizing regeneration. A common approach involves supplementing woody plant medium (WPM) with zeatin (ZT) and indole-3-butyric acid (IBA) to induce callus formation in leaf explants (Qiu et al. 2018). Additionally, high concentrations of cytokinins, such as ZT and 2-isopentenyladenine (2-iP), promote shoot regeneration in cultivars including *V. corymbosum* ‘Aurora,’ ‘Brigitta,’ ‘Elliott,’ and ‘Bluecrop’ (Song and Sink, 2004). Debnath et al. (2009) demonstrated that thidiazuron (TDZ) alone could effectively induce regeneration in wild lowbush blueberry (*V. angustifolium*).

Agrobacterium infection and foreign gene introduction are the most critical, yet technically challenging, steps in *Agrobacterium*-mediated genetic transformation. Over the past two decades, significant efforts have been devoted to establishing and optimizing efficient blueberry genetic transformation protocols. Studies have suggested that diploid species (e.g. *V. myrtillus* and *V. reticulatum*) exhibit high *Agrobacterium* infectivity (Wang et al. 2022; Song et al. 2023). Nevertheless, the tetraploid highbush blueberry (*V. corymbosum*), the predominant commercially cultivated species, remains a major focus of transgenic research. Cao et al. (1998) evaluated transformation parameters affecting transient β -glucuronidase (*GUS*) expression in *V. corymbosum* leaf disc assays, but stable transgenic lines were not obtained. The first successful *Agrobacterium*-mediated genetic transformation of *V. corymbosum* was reported by Song and Sink (2004), who utilized a leaf-disc method with optimized parameters, which included the *Agrobacterium* strain, infection duration, co-cultivation period, and kanamycin selection stress. However, the transformation efficiency remained relatively low. Subsequent studies

explored strategies to enhance *Agrobacterium* infection in blueberry leaf explants, such as optimizing explant age (e.g. using younger leaves) and mechanical wounding (Liu 2018; Qin 2020; Qin et al. 2023). These investigations primarily assessed transient reporter gene expression or the number of antibiotic-resistant shoots; however, none succeeded in regenerating stable transgenic plants.

The Citrus *Ruby* locus encodes the R2R3 MYB transcription factor (Butelli et al. 2012, 2019; Huang et al. 2018), which is a component of the highly conserved core regulatory MYB-bHLH-WD40 (MBW) complex that controls anthocyanin biosynthesis in plants (Koes et al. 2005; Ramsay and Glover 2005; Xu et al. 2015). In pummelo (*Citrus grandis*), the full-length *Ruby1* (*MYB1*) gene confers the distinctive purple/red pigmentation observed in the fruits of a wild pummelo accession (designated as ‘Purple Pummelo’), in which anthocyanins accumulate abundantly (Butelli et al. 2017; Huang et al. 2018). Owing to their visually discernible color phenotype, Citrus *Ruby1* and its orthologs in other plant species are occasionally employed as reporter genes in transgenic studies.

Establishing an efficient, rapid, and stable transformation system for blueberries is currently challenging (Song and Hancock 2012; Song et al. 2012). In addition, genetic transformation systems often exhibit low reproducibility across different laboratories. Therefore, a stable genetic transformation platform for institutions committed to long-term fundamental blueberry research must be established. In this study, we used ‘Legacy’ (*V. corymbosum*), a highbush blueberry variety widely cultivated in southern China, as experimental material. By employing a transformation vector carrying the *enhanced green fluorescent protein* (*Egfp*) reporter gene and *CgRuby1* gene, we optimized both the adventitious shoot regeneration system and *Agrobacterium*-mediated transformation. This study lays the foundation for advancing biotechnological blueberry breeding.

Materials and methods

Plant materials and growth conditions

The highbush blueberry cultivar ‘Legacy’ (*V. corymbosum*) was used in this study. Healthy and pest-free new shoots from the spring were collected as primary explants from two-year-old trees in April and May. The trees were planted in a blueberry orchard in Chongzhou City, Chengdu, Sichuan Province, China. Moderately lignified stems were trimmed to 5–7 cm, soaked in carbendazim for 30 min, and rinsed for 2–3 h. Under sterile conditions, the trimmed stems were sterilized with 75% ethanol for 30 s, followed by 3% sodium hypochlorite for 10 min, and then washed 4–5 times with sterile water. The stems were cut into 1-cm segments, each containing one bud, and inoculated onto the primary culture

medium (WPM + ZT, 4 mg/L) base-down. The medium was supplemented with 30 g/L sucrose, and the pH was adjusted to 5.2 (the same procedure was followed for all subsequent media). For subculturing, 8–10-week-old in vitro-grown plantlets were used. The stems were cut into 1-cm segments and inoculated onto the subculture medium (WPM + ZT, 1 mg/L). After 8–10 weeks at 25°C, the plantlets were ready for further regeneration experiments.

Optimization of culture medium and culture conditions for adventitious shoot regeneration

The leaf-regeneration system was established using sterile leaves collected from 8–10-week-old ‘Legacy’ plantlets maintained on subculture medium. The hormone combinations in adventitious shoot regeneration medium were optimized, in which ZT (2, 3, or 4 mg/L) and 6-benzylaminopurine (6-BA; 1, 2, or 3 mg/L), combined with naphthaleneacetic acid (NAA) 0.5 mg/L, and TDZ (1, 2, or 3 mg/L) combined with NAA 0.5 mg/L were compared. The median codes are listed in Table 1. Furthermore, the following conditions were tested: (1) leaf explant position (top, middle, or basal leaves of in vitro plantlets), (2) leaf-cutting methods (slight wounding/Method 1, moderate wounding/Method 2, and excessive wounding/Method 3), (3) leaf explant orientation (adaxial side or abaxial side facing upward), and (4) dark incubation duration (8, 10, or 12 d). Following dark incubation, explants were transferred to the illuminated greenhouse with a 16/8-h (light/dark) photoperiod at 25°C. Adventitious shoot regeneration rates were quantified after 30 d

as (regenerating explants/total explants) × 100%. Three biological replicates were performed for each treatment, with each replicate containing thirty explants.

Screening of kanamycin concentration

Prior to infection experiments, the optimal kanamycin screening concentration for adventitious shoot regeneration medium was determined using non-infected explants. Gradient concentrations of kanamycin (0, 7.5, 10, and 12.5 mg/L) were added to adventitious shoot regeneration medium for screening. Three biological replicates were performed for each treatment, with each replicate containing thirty explants.

Optimization of conditions for Agrobacterium-mediated transformation

A pK2GW7 vector containing the *CgRuby1* kindly provided by Prof. Xu (Huang et al.), *Egfp* (Bierhuizen et al. 1997), *spectinomycin resistance (SpR)*, and *kanamycin resistance (Km)* genes was used. These four genes are connected to independent promoters and terminators. The *A. tumefaciens* strain was prepared as previously described (Song and Sink 2004). Blueberry leaves were prepared according to the regeneration system optimized in the early stages of the experiment. We systematically evaluated five critical transformation parameters: (1) *Agrobacterium* culturing on solid or liquid Luria–Bertani (LB) medium, (2) *Agrobacterium* infection duration for 1, 2, or 3 h, (3) *Agrobacterium* infection suspension density at OD₆₀₀ of 0.7, 0.8, 0.9, 1.0, or 1.1, (4) various vacuum infiltration (–80 kPa) duration, and (5) co-cultivation for 4, 6,

Table 1 Effect of hormone combinations on adventitious shoots regeneration

Medium	Hormone combinations	Regeneration rate (%)	Growth status
Z2	ZT 2 mg/L	14.8 ± 1.44 h	Some leaves with yellowish healing at the edge, a few regenerating adventitious buds, average growth and reddish color
Z3	ZT 3 mg/L	22.2 ± 1.33f	Some leaf blade edges with yellowish healing, indeterminate buds crowded, good growth, a few reddish
Z4	ZT 4 mg/L	33.6 ± 1.67d	Part of the leaf blade with yellowish healing at the edge, dense indeterminate buds, but average growth, a few reddish
B1N	6-BA 1 mg/L + NAA 0.5 mg/L	19.5 ± 1.11 g	Green healing wounds on the whole leaf blade, a few regenerating adventitious shoots, tender green, average growth
B2N	6-BA 2 mg/L + NAA 0.5 mg/L	44.1 ± 1.16c	Green healing wounds on the entire leaf blade, indeterminate buds crowded but of average growth, a few reddish, a few regenerating adventitious roots
B3N	6-BA 3 mg/L + NAA 0.5 mg/L	31.2 ± 1.11e	Dark green healing throughout, indeterminate buds crowded, but poor growth, tender green
T1N	TDZ 1 mg/L + NAA 0.5 mg/L	58.9 ± 1.83b	Entire leaf blade with green healing, indeterminate buds crowded, but poor growth, young green
T2N	TDZ 2 mg/L + NAA 0.5 mg/L	72.1 ± 1.88a	Green healing wounds on the whole leaf blade, indeterminate buds crowded, but poor growth, young green
T3N	TDZ 3 mg/L + NAA 0.5 mg/L	–	All leaves browned

ZT zeatins, 6-BA 6-benzylaminopurine, TDZ thidiazuron, Z2–Z4 zeatin (ZT) 2–4 mg/L, B1N–B3N 6-BA 1–3 mg/L + naphthaleneacetic acid (NAA) 0.5 mg/L, T1N–T3N TDZ 1–3 mg/L + NAA 0.5 mg/L

Different letters next to the values denote statistically significant differences (p < 0.05)

or 8 d. To compare the differences between treatments, the resistant shoot regeneration rate was quantified after approximately 40 d on selection medium (adventitious shoot regeneration medium supplemented with kanamycin) as the percentage of explants producing resistant shoots (regenerating explants/total explants $\times$ 100%), with three biological replicates of 30 explants per treatment. Resistant shoots were transferred to rooting medium (1/2 WPM + IBA 1 mg/L) to induce roots.

Green fluorescence observation

At the third week post-infection, the green fluorescence of the EGFP protein was observed. Culture dishes containing *Agrobacterium*-infected blueberry leaf discs were observed under a stereo fluorescence microscope (M205FA, Leica Microsystems, Wetzlar, Germany). Green fluorescence was observed under the EGFP light mode, with uninfected blueberry leaf discs from the same period serving as the control.

Polymerase chain reaction (PCR)

When the resistant shoots reached an appropriate height of >2 cm before rooting, 2–3 leaves from each independent transgenic event were cut and placed in labeled grinding tubes. The wild-type (WT) was an uninfected control group collected during the same period. DNA was extracted from leaves using a Modified CTAB Plant DNA Kit (Aidlab Biotech, Beijing, China). PCR was performed to detect the *CgRuby1* gene using the primers *CgRuby1*-F 5'-AAAAAGCAGGCTCCATGGCGGATTCCTTAGGAGTTC-3' and *CgRuby1*-R 5'-AGAAAGCTGGGTTTACTTTGCATTGAGAAGATCCCA-3' (the vector-derived sequence is underlined). Additionally, to confirm that the *CgRuby1* PCR band was not from *Agrobacterium* contamination, a *VirD2* nucleotide sequence, a component of the T-DNA border endonuclease in *Agrobacterium* (Eric and Wayne 1988; Padavanil et al. 2014), was amplified using the aforementioned DNA template. The primers used to amplify *VirD2* were *VirD2*-F: 5'-ATGTCGAAGATCACCGCTCA-3' and *VirD2*-R: 5'-TCAGGCTTCGTCGTACTGGT-3'. A PCR-positive plant derived from a single somatic embryo was considered an independent transformation event, and the transformation efficiency was defined as the number of independent transformation events obtained per batch of transformations (using approximately 200 leaf explants).

Determination of transgenic plant anthocyanin contents

Anthocyanin accumulation and content were analyzed one month after transferring the transgenic seedlings to the soil. Anthocyanin extraction and determination were performed using the pH differential method

(Sinelli et al., 2008). Approximately ten leaves from the top of each plant were collected as samples, ground into a fine powder using liquid nitrogen, and divided into three aliquots as replicates. The leaf powder was mixed with a methanol-based solution containing 1% hydrochloric acid in the dark with shaking. Following centrifugation, the supernatant was collected and the extraction process was repeated before combining and diluting the extracts. Aliquots of the extract were mixed with 4 mL of KCl (pH 1.0) and sodium acetate trihydrate (NaAc) (pH 4.5) buffer, and absorbance was measured at 520 and 700 nm using distilled water as a blank. The anthocyanin content was calculated based on the absorbance differences between the two pH conditions, applying the molecular weight of cyanidin-3-glucoside (449.2 g/mol), dilution factor, molar extinction coefficient of cyanidin-3-glucoside (26,900 L·mol⁻¹·cm⁻¹), and sample weight.

Quantitative real-time PCR (qRT-PCR)

RNA was extracted from transgenic leaves and used to determine the transcript levels of anthocyanin biosynthetic genes, including *chalcone synthase* (*VcCHS*), *flavanone-3-hydroxylase* (*VcFHT*), *dihydroflavonol 4-reductase* (*VcDFR*), *anthocyanidin synthase* (*VcANS*), and *UDP-glucose: flavonoid 3 glucosyl transferase* (*VcUFGT*). A total RNA extraction kit (RNAsimple Total RNA Kit, Tiangen Biotech, China) was used. The extracted RNA was reverse-transcribed into cDNA using the TransScript Reverse Transcription Kit (TransGen Biotech, Beijing, China). qRT-PCR was performed using TransStart Top Green qPCR Super-Mix (TransGen Biotech, Beijing, China) on a real-time fluorescence quantitative PCR instrument (CFX96, BioRad, Shanghai, China). The *Actin* gene was used as an internal reference. The relative expression levels of target genes were calculated using the 2^{- $\Delta\Delta C_t$} method. The primers used for qRT-PCR are listed in Table S1.

Statistical analysis

Data for callus induction and stable transformation were analyzed for significance using the Student–Newman–Keuls (SNK) test in ANOVA using SPSS 19.0 (IBM Corporation, Armonk, NY, USA). Figures were produced using Microsoft Excel 2021 software.

Results

Effect of hormone combinations on adventitious shoot regeneration

Adventitious shoots were successfully regenerated in all 6-BA and TDZ treatments combined with NAA, but

not in T3N. Under treatments T1N and T2N, regeneration rates were significantly higher than those under the other combinations, with values of 58.9% and 72.1%, respectively. However, during the culturing process, the regenerated shoots in T1N and T2N remained as buds and failed to grow further. In contrast, in the medium containing ZT, the regenerated shoots maintained a relatively low regeneration rate, but the state of the regenerated shoots was better, such as in Z3, with a regeneration rate of 22.2% (Table 1; Fig. 1). Therefore, a further test was conducted in which adventitious buds induced in T2N for 20 d were transferred to Z3. As a result, all adventitious buds continued to grow into robust shoots (Fig. 1).

Conditions optimization for adventitious shoot regeneration

We examined the effects of different leaf explant positions on adventitious shoot regeneration. The top leaves of in vitro-grown plantlets exhibited better induction of adventitious shoots, with a regeneration rate of 25.3%, which was significantly higher than the values of 13.2% achieved using the middle part and

10.6% achieved using the basal part (Fig. 2a). Next, we investigated the effects of the leaf explant cutting method on adventitious shoot regeneration (Fig. 2b). Moderate wounding (method 2) induced more callus formation, thereby differentiating more regenerated shoots (34.2%). In contrast, slight (method 1; 17.1%) and excessive wounding (method 3; 7.0%) were both unfavorable for shoot regeneration. Third, we examined the effect of leaf explant orientation on adventitious shoot regeneration (Fig. 2c). When the leaves were positioned with the adaxial side facing upward (abaxial/back side in contact with the medium), adventitious shoots were more effectively induced (33.3%). Conversely, no adventitious shoots were regenerated when the leaves were placed with the adaxial surface in contact with the medium. The effect of dark cultivation duration on adventitious shoot regeneration was also studied (Fig. 2d). When leaves were cultured on the induction medium in the dark for 12 days, the induction of adventitious shoots was more effective, with a regeneration rate of 27.7%, which was significantly higher than that of leaves cultured in the dark for 10 or 8 days.

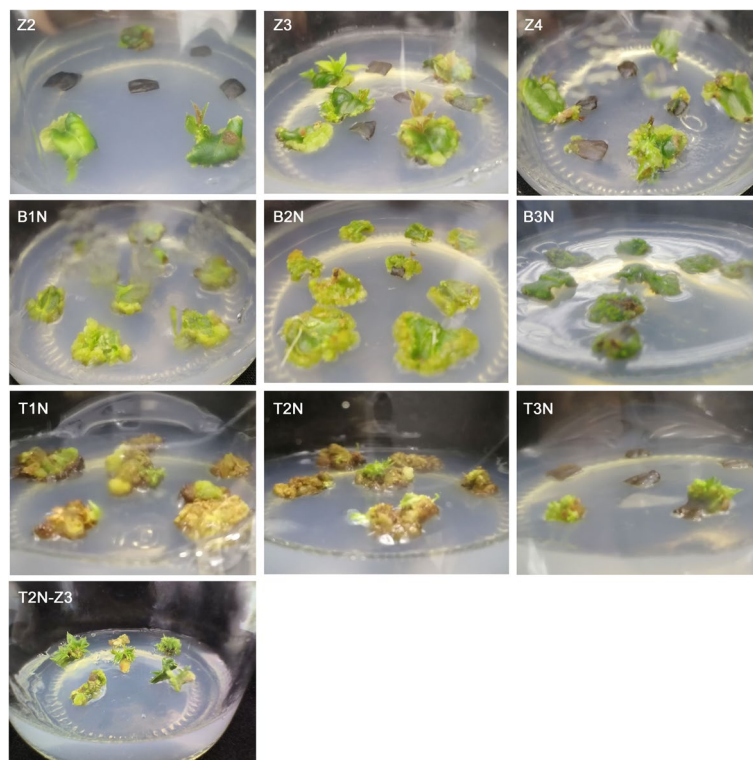


Fig. 1 Effect of hormone combinations on adventitious shoot regeneration. Z2–Z4, zeatin (ZT) 2–4 mg/L; B1N–B3N, 6-benzylaminopurine (6-BA) 1–3 mg/L + naphthaleneacetic acid (NAA) 0.5 mg/L; T1N–T3N, thidiazuron (TDZ) 1–3 mg/L + NAA 0.5 mg/L; T2N–Z3, adventitious buds induced in T2N for 20 d were transferred to Z3

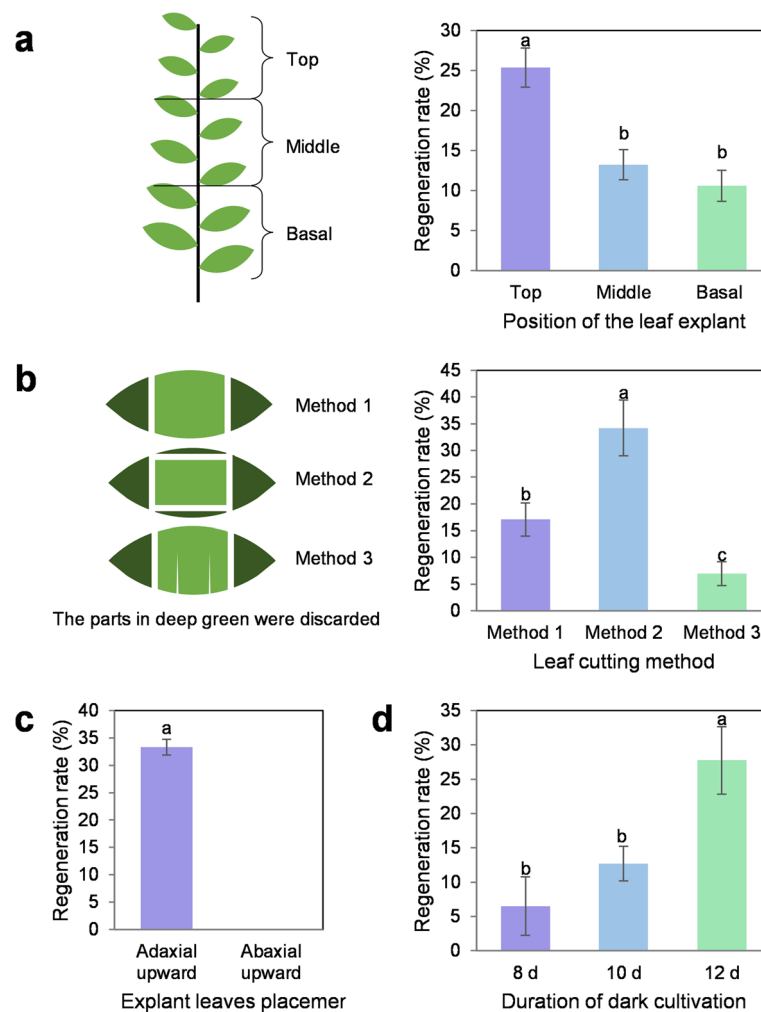


Fig. 2 Effect of different conditions on adventitious shoot regeneration rate. **a–d** Effect of the leaf explant position (**a**), leaf-cutting method (**b**), leaf explant orientation (**c**), and dark incubation duration (**d**). Different lower case letters denote statistically significant differences ($p < 0.05$)

Screening of kanamycin concentration

In kanamycin-free medium, the resistant shoot regeneration rate reached 83.4% (Fig. 3a). When supplemented with kanamycin at 7.5 mg/L, the regeneration rate decreased to 39.4%. At 10-mg/L kanamycin, the regeneration rate dropped to zero, with white calli and stunted purple–red shoots that failed to develop normally, indicating a strong inhibitory effect. Therefore, 10-mg/L kanamycin is recommended as a suitable selective pressure for screening unambiguously resistant regenerated shoots.

Conditions optimization for *Agrobacterium*-mediated transformation

The first was the effect of the method of culturing *Agrobacterium* on resistant shoot regeneration (Fig. 3b). Liquid-cultured *Agrobacterium* achieved a higher infection efficiency, yielding a resistant shoot regeneration rate of

8.5%, significantly surpassing the 2.6% observed in solid culture. Next, we examined the effect of *Agrobacterium* infection duration on the regeneration of resistant shoots (Fig. 3c). One-hour treatment led to a maximum regeneration rate of 6.3%, whereas a three-hour infection completely inhibited shoot formation. Third, we examined the effect of *Agrobacterium* infection suspension density on resistant shoot regeneration (Fig. 3d). An OD_{600} of 0.8 produced the highest regeneration rate (7.4%), whereas rates slightly declined at an OD_{600} of 0.9 (6.2%) and sharply dropped to 1.7% at an OD_{600} of 1.1. In addition, we examined the effects of vacuum infiltration on resistant shoot regeneration, and found that the vacuum infiltration enhanced the regeneration rate to 7.2% (Fig. 3e), better than that without a vacuum process. Finally, we examined the effect of the co-cultivation duration on resistant shoot regeneration. Co-cultivation for six days

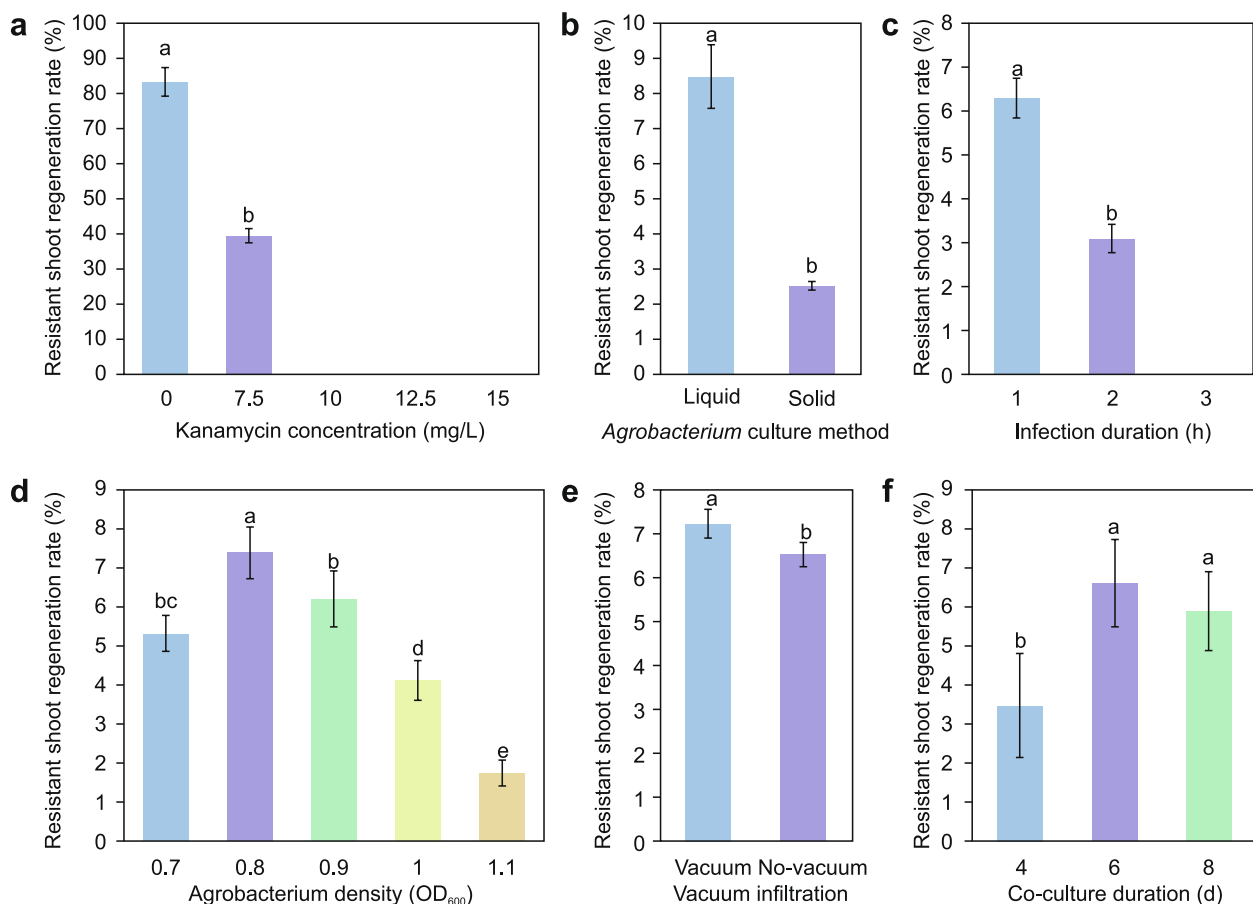


Fig. 3 Screening of kanamycin concentration and optimization of conditions for *Agrobacterium*-mediated transformation. **a–f** Screening of kanamycin concentration (**a**), *Agrobacterium* culture method (**b**), *Agrobacterium* culture density (**c**), infection duration (**d**), vacuum infiltration during infection (**e**), and co-culture duration (**f**). Different lower case letters denote statistically significant differences ($p < 0.05$)

yielded the highest regeneration of 6.5%, followed by 5.8% for eight days, and only 3.4% at four days (Fig. 3f).

Observation of EGFP fluorescence and PCR analysis of CgRuby1

The green fluorescence signal of the *Egfp* gene was observed in kanamycin-resistant adventitious shoots three weeks after infection. Under the EGFP light mode, no green fluorescence was observed in WT adventitious shoots (Fig. 4a, b). In contrast, green fluorescence signals were observed in resistant shoots through the filter, indicating the possible integration of the *Egfp* gene into kanamycin-resistant adventitious shoots (Fig. 4c, d).

Based on the optimized transformation conditions, we inoculated 200 leaf discs and cultured them on selective regeneration medium containing kanamycin. After approximately six months of subculture, we obtained 13 antibiotic-resistant plantlets (all > 2 cm in height). PCR analysis of the *CgRuby1* gene confirmed that all 13 resistant

plantlets were transgenic (Fig. 4e) with a transformation efficiency of 6.5% (13/200). PCR verification of the *Agrobacterium*-derived *VirD2* gene showed no amplified bands in the kanamycin-resistant adventitious shoots (Fig. S1). This confirmed that the PCR bands of the *Egfp* gene were amplified from those integrated into the plants rather than from *Agrobacterium* contamination.

Enhanced anthocyanin contents and expression levels of CgRuby1 and blueberry anthocyanin biosynthetic genes in transgenic plants

The anthocyanin content was analyzed in the five transgenic lines after one month of growth in the soil. The leaves exhibited a purplish-red phenotype, whereas the WT shoots showed a normal green phenotype (Fig. 4f). Transgenic leaf extracts showed more intense anthocyanin pigmentation than the WT (Fig. 4g). Anthocyanin content analysis demonstrated that *CgRuby1*-overexpressing transgenic plants had significantly higher anthocyanin levels than WT plants (Fig. 4h). Moreover,

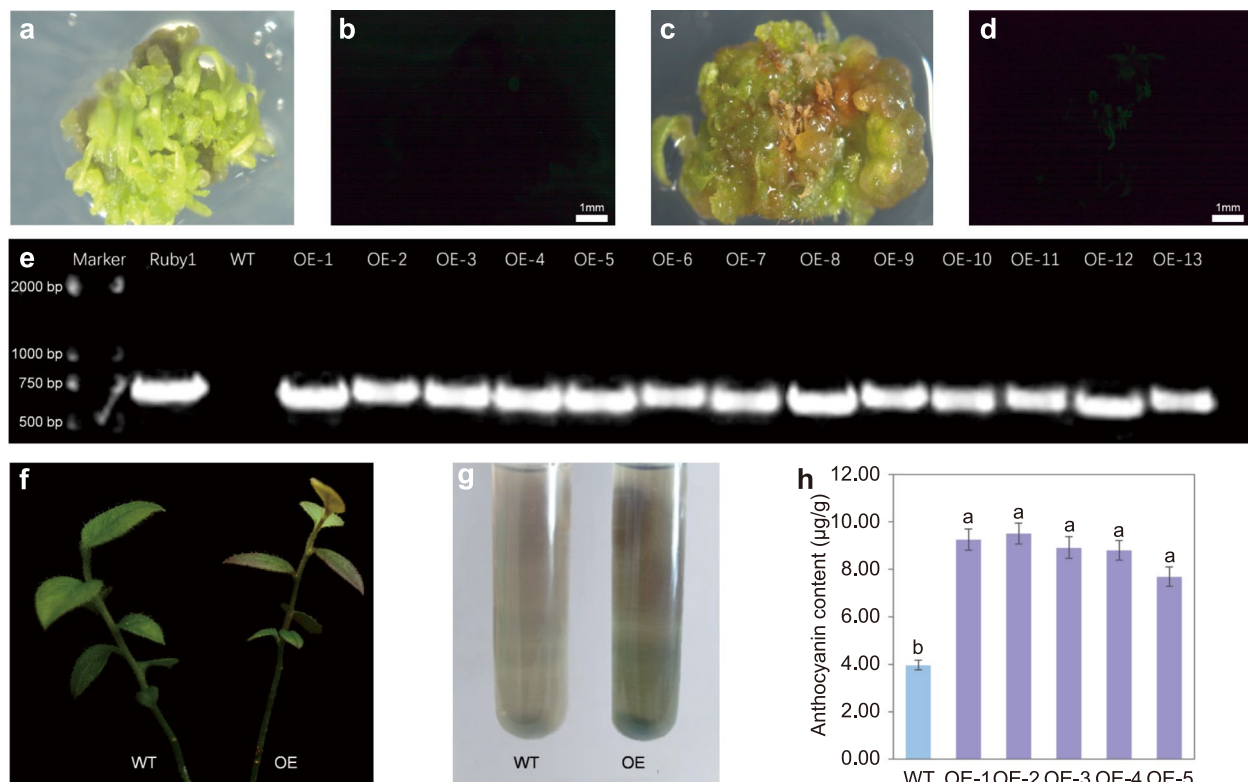


Fig. 4 Screening of transgenic plants and analysis of anthocyanin accumulation. **a–d** Green fluorescence observation. Wild-type (WT) adventitious shoots in natural (**a**) and fluorescent light (**b**). Kanamycin-resistant adventitious shoots in natural (**c**) and fluorescent light (**d**). **e** Polymerase chain reaction (PCR) amplification of resistant shoots. **f–h** Anthocyanin concentration determination in transgenic plants. Leaf phenotype (**f**), leaf extracts (**g**), and anthocyanin contents (**h**) in WT and transgenic plants. Different lower case letters denote statistically significant differences ($p < 0.05$). Ruby1, plasmid pK2GW7; WT, wild-type; OE, over-expressing lines

CgRuby1 and five anthocyanin biosynthesis pathway genes in blueberry, including *VcCHS*, *VcFHT*, *VcDFR*, *VcANS*, and *VcUFGT*, were quantified in WT and five transgenic lines (Fig. 5). The results revealed that *CgRuby1* and the five structural genes were significantly upregulated in the transgenic plants relative to WT.

Discussion

Previous studies on blueberry in vitro adventitious shoot regeneration demonstrated that a high cytokinin-to-auxin ratio is the most effective hormonal combination for regeneration media. Commonly employed cytokinins include ZT, TDZ, and 6-BA (Qiu et al 2018; Song & Sink, 2004; Debnath 2009). In this study, we evaluated various hormone combinations in the blueberry cultivar ‘Legacy’. Initial experiments revealed that a medium supplemented with TDZ (2 mg/L) and NAA (0.5 mg/L) induced prolific shoot differentiation. However, the regenerated shoots exhibited suboptimal growth. In contrast, media containing ZT (Z2, Z3, and Z4) promoted reduced, but more robust, shoot regeneration. To capitalize on these observations, we developed a two-stage regeneration

protocol: first, explants were cultured on T2N medium (TDZ 2 mg/L + NAA 0.5 mg/L) to maximize shoot initiation, followed by subculture onto Z3 medium (ZT 3 mg/L) to enhance shoot elongation. This sequential approach yielded numerous regenerated shoots exhibiting improved growth characteristics.

To date, only a limited number of studies have reported successful genetic transformation of tetraploid blueberry (*V. corymbosum*), with varying protocols depending on cultivar-specific requirements (a brief review is provided in Table S2). Earlier work by Cao et al. (1998), based on *GUS* transient expression assays, recommended a protocol that included infection duration, co-cultivation period, and kanamycin selection pressure. Liu (2018) introduced several methodological refinements, including the use of young leaves (from 40-day-old subcultured shoots) as explants, mechanical wounding of leaf tissues, and placement of the abaxial side of the leaves in contact with the culture medium to enhance transformation efficiency. Qin (2020) optimized transformation conditions for two cultivars, ‘Northland’ and ‘Millennia’, and determined the optimal *Agrobacterium* infection suspension

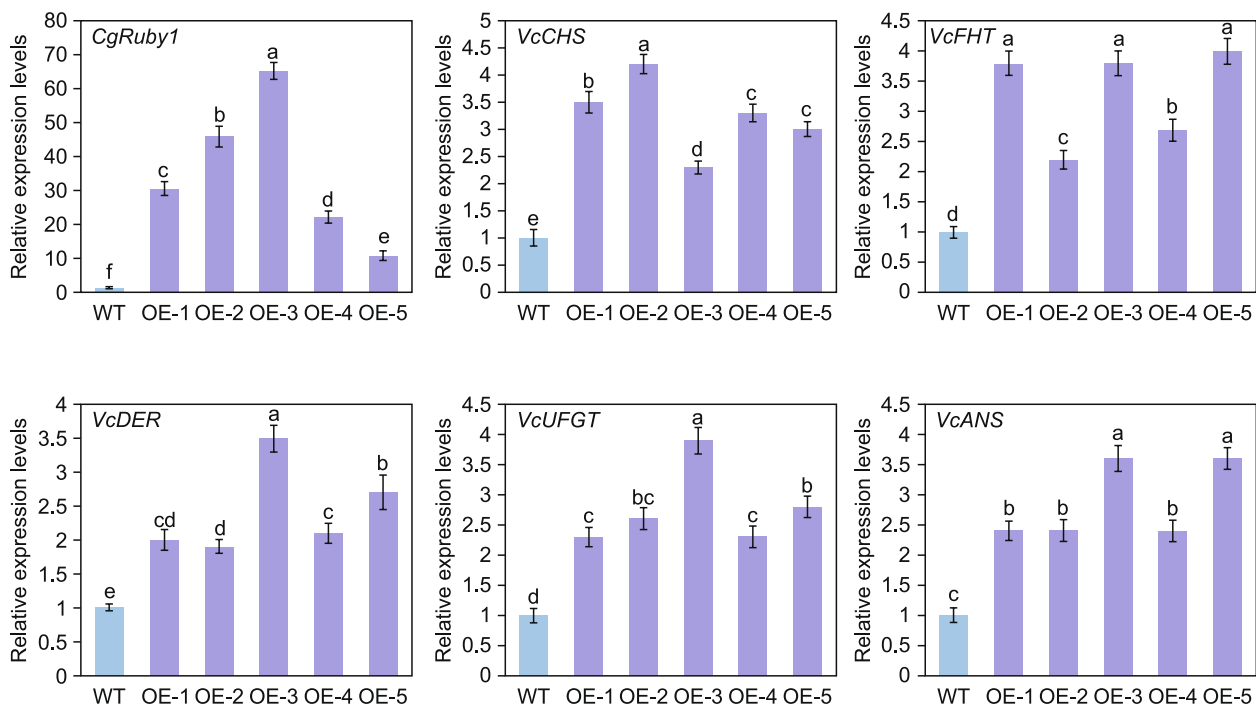


Fig. 5 Relative expressions of *CgRuby1* and anthocyanin biosynthesis pathway genes in wide-type (WT) and over-expressing (OE) plants. Different lower case letters denote statistically significant differences ($p < 0.05$). *VcCHS*, chalcone synthase; *VcFHT*, flavanone-3-hydroxylase; *VcDFR*, dihydroflavonol 4-reductase; *VcANS*, anthocyanidin synthase; *VcUFGT*, UDP-glucose: flavonoid 3 glucosyl transferase

density and the most effective kanamycin selection concentrations. In an alternative approach, Qin et al. (2023) employed callus tissues as explants (as opposed to leaf discs) and assessed the transformation efficiency via *GFP* transient expression. However, these studies did not successfully generate true transgenic plants.

The first successful generation of transgenic plants using tetraploid materials began with the work of Song and Sink (2004) and their subsequent studies (Song and Sink 2015; Song 2015; Song and Chen 2018; Walworth and Song 2018; Song et al. 2019; Lin et al. 2019; Li et al. 2024). The established key parameters achieved transformation efficiencies ranging from 5.0% to 15.3% across four cultivars (Song and Sink 2004). In this study, we used the widely cultivated highbush cultivar ‘Legacy’ to yield a transformation efficiency of 6.5%, representing a modest but statistically significant improvement over the 5.6% efficiency previously reported for this cultivar (Song and Sink 2004). This enhancement can be primarily attributed to the establishment of a two-stage regeneration protocol (mentioned earlier), the implementation of wounding techniques, and vacuum infiltration during *Agrobacterium* infection. The wounding strategy likely facilitated more extensive *Agrobacterium* access to the meristematic tissues without compromising explant viability (Hwang et al. 2017). The application of vacuum

infiltration represents a particularly important modification, consistent with previous reports on other plant systems (Bechtold & Bouchez, 1995; Desfeux et al. 2000; Tague & Mantis, 2006; Hada et al. 2018). This technique promotes the removal of intercellular air from the stomatal chambers and cell wall matrix, thereby enhancing bacterial penetration into target tissues during pressure normalization.

In previous studies, the kanamycin concentration used to select positively transformed plants varied. For instance, Song (2004) employed 10-mg/L kanamycin, whereas Liu (2018) applied different concentrations within the range of 15–30 mg/L depending on the cultivar. Other studies reported the use of up to 50 mg/L kanamycin (Cao et al. 1998; Song et al. 2012). In this study, we evaluated the selective pressure of kanamycin on non-infected leaf explants. Within the tested range of 1–15 mg/L, explants were still capable of growth at 7.5 mg/L, but failed to survive at concentrations of 10 mg/L or higher. Therefore, a concentration of 10 mg/L was selected for antibiotic screening, as it resulted in relatively few potential escape events. Owing to the inherently weak root systems of blueberry plants, kanamycin selection pressure was not applied during the rooting stage.

Table 2 The medium formula in this study

Number	Formula	Status
1	LB + Spec 100 mg/L + Rif 20 mg/L	Solid
2	LB + Spec 100 mg/L	Liquid
3	WPM + AS 100 μM	Liquid
4	WPM + ZT 1 mg/L	Solid
5	WPM + AS 100 μM	Solid
6	T2N (WPM + TDZ 2 mg/L + NAA 0.5 mg/L) + Kan 10 mg/L + Cef 400 mg/L	Solid
7	Z3 (WPM + ZT 3 mg/L) + Kan 10 mg/L + Cef 400 mg/L	Solid
8	1/2 WPM + IBA 1 mg/L	Solid

LB Luria–Bertani medium, Spec Spectinomycin, Rif Rifampicin, WPM Woody Plant Medium, AS Acetosyringone, ZT zeatin, TDZ thidiazuron, NAA 1-naphthlctic acid, Kan kanamycin, Cef cefotaxime, IBA indole-3-butyric acid

If adventitious shoots are regenerated from single cells, homohistic transgenic plants can be obtained directly (Geier 2012). Shoot regeneration typically occurs in a group of cells (Marcotrigiano 1986). When multiple cells from the original explant contribute to the shoot apical meristem (SAM) of regenerants, the regenerating shoots become sectorial chimeras (Geier 2012). This chimeric state is unstable and prone to spontaneous dissociation, potentially leading to the formation of homomorphic organs or tissues (Geier 2012). Consequently, as shoots regenerate from a single plant, sectorial chimerism may dissociate, resulting in the coexistence of transformed and untransformed branches (Chhabra et al. 2024). Song et al. (2012) confirmed the stable presence of transgenes

in blueberries through PCR detection of in vitro-cultured transgenic shoots maintained for five years and greenhouse-grown transgenic plants cultivated for three years. These findings demonstrated the stability of transgenic expression in blueberry. However, because this study did not sample different tissues or organs, whether the transgenic plants were chimeric remains uncertain. In this study, PCR analysis of leaf samples from kanamycin-resistant regenerants tested positive for the transgene. Nevertheless, the possibility of chimerism cannot be ruled out. To obtain homologous (non-chimeric) transgenic plants, the following approaches are recommended: First, regular systematic sampling of different tissues and organs from transgenic plants should be performed for detection. Second, methods such as seed propagation, vegetative propagation, and single-cell regeneration systems should be utilized to eliminate chimerism (Geier 2012).

MBW complex is a highly conserved core module that regulates anthocyanin biosynthesis in plants (Koes et al. 2005; Ramsay and Glover 2005; Xu et al. 2015). Within this complex, the MYB transcription factor serves as the key determinant that confers DNA-binding specificity to the structural genes involved in the anthocyanin biosynthesis pathway (Xu et al. 2015; He et al. 2023). In citrus species, Ruby1 (also designated as MYB1) has been identified as an integral component of the MBW complex (Oh et al. 2021; Huang et al. 2018). Ectopic expression of citrus *Ruby1* significantly enhances anthocyanin

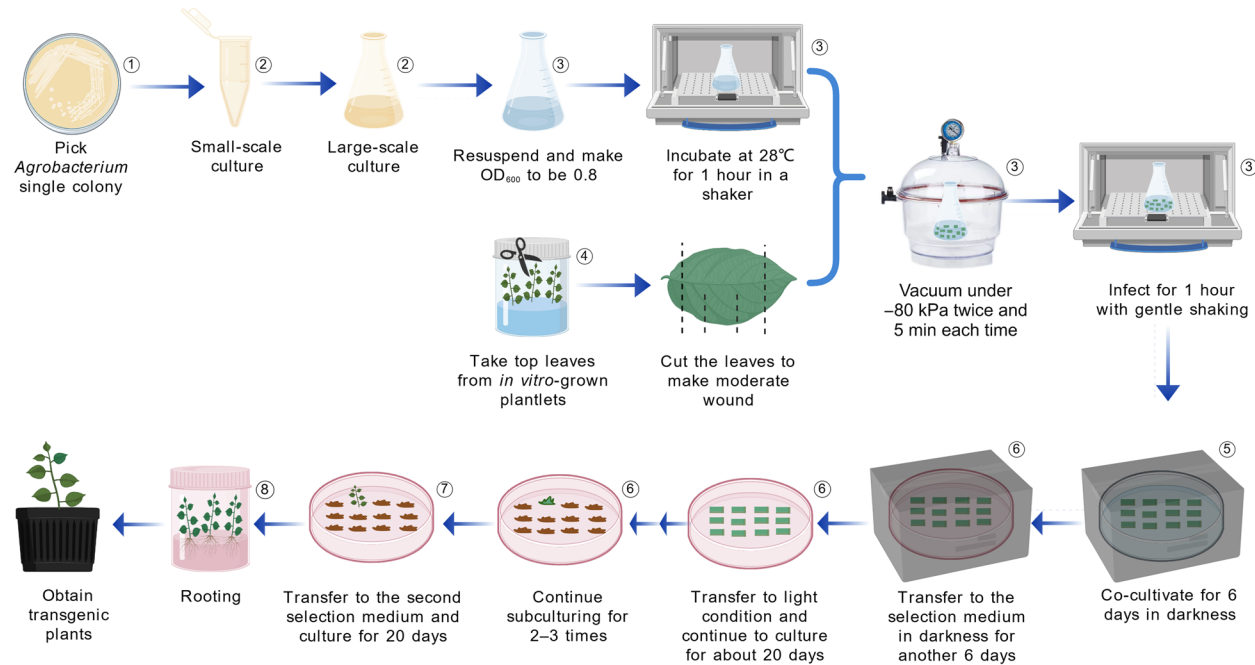


Fig. 6 Blueberry genetic transformation system used in this study. The numbers in the circles indicate the code of the culture medium used in that step, and the medium formula is listed in Table 2

accumulation in *Arabidopsis* seedlings, Mexican lime (*C. aurantifolia*), and an anthocyanin-deficient yellow strawberry mutant (*Fragaria vesca*) (Huang et al. 2018; Oh et al. 2021; Thilmony et al. 2022; Dutt et al. 2023). Owing to its visually scorable pigmentation phenotype, we employed *CgRuby1* from purple pummelo as a supplementary reporter gene to optimize transgenic procedures for blueberries. Anthocyanin accumulation was visually evident and analytically confirmed in the leaves of the transgenic blueberry plants. qRT-PCR further revealed the drastic upregulation of key anthocyanin biosynthetic genes (*VcCHS*, *VcFHT*, *VcDFR*, *VcANS*, and *VcUFGT*), suggesting that ectopically expressed *CgRuby1* may also activate the anthocyanin pathway in blueberry.

Conclusion

Taken together, we successfully established a blueberry genetic transformation system (Fig. 6) that resulted in slightly higher transformation efficiency than those reported in previous studies. The advantages of our system may be primarily attributed to the two-stage regeneration protocol, implementation of wounding techniques, and vacuum infiltration. Furthermore, the use of the *Ruby1* gene as an assistant reporter gene provided a visible phenotypic marker and confirmed the functionality of the transgene. Therefore, this system can be applied to introduce other functional genes and provide a foundation for the genetic modification of the blueberry germplasm through techniques such as CRISPR-based gene editing. However, this system has some limitations. For instance, compared with established transformation systems in model species, the efficiency requires further enhancement, potentially through the incorporation of elicitor peptide-based strategies (Yang et al. 2024). Such improvements are essential for supporting large-scale functional genomic studies of blueberries and enable genetic transformation in other tetraploid cultivars. Moreover, further investigations are needed to determine whether the transgenic plant is a chimeric nature, the stability of the transgene after long-term subculture or propagation.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1007/s44281-025-00092-5>.

Supplementary Material 1: Figure S1. PCR amplification of *VirD2*. WT, wild-type; Plasmid, pK2GW7. Table S1. Primers for qRT-PCR. CHS, chalcone synthase; FHT, flavanone-3-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; UFGT, UDP-glucose: flavonoid 3 glucosyl transferase. Table S2. A brief review of references involved in the *Agrobacterium*-mediated transformation of tetraploid blueberry (*Vaccinium corymbosum*). PGR, plant growth regulator; TDZ, thidiazuron; NAA, naphthaleneacetic acid; GFP, green fluorescent protein.

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Authors' contributions

MFZ composed the draft. RMD, LF, XYS, and CYH performed the experiment. XW and YGX directed the experimental operation. CL supervised the research. XW designed the experiments and revised the manuscript.

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Data availability

Data supporting the findings are available in the article and supplementary materials.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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