

A novel strategy for enhancing anthocyanins content in VdMYB56-overexpressing rabbiteyes blueberry lines

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Abstract

As a horticultural plant with high nutritional and commercial value, blueberries (*Vaccinium ashei*) suffer from inefficient genetic transformation systems, which highly constrain the development of biotechnology breeding in this fruit crop. The central method involved transferring *VdMYB56* into *Agrobacterium*, thereby establishing the *Agrobacterium*-mediated genetic transformation system, which was started from an infection with *Agrobacterium* ($OD_{600} = 0.6$) for a 10 min, followed by co-cultivation for three days. The main results indicated that the transformation efficiency detected in red *VdMYB56*-overexpressing calli was as high as 10.95%, which was consistent with the PCR assay results. In the *VdMYB56*-overexpressing calli, the expression levels of *VdMYB56* and *Vd3GT* were higher than those of the WT; The total anthocyanin content (TAC) and *VdUFGT* enzymatic activity were both superior to those of the wild type (WT). Subsequently, the *VdMYB56*-overexpressing adventitious buds exhibiting red were obtained, and the expression levels of *VdMYB56* and *Vd3GT* in the *VdMYB56*-overexpressing lines were 2.75–24.15 and 10.56–20.69 folds as high as those of the WT, respectively; the TAC and *VdUFGT* activity in three lines were 1.78–4.52 and 2.25–4.35 times also higher than those of the WT, respectively. Collectively, an efficient genetic transformation system was established, and the *VdMYB56*-overexpressing rabbiteye blueberry lines with enhanced anthocyanin content was gained herein, which provided an appropriate methodology for new germplasm creation in blueberry, and the *VdMYB56*-overexpressing lines may facilitate the breeding in elevating anthocyanin.

1. Instruction

Blueberry (*Vaccinium ashei*) is recognized as a 'superfruit' due to their sweet flavor and nutrient-dense composition (Pires et al., 2020), and a functional food, primarily attribute to their abundant health-promoting multiple nutrients, e.g., soluble sugar, vitamins, and various secondary metabolites, etc (Krishna et al., 2023). Among its complex secondary metabolites, anthocyanins possesses many physiological roles and widely potential application (Jaakola, 2013; Wu et al., 2022).

During the past few decades, regulatory genes regulating the anthocyanins biosynthesis had been extensively unraveled in various plant species such as *Arabidopsis thaliana* (Cominelli et al., 2008), *Vaccinium ashi* (Deng et al., 2026), *Pyrus persica* (Liu et al., 2023). Also, several genes encoding key enzymes within the anthocyanin biosynthesis pathway, e.g., chalcone synthase (CHS), dihydroflavonol-4-reductase (DFR), and uridine diphosphate glucose: flavone-3-glucosyl transferase (UF3GT) were also identified (Tanaka et al., 2008; Yang et al., 2018), particularly, the functions of the *VaLAR1*, *VaLAR2*, *VaANS*, and *VaANR* genes were validated from the bilberry cultivar 'Herbert' (Li, 2017). In *Vaccinium myrtillus*, the roles of MYBPA1 (Karppinen et al., 2021) and MYBA1 (Song et al., 2023) had been justified to promote anthocyanin biosynthesis via *agrobacterium*-mediated genetic transformation. Besides, *3GT* acts as a key downstream enzyme-encoding gene in the anthocyanin biosynthetic pathway; it catalyzes the glycosylation of flavonoid compounds and consequently enhances anthocyanin stability (Liu et al., 2020; Luo et al., 2025). For instance, the regulatory roles of *Freesia hybrida* *Fh3GT1* (Sun et al., 2016) and *Rhododendron agastum* *Ra3GT2* (Luo et al., 2025) had been verified to regulate anthocyanin biosynthesis.

Deng et al.(2026) found that VdMYB56 promoted the anthocyanins accumulation by up-regulating the expression of *Vd3GT*. These available genes considerably facilitate the creation in new germplasms for anthocyanin improvement via biotechnology breeding.

To date, however, the low transformation efficiency in woody plants may be considerably ascribed to backward regeneration system as well as several factors such as *Agrobacterium* concentration, infection and co-culture time, and antibiotic concentration, etc (Qin et al., 2023; Gong et al., 2024). The regeneration ability remarkably varied among species, even highly demonstrated genotype-dependence. The transgenic adventitious buds were derived from the transformed calli of blueberry 'Red Button' with the combination of Zeatin (ZT) and indole-3-acetic acid (IAA), which was specifically constrained among blueberry species (Wang et al., 2022). To improve the adventitious buds, appropriate plant growth regulators (PGRs), *i.e.*, 6-Benzylaminopurine (6-BA), Thidiazuron (TDZ), and Naphthaleneacetic acid (NAA), are usually used to optimize the regeneration (Long et al., 2022). However, no efficient regeneration system has been available in rabbiteye blueberry so far, which leads to a hard nut for the genetic transformation as well as function validation of anthocyanin-related genes via homologous transformation in this fruit species (Fan et al., 2017; Gong et al., 2024). Therefore, optimizing the regeneration system is crucial for the differentiation of adventitious buds (Yang et al., 2018; Wei et al., 2023).

To establish an efficient genetic transformation system as well as gain the new lines with elevated anthocyanins in rabbiteye blueberry, currently, the efficient transformation methodology was established in rabbiteye blueberry by optimizing the several important factors including bud regeneration using 'Brightwell'. Sequentially, the *VdMYB56*-positive callus and *VdMYB56*- overexpressing lines with the ameliorated anthocyanin accumulation were created using the available methodology, which considerably facilitate the new germplasm creation in blueberry, particularly for anthocyanin improvement in this fruit species.

2. Materials and methods

2.1 Plant materials

Sterile tissue-cultured seedlings of 'Brightwell' were obtained by germinating seeds on WPM induction medium containing 2 mg·L⁻¹ TDZ + 0.1 mg·L⁻¹ NAA (Table.S1). Subsequently, the leaves (2 cm × 2 cm) of the sterile seedlings were placed on the WPM medium, which was optimized and improved according to the research of Liu et al. (2023). Briefly, callus was induced from sterile leaves on WPM medium containing 2 mg·L⁻¹ TDZ + 0.20 mg·L⁻¹ 2,4-D + 30 g·L⁻¹ sucrose + 8 g·L⁻¹ agar (Table.S1). Subsequently, it was transferred to WPM medium containing 2 mg·L⁻¹ TDZ + 0.10 mg·L⁻¹ IBA + 30 g·L⁻¹ sucrose + 8 g·L⁻¹ agar for subculture (Table.S2). The above-mentioned medium (pH 5.2–5.5) was used for callus induction and was cultured in darkness for 25 days at 25 °C (Table.S1).

2.2 Induction of adventitious bud of rabbiteye blueberry

The well-grown callus was selected from rabbiteye blueberry and cut into small pieces (0.6 cm × 0.6 cm). Then, approximately 30 segments were inoculated onto WPM medium supplemented with 30 g·L⁻¹ sucrose, 8 g·L⁻¹ agar, and various concentrations of TDZ (1.0, 1.5, 2.0, 2.5, and 3.0 mg·L⁻¹) and NAA (0.10, 0.15, 0.20, 0.25, and 0.30 mg·L⁻¹). Next, these calli were cultured in the dark at 25°C for 5 days, which were transferred to light conditions (16 h / 8 h, light/dark) for another 25 days (Table.S1). The induction rate and browning rate of adventitious buds and their growth dynamics were continuously observed and meticulously recorded. The induction and browning rate of adventitious buds were calculated as follows: (number of adventitious buds or browning of adventitious buds / total number of inoculated calli) × 100% (Wang et al., 2022). The experiment was repeated three times. Meanwhile, all experimental results are evaluated in accordance with the specified standards for each treatment.

2.3 Antibiotic identification and screening

To screen out the appropriate antibiotic concentration suitable for the *Agrobacterium*- mediated genetic transformation system, the wild type calli (WT, untransformed callus) of 'Brightwell' and transformed calluses were severally treated with different concentrations of Kanamycin (Kan: 0, 60, 80, 100, and 120 mg·L⁻¹) and Cefotaxime (Cef: 0, 100, 200, 300, and 400 mg·L⁻¹). Meanwhile, the above-mentioned culture media with different concentrations of antibiotics were placed in the dark at 25°C to determine the appropriate concentration of antibiotics (Table.S1). Each treatment included 50 calluses and was replicated three times. After 30 days, the growth status of callus cultivation, tolerance values and survival rate of the transgenic calli were recorded and calculated as follows: (number of proliferation or browning of calluses / total number of inoculated calli) × 100% (Wang et al., 2024). All treatments were performed in triplicate, and the results for each treatment were evaluated based on the corresponding criteria.

2.4 Construction of overexpression vector

The overexpression vector pCAMBIA2300-35s-GFP had the Kan resistance gene and the green fluorescent protein (GFP) gene. Meanwhile, the coding sequence (CDS) of the *VdMYB56* gene was cloned from the rabbiteye blueberry using specific cloning primers (Table.S3). Further, the CDS sequence of *VdMYB56* was inserted into the pCAMBIA2300-35s-GFP vector (containing a 35S promoter) to construct an overexpression recombinant plasmid *VdMYB56* :: pCAMBIA2300-35s-GFP (Deng et al., 2026). The above recombinant plasmid was transformed into *A. tumefaciens* strain GV3101 to obtain *A.tumefaciens* with the recombinant plasmid.

2.5 Optimizing *Agrobacterium*-mediated transformation system for rabbiteye blueberry

According to the research of Zhong et al. (2020), a genetic transformation system mediated by *Agrobacterium* was optimized and established. Specifically, the *A. tumefaciens* strain GV3101 was cultured overnight in YEB liquid medium, which supplemented with 100 mg·L⁻¹ rifampicin (Rif) and 100 mg·L⁻¹ Kan at 28°C, with shaking at 120 rpm, until the OD₆₀₀ value of the bacterial solution reached 1.2. Subsequently, the *Agrobacterium* suspension was transferred into a 50 ml centrifuge tube and

centrifuged at 5000 rpm for 10 min. It was then resuspended in WPM liquid medium containing 30 g·L⁻¹ sucrose and 100 µM AS, and this process was repeated three times (Table.S1). The OD₆₀₀ value was adjusted to 0.3 - 1.2. Next, the obtained calli were inoculated with *Agrobacterium* solution at different concentrations (OD₆₀₀ = 0.3, 0.6, 0.9, and 1.2, respectively) and then infected for 5, 10, 15, and 20 min, respectively. After infection, the calli were transferred to co-medium supplemented with 100 µM AS. Cultures were incubated in the dark for 3, 5, 7, and 9 days, followed by the procedures described above. Further, it was transferred onto selection medium supplemented with 100 mg·L⁻¹ Kan and 300 mg·L⁻¹ Cef to screen for *VdMYB56*-overexpressing calli. These media were renewed on the 16th day of selection. After 30 days, the proliferation and browning status of both resistant and non-resistant calli were observed and analyzed. Next, *VdMYB56*-overexpressing calli were obtained and cultured on WPM supplemented with 2.5 mg·L⁻¹ TDZ, 0.2 mg·L⁻¹ NAA, 100 mg·L⁻¹ Kan, and 300 mg·L⁻¹ Cef for 45 days. Then, the induction and browning conditions of both resistant and non-resistant adventitious buds were analyzed.

2.6 qRT-PCR validation of *VdMYB56*-overexpressing (*VdMYB56*-OE) lines

Total RNA was extracted from the samples using a plant RNA extraction kit (Tiangen, Beijing, China), and complementary DNA (cDNA) was synthesized from 1 µg of total RNA by reverse transcription. Meanwhile, WT plants served as the control for quantitative real-time polymerase chain reaction (qRT-PCR) analysis. The primers of *VdMYB56* and *Vd3GT* are listed in Table.S3. The qRT-PCR reaction conditions were set as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 60°C for 20 s, extension at 72°C for 70 s, and a final extension at 72°C for 5 min. These PCR products were analyzed using 1% agarose gel electrophoresis. The qRT-PCR was performed on an ABI- StepOnePlus system, with the reaction conditions following the protocol for PowerUp™ SYBR™ Green Master Mix (ABI system). The relative transcription levels of *VdMYB56*-overexpressing calli were calculated utilizing the 2^{-ΔΔCt} method. Simultaneously, the primers for the reference gene *VdGAPDH* (AY123769) used for qRT-PCR validation are also listed in Table S3 (Jaakola, 2013).

2.7 Determination of total anthocyanin content in *VdMYB56*-overexpressing lines

The colour and growth status of the *VdMYB56*-overexpressing (*VdMYB56*-OE) lines were recorded and observed. Further, total anthocyanin content (TAC) in the lines were extracted using an anthocyanin extraction solution (methanol : water : formic acid : trifluoroacetic acid = 70 : 27 : 2 : 1, v/v/v/v) (Hashimoto et al., 2002). The TAC of transgenic and WT lines were determined using the pH differential method according to Sun et al.(2011). The fresh weight of these lines was approximately 1.02g. The samples were ground into powder, followed by the addition of 5 mL anthocyanin extraction solution. The mixture was soaked and incubated at 4°C for 24 h to extract anthocyanin. After centrifugation, the supernatant was filtered through a 0.45 µm membrane and transferred to a sample vial for TAC determination.

2.8 Determination of VdUGT enzymatic activity in VdMYB56-overexpressing lines

The activity of UDP-glucose: flavonoid-3-O-glucosyltransferase (VdUGT) in the anthocyanin biosynthetic pathway of *VdMYB56*-overexpressing lines was determined using a double-antibody, one-step sandwich enzyme-linked immunosorbent assay (ELISA) kit (Schraer et al., 2000). Briefly, 1.0 g of sample from transgenic lines was homogenized in 0.01 M PBS buffer (pH 7.4) at a 1:9 (w/v) ratio, with all grinding procedures performed on ice. Next, the mixture was centrifuged at 5000 rpm for 10 min, after which the supernatant was collected for the determination of VdUGT enzymatic activity ($\text{U}\cdot\text{g}^{-1}$).

2.9 Statistical analysis

The measurement data were analyzed by utilizing GraphPad Prism 8.0 (Mavrevski et al., 2018) and Excel. Meanwhile, the data in this study were presented as mean $\pm$ standard deviation (SD). All experiments were performed in triplicate.

3. Results

3.1 Optimization of adventitious bud induction in the rabbiteye blueberry

To obtain well-developed calli, different combinations of TDZ and IBA were optimized for callus induction. In the present study, a green and hard callus was induced on WPM medium containing $2.0 \text{ mg}\cdot\text{L}^{-1}$ TDZ and $0.05 \text{ mg}\cdot\text{L}^{-1}$ IBA, with an induction rate of 15.26 % (Table.S2; Fig.S1A). Yellow, friable, and soft calli were induced on WPM medium supplemented with $2.0 \text{ mg}\cdot\text{L}^{-1}$ TDZ and $0.1 \text{ mg}\cdot\text{L}^{-1}$ IBA, with an induction rate as high as 93.41% (Table.S2; Fig.S1B). Yellow and compact calli were induced on WPM medium supplemented with $2.0 \text{ mg}\cdot\text{L}^{-1}$ TDZ and $0.15 \text{ mg}\cdot\text{L}^{-1}$ IBA, with an induction rate of 47.56% (Table S2; Fig.S1C). Partially dark yellow compact calli were obtained on WPM medium containing $2.0 \text{ mg}\cdot\text{L}^{-1}$ TDZ and $0.2 \text{ mg}\cdot\text{L}^{-1}$ IBA, with an induction rate of 40.23% (Table S2; Fig.S1D).

Next, to optimize medium for adventitious bud differentiation system from rabbiteye blueberry callus, the further assays were conducted. In the current case, yellow friable calli were induced on WPM medium containing $2.0 \text{ mg}\cdot\text{L}^{-1}$ TDZ and $0.1 \text{ mg}\cdot\text{L}^{-1}$ IBA, with an induction rate of 93.41% (Table.S2; Fig.S1B). Subsequently, these mentioned well-growing calli were cultured on medium with different combinations of TDZ and NAA concentrations to induce adventitious buds (Table.1). The results showed that under the combination of a TDZ concentration of $1.5 \text{ mg}\cdot\text{L}^{-1}$ and an NAA concentration of $0.2 \text{ mg}\cdot\text{L}^{-1}$, few adventitious buds were formed, the induction rate of adventitious buds reached 34.81%, and the browning rate was 65.19% (Table.1; Fig.1A). Additionally, under the combined concentrations of $2 \text{ mg}\cdot\text{L}^{-1}$ TDZ and $0.2 \text{ mg}\cdot\text{L}^{-1}$ NAA, the number of adventitious buds increased, with an induction rate of 39.82% (Table.1; Fig.1B). Subsequently, the number of adventitious buds increased significantly under the combined treatment of $2.5 \text{ mg}\cdot\text{L}^{-1}$ TDZ and $0.2 \text{ mg}\cdot\text{L}^{-1}$ NAA, with an induction rate of 52.41%. However, the adventitious buds grew poorly, and the browning rate reached 47.59% (Table.1; Fig.1C). When cultured on medium supplemented with $3.0 \text{ mg}\cdot\text{L}^{-1}$ TDZ and $0.2 \text{ mg}\cdot\text{L}^{-1}$ NAA, the number of adventitious buds decreased significantly (Table.1; Fig.1D). Its induction rate was 20.92%, whereas the browning rate

reached 79.08% (Table.1; Fig.1D). Collectively, 2.5 mg·L⁻¹ TDZ and 0.2 mg·L⁻¹ NAA are suitable combinations of plant growth regulators for the formation of adventitious buds in rabbiteye blueberry callus. Collectively, 2.5 mg·L⁻¹ TDZ and 0.2 mg·L⁻¹ NAA are a suitable combination of plant growth regulators (PGRs) for the induction of adventitious buds in rabbiteye blueberry callus.

Table.1 The effects of different hormone combinations on bud induction in rabbiteye blueberry

Medium number	TDZ Concentration (mg·L ⁻¹)	NAA Concentration (mg·L ⁻¹)	Bud induction rate, % (Mean ± standard error)	Browning rate, % (Mean ± standard error)
M1	1.5	0.10	5.94 ± 0.39e	94.05 ± 6.81A
M2	1.5	0.15	13.93 ± 1.73c	86.07 ± 1.73B
M3	1.5	0.20	34.81 ± 3.94a	65.19 ± 3.94E
M4	1.5	0.25	8.02 ± 2.44d	81.98 ± 2.54C
M5	1.5	0.30	27.71 ± 2.36b	72.29 ± 2.36D
M6	2	0.10	16.95 ± 2.48d	83.05 ± 1.37B
M7	2	0.15	12.79 ± 1.62e	87.21 ± 6.16A
M8	2	0.20	39.82 ± 1.75a	60.18 ± 1.76E
M9	2	0.25	33.76 ± 4.67b	66.24 ± 4.76D
M10	2	0.30	20.59 ± 1.02c	79.41 ± 1.09C
M11	2.5	0.10	43.45 ± 5.04b	56.55 ± 1.45D
M12	2.5	0.15	18.47 ± 2.33e	81.53 ± 4.46A
M13	2.5	0.20	52.41 ± 2.07a	47.59 ± 2.61E
M14	2.5	0.25	30.90 ± 1.14c	69.10 ± 1.15C
M15	2.5	0.30	23.83 ± 3.36d	76.17 ± 3.36B
M16	3	0.10	18.58 ± 1.38b	81.42 ± 1.52D
M17	3	0.15	14.60 ± 1.55dc	85.40 ± 3.06C
M18	3	0.20	20.92 ± 3.86a	79.08 ± 3.86E
M19	3	0.25	12.39 ± 1.34d	87.61 ± 1.36B
M20	3	0.30	9.55 ± 0.52e	90.45 ± 2.06A

Note: The data were as mean ± SD of three biological replicates (n = 3). Values followed by different lowercase or capital letters within the same column denote statistically significant differences at

the $p < 0.05$ level.

3.2 Screening of Kanamycin and Cefotaxime concentration

To further optimize the *Agrobacterium*-mediated genetic transformation system of rabbiteye blueberry, it is important to screen the optimal concentration of Kan and Cef. In a previous study, it was found that Cef was selected as the antibiotic to inhibit the growth of *A.tumefaciens*, while Kan was used to identify transgenic calli (Wang et al., 2024). In the present study, On WPM medium supplemented with 100 mg·L⁻¹ Cef and 100 mg·L⁻¹ Kan, the yellowish-white and friable transgenic calli were obtained, with an induction rate of 6.39%, and a callus browning rate was 93.61% (Table.2; Fig.2A). Subsequently, at 100 mg·L⁻¹ Kan and 200 mg·L⁻¹ Cef, the growth of transgenic calli was partially inhibited (Fig.2B). The induction and browning rates were 16.34% and 83.66%, respectively (Table.2). The growth of transgenic calli was significantly inhibited by combined treatment with 300 mg·L⁻¹ Cef and 100 mg·L⁻¹ Kan, reducing the induction rate to 43.34% (Table.2; Fig.2C). When the Cef concentration was increased to 400 mg·L⁻¹ with the Kan concentration remained unchanged, the growth of transgenic callus was severely inhibited. The induction rate decreased to 4.39%, and the browning rate increased to 95.61% (Table.2; Fig.2D). The growth of transgenic callus was obviously affected in WPM medium containing 100 - 400 mg·L⁻¹ Cef due to the inhibition of *A.tumefaciens* growth. Therefore, the appropriate 300 mg·L⁻¹ Cef was selected as the appropriate antibacterial concentration for the resistant transgenic callus.

Additionally, under the conditions of 300 mg·L⁻¹ Cef and 60 mg·L⁻¹ Kan, callus growth was somewhat affected, and some of the callus began to brown (Table.2; Fig.2E). In WPM medium with 300 mg·L⁻¹ Cef and 80 mg·L⁻¹ Kan, the growth of transgenic calli was obviously limited and exhibited visible browning; the induction rate was about 18.63% (Table.2; Fig.2F). When the Kan concentration was increased to 120 mg·L⁻¹, the induction rate of transgenic calli decreased to 13.35%; however, the browning rate increased to 86.65% (Table.2; Fig.2H). Consequently, 100 mg·L⁻¹ Kan was selected as the screening concentration for kanamycin- resistant calli (Table 2; Fig.2G). Taken together, 300 mg·L⁻¹ Cef was suitable for this genetic transformation system, while 100 mg·L⁻¹ Kan served as the optimal screening concentration for the same system.

Table.2 Effects of different concentration combinations of Kan and Cef on transgenic lines

Cef concentration (mg·L ⁻¹)	Kan concentration (mg·L ⁻¹)	Callus induction rate, % (Mean ± standard error)	Callus browning rate, % (Mean ± standard error)
0	0	88.61 ± 1.11a	1.39 ± 0.16E
100	60	2.39 ± 0.35c	97.65 ± 3.53A
100	80	3.83 ± 0.92c	96.17 ± 2.29A
100	100	6.39 ± 1.23b	93.61 ± 3.32B
100	120	4.36 ± 0.67d	95.64 ± 4.67A
200	60	8.94 ± 0.79e	91.06 ± 3.79A
200	80	14.16 ± 0.90d	85.84 ± 4.90B
200	100	16.34 ± 0.63b	83.66 ± 2.63B
200	120	15.85 ± 0.75c	84.15 ± 3.75B
300	60	10.39 ± 1.35e	89.61 ± 5.53A
300	80	18.63 ± 0.85c	81.37 ± 1.85C
300	100	43.34 ± 0.67b	56.66 ± 2.67D
300	120	13.35 ± 1.46d	86.65 ± 3.46B
400	60	9.31 ± 0.85b	80.69 ± 3.58D
400	80	8.86 ± 0.41c	91.14 ± 4.47C
400	100	4.39 ± 0.81d	95.61 ± 5.81B
400	120	1.46 ± 0.45e	98.54 ± 5.84A

Note: Values were indicated as mean ± SD of three biological replicates (n = 3). Different letters expressed a significant differences at $p < 0.05$ according to ANOVA.

3.3 Optimization of the *Agrobacterium*-mediated genetic transformation system in blueberry

To establish an *Agrobacterium*-mediated genetic transformation system for rabbiteye blueberry, main influencing factors were optimized. The 813 bp CDS of *VdMYB56* was cloned using specific primers and inserted into an overexpression vector to generate the recombinant overexpression plasmid. Subsequently, this recombinant plasmid was transformed into *Agrobacterium* to facilitate the establishment of a genetic transformation system for rabbiteye blueberry (Fig.1A). Meanwhile, several key factors, e.g., *Agrobacterium* concentration, infection time, co-cultivation time, as well as the type and concentration of antibiotics (Sutradhar and Mandal, 2023), were optimized to establish a genetic transformation system of rabbiteye blueberry. Next, the results of the $L_{16}(4^3)$ orthogonal assay indicated

that the influence of the three important factors on the genetic transformation efficiency of rabbiteye blueberry were as follows: infection time > *Agrobacterium* concentration > co-cultivation time (Table S2; Table.3). Thus, the infection time might serve as the primary factor affecting the genetic transformation efficiency of rabbiteye blueberry callus (Table.3). Therefore, infection time may be the main factor affecting the genetic transformation efficiency of rabbiteye blueberry. Moreover, this study found that when the infection time exceeded 10 min, the transformation efficiency of the callus gradually declined (Table.4). When the infection time was increased to 20 min, the lowest transformation efficiency recorded was 1.57% (Table.4). The above results indicated that the optimal conditions were an *Agrobacterium* OD₆₀₀ of 0.6, an infection time of 10 min, and a subsequent 3 days co-cultivation for the VdMYB56- overexpressing calli (Table.3). The calli were then transferred to WPM medium for proliferation induction. Meantime, the transformation efficiency of VdMYB56-overexpressing callus was 10.95% (Table.S2).

Table.3 Variance analysis was conducted on the factors affecting the genetic transformation efficiency

Source of Variation	Quadratic Sum	Degree of Freedom	Mean Square	F	p
Error correction model	142.163 ^a	9	15.796	10.111	0.005
Intercept	270.932	1	270.932	173.417	0
A : OD ₆₀₀	27.367	3	9.122	5.839	0.033
B : Infection time	101.318	3	33.773	21.617	0.001**
C: Co-cultivation time	13.478	3	4.493	2.876	0.125
Error	9.374	6	1.562		
Summation	422.469	16			

Note:Values indicate the mean (± SD) of three independent experiments. “***” indicates a significant difference at *p* < 0.001. “a” represented R² was 0.938 (adjusted R² was 0.845).

Table.4 ANOVA analysis of infection time influence on transformation frequency for rabbiteye blueberry.

	Infection Time (min)				F	p
	5	10	15	20		
Transformation efficiency (%)	6.12 ± 1.89	7.09 ± 0.35	1.67 ± 0.62	1.57 ± 0.55	8.07	0.003**

Note:Values represent the mean(±SD) of three independent experiments.“***”denotes a remarkable difference (*p* < 0.01). The data were the means ± SD (n=3).

3.4 qRT-PCR validation and physiological indicator analysis of VdMYB56-overexpressing lines

To obtain the *VdMYB56-overexpressing* line with elevated anthocyanin accumulation in rabbiteye blueberry, qRT-PCR verification was conducted on the transgenic calli, and physiological indicators were determined and analyzed. In the present study, eight transgenic calli were obtained through qRT-PCR analysis, and three of these calli (OE1, OE4, and OE7) with varying expression levels were selected for further study (Fig.3A, B, C, D, E). qRT-PCR validation was performed on selected red transgenic calli that were uniform sized. The results indicated that the expression level of *VdMYB56* in *VdMYB56-overexpressing* (*VdMYB56-OE*) lines was approximately 3.57-30.18 times higher than that of the WT (Fig.3F). The red transgenic calli were obtained after 25 days of selection on WPM medium containing $2.0 \text{ mg}\cdot\text{L}^{-1}$ TDZ, $0.1 \text{ mg}\cdot\text{L}^{-1}$ IBA, $100 \text{ mg}\cdot\text{L}^{-1}$ Kan, and $300 \text{ mg}\cdot\text{L}^{-1}$ Cef, while the WT still remained yellow under the identical screening conditions (Fig.3E). The expression of *Vd3GT* in the *VdMYB56-OE* lines was approximately 13.62-20.10 folds higher than that of the WT (Fig.3G). Meantime, the TAC of *VdMYB56-OE* lines was approximately 1.25 - 3.56 folds higher than that of WT (Fig.3H). The *VdUFGT* enzymatic activity in transgenic lines was approximately 2.25 - 4.35 times as high as that of the WT (Fig.3I). These results indicated that eight transgenic calluses were selected from those obtained by the genetic transformation system, and three red lines, *i.e.*, OE-1, OE-4, OE-6, OE-7, and OE-8, were chosen based on the expression levels of *VdMYB56* in the transgenic calluses, which could be used as materials for the induction of transgenic buds.

3.5 Analysis of adventitious bud induction of *VdMYB56-overexpression* lines

To obtain *VdMYB56-OE* adventitious bud lines, *VdMYB56-OE* calli of rabbiteye blueberry were induced and identified. According to the above screening results of aforementioned screening results of *VdMYB56-OE* callus, eight *VdMYB56-OE* callus lines were transferred to WPM medium supplemented with $2.5 \text{ mg}\cdot\text{L}^{-1}$ TDZ, $0.2 \text{ mg}\cdot\text{L}^{-1}$ NAA, $100 \text{ mg}\cdot\text{L}^{-1}$ Kan, and $300 \text{ mg}\cdot\text{L}^{-1}$ Cef after 45 days of light incubation at 25°C . Subsequently, semi-quantitative PCR and qRT-PCR analyses indicated that five positive transgenic lines, *i.e.*, OE1, OE4, OE6, OE7, and OE8, were obtained (Fig.4A,B). Meanwhile, in the *VdMYB56-OE* buds, the expression of *VdMYB56* was 2.75-24.15 folds higher than that of the WT adventitious buds (Fig.4A, B). The red *VdMYB56-OE* adventitious buds were obtained, whereas adventitious buds derived from WT callus lines remained green (Fig.4C, D). Further, the expression level of *Vd3GT* in transgenic adventitious buds was 10.56-20.69 times as high as those of the WT (Fig.4E). Simultaneously, the TAC of *VdMYB56-OE* adventitious buds was approximately 1.78-4.52 folds higher than that of the WT (Fig.4F). Furthermore, the enzymatic activity of *VdUFGT* in *VdMYB56-overexpressing* adventitious buds was 2.25-4.35 times higher than that of WT (Fig.4G). These results declare that five *VdMYB56-overexpressing* lines were obtained and markedly enhanced anthocyanin accumulation in the adventitious buds of rabbiteye blueberry.

4. Discussion

A reliable and efficient genetic transformation system can be served as an effective tool for plant genetic breeding and gene function identification. Unfortunately, the transformation efficiency of blueberry highly

displays genotype-dependence and -recalcitrance due to low bud regeneration, which is a dilemma for biotechnology breeding in this crop (Popelka et al., 2006; Wang et al., 2023a).

4.1 Efficient *Agrobacterium*-mediated genetic transformation in rabbiteye blueberry

Blueberries are served as small shrubs and favored for their rich in highly valuable anthocyanin (Ranwala and Naylor., 2004; Krishna et al., 2023). However, the genetic improvement in this fruit crop has been highly constrained due to the hard nut for *in vitro* regeneration. The available evidences confirm that cytokinins and auxins are imperative for callus differentiation *via* synergistic and antagonistic interactions (Wang et al., 2023b; Xu et al., 2024), and the PGRs used for adventitious bud regeneration is considerably genotype-dependent (Wang et al., 2022; Qin et al., 2023). In blueberry, leaf explants of 'Northland' and 'Legacy' (Qin et al., 2023), as well as 'Red Button' (Wang et al., 2022) all were dedifferentiated into calli with a combination of different PGRs in genetic transformation. TDZ was justified to be critical for advancing genetic enhancement in *Vaccinium corymbosum* (Cappelletti et al., 2014) and *Fragaria ananassa* (Akter et al., 2024). In the current case, yellow and loose calli could be induced from WPM medium containing 2.0 mg·L⁻¹ TDZ and 0.1 mg·L⁻¹ IBA, with an induction rate of 93.41% (Table.S2; Fig.S1B,b), and a combination of 2.5 mg·L⁻¹ TDZ and 0.2 mg·L⁻¹ NAA gave the best effect for the adventitious bud regeneration (52.41%, bud induction rate) in rabbiteye blueberry (Table.1; Fig.1C,c), which was similar to the result in 'Pinkblue', despite the concentrations of PGRs were somehow different (Wu et al., 2016).

Hygromycin (hyg) and Kan resistance genes are commonly used as markers for screening transformed lines, and can also be utilized to verify the function of important genes, in the construction and application of genetic transformation system (Zhang et al., 2023). Also, to reduce the impact of *Agrobacterium* on the transformation efficiency a certain amount of antibacterial agents (e.g., Tim and Cef) needs to be added (Wang et al., 2024). Among which, Cef has a significant effect on the oxidative stress system of plant cells (Panathula et al., 2014). In the present study, to construct an efficient genetic transformation system in rabbiteye blueberry, it confirmed that the selection concentration of Kan was 100 mg·L⁻¹, and the appropriate concentration of Cef was 300 mg·L⁻¹ (Table.2; Fig.2). The above results were also observed in studies of *Malus domestica* (An et al., 2019), *Vitis vinifera* (Zhang et al., 2023) and *Prunus persica* (Wang et al., 2024).

Previous researches have documented that shorter infection time will reduce the transformation efficiency of *A. tumefaciens*, which may be due to the fact that *A.tumefaciens* can't effectively infection. Conversely, low transformation efficiency was also ascribed to the significantly decreased vitality of the explants after infection, or even death (Sutradhar and Mandal, 2023; Qin et al., 2023). Short-term co-cultivation highly decreased transformation efficiency; and the observed low efficiency might be attributed to cellular damage or the formation of an unfavorable microenvironment (Zhang and Zhi, 2021; Sutradhar and Mandal, 2023). In the current case, the best transformation efficiency was reduced when the infection time was investigated from the 10 min infection, when co-cultivation time strayed from 3 days (Table.S4). Moreover, variance analysis declared that the infection time was the most

important factor for the transformation efficiency of rabbiteye blueberry (Table.3; Table.4). These results corroborate those observed in other horticultural crops such as *Pyrus armeniacaefolia* (Wang et al., 2021) and *Prunus persica* (Wang et al., 2024). Currently, the transformation efficiency got the ceiling (10.95%) from the methodology with an OD₆₀₀ value of 0.6, 10 min infection, and 3 days co-cultivation (Table.S4). This genetic transformation system were simple and easy to operate is superior to the transformation verification methods of *Vaccinium corymbosum* VcMYB4a (Zhang et al., 2020), *Vaccinium myrtilus* VcMYBA1 (Song et al., 2020), and *V. corymbosum* VcCHS (Qin et al., 2023). Furthermore, this transformation efficiency is markedly higher than that of *V. corymbosum* (Song et al., 2004) and *V. myrtilus* (Song et al., 2023). Therefore, the genetic transformation system optimized and established in this study is suitable for breeding new rabbiteye blueberry varieties with high nutritional value. Nevertheless, the intrinsic unique genetic traits of woody plants act as a key factor, leading to a lack of universality in genetic transformation systems across different varieties (Litz and Padilla, 2012; Orbović, 2019).

4.2 The VdMYB56-overexpressing adventitious buds of rabbiteye blueberry may facilitate the breeding in anthocyanin biosynthesis

To data, heterologous genetic transformation is the main method to identify the function of important genes in woody plants due to low efficiency in homologous transformation. For example, the role of *Actinidia chinensis* AcMYB75 (Li et al., 2017) and *Gerbera hybrida* GhMYB1a (Zhong et al., 2020) in anthocyanin biosynthesis had both been confirmed *via* genetic transformation method in model plants, *e.g.*, *A.thaliana* and *Nicotiana tabacum*. However, the transformation results may deviate or interfere with the function of the target genes, which may be attributed to the differences of regulatory networks among species, thereby reducing the accuracy of the functions of target genes (Zhang and Zhi, 2021). Meanwhile, homologous genetic transformation may substantially improve the accuracy of gene function verification and reliability (He et al., 2023; Gao et al., 2023). Therefore, the gene function validation in numerous woody plants is gradually transitioning from heterologous to homologous genetic transformation with the advances in optimizing regeneration methodology (Altpeter et al., 2016; Orbović, 2019). For instance, blueberry VcCHS promotes anthocyanin biosynthesis by homologous identification (Qin et al., 2023).

In the current case, the yellow, soft and loose callus from rabbiteye blueberry could be served as genetic transformation recipients (Fig.S1B, b). After the VdMYB56-transformed callus was further screened and cultured, the proliferating callus gradually changed from yellow to red (Fig.3E). Interestingly, in VdMYB56-overexpressing calli, the expression levels of VdMYB56 and Vd3GT was significantly higher than that of WT (Fig.3F, G). Further, the TAC and VdUFGT enzymatic activity of the VdMYB56-overexpressing calli also were significantly superior than that of the WT (Fig.3H, I). Therefore, VdMYB56 substantially up-regulates Vd3GT to elevate anthocyanin accumulation leading to form the red color in the transgenic callus. This result is consistent with the research results of Deng et al.(2026). Additionally, previous studies have found that there are significant differences in the molecular regulatory networks of MYB transcription factors involved in anthocyanin biosynthesis. For example, *Rosa hybrida* RcMYB1 (He et al., 2023) and

Camellia sinensis CsMYB75 (Zuo et al., 2025) can both activate the expression of the structural genes *DFR* and *ANS*, thereby positively regulating anthocyanin accumulation. Likewise, in *V. corymbosum*, VcMYB1 promotes anthocyanin biosynthesis by activating the expression of VcDFR (Wang et al., 2023c). In *Capsicum annuum*, CaMYB5 positively regulates anthocyanin biosynthesis by promoting the up-regulation of CaPAL expression (Zhou et al., 2025). In the present study, the 25-day-old VdMYB56-overexpressing calli were cultured and differentiated into red adventitious buds, sequentially, five VdMYB56-OE lines with red phenotypic feature were obtained herein (Fig.4A-D), and the expression of VdMYB56 and Vd3GT in transgenic lines were higher than those of the WT (Fig.4B, E). Further, the TAC and enzymatic activity of VdUFGT in VdMYB56-overexpressing lines attained markedly stronger than that of the WT (Fig.4F, G). Therefore, VdMYB56 promoted anthocyanin accumulation by activating the expression levels of Vd3GT in VdMYB56-OE adventitious buds of rabbiteye blueberry. Meanwhile, the gene VdMYB56 exhibits functions similar to those of *V. myrtillus* MYBA1 (Song et al., 2023) and *V. corymbosum* VcSRL12 (Li, H. X., 2021) in anthocyanin biosynthesis. Besides, the acquisition of five anthocyanin-rich VdMYB56-OE lines further confirms that VdMYB56 plays a positive regulatory role in anthocyanin biosynthesis of rabbiteye blueberry, and provides a new anthocyanin-rich germplasm.

Conclusively, the *Agrobacterium*-mediated genetic transformation system established herein was efficient, and the role of VdMYB56 in positively regulating anthocyanin accumulation was further justified; the VdMYB56-overexpression rabbiteye blueberries were obtained and demonstrated the enhanced anthocyanin contents (Fig.5). The optimized genetic transformation methodology furnishes an appropriate technique for new germplasm creation in blueberry, and the VdMYB56-overexpressing lines may facilitate the breeding in elevating anthocyanin.

5. Conclusions

In the present study, an effective genetic transformation system was established, as well as obtained the VdMYB56-overexpressing lines with elevated anthocyanin accumulation in rabbiteye blueberry. The red VdMYB56-overexpressing calli were obtained by utilizing the *Agrobacterium* -mediated genetic transformation system, which involves using *Agrobacterium* with an OD₆₀₀ value of 0.6 for a 10 min infection, followed by co-cultivation for 3 days. Subsequently, the transformation efficiency of 10.95% was consistent with that of the red VdMYB56-overexpressing calli. The VdMYB56-overexpressing calli were then cultured on WPM + 2.5 mg·L⁻¹ TDZ + 0.2 mg·L⁻¹NAA + 300 mg·L⁻¹ Cef + 100 mg·L⁻¹ Kan for 45 days, and red VdMYB56-overexpressing adventitious buds were obtained. Simultaneously, in VdMYB56-overexpressing calli and adventitious buds, TAC and enzymatic activity of VdUFGT were superior to those of the WT, respectively; the expression levels of VdMYB56 and Vd3GT were as high as those of the WT, separately. Collectively, an efficient genetic transformation system was established, and the VdMYB56-overexpressing rabbiteye blueberry lines with enhanced anthocyanin content were gained herein, which provides an appropriate methodology for new germplasm development in rabbiteye blueberry.

Declarations

Conflict of interest

According to the authors, there are no financial interests or personal relationships that might have affected the work described in this paper.

Credit authorship contribution statement

Lin Deng: Writing – original draft, Validation, Methodology, Investigation, Data curation. **Luonan Shen:** Writing – review & editing, Investigation. **Kui Zhou:** Writing – review & editing. **Guang Qiao:** Writing – review & editing, Validation. **Yi Min:** Writing – review & editing, Methodology, Validation. **Lei Peng:** Writing – review & editing, Methodology, Validation. **Sulin Wen:** Writing- review & editing, Validation. **Huiying Chong:** Writing – review & editing, Validation. **Xiaopeng Wen:** Writing– review & editing, Funding acquisition, Conceptualization.

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Data availability

Upon request, the data will be provided.

Ethical applicable Not applicable.

Competing interests The authors declare no competing interests.

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Figures

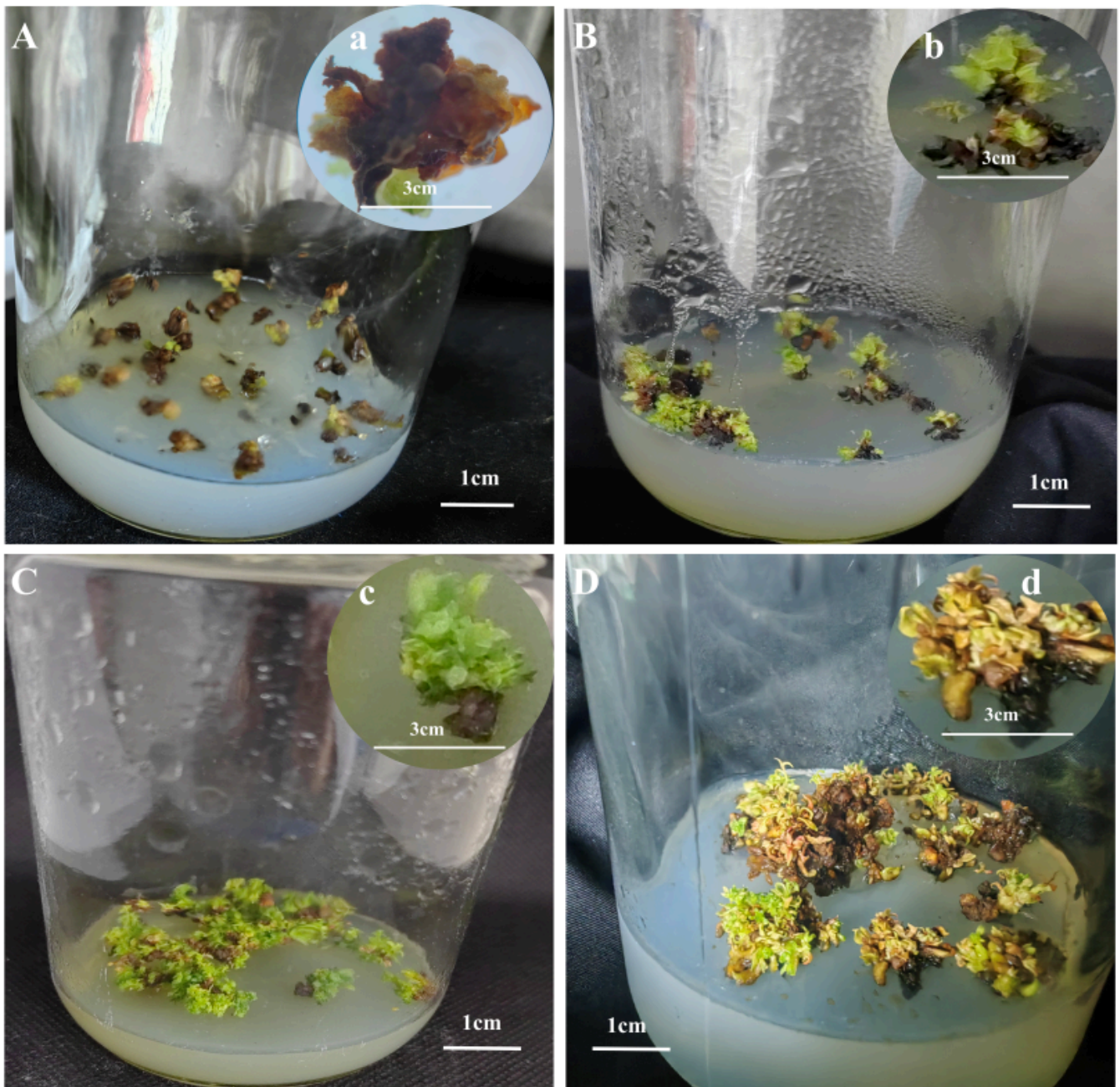


Figure 1

The growth status of buds under the condition different concentrations of TDZ and NAA. (A,a) The growth state of buds under induction with 1.5 mg·L⁻¹ TDZ and 0.20 mg·L⁻¹ NAA; (B, b) The growth status of buds under the conditions of 2.0 mg·L⁻¹ TDZ and 0.20 mg·L⁻¹ NAA; (C,c) The growth status of buds induced by 2.5 mg·L⁻¹ TDZ and 0.20 mg·L⁻¹ NAA; (D, d) The growth status of buds under the conditions of 3.0 mg·L⁻¹ TDZ and 0.20 mg·L⁻¹ NAA.

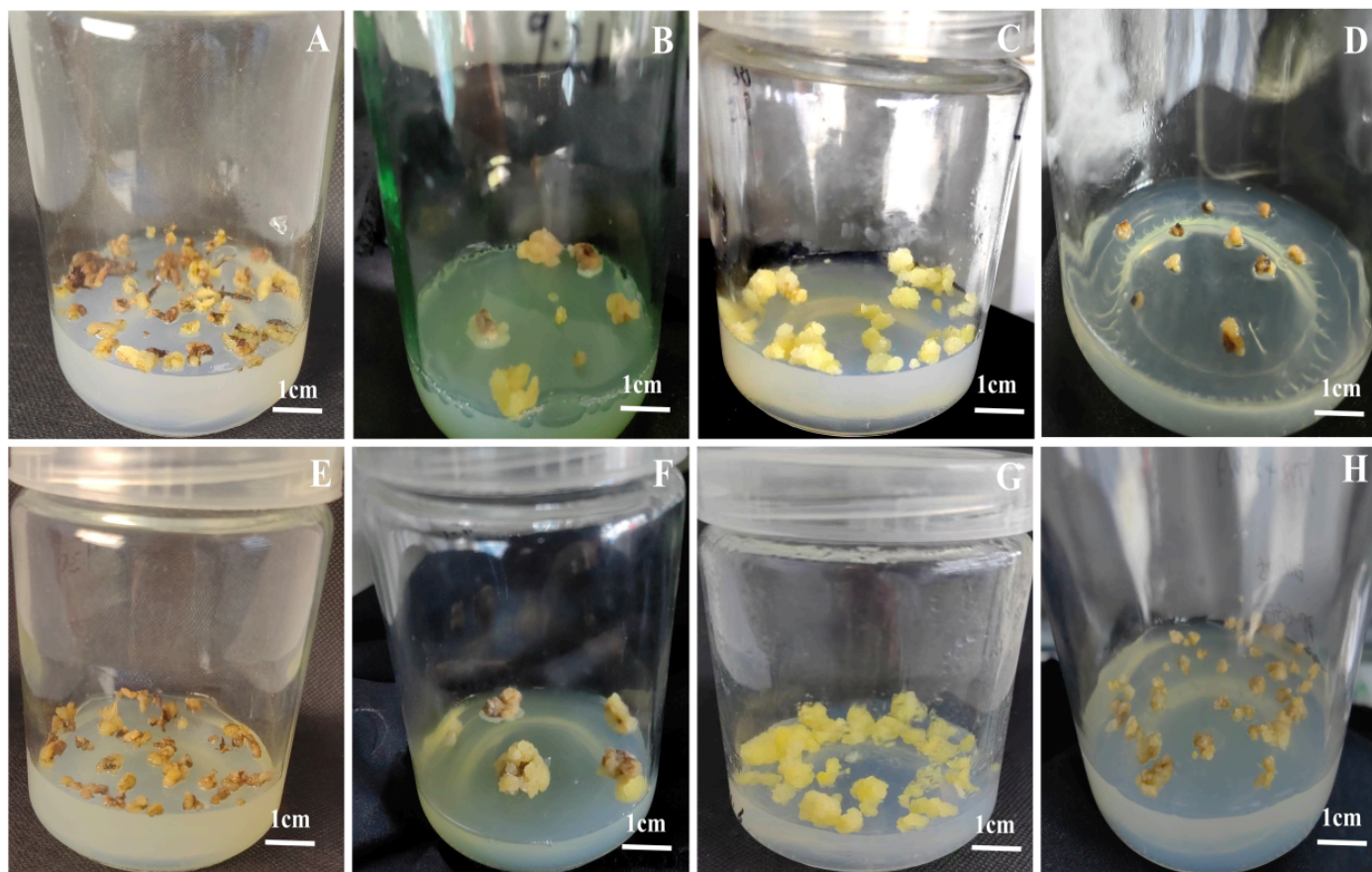


Figure 2

Effects of different concentration combinations of Kan and Cef on the growth of transgenic calli. (A) Effect of $100 \text{ mg}\cdot\text{L}^{-1}$ Kan and $100 \text{ mg}\cdot\text{L}^{-1}$ Cef on the growth status of transgenic calli; (B) The growth status of transgenic calli was influenced by the combination of $100 \text{ mg}\cdot\text{L}^{-1}$ Kan and $200 \text{ mg}\cdot\text{L}^{-1}$ Cef; (C) Effects of the combination of $100 \text{ mg}\cdot\text{L}^{-1}$ Kan and $300 \text{ mg}\cdot\text{L}^{-1}$ Cef on the growth state of transgenic calli; (D) The growth state of transgenic calli was influence by the combination of $100 \text{ mg}\cdot\text{L}^{-1}$ Kan and $400 \text{ mg}\cdot\text{L}^{-1}$ Cef ; (E) Effects of the combination of $60 \text{ mg}\cdot\text{L}^{-1}$ Kan and $300 \text{ mg}\cdot\text{L}^{-1}$ Cef on the growth of transgenic calli; (F) The growth state of transgenic calliEffect of the combination of $80 \text{ mg}\cdot\text{L}^{-1}$ Kan and $300 \text{ mg}\cdot\text{L}^{-1}$ Cef; (G) Effects of the combination of $100 \text{ mg}\cdot\text{L}^{-1}$ Kan and $300 \text{ mg}\cdot\text{L}^{-1}$ Cef on the growth of transgenic calli; (H) The growth status of transgenic calli was influenced by the combination of $120 \text{ mg}\cdot\text{L}^{-1}$ Kan and $300 \text{ mg}\cdot\text{L}^{-1}$ Cef.

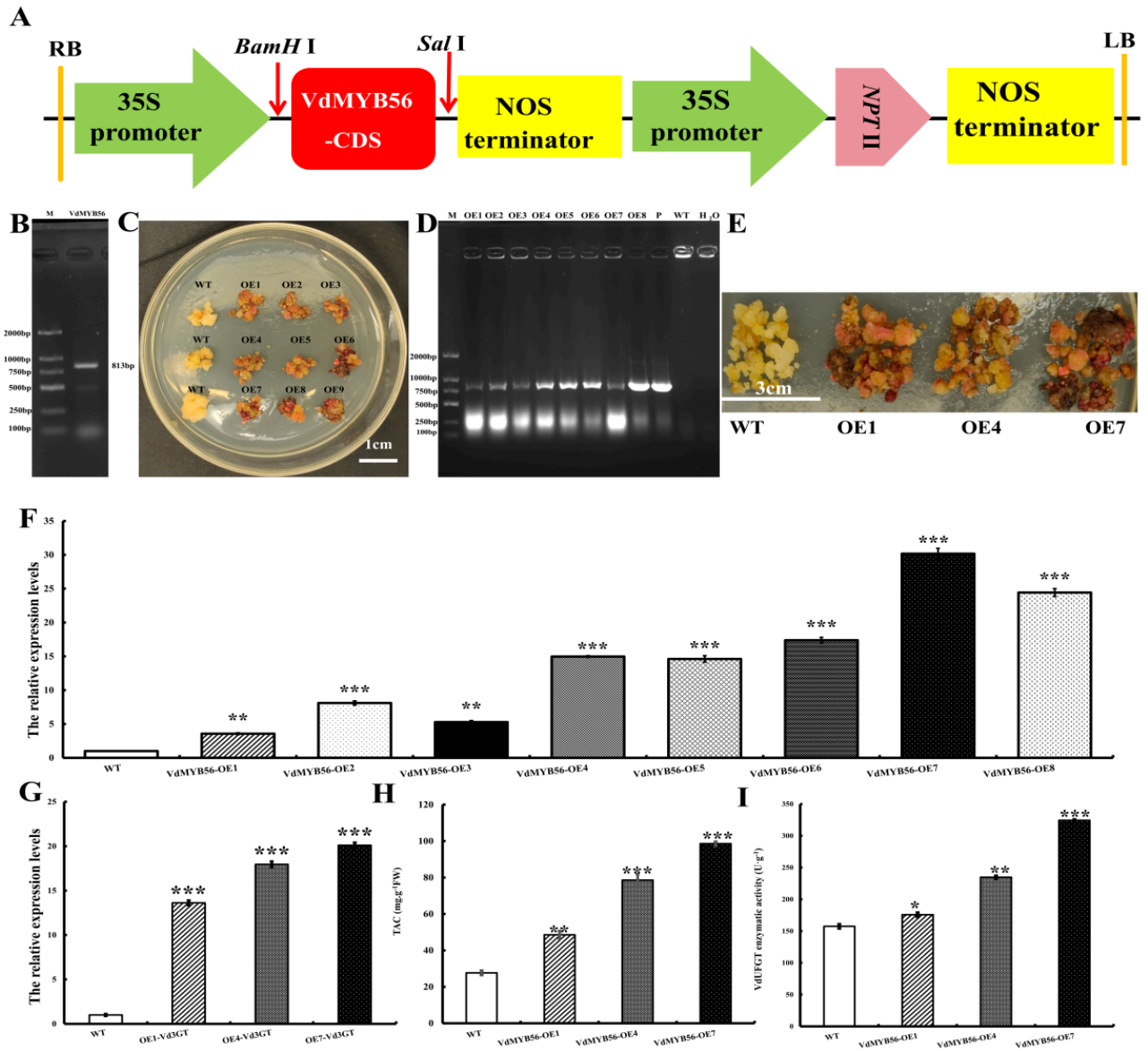


Figure 3

qRT-PCR verification, phenotype traits and total anthocyanin content (TAC) change trends of *VdMYB56* -overexpressing (*VdMYB56*-OE) callus. (A) Schematic diagram of *VdMYB56*-OE vector. (B) Cloning of *VdMYB56* gene. (C) *VdMYB56*-OE calli in rabbiteye blueberry. (D) PCR identification of *VdMYB56*-OE callus lines. (E) qRT-PCR verification of *VdMYB56*-OE rabbiteye blueberry calli. (F) Phenotypic traits of *VdMYB56*-OE calli in rabbiteye blueberry. (G) The expression level changes of *Vd3GT* in *VdMYB56*-OE calli. (H) TAC changes in *VdMYB56*-OE calli. (I) Changes in *VdUFGT* enzymatic activity of *VdMYB56*-OE calli. RB and LB are separately the right and left boundaries of the recombinant vector. NPT II is a screening marker gene used to detect transformed cells. WT: Wild type callus. M: Marker. P: *VdMYB56* plasmid. ddH₂O and WT were served as negative controls. OE-1 to OE-9 indicate eight positive transgenic lines. OE-1 to OE-8 represent eight positive transgenic lines. OE1-OE8: *VdMYB56*-overexpressing lines. "*" indicates a remarked difference ($p < 0.05$), "***" shows a significant difference ($p < 0.01$), "****" means a marketed difference ($p < 0.001$).

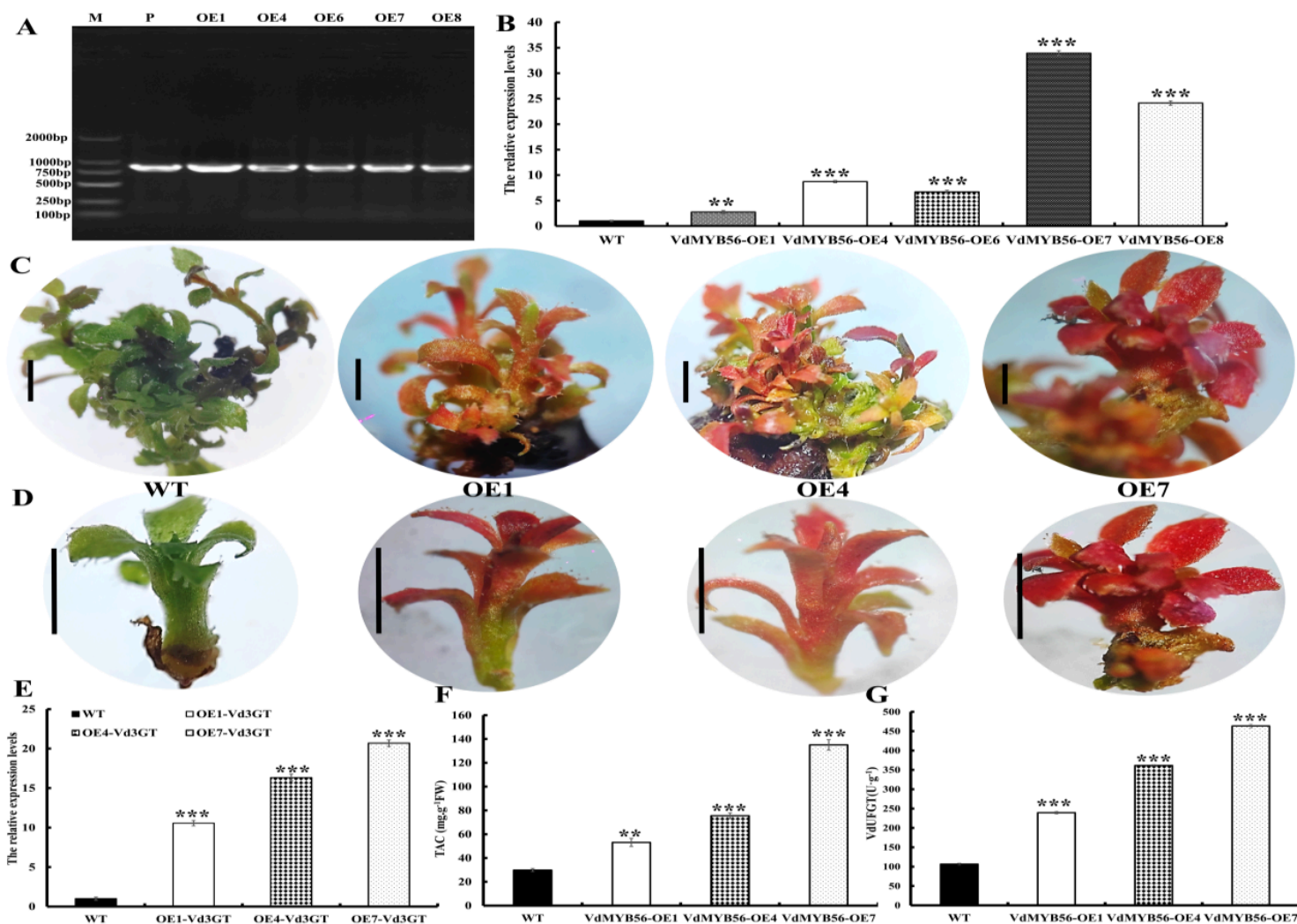


Figure 4

qRT-PCR verification, coloration changes, *Vd3GT* expression levels, and physiological index determination and analysis of *VdMYB56*-overexpressing (*VdMYB56*-OE) adventitious buds. (A) qRT-PCR verification of *VdMYB56*-OE adventitious buds. (B) Expression levels of *VdMYB56* in *VdMYB56*-OE adventitious buds. (C) The phenotypic trait of WT and *VdMYB56*-OE adventitious buds on 45-day-old. (D) Phenotypic trait of WT and *VdMYB56*-OE adventitious buds. (E) Changes in the expression level of *Vd3GT* in *VdMYB56*-OE lines. (F) The variation pattern of TAC in *VdMYB56*-OE adventitious lines. (G) The change of *VdUFGT* enzymatic activity in *VdMYB56*-OE adventitious buds. WT: Wild type. M: Marker. OE1, OE4, OE6, OE7, OE8: *VdMYB56*-OE adventitious buds. C: Bar = 100μm; D: Bar = 500μm. According to Duncan's test, means $\pm$ SD were labeled with different significantly at the $p < 0.05$ level. "*" indicates a difference ($p < 0.05$), "**" represents a notable difference ($p < 0.01$), "***" means more obvious difference ($p < 0.001$).

