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Review

Genetic Breeding to Improve Freeze Tolerance in Blueberries, a Review

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Abstract: The abiotic stresses associated with spring/fall freezes and extreme winter cold cause significant economic losses in blueberry production. These problems are exacerbated by climate change and increasingly erratic weather patterns. Developing freeze-tolerant blueberry cultivars with optimized cold hardiness, chilling requirement, and flowering and fruiting phenology holds promise for mitigating the risk of these weather-related damages. These weather-resilient cultivars will ensure the long-term productivity and sustainability of the blueberry industry. The focus of this review is to present the current understanding of the major components of genetic breeding for blueberry freeze tolerance, i.e., phenotyping, genotyping, genetic association analysis, and marker development. The advancement in gene regulation and corresponding proteomic changes upon cold acclimation, dormancy, de-acclamation, and flowering and fruiting aids in the understanding of the adaptive stress response in blueberries. A wide range of genetic diversity in freeze tolerance and phenological traits has been identified among cultivated and wild blueberry relatives. Significant efforts have been made to phenotype freeze tolerance, chilling requirement, and flower and fruit development in both field and controlled environmental conditions. Recent studies emphasize the need for high-throughput, image-based phenotyping of blueberry flower development to improve the precision and efficiency of selecting freeze-resilient genotypes. In addition, advancements in blueberry genomics and pangenome resources expanded the potential of variant calling and high-density linkage map construction. Genetic association studies have identified QTL regions linked to freeze tolerance in blueberries, providing valuable targets for selection. The implementation of these advanced genomic tools and high-throughput phenotyping methodology will accelerate the development of weather-resilient blueberry cultivars.

Keywords: blueberry; freeze damage; phenology; phenotyping; genetics

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1. Introduction

Blueberry (*Vaccinium* sect. *Cyanococcus*, Ericaceae) is a high-value fruit tree crop with 0.3 million tons of production and over USD 1 B in sales value in the US in 2023 (www.nass.usda.gov, access date 25 January 2025). Consumer's appreciation of blueberries' flavor, versatility, and health benefits has been the major driver for the expansion of blueberry production worldwide [1]. Cultivated blueberries in the US include northern highbush (NHB, $2n = 4x = 48$), southern highbush (SHB, $2n = 4x = 48$), lowbush

(LB, 2*n* = 4*x* = 48), and rabbiteye (RE, 2*n* = 6*x* = 72) [2]. Originating in North America, NHB and LB are adapted to the US northwest and northeast growing environment [3]. RE is the other cultivated blueberry species endogenous to the southeast US [4]. SHB was developed through interspecific hybridization among NHB, RE, and diploid blueberry species such as *V. darrowii* and *V. fuscatum* [5–7]. Traditionally, blueberries grown in the temperate and boreal regions are deciduous and produce one crop within one or two months from spring to summer. The introgression of the low-chill requirement from wild diploid blueberries rendered the adaptation of blueberries to warmer growing regions such as California, Florida, Spain, Portugal, Hawaii, Mexico, and Morocco [8,9]. In regions with mild winters, evergreen production systems with low-chill SHB varieties produce fruit for more than half a year [10–12]. Although the blueberry plants grown in evergreen production systems appear to circumvent endodormancy, early flowering cultivars are at risk of spring freeze damage [13]. This review focuses on genetic breeding addressing freeze damages threatening blueberry production in both deciduous and evergreen production systems.

The active growth/dormancy cycle is critical for the survival of blueberry plants [14]. This cycle is regulated by photoperiod and temperature changes [15]. Freezing temperatures in both fall and spring can cause injury to the buds [16,17]. In fall, the freezing temperature kills buds before they fully enter winter dormancy. In spring, after buds are initiated upon a warm spell, the freezing temperature causes damage to the buds. In addition, the insufficient cold tolerance of blueberries to midwinter freezes also critically reduces blueberry production in the northern US growing regions [18,19]. Therefore, developing blueberry cultivars with a proper dormancy period, cold hardiness, flowering time, and fruiting time that are adapted to the local growing conditions is important to prevent freeze damage [20,21].

In both evergreen and deciduous production systems, blueberry flower buds form in late summer to fall in response to short-day photoperiods [10,22]. Subsequently, the buds enter dormancy to survive winter conditions for the deciduous system [23] whereas proper fertilization and disease management in the evergreen production system allows for leaf retention and early flowering. Like other perennial woody plants, blueberries acclimate to winter cold and maintain freeze tolerance until warm weather comes. Cold hardiness measures how well a plant can survive freezing temperatures. In blueberries, this characteristic is quite different across genotypes. Cold hardiness was measured by the lethal temperature where 50% of the plant tissue was subjected to freeze damage in blueberries [24]. The lower the lethal temperature a blueberry plant tolerates, the higher the level of cold hardiness the plant attains. The genotypic difference in cold hardiness was reported in a controlled freeze chamber or a glycol freezing bath [24]. For instance, half highbush 'Northsky' was found to have the highest level of cold hardiness compared with RE, NHB, and SHB genotypes [19]. In the meantime, genotypic variation in cold tolerance among NHB cultivars was also detected [24]. To come out of the winter dormancy, most blueberries have a chilling requirement which is the duration of time in low temperatures (between 0 and 7 °C) needed for the flower bud to resume growth [25]. Insufficient chilling leads to delayed flowering, leaf bud breaking, and fruit development in blueberries [26,27]. This internally regulated requirement of exposure to low temperature to exit dormancy is defined as endodormancy [28,29]. Upon satisfying the chilling requirement, the activation of meristem cell growth or de-acclimation [30] requires exposure to warm temperatures quantified as growing degree days [31]. Unfavorable environmental conditions that adversely affect the resumption of the growth of flower and leaf buds are deemed as eco-dormancy [29]. Therefore, eco-dormancy is regulated by external factors. Cultivated blueberries have demonstrated a highly diverse genotypic response to environmental cues [9,32–34]. The chilling hours required for SHB and RE blueberries range from 100 to 600 h whereas NHB

requires over 800 chilling hours for dormancy to break [35,36]. Upon dormancy break, blueberries produce flowers followed by the emergence of new leaves. Blueberry flowering time, fruiting time, and flower-to-fruit interval were reported to be genetically controlled and influenced by the environment [37] cognate with other woody plant species [14,38].

To provide a comprehensive view of the current advancements in phenotyping, genotyping, marker-traits association analysis and expression profiling of blueberry flowering time, and chilling requirement and cold tolerance, the objectives of this study include (1) reviewing the damage from spring and winter freeze in blueberries; (2) presenting phenotyping efforts addressing blueberry cold hardiness and phenological development; (3) discussing the current advancements in QTL analysis and genetic control of freeze tolerance in blueberries.

Spring and winter freeze injuries in blueberries are distinctly different. In early spring, the temperature fluctuation in most of the blueberry growing regions triggers flower bud initiation and subsequent early freeze damages the actively growing buds (covered in Section 2). On the other hand, winter freeze damage occurs in colder USDA plant hardiness zones of 1 to 8 (https://planthardiness.ars.usda.gov/, access date: 6 May 2025) where dormant blueberry bushes are exposed to prolonged low temperatures (covered in Section 3).

2. Spring Freeze Causes Damage to Blueberries

Climate change in the past half-century has contributed to earlier warm weather accompanied by erratic spring freeze events [31,39]. Based on over 100 years of records of temperature and woody plants' phenological development, it was found that there was an increase in the frequency of spring freeze damage [40]. The unpredictable distribution of spring freeze is associated with the complex interaction of changes in temperature, precipitation, and CO_2 concentration in the atmosphere [41]. The warming temperature in spring (false spring) results in the early onset of blooms and leaf growth in fruit trees, which predisposes them to early freeze damage [42,43]. The ensuing subfreezing temperatures cause ice crystal formation intra- and extra-cellularly in the young leaves and flower buds resulting in tissue damage and yield loss [44]. The degree of susceptibility to spring freeze is one of the most important genetic factors that need to be optimized in highbush blueberries [20].

Spring freeze occurs when blueberry plants are exposed to subfreezing temperatures followed by warm spells that initiate flower development [45] (Figure 1). Oxidative browning of the ovaries and placental tissue was found in both highbush and LB blueberries subjected to spring freeze damage [46,47]. The freeze damage to flower buds was determined by the percentage of oxidative browning ovaries [47]. Once dormancy breaks upon de-acclimation, the developing flower buds are highly susceptible to freeze damage [48]. The susceptibility of blueberry floral buds to freeze damage is inversely associated with developmental stages [49,50]. Tight buds can tolerate a temperature lower than −12 °C, but swelled buds and buds at tight cluster stages are sensitive to temperatures ranging from −5 to −7 °C. Furthermore, flowers at the pink bud and full bloom stages are sensitive to temperatures ranging from −4.4 to −2.2 °C. Fruit tissue is more susceptible to cold damage than floral tissue [51]. From petal fall to fruit maturation, freeze damage occurs at 0 °C [48,52]. On the same plant, lower buds formed in fall woods were found to have less cold injury than the buds formed during the spring growth [47]. A significant reduction in fruit set was reported from cold injured flowers from low bush [53] and RE blueberries [54]. In addition, the unopened flower buds had a diminished potential to bloom after exposure to the freezing temperature [53]. Spring freezes have brought frequent damage to blueberry production regions around the world [45,51,55,56] (Table 1). An early season freeze in

South Georgia, USA, in March of 2022 resulted in more than 50% total crop loss, including 100% loss of SHB without overhead freeze protection and a loss of more than 15% with protection [57,58]. The southeastern US recorded more than 50% loss of SHB crops in 2017 due to a deep freeze event [59]. During the event, warm March temperatures were followed by a broad invasion of arctic cold air in April 2007, resulting in a catastrophic spring freeze [60–62]. A 50 to 100% crop loss of NHB was reported in southern Iowa, Kansas, Missouri, Arkansas, Illinois, Kentucky, Michigan, North Carolina, and Tennessee from the three days of freezing temperatures accompanied by desiccating wind [52]. Additionally, post-freeze fungal infection on the damaged limbs was reported.

Major freeze events that have significantly damaged blueberry flowers and fruits have been reported in the US, central Poland, and Canada [45–63].

Active practices to mitigate spring freeze damage such as overhead irrigation, wind machines, helicopters, etc., were reviewed elsewhere [57,64]. These reviews focused on the methodology adopted by growers to mitigate freeze damage which is different from the objectives of the current review. The effectiveness of these active measures of freeze protection depends on environmental factors such as freeze types, wind speed, dew point, temperature, etc. [65]. Overhead irrigation is one of the most prevalent measures for freeze protection but, not only is it very costly to install and operate, it also instigates additional biotic stresses such as Phytophthora and other root rot diseases due to soil saturation in the fields caused by the large amount of water applied [66]. Alternatively, the application of plant growth regulators provides yield protection against spring freeze damage. For instance, the application of ethephon, a plant growth regulator that releases ethylene in plant cytoplasm, was found to delay blooms by up to 14 days in both HB and RE blueberries [67]. Gibberellic acid (GA3) application on blooms suffering from mild freeze damage was found to improve fruit set in RE blueberries by inducing parthenocarpy fruit formation after the adverse freeze damage [54,68]. The responsiveness to these growth regulators varies across blueberry genotypes [69] and the long-term effect of repetitive applications of growth regulators to blueberry plant growth is unclear [70].

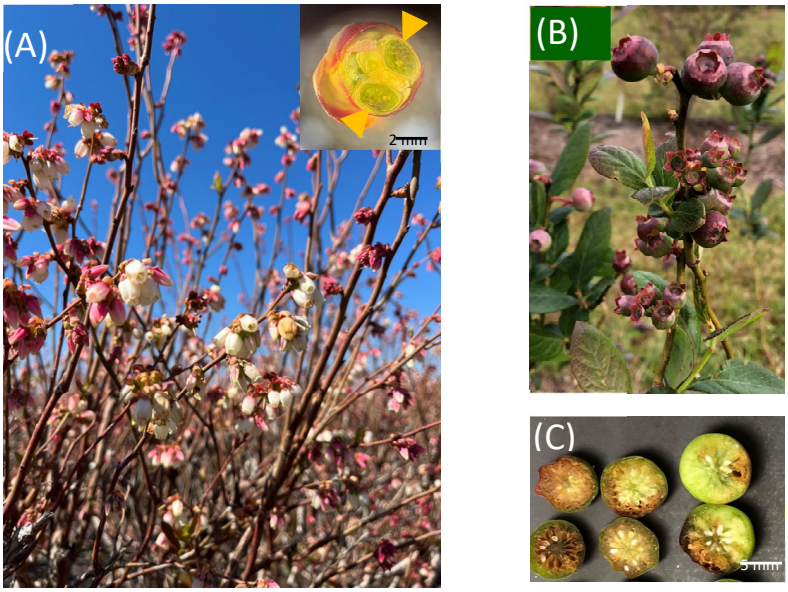

Figure 1. Blueberry flower and fruit damages caused by the spring freeze. (**A**) SHB blueberry flowers were damaged by an early spring freeze event in February 2025. In the top right corner: an un-opened flower bud dissected transversely demonstrating inflorescence tissue browning from the freeze damage. Blue arrowheads point to the damaged tissues. (**B**) Immature blueberry fruits subjected to early freeze damage in March 2024. (**C**) Dissected fruits showed varied levels of tissue damage from the freeze. These images are original to this study.

Table 1. Hazardous weather conditions have damaged blueberry production.

Starting Date	Duration	Temperature	Type of Weather	Location	Blueberry Genotypes	Damages	Ref.
Spring freeze							
11 March 2022	3 days	−5 °C	Advective with high wind	Alabama, Georgia, and North Carolina	SHB and RE cultivars	50% yield reduction	[57,58]
15 March 2017	3 days	−6 °C	Deep freeze event	Georgia	SHB cultivars	over 60% yield reduction	[59]
11 February 2012	2 days	−2 to 4 °C	Advective with high wind	North and central FL	SHB cultivars	10% to 50% loss of green fruit	[56]
8 March 2008		−3.5 to 4.2 °C		Griffin, GA	4 RE cultivars	50% to 90% reduction in fruit set	[63]
7 April 2007	3 days	−2 to 10 °C	Freezing temperature with desiccating wind	Widespread including central, mideast, and southeast US	NHB and SHB cultivars	90% to 100% loss of NHB and 40 to 90% loss of SHB in affected production regions; post freeze fungal infection	[52]
31 March 2003	3 days	−1.9 °C	N/A	Stone County, MS	11 RE cultivars	Both blooms and green fruits were damaged	[51]
3 May 2000	3 h	−6 °C	N/A	Central Poland	9 NHB cultivars	Flower damage and fruit set reduction	[55]
6 May 1996	3 days	−6 to 7 °C	N/A	Nova Scotia	*V. angustifolium*	Oxidative browning of ovaries, yield reduction	[46]
4 February 1989	5 days	−6 to 13 °C	N/A	Overton, TX, and Clarksville, AR	4 SHB, 4 RE, and 1 NHB cultivars	Oxidative browning of ovaries, yield reduction	[47]
9 March 1982	3 h	−2 °C	N/A	USDA Small Fruit Research Station, Poplarville, MS	5 RE cultivars	Browning of flower petal, yield reduction	[45]
15 April 1972	N/A	−23.4 °C	N/A	Central Poland	9 NHB cultivars	Flower bud damage and fruit set duction	[62]
Winter freeze							
January 1972	one month	−7.6 °C	N/A	Central Poland	9 NHB cutlivars	Shoots were killed	[62]
19 February 1927	3 h	−32 °C	N/A	Minnesota Agricultural Experiment Station, Colquet, MN	*V. pennsylvanicum*, *V. canadense*, and *V. corymbosum*	Most of the shoots were killed	[63–77]

N/A stands for not available.

Early flowering blueberry cultivars were reported to suffer more than 70% crop loss whereas late-blooming varieties escaped damage during an early freeze event [55]. This indicates that delaying bloom time can reduce the risk of early freeze damage. However, if the bloom time is too late, the growing season will be shortened. Therefore, staggering delayed blooming time and a shortened fruiting period through breeding holds promise to reduce spring freeze damage while maintaining fruit production.

3. Winter Freezing Brings Injuries to Blueberries

In the northern production regions, winter cold damage to blueberry buds and stems is a major constraint to blueberry production [30,71]. When the winter cold exceeds the limit of plant tolerance, tissue damage occurs. Damage to the blueberry buds from cold temperatures was found to be associated with browning of the ovaries with increased susceptibility to cold stress in certain positions [17,72]. Blueberry buds developed on the distal position of the twigs are found to be less hardy than the buds located at the proximal positions [73]. Besides tissue damage, disease progression such as stem blight caused by *Botryosphaeria dothidea* on cold injured stems was reported, which compounded the yield

loss to freeze damage [59,74]. Further collateral damage from Phytophthora root rot disease occurs in blueberry fields utilizing overhead irrigation to mitigate freeze damage. When large volumes of water are applied to the field for freeze protection, insufficient drainage in low land instigates the root infection from *Phytophthora cinnamomic*, resulting in plant death [57,75]. Low-temperature exothermic reactions, supercooling, and dehydration play roles in the freeze tolerance of blueberry flower buds [72].

Woody plants increase cold hardiness by developing mature hardwood that can sustain freezing temperatures during the gradual decline in temperature in the fall. In Northern America, blueberry production regions include Canada and Minnesota, where winter temperatures down to −40 °C can cause injury to canes and bud-bearing lateral shoots exposed to the air without snow covering [76]. Immature woods developed in fall are particularly susceptible to cold damage. Injury to the canes can be detected by browning in the phloem and cambium regions. In a survey conducted by Brierly and Hildreth in 1928, an extremely cold temperature of −32 °C for three hours in the field killed most of the testing species including *V. pennsylvanicunm*, *V. canadense,* and *V. Corymbosum* [77]. Similarly, 3 to 20% of NHB shoots were killed when exposed to continuous cold temperatures as low as −23 °C in January, 1972 in central Poland [63]. The native wild blueberry relative *V. augustifolium* ($2n = 4x = 48$) is cold hardy [76] and readily crossable with highbush blueberries. It has been used extensively to produce half-highbush cultivars with improved cold hardiness [78]. Other cold hardy germplasms used to improve blueberry hardiness through interspecific hybridization include *V. uliginosum* [79] and *V. lamarckii* [80]. The diverse levels of cold hardiness in the tested materials were found to be associated with the genetic composition of the tested lines [18].

Genotypic diversity in the winter cold hardiness of blueberry flower buds was identified among NHB and SHB cultivars determined by lethal freezing temperature (LT_{50}) in the controlled freezing test [81,82]. Similarly, RE and interspecific hybrids with cold-adapted native germplasm *V. augustifolium* and *V. constablaei* in the pedigree demonstrated genotype-dependent variation in cold hardiness [15,20]. On the other hand, the de-acclimation rates of flower buds exhibited genotype-specific kinetics among five HB and RE cultivars [83]. No association was found between de-acclimation kinetics with mid-winter cold hardiness suggesting that these phenotypic characteristics are conditioned by separate genetic factors [30].

To mitigate winter freeze damage, it is essential to develop blueberry cultivars that would enter dormancy in response to the cold temperature in fall and maintain its winter cold hardiness until consistent warm spring temperatures arrive.

4. Advancement in Phenotyping Blueberry Phenological Development and Cold Hardiness

4.1. Phenological Development

Traditional phenotyping work on blueberry phenological development was performed in the field for multiple years [37,84,85]. Visual ratings of the percentages of full blooms and mature fruits were documented at 3- to 7-day intervals throughout the season. These data were used to estimate the date of 50% flowering, 50% ripening, and flowering-to-ripening interval [37,86]. It was found that the blueberry flowering time and fruit ripening period were highly genetically heritable, demonstrating additive genetic variance, and were influenced by the environment [84,85]. Although visual ratings provide valuable insights about blueberry phenological development, they often lack objectivity and precision. The advancement in high-throughput phenotyping by leveraging sensors, data analytics, and phenotyping platforms such as gantry [87], ground mobile platforms [88–91], and aerial platforms [92–94] has shown promise in replacing humans to objectively measure

phenotypic traits. Some of these automated phenotyping systems have been applied to the detection of blueberries.

One early study presented the feasibility of adopting machine vision technology by using an RGB (red, green, and blue) camera to estimate the yield of wild lowbush blueberries in the field using top-view observations [95]. The lowbush blueberry, however, has a much simpler architecture (in two dimensions on the ground) than highbush blueberry cultivars, where the three-dimensional spatial distribution of the berries is more complex. Machine learning and deep learning have further advanced automated outdoor blueberry detection and phenotyping in the past decade. For instance, the color feature was used for training a K-nearest neighbor (KNN)-based classifier to distinguish different maturity levels of blueberries captured by RGB images [96], but the method could not count the number of berries. A Histogram Oriented Gradients (HOG)-based KNN classifier was developed to make the model robust in different illuminations, but it could only distinguish individual fruit cropped within ROIs (region of interest) rather than detecting all berries in an image [97]. Deep learning models, such as You Look Only Once (YOLO), achieved higher accuracy in outdoor blueberry fruit detection [98–100]. Its early versions, such as YOLO*v*3 and YOLO*v*4, have demonstrated real-time detection of lowbush wild blueberries at various maturity stages with over 90% accuracy [101,102]. Another study modified the YOLO*v*5 model to enhance blueberry fruit recognition within the fruit clusters of the commercial highbush plants [103].

Hyperspectral imaging was employed to determine freeze damage to blueberry flower buds at bud break and early pink developmental stages—although the study was carried out in a lab setting [104]. Furthermore, some studies have investigated deep learning instance segmentation models such as Mask RCNN to count and evaluate blueberry clusters for quantification [105] and extract fruit traits associated with harvestability and yield, such as fruit number, maturity, and cluster compactness [106,107]. Compared with the detection model, the instance segmentation models provided more detailed information about blueberries, such as boundaries, which made it possible to derive other valuable traits, such as cluster compactness. However, their high performance relied on the imaging quality of the fruit clusters and the images in the studies were captured by hand-held phones or cameras with specific angles near the fruit, which is still a tedious process. To address these limitations, a recent study deployed a custom-made phenotyping robot MARS-Phenobot to collect images autonomously in the field and a customized deep learning model BerryNet achieved 86% mean average precision in berry segmentation (Figure 2). The segmentation results were used to successfully estimate yield, maturity, and cluster compactness of berries from 17 different genotypes [91].

Although multiple studies have focused on assessing blueberry fruit yield and maturity levels, image-based analysis of blueberry flower development remains limited. Understanding flower development is essential for accurately determining bud set, flowering time, and the fruiting period—key phenological traits for breeding blueberries that can avoid freeze damage. Machine learning and artificial intelligence offer promising tools for recognizing and quantifying flowers at different developmental stages. However, further advancement in automated image collection and analysis are needed to enable objective, high-throughput monitoring of blueberry phenological development.

Figure 2. Cluster-level compactness among five different genotypes using a robotic system for data collection and deep learning models for data analysis (each column shows four examples from a particular genotype and the numeric number in each image is the compactness value [91]). Permission to include these images was obtained from the authors of the original publication.

4.2. Winter Hardiness

Phenotyping for winter hardiness, chilling requirement, and freeze injuries has been conducted by traditional approaches under both field and controlled environmental conditions [108,109]. Although winter survival and chilling requirements in the field are important for breeding, the complex factors in the field such as seasonal temperature fluctuation, wind speed, and snow cover often lead to inconclusive genetic analysis [110,111]. Therefore, controlled environmental studies were conducted to provide precise measurements of the component traits associated with winter survival [109,110].

The chilling requirement of blueberries was determined by exposing cold-acclimated shoots with 3 to 8 dormant flower buds to 0 to 7 °C temperatures in a stepwise increment of chilling units [49,112]. At each step of chilling unit accumulation, the flower buds on the shoots were forced to bloom at 24 °C for three weeks. The chilling unit at which 50% of the flower buds developed beyond the bud break stage was considered the chilling requirement of the tested genotype. As for the freeze tolerance, blueberry cold hardiness was evaluated by rating the freeze injury caused by the controlled environment. Artificial testing of blueberry cold hardiness was conducted by immersing blueberry shoots in a glycol bath with a temperature gradient between −10 and −28 °C overnight followed by thawing at 20 °C [85]. The temperature that caused 50% bud damage (LT_{50}) was used to differentiate the level of cold tolerance among the tested genotypes. Genotypic variation in cold hardiness was identified among RE, NHB, and SHB blueberries and testing populations segregating for cold hardiness [18,19,109]. As a replacement for the visual ratings of freeze damage in blueberry tissue, electrolyte leakage analysis was used to determine the cold hardiness of the blueberry shoots [113]. In this method, the blueberry shoots were frozen under a controlled freezing temperature gradient and allowed to thaw

overnight at 4 °C. The electrical conductivity of the thawed shoot was measured by a conductivity meter. The temperature at which 50% tissue damage occurred was derived from the Gompertz asymmetric sigmoid function [114]. Thermal analysis allowed the detection of deep supercooling in which the cold-hardy plant tissue, such as dormant flower buds and stems, exhibits freeze resistance by forming ice in the extracellular space while maintaining the integrity of cellular structures [115]. During ice formation in the plant tissue, the temperature of the tissue increases abruptly (exotherms) due to the release of latent heat. The detection of exotherms was performed using a small thermocouple probe embedded on the stem or bud during a steady drop in temperature to −40 °C [76,116]. Beside these methods, the increments of free water detected by nuclear magnetic resonance were found to be associated with the release of dormancy [28] and was used to distinguish ice crystal and free water amounts in the blueberry plant tissue [117]. Freeze damage in blueberry fruit was detected by magnetic resonance imaging [118]. Infrared thermography can detect ice nucleation and propagation [119] and was used to determine freeze injuries in other woody plant species [120,121], which could potentially be useful for determining blueberry cold hardiness.

These conventional and high-throughput methods are useful in phenotyping blueberry mapping populations for marker-trait association analysis. In addition, comparison of gene expression profiles of the plant tissue subjected to the above treatments informs the gene networks responsive to cold treatment and blueberry phenological development. Progress in these areas is discussed in the next section.

5. Genetic Analysis of Freeze Tolerance and Flowering Phenology in Blueberries

5.1. Gene Regulation Associated with Flowering and Chilling Requirements

It is well established that flower induction and fruit formation are closely regulated by both genetic and environmental factors including photoperiod, temperature, and endogenous hormonal levels [122]. The genetic regulation of bud dormancy and flower development through the common floral pathway integer FLOWERING LOCUS T (*FT*) coordinating the complex crosstalk among the genetic pathways including photoperiod, circadian clock, abscisic acid, gibberellic acid, autonomous, and age was reviewed recently [123–125]. These comprehensive reviews presented the gene regulatory pathways related to blueberry flower initiation which provided the foundation of gene discovery for genetic association analysis. Of particular importance is the advancement of biological pathways in the flowering induction of Arabidopsis [123]. Most of the genes associated with blueberry flower development were discovered through comparative genetic analysis with the flowering genes discovered in Arabidopsis. Transgenic NHB blueberries with constitutively overexpressed *vcFT* (vc: *Vaccinium corymbosum*) exhibited early flowering [36], dwarf architecture [126], and the upregulation of flowering genes including the suppressor of overexpression of Constans 1 (*SOC*1), APETALA1 (*AP*1), LEAFY(*LFY*), and squamosa promoter binding protein-like genes (*SPLs*) [127]. These transgenic NHB plants failed to produce blooms without chilling treatment [127]. In addition, the *vcFT*-overexpressed transgenic blueberry seedlings also had a shorter transitional period from the juvenile to reproductive phases [125]. SOC1 was found to be the key regulator of blueberry chilling requirements. Transgenic blueberries constitutively express *SOC*1-produced flowers under no chilling conditions [127]. The overexpression of the K-domain of blueberry *SOC*1 in tobacco resulted in early flowering [128,129] and increased yield potential [129] in transgenic blueberry plants. The constitutive expression of an apple *mdFLC3*, another MADS-box gene with a K-domain, also produced flowers under non-chilling conditions [130]. The extensive genetic analysis of a no-chill transgenic line mu-'Legacy' revealed the poten-

tial regulatory role of the *RESPONSE REGULATOR 2-like* gene (*VcRR2*) in mediating the chilling requirement in blueberries [131]. Besides these extensive functional analyses of major genes through forward genetic transformation experiments, RNAseq analysis on blueberry flower buds subjected to diverse chilling treatments revealed the genes and gene networks responsive to chilling treatment [132]. These genes include upregulated major flowering MADS-box genes such as *AP*1, *FURITFUL* (*FUL*), *SOC*1, *CALUFLOWER* (*CAL*), *FLOWERING LOCUS C* (*FLC*), *AGL*24, and *SHORT VEGETATIVE PHASE (SVP)* in addition to the downregulated proteins *FD* (FD), *TERMINAL FLOWER* 1(TFL1), and *LEAFY* (LFY).

The discovery of these genes and gene networks associated with blueberry flowering provides the metabolic pathways conditioning blueberry flowering phenology and chilling requirement. This information enables candidate gene identification for genetic association analysis.

5.2. Gene Regulation Associated with Dormancy and Freeze Tolerance

The gene networks associated with plant dormancy and response to low temperature and freeze tolerance in Arabidopsis and other wood plant species were reviewed and provide reference genes and gene networks for comparative analysis in blueberries [124,133]. Several major dehydrins (14, 60, and 65 kDa) in blueberry flower buds were found to increase in expression as chilling units accumulated and decrease in the process of dormancy release [83,134–137]. These highly expressed proteins formed saliant protein bands when separated by SDS-PAGE. Both 14 and 60 KDa dehydrins are inducible by cold stress and their expression levels correlated positively with cold tolerance in the tested blueberry genotypes [138,139]. Dehydrins are heat-stable glycine-rich proteins [140]. They are hydrophilic with an amphiphilic α-helix domain which may interact with endomembranes and stabilize cellular proteins in sub-zero temperatures and/or prevent freeze-induced desiccation [141]. The C-repeat binding factor (CBF) is another well-characterized transcription factor that is responsive to the dynamic changes in the dormancy status in blueberries. The induction of CBF by a short photoperiod was found to be four times higher in the flower buds from the cold-tolerant NHB cultivar 'Bluecrop' than the RE cultivar 'Tifblue' in fall [142]. The overexpression of a C-repeat binding factor in the SHB cultivar 'Legacy' increased the cold tolerance of the flower buds and leaves but not in opened flowers [143]. The CBF transgene was later renamed as DWARF AND DELAYED FLOWERING gene (*vcDDF*1) due to the higher sequence similarity to the Arabidopsis ortholog of the latter [144]. *vcDDF*1 was found to co-regulate with cold-responsive genes (COR) in blueberry buds subjected to differential cold treatments and has the potential to increase blueberry cold hardiness [145].

Besides these well-characterized genes, other genes associated with freeze tolerance were revealed through 2D gene electrophoresis, microarray, and RNAseq analysis. The sequencing of cDNA libraries constructed from flower buds of 'Bluecrop' exposed to field acclimation and non-acclimation revealed a differential distribution of genes involved in chloroplast biogenesis, chromatin modification, sugar metabolism, fruit and flower development, stress response, and transposase involved in cell growth and divisions [146]. The upregulation of dehydrin, early light-inducible protein, and beta-amylase upon chilling accumulation was confirmed by Northern blot analysis [146]. Besides the discovery of these high abundance genes responding to cold acclimation, 374 low abundance genes responsive to cold acclimation were identified by subtractive hybridization [147]. The majority of these genes are related to cold, light, and drought stress. A comparison of cDNA expression profiles from cold-acclimated flower buds induced by cold room and field conditions was reported using microarray analysis [148]. More than 50 genes, such as dehydrin, early light-inducible proteins, and beta-amylase were found to be simultaneously upregulated under both field and cold room treatments. Genes induced only under cold room treatment are associated with stress tolerance, glycolytic pathways, and

protein synthesis whereas light stress-related genes were only upregulated from field cold-acclimated samples [148]. A global proteomic analysis of flower buds from 'Bluecrop' subjected to 0 to 800 chilling units treatment revealed protein expression changes in the pathways associated with plant stress response, photosynthesis, and cell growth, which corroborated the transcriptomic data [149]. An RNAseq analysis of blueberry flower buds collected at multiple time points of chilling accumulation also confirmed the findings from a microarray analysis and identified additional unique genes responding to environmental cues [150].

Gene expression profiling of the responsive gene networks from diverse blueberry species with a broad range of cold tolerance is informative. These gene networks can be further utilized to improve cold tolerance in blueberries.

5.3. Marker Discovery of Chilling Requirement, Freeze Tolerance, and Phenology in Blueberries

Developing genetic markers for major quantitative genetic traits and determining breeding values through genomic prediction for small effect QTLs will enable molecular breeding to improve freeze tolerance traits in blueberries (Figure 3). However, few linkage maps are available for tetraploid blueberries which limits genomic-assisted selection for breeders [151]. This is in part due to the early-acting inbred depression and self-sterility of blueberries [152]. For marker discovery, pseudo-backcross populations were constructed by generating progenies from the test cross between a hybrid F_1 and a sibling of the parental line [140]. Alternatively, diversity populations consisting of blueberry selections from multiple crosses were utilized for genome-wide association analysis (GWAS) (Table 2). As for genetic markers, randomly amplified polymorphic DNA has been utilized.

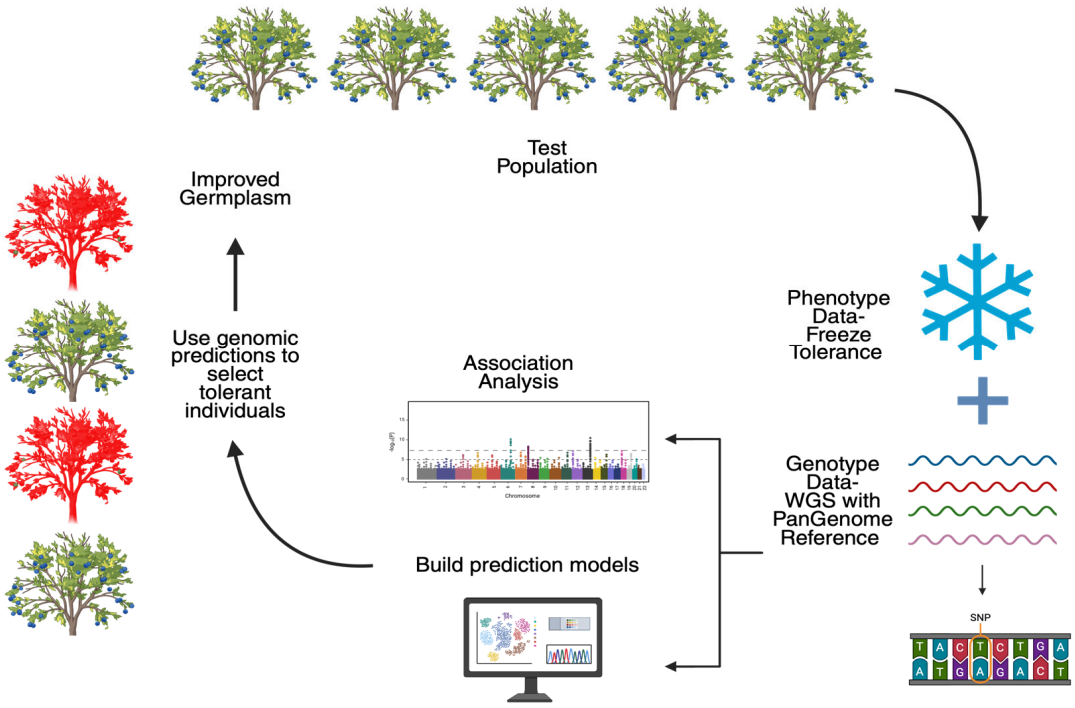

Figure 3. Schematic representation of the genomic selection pipeline for improving freeze tolerance in blueberries.The process begins by collecting DNA from a test population, which is also evaluated for freeze tolerance under controlled or field-based conditions. High-quality genotype data are generated using short-read whole-genome sequencing (WGS) and aligned to a pangenome reference graph. The resulting genomic and phenotypic data are used to train a genomic prediction model that estimates genomic estimated breeding values (GEBVs) for freeze tolerance. This trained model enables the prediction of freeze tolerance in individuals without phenotype data, facilitating early selection of superior genotypes based on GEBVs. GEBV is an integrated genomic method that accurately predicts the breeding value of the genetic material. Marker identification through genome-wide association

studies (GWAS) can further inform selection, which may also be implemented using marker-assisted selection (MAS). MAS allows the breeder to select breeding lines with marker profiles associated with traits of interest. Selected individuals are advanced through clonal propagation, further breeding, or incorporation into the breeding pipeline. Figure created using BioRender.com.

DNA (RAPD), expressed sequence tags (ESTs), and cleaved amplified polymorphic sequences (CAPS) markers were developed to genotype blueberry populations or to determine genetic relatedness among blueberry genotypes [153–155]. More recently, high-density single nucleotide polymorphic (SNP) markers developed from capture sequencing (capture-seq) [156] and double digest restriction site-associated DNA sequencing (ddRAD-seq) [157] greatly improved genome coverage for marker-trait association analysis.

Chilling requirement is a quantitatively inherited trait in fruit trees suitable for QTL analysis [124,158,159]. Blueberry chilling requirement was found to be dominant or partially dominant inherited in a study using a testcross population derived from *V. darrowii* and diploid *V. corymbosum* crosses [112,140]. The genetics mechanism controlling chilling requirement was found to be conditioned by additive gene action [140]. Three QTLs of chilling requirement located on linkage groups (LGs) 5, 6, and 8 were identified [112,160]. Using an SHB diversity population, 12 SNP markers distributed on chromosome 4 and 14 in reference to the 'Draper' genome [161] were found to be associated with chilling requirement [157]. Cold hardiness was mapped with the same diploid test population [140]. A controlled freeze–thaw analysis of the test population indicated that freeze tolerance was controlled by an additive gene effect [110]. The parental lines from *V. darrowii* were significantly more freeze-sensitive than the diploid *V. corymbosum* parents. Three QTLs on LG 2, 4, and 10 were identified based on two years of phenotyping data sets [112,160]. With regard to flowering time, a QTL region on LG5 was found using capture-based sequencing for SNP variant calling [160]. Nagasaka et al. reported 67 SNP markers distributed on 12 chromosomes associated with flowering date in an SHB diversity population [157]. In addition, 18 SNP markers distributed on 9 chromosomes were associated with fruit ripening date and 3 SNP markers on chromosome 9 and 10 were associated with fruiting period [157]. In a separate SHB diversity population, 17 SNP markers were found to be associated with off-season flowering [157]. These SNP markers were recovered by aligning partial genome sequences to the linear reference genome [161] which resulted in partial variants recovery. The most recently developed genotyping platforms such as DArTag panel of 3000 loci [162] and the Flex-Seq platform covering 22,000 loci [163] offer the opportunity to unify genotyping efforts from the blueberry breeding community. However, both genotyping platforms suffer from ascertainment bias, with limited genome coverage and an inability to detect structural variants.

Key regulators of flowering time were identified in large presence/absence variants (PAVs) in other crop species such as *Brassica napus* [164] and *Gossypium hirsutum* [165]. PAVs can only be readily curated using a pangenome graph-based approach which allows for accurate allele mining and pinpointing of causal alleles responsible for phenotypic variations. The published blueberry pangenome [166] offers the opportunity to expand the marker types to structural variants (SVs) such as insertion/deletions (indels), presence/absence variants (PAVs), and copy number variants (CNVs). Whole genome sequencing (WGS) captures genome-wide variation and is unhampered by ascertainment bias, but is cost prohibitive with large sample sizes, precluding its use for breeding and functional variation discovery. However, the advent of highly multiplexed, cost-effective sequencing library preparation has made the use of WGS for large populations possible.

Table 2. QTLs discovered for blueberry phenology and cold hardiness. N/A stands for not available.

Traits	Populations	Population Size	Marker Types	Marker Number	Linage Map Size (cm)	QTLs	Associated Markers from GWAS	Ref.
Chilling requirement	*V. darrowii* (2*x*) × *V. corymbosum* (2*x*)	82	EST-PCR, SSR, RAPD, SNP	265	1740	LG 6 and 8	N/A	[112]
Chilling requirement	*V. darrowii* (2*x*) × *V. corymbosum* (2*x*)	117	SNP markers from capture Seq	17,468	1539.4	LG 5	N/A	[160]
Chilling requirement	SHB diversity population	95	SNP markers from ddRADseq	65,145	N/A	N/A	12 SNPs on chr. 4 and 12	[157]
Cold hardiness	*V. darrowii* (2*x*) × *V. corymbosum* (2*x*)	82	EST-PCR, SSR, RAPD, SNP	265	1740	LG 4	N/A	[112]
Cold hardiness	*V. darrowii* (2*x*) × *V. corymbosum* (2*x*)	117	SNP markers from Capture Seq	17,468	1539.4	LG 2 and 10	N/A	[160]
Flowering time	*V. darrowii* (2*x*) × *V. corymbosum* (2*x*)	117	SNP markers from Capture Seq	17,468	1539.4	LG 5	N/A	[160]
Flowering time	SHB diversity population	95	SNP markers from ddRADseq	65,145	N/A	N/A	67 SNPs on 12 chr.	[157]
Fruit ripening date	SHB diversity population	95	SNP markers from ddRADseq	65,145	N/A	N/A	18 SNPs on 9 chr.	[157]
Off-season flowering	SHB diversity population	536	SNP markers from Capture Seq	59,910	N/A	N/A	17 SNPs	[156]
Fruiting period	SHB diversity population	95	SNP markers from ddRADseq	65,145	N/A	N/A	3 SNPs on chr. 9 and 10	[157]

Comprehensive genetic marker curation along with the phenotypic data collected by high-throughput phenotyping methods will enable efficient and accurate QTL discovery associated with blueberry phenological traits. Translating these advances in blueberry genomes and genetics to breeder-friendly genomic selection platforms will accelerate the development of resilient blueberry varieties adapted to diverse growing environments.

In addition, gene editing through the CRISPR/Cas9 system was used to knock out the *CENTRORADIALIS*, a homologous gene of *TFL1* [167] and phytoene desaturase [168] in highbush blueberries. The efficiency of the genome editing system was improved by using endogenous promoter sequences [169]. Candidate genes for chilling requirement and cold tolerance [160] can be functionally tested using the CRISPR/Cas9 system. Furthermore, mutant lines with a desirable chilling requirement and cold tolerance can be further integrated into breeding cycles.

6. Conclusions

The continued rise in blueberry consumption is expected to drive production into new regions where environmental conditions, including early spring freezes and mid-winter cold events, pose significant challenges. Adapting blueberry cultivation to these diverse and often harsh climates will require intensified and more efficient breeding strategies. While major QTLs and SNP markers associated with chilling requirement, cold hardiness, flowering time, and ripening period have been identified—primarily from one diploid population and several highbush diversity populations—these findings are based on phenotyping data from a single location. The implementation of these markers in breeding programs remains limited. Moreover, the transferability of these QTLs across populations has yet to be validated. To address climate variability across geographical regions, multi-year and multi-location evaluations of blueberry cultivars and mapping populations are essential. Such efforts will enhance our understanding of phenological plasticity and genotype resilience under unpredictable weather conditions. These studies will not only

support further marker discovery but also inform cultivation and orchard management practices aimed at mitigating climate-related yield loss. Advancements in high-throughput phenotyping are highly promising for improving the efficiency and accuracy of phenotypic data collection. However, these approaches require substantial investments in robotics, imaging equipment, and computing infrastructure. Machine learning and deep learning models provide powerful tools for automated image analysis and novel trait extraction, while leveraging pangenome, genomic, and genetic resources demands personnel with strong bioinformatic expertise. In addition to technological needs, the blueberry breeding community must develop frameworks for storing and sharing the vast amounts of data generated from genome sequencing and high-throughput imaging. Implementing advanced genotyping and phenotyping platforms requires substantial investment in equipment, consumables, cloud storage, and computational infrastructure–resources that are impractical for individual breeding programs to acquire and maintain independently. A more feasible and sustainable approach is for universities or research institutions to consolidate these resources, offering them as centralized services. Such shared facilities can support and collaborate with multiple breeding programs, enabling broader access to cutting-edge technologies while fostering interdisciplinary collaboration and efficiency in cultivar development. Regardless of these hurdles, embracing modern genomic and phenomic tools will be pivotal to accelerating the development of climate-resilient blueberry cultivars capable of thriving in an era of increasing environmental unpredictability.

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