Quantitative Trait Loci Analysis of Low Temperature Responses in Grapevine F2 Population

Mani Awale
South Dakota State University

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QUANTITATIVE TRAIT LOCI ANALYSIS OF LOW TEMPERATURE RESPONSES IN GRAPEVINE F$_2$ POPULATION

BY

MANI AWALE

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Master of Science

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QUANTITATIVE TRAIT LOCI ANALYSIS FOR LOW TEMPERATURE RESPONSES IN GRAPEVINE F₂ POPULATION

This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science degree in Plant Sciences and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Anne Fennell, PhD
Thesis Advisor

David Wright, PhD
Head, Plant Science

Dean, Graduate School
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ABSTRACT

QUANTITATIVE TRAIT LOCI ANALYSIS OF LOW TEMPERATURE RESPONSES IN GRAPEVINE F2 POPULATION

MANI AWALE

2016

Freezing injury, caused by freezing temperatures in the late fall, midwinter, or early spring, can result in significant loss to grape growers. The damage may range from the partial damage of parts of the plants to the total death of the plant, and may vary between years. Freezing tolerance is a multi-genetic, complex quantitative trait that involves many related traits like dormancy induction, growth cessation, acclimation, deacclimation and bud break. Developing an understanding of the genetics behind the complex trait requires connecting the phenotype with the genotype to enable discovering the underlying genes that can contribute to quantifiable differences between individuals.

The objective of this thesis is to detect the genomic location(s) underlying genetic variation in low-temperature response traits like freezing tolerance and bud break using quantitative trait loci analysis (QTL).

An F2 population developed by selfing a single F1 from a cross between American species Vitis riparia and a hybrid grapevine Seyval, was used to conduct QTL analysis for subzero temperature tolerance. The low temperature exotherms (LTE) obtained from differential thermal analysis (DTA) were used as phenotype data for the QTL analysis. The phenotyping was conducted in multiple months and dormant seasons. LTE results when the supercooled water inside the bud freezes and identifies the
temperature at which buds are killed. Best linear unbiased predictors (BLUP) was also calculated using the LTEs to calculate random genetic effects. Composite interval mapping (CIM) was conducted using either the average LTEs or BLUP values in R/QTL with 1000 permutations and error rate of 5%. The LTEs for individual genotypes varied in the different months. QTLs were identified using either average LTEs or BLUP values for all the months and dormant seasons. QTLs appeared on Chromosome 1, 5, 9, 13 and 16 in different months or dormant seasons. Many potential candidate genes associated with calcium signaling, ethylene signaling, ABA signaling, cellular metabolism and dehydration response were found underlying the 1.5 LOD interval of these QTLs.

The bud break phenology was studied at three chilling periods to identify bud break related QTL. Bud break occurs upon the exposure to optimal growth conditions, after the chilling requirement fulfillment transitions the bud from endodormancy to ecdormancy. Three one node cuttings of the grapevines were forced at 13 hours of daylength and 25°C/20°C thermoperiod in the growth chamber for four weeks. Each week the growth stage of the cuttings was scored using Modified Eichhorn-Lorenz (Modified E-L) phenology scale. This was repeated in multiple dormant seasons and months. The bud break data was ordinal, monotonic and based on repeated measurements, thus similar to data in disease epidemiological studies in which disease severity is scored repeatedly over a time period. Therefore, the concept of area under the curve (AUC) was applied to the bud break data. The area under the bud break progression curve (AUBPC) was calculated for the bud break data of each individual for an individual month. CIM was conducted with the average week 3 phenology score data or AUBPC data in R/qtl using 1000 permutations. Both data processing approaches provided similar
QTL results on chromosome 3, 7, 8, 9, 13, 18 and 19. These QTLs encompassed candidate genes involved in calcium signaling, auxin signaling, ethylene signaling, circadian clock signaling, cellular metabolism, primary and secondary metabolism, dehydration stress response, drought stress response, fruit ripening and many transcription factors.

The results from this study can be useful in developing low temperature response trait markers that can be applied in marker-assisted breeding and selection to develop the grapevine cultivars suitable for northern continental climates where freezing temperatures are common. This will enhance the sustainability of cold climate grape production and promote expansion of grape growing regions.
Chapter 1 General introduction and literature review

Grapes are one of the most valued cultivated fruit crops in the world ranking second in global production. Currently, there are ~8 million hectares of vineyards worldwide, with the majority of fruit processed into wine and the remainder consumed fresh as table grapes, dried into raisins, processed into juices and distilled into spirits. Italy is the largest producer of grapes followed by France, United States, Spain, and China. United States produced ~7.7 million tons of grapes in 2013 (http://faostat3.fao.org/). Grapes are highest value fruit crop produced in the United States. The grape industry contributes about $162 billion to US economy ($33 billion in wages and $17 billion in state and local tax revenues) (www.ngwi.org).

There are around 60 inter-fertile species of Vitis in the Northern hemisphere, however, Vitis vinifera is the most popular and extensively used in wine production (This et al. 2006). V. vinifera is a Eurasian domesticated grapevine that is widely cultivated, and is adapted to areas with moderate winter temperatures, as it is susceptible to winter injury. Freezing injury is one of the major factor affecting the grapevine productivity and sustainability as well as a major factor limiting the production of grapes in areas in northern continental climate of North America (Fennell 2004). Some of the most destructive cold events in history include the “Easter freeze” (April 2007) (Warmund et al. 2008), the “killer frost” (April 2012) (http://www.producenews.com/markets-and-trends/8173-mi-apples-cherries-bear-brunt-of-killer-frost) and the “polar vortex” (January 2014) (http://cfaes.osu.edu/news/articles/most-ohio%E2%80%99s-2014-wine-grape-crop-lost-due-polar-vortex-ohio-state-survey-finds) that devastated large growing areas of the United States causing great loss to the growers. The “polar vortex” in 2014
caused widespread, significant damage and vine loss in the continental part of the U.S. (Dami et al. 2016). The wild North American species *Vitis riparia*, *Vitis labrusca*, *Vitis rupestris* are more freezing tolerant than *V. vinifera* grapes. *V. riparia*, also known as riverbank grape, can survive temperatures as low as -40°C in continental climates of North America (Pierquet et al. 1977; Pratt 1996). Due to its cold hardiness and disease resistance, this species has been extensively used in scion and rootstock breeding programs (Luby 1991). Understanding the genetics of cold hardiness/subzero temperature tolerance is thus very important for breeders to select the cultivars to promote survival and productivity of grapes in variable climatic conditions.

The objective of this study was to understand the genetics of low temperature responses including acclimation to subzero temperatures and bud break. Low temperature response traits were phenotyped in a F2 population derived from a single F1 developed from a cross between native cold hardy grapevine species, *V. riparia* and Seyval, a moderately cold hardy cultivar (Fennell et al. 2005; Garris et al. 2009).

**Subzero temperature tolerance**

The ability of plants to adapt to and withstand subzero temperatures during autumn and winter, is a complex, multi-genetic trait that involves physiological, biochemical and molecular processes (Gusta and Wisniewski 2013; Wisniewski et al. 2003; Fennell 2004; Guy 1990). The transition of the plant from cold tender state to cold hardy state at the end of a growing season is characteristic of woody perennials including grapevines, and it is a survival strategy in continental climates, where the temperature often goes below freezing (Wisniewski et al. 2003). Many related traits promote subzero temperature tolerance in grapevine including dormancy, growth cessation, periderm
development, early acclimation, mid-winter freezing tolerance and timing of
deacclimation, chilling fulfillment and bud break (Fennell 2004; Fennell and Hoover
1991; Howell and Shaulis 1980; Stushnoff 1972). In grapevines, the bud enters
endodormancy and acclimates to low temperatures during autumn and winter as a
mechanism to withstand subzero temperatures. Lang (1987) classified bud quiescent
cycle into paradormancy, endodormancy, and ecodormancy. Paradormancy has been
defined as the inhibition of growth imposed on bud by other organs of the plant besides
the bud. Endodormancy is the temporary suspension of bud meristem outgrowth due to
endogenous bud factors. In ecodormancy, outgrowth of the bud is prevented by
unsuitable environmental conditions like low temperatures. Endodormancy and
acclimation in grapevines and woody perennials are triggered by the environmental cues
like low temperature, day length and water stress (Fennell 2004; Fennell and Hoover
1991; Kalberer et al. 2006; Wake and Fennell 2000; Wisniewski et al. 2003). These
environmental cues result in morphological and physiological changes like shoot tip
abscission, periderm development, growth cessation, leaf senescence, failure to bud break
and dormancy induction in addition to the biochemical changes (Wake and Fennell
2000).

Cold acclimation is a dynamic process that changes with time (Fennell 2004). It is
the response of plants to exposure to low temperature and results in the induction of
subzero temperature tolerance. Species that are unable to cold acclimate are killed by the
subzero temperatures and thus are not suitable for cultivation in the continental climate.
The grapevine bud, cane and trunk tissues respond differently to temperature extremes
during the dormant period in winter (Fennell 2004). The freezing tolerance differs
Variability in subzero temperature tolerance has been found in different genotypes in grapevines (Dami et al. 2016; Fennell and Hoover 1991; Ferguson et al. 2014; Hamman et al. 1996; Mills et al. 2006; Pierquet and Stushnoff 1980; Wake and Fennell 2000). However, environmental cues like low temperature and decreasing photoperiod play a vital role in stimulating plant’s capacity to develop subzero temperature tolerance (Fennell 2004; Schnabel and Wample 1987). The winter injury caused by the subzero temperatures depends on the intensity and duration of the low temperature as well as the phenological stage of the plant (Stushnoff 1972). A gradual drop in temperature promotes acclimation and the plants ability to tolerate subzero temperatures whereas the sudden decrease in temperature from warm to extreme cold can be very dangerous to the plants. A prolonged exposure to low nonfreezing temperatures increases the plants ability to tolerate subzero temperature. There is also genotypic differences in responsiveness to decreasing day length (Fennell and Hoover 1991; Schnabel and Wample 1987). Decreased photoperiod promotes greater subzero temperature tolerance and endodormancy in some genotypes. Cold hardy genotypes have been found to be more responsive to short day length prior to exposure to short photoperiod, resulting in cold acclimation (Fennell and Hoover 1991; Grant et al. 2013).

**Mechanisms of subzero temperature tolerance**

Grapevine is a temperate perennial plant. It transitions from a cold-tender state to cold-hardy dormant state in response to decreasing temperatures and photoperiod. The mechanism responsible for subzero temperature tolerance differs spatially and temporally.
within the same plant (Gusta and Wisniewski 2013). There are two mechanisms that help the grapevines tolerate subzero temperatures, freezing tolerance and freezing avoidance. Freezing tolerance involves tolerating the presence of ice in tissue apoplast and the dehydrative stress as the water moves from the cell to ice in the extracellular spaces (Levitt 1980). The cane and trunk tissues can withstand extracellular ice formation (i.e. ice outside the living cells) resulting in desiccation of cytoplasm inside the cells (Levitt 1980; Zabadal et al. 2007). Tolerance of desiccation is the result of biochemical changes brought about by a specific set of genes, proteins and metabolites (Wisniewski et al. 2014). In grapevine, the ice crystals can grow, without significant tissue stress and strain in the void space of the xylem in contrast to the phloem that does not have enough space to accommodate ice crystal growth (Paroschy et al. 1980).

Freezing avoidance involves mechanisms that allow the plant to avoid the ice nucleation in tissues (Ashworth 1992; Wisniewski et al. 2003). Supercooling is defined as the ability of a liquid to remain liquid at subzero temperatures and isolate itself from the ice nucleators. Supercooling in plants allows tissues to survive subzero temperatures without ice formation which is possible due to the presence of barriers to ice propagation, for example, development of incomplete vascular connection in case of grapevines prevent the propagation of ice from the canes to the bud (Andrews et al. 1984; Pierquet and Stushnoff 1980; Pierquet et al. 1977; Quamme 1978; Wisniewski et al. 2003). The ability to supercool is a dynamic characteristic and depends on the water content in tissues, bud phenology, shoot morphology, the level of acclimation attained, the development of barriers to ice propagation and the absence of extrinsic and intrinsic nucleators (Ashworth 1992; Fennell 2014; Gusta and Wisniewski 2013; Levitt 1980).
phenomenon of supercooling occurs in grapevines and many other fruit crops such as apple, apricot, blackberry, cherry, pear, plum and raspberry and in different tissues like xylem and flower buds (Quamme 1991). In apple (*Malus*), the mechanism of supercooling occurs in xylem parenchyma cells, whereas the bark and phloem tissue tolerate extracellular ice formation (Gusta et al. 2009; Quamme et al. 1973). In grapevines, supercooling occurs in buds and the cane tissue tolerate extracellular freezing (Quamme 1986). The bud isolates itself from ice nucleation, particularly from extracellular freezing that occurs in the adjacent cane tissues because of the incomplete functional vascular connection to the cane, which is a major way for ice nucleation propagation (Andrews et al. 1984; Fennell 2004; Pierquet and Stushnoff 1980). When the temperature decreases further, the supercooled water inside the bud freezes resulting in lethal injury to the cells (Quamme 1978). Ice formation in the tissues causes volumetric expansion that causes stress and damage to the membranes and cell wall. Understanding the physical properties of water and its interactions with cellular components is vital to understand the mechanisms of subzero temperature tolerance of cells. The mechanism of ice formation and the kinetics of water movement is important for plant freezing response (Fennell 2004).

Many methods have been developed to assess subzero temperature tolerance of grapevines dormant buds and cane tissues. Electrolyte leakage, tissue staining, chlorophyll fluorescence, oxidative browning, and thermal analysis are the major techniques used (Fennell 2004; Zabadal et al. 2007). Among these, the differential thermal analysis (DTA) is used to detect and quantify the transition of supercooled water to ice (Fennell 2004; Gao et al. 2014; Mills et al. 2006). Deep supercooling in plants,
which is one of the mechanisms to tolerate subzero temperatures, is quantified using
differential thermal analysis (DTA). DTA uses thermocouples to detect and measure the
heat of fusion released by the water in the tissues when it undergoes a transition from
liquid to solid phase change (Mills et al. 2006). DTA was first used in Prunus by
Quamme et al. (1973). Low temperature exotherms (LTE) correspond to the killing
temperature of the bud or bud death (Pierquet and Stushnoff 1980) due to the volumetric
expansion associated with the ice crystal formation that causes large stress in the cell
wall, resulting in cell death (Paroschy et al. 1980). The initial high temperature exotherms
(HTEs) are non-lethal temperature and represent the freezing of water in the stem tissues
adjacent to the buds (Burke et al. 1976; Mills et al. 2006; Quamme 1986; Wolf and Cook
1994). In mid-winter, the lack of sufficient water inside the bud results in a lack of
detection of LTEs (Lipe et al. 1992; Pierquet and Stushnoff 1980).

The overwintering buds are more susceptible to winter injury than the canes and
the trunks (Andrews et al. 1984; Quamme 1986). Grapevine has a compound bud
consisting of primary, secondary and tertiary meristems with the primary and secondary
meristems having both leaf and flower primordia (Mullins et al. 1992). The tertiary bud
meristem, which is predominately vegetative, is the hardiest bud followed by secondary
and the primary bud meristems (Pierquet and Stushnoff 1980; Wolf and Cook 1994).
Subzero temperature tolerance develops basipetally, from cane origin towards the tip
(Fennell 2004; Zabadal et al. 2007). The basal buds are more freeze tolerant (Fennell and
Hoover 1991; Grant et al. 2013). Thus, subzero temperature tolerance of the buds and
canes differs even within the same plant. Bud subzero temperature tolerance is dynamic,
varying by cultivar and dormant season environmental conditions. (Howell and Shaulis 1980).

**How do plants start to acclimate subzero temperatures?**

In grapevines, two major environmental cues that promote cold acclimation, are low temperature and decreasing daylength (Fennell 2004; Fennell and Hoover 1991; Fennell et al. 2005; Wolf and Cook 1994). During autumn and winter, grape tissues develop the ability to survive freezing temperature stress. There are species specific and genotype-specific differences in response to low temperature and/or photoperiod for developing subzero temperature tolerance in grapevines which may influence the subzero temperature tolerance of the genotypes (Fennell and Hoover 1991; Fennell and Mathiason 2002; Fennell et al. 2005). Some genotypes initiate the process of acclimation in response to decreasing photoperiod whereas some genotypes require synergistic response of decreasing daylength and low temperature for acclimation whereas the other genotypes acclimate in response to low non-freezing temperatures (Fennell and Mathiason 2002). Cold hardy cultivars are found to be more responsive to short photoperiod prior to exposure to low temperatures (Fennell and Hoover 1991; Fennell and Mathiason 2002; Schnabel and Wample 1987; Wake and Fennell 2000). Exposure to low temperature increases the subzero temperature tolerance in photoperiod-responsive genotypes and induces dormancy and acclimation in nonphotoperiod-responsive genotypes (Paroschy et al. 1980). These environmental cues stimulate the grapevine to transition to an endodormant state which is marked by growth cessation, leaf senescence, periderm development, tip abscission and endodormancy induction (Fennell 2004; Fennell and Hoover 1991; Fennell and Mathiason 2002; Wake and Fennell 2000).
Besides these morphological and physiological changes, the acclimation to low freezing temperature involves many biochemical and metabolic changes in the plant (Fennell and Mathiason 2002). Endogenous levels of glucose, fructose, raffinose and stachyose were strongly associated with subzero temperature tolerance, increasing from the onset of endodormancy to maximum subzero temperature tolerance, whereas, the decrease in the endogenous level of sugars is found in deacclimated grapevines (Hamman et al. 1996). Bud soluble proteins have been reported to increase under short photoperiod during dormancy induction (Wake and Fennell 2000). The bud starch levels decreased whereas the soluble carbohydrates increased with cold acclimation (Hamman et al. 1996). Bud water content also decreases with the dormancy induction (Fennell and Hoover 1991; Fennell and Line 2001; Fennell et al. 1996; Paroschy et al. 1980; Wolpert and Howell 1984). Increase in cell wall strength and pore size also occurred at the time of acclimation in grapevines (Rajashekar and Burke 1996). This decrease in bud water content and increase in soluble carbohydrates and proteins contributes to the ability of the buds to supercool (Fennell and Mathiason 2002).

There are two stages of cold acclimation in grapevines. The first stage occurs in late summer to early fall when the vines start acclimating in response to low but above freezing temperatures. In some species like the American native *Vitis labrusca* and *V. riparia*, decreasing photoperiod period plays a vital role in the induction of cold acclimation, whereas *V. vinifera* grapevines begin cold acclimating in response to low temperatures and decreasing photoperiod (Fennell, 2004; Zabadal, 2007). At this stage the grapevines do not attain maximum subzero temperature tolerance, however, they can survive temperatures below freezing. The second stage of cold acclimation occurs in mid-
December to mid-February in response to sub-zero temperatures. The bud develops maximum subzero temperature tolerance as a result of prolonged exposure to subzero temperatures.

Figure 1.1 Graph showing different stages of subzero temperature tolerance in grapevines. The data is obtained from Fennell (2004).

Deacclimation is the loss of subzero temperature tolerance upon exposure to warm temperatures in the spring. As the temperatures start increasing, deacclimation occurs more rapidly than acclimation. It is the transition from cold-hardy state to cold tender state. Deacclimation may be reversible by subsequent exposure to low temperature or irreversible with the total loss of subzero temperature tolerance upon bud break (Kalberer et al. 2006). It depends on the genotype and the temperature (Wisniewski et al. 2014; Zabadal et al. 2007). At this stage, the buds are easily injured when the temperatures return back to subzero conditions. The buds that have not begun to break
can reacclimate in response to low temperatures after short warm spells (Kalberer et al. 2006).

**Genomics of low-temperature responses:**

Many biochemical changes take place during low-temperature response of plants that cause changes in the physiology of the plant. The changes also occur in the gene expression and protein that adjust the plant to the presence of ice in the apoplast and freezing temperature (Guy 2003). Many transcriptomic studies relating to low-temperature response traits have been done in woody plants including grapevines. Mathiason et al. (2009) found the upregulation of transcripts related to responses to external stimuli and PR-proteins such as chitinases and thaumatin-like proteins during acclimation under short photoperiod. Calcium and reactive oxygen signaling have been found to be involved in low-temperature responses, resulting in the production of protective proteins and metabolites (Theocharis et al. 2012). Dehydrins are the common proteins associated with the low temperature responses (Wisniewski et al. 2014). The soluble carbohydrates have been found to increase as a response to low temperature and short photoperiod, which serve as an osmoticum to reduce dehydration, as well as serve as a nutritional source during acclimation (Wisniewski et al. 2014). The accumulation of sugar, as well as the cold-regulated transcripts, increased as a response to low-temperature stress. Cold-inducible CBF or DREB transcriptional factors that belong to small sub family of AP2/ERF family of transcription factors has been found to be associated with a number of genes regulating the subzero temperature tolerance of woody plants. These genes are also regulated by the circadian clock. The low-temperature responses are thought to be perceived by the plasma membrane, leading to an increase in
calcium level. Many genes related to calcium signaling and sensors are involved in low-temperature response. In grapevines, seven CBF genes have been reported which are located on chromosome 6, 16, 17, 2 and 19 (Wisniewski et al. 2014). Upregulation of transcripts related to abscisic acid signaling, and ethylene signaling have been found in plants exposed to low temperature (Fennell 2004, 2014, 2015).

**Bud break**

Bud break and shoot growth provide a very good estimate of winter injury in the grapevines; however, this assay of viability requires several weeks to complete (Fennell 2004). Bud break is defined as the first day when green tissue appears between the bud scales (Coombe 1995; Lorenz et al. 1995). Bud break occurs when the chilling requirement is fulfilled and warm temperatures promote growth. Chilling requirement is the amount of low temperature exposure required by the dormant overwintering buds to transition from endodormancy to ecodormancy (Dokoozlian 1999; Lavee and May 1997). Ecodormancy is released in the presence of favorable environmental growth conditions (warm temperature >15°C). *Vitis vinifera* typically requires between 50 and 400 hours of chilling to satisfy endodormancy (Londo and Johnson 2014) at a temperatures between 0 and 7°C (Dokoozlian 1999). The chilling hours required to break endodormancy range from 250-2250 for other species/genotypes (Dokoozlian 1999; Londo and Johnson 2014). Increased exposure to chilling temperatures increases the number and the rate of bud breaks (Dokoozlian 1999; Londo and Johnson 2014). The chilling requirement plays a protective role against short intervals of unseasonably warm temperatures followed by resumed cold (Bailey and Hough 1975). Insufficient chilling as a result of temperature greater than 7°C results in delayed and desynchronized bud break. The productivity of
vineyards is negatively affected by the delayed and desynchronized bud break as it
directly impacts the number of shoots and clusters and the fruit ripening rates of the vines
(Dokoozlian 1999; Lavee and May 1997).

The ecodormant bud breaks under permissible environmental conditions. There
are many published scales describing the grapevine bud phenological stages, the first by
Baggiolini (1952) was then revised by Baillod and Baggiolini (1993). Additional scales
include biologische bundesantalt, bundessortenamt and chemische industrie scale (BBCH
scale), Eichhorn and Lorenz (Eichhorn and Lorenz 1977, Lorenz 1994) and the modified
Eichhorn and Lorenz scale (modified E-L scale). The modified E-L scale developed by
Coombe (1995) is one of the scale to describe the phenological stages of bud break. Early
genetic studies in perennial woody species demonstrated that a number of dormancy-
related traits are under the genetic control, including chilling requirement. These traits are
quantitative in nature and under multigene control along with environmental effect.

**Quantitative trait loci (QTL) analysis:**

Subzero temperature tolerance is known to be a complex trait governed by many
genes with small additive effects. Multiple genes cannot be studied individually using the
methods for classical Mendelian genetics since their small effects are lost in the
background variation (Falconer et al. 1996). These small effect genes, also known as
quantitative trait loci (QTL), are the small segments of the chromosome that contain the
genes affecting the trait of interest. QTL analysis is a statistical tool that aims at
identifying regions of the genome that is contributing to the variation in the trait of
interest. It is a popular tool to dissect the genetics behind complex traits by the combined
use of molecular markers and phenotype data so as to explore the individual genes
concerned with quantitative traits (Kearsey 1998). QTL mapping is based on the principle that genes and markers segregate by recombination during meiosis resulting in variability in the quantitative traits in the progeny or segregating population (where there is a mixture of parental and recombinant genotypes) which is then analyzed using statistical methods. The two requirements for conducting QTL analysis are a linkage map of polymorphic marker loci that adequately covers the whole genome and variation for the quantitative trait within or between populations or strains. There are different methods for doing QTL analysis, for example: single marker analysis, simple interval mapping (SIM), composite interval mapping (CIM) and multiple QTL mapping (MQM) (Jansen 1994; Lander and Botstein 1989; Zeng 1994). In CIM, a LOD score peak profile that either equals or exceeds a predecided value indicates a QTL position (LOD score threshold). A LOD score threshold depends on many factors like size of the genome, the density of markers and the amount of missing data. The permutation test is used to obtain marker threshold, where, the trait phenotypes are randomly shuffled, keeping the marker genotypes for the individuals of the sample fixed. This results in total disruption of original trait phenotype and marker association. The QTL analysis is performed with the marker genotype and the shuffled phenotype data, and the LOD score is determined for a given position in the genome. When 1000 permutations are performed, the process is repeated for 1000 times for a given genomic position, and the LOD scores so obtained are examined to obtain the LOD score threshold value. The above process is repeated for every genomic position at which the presence of a QTL is to be detected.

The power of QTL detection increases when combining the information of correlated traits and multiple environments (Singh and Singh 2015). The inclusion of
information from the correlated traits can increase the detection of relevant QTLs, and also increases the precision of QTL effect estimates. It also provides the necessary background to study QTL pleiotropy or a close linkage among QTLs affecting individual traits (Singh and Singh 2015). The power of QTL detection also increases when the same mapping population is evaluated in several environments. The effect size estimates for the same QTL may vary from one environment to another and some of the QTLs may not be detected in some of the environments because of QTL by environment interaction. However, the failure to detect a QTL in some of the environments may not necessarily be due to QTL by environment interaction, but may be the result of an unusually high error variance in the concerned environments (Bernardo 2008). This results in a reduction of the transferability of results from QTL analyses and heritability and effectiveness of selection for the trait. The QTLs involved in the control of different correlated traits usually map in the same genomic region; such a genomic region is QTL hotspot. A QTL hotspot may contain hundreds of different genes (Singh and Singh 2015).

**QTL mapping in Grapevines**

Genetic mapping and QTL analysis are one of the popular and effective methods for studying the genetics behind the quantitative traits (Collard et al. 2005; Falconer et al. 1996; Grattapaglia et al. 1995; Kearsey 1998). In grapevines, various types of markers have been used for QTL analysis over the past two decades. The first genetic map of grapevine was published in 1995 (Lodhi et al. 1995). The first maps used random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) to construct genetic maps (Dalbo et al. 2000; Doligez et al. 2002; Doucleff et al. 2004; Fanizza et al. 2005; Fischer et al. 2004; Grando et al. 2003; Lodhi et al. 1995).
Although these markers are convenient to use, these are dominant markers and are difficult to transfer and compare between mapping populations (Adam-Blondon et al. 2004). Thus, the development of genetic maps using co-dominant marker microsatellites or simple sequence repeats (SSR) markers became popular (Adam-Blondon et al. 2005; Adam-Blondon et al. 2004; Bowers et al. 1996; Costantini et al. 2008b; Di Gaspero et al. 2007; Doligez et al. 2002; Garris et al. 2009; Grando et al. 2003; Lowe and Walker 2006; Riaz et al. 2004; Zhang et al. 2009). SSR is a low throughput marker platform and though it has the advantage of being able to transfer across diverse germplasm, it is expensive and time-consuming and results in low-resolution genetic map using 100 to 600 markers per genome (Adam-Blondon et al. 2005; Duchene et al. 2010; Mejía et al. 2007; Vezzulli et al. 2008). In 2007, a dense genetic map of V. vinifera anchored to ‘Pinot Noir’ genome was described using 483 SNP-based markers (Troggio et al. 2007). SNPs have been popularly used in constructing dense genetic map because of its co-dominance nature, high level of polymorphism and their abundancy (Troggio et al. 2007). Along with the genetic map, grapevine physical maps have also been constructed (Jaillon et al. 2007; Moroldo et al. 2008; Scalabrín et al. 2010; Velasco et al. 2007). With the advancement in whole genome sequencing and next generation sequencing (NGS) technology, a genetic map using 1643 SNPs was developed along with SNP chip array (Wang et al. 2012; Myles et al. 2011; Myles et al. 2010). With the development of genotyping by sequencing (GBS), one of the reduced representation library (RRL) approach, the genotyping cost per sample has reduced drastically and the thousands of markers with low coverage can be easily obtained (Elshire et al. 2011).
GBS is a simple and cost-effective approach used prior to next generation sequencing that is useful for genotyping highly diverse and complex genome. The ability of GBS to reduce genome complexity and effectively sequence low copy region of the genome is made possible with the use of methylation-sensitive restriction enzymes that avoids the repetitive regions of the genome (Elshire et al. 2011). Barba et al. (2014) successfully applied GBS to discover 16833 SNPs with an average density of 36 SNPs/Mbp to develop a heterozygous map of grapevine. There are some problems with GBS due to its high level of multiplexing and shallow sequencing such as missing data, heterozygote under calling and genotyping error that makes this platform difficult to use for heterozygous or highly diverse species like grapes or for species that do not have a reference genome available (Yang et al. 2016). Various computational strategies have been applied to overcome the drawbacks of GBS approach in grapevine among which heterozygous mapping strategy (HettMappS) has the ability to construct genetic maps based on synteny (reference genome provided) and de novo assembly (for which reference genome is not available) (Hyma et al. 2015).

Grapes are highly heterozygous crop and are severely affected by inbreeding depression resulting in poor seed viability and stunted growth. Therefore, QTL analysis in grapevines and other woody fruit crops commonly uses F1 mapping population and pseudo-testcross strategy (Grattapaglia et al. 1995). However, segregating F2 population has been used to develop genetic map and perform genetic analysis in many woody fruit crops including grapevines (Bielenberg et al. 2015; Yang et al. 2016; Zhebentyayeva et al. 2013). QTL analysis has been performed for many traits in different grapevine progenies to gain understanding of the genetic determinants related to these traits.
Grapevine QTL analyses have focused mainly on disease resistance downy mildew, powdery mildew, Pierce’s disease and other diseases (Barba et al. 2014; Blanc et al. 2012; Blasi et al. 2011; Di Gaspero et al. 2007; Fischer et al. 2004; Krivanek et al. 2006; Luo et al. 2001; Moroldo et al. 2008; Riaz et al. 2011; Riaz et al. 2008; Venuti et al. 2013; Wang et al. 2012). Similarly, QTL analysis has been equally employed to study the genes related to insect resistance (Hwang et al. 2010; Kuczmog et al. 2012; Xu et al. 2008). QTL analysis has also been conducted for agronomical traits like berry size, seed number, berry weight, inflorescence, flower morphology, flowering date, aroma profile, anthocyanin content, berry color, cluster architecture and number of clusters per vine, sexuality, sugar and acid production, pH. and titrable acidity (Battilana et al. 2013; Cabezas et al. 2006; Carrier et al. 2013; Chen et al. 2015; Correa et al. 2014; Costantini et al. 2008b; Costantini et al. 2015; Dalbo et al. 2000; Doligez et al. 2013; Doligez et al. 2002; Fanizza et al. 2005; Fernandez et al. 2006; Fournier-Level et al. 2011; Fournier-Level et al. 2009; Grzeskowiak et al. 2013; Houel et al. 2015; Huang et al. 2013; Huang et al. 2012; Hyma et al. 2015; Marguerit et al. 2009; Viana et al. 2013; Yang et al. 2005; Zhao et al. 2015). Though abiotic stress tolerance like drought tolerance, salt tolerance and subzero temperature tolerance are necessary for the survival and good performance of grapevines, there are inadequate studies to determine the genes regulating those stresses (Bert et al. 2013). Until now, no QTL study regarding the subzero temperature tolerance and other cold stresses has been done on grapevines. Garris et al. (2009) performed QTL analysis for the photoperiod-induced growth cessation that promotes dormancy and early acclimation. The F2 population was developed by selfing a single F1 (16_9_2) from a cross between V. riparia and hybrid cultivar Seyval (Fennell et al.
This population was used to develop genetic map with 115 SSR markers (Garris et al. 2009). Yang et al. (2016) developed a dense genetic map from 424 F2 progeny using 1449 single nucleotide polymorphism (SNP) markers generated from using genotype by sequencing (GBS). The SNP map covers 95% of the genome, with a genetic length of 2424 cM across 19 linkage groups averaging 1.67cM between adjacent markers (Yang et al. 2016). The F2 GBS SNP map has been used for QTL analysis of fruit quality traits sing R/qtl (Yang et al. 2016).

Genetic studies of freezing and low temperature related stresses are well studied in other plants such as barley, Arabidopsis, Medicago truncatula, Festuca, Crucifers, winter wheat and Salix (Alm et al. 2011; Alonso-Blanco et al. 2005; Avia et al. 2013; Baga et al. 2007; Bartos et al. 2011; Francia et al. 2004; Gery et al. 2011; Hayes et al. 1993; Heo et al. 2014; Knox et al. 2008; Meissner et al. 2013; Tayeh et al. 2013; Teutonico et al. 1995; Tsarouhas et al. 2004). In grapevines, most of the studies are focused on the physiological and biochemical aspect of subzero temperature tolerance rather than on genetic analysis of the traits. Locating the position of QTL and identifying potential candidate genes involved in subzero temperature tolerance is very important to further study the magnitude of their effects on phenotype, the parental origins of the favorable QTL alleles, and the relationships between QTLs controlling different low temperature response processes. For a quantitative trait like subzero temperature tolerance and other low temperature response tolerance traits, QTL mapping is an appropriate approach. Studying the genetics behind such complex traits is very important to select for the genotypes that can perform well even in continental climates and QTL analysis is one of the appropriate ways to do so.
**Processing phenotypic trait data for QTL analysis:**

Before QTL analysis, the phenotypic data is checked for the normal distribution. Violation of normality can increase type -1 error. Severity of a deviation from normality can be in graphic distribution analysis of the data. Sometimes, the data is skewed and unbalanced. Depending on the nature of the data, the data need to be transformed with different mathematical approaches before doing QTL analysis. This will improve the accuracy of QTL prediction and reduce type-1 error. For the unbalanced data, best linear unbiased predictors (BLUPs) are often used to predict random genotypic effects.

Similarly, in disease studies, area under curve (AUC) is also popular.

**Best linear unbiased predictors**

Best linear unbiased predictors (BLUP), is a statistical approach for estimating random effects of a mixed model whereas the fixed effects are calculated with best linear unbiased estimators (BLUE). This technique was developed by Charles Henderson (1984) to process highly unbalanced cattle data. It is commonly used in animal breeding to estimate the breeding value of parents, however, this technique has gained popularity in the field of plant research (Piepho et al. 2008). This technique treats the genetic effects as a random effect to understand the breeding value of the parents. It incorporates best linear unbiased estimates of the fixed effects through generalized least squares with the best linear unbiased prediction of the random genetic effects (Soh 1994). It has the ability to improve the prediction of genetic values by incorporating information from relatives and to estimate and remove genetic trends and selection bias by treating them as fixed effects. BLUPs are obtained by fitting the phenotypic data in mixed model. These BLUPs
are adjusted for the fixed effects and regress the phenotypes toward the mean as a function of the repeatability of the data.

**Area under bud break progression curve**

The concept of area under bud break progression curve (AUBPC) is borrowed from area under the curve (AUC), often used in plant epidemiological studies (Simko and Piepho 2012). The idea of AUC is to incorporate multiple observations of disease progress into a single value. AUC or area under the disease progression curve (AUDPC) is one of the most popular measurement used to study disease intensity over time where quantitative resistance is conferred by multiple genes of minor effects (http://www.apsnet.org/EDCENTER/ADVANCED/TOPICS/ECOLOGYANDEPIDEMIOLOGYINR/DISEASEPROGRESS/Pages/AUDPC.aspx). This method allows quantification of temporal increase of the disease as well as allows comparison of disease development in different years, locations and management practices (Jeger and Viljanen-Rollinson 2001). It entails repeated disease assessment of the plants using a certain scale. In nature, the disease typically starts at a low level, gradually increasing in incidence and/or severity over time. During pathogen-epidemiological studies, a number of observations are made to evaluate the progress of disease on plants and the extent of disease progression is assessed at each observation using scales that are based on disease incidence, severity, or a combination of both. Van der Plank proposed a method to combine these repeated observations into a single value by calculating the area under the disease progress curve (AUC) (Simko and Piepho 2012). The advantage of using AUC is it is simple to calculate, incorporates multiple evaluations and does not rely on transformation of the data. A common approach to determine AUC is through a simple
midpoint (trapezoidal) rule that breaks up a disease progress curve into a series of trapezoids, calculating the area of each, and then adding up the areas

$$AUC = \frac{\sum_{i=1}^{n-1} y_i + y_{i+1}}{2} \times (t_{i+1} - t_i)$$

Where $y_i$ is an assessment of a disease (percentage, proportion, ordinal score, etc.) at the $i^{th}$ observation, $t_i$ is time (in days, hours, etc.) at the $i^{th}$ observation, and $n$ is the total number of observations.

The idea of AUC is applied with the bud break data in this study (AUBPC). The bud break data that we collected was ordinal data, scored on the basis of a scale and monotonic which is unidirectional, and involves repeated measurements in the time period, which can be compared with disease progress. The disease progress is also ordinal, monotonic and based on repeated measurements on the same entity. In the same way, the same cane with one bud was used for measurement in each week, up to four weeks. The AUC concept is applied in our bud break study to account the changes that take place in the time period (as the stages of bud growth continue to increase (grow) as the time passes). Similar to disease development over time, bud break stages increase with time.
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Chapter 2 Mapping subzero temperature tolerance quantitative trait loci in F2 grapevine population.

Abstract

Cold acclimation is an important trait enabling grapevines to survive freezing injury in northern continental climates. It is a complex quantitative trait that is influenced by the genetic characteristics of grapevines as well as environmental interactions. To explore the genetic components of freezing tolerance in grapevine, an F2 population was phenotyped for freezing tolerance during multiple months in a dormant season and in multiple dormant seasons using differential thermal analysis (DTA) to identify low temperature exotherms (LTE). The average LTEs were fitted into linear mixed models to obtain best linear unbiased predictors (BLUP), which were run using composite interval mapping (CIM) to find quantitative trait loci (QTL) for subzero temperature tolerance. Genotypes that were slow to acclimate exhibited LTE at higher temperatures than genotypes that acclimate early. Increased exposure to low nonfreezing temperatures increased subzero temperature tolerance and the number of genotypes capable of surviving lower temperatures increased. Subsequently, as the temperatures increased in late winter, vines deacclimated and buds were killed at higher temperatures. QTLs were identified for all the months and dormant seasons. The QTLs demonstrated instability from year to year; however, QTLs were identified in more than one month or dormant season on chromosomes 1, 5, 9, 13 and 16. The QTLs explained 7% to 17% of the phenotypic variation. QTL on chromosome 5 and 13 were identified for December and November using both average LTE and genotypic BLUPs, overlapping the same genomic region and same nearest marker. The QTLs explained 6.7% to 16.52% of the phenotypic variation.
variation. The additive effects showed that female grandparent *V. riparia* has been found to be contributing the subzero temperature tolerance to the F2 genotypes. A search of candidate genes for these QTLs rendered different genes associated with calcium signaling, dehydration response, ethylene signaling, ABA signaling, cell wall synthesis and cellular metabolism. A better understanding of the genetic factors affecting subzero temperature tolerance will help the breeders to select genotypes that can perform well in low temperatures.
Introduction

Freezing temperature can cause severe injury of plant parts to plant death, resulting in low productivity and economic loss (Fennell 2004). Upon exposure to low, non-freezing temperatures, temperate perennial plants including grapevines develop an ability to acclimate and tolerate freezing temperature stress during autumn and winter (Fennell 2004). Grapevines undergo several gradual developmental, physiological and molecular changes before the onset of winter including endodormancy induction, periderm development, growth cessation, leaf senescence along with various physiological and molecular changes, in response to environmental cues like photoperiod and low temperature (Fennell 2004; Fennell and Hoover 1991; Fennell and Mathiason 2002; Grant et al. 2013; Wake and Fennell 2000). The ability to tolerate freezing temperature is a combination of genetic potential of the crop and interaction with environment (Fennell 2004; Palonen and Buszard 1997; Stushnoff 1972; Wisniewski et al. 2003). Understanding the genetics of this complex multi-trait phenomena of subzero temperature tolerance will help to develop the cultivars that survive winters with severe temperatures.

Grapes rank second in terms of production of fruit crops (http://faostat3.fao.org/) due to its multiple uses in wine, raisins, table grapes, juices and much more. The grape industry contributes about $162 billion to US economy ($33 billion in wages and $17 billion in state and local tax revenues) (www.ngwi.org). The freezing injury causes significant loss to the grapes and wine production. Most of the grapevines cultivated in the world belong to Vitis vinifera, which is not much freezing tolerant (Pratt 1996). A fully acclimated grapevine of V. vinifera can tolerate -15°C temperatures without
significant winter injury (Fennell 2004; Pratt 1996). In South Dakota, the temperature often goes well below that, frequently reaching -30°C and lower. Wild North American native species like *Vitis riparia*, are resistant to phylloxera and most fungal diseases, and can withstand freezing temperatures up to -40°C (Fennell 2004; Pratt 1996). Thus, breeding new cultivars for the continental climates requires the combination of adaptive genes from wild species and other quality traits from *V. vinifera* to assure the survival and productivity in the cold regions (Garris et al. 2009).

In woody plants, cold acclimation is triggered by the environmental cue of shortening days and/or decreasing temperature that leads to growth cessation and the induction of dormancy (Fennell 2004; Sakai and Larcher 1987). Low temperature is the major factor to induce acclimation and dormancy in apple (*Malus pumila*), pear (*Pyrus communis*), red osier dogwood (*Cornus sericea*), birch species, raspberry whereas the photoperiod plays a significant role in cold acclimation in poplars (*Populas sps*) (Howe et al. 2000; Palonen and Buszard 1997). In grapevines, two major environmental cues found responsible for the induction of cold acclimation, are low temperature and decreasing day length (Fennell 2004; Fennell and Hoover 1991; Fennell et al. 2005; Grant et al. 2013; Schnabel and Wample 1987; Wolf and Cook 1994). These initiate a cascade of developmental changes in fall that transitions a plant from cold tender to cold hardy state through the process of acclimation and endodormancy induction. Genetic variability to the sensitivity to low temperature and/or photoperiod for developing subzero temperature tolerance in grapevines can be observed at species level and genotype level (Fennell and Hoover 1991; Fennell et al. 2005; Wake and Fennell 2000). In some genotypes, acclimation begin in response to decreasing photoperiod whereas in
some low temperature is required to initiate the process of acclimation. Some genotypes require synergistic response to decreasing photoperiod and low temperatures for the start of acclimation. Grapevines do not set terminal bud which is a characteristic of woody perennials, rather shoot tip abscission occur in response to growth cessation and cold acclimation (Wake and Fennell 2000). Subzero temperature tolerance is a multi-trait phenomena, dependent on different interacting factors like the timing of dormancy induction, the acclimation rate, the degree of tolerance attained, and the rate of deacclimation (Rowland et al. 2008; Wisniewski et al. 2003). Subzero temperature tolerance is a complex trait that is found to be heritable and controlled by a number of quantitative trait loci (Fennell et al. 2005; Howe et al. 2000).

The mechanism of subzero temperature tolerance differs in various tissues. The grapevines dormant buds protect itself from the freezing temperatures through the mechanism of supercooling, whereas the canes tolerate extracellular ice formation (Andrews et al. 1984; Fennell 2004; Pierquet et al. 1977). The subzero temperature tolerance of the grapevines buds and canes are assessed using differential thermal analysis (DTA), a technique to measure/quantify subzero temperature tolerance of the tissues based on the release of heat when the supercooled water that is present inside the buds/tissues freeze (Quamme 1978, 1986). DTA was first used in Prunus sps by Quamme et al. (1973) and the relationship between LTE and injury in grapevines was confirmed in V. riparia and V. vinifera (Ferguson et al. 2014; Mills et al. 2006; Pierquet and Stushnoff 1980). The high and low temperature exotherms identified using DTA are associated with initial freezing or non-lethal formation of extracellular ice (Andrews et al. 1984) and freezing of supercooled water inside the bud cells respectively (Quamme 1978,
Low temperature exotherms (LTE) correspond to the killing temperature of the bud or bud death (Pierquet and Stushnoff 1980).

The objective of this study was to investigate the genetic determinism of subzero temperature tolerance in grapevines and find the genomic location associated with those traits. QTL analysis has been performed successfully to identify genomic regions associated with many traits such as disease resistance, biotic stresses, abiotic stress, fruit quality traits and phenological traits using F1 grapevine progenies. Garris et al. (2009) performed QTL analysis for the photoperiod-induced growth cessation in F2 population using simple sequence repeats (SSRs), which is an important process in cold acclimation. The F2 population has been successfully used for the QTL analysis in grapevines and also other woody fruit crops (Bielenberg et al. 2015; Yang et al. 2016; Zhebentyayeva et al. 2013). Genetic studies of freezing and low temperature responses has also been studied in other crops such as poplars, citrus, Salix and Douglas-fir (Alonso-Blanco et al. 2005; Anekonda et al. 2000; Jermstad et al. 2001; Tsarouhas et al. 2004; Weber et al. 2003). Until now, no QTL study regarding the subzero temperature tolerance and other cold responses have been conducted on grapevines. Knowing the genetics behind subzero temperature tolerance will help breeders to develop the cultivars that have the potential to tolerate low winter temperatures and thereby expand grape production in continental climates. Identifying the loci and underlying genes related to subzero temperature tolerance is very important to further study the magnitude of their effects on phenotype, the parental origins of the favorable QTL alleles, and the relationships between QTLs controlling different acclimation and subzero temperature tolerance process.
Materials and Methods

Population development

F2 progeny developed by selfing a single hermaphrodite F1 (16_9_2) from the cross *V. riparia* (USDA PI 588289) X *Vitis* hybrid “Seyval” (Seyval Villard 5-276) were used in the study (Fennell et al. 2005). The parent *V. riparia*, F1 and 113 F2 progenies were clonally propagated and planted in the vineyard at the NE Hansen Research Farm, Brookings, South Dakota (44°19’N) in 2005.

Differential thermal analysis

Three canes were harvested randomly from each genotype in two or three months of each dormant season from 2011 to 2015 (Table 2.1). The size of the vines limited the number of sample times in each year. Each cane consisted of 4-5 buds. After the canes were harvested from the field, bud viability was checked visually after longitudinal sectioning to expose the meristems. A green bud meristem indicated the bud is alive, whereas the brown bud indicated that the bud is injured or dead. Only live buds were used for freezing test differential thermal analysis (DTA). The number of plants phenotyped varied from 48 to 95 (Table 2.4).

A DTA unit consisting of a programmable freezer (Tenney International, Environment Test chamber, Model no. BTC), thermoelectric modules (TEM) and data acquisition system (DAS, Keithley 2700 Multimeter system) as described by Mills et al. (2006) was used to acquire low temperature exotherms (LTEs) of the buds. The samples were placed in trays containing nine TEMS and a thermistor. Three to four buds were placed in each TEM. The trays were placed in the programmable freezer at 4°C for 1 hour and then temperature decreased at 4°C per hour. Voltage change in TEM was
recorded by the DAS and thermistor temperature monitored for each tray. Temperature of exotherm peaks were identified by plotting TEM voltage against the temperature. Two types of exotherms are recorded by the DTA depending on the tissue where the supercooled water freezes. The exotherms that occurred at higher temperature below 0°C were considered the high temperature exotherms (HTE) and are associated with non-lethal formation of ice in bud scales or the cane tissues attached with the buds whereas the low temperature exotherms (LTE) occurred at lower temperature and are associated with the freezing of the supercooled water inside the bud. The HTEs and LTEs were differentiated based on the distribution of the exotherms collected in the particular month.

**Phenotypic evaluation**

All phenotypic diagnostic analysis was performed with RStudio (software version 0.99.902; (Team 2015a)). The average DTA data for each genotype in each month and year were used for all the analyses. The maximum and minimum LTE obtained among the LTEs of the genotypes, referred as the high LTEs and low LTEs respectively, were also used to perform the phenotypic analysis (data not shown). Since, HTEs occurred when the water in the cane tissues attached with the bud froze, we did not use the HTEs in our analysis. The distribution of the data was checked using the histogram in R (Team 2015b). The normality of the data was checked with Shapiro-Wilk test and gvlma package in R. Boxplot was used to see the distribution of variance in the data. The Pearson correlation coefficient was calculated to see the strength and direction of the data. Data transformation was tried but did not improve the distribution of the data, so, the untransformed data was used for the analysis. Transgressive segregants, that showed
higher/lower LTEs than the parents were also identified. The boxplot was also plotted to see the variation in the data between different months and different dormant seasons.

**Best linear unbiased predictors analysis**

Best linear unbiased predictors (BLUP)s were calculated for the average LTEs of the F2 population. These BLUPs were adjusted for the fixed effects and they regress the phenotypes towards the mean as a function of the repeatability of the data (Eckard et al. 2015; Soh 1994). We identified the best-fit model for LTEs for each year through the linear mixed model in order to extract the BLUPs for genotypic values. The LTEs were fitted in the following mixed model:

\[ Y_{ij} = \mu + G_i + T_j + e_{ij} \]

Where,

\( Y_{ij} \) is the average low temperature exotherms obtained from bud through DTA for \( i^{th} \) genotype in \( j^{th} \) time point/month. \( \mu \) refers to the population mean, \( G_i \) is the genotype of the \( i^{th} \) plant and \( T_j \) refers to the \( j^{th} \) month when the samples were collected from the field and analyzed. \( e_{ij} \) refers to the residual errors. Since there were no replications in a particular time point; we cannot consider the G X E interaction in our model. The genotypes and the time points were considered as the random effects since the genotypes were sampled from a large population of the F2 plants in the specific months.

The “minqué” package in R (Wu 2014) was used to fit the model and estimate the BLUPs for LTEs of the F2 populations for each dormant season.. For example, in 2011, the phenotyping was done in November 2011, December 2011 and February 2012. Similarly, BLUPs were extracted in two ways:
i. All months within a dormant season.
ii. Individual months across different dormant seasons.

**Quantitative trait loci analysis**

Quantitative trait loci (QTL) analysis was performed on average LTE data for each month and dormant season individually and the genotypic BLUPs with the R/qtl package (Broman et al. 2003). The average of the same month across all dormant seasons was also used for the QTL analysis. The F2 GBS-SNP genetic map developed by Yang et al. (2016) was used for the analysis. The map was developed using 1449 SNP markers and 424 F2 progeny. QTL analysis was performed using single QTL scan (“scanone” function, “Normal” model) and composite interval mapping (CIM) (Zeng 1994) with R/qtl (the “scanone” function, map function “Kosambi”, method=”hk”, n.perm=1000) and using F2 as the cross type. CIM uses forward selection to identify the markers and then runs interval mapping with the selected markers as covariates. The threshold was determined with 0.05 level of significance and grapevine standards of 1000 permutations. All the QTLs that crossed the LOD score of 3 (standard LOD threshold) were considered a significant QTL (Singh and Singh 2015; Wu et al. 2014). The QTL with the largest LOD was identified as the most probable QTL. After the QTL was identified, we obtained the phenotypic variance ($R^2$) of the trait explained by the QTL identified by calculating the genotypic probabilities for the marker linked to the QTL. The QTL identified was then fitted in a model, LTE~QTL+e to obtain the genotypic additive and dominance effects (“fitqtl” function). The “lodint” function was used to derive the 1.5 LOD support interval. Genome wide scan was performed with a 1cM step. Reports were generated for maximum LOD score, 1.5-LOD support interval in cM, the flanking
markers, the physical location in the reference genome in Mbp, as well as the variation explained.

**Results**

**Phenotypic evaluation**

Phenotypic data distributions were different for different dormant seasons and months. The distribution of LTE dataset was more or less normal (Appendix 1). In some dormant seasons, the datasets did not fulfill all the assumptions of normality and homogeneous variance, in such cases data transformation was tried, but that did not improve the data distribution. Transgressive segregation was observed for all the months and dormant seasons. The F2 genotypes that showed lower LTEs than that of the female grandparent, V. riparia were considered a transgressive segregant. Since, the male grandparent, Seyval cannot survive in the field conditions in Brookings, we were not able to identify the transgressive segregants that had higher LTEs than the Seyval as they were killed in the field conditions. For subzero temperature tolerance, the transgressive segregants that had a phenotypic value higher than the high-value parent ranged from 7.8% to 51.4%, shown in Table 2.2. The variation in LTEs in different dormant seasons and months is shown with the help of boxplot (Figure 2.2 and 2.3). The median value for the December was lower than other months, which may be due to December being the coldest month of the year and February showed the highest level of variability in Figure 2.2.

The Pearson correlation coefficients between November of dormant season 2011-2012 and November of dormant season 2012-2013 was strong and significant, whereas the correlation between the same months in two different dormant seasons was weak.
The positive correlation between the same month in two different
dormant seasons showed the similar response of genotypes in different dormant seasons
(Figure 2.7). In dormant season of 2011-2012 and 2012-2013, the correlation coefficient
between the month of November and December was observed positive with stronger
correlation in 2011. The correlation between December 2012 and February 2013, as well
as January 2015 and February 2015, were observed to have a negative correlation,
whereas a positive correlation was observed for January 2014 and February in 2014, and
December 2014 and January 2015.

The F2 population showed different LTEs in different months and dormant
dormant seasons. The frequency distribution of average LTE of the plants across 2011-
2012 and 2012-2013 dormant season are shown in Figure 2.4. and Figure 2.5,
respectively. The mean, maximum and minimum temperature of the field one week
before the sampling are shown in Appendix 5. The LTE of F2 occurred at higher
temperature in November of dormant season 2011-12 when the average temperature one
week before sampling was 4.7°C in comparison to the month of December (-6.8°C). In
dormant season 2012-2013, the average temperature in November, December, and
February were 4.7°C, -6.8°C in and -12.3°C respectively (Appendix 5). A greater
percentage of genotypes had higher LTEs at in November and December in comparison
to February. In February, the grapevines had already developed maximum subzero
temperature tolerance and thus more buds were able to survive the freezing temperature.
This showed an increase in subzero temperature tolerance with increased low-
temperature exposure. The number of genotypes with a bud LTE below -27°C increased
as winter progressed.
In January 2014, the bud LTEs occurred at lower temperatures than in February, which was indicated by a greater number of genotypes that were able to survive low freezing temperature in January than in February. The average temperature one week before sampling in January 2014 was -18.4°C and in February 2014 was -2.9°C (Appendix 5). The buds were killed at relatively higher temperatures in February. Exposure to increasing temperatures in February resulted in a greater number of genotypes with an average LTE > -26°C in February 2014 than in January 2014 (Figure 2.6). As the temperature started to increase in February, the plants began to lose the subzero temperature tolerance.

The F₂ plants showed greater subzero temperature tolerance in all the months phenotyped than *V. vinifera*. This may be due to the contribution of inherent subzero temperature tolerance the female grandparent *V. riparia*. An overview of the events occurring in different months across different dormant seasons from 2011 to 2016 shows variation in the response of the F2 genotypes (Figure 2.7). The bud LTEs occurred at higher temperature in November than any of the months. The LTEs in December and January occurred at similar temperature and the bud LTEs were the lowest in these months. February also demonstrated the LTEs at low temperatures. The different levels of subzero temperature tolerance: acclimation, mid-winter hardiness, and deacclimation, which is characteristics of grapevine are illustrated in the figures 2.3, 2.4 and 2.5.

**QTL analysis using Low Temperature Exotherms**

QTLs for subzero temperature tolerance, detected by CIM and Kosambi map function in R/qtl, including their LOD score, the physical location of the nearest marker, flanking markers, the phenotypic variation explained by the QTL (R²), the physical
location of the flanking markers, the additive and dominant effect of the QTLs are presented in Table 2.3, 2.4 and 2.5. The QTL that appeared in multiple dormant seasons and/or more than one approach and that overlap the same genomic region were considered a dependable QTL and then annotated. Six QTLS for average LTE were identified on chromosome 1, 5, 9, 13 and 16. A QTL for average bud LTE was observed on chromosome 1 in February 2013 and January 2015 that explained 15.5% and 7.88% of phenotypic variation respectively. This QTL occupied a similar genomic region in both the months, however, the nearest markers were different. Two QTLS were identified on chromosome 5 using individual months and BLUPs. Analysis using average bud LTE identified a QTL on chromosome 5 in November 2011, November 2012 and January 2016 that overlapped the same genomic region and explained 8.67%, 20.22% and 17.56% of the phenotypic variation respectively. The additive effect of the QTL was negative indicating that the greater subzero temperature tolerance came from the female V. riparia grandparent. Another QTL was identified on chromosome 5 using BLUP for the dormant season 2011 and 2016, explaining 17.58% and 15.5% of the phenotypic variation. A QTL was identified on chromosome 9 using average LTE for December 2011 and genotypic BLUPs for February. In both the approaches, the 1.5 LOD support interval overlapped the same genomic region and explained 14.11% and 17.39% of the phenotypic variation for December 2011 and BLUP February. A QTL was identified on chromosome 13 for November using average LTE data and genotypic BLUPs for November. This QTL occupied the same genomic location and had the same nearest marker, and explained 12.7% and 16.5% of the phenotypic variation. QTL analysis using average LTE for November 2011 also detected a QTL on chromosome 13, which co-localized in the same
genomic region, but had different nearest marker. The negative additive effect of the QTL suggests that the greater subzero temperature tolerance is contributed by the female V. riparia grandparent. A QTL was identified on chromosome 16 using genotypic BLUPs for the dormant seasons 2011-2012 and 2015-2016, covering the same genomic location and associated with the same marker. The QTL contributed 12.3% and 11.5% variation to the phenotype in dormant season 2015-2016 and 2011-2012 respectively. The additive effect of the QTL was negative, suggesting the greater subzero temperature tolerance was contributed by the female V. riparia grandparent.

**Discussion**

The dormant bud is the most vulnerable part of grapevines to winter injury (Clore et al. 1974). The high temperature exotherms identified using DTA are usually associated with non-lethal formation of extracellular ice and low temperature exotherms are associated with freezing of supercooled water inside the bud cells (Quamme 1978, 1986). Since, the LTE corresponds to the killing temperature of the bud, the average LTE of the F2 buds (below -20°C) were used to quantify the subzero temperature tolerance attained by grapevine F2 progeny in different months and dormant seasons (Pierquet and Stushnoff 1980). As characteristic of the quantitative traits, the average LTE showed a more or less normal distribution (Appendix 1). However, in some of the months the distribution was skewed. In December 2014, the number of samples was very low (46) because of limited materials in the field caused by winter injury in the previous year. Data transformation did not help with the distribution, therefore, the average bud LTE data was used for the analysis.
The bud LTE and the ambient air temperature have been found to have a very high correlation in grapevines (Jiang and Howell 2002). As the ambient temperature begins to decrease in October and November and day length becomes shorter, the grapevines start to prepare winter temperatures by developing periderm and entering into dormancy (Fennell 2004; Wake and Fennell 2000). The greater exposure to low non-freezing temperatures, the greater cold acclimation. In November when the average air temperature was above freezing (5°C), the LTE occurred at relatively higher subzero temperatures (Figure 2.3). The number of genotypes that survived temperatures below -26°C was higher in the month of December than in the month of November (Figure 2.3). The average air temperature was below -6°C in December (Appendix 4). During November, the genotypes responded to the decreasing photoperiod prior to exposure to the sub-freezing temperatures, which resulted in early acclimation (Fennell 2004; Fennell and Hoover 1991; Wake and Fennell 2000). The buds were endodormant but had cold acclimated extensively in November. The sensitivity to short photoperiod for endodormancy induction and early acclimation in some F2 must have come from V. riparia, which is very responsive to decreasing photoperiod and has greater freezing tolerance (Fennell and Hoover 1991; Fennell et al. 2005). The identification of transgressive segregants and negative additive effect shown by the QTL also supported that the subzero temperature tolerance in F2 came from female grandparent V. riparia.

The prolonged exposure to the low non-freezing temperatures increases the grapevines ability to survive in the freezing temperatures (Fennell 2004; Fennell and Hoover 1991; Ferguson et al. 2014; Miller et al. 1988; Schnabel and Wample 1987). The correlation between December and February was found to be very weak or negative. This
may be explained by the fact that the plants attain greatest subzero temperature tolerance in December and in late February, the occasional warm temperatures promoted deacclimation which resulted in a decrease in subzero temperature tolerance (Figure 2.6). The decrease in subzero temperature tolerance has also been observed with the occasional warm spells in the spring (Ferguson et al. 2011). The level of subzero temperature tolerance is altered at the time of transition between dormant and growing seasons, which are found to be associated with acclimation and deacclimation (Ferguson et al. 2014; Gusta and Wisniewski 2013; Kalberer et al. 2006).

QTLs in Chromosome 13 and 11 were found to be associated with the photoperiodic induction of dormancy in a previous study using the same F2 population (Garris et al. 2009), suggesting that photoperiod may be a key factor in cold acclimation in November when the air temperature is not that low. The occurrence of an average LTE QTL on chromosome 13 and the occurrence of a photoperiod response QTL on chromosome 13 suggested that the subzero temperature tolerance in the month of November may be correlated with photoperiod responsiveness promoting early acclimation with dormancy induction (Garris et al. 2009).

A QTL on chromosome 1 was identified using average LTE in February 2013 and January 2015. Marker position was used to identify genes underlying loci. The functional annotation indicated the genes are associated with leaf senescence, calcium signaling and sensors, carbohydrate metabolism, calmodulin binding protein, basic helix-loop-helix family, ABA signaling/ ABA signaling pathway, heat shock proteins, Myb transcription factors, cell wall organization and biogenesis, cell wall metabolism. Xyloglucan modification, PRR transcription factors, WRKY transcription factors, and many other
genes related to stress tolerance. In grapevines, heat shock proteins have been reported to be upregulated in short photoperiod. Ethylene has been found to be highly responsible for the cold tolerance in grapevines (Sun et al. 2016).

Two QTLs were identified on Chromosome 5 in December in two different analyses using BLUP and year wise average. Many potential candidate genes relating to circadian clock signaling, calcium signaling, heat shock proteins, photosystems and light signaling, calmodulin binding region, cellular metabolism, cell wall biosynthesis, ethylene signaling and ethylene-mediated signaling, calmodulin, metallothionein 2A, COP1-interacting protein 7, and transposons were found in the confidence interval suggesting the probable locations of the QTL hotspots. A QTL on Chromosome 13 was detected in November using average LTE and BLUP showing the presence of many potential genes related to cellular metabolism, primary and secondary metabolism, WRKY transcription factors, ABA signaling, ethylene signaling and so on. Functional annotation of genes underlying QTL on chromosome 9 and 16 also showed potential gene families similar to the QTL on chromosome 13. The presence of these potential genes suggest a relation with subzero temperature tolerance and potential biochemical changes like reduction in water content in cells, fluxes of calcium, membranes modifications, metabolomics reprogramming, synthesis and storage of carbohydrates, amino acids, proteins and secondary metabolites and changes in cell wall structure occur in plant (Fennell 2004, 2014; Gusta and Wisniewski 2013; Guy 2003). In grapevines, during the endodormant state, starch catabolism, ABA catabolism, CCAAT family transcription factor, HSP-mediated protein folding, stress responsive genes are upregulated whereas, the photosynthesis and transport genes are downregulated. High
level of expression of dehydrins and metallothioneins were found along with protein synthesis, translation, cellular processes and response to external stimuli (Fennell 2014, 2015). The identification of the same loci and/or confidence interval on chromosome in multiple months with two methods strengthened the QTL identity. The presence of previously identified cold acclimation genes within these QTLs also strengthened their importance in their potential association with the genetic regulation of subzero temperature tolerance, further indicating that subzero temperature tolerance is a complex trait governed by many genes.

Grapevine subzero temperature tolerance QTL analysis has not previously been reported although many physiological and molecular studies have been conducted. Subzero temperature tolerance is governed by many different physiological processes including endodormancy induction, chilling fulfillment and timing of bud break, which makes the trait a challenging one to phenotype. The timing, duration, rate of temperature decline and rise, minimum temperature reached, nucleation temperature, repeated freeze-thaw cycles, snow cover, no snow cover, other abiotic factors including light, nutritional status, and other biotic factors, all determine if the plant has a genetic ability to implement tolerant mechanisms that will permit the survival in the cold freezing temperature (Guy 2003). As no two winters are alike, the subzero temperature tolerance developed by the plants in the same months in different dormant seasons are different and different factors may have played the key role that caused the QTLs to appear and disappear. In contrast to annual crops like maize, wheat, soya and rice, in which biological replications of each genotype in one growth environment is common, only one vine per genotype was used in multiple dormant seasons in this experiment. This may
lead to bias in phenotypic value assessment, leading to increase in the likelihood of error and underestimated LOD values. The environmental factors like temperature and rainfall, are more variable over time. Other reasons may be due to small size population, individual QTL effects are underestimated and less predictable. Due to the complexity of the trait and yearly temperature variation, many QTLs appeared in individual month and dormant season fail to be detected in the other dormant seasons, which shows the QTL instability as a factor of environment instability. QTLs were detected for all months and dormant seasons for the average LTE data. Many of the QTLs detected in one dormant season did not appear in the next dormant season (Table 2.3, 2.4 and 2.5). Similar instability of QTLs across dormant seasons has been widely reported for grapevines (Chen et al. 2015; Costantini et al. 2008b; Fanizza et al. 2005; Grzeskowiak et al. 2013), and also for other fruit tree species. The majority of these studies identified QTLs in a single environment and are not repeated in other dormant seasons which may be due to the widely varying and less controlled environment. In contrast, this study employed multiple months in dormant season and multiple dormant seasons, providing greater opportunity to identify repeat QTLs. This study also identified the QTLs using two methods, strengthening the identification of pertinent QTL.

**Conclusions**

Freezing injury poses an environmental challenge that limits the production, productivity and sustainability of the grapevines in continental climates. Understanding the genetics behind the complex trait like subzero temperature tolerance and knowing how the environmental cues regulate the subzero temperature tolerance in plants is an essential part of dealing with the problem of freezing injury. Long generation time and
the high heterozygosity in grapevines poses many constraints in the genetic analysis of quantitative trait like subzero temperature tolerance. Therefore, multiple measures of subzero temperature tolerance trait were performed in multiple months and multiple dormant seasons (2011-2016) in the F2 population for reliability of QTL detection. Our results confirmed that subzero temperature tolerance is a quantitatively inherited trait controlled by the combined effect of numerous gene loci. Subzero temperature tolerance of the F2 genotypes changed with time and environmental conditions as the plant acclimated in response to decreasing daylength and low temperature. The F2’s demonstrated genetic variation in subzero temperature tolerance in response to environmental conditions. QTL analysis of the average LTE data for each month individually and same month across dormant seasons and genotypic BLUPs allowed us to identify 6 QTLs: one on chromosome 1, 7, 13 and 16 and two on chromosome 5. These QTLs were identified in multiple months or dormant seasons or using more average LTE or genotypic BLUP, increasing our confidence on the QTL detected. Relevant candidate genes regulating the calcium signaling, cellular metabolism, ethylene signaling, dehydration responsive genes, heat shock proteins and ABA signaling were found in the confidence intervals of these QTLs. Studying the genetics of multi-faceted trait of subzero temperature tolerance will help to understand the genetics underlying subzero temperature tolerance and help the breeders to select genotypes that are suite to low temperature regions.
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located in a major freezing tolerance QTL region on *Medicago truncatula* chromosome 6. BMC Genomics 14:814


Table 2.1 Sampling months and dormant seasons.

<table>
<thead>
<tr>
<th>Year/Month</th>
<th>November</th>
<th>December</th>
<th>January</th>
<th>February</th>
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<td>2011-12</td>
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<td>2015-16</td>
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</table>
Table 2.2 Observation of transgressive segregants in the F2 population, phenotyped for subzero temperature tolerance.

<table>
<thead>
<tr>
<th>Month</th>
<th>Total individuals phenotyped</th>
<th>No. of transgressive segregants observed in the population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nov-2011</td>
<td>90</td>
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<td>Dec-2011</td>
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<tr>
<td>Nov-2012</td>
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<tr>
<td>Dec-2012</td>
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<td>Feb-2013</td>
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<tr>
<td>Jan-2014</td>
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<tr>
<td>Feb-2014</td>
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<tr>
<td>Dec-2014</td>
<td>48</td>
<td>15</td>
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<tr>
<td>Jan-2015</td>
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<tr>
<td>Feb-2015</td>
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<td>10</td>
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</table>
Table 2.3: Summary of QTL results for individual months and dormant seasons.

<table>
<thead>
<tr>
<th>Trait</th>
<th>No of individuals phenotyped</th>
<th>Linkage group</th>
<th>Physical position of the nearest marker</th>
<th>LOD score</th>
<th>Phenotypic variation (R²)</th>
<th>Additive effect</th>
<th>Dominance effect</th>
<th>Physical position of flanking markers</th>
<th>1.5 LOD interval (cM)</th>
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<tbody>
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<td>LTE_avg_Nov_2011</td>
<td>85</td>
<td>11</td>
<td>6607300</td>
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<td>10.71</td>
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<td>69.99-80.3</td>
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<td>13</td>
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<td>5</td>
<td>4617260</td>
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<td>LTE_avg_Dec_2011</td>
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<td>9694212</td>
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<td>16</td>
<td>22920762</td>
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<td>15</td>
<td>1644882</td>
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<td>24242954</td>
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<td>21192525-24448098</td>
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<td>LTE_avg_Jan_2015</td>
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Table 2.4 Summary of QTLs across dormant seasons (average bud LTE and BLUP analysis).

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<th>Phenotypic variation (R²)</th>
<th>Additive effect</th>
<th>Dominance effect</th>
<th>Physical position of flanking markers</th>
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Table 2.5 Summary of QTL using BLUP for individual dormant season.

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<th>Trait</th>
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<th>Linkage group</th>
<th>Physical position of the nearest marker</th>
<th>LOD score</th>
<th>Phenotypic variation</th>
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Figure 2.1 Summary of the methods used in this study.
Figure 2.2 Box plot showing distribution of LTEs in different dormant seasons.
Figure 2.3 Boxplot of distribution of LTE's by months.
Figure 2.4 Frequency distribution of LTE in dormant season 2011-2012 showing plant acclimation.

Figure 2.5 Frequency distribution of LTE in dormant season 2012-2013.
Figure 2.6 Frequency distribution of LTE in dormant season 2013-2014 showing deacclimation response in plants.

Figure 2.7 Frequency distribution of the LTEs for different months across dormant seasons.
Figure 2.8 Comparison of the same month between dormant seasons.
Figure 2.9 QTL for average LTE for the month of November 2011 on chromosome 13.

The peak was generated using CIM in R/qtl based on 1000 permutations.
Figure 2.10 QTL of average bud LTE for dormant season 2011-2012 using BLUP.
Chapter 3 Mapping of quantitative trait loci controlling bud break in F2 grapevine population.

Abstract

Bud break occurs when the chilling requirement is fulfilled, the buds have transitioned to ecdormancy, and grapevines are exposed to favorable environmental conditions. In the current prospect of global climate change, understanding of the genetics of bud break aids in breeding perennial grapevines that can tolerate sudden warm temperature and subsequent low freezing temperatures. The objective of this study was to identify QTLs associated with bud break in a segregating population in grapevines. The bud break in *Vitis* is studied with an F2 grapevine population. The bud break growth stages were monitored for four weeks according to the modified Eichhorn and Lorenz (modified E-L) scale. The concept of area under curve (AUC), used in plant epidemiological studies, was used to analyze the bud development in four weeks’ time period. The phenotypic data obtained was transformed using area under bud break progression curve (AUBPC) because of the ordinal nature and monotonicity of the measurements. QTLs were identified using a F2 genotype by sequenced single nucleotide polymorphism (F2 GBS SNP) linkage map, using composite interval mapping (CIM), with a 1000 permutations. QTLs were identified in chromosomes 3, 7, 8, 9, 13, 18 and 19, contributing up to 33% of the phenotypic variations. Negative additive effects were identified in samples with low chilling and positive additive effect were found with greater chilling suggesting contributions by the female grandparent and male grandparent, respectively. About 20 QTLs were identified with LOD higher than 3, providing evidence of multi-genic control of the trait. The markers associated with the
QTL regions can be used to develop markers for marker-assisted selection and breeding varieties that are suitable in cold regions with transient warm temperatures in the spring.

**Introduction**

In regions with potentially damaging winter temperatures, the plants develop an ability to undergo into the state of endodormancy which is central to a perennial life strategy. Endodormancy is defined as the inability of the meristem to resume growth under favorable conditions (Rohde and Bhalerao 2007). Woody perennials, including grapevines, enter into the state of endodormancy in response to decreasing daylength and temperatures in fall (Fennell 2004; Fennell and Hoover 1991; Fennell and Mathiason 2002; Wake and Fennell 2000). The endodormant grapevines are unable break bud and grow even under favorable growth conditions (Lavee and May 1997). Upon exposure to chilling temperatures, the grapevine transitions from endodormancy to ecodormancy. An ecodormant bud can burst and resume growth after exposure to warm temperature (Dokoozlian 1999). The chilling temperature required to fulfill chilling requirement is reported between 7.2°C and 0°C (Dokoozlian 1999). There is genotypic variation in the amount of chilling required to break endodormancy, ranging from 50-400 hours for *V. vinifera* and 250 to 2250 hours for other species (Dokoozlian 1999; Londo and Johnson 2014). However, prolonged exposure to chilling temperature increases the rate and number of bud breaks (Dokoozlian 1999; Londo and Johnson 2014). The chilling requirement plays a protective role against short intervals of unseasonably warm temperatures followed by resumed cold.

Bud break is defined as the first day when green tissue beneath the bud scales appeared in grapevines (Coombe 1995; Lorenz et al. 1995). The plant also transitions
from cold hardy to cold tender state losing subzero temperature tolerance. It is a reliable estimate of the damage incurred due to low winter temperatures (Fennell 2004). Bud break is an intrinsic characteristic of Vitis which varies between and within species and greatly impacts the fruiting of the next season (Fennell 2004). It also determines the production and vegetative growth of the plants. Thus, understanding the genetics behind bud break is necessary to select and develop cultivars that are suitable to the environmental conditions.

Grapevine production is expanding in higher latitudes in N. America, where winter can be very cold. The widely cultivated, European grapevine, V. *vinifera* is a low chill species requiring 50 to 400 hours of chilling to satisfy endodormancy (Dokoozlian 1999). The native N. American grapevines species are typically high chill species and require 250 to 2250 chilling hours to satisfy their endodormancy (Londo and Johnson 2014). Breeding grapevines suitable for this region requires combination of adaptive genes from native species and fruit quality from *V. vinifera*. Identifying the genes and markers associated with the mechanism of bud break and chilling requirement will contribute in selecting and developing the genotypes that match the climatic conditions.

Early genetic studies in perennial woody species demonstrated that a number of dormancy-related traits are under the genetic control, including chilling requirement and bud break. These traits are quantitative in nature and under multigene control along with environmental effects (Abbott et al. 2015; Fabbrini et al. 2012; Fan et al. 2010a; Frewen et al. 2000; Howe et al. 2000; Olukolu et al. 2009a; Rohde et al. 2011; Zhebentyayeva et al. 2013). QTL analysis has also been done for chilling requirement and bloom date in apricot (*Prunus armeniaca*), peach, almond and sweet cherry (Dirlewanger et al. 2012;
These studies suggest that bud break in woody fruit crops is a complex genetic character and is genotype dependent varying from a few strong QTLs to numerous small contributing QTLs. Many studies regarding bud break has been done in the *Vitis* but none has been focused on the genetics of this trait (Londo and Johnson 2014).

The aim of this work was to investigate the genetic determinism of bud break in grapevines and find the loci of the candidate genes regulating this trait. Understanding the genetic foundations of bud break is very important to protect grapevines from the freezing injury and to ensure synchronous production, since, the timing of bud break is important in grape production to protect the plants from early warming and subsequent cold episodes, as well as to prevent delayed bud break.

**Materials and Methods**

**Population development**

The F2 mapping population which comprised of 113 individuals was developed by selfing a single hermaphrodite F1 (16_9_2) from the cross *V. riparia* (USDA PI 588289) X Vitis hybrid “Seyval” (Seyval Villard 5-276) (Fennell et al. 2005). The *V. riparia* grandparent F1 and F2 progenies were clonally propagated and evaluated under field conditions in NE Hansen Research Farm, Brookings, South Dakota (44°19’N). The *V. riparia* grandparent is known to be a high chill species requiring 1500-2000 hours of chilling hours to break endodormancy (Mathiason et al. 2009).
**Phenotypic evaluation**

Bud break data acquired using the same canes that were used to sample buds for low temperature exotherms, thus the bud break activity and subzero temperature tolerance can be compared. Canes of each genotype were collected from the vineyard at Brookings, SD, USA for each month tested for a particular dormant season. For each genotype, 3 cuttings of 4-5 nodes, were harvested from the vine. The most apical bud was dissected to see if each cane sample was alive or dead and only canes with live buds were used (Zabadal et al. 2007). A 5-6 cm cane section with overwintering bud in the center (one node cutting) was placed in water under 13h photoperiod and 25/20°C thermoperiod in growth chamber (Conviron, Controlled Environments Limited, Model no PGW36, Canada). Water was added to forcing containers daily so that the water level remained just below the bud. Each week the growth stage of the bud was recorded according to modified Eichhorn-Lorenz bud phenology scale (modified E-L scale) (Coombe 1995) as shown in Fig.1. Bud break was monitored for consecutive 4 weeks, after which the buds that did not show any swelling were cut in half to see if the meristems were alive or dead. Browning of the bud tissue indicated that the bud is dead. The bud break study was conducted in multiple months for five dormant seasons (2011-12, 2012-2013, 2013-2014, 2014-2015 and 2015-2016) (Table 3.1). The availability of plant materials in the field limited the sampling to two months in some dormant seasons.

**Statistical Analysis**

The phenotypic data was analyzed using R (Team 2015a). The bud break growth stages were averaged for all the replicates of the same genotype. The average data from each month and dormant season were used for all the analysis. A histogram was used to
check the data distribution. Data that were skewed and did not follow a normal
distribution were log transformed. Bud break phenology data was ordinal, monotonic
data and was based on repeat measures. Each measure was dependent on the previous
stage of development. The Spearman correlation coefficient was calculated for week 3
phenological stages for each month and year. Spearman’s rank correlation coefficient is a
non-parametric method of estimating correlation in the case of ordinal and monotonic
data. Week 3 phenological data was used in our analysis as week 3 accounted changes in
the bud phenology and growth stages in most of the F2 genotypes. Week 3 was more
informative than other weeks because variability in the genotypes was observed at this
stage so that we can account the genetic variation in the bud break in our genetic analysis.
Data analysis was conducted using the following phenotypes:

i. Phenological stage at week 3.

ii. Phenological stage at week 4.

iii. Average of phenological stages at all four weeks.

iv. Area under bud break progression curve (AUBPC) method.

Here, the data analysis using week 3 phenological stage and AUBPC are
presented and discussed.

**Area under bud break progression curve (AUBPC)**

Area under curve (AUC) is one of the most popular measurements used in plant
disease epidemiological studies to study for disease intensity over a time period
([http://www.apsnet.org/EDCENTER/ADVANCED/TOPICS/ECOLOGYANDEPIDEMI
OLOGYINNR/DISEASEPROGRESS/Pages/AUDPC.aspx](http://www.apsnet.org/EDCENTER/ADVANCED/TOPICS/ECOLOGYANDEPIDEMI
OLOGYINNR/DISEASEPROGRESS/Pages/AUDPC.aspx)). This method allows
quantification of temporal increase of disease as well as allows comparison of disease
development in different years, locations and management practices (Jeger and Viljanen-Rollinson 2001). The concept of disease progress with time was applied in our bud break. As disease intensity increases with time, the phenological stage of bud also increases with time. The bud in stage 4 in week 2 will grow more to be in stage 5 or higher in next week if given appropriate conditions for growth. This measure has been used to average out the variation as well as integrate all aspects of bud development in relation to genotype and phenology. The AUBPC was calculated in R using the function \texttt{audpc} under the package \textit{Agricolae}. The area under the trapezoid under the curve made from the different growth stages with time is calculated. Both relative AUBPC as well as absolute AUBPC were calculated in R. The Spearman rank correlation coefficient was calculated in R for both the relative and absolute AUBPC to see the relationship between different months and dormant seasons to see the strength of association and direction between bud break in different months and dormant seasons.

**Quantitative trait loci analysis**

Quantitative trait loci (QTL) analysis was performed on average week 3 phenological stage data for each month and dormant season individually and both relative and absolute AUBPCs with the R/qtl package (Broman et al. 2003). The F2 GBS-SNP genetic map containing 1449 SNP markers as described developed by Yang et al. (2016) was used for the analysis. A total of 424 plants were genotype by sequenced (GBSeq) and the F2 GBS-SNP was developed and published in 2016. The F2 GBS map has a genetic length of 2424 cM with an average distance of 1.67 cM between markers, covering all 19 linkage groups and 95% of the genome (Yang et al. 2016). QTL analysis was performed in R using single QTL scan (“\texttt{scanone}” function, “Normal” model) and composite
interval mapping (CIM) (Zeng 1994) with R/qtl (the “scanone” function, map function “Kosambi”, method=”hk”, n.perm=1000) and using F2 as the cross type. CIM uses forward selection to identify the markers and then runs interval mapping with the selected markers as covariates. The threshold was determined with 0.05 level of significance and grapevine standards of 1000 permutations. All the QTLs that crossed the LOD score of 3 were considered valid QTL (Wu et al. 2014). Once the QTL was identified, we obtained the phenotypic variance ($R^2$) of the trait explained by the QTL identified by calculating the genotypic probabilities for the marker linked to the QTL. Then, the QTL identified was fitted in a model, LTE~QTL+e to obtain the phenotypic variation contributed by the QTL (“fitqtl” function). The “lodint” function was used to derive the 1.5 LOD support interval. Genome wide scan was performed with a 1cM step. Reports were generated for maximum LOD score, 1.5-LOD support interval in cM, the flanking markers, the physical location in the reference genome in Mb, as well as the variation explained.

**Results**

**Phenotypic evaluation**

Bud break was evaluated for multiple months and dormant seasons (Figure 3.1). The month where bud break was evaluated differed among dormant seasons, however, each month is evaluated at least three times. Thus, the results may not be comparable across dormant seasons, however, can be compared among months in different dormant seasons.

The budbreak phenological score data is ordinal and monotonic in nature, which means as the value of one variable increases, the value of the other variable also increases. The Spearman rank correlation coefficient used to estimate the correlation
between different months of the same dormant season ranged from weak to strong correlation (Table 3.2). The correlation between December 2012 and February 2013 was strong (0.7) with p-value $2.2 \times 10^{-16}$ whereas the correlation between November 2011 and December 2011 was a weak correlation (0.34) with p-value 0.00065. The correlation between two months in the same dormant season is medium (0.5) and significant. When we compared same months in different dormant seasons, it exhibited weak to medium correlation, mostly equal or less than 0.5. There was also a negative correlation observed between November 2011 and November 2015 ($r=0$).

Evaluation of the bud break data showed that increased chilling increased the number of genotypes breaking bud after 3 weeks of forcing. The phenological growth score of the bud is lower in November in most of the genotypes in comparison to December, January, and February. Week 3 growth stage was taken as a standard for comparison since week 3 encompasses the greatest range of bud phenology responses across genotypes. In November, the number of genotypes breaking bud after three weeks of forcing are limited, whereas, in February, bud break started in week 2. The number of genotypes breaking bud and the stage of bud break increased with increased chilling between December and February.

AUBPC results

The absolute and relative AUBPC both were calculated in R. The area under the growth curve was different for different genotypes and varied in different months and dormant seasons. Generally, the area was higher in January and February as the buds broke sooner and grew continuously with the fulfillment of the chilling requirement. Whereas, in the case of November, the area was relatively less since the bud break and
growth was delayed as the chilling requirement was not fulfilled. The AUBPC data processing encompassed all the activities that took place within forcing period in contrast to taking just one week and, thus helped to consider the total sample variation. The Spearman correlation was more or less the same in the case of both absolute and relative AUBPC. The correlation among different months in the same dormant season was generally greater than the correlation between same months in different dormant seasons. For example, the correlation between December 2012 and February 2013 is strong (0.72) whereas the correlation is low between February 2013 and February 2015 (0.16) (Figure 3.3). The correlation between January and February is relatively limited.

**QTL Analysis of Week 3 bud break data**

The QTL summary for the average week 3 data for different months and dormant seasons are presented in the table 3.4. Three QTLs passed the threshold of 1000 permutations using week 3 phenological data (Figures 3.3, 3.4 and 3.5). QTLs on Chromosome 13 and 12 were identified in December 2011, chromosome 18 was identified in December 2012 and December 2015. The number of observations ranged from 84 to 94 in the analysis. About 20 QTLs were observed when the threshold was considered as LOD 3 or greater. QTL on chromosome 18 was detected multiple times in November 2011, November 2012, December 2012, February 2013, February 2014 and December 2015, contributing up to 29.5% of the phenotypic variation. QTL on chromosome 13 and 19 was identified in December 2011, explaining about 25% and 17% of phenotypic variation respectively; QTL on chromosome 7 also appeared once in November 2012, explaining about 22% of the phenotypic variation; and QTL on chromosome 17 appeared in January 2014, explaining about 15% of the phenotypic

**QTL analysis of AUBPC results**

The QTL analysis was conducted using composite interval mapping (CIM) in R/qtl (Broman et al. 2003). The LOD threshold was determined with 1000 permutations and 0.05 level of significance. The QTL are summarized in the Table 3.5. There are about 20 QTLs controlling bud break found in the F2 populations on chromosome 3, 4, 7, 8, 9, 11, 13, 17, 18, and 19. Only four QTLs crossed the threshold of 1000 permutations. They were chromosome 13 in December 2011, chromosome 12 in February 2015, chromosome 3 in November 2015 and chromosome 18 in December 2015. The peaks that crossed the LOD of 3 were also considered a QTL. Chromosome 3 and 18 appeared multiple times in different months and dormant seasons. Chromosome 18 appeared in November 2012, December 2012, February 2013 which coincided with each other in 1.5 LOD support interval. Also, A QTL on chromosome 18 appeared in January 2014, February 2014, January 2015, February 2015 and December 2015, explaining up to 33% of the phenotypic variation. A QTL on chromosome 3 appeared three times in December 2012, February 2015 and December 2015, explaining up to 19% of the phenotypic variation. Similarly, QTLs on chromosome 13 and chromosome 12 appeared just once in December 2011 and February 2015 respectively.

The QTL analysis of both data processing approaches were found to have similar results. A QTL on chromosome 3 was identified in November 2015 with average phonological week 3 data and AUBPC, contributing up to 21% of the phenotypic
variation. This QTL had the same nearest marker location and covered the same genomic region. A QTL on chromosome 7 was also identified for November 2012 using both week 3 phenological stage and AUBPC, explaining about 22% of the phenotypic variation. This QTL had the same nearest marker loci and covered the same genomic loci. QTLs on chromosome 3 and 7 had positive additive effect which indicated that the lower chilling requirement was contributed by the male grandparent Seyval.

A QTL on chromosome 3 was also identified on December 2015 with the AUBPC data. This QTL covered the same genetic location but the nearest marker loci was different. The additive effect for this QTL was positive. This indicates that low chilling requirement in F2 is contributed by the male grandparent Seyval.

A QTL on chromosome 8 was identified for week 3 phenological stage in November and December 2012, covering the same genomic location. A QTL on chromosome 9 was identified in January 2014 using both week 3 phenological data and AUBPC, covering the same genomic loci. This QTL contributed up to 15.4% of the phenotypic variation and showed negative additive effects with high chilling requirement contributed by the female grandparent. A QTL was identified on chromosome 13 for December 2011 using both week 3 phenological stage and AUBPC, contributing the highest 31.6% of the phenotypic variation. This QTL also showed the positive additive effects. A QTL on chromosome 18 was detected in December of dormant season 2011-2012, 2012-13 and 2015-16 with the week 3 phenological stage and AUBPC, contributing the highest 33% of the phenotypic variation. A QTL was also identified on chromosome 18 for February 2013 using both week 3 average data and AUBPC, explaining 21% of the phenotypic variation. Four QTLs identified in chromosome 18
occupy the same genomic location and had different nearest marker. These QTLs all had positive additive effects.

Discussions

A grapevines bud enters into the state of endodormancy in response to the decreasing daylength and temperature in the fall leading to acclimation. After the vines are exposed to chilling temperature below 7°C for specific hours, the endodormant transitions into the state of ecodormancy. The speed of bud break increased gradually when an ecodormant bud is exposed to warm temperature. In December, January and February, the number of genotypes that showed bud break increased. In these months, the buds had already fulfilled the chilling requirement to satisfy endodormancy. When the node samples are collected later in the dormant season, the incubation time required for bud break is shorter (Kovács et al. 2003). The number of genotypes that break bud after three weeks of forcing in November was less than other months (Figure 3.1). The sub-freezing temperatures in November is not effective at satisfying chilling requirements and thus number of genotypes exhibiting bud break is less in early endodormancy (Hauagge and Cummins 1991; Kovács et al. 2003). The timing of bud break is closely related to bud chilling requirement (Hauagge and Cummins 1991). The initial chilling in November did not contribute to the breaking of endodormancy as higher number of genotypes did not show any growth activity at three weeks of forcing and thus, resulted in few and delayed bud breaks. The number of bud breaks increased with increased chilling exposure. At the end of endodormancy and during deacclimation, the bud break occurs upon exposure to temperatures as chilling requirement has been fulfilled (Andreini et al. 2009). A greater number of genotypes showed faster bud break in February. The negative
correlation observed in the same month across different dormant seasons showed that each year, the environment was different and the buds received different levels of chilling fulfillment in different dormant seasons. This may also be attributed to the difference in a number of observations in each year.

Bud break is a heritable complex quantitative trait involving the action of many genes (Fennell and Hoover 1991; Hauagge and Cummins 1991; Wake and Fennell 2000). Study of the genetics of endodormancy and bud break is complicated by the multiple processes such as cold acclimation, dormancy induction and chilling requirement. Both early and late bud break can be harmful to the plants since early bud break may increase the vulnerability of the plants to the cold episodes and frosts that are very common in the Northern climates (Lavee and May 1997). Whereas, late bud break may affect the maturity, uniformity and productivity of the plants and affect the growers in terms of profitability. Dokoozlian (1999) found that chilling is a facultative rather than the absolute requirement for the bud break growth in grapevines, however, the bud break accelerates in cold climate genotypes with the increased chilling in grapevines.

QTL mapping is one of the most successful approaches for the finding marker-trait associations. Thus, we evaluated bud break using a F2 population and a GBS-SNP map (Yang et al. 2016). Woody fruit crop breeding and QTL analysis typically uses small population size due to the long generation time and large space for maintenance (Bielenberg et al. 2015; Luby 1991; Yang et al. 2016). The lack of replications, as well as low number of samples, may present complications in the analysis. Also, the number of samples varied in different dormant season and months leading to unbalanced data. The
severity of winter temperatures limited the sampling in some dormant seasons. Therefore, we analyzed five years of data to provide replications through time.

Integration of the concept of area under curve (AUC) in the bud break data (AUBPC) analysis was very helpful for the quantitative and comparative studies of the bud break over a time period in different dormant seasons. The ability to use all the four weeks in the analysis allowed the differences in rate of break to be considered in the bud break value. Although different data processing approaches were used to perform QTL analysis, there was not remarkable difference between the QTLs found using different data processing methods. The most consistent QTL in this study using these approaches was a region on chromosome 18, which was identified for December in dormant seasons 2011-12, 2012-13 and 2015-16. The genomic location for the QTL coincided in all the dormant seasons. Functional annotation of genes underlying this loci showed potential candidate genes involved in Circadian clock signaling, GA-mediated signaling, drought stress response genes, ERS type ethylene receptors, AP2-like ethylene-responsive transcription factors, late embryogenesis abundant (LEA) proteins (desiccation stress response), Constan-like 13 (light signaling), flowering genes, transport inhibitor response genes (auxin-mediated signaling pathway) and many other cellular metabolism related genes and transcription factors. Various potential genes like dehydration responsive element DREB2F, jasmonate salicylate signaling, ethylene signaling, heat shock transcription factors, calcium sensors and signaling, calmodulin-binding region, dehydration induced protein (ERD15) were found underlying the QTL on chromosome 8. Similarly, transcripts related to temperature stress response, starch catabolism, ABA signaling, HSP-mediated protein signaling and stilbenoids biosynthesis were upregulated
and gene pathways related to photosynthesis, primary and secondary metabolism (fatty acid, carbohydrate, cell wall, flavonoid biosynthesis and cell cycle) were down-regulated in endodormant grapevine buds (Fennell 2014; Fennell et al. 2015). Many genes related to calcium signaling and reactive oxygen signaling were found which results in a cascade of cold-mediated transcription regulation (Fennell 2014; Wisniewski et al. 2014). QTL on chromosome 9 was identified in January 2014 using week 3 phenological stage and AUBPC. Similarly, a QTL on chromosome 13 was identified in December 2011 using week 3 phenological stage and AUBPC. A QTL on chromosome 13 was also identified for freezing tolerance in our studies of freezing tolerance and growth cessation by Garris et al. (2009). Many genes related to dehydration response, calcium signaling, ethylene signaling, auxin signaling, light signaling, desiccation stress response, jasmonate salicylate signaling and cellular metabolism were found in the QTL confidence interval. The presence of these genes suggested the structural changes in plants that occurred as the endodormant plants transitions to ecdormancy and then to growth resumption. Genome assembly is a good way to locate the position of genes. The loci point to chromosome region where gene annotation can be used to describe the underlying genes. QTLs on chromosome 9 and 13 were also observed in freezing tolerance studies suggesting the role of similar processes like endodormancy, acclimation, ecdormancy and deacclimation on bud break and freezing tolerance.

The QTL affecting the berry and phenology-related traits have been found in chromosome 18 (Cabezas et al. 2006; Costantini et al. 2008a; Doligez et al. 2002; Mejía et al. 2007). QTLs related to phenology related traits have been also found on chromosome 1, 6, 7, 8,12, 16 and 18 (Cabezas et al. 2006; Costantini et al. 2008a;
Doligez et al. 2002; Fechter et al. 2014; Mejía et al. 2007). The occurrence of bud break QTL on chromosome 18 suggests the relationship between bud break and phenology related traits. The timing and speed of bud break is directly or indirectly related to the berry and phenology related traits in grapevines. Exposure to sufficient chilling increased the speed and number of bud breaks. The uniformity in bud break is very important to determine the quality and production of the grapes. Early bud break speeds up flowering, fertilization and fruit ripening.

Conclusions

The study of bud break is an important parameter to study the winter survival and adaptability of plants in various temperature zones. Bud break increases with increased exposure to chilling, as shown by the increased number of genotypes exhibiting bud break in December, January and February after three weeks of forcing in natural conditions in comparison to the number of genotypes showing bud break in November. CIM detected six QTLs on chromosome 3, 7, 8, 9, 13 and 18, contributing up to 33% of the phenotypic variation. The detection of the same QTLs using week 3 bud break stage and AUBPC increased the strength and reliability of our analysis. The presence of many genes related to dehydrative stress response, ethylene response signaling, calcium sensors and signaling, cell metabolism and cell wall biosynthesis, light signaling and various transcription factors suggest the presence of QTLs. These QTLs point to loci in the chromosome which encompass the genes regulating the bud break in grapevines. Understanding the genetics behind the complex trait will help in breeding grapevines that are suitable to the environment.
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Table 3.1 Sampling months and dormant seasons. Due to inclement weather and lack of materials in the field, the sampling is done in different months in different dormant seasons, however, every month has at least 3 replications.

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<thead>
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<th>February</th>
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<td>2015-16</td>
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</table>
Table 3.2 Spearman’s rank correlation coefficient of week 3 bud break stages between different dormant seasons and months.

The dormant seasons are separated by grey and white backgrounds.

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<th>Nov-12</th>
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Table 3.3 Spearman rank correlation coefficients between absolute AUBPC bud break data among different dormant seasons and months. The dormant seasons are separated by grey and white backgrounds.

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Table 3.4: Summary of bud break week 3 QTL for different months and dormant seasons for *V. riparia* X “Seyval” F$_2$ population. The QTLs were calculated in R/qtl using CIM, 1000 permutations.

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Table 3.5: QTL summary for AUBPC absolute for different dormant seasons and months for *V. riparia* X “Seyval” F₂ population. The QTLs were calculated in R/qtl using CIM, 1000 permutations.

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Figure 3.1 Bud break assay in growth chamber.
Figure 3.2 Comparison of bud breaks and growth stages in different months of the dormant season 2012-2013 using histogram.
Figure 3.3 QTL showing bud break using AUBPC in December 2011. QTL were calculated using CIM in R/qtl, based on 1000 permutations and 0.05 level of significance.
Figure 3.4 A QTL in chromosome 12 showing bud break in February 2015 using AUBPC. QTL peak on chromosome 12 passes the LOD threshold, based on 1000 permutations and 0.05 level of significance generated by CIM in R/qtl.
Figure 3.5 A bud break QTL on chromosome 18 for December 2015 using AUBPC. The QTL peak in chromosome 18 passed the LOD threshold, based on 1000 permutations and 0.05 level of significance using AUBPC.
Figure 3.6 A bud break QTL in November 2015 using AUBPC. Chromosome 3 passed the LOD threshold based on 1000 permutations and 0.05 level of significance.
Chapter 4 Conclusion and future works

Subzero temperature tolerance and bud break are complex, quantitative traits governed by many genes. Due to the high level of heterozygosity in the grapevine genome, genetic analysis of the complex traits is onerous. Freezing tolerance is a multi-trait that is highly influenced by many related traits like acclimation, growth cessation, dormancy induction, periderm development, chilling requirement and bud break. Many structural, physiological and biochemical changes occur in plants as the plant prepare itself to adapt to the freezing temperature stresses including changes in signal transduction. The environmental cues play a very important role for the start of these changes in the plants, among which low temperature sensitivity and short photoperiod are the major ones. There is genotypic variation in the sensitivity to the photoperiod and low temperature. Usually, the cold hardy genotypes are more responsive to decreasing day length for dormancy induction, whereas low nonfreezing temperature play a major role in cold susceptible cultivars. Some cultivars require both of these environmental cues for the process of adaptation to begin. Prolonged exposure to low temperature increased the level of subzero temperature tolerance in the F2 genotypes, which was manifested by the high number of buds with extreme low temperature exotherms (LTE). In addition, the number of genotypes breaking bud and the bud break growth stages increased as the plants were exposed to low temperature.

Differential thermal analysis (DTA) detection of LTE provided a very good method for phenotyping subzero temperature tolerance. November was a good month to phenotype subzero temperature tolerance because it captured the early acclimation period when the plant prepared itself for low temperature stresses. It is also important since the
subzero temperature tolerance achieved by the plant is predominately dependent on acclimation in response to decreasing photoperiod. In November, the grapevines have not attained the maximum level of hardiness and are killed at relatively higher temperatures. The low number of genotypes exhibiting bud break in November indicate that most of the genotypes had not achieved chilling requirement fulfillment; however, but some genotypes were beginning to break bud suggesting lower chilling requirements among the genotypes. The lowest LTE were observed in late December and January. In late February, the field temperatures often increased and the bud subzero temperature tolerance decreased.

Quantitative trait loci analysis (QTL) using composite interval mapping (CIM) allowed genetic analysis of the complex low temperature response traits. QTLs were observed on chromosome 13, 3, 5, 8, 9, and 18 for subzero temperature tolerance (low temperature exotherms). QTL support interval showed the presence of potential candidate genes related to calcium signaling, cell wall organization and biogenesis, ethylene signaling, dehydration stress response, circadian clock signaling, jasmonate salicylate signaling, transposons which are found to be directly and indirectly related to low temperature responses.

These studies were conducted using a single unreplicated F2 population; however, phenotyping in five dormant seasons provided replication in time. The LTE data was fitted into mixed model to extract BLUP values to handle the unbalanced data before QTL analysis. Similarly, to account all the changes taking place throughout the time period, the bud break data was used in calculating AUBPC. The analysis of the data through different approaches and obtaining the same result also strengthen the results.
However, the results need to be verified in a much larger population. Identifying the candidate genes by fine mapping and then gene cloning can be done to further find the important genes regulating this complex trait.

The results from my study can be used in identifying molecular markers associated with the low temperature stress. This will help in selection and development cold tolerant cultivars and genotypes with the help of marker assisted selection and breeding. Development of cultivars with subzero temperature tolerance and good fruit quality traits will enhance viticulture and enology in the areas where winter temperatures are low, resulting in expansion of grape growing regions.
Appendix 1 Distribution of average LTEs using histogram.

Appendix 3 Frequency distribution in dormant season 2015-2016.
Appendix 4 Pearson’s product moment correlation coefficient between different months and dormant seasons.

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Appendix 5 Field temperature one week before sampling.

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