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Physiological and Molecular Basis of Delayed Bud Dormancy Release by Exogenous Ethylene Treatment in Blueberry

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Abstract

Global warming leads to premature dormancy release and untimely flowering in southern highbush blueberry during winter, resulting in chilling injury and yield losses. However, effective strategies to delay flowering by modulating dormancy progression without compromising fruit quality remain lacking. This study demonstrated through field trials that spraying 1 mg/mL ethephon (ETH) during the early endodormancy stage effectively delayed dormancy release and reduced the bud break rate of spring shoots by approximately 33.92% relative to the control, with no adverse effects on fruit quality. The treatment also reduces sucrose content in floral buds, a change potentially associated with dormancy maintenance. To explore the molecular basis of this process, we examined two ethylene-responsive transcription factors, VcERF112 and VcERF115, previously identified in our laboratory. Their expression was rapidly upregulated following ETH treatment. Heterologous expression of either gene in *Arabidopsis* delayed both seed germination and flowering, suggesting a conserved growth-suppressive function. Dual-luciferase reporter assays confirmed that VcERF112 and VcERF115 bind to the T2 region (−2310 to −1595 bp) of the *VcBRC1* (*VcBRANCHED1*) promoter and enhance its expression. In contrast, sucrose treatment suppressed *VcBRC1* expression. Collectively, these results propose that ethylene may sustain bud dormancy through a coordinated mechanism that operates independently of the classic abscisic acid (ABA)/gibberellins (GA) balance, a relationship not addressed in this study. This mechanism involves the induction of *VcERF112/115* to activate *VcBRC1*, coupled with the reduction in sucrose levels to alleviate its repressive effect on *VcBRC1*. These findings provide new molecular insights into the ethylene-mediated regulatory network underlying bud dormancy in blueberry.

Keywords: *Vaccinium*; endodormancy; ethephon; sucrose; *ERF*; *BRC1*

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1. Introduction

Blueberry is a perennial shrub with high economic value, cultivated worldwide for its fruits rich in anthocyanins and polyphenols [1]. Based on ecological habits and chilling requirements, blueberry cultivars are classified into several groups, including northern highbush, southern highbush, half-high, lowbush, and rabbiteye [2]. Among them, southern highbush blueberry is a major commercial group due to its low chilling requirement [3],

large fruit size, and high yield potential. However, in the context of global warming, frequent warm winters in the Jiangnan region of China have induced early winter flowering in leading cultivars such as 'Emerald'. This phenomenon occurs when flower buds break endodormancy prematurely, leading to bloom periods that coincide with late frost events and result in severe blossom damage and yield loss. Extending bud dormancy to delay flowering has long been considered an effective strategy to avoid frost damage. Nevertheless, a core challenge for the industry remains how to precisely regulate the dormancy process while maintaining fruit quality, thereby developing effective flowering control strategies.

Plant bud dormancy is a complex physiological process regulated by both internal and external factors. It is primarily classified into three types: paradormancy, endodormancy, and ecodormancy [4]. Endodormancy, also known as true dormancy, is controlled by internal factors within the bud. Buds in this state cannot break dormancy even under favorable environmental conditions until their chilling requirement is satisfied [5]. Furthermore, the transition from dormancy release to flowering requires the accumulation of both chilling and heat units, known as chilling and heat requirements [6]. While models such as the 0~7.2 °C model and the Utah model are widely used to estimate chilling requirements, their applicability and accuracy across different geographical and climatic regions remain limited [7].

The induction and release of bud dormancy are regulated by environmental factors and endogenous hormone signals. Photoperiod serves as a key environmental cue for plants to perceive seasonal changes. In poplar (*Populus tremuloides*), for instance, short-day signals in autumn trigger the cessation of shoot growth by regulating the FLOWERING LOCUS T/CONSTANS (FT/CO) module [8,9]. Additionally, low temperatures induce the expression of core circadian clock components, such as LATE ELONGATED HYPOCOTYL 1/2 (LHY1/2) in poplar, which interacts with photoperiod signaling to integrate into the dormancy regulatory network [10,11]. Regarding endogenous hormones, the antagonistic balance between ABA and GA is well known to precisely regulate dormancy progression [12,13]. Meanwhile, dynamic changes in carbohydrate metabolism, particularly sucrose levels within buds, can serve both as an energy source and as signaling molecules. In grapevine (*Vitis vinifera* L.), for example, bud dormancy release is accompanied by significant sucrose accumulation. This sucrose acts as a signal to promote polar auxin transport, thereby driving bud break, demonstrating the key regulatory role of sugar signaling [14]. Notably, ethephon, an ethylene-releasing compound, shows potential in regulating plant dormancy. For example, in the woody plant peach (*Prunus persica*), ethephon delays budburst to avoid early spring frost damage [15]; similarly, in another woody fruit tree, litchi (*Litchi chinensis*), foliar application of ethephon can induce bud dormancy and up-regulate dormancy-related genes [16]. In contrast, in potato (*Solanum tuberosum*) tubers, ethephon breaks dormancy by remodeling the GA/ABA balance, activating reactive oxygen species signaling, and enhancing energy metabolism [17]. This divergence in regulatory function may originate from fundamental differences in dormancy physiology, metabolic demands, and environmental response strategies between above-ground bud organs and below-ground storage organs. Nevertheless, the precise molecular mechanisms underlying ethylene-mediated bud dormancy regulation in *Vaccinium* species, such as blueberry, remain largely unexplored.

Within the complex regulatory network of plant bud dormancy, diverse upstream signaling pathways ultimately converge onto key molecular nodes to coordinately regulate growth transitions. Among these, the transcription factor BRANCHED1 (BRC1), a core member of the TCP family, acts as a central signaling hub. Its expression is precisely regulated by multiple internal and external signals. Under low red/far-red light conditions, inactivated phytochrome B (PHYB) upregulates *BRC1* expression, thereby suppressing

axillary bud growth [18]. In rose (*Rosa hybrida*), darkness similarly induces high-level transcription of *RhBRC1* and inhibits bud outgrowth [19]. Beyond light signals, metabolic cues are deeply involved. Elevated sugar levels in buds correlate with decreased *BRC1* expression, and sucrose analogs (e.g., palatinose and turanose) can suppress its expression and promote bud break, confirming the key regulatory role of sugar signaling in bud dormancy [20,21]. Furthermore, BRC1 integrates multiple hormone signals, including cytokinins, strigolactones, and gibberellins, to precisely control the growth program within buds [22–24]. In blueberry, the *BRC1* homolog *VcTCP18* has been shown to regulate bud dormancy [25], strongly suggesting that *VcBRC1* is an ideal candidate for linking upstream complex signals to downstream growth responses. However, in blueberry flower buds, it remains unknown which upstream signals can directly respond to primary cues (such as hormones) and precisely initiate the transcriptional regulation of *VcBRC1*, and particularly, whether the key gaseous hormone ethylene is involved in this process.

The role of ethylene in regulating plant dormancy is complex and species-specific. Extensive studies have shown that ethylene is a key signal in breaking seed dormancy. For example, in *Arabidopsis thaliana*, ethylene can break seed dormancy even in cases of coat-imposed dormancy [26]. However, this germination-promoting mode is not universally applicable. Research in the perennial fruit tree lemon (*Citrus limon* L. Burm) revealed that the ethylene biosynthesis gene *CiACS4* delays flowering by suppressing gibberellin biosynthesis, highlighting a divergent role for ethylene in regulating plant developmental cycles [27]. Ethylene signaling output largely depends on its downstream AP2/ERF transcription factor family. These factors widely participate in plant growth, development, and abiotic stress responses by binding to cis-elements such as the GCC-box in the promoters of target genes [28–30]. Evidence indicates that ERF transcription factors directly regulate dormancy and germination. For instance, in *Arabidopsis*, *AtERF55* and *AtERF58* inhibit light-induced seed germination [31]. In European beech (*Fagus sylvatica*), *FsERF1* expression is minimal in dormant embryos but increases during moist-chilling, thereby breaking dormancy [32]. In sunflower (*Helianthus annuus* L.), *ERF1* expression is five-fold higher in non-dormant embryos compared to dormant ones, and its expression is significantly stimulated by HCN, which promotes dormancy break [33].

Numerous studies have demonstrated profound similarities in the regulatory mechanisms underlying seed dormancy and bud dormancy. Both processes share core physiological and molecular frameworks, exhibiting a high degree of conservation—particularly in hormonal dynamics, cell cycle reactivation, and the perception of and response to environmental signals such as low temperatures [34]. In perennial fruit trees such as peach, chilling accumulation during seed stratification and endodormancy release in floral buds is accompanied by coordinated changes, including the attenuation of ABA signaling, enhancement of GA signaling, and altered expression of cell cycle-related genes [35]. These parallels provide a theoretical foundation for using model plant systems—such as *Arabidopsis thaliana* for seed dormancy studies—to explore the mechanisms underlying bud dormancy in woody plants.

Building on the conserved regulatory framework described above, and inspired by the finding that *CiACS4* in citrus delays flowering by suppressing gibberellin biosynthesis through ethylene [27], we propose that in perennial woody plants, ethylene may similarly antagonize certain germination-promoting factors and thereby extend dormancy. Based on this, we hypothesize that a transient ethylene signal during early endodormancy in blueberry may also prolong floral bud dormancy. To test this, the present study combines field treatments with ethephon to examine whether ethylene delays budbreak and modulates sucrose levels, while employing heterologous expression and promoter analysis to validate the activation of *VcBRC1* by VcERF112/115.

2. Materials and Methods

2.1. Plant Materials and Experimental Design

This study used five-year-old plants of the southern highbush blueberry cultivars 'Emerald' and 'O'Neal', cultivated at the blueberry base of the Zhejiang Provincial Key Laboratory of Biotechnology for Specialty Economic Plants. During the experimental period, plants were maintained according to standard orchard management practices. Over three consecutive winters, samples were collected according to the schedule detailed in Table S1. Spring shoots (SS) and autumn shoots (AS) bearing flower buds, each approximately 20 cm in length, were collected for budbreak rate assessment, chilling requirement analysis, paraffin section observation, and physiological index measurement.

2.2. Budbreak Rate and Chilling Requirement Determination

Collected shoots with flower buds were placed in culture bottles containing 250 mM sucrose (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) solution and maintained in a growth chamber (23/20 °C, 16/8 h photoperiod, 75% relative humidity). The sucrose solution was replaced every two days, and budbreak was monitored throughout the incubation period. Budbreak was defined as the stage when flower buds swelled and showed visible green tip emergence. The budbreak rate was recorded accordingly [36]. Endodormancy release was considered achieved when the budbreak rate first exceeded 50% [37]. Chilling requirements were estimated using three different models: the 0~7.2 °C model, the 7.2 °C model, and the Utah model [7].

2.3. Paraffin Sectioning and Morphological Observation

Flower buds from SS and AS of 'Emerald' blueberry at different developmental stages were collected. The outer scales of the buds were carefully removed with forceps, and the samples were fixed in FAA fixative (containing 5% formaldehyde, 7% glacial acetic acid, and 88% ethanol at a concentration of 70%). Following fixation, the samples underwent dehydration through a graded ethanol series, clearing in xylene and embedding in paraffin. Sections of 10 μm thickness were prepared longitudinally and stained using the safranin-fast green method. After mounting with neutral balsam, the sections were observed and photographed under an inverted microscope to compare developmental differences among flower buds.

Formaldehyde (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), glacial acetic acid (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), ethanol (YONGHUA Chemical Co., Ltd., Changshu, China), xylene (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China).

2.4. Field ETH Treatment

On 20 October 2020, plants were sprayed with different concentrations of ETH (Beijing Green Agricultural Science and Technology Group Co., Ltd., Beijing, China) solution using a handheld sprayer to screen for the optimal concentration. The concentrations were set at 0 (control), 0.33, 0.4, 0.5, 0.67, 1, and 2 mg/mL. All solutions contained 0.1% (*v*/*v*) Tween-20 as a surfactant to ensure uniform adhesion and wetting on the surfaces of buds and leaves. For each concentration, ten plants with consistent growth status were sprayed until slight run-off was observed from the canopy. To avoid cross-contamination between treatments due to spray drift, the control and each concentration were applied in separate blocks. On 15 December of the same year, SS and AS bearing flower buds were randomly collected, each approximately 20 cm in length, for subsequent analysis. All shoot samples were randomly divided into three biological replicates and placed in a growth chamber under standard conditions: day/night temperature of 23/20 °C, relative humidity of 75% [38],

photoperiod of 16 h light/8 h dark, and a light intensity of 320 μmol·m^{-2}·s^{-1}. These samples were used for bud break rate assessment and sugar content measurement.

In 2021, to determine the optimal treatment timing, 1 mg/mL ETH solution was applied at different dormancy stages (from 24 September to 3 December, at 15-day intervals). The spraying method and solution preparation were the same as described above. On 17 December of the same year, SS and AS bearing flower buds were similarly collected and incubated in the growth chamber for bud break rate and sugar content determination.

2.5. Fruit Quality and Sucrose/Starch Content Measurement

Mature fruits harvested after ETH treatment were analyzed for fruit weight, longitudinal and transverse diameters, fruit shape index (calculated as the ratio of longitudinal to transverse diameter), soluble solid content, and titratable acid content. Fruit weight was measured using an electronic balance, while fruit dimensions were recorded with a digital caliper. Soluble solids and titratable acid contents were determined using a portable refractometer (PAL-BX | ACID7, ATAGO, Tokyo, Japan). Sucrose and starch contents were determined according to the method described in [39].

2.6. RNA Extraction and Quantitative Real-Time PCR (qRT-PCR) Analysis

'Emerald' blueberry shoots that had entered endodormancy were treated with ETH. Flower bud samples were collected at 0, 12, and 24 h post-treatment and immediately frozen at −80 °C. Total RNA was extracted from blueberry flower buds using an improved CTAB method [40]. The cDNA, serving as a template for qRT-PCR, was reverse transcribed using the HiScript III RT SuperMix for qPCR (+gDNA wiper) obtained from Nanjing Nuoweizan Biotechnology Co., Ltd. Quantitative primers were designed using Primer Premier 5.0 software (Table S2) and synthesized by Tsingke Biotechnology Co., Ltd. (Hangzhou, China). A two-step amplification protocol was used for qRT-PCR. The blueberry *GAPDH* gene served as the internal control. Relative expression levels of *VcERFs* in flower buds were calculated using the 2ˆ(−ΔΔCt) method. Statistical significance was analyzed with SPSS Statistics 21, and graphs were generated using GraphPad prism 8 software.

2.7. Gene Cloning, Plant Transformation and Screening of Transgenic Lines

Wild-type (WT) and transgenic *Arabidopsis* seeds were germinated on 1/2 MS medium. After one week of incubation under controlled environmental conditions, seedlings were transplanted to soil. Subsequent growth was monitored under a 16/8 h light/dark cycle, 23/20 °C day/night temperatures, and 70% relative humidity.

Full-length *VcERF112* and *VcERF115* sequences were amplified by PCR using blueberry flower bud cDNA as a template, with primers listed in Table S3. The amplified cDNA fragments were cloned into the pMD19-T vector and transformed into *Escherichia coli* DH5α competent cells. After sequence verification, the coding sequences were subcloned into the pCAMBIA1300-35S-GFP overexpression vector. The recombinant expression vector was constructed through restriction digestion, ligation, and transformation steps and subsequently introduced into *Agrobacterium tumefaciens* GV3101. The recombinant vector was transformed into *Arabidopsis* (Col-0) via the floral dip method [41]. T0 transgenic plants were selected on hygromycin-containing medium, and homozygous lines (T3) were subsequently isolated. Seed germination rate, flowering time, rosette leaf number and other phenotypes were observed and recorded.

2.8. Dual-Luciferase Reporter Assay and Promoter Analysis

To analyze the activity of the *VcBRC1* promoter and its response to low temperature and ethylene signals, and to verify the regulatory roles of VcERF112 and VcERF115 on the *VcBRC1* promoter, the full-length *VcBRC1* promoter and its deletion fragments (−2860

to −2310 bp, −2310 to −1595 bp), designated as T1 and T2, respectively (Figure S1), were constructed. The full-length promoter (pro*VcBRC1*, −2860 to −1 bp) and truncated promoter sequences (p1*VcBRC1*, −2310 to −1 bp; p2*VcBRC1*, −1595 to −1 bp) were cloned (primers listed in Table S4) into the pGreenII 0800-LUC vector. The T2 fragment was also cloned into pGreenII 0800-LUC, while *VcERF112* and *VcERF115* were cloned into the pGreenII 0029 62-SK vector. The reporter and effector constructs were co-infiltrated into leaves of tobacco via *Agrobacterium*-mediated transient transformation. After infiltration, tobacco plants were subjected to low-temperature (4 °C) treatment, ETH spray treatment, or maintained at room temperature as a control. Samples were collected 72 h post-treatment to measure LUC and REN luminescence intensities. The LUC/REN ratio was used to evaluate promoter activity and transcription factor regulatory effects.

2.9. Quantitative Analysis of VcBRC1 Expression Under Sucrose Treatment

The basal ends of 'Emerald' blueberry shoots were immersed in 250 mM sucrose solution, using water as a control. After 24 h of treatment, flower buds were collected for RNA extraction and subsequent cDNA synthesis. The expression change in VcBRC1 was detected by qRT-PCR using the method described in Section 2.6 to analyze the regulatory effect of sucrose on *VcBRC1* expression.

2.10. Data Analysis

Experimental data were analyzed for significant differences using SPSS Statistics 21. Prior to analysis, data normality was confirmed by the Shapiro–Wilk test ($p > 0.05$), and homogeneity of variance was verified by Levene's test ($p > 0.05$). Statistical significance was assessed by an independent samples *t*-test (confidence interval = 95%) using Statistics 21 software. Significant differences are indicated at $p < 0.05$. Graphs were generated using GraphPad Prism 8.

3. Results

3.1. Early Flowering in 'Emerald' Blueberry and Differential Dormancy Release Between SS and AS

The 'Emerald' blueberry exhibits two distinct types of fruiting shoots. Shoots emerging from February to April are termed SS. SS typically carry more flower buds, exhibit curved growth, and have moderate growth vigor. The more upright shoots emerging from August to October are termed AS. AS generally bear fewer flower buds. Field observations revealed that flower buds on SS underwent early flowering throughout the winter (Figure 1A). In contrast, AS, being upright and developing later, were less prone to early flowering (Figure 1B). On 29 December 2020, the budbreak rate of flower buds on SS and AS was assessed in the field. The budbreak rate of SS flower buds reached 51.8%, significantly higher than the 16.2% observed for AS (Figure 1C).

The budbreak rate of flower buds on SS and AS increased progressively with the accumulation of chilling. Field data collected over three consecutive years showed that the budbreak rate of SS flower buds in 'Emerald' blueberry (an evergreen southern highbush type) consistently exceeded 50% in late November or early December, indicating endodormancy release. In contrast, the budbreak rate of AS flower buds surpassed 50% in early December, 7~15 days later than SS (Figure S2A,C,E). For 'O'Neal' blueberry (a deciduous southern highbush type), the budbreak rates of both SS and AS flower buds nearly all exceeded 50% in mid-December or later (Figure S2B,D,F). These results indicate that SS flower buds of 'Emerald' exhibit early flowering characteristics, whereas those of 'O'Neal' do not. To further investigate this difference, we subsequently determined the chilling requirements of SS and AS flower buds in blueberry.

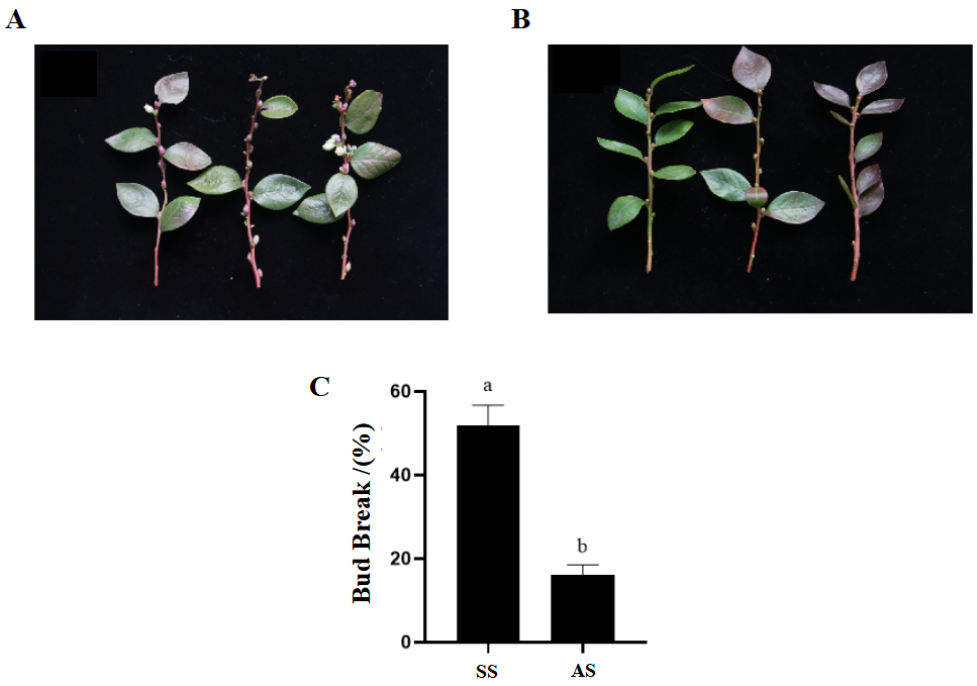

Figure 1. Budbreak status of flower buds on different shoot types in the field, December 2020. Budbreak of SS (**A**) and AS (**B**) flower buds in 'Emerald' blueberry; (**C**) Budbreak rates of SS and AS flower buds recorded on 29 December 2020. Data are presented as mean ± SD (n = 3). Significant difference analysis used Duncan's test, different lowercase letters indicate significant difference ($p < 0.05$).

Both 'Emerald' and 'O'Neal' are southern highbush cultivars. However, SS flower buds of 'Emerald' are prone to early flowering, whereas those of 'O'Neal' rarely flower in winter. These two cultivars were therefore selected for comparative analysis. Based on field temperature records, we estimated the chilling accumulation required for endodormancy release in their flower buds over three consecutive years (2020–2022) using three different models (Table 1). According to the Utah model, the chilling requirement for SS flower buds of 'Emerald' ranged from 0 to 47.9 C.U., while that for AS flower buds ranged from 76.5 to 137.5 C.U. during this period. For 'O'Neal', the chilling requirement for SS flower buds ranged from 137.5 to 381.7 C.U., and for AS flower buds from 243.5 to 454.5 C.U. The estimated chilling accumulation varied considerably across years, with ranges exceeding 100 C.U., indicating that the Utah model is unsuitable for accurate estimation of low-chill cultivars in the Central Zhejiang region. Using the <7.2 °C model, the chilling requirement for SS flower buds of 'Emerald' was estimated at 0–56.9 C.U., and for AS flower buds at 45.3–112 C.U., showing relatively minor variation. In contrast, for 'O'Neal', the chilling requirement for SS flower buds ranged from 116 to 229.3 C.U., and for AS flower buds from 164 to 272.7 C.U., with interannual variations exceeding 100 C.U.

Table 1. Chilling requirements of SS and AS flower buds in 'Emerald' and 'O'Neal' blueberry cultivars under different estimation models.

Year	Shoot Type	Cultivar	Bud Break (%)	Utah Model	0~7.2 °C Model	<7.2 °C Model
2020	SS	'Emerald'	66	0	0	0
		'O'Neal'	51.6	381.7	166	229.3
	AS	'Emerald'	67.8	84.9	13.7	45.3
		'O'Neal'	50	454.5	207.7	272.2
2021	SS	'Emerald'	58.2	47.9	56.9	56.9
		'O'Neal'	50.5	203.7	195.1	205.9
	AS	'Emerald'	50.7	76.5	100.4	109.6
		'O'Neal'	64.1	283.9	247.9	272.7
2022	SS	'Emerald'	50.1	0	49	49
		'O'Neal'	50	137.5	116	116
	AS	'Emerald'	50.8	137.5	112	112
		'O'Neal'	57.7	243.5	164	164

Note: All experiments were repeated three to four times. Based on field temperature records, the chilling accumulation required for endodormancy release in blueberry during 2020–2022 was quantified using three different chilling models.

According to the 0~7.2 °C model, the chilling requirements for SS flower buds of 'Emerald' blueberry were 0 C.U., 56.9 C.U., and 49 C.U. in 2020, 2021, and 2022, respectively. For AS flower buds of 'Emerald', the chilling requirements were 13.7 C.U., 100.4 C.U., and 112 C.U. over the same three years. For 'O'Neal' blueberry, the chilling requirements for SS flower buds were 166 C.U., 195.1 C.U., and 116 C.U. in 2020, 2021, and 2022, respectively, while those for AS flower buds were 207.7 C.U., 247.9 C.U., and 164 C.U. Under this model, both cultivars exhibited the smallest variation in chilling requirements for the same shoot type across the three years.

In summary, the 0~7.2 °C model demonstrated smaller interannual variation and greater stability in estimating chilling requirements for flower buds and can be considered the optimal model for evaluating blueberry chilling requirements in the Central Zhejiang region. Furthermore, regardless of the model used, the chilling requirements of SS flower buds in both 'Emerald' and 'O'Neal' were consistently lower than those of AS flower buds. Consequently, SS flower buds underwent earlier dormancy release. Among these, 'Emerald' SS flower buds exhibited the lowest chilling requirement, making them the most prone to breaking endodormancy and transitioning into ecodormancy.

Floral bud development stage significantly influences flowering time. Paraffin section analysis revealed that on 24 September 2021, pistil primordia were visible around the meristem of both SS and AS flower buds, with stigmas, styles, and ovaries present, and stamens surrounding the pistils. No significant morphological differences were observed between SS and AS at this stage (Figure S3A,B). By 29 October, both exhibited a complete floral structure with ovules visible inside the ovaries, and the difference remained minimal (Figure S3C,D). However, by 26 November, SS flower buds had broken endodormancy, showing significantly elongated styles and more advanced development compared to AS flower buds, which remained endodormant (Figure S3E,F). These results indicate that SS flower buds develop more rapidly, while AS flower buds require more chilling accumulation to break endodormancy, demonstrating a close relationship between floral bud development and chilling requirement.

3.2. Screening of Optimal Concentration and Application Timing for Suppressing Early Flowering with ETH

On 20 October 2020, blueberry plants in different treatment groups were sprayed with varying concentrations of ETH. After 56 days of treatment (15 December), shoots were collected and transferred to the laboratory for cultivation. Assessment of budbreak rate revealed that the SS flower buds in the control group exhibited a budbreak rate of approximately 68.87%, indicating the release of endodormancy. Only the 1 mg/mL and 2 mg/mL ETH treatment groups showed budbreak rates lower than that of the control (Figure 2A). No significant differences were observed between the control and the other treatment groups. AS flower buds responded to ETH treatment in a similar manner.

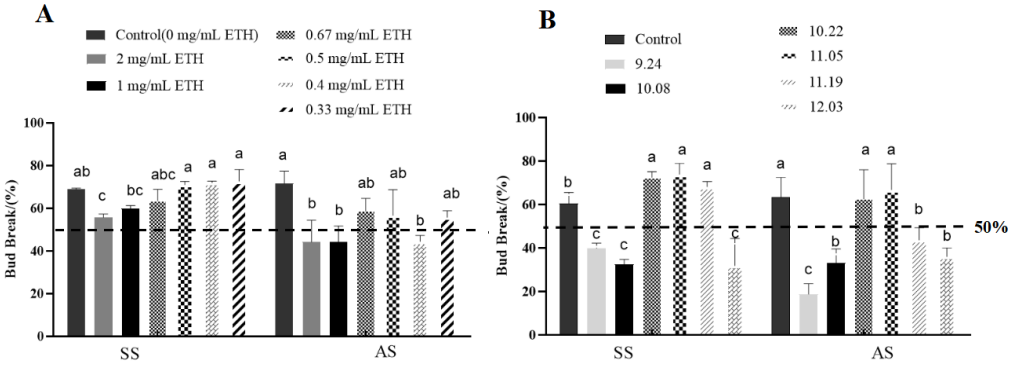

Figure 2. Bud break rate of 'Emerald' blueberry flower buds under ETH treatment. (**A**) Bud break rates under different ETH concentrations (Note: plants were sprayed on 20 October 2020, and shoots were uniformly collected after 56 days); (**B**) Bud break rates following ETH application at different time points (Note: ETH was applied at the six time points shown in 2021, and shoots were uniformly collected on 17 December of the same year). Data are presented as mean ± SD (n = 3). Significant differences were analyzed using Duncan's test; different lowercase letters indicate significant differences ($p < 0.05$). The horizontal dashed line indicates the 50% bud break threshold, above which endodormancy is considered released.

While both the 1 mg/L and 2 mg/L ETH treatments significantly suppressed bud break, field observations indicated that at the 2 mg/L concentration, 4–5 out of 10 monitored (approximately 45%), normally growing 'Emerald' blueberry plants exhibited symptoms such as shoot withering and apical necrosis within 20 days after application. In contrast, no obvious phytotoxicity was observed at the 1 mg/L concentration or lower. Therefore, 1 mg/L was identified as the optimal, non-phytotoxic dosage and was consistently used in all subsequent experiments.

Statistical analysis showed that different concentrations of ETH treatments had no significant effects on fruit weight, transverse diameter, longitudinal diameter, fruit shape index, or soluble solid content of the subsequent year's fruits from both SS and AS of 'Emerald' blueberry (Table 2). These results indicate that ETH application does not adversely affect blueberry fruit quality and can serve as an effective measure for controlling early winter flowering in blueberry.

To determine the optimal application timing for ETH, blueberry plants were treated with 1 mg/mL ETH at six time points spaced 15 days apart from 24 September to 3 December 2021. Shoots were collected on 17 December for indoor cultivation. Results showed that the budbreak rate of both SS and AS flower buds in the control group exceeded 50%, while the budbreak rate in the 24 September treatment group remained below 50%. For SS flower buds specifically, the budbreak rate was reduced by approximately 33.92% compared to the control. When ETH was applied on 3 December, some SS flower buds had already bloomed; although this treatment group exhibited a low budbreak

rate, its regulatory significance was limited (Figure 2B). These findings indicate that the late-September to early-October period, corresponding to the early endodormancy stage of flower buds, represents the optimal window for ETH application. Treatment during this period effectively prolongs endodormancy in both SS and AS flower buds, increases their chilling requirement, and thereby successfully suppresses early winter flowering.

Table 2. Effects of different ETH concentrations on fruit quality of 'Emerald' blueberry.

	Concentration/ (mg/mL)	Fruit Weight/(g)	Longitudinal Diameter/ (mm)	Transverse Diameter/ (mm)	Fruit Shape Index	Soluble Solid%
Fruits on SS	Control	2.34 ± 0.37 ^{a}	16.75 ± 1.77 ^{a}	13.31 ±0.74 ^{ab}	0.80 ± 0.09 ^{a}	9.43 ± 0.68 ^{a}
	1.0	2.47 ± 0.60 ^{a}	17.55 ± 1.05 ^{a}	13.45 ± 0.52 ^{a}	0.77 ± 0.04 ^{ab}	9.38 ± 0.68 ^{a}
	0.67	2.12 ± 0.38 ^{a}	16.86 ± 1.20 ^{a}	12.81 ± 0.68 ^{b}	0.76 ± 0.05 ^{ab}	8.85 ± 0.79 ^{a}
	0.5	2.18 ± 0.45 ^{a}	16.98 ± 1.17 ^{a}	12.69 ± 0.81 ^{b}	0.75 ± 0.03 ^{b}	8.93 ± 0.61 ^{a}
	0.4	2.18 ± 0.36 ^{a}	17.11 ± 1.12 ^{a}	12.72 ± 0.58 ^{b}	0.75 ± 0.04 ^{b}	9.03 ± 0.53 ^{a}
	0.33	2.11 ± 0.18 ^{a}	16.89 ± 0.66 ^{a}	12.97 ±0.48 ^{ab}	0.77 ± 0.03 ^{ab}	9.20 ± 1.41 ^{a}
Fruits on AS	Control	2.46 ±0.36 ^{abc}	17.51 ± 0.97 ^{b}	13.35 ±1.10 ^{ab}	0.76 ± 0.05 ^{a}	9.90 ± 0.90 ^{a}
	1.0	2.83 ± 0.54 ^{a}	18.62 ± 1.41 ^{b}	13.56 ± 0.67 ^{a}	0.73 ± 0.04 ^{a}	10.03 ± 0.26 ^{a}
	0.67	2.46± 0.39 ^{abc}	17.60 ± 1.12 ^{a}	13.37 ±0.67 ^{ab}	0.76 ± 0.04 ^{a}	9.27 ± 0.86 ^{a}
	0.5	2.48 ± 0.35 ^{ab}	17.86 ± 0.89 ^{ab}	13.38 ±0.69 ^{ab}	0.75 ± 0.04 ^{a}	9.38 ± 0.55 ^{a}
	0.4	2.27 ± 0.40 ^{bc}	17.24 ± 0.99 ^{ab}	12.94 ±0.87 ^{ab}	0.75 ± 0.03 ^{a}	9.85 ± 0.98 ^{a}
	0.33	2.07 ± 0.29 ^{c}	16.75 ± 1.16 ^{b}	12.77 ± 0.30 ^{b}	0.76 ± 0.05 ^{a}	9.70 ± 1.04 ^{a}

Note: Different letters within the same column indicate significant differences at the 0.05 level.

3.3. Dynamic Changes in Sugar Content in Flower Buds and Their Response to ETH Treatment

Sucrose and starch levels in flower buds were measured during dormancy release. In 'Emerald' blueberry, sucrose content in both SS and AS flower buds was highest during early endodormancy and gradually decreased as dormancy deepened. As chilling accumulated and endodormancy was released, sucrose content increased (Figure 3A), suggesting its involvement in dormancy release. Sucrose content in SS flower buds increased earlier and more markedly than in AS, potentially associated with early flowering. Before dormancy release, sucrose levels in SS were consistently lower than in AS, but this relationship reversed after release. Starch content showed an opposite trend: it accumulated during dormancy and declined after release, with SS flower buds maintaining higher starch levels than AS throughout (Figure 3B). These results indicate that chilling accumulation drives starch mobilization and sucrose synthesis, enhancing metabolic activity to provide the material and energy basis for budbreak.

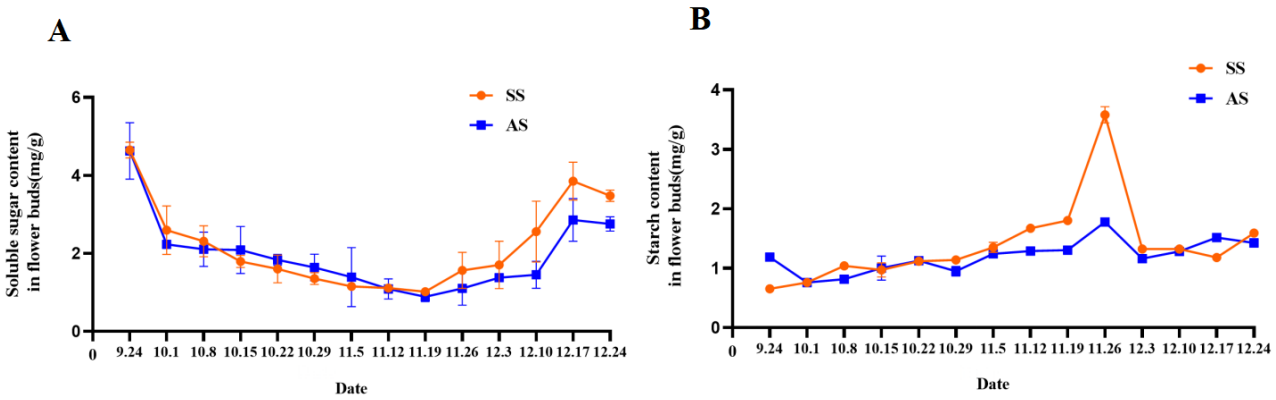

Figure 3. Changes in sucrose and starch content in flower buds of 'Emerald' blueberry. (**A**) Dynamic changes in sucrose content in flower buds at different stages; (**B**) Dynamic changes in starch content in flower buds at different stages. Data are presented as mean ± SD (n = 3).

To investigate whether ETH influences dormancy by regulating sugar metabolism, we analyzed its mechanism of action. Using the aforementioned SS and AS flower buds, we measured changes in sucrose content following ETH treatment. Results showed that when ETH was applied on 24 September (early endodormancy stage) and samples were analyzed two weeks later, sucrose content in treated flower buds was significantly lower than in the control (Figure 4). Further analysis of ETH application at different endodormancy stages revealed that sucrose levels in all treatment groups were generally lower than those in corresponding controls across most time points. Notably, spring shoot flower buds treated on 24 September exhibited the most pronounced reduction in sucrose content (Figures 3A and 5), which is consistent with the observed bud break rates. These results demonstrate that ETH treatment is associated with a decrease in sucrose content in blueberry flower buds. This physiological change may contribute to the ability of ETH to delay endodormancy release and suppress early flowering.

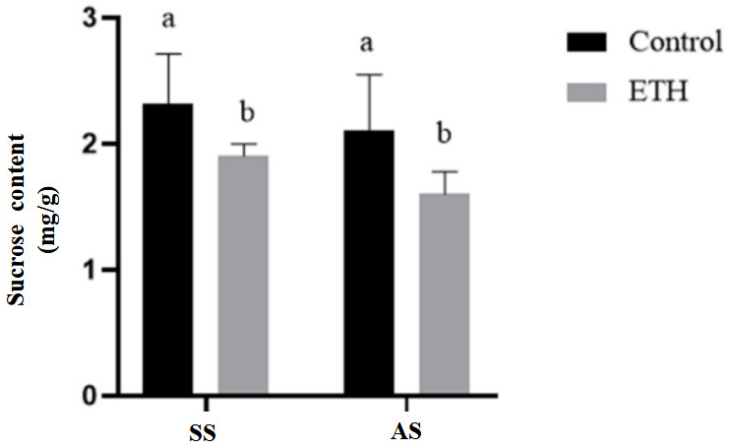

Figure 4. Changes in sucrose content in spring and autumn shoot flower buds of 'Emerald' blueberry two weeks after ETH treatment. Note: Treatment was applied on 24 September. Data are presented as mean ± SD (n = 3). Significant differences were analyzed using Duncan's test; different lowercase letters indicate significant differences ($p < 0.05$).

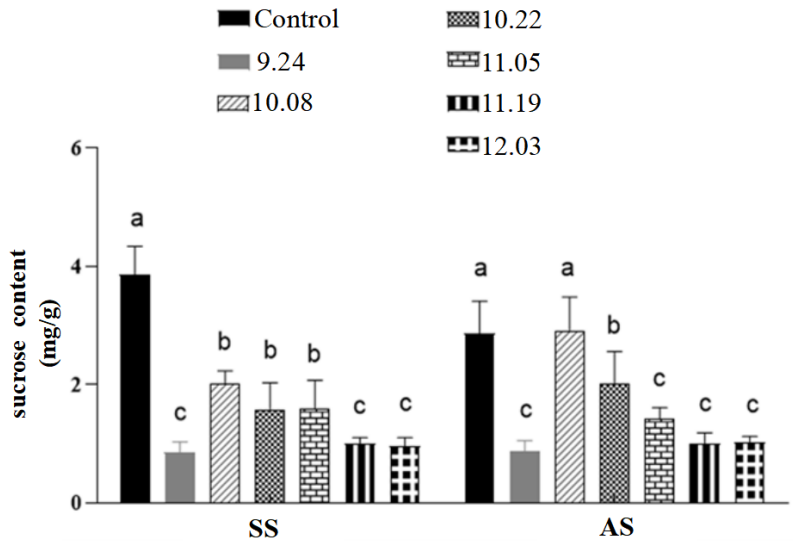

Figure 5. Changes in sucrose content in spring and autumn shoot flower buds of 'Emerald' blueberry following ETH treatment at different time points. Note: Sampling was conducted on 17 December. Data are presented as mean ± SD (n = 3). Significant differences were analyzed using Duncan's test; different lowercase letters indicate significant differences ($p < 0.05$).

3.4. Expression Patterns of VcERFs Before and After ETH Treatment

Based on our previous research, we focused on two AP2/ERF transcription factors, VcERF112 (VaccDscaff31-augustus-298.26) and VcERF115 (VaccDscaff33-processed-241.9), which are closely associated with ethylene signaling and ethylene content changes [25,42]. Our prior work confirmed that ethylene and *VcBRC1* play crucial roles in regulating blueberry bud dormancy, and suggested that VcERF112 and VcERF115 may function as ethylene response factors modulating *VcBRC1* expression [25]. To further investigate this potential regulatory mechanism, we selected these two genes for functional analysis.

Using cDNA from blueberry flower buds treated with ETH for 0 h, 12 h, and 24 h as templates, we analyzed the expression dynamics of these two genes before and after ethylene treatment by qRT-PCR. The results showed that both *VcERF112* and *VcERF115* responded to ETH, but with distinct temporal patterns: *VcERF112* expression increased significantly at 12 h post-treatment, while *VcERF115* showed an initial increase followed by a decrease within 24 h (Figure 6). These findings indicate that both *VcERF112* and *VcERF115* are likely involved in ethylene signal transduction and were therefore selected as candidate genes for further functional studies.

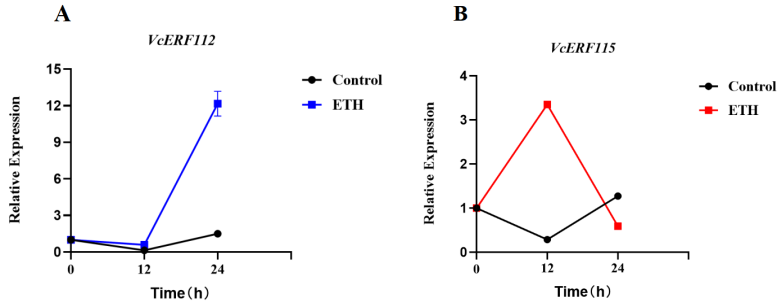

Figure 6. Expression levels of *VcERF112* (**A**) and *VcERF115* (**B**) in 'Emerald' blueberry flower buds at 0, 12, and 24 h after ETH treatment. Data are presented as mean ± SD (n = 3).

3.5. VcERF112 and VcERF115 Inhibit Seed Germination and Delay Flowering in Arabidopsis

Seeds of homozygous T3 transgenic *Arabidopsis* lines (verified by hygromycin selection) and WT were sown simultaneously on 1/2 MS medium. After 2 days of cold treatment, they were transferred to normal growth conditions for germination monitoring. WT seeds reached approximately 45% germination at 36 h, while transgenic seeds showed only about 25% germination (Figure 7), indicating that both *VcERF112* and *VcERF115* inhibit *Arabidopsis* seed germination, with *VcERF115* exhibiting a slightly stronger effect than *VcERF112*. Compared to WT, *Arabidopsis* plants overexpressing *VcERF112* or *VcERF115* bolted approximately 6 days later, displaying a late-flowering phenotype (Figure 8). Further comparison revealed that at bolting, WT *Arabidopsis* had significantly fewer rosette leaves than transgenic lines overexpressing *VcERF112* or *VcERF115* (Figure 9).

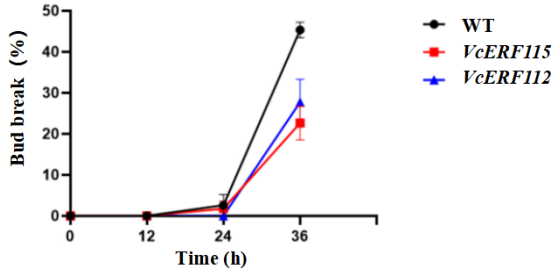

Figure 7. Seed germination rates of WT and overexpressed *VcERF* in *Arabidopsis*. Data are presented as mean ± SD (n = 3).

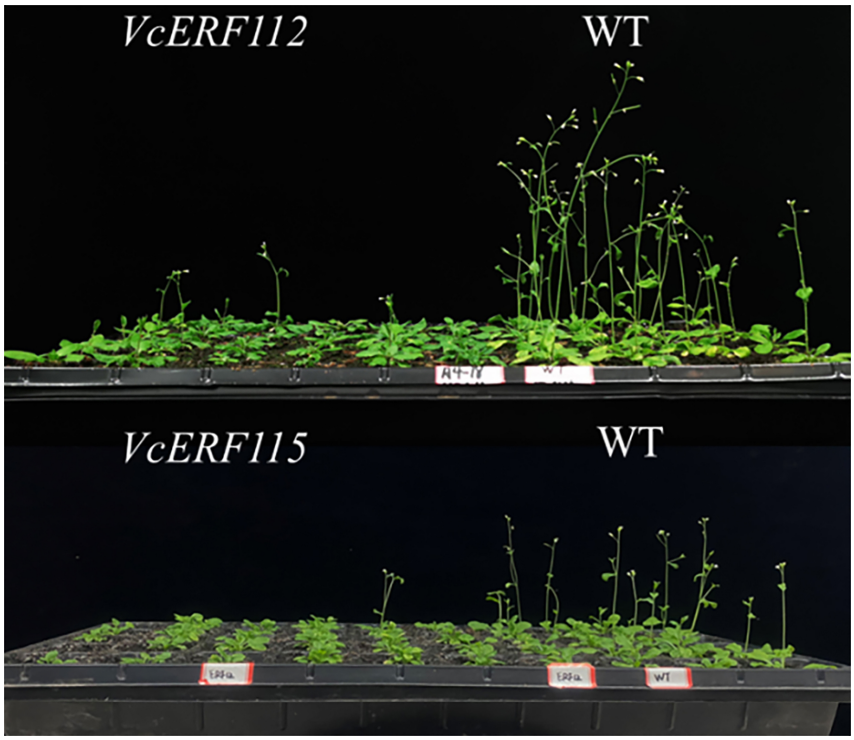

Figure 8. Comparison of flowering time between WT and overexpressed *VcERF Arabidopsis*.

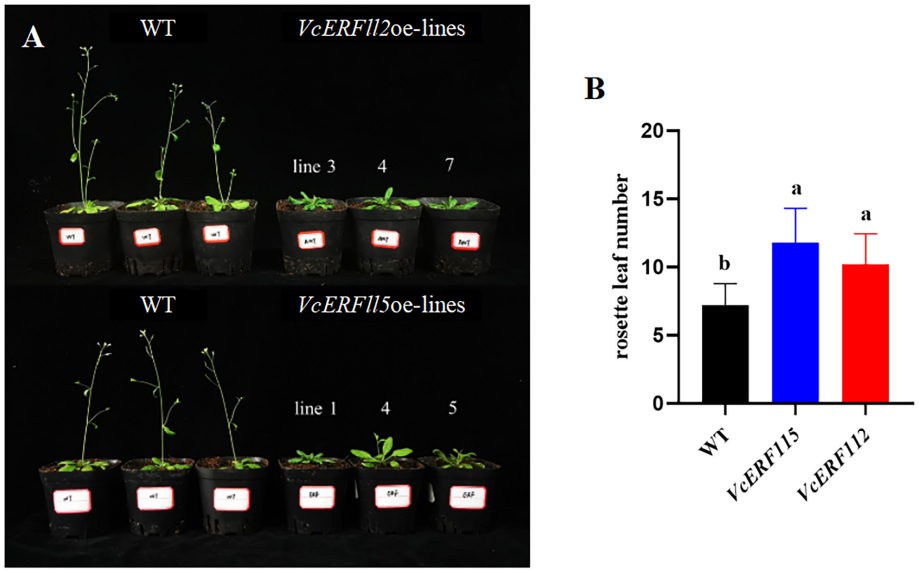

Figure 9. Comparison of the number of *Arabidopsis* rosette leaves between WT and overexpressed *VcERF*. (**A**) *Arabidopsis* rosette leaf phenotype; (**B**) The number of rosette leaves in *Arabidopsis thaliana*. Data are presented as mean ± SD (n = 3). Significant difference analysis used Duncan's test, different lowercase letters indicate significant difference ($p < 0.05$).

3.6. The VcBRC1 Promoter Responds to Low Temperature and ETH

To verify the transcriptional activity of the *VcBRC1* promoter fragments, *Agrobacterium* suspensions carrying the recombinant plasmids pro*VcBRC1*-pGreen0800-LUC, p1*VcBRC1*-pGreen0800-LUC, and p2*VcBRC1*-pGreen0800-LUC were injected into tobacco (*Nicotiana benthamiana*) leaves for transient expression. Luminescence detection showed that the *VcBRC1* promoter possesses transcriptional activity (Figure 10A). With progressive 5′ deletions, the LUC/REN ratios in tobacco leaves infiltrated with Pro*VcBRC1*, P1*VcBRC1*, and P2*VcBRC1* decreased significantly (Figure 10B), indicating that the truncated *VcBRC1* pro-

moters could initiate normal LUC gene expression but exhibited substantial differences in promoter activity as reflected by the LUC/REN ratios. To further substantiate the involvement of *BRC1* in low temperature-mediated dormancy release and to examine whether low temperature affects *VcBRC1* promoter activity, tobacco leaves infiltrated with different promoter constructs were treated at 4 °C for 72 h. Luminescence analysis revealed that LUC gene expression remained active but showed lower intensity compared to the room temperature control (Figure 10A). Further measurement of LUC/REN ratios demonstrated that the promoter activities of pro*VcBRC1* and p1*VcBRC1* were significantly suppressed under low temperature, while p2*VcBRC1* activity showed no significant change compared to the control (Figure 10B). These results indicate that the T1 (−2860 bp to −2310 bp) and T2 (−2310 bp to −1595 bp) regions are essential for the low-temperature response of the *VcBRC1* promoter. The findings indirectly confirm that low temperature inhibits *VcBRC1* expression, thereby reducing the suppression of bud break and consequently promoting dormancy release.

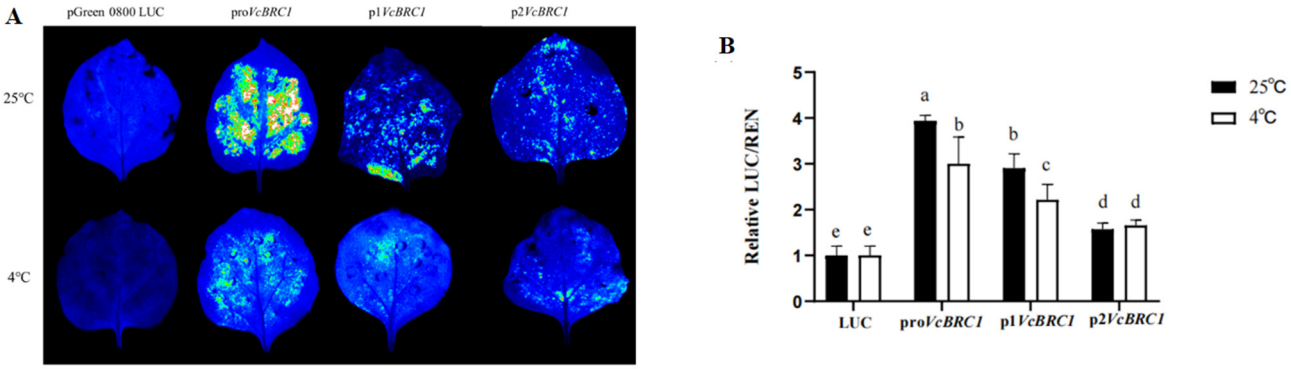

Figure 10. Analysis of low-temperature-responsive promoter activity of *VcBRC1*. (**A**) Luminescence imaging of transient expression of different *VcBRC1* promoter deletions under normal and low temperature conditions; (**B**) Relative luminescence intensity of different *VcBRC1* promoter deletions under normal and low temperature conditions. Data are presented as mean ± SD (n = 3). Significant difference analysis used Duncan's test, different lowercase letters indicate significant difference ($p < 0.05$).

To further elucidate the complex regulatory pathway from ethylene to bud break and verify whether *VcBRC1* is positively regulated by ethylene, tobacco leaves infiltrated with pro*VcBRC1*-LUC, p1*VcBRC1*-LUC, and p2*VcBRC1*-LUC were treated with ETH. After 3 days of cultivation, luminescence and dual-luciferase activity were measured. Results showed that ETH treatment significantly increased the LUC/REN ratio in leaves expressing pro*VcBRC1*-LUC and p1*VcBRC1*-LUC (Figure 11A,B), demonstrating that *VcBRC1* responds to ethylene.

3.7. Analysis of the Interaction Between the VcBRC1 Promoter and VcERFs

To further demonstrate that VcERF112 and VcERF115 directly regulate *VcBRC1*, *Agrobacterium* strains carrying *VcERF112*-pGreen0029 62-SK and *VcERF115*-pGreen0029 62-SK were co-infiltrated with *Agrobacterium* carrying *VcBRC1* T2-pGreenII 0800-LUC into the abaxial side of tobacco leaves. After 3 days of cultivation, luminescence detection and dual-luciferase assays were performed.

Luminescence results showed that compared to the empty vector control, tobacco leaves co-infiltrated with *VcBRC1* T2 and either *VcERF112*-pGreen0029 62-SK or *VcERF115*-pGreen0029 62-SK exhibited enhanced luminescence intensity (Figure 12A). Measurement of LUC/REN ratios revealed that leaves co-expressing *VcERF112*-pGreen0029 62-SK and *VcBRC1* T2 showed a 15.2-fold increase in the LUC/REN ratio compared to the control co-infiltrated with *VcBRC1* T2 and the empty pGreen62-SK vector. Similarly, leaves co-

expressing *VcERF115*-pGreen0029 62-SK and *VcBRC1* T2 exhibited a 6-fold increase relative to the control (Figure 12B). These results demonstrate that both VcERF112 and VcERF115 proteins can bind to the T2 fragment of the *VcBRC1* promoter and regulate its expression.

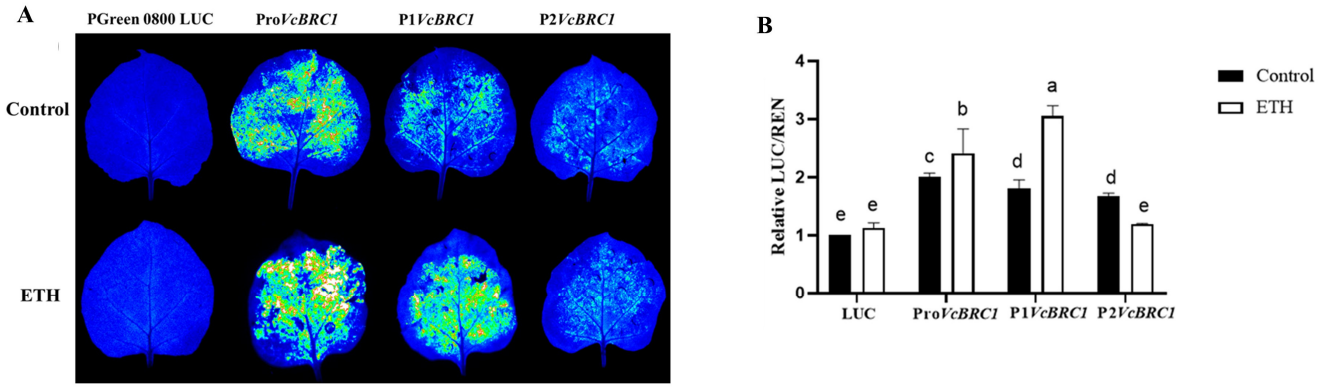

Figure 11. Analysis of ethylene-responsive promoter activity of *VcBRC1*. (**A**) Luminescence imaging of pro/p1/p2-*VcBRC1*-LUC after 3 days of ETH treatment. (**B**) Relative luminescence intensity of pro/p1/p2-*VcBRC1*-LUC after 3 days of ETH treatment. Data are presented as mean ± SD (n = 3). Significant difference analysis used Duncan's test, different lowercase letters indicate significant difference ($p < 0.05$).

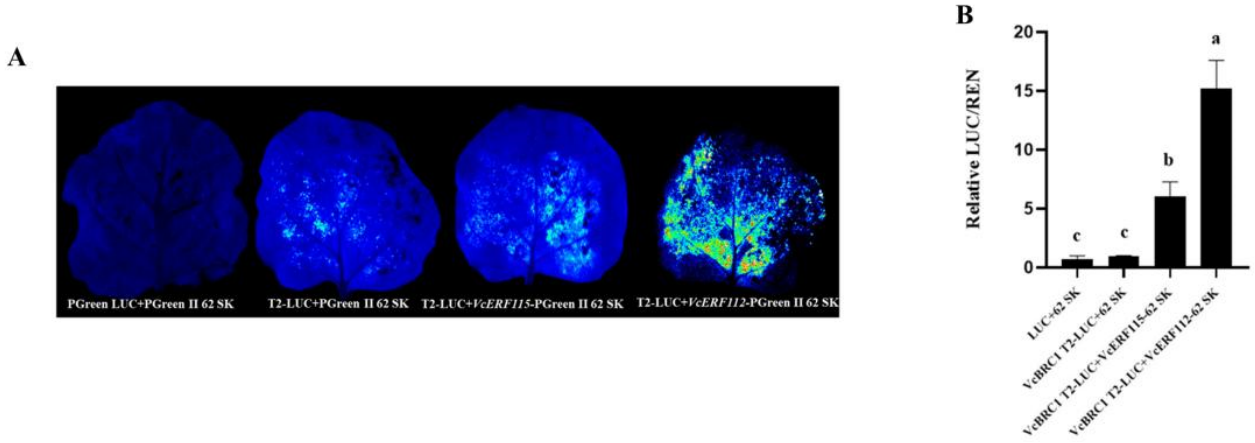

Figure 12. Analysis of the interaction between the *VcBRC1* promoter and VcERFs. (**A**) Luminescence imaging for interaction analysis between *VcBRC1*-T2-LUC and *VcERFs*-62SK. (**B**) Relative luminescence intensity for interaction analysis between *VcBRC1*-T2-LUC and *VcERFs*-62SK. Data are presented as mean ± SD (n = 3). Significant difference analysis used Duncan's test, different lowercase letters indicate significant difference ($p < 0.05$).

3.8. Expression Analysis of VcBRC1 in Response to Sucrose

Our previous results showed that ETH significantly reduces sucrose content in flower buds (Figures 4 and 5), a change closely associated with deepened dormancy. Since studies in *Arabidopsis* have reported that *BRC1* expression is sensitive to sucrose [43], we investigated whether sucrose affects *VcBRC1* expression in blueberry flower buds. To test this hypothesis, we conducted sucrose treatment experiments on blueberry shoots bearing flower buds. qRT-PCR analysis revealed that after 24 h immersion in 250 mM sucrose solution, *VcBRC1* expression levels decreased significantly by 37.8% compared to the water control (Figure 13), indicating that sucrose negatively regulates *VcBRC1* expression.

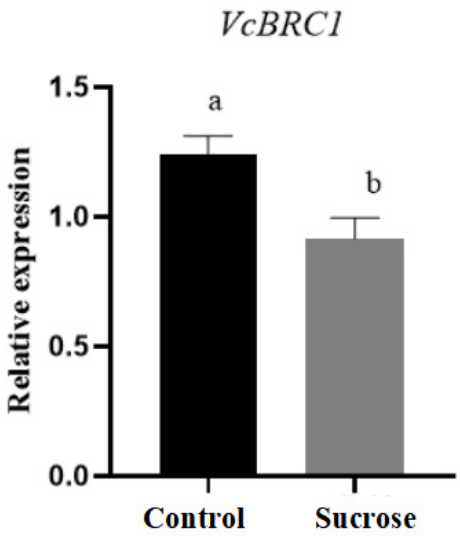

Figure 13. Relative expression level of *VcBRC1* in 'Emerald' blueberry flower buds after 24 h of sucrose treatment. Data are presented as mean ± SD (n = 3). Significant differences were analyzed using Duncan's test; different lowercase letters indicate significant differences ($p < 0.05$).

3.9. Putative Mechanism of ETH in Delaying Bud Dormancy in Blueberry

Based on the findings described above, we propose a preliminary working model for how ETH may regulate floral bud break in blueberry (Figure 14). The model integrates two potentially coordinated pathways. First, ETH treatment significantly reduces sucrose levels in floral buds, and separate sucrose-treatment experiments show that sucrose suppresses *VcBRC1* expression. Thus, ETH may indirectly attenuate the transcriptional repression of *VcBRC1* by lowering sucrose content. Second, ETH treatment rapidly induces the expression of *VcERF112* and *VcERF115*, and dual-luciferase reporter assays confirm that both proteins can activate the *VcBRC1* promoter, suggesting that *VcERF112/115* may function as transcriptional activators of *VcBRC1*. Collectively, these results indicate that ETH likely promotes *VcBRC1* expression through both pathways, which may together contribute to the observed suppression of bud break.

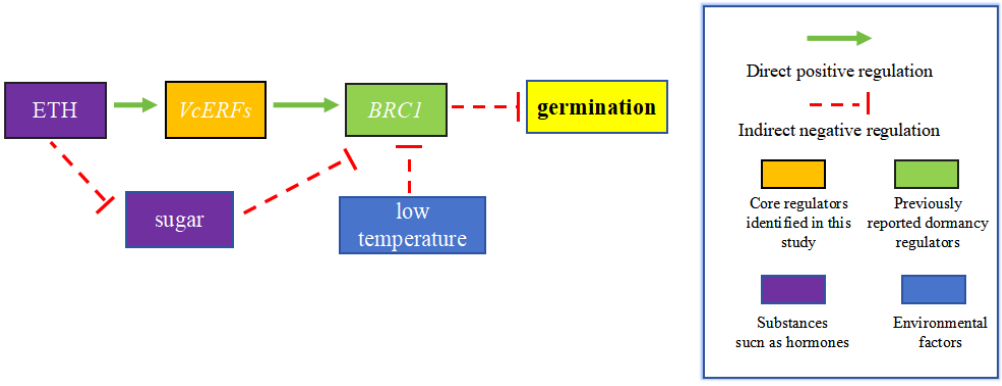

Figure 14. Schematic model illustrating the proposed mode of action of *VcERFs*. This diagram summarizes two molecular pathways that converged on *VcBRC1* and mediate ethylene regulation of bud break in 'Emerald' blueberry. Pathway 1 (Metabolic signaling route): Ethylene signaling may indirectly promote *VcBRC1* expression by negatively regulating sucrose content in buds, as sucrose itself represses the expression of the bud-break repressor *VcBRC1*. Pathway 2 (Direct transcriptional route): Ethylene directly up-regulates the expression of *VcERF112* and *VcERF115*, which act as transcriptional activators that directly enhance the transcription of their downstream target gene *VcBRC1*. Together, these two pathways lead to elevated *VcBRC1* expression, ultimately suppressing bud break.

4. Discussion

4.1. Chilling Requirement and Floral Bud Development

Global warming has created a compounding effect where the widespread advancement of fruit tree phenology coincides with frequent late spring frosts, making "early flowering frost damage" a significant threat to deciduous fruit production. Early flowering in fruit trees typically refers to bloom occurring before late spring frosts, where premature blossoming leads to frost injury, reducing fruit quality and yield. This phenomenon is not unique to blueberry but has also been reported in other deciduous fruit trees such as apple and pear [44]. The underlying mechanism is complex, involving interactions between environmental factors and genetic regulation [45].

The depth of endodormancy directly influences flowering time. After entering endodormancy, plants require sufficient chilling accumulation to break dormancy and resume normal growth; following dormancy release, a certain amount of effective heat accumulation is needed for budbreak and flowering [46]. Due to varying natural environments across regions, the timing of dormancy release and the specific chilling requirements for the same cultivar can differ considerably [47,48]. In this study, using temperature loggers, we compared the chilling accumulation required for endodormancy release in SS and AS flower buds of 'Emerald' and 'O'Neal'. The chilling requirement of SS flower buds was consistently lower than that of AS in both cultivars, leading to earlier dormancy release in SS. Notably, 'Emerald' SS flower buds had the lowest chilling requirement, making them most prone to breaking endodormancy and transitioning into ecodormancy. This aligns with previous reports that low-chill cultivars are more susceptible to dormancy release during warm winters [49]. Furthermore, these findings indicate that dormancy physiology can vary even within the same plant across different organs, providing new insight into the complexity of early flowering mechanisms.

Insufficient chilling accumulation during winter is a key environmental factor limiting normal floral organ development in perennial woody plants. In kiwifruit (*Actinidia chinensis* Planch. and *A. deliciosa* A. Chev.), warm temperatures during overwintering have been found to significantly reduce effective chilling accumulation, leading to delayed floral bud development and disrupted flowering phenology [50]. Similarly, when peach trees experience insufficient chilling, they exhibit retarded floral organ development, delayed flowering, and abnormal fruit morphology [51]. These studies collectively demonstrate that adequate chilling accumulation is essential for the transition of flower buds from dormancy to normal development. Our morphological observations support this conclusion: under the same environmental conditions, SS flower buds that had broken endodormancy showed significantly more advanced development of ovules and styles compared to AS flower buds that remained dormant. This strongly suggests that normal development of AS flower buds may require greater chilling accumulation and that floral bud morphogenesis is closely linked to the fulfillment of chilling requirements. Therefore, insufficient winter chilling directly delays endodormancy release and may cause delayed or abnormal floral organ development, ultimately affecting fruit tree yield and quality through multiple pathways.

4.2. Role of Sugars in Bud Dormancy

In perennial woody plants, bud dormancy release requires not only environmental signals but also the reactivation of internal metabolic activities. Among these, the dynamics of carbohydrate metabolism—including starch mobilization and the resynthesis of soluble sugars, particularly sucrose—are considered the central driving force [52]. Sucrose, as the primary soluble sugar, plays a crucial role in energy and carbon storage. Moreover, it acts as a signaling molecule regulating various physiological processes, such as flower bud differentiation and fruit development [53,54]. In flower buds of 'Cuiguan' pear (*Pyrus*

pyrifolia 'Cuiguan'), transcriptomic and proteomic analyses revealed that key genes in the sucrose metabolism pathway were significantly upregulated during dormancy release, promoting sugar accumulation and energy supply to drive budbreak [55]. Similarly, studies on *Lilium brownii* var. *viridulum* Baker bulbs showed high starch and low soluble sugar (sucrose, glucose, and fructose) levels during dormancy maintenance, followed by rapid starch hydrolysis and a simultaneous increase in sucrose, glucose, and fructose during dormancy release [56]. Our study observed a similar pattern in blueberry flower buds: sucrose levels increased significantly during dormancy release, accompanied by a decline in starch content. This indicates that starch hydrolysis provides the necessary substrates for sucrose synthesis and accumulation, which may represent a conserved metabolic feature during dormancy release across species. This process not only supplies essential carbon skeletons and energy for cell reactivation and floral organ development but also positions sucrose as an important signaling molecule regulating key developmental transitions.

Another important finding of this study is that sucrose accumulation in SS flower buds began earlier and was more pronounced than in AS flower buds. This temporal difference perfectly corresponds to the lower chilling requirement and stronger early flowering tendency of SS, suggesting that premature sucrose accumulation may be a key physiological signal determining early flowering in 'Emerald' blueberry. This pattern is corroborated by research on autumn flower formation in tree peony (*Paeonia suffruticosa* 'Ao-Shuang'), where early accumulation of sucrose and glucose was critical for successful induction of floral organ differentiation [57]. Together, these findings indicate that the temporal pattern of sugar accumulation likely serves as a determinant of flowering time and that SS flower buds may initiate this sugar metabolism program earlier, thereby establishing both the material and signaling basis for early flowering. The role of sucrose in this process is further elucidated in tree peony. Metabolomic analysis of dormancy transition in tree peony buds showed that sucrose was the most abundant component among 13 sugars measured. Its change during dormancy release did not follow a linear increase but exhibited a sharp peak, rising abruptly during mid-chilling and then declining rapidly [58]. This dynamic pattern suggests that sucrose may function not only as a basic carbon source and energy reserve for budbreak, but also that its concentration peak during a specific time window may itself constitute a critical endogenous signal triggering downstream budbreak programs.

4.3. Regulation of BRC1 by ERFs and Sucrose Metabolism

In woody plants, the TCP transcription factor BRC1 has been widely demonstrated as a key repressor controlling bud dormancy. BRC1 is renowned for its role in branching and functions by integrating various upstream signals that influence bud growth potential [18,22,59]. For instance, *Arabidopsis* mutants of *BRC1* in axillary buds exhibit increased bud outgrowth [60]. In hybrid poplar, *BRC1* expression in axillary buds is directly suppressed by *LAP1* (*Like-APETALA1*). Short-day conditions inhibit *LAP1* expression, thereby releasing this suppression and leading to *BRC1*-mediated growth arrest [59]. Another study showed that in poplar apical meristems, *SPL16/23* directly repress *FT2* expression while activating *BRC1* orthologs (*BRC1.1* and *BRC1.2*) to coordinate seasonal growth cessation in lateral buds [61]. In *Brassica napus*, WRKY28 directly targets multiple *BRC1* gene copies, suppresses their expression, and reduces ABA accumulation in axillary buds, thereby promoting dormancy release and excessive bud outgrowth [62]. Collectively, these studies illustrate that *BRC1* serves as an integration hub for multiple upstream signals (e.g., photoperiod, hormones) across species, precisely regulating bud dormancy and outgrowth. Our previous work also showed that heterologous expression of *VcBRC1* in *Arabidopsis* results in a late-flowering phenotype, further supporting its conserved role in growth repression [38].

ERF transcription factors, as key components downstream of ethylene signaling, play important roles in regulating plant dormancy and germination. However, their regulatory relationship with *BRC1* remains unclear in woody plants. In *Arabidopsis* seeds, *ERF50* was identified as a key germination promoter that reduces levels of the germination inhibitor *DOG1* through negative feedback and activates the expansin gene *EXPA2* by antagonizing *RGL2*, thereby coordinately promoting germination [63]. In maize (*Zea mays*), transcriptional repression of *ZmEREB92* under suitable conditions enhances expression of *ZmEIL7* and *ZmAMYa2*, promoting ethylene signaling and starch mobilization to facilitate timely germination [64]. In *Cymbidium sinense*, ERF transcription factors precisely regulate floral organ development and morphogenesis by directly controlling downstream *MADS-box* gene expression [65]. These studies suggest that ERFs may regulate dormancy processes through different target genes across species and tissues. This study employed the *Arabidopsis* model system to investigate the biological functions of the ethylene-responsive factors VcERF112 and VcERF115, identified in blueberry. As a perennial woody species, blueberry presents challenges for genetic transformation due to its low efficiency and extended experimental timelines [66,67]. In contrast, *Arabidopsis* serves as an efficient and well-established platform for rapid functional validation of plant genes [68]. Furthermore, the high conservation between seed dormancy and bud dormancy in hormone-regulatory networks and signal-integration mechanisms provides a theoretical rationale for using *Arabidopsis* seed germination and flowering time phenotypes to infer the potential roles of candidate genes in blueberry bud dormancy [69]. Our results showed that heterologous expression of either *VcERF112* or *VcERF115* significantly delayed seed germination and flowering in *Arabidopsis*. These observations suggest that *VcERF112* and *VcERF115* possess conserved growth-suppressive functions and may be involved in dormancy maintenance. It should be noted, however, that seed dormancy in *Arabidopsis* and floral-bud dormancy in blueberry represent distinct physiological processes, differing in organ ontogeny, predominant environmental cues, and certain aspects of their regulatory networks. Consequently, these heterologous phenotypes serve primarily as preliminary functional clues; the precise physiological roles and molecular mechanisms of these genes in blueberry floral buds remain to be directly validated in the native system.

Building on this knowledge, our study further reveals that *VcERF112* and *VcERF115* not only respond rapidly to ethylene signaling with upregulated expression but also directly bind to the T2 region of the *VcBRC1* promoter and activate its transcription. This T2 region also contains low-temperature response elements. This finding aligns with reports in poplar demonstrating that *SVL* regulates *BRC1* downregulation under low temperature, collectively forming a temperature-responsive transcriptional module involved in bud dormancy release [70]. This suggests that *BRC1*, as a conserved hub in dormancy regulation, may integrate dual inputs from both ethylene and low-temperature signals.

Beyond direct transcriptional regulation, our study reveals an additional pathway through which ethylene indirectly modulates *VcBRC1* expression via sucrose metabolism. ETH treatment significantly reduced sucrose content in flower buds, whereas sucrose treatment itself suppressed *VcBRC1* expression. This suggests that sucrose may act as a negative regulatory signal controlling *VcBRC1* transcription. It should be noted, however, that the inverse correlation between sucrose level and *VcBRC1* expression is currently based on phenotypic association and indirect evidence; direct causal linkage remains to be established through metabolic or genetic perturbation experiments. A key mechanistic question that arises is how ethylene mediates the decrease in sucrose content. This may involve two non-mutually exclusive routes. One possibility is a direct metabolic effect, analogous to the ethylene-induced respiratory climacteric in ripening fruits, whereby ethylene accelerates starch-to-soluble-sugar conversion and respiratory consumption of sugars [71]. Alterna-

tively, ethylene may exert an indirect signaling effect by reprogramming the expression or activity of key sucrose-metabolizing enzymes through downstream transcription factors, thereby shifting the balance between sucrose synthesis and degradation or altering transport efficiency. At present, direct evidence distinguishing these possibilities in blueberry buds is lacking. Relevant insights come from melon fruit, where the ethylene-responsive transcription factor CmERFI-2 relieves repression of sucrose-metabolism genes by inhibiting *CmMYB44* [72], illustrating how ethylene can reshape sugar homeostasis through transcriptional networks. Therefore, future work should proceed systematically on two fronts: First, respiratory rates, sucrose-metabolizing enzyme activities, and dynamic gene expression profiles should be measured in ethylene-treated blueberry flower buds to clarify the dominant pathway through which ethylene lowers sucrose levels. Second, metabolic or genetic perturbation experiments are needed to directly test in the native system whether sucrose signaling must act through *VcBRC1* to execute its dormancy-regulatory function. Together, such approaches will refine and ultimately establish the functional and causal relationships within the "ethylene–sucrose–*VcBRC1*" regulatory axis.

5. Perspectives

While this study reveals a novel mechanism by which ethylene regulates blueberry bud dormancy through the VcERF-*VcBRC1* module, several important questions remain to be explored. First, the interaction between ethylene signaling and the core ABA/GA hormonal module remains unclear. Concurrent profiling of the dynamics of these hormones together with the expression of key biosynthesis- and signaling-related genes would help delineate their hierarchical regulatory relationships. Second, validating the functions of *VcERF112* and *VcERF115* in their native context using a stable genetic transformation system in blueberry will be crucial for confirming the necessity of this regulatory pathway. As a complementary approach, transient gene-silencing techniques in blueberry flower buds could be prioritized for preliminary functional assessment, while continued efforts should be directed toward optimizing CRISPR/Cas9-mediated stable genome-editing systems to generate targeted mutants. Phenotypic analysis of ethylene responses in such genetic materials will ultimately establish the biological role of the VcERF-*VcBRC1* module. In addition, the phytotoxicity observed at higher treatment concentrations underscores the need for future studies to systematically determine the effective concentration threshold of ETH application, thereby achieving reliable dormancy delay while minimizing adverse effects on plant growth and health.

In southern highbush blueberry production regions with warm winters, applying 1 mg/L ethephon during the early endodormancy phase could be developed into a feasible agricultural strategy. This approach may enable growers to proactively adjust flowering timing, thereby reducing the risk of late-spring frost damage to blossoms under mild winter conditions without compromising fruit quality. However, before large-scale implementation can be recommended, further multi-year optimization and evaluation across other major cultivars and different climatic zones are necessary. This includes assessing potential cumulative effects on overall plant vigor and long-term reproductive performance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae12020154/s1, Figure S1: Diagram of *VcBRC1* promoter 5 '-end deletion; Figure S2: Budbreak rates of SS and AS flower buds in 'Emerald' and 'O'Neal' blueberry cultivars. (A,B) Budbreak rates of SS and AS flower buds in 'Emerald' and 'O'Neal' blueberry, 2020; (C,D) Budbreak rates of SS and AS flower buds in 'Emerald' and 'O'Neal' blueberry, 2021; (E,F) Budbreak rates of SS and AS flower buds in 'Emerald' and 'O'Neal' blueberry, 2022. The horizontal dashed line indicates the 50% budbreak threshold, above which endodormancy is considered released. Significant difference analysis used Duncan's test, different lowercase letters

indicate significant difference ($p < 0.05$). Data are presented as mean ± SD ($n = 3$). Figure S3: Morphological development of SS and AS flower buds in 'Emerald' blueberry. (A,B) Floral bud development observed on 24 September; (C,D) Floral bud development observed on 29 October; (E,F) Floral bud development observed on 26 November; Note: (A,C,E) represent SS; (B,D,F) represent AS. Table S1: Sequential sampling time points for blueberry flower bud dormancy study. Table S2: qRT-PCR primers sequence of related genes. Table S3: Full length cloned primer sequence of *VcERFs*. Table S4: Primer sequence for cloning.

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