

# Mapping Freezing Tolerance QTL in Alfalfa Based on Indoor Phenotyping

**Laxman Adhikari**

University of Georgia

**Shiva O. Makaju**

University of Georgia

**Orville M. Lindstrom**

University of Georgia

**Ali Mekki Missaoui** (✉ [cssamm@uga.edu](mailto:cssamm@uga.edu))

University System of Georgia <https://orcid.org/0000-0003-1710-1142>

---

## Research article

**Keywords:** Alfalfa, GBS, SNP, CBF, freezing tolerance, winter survival, germplasms

**Posted Date:** October 6th, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-78840/v1>

**License:** © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

**Version of Record:** A version of this preprint was published at BMC Plant Biology on September 6th, 2021.

See the published version at <https://doi.org/10.1186/s12870-021-03182-4>.

# Abstract

**Background** Winter freezing temperature impacts alfalfa (*Medicago sativa* L.) persistence and seasonal yield and can lead to the death of the plant. Understanding the genetic mechanisms of alfalfa freezing tolerance (FT) using high-throughput phenotyping and genotyping is crucial to select suitable germplasm and develop winter-hardy cultivars. Several clones of an alfalfa F1 mapping population (3010 x CW 1010) were phenotyped for FT using a cold chamber. The population was genotyped with SNP markers identified using genotyping by sequencing (GBS) and the QTL associated with FT were mapped on the parent-specific linkage maps. The ultimate goal is to develop non-dormant and winter-hardy alfalfa cultivars that can produce extended growth in the areas where winters are often mild.

**Results** Alfalfa FT screening method optimized in this experiment comprises three major steps; clone preparation, acclimation, and freezing test. Twenty clones of each genotype were tested, where 10 samples were treated with freezing temperature, and 10 were used as controls. A moderate positive correlation ( $r \sim 0.36$ ,  $P < 0.01$ ) was observed between indoor FT and field-based winter hardiness (WH), suggesting that the indoor FT test is useful as an indirect selection method for winter hardiness of alfalfa germplasm. We detected a total of 20 QTL for four traits; visual rating-based FT, percentage survival (PS), treated to control regrowth ratio (RR), and treated to control biomass ratio (BR). Some QTL overlapped with WH QTL reported previously, suggesting a genetic relationship between FT and WH. Some favorable QTL from the winter-hardy parent (3010) potentially represented the genic region of a cold tolerance gene, the c-repeat binding factor (*CBF*). These QTL were located on the terminal end of chromosome 6 which is considered a location of the *CBF* homologs in alfalfa.

**Conclusions** The indoor freezing tolerance selection method reported here is valuable for alfalfa breeders to accelerate breeding cycles through indirect selection. The QTL and associated markers add to the genomic resources needed by the alfalfa research community and can be used in marker-assisted selection (MAS) for alfalfa cold tolerance improvement.

## Background

Most of the U.S. states experience freezing to extremely low temperatures in winter posing a serious threat to the survival of herbaceous forage species. Severe winter injury in alfalfa is frequent especially in the northern climate [1, 2]. Harsh winters affect alfalfa (*Medicago sativa* L.) growth leading to reduced biomass yield, low stand persistence, and eventually death [3]. Improvement of alfalfa for winter hardiness (WH) has traditionally been achieved via mass and recurrent selection of superior genotypes in field nurseries in cold regions[4]. However, selection for cold hardiness in nurseries has often low efficiency because of the unpredictable winter season and requirement of data from multiple locations and years for the efficient selection [4]. Recording several data for multiple seasons is costly and laborious [5]. The North American Alfalfa Improvement Conference (NAAIC) also recommends data collection for a minimum of two locations and two years for winter survival evaluation [6]. The NAAIC also recommends some guidelines for the collection and interpretation of alfalfa winter survival data as

the winter survival ratings recorded too early can underestimate the WH of dormant genotypes. Also, alfalfa selection against winter damage based on conventional breeding is too slow as the trait is quantitative and has substantial interaction with the environment [7]. Therefore, the assessment of WH in the field is a relatively tedious and long process.

Alfalfa is an autotetraploid ( $2n = 4x = 32$ ) and the most widely cultivated forage globally [8]. Although recurrent phenotypic selection is still a widely accepted method for improving winter survival in alfalfa [4], the method still requires several cycles and years. Freezing tolerance (FT) is an important factor for predicting alfalfa winter hardiness (WH) [9]. Therefore, the evaluation of plants tolerance to freezing temperatures in a simulated environment is deemed as an alternative to field phenotyping. The indoor freezing tolerance tests are commonly carried out using a temperature bath [10], as electrolytic leakage assessment [11], using chlorophyll fluorescence assays [12] and freezer chambers with programmed temperatures [4]. The better nursery performance of plants selected from the indoor freezing test has been described in alfalfa [4, 13] and other species. Adkins et al. (2002) evaluated the cold hardiness of ten species of the genus *Hydrangea* in the lab, where they observed the performances of *H. macrophylla* cultivars corresponding to their landscape performance [14]. A strong positive correlation between freezing test and plant cold hardiness was reported for St. Augustinegrass [*Stenotaphrum secundatum* (Walt.) Kuntze] [15].

Low temperature induces several genes and associated signal transduction pathways to synthesize essential biomolecules (proteins, soluble sugars, osmoprotectants) for cold acclimation [16]. The *CBF* (C-repeat binding factor) genes are transcription factors that play a key role in cold acclimation regardless of the source of temperatures [17, 18]. Cold-sensitive plants often lack the associated genes, or the genes get inactivated due to mutations thereby dysregulating the formation of regulatory and signaling molecules for cold acclimation. Other factors such as water stress, pesticide application, fertilizer treatment, bacterial colonization, and planting date [10] also affect plant cold hardiness. However, low temperature is the major factor bringing cellular changes and winter injuries in sensitive plants. Therefore, dissecting the genomics features associated to alfalfa freezing tolerance is crucial.

Incorporation of cold tolerance in non-dormant alfalfa germplasm through genetic manipulation could be ideal for developing alfalfa cultivars that have high biomass yield and winter survival.

Winter-hardy non-dormant alfalfa with wider adaptation can fill the forage gap through the extension of regrowth time in winter and early spring. Seasonal forage gaps exist because of partial to complete growth cessation of warm-season species when cool-season forages are not productive yet [19]. Winters in Georgia and the southeast USA are overall mild but these locations often experience a few freezing days per season depending on the latitude and elevation. Therefore, non-dormant and winter-hardy alfalfa are desirable to the region. In our previous study, we reported QTL associated with alfalfa WH and fall dormancy in field-grown plants [19].

In this study, we investigated the association of freezing tolerance with genetic loci using indoor phenotyping in freeze chambers. Accordingly, the objectives of this experiment were i) to optimize a

protocol for alfalfa freezing tolerance test in a walk-in freezer ii) to identify alfalfa QTL associated with freezing tolerance, and iii) to select freeze tolerant advanced alfalfa breeding lines for the development winter-hardy cultivars.

## Results

### Testing Freezing Tolerance

The standard check seedlings in control and treatment sets of cultivars 5262 (WS = 2) and G-2852 (WS = 4) showed significant differences ( $P < 0.05$ ) when the freezing environment was adjusted in such a way that the minimum testing temperature was  $-8\text{ }^{\circ}\text{C}$  (Figure 1). The optimized protocol included a series of combinations of temperatures and durations of exposure. After acclimation, both treatment and control plant sets were maintained at  $0\text{ }^{\circ}\text{C}$  for 8 hours. Then, the control set was removed from the freezing chamber and transferred to normal growing conditions at 14 hr. of light ( $23\text{ }^{\circ}\text{C}$ ) and 10 hr. of dark ( $15\text{ }^{\circ}\text{C}$ ). Plants in the treatment sets were treated in such a way that the temperature was decreased by  $2\text{ }^{\circ}\text{C/hr}$  until it reached  $-8\text{ }^{\circ}\text{C}$ . The plants were maintained for 90 min at  $-8\text{ }^{\circ}\text{C}$ , then the temperature in the chamber was raised gradually by  $2\text{ }^{\circ}\text{C/hr}$  until it reached  $2\text{ }^{\circ}\text{C}$ . The treated plants were then transferred to the normal greenhouse condition and phenotyped.

### Phenotypic Variation and Distribution

Phenotypic variability was noticed among the F1 individuals for all four traits; FT, PS, RR, and BR (Figure 1). We also noticed transgressive segregants for all four traits. The PS of cold treated genotypes ranged from 7% to 100%. The mean RR of surviving plants in treatment versus control ranged from completely sensitive (near 0) to almost completely tolerant ( $\sim 1$ ) genotypes. Similarly, the mean BR of surviving plants in treatment and control sets ranged from 0.01 to 0.99. The average visual rating based freezing tolerance (FT) of F1 plants varied from 1 to 4.9, indicating sufficient variation is present in the F1 genotypes for freezing temperature tolerance. The genotypes with higher PS, RR, and BR, and lower FT were considered cold hardy genotypes. The PS showed a strong negative correlation ( $r = -0.91$ ,  $P < 0.01$ ) with mean FT, which means that the higher the % of the surviving clones, the better is the freezing tolerance of the genotypes (Table 1). The significant positive correlations ( $r = 0.55$ ,  $P < 0.001$ ) between PS and RR as well as PS and BR ( $r = 0.40$ ,  $P < 0.01$ ) indicated that the genotypes with higher % survival produced higher regrowth and subsequently higher biomass. Strong negative correlations were also observed between variables FT and RR, suggesting that the cold-sensitive genotypes had a low regrowth. Similarly, significant negative correlations ( $r = -0.46$ ,  $P < 0.01$ ) were obtained between BR and FT as with moderate  $r$  values (Table 1).

We found a significant positive correlation ( $r = 0.36$ ,  $P < 0.01$ ) between mean FT and the LS mean of WH scores collected at the JPC field location in 2017 (WH017JPC). A significant positive correlation ( $r = 0.26$ ,  $P < 0.01$ ) was also observed between FT and WH data from Blairsville location (BVL) in 2017 (WH017BVL) (Table 1). Besides FT, other variables, such as PS, RR, and BR, from indoor testing also displayed significant correlations ( $P < 0.05$ ) with WH data from the field (Table 1). However, we could not

find significant correlations ( $P < 0.05$ ) between field data for BVL and the variables PS, RR, and BR. But, the direction of the relationship between them was similar to that exhibited by the JPC field data. These relationships among the traits indicated the values of the phenotyping method used.

## QTL Mapping

As we generated two groups of linkage maps specific to each parent, we mapped QTL separately on them. In this experiment, we detected a total of 20 QTL for four traits (Table 2). The QTL that were detected on the linkage map of the dormant parent (3010) were coded as trait name followed by -d1 to -dn (e.g. FT-d1). Of five QTL detected for FT on 3010 linkage maps, two were on homolog 3C, one on 4B, one each on 6B, and 6D (Table 2). Among five FT QTL from the 3010 parent, the QTL FT-d1 ( $R^2 = 0.19$ ) explained the highest phenotypic variation. . Since we used only a single dose allele locus for linkage grouping that represents only a portion of the loci responsible for the trait in autotetraploid species, here we reported only the direction of allelic effects instead of actual additive effects. Of the five FT related QTL from the 3010 parent, only two QTL had positive effects for freezing tolerance while the other three were enhancing freezing sensitivity. We detected two QTL for RR (RR-d1 and RR-d2), two QTL for BR (BR-d1 and BR-d2), and only one QTL for PS (PS-d) on the 3010 linkage maps (Table 2). Of the 10 QTL reported for the 3010 (winter hardy) parent, five QTL exhibited favorable loci with positive impacts on the freezing tolerance related traits (Table 2).

The QTL detected on the linkage maps of the non-dormant (CW 1010) parent were designated as trait names followed by -n1, -n2, and so on (e.g. FT-n1, RR-n1) (Table 2). We identified four QTL for FT, four for PS, and two for RR on the linkage groups of CW 1010 (Table 2). For FT, we identified three of four QTL on 5B of CW 1010, where a QTL (FT-n3) explained the phenotypic variation up to 29%. Also, two QTL for PS (PS-n1 and PS-n2) were reported in the same region on 5B for CW 1010. These five overlapping QTL on 5B of CW 1010 with negative effect suggested that the chromosomal segment is crucial for freezing sensitivity in alfalfa. We also observed two QTL for regrowth ratio (RR-n1 and RR-n2) for this paternal parent on chromosome 4D and 8D (Table 2). Of the total 10 QTL identified for CW 1010 (cold-sensitive parent), only one QTL (FT-n1) had a favorable (+) effect for alfalfa freezing tolerance that explained only 11% ( $R^2 = 0.11$ ) of the phenotypic variation. This output affirms the reliability of the trait value used.

Some QTL identified in this experiment overlapped with genomic regions of WH related QTL reported previously. The QTL RR-n1 on chromosome 4D was detected in the same chromosomal region where the QTL ws10 was detected [19]. The QTL RR-n2 reported here also overlapped with ws5 on chromosome 8D of CW 1010 parent [19]. Another QTL BR-d1 of 3010 on chromosome 2B was identified in the proximal region where winter hardiness QTL wh15 and dormancy related QTL dorm16 was detected [19]. The direction of the allelic effect of these QTL overlapped and matched in both phenotyping conditions. Also, in this study, we detected major QTL on various homologs of chromosomes 2, 3, 4, 5, 6, and 7. Another experiment also reported major winter injury-related QTL on linkage groups 2, 3, 4, 5, 6, and 7 [20]. These pieces of evidence support the genetic relationship of freezing tolerance and field cold survival. The tag

sequences of flanking and peak markers of the QTL are provided in the supplementary file (File S) that can be used as potential markers for marker-assisted selection (MAS).

### **Indoor Screened Breeding Materials**

After consecutive cycles of freezing tolerance testing, selection, and polycrossing, we developed 177 F2:3 advanced half-sib breeding lines. These lines have been screened in the field for cold hardiness selection at Blairsville, GA. The next cycle of selection will be made based on the field performance of the genotypes for WH and marker-assisted selection (MAS).

## **Discussions**

### **Alfalfa Indoor Screening for Freeze Tolerance**

The indoor freezing tolerance selection method we reported here is relatively faster and cheaper than field selection as it takes only a few hours in a freezer after acclimation. All the steps are conducted in a controlled environment and hence the freezing test can overcome the difficulties associated with the field phenotyping mainly environmental variability and the unpredictable winters. Past studies have established that alfalfa winter hardiness in the field is influenced by the ability of genotypes to tolerate and survive freezing [9], suggesting an optimized freezing test is valuable. This indoor FT selection also enables the screening of a large number of accessions at the same time ; normally , we tested a set of 98 X 2 clones grown in cones that included both control and treatment groups. Nonetheless, the indoor selected alfalfa may also require a few cycles of field selection prior to the release as a cold-tolerant cultivar. A past study indicated that indoor selected alfalfa plant progeny showed an increase in freezing tolerance up to 5 °C and superior winter survival [4].

In mild winter areas where the winters have occasional frost and fluctuating temperatures, the condition can be detrimental to perennial forages such as alfalfa with the non-dormant types being especially more vulnerable. When winters are warm with a temperature near 13 °C, over-wintering alfalfa breaks dormancy and starts new growth with elongated crown buds [3], the phenomenon is known as deacclimation [21]. The process depletes alfalfa root reserves, which eventually makes the plant susceptible to low temperatures [3]. Thus, the sensitive genotypes fail to reacclimatize when frost returns in late winter and early spring (Figure 2). Therefore, it seems that the efficiency of the freezing test would be enhanced by the freezing of the already tested (deacclimated) clones one more time. In other words, a simulated environment that involves a series of acclimation-deacclimation-reacclimation process could be ideal for future freezing tests in alfalfa.

### **Clonal Variations**

One of the challenges encountered in the alfalfa indoor cold test was to generate identical clones by stem cuttings. The clonal variation is not unusual and such variation among clones of stem cuttings of the same genotype is commonly experienced in alfalfa. Perhaps the uncontrolled source of variability is

attributable to the vigor of establishment and the interaction with the environment (watering, location in the growth chamber, etc.) (Yves Castonguay, personnel communication, 2016). This effect can be minimized by making multiple clonal propagules for each genotype and using the vigorous clones of uniform size for freezing treatment. When resources are not limited, producing a large number of cuttings and testing more uniform clones could be effective to avoid the uncontrolled source of variability among clones. However, the phenotypic variation present in clones, which is also known as somaclonal variation, could be because of other factors such as epigenetic changes [22].

## **FT and WH Relationship**

The moderate positive correlation ( $r = 0.24$  to  $0.36$ ) observed between indoor cold screening and field WH rating (Table 1) indicated that WH selection in the field can be accelerated by screening alfalfa in the freezing chambers. Some past studies also reported similar information. Brouwer et al. (2000) found a positive correlation ( $r = 0.34$  to  $0.58$ ) between freezing injury and winter injury in field conditions and suggested that freezing tolerance and WH are potentially controlled by some common genetic mechanism [9]. A breeding population developed using indoor selected parents accelerated the breeding process [4]. For instance, superior freeze-tolerant cultivars like Apica (ATF0) and (ATF5) were developed using recurrent selection for up to five cycles [23]. The results from this study and past reports indicate that indoor freezing testing can be useful in indirectly selecting alfalfa winter hardiness.

Winter hardiness is a broad term that refers to the plant's ability to withstand harsh winters, which encompasses freezing temperature, diseases, high moisture level, ice formation, and frost-heaving [24, 25]. Therefore, selecting only based on FT represents only a part of the spectrum of variables and breeders also need to test the freeze selected plants in the field. For instance, different levels of carbohydrate accumulation in the alfalfa crown after artificial freezing and natural hardening was reported [26]. Artificial freezing tolerance was related to the accumulation of sucrose, stachyose, and raffinose and decreased levels of glucose, fructose, and starch. In contrast, alfalfa natural hardening triggered the accumulation of raffinose and stachyose and was less relevant to sucrose accumulation [26]. Perhaps, these physiological and molecular factors are behind the moderate relationship between indoor FT and field WH we have seen in the present study.

## **FT QTL and Potential Application**

In this experiment, we found six favorable QTL (Table 2) that may have the potential to be used for marker-assisted selection (MAS) for alfalfa freezing tolerance as they individually explain more than 10% of the phenotypic variance ( $R^2 \geq 10$ ). Several other QTL with negative effects on freezing tolerance could be an interesting region to investigate the molecular basis of FT in alfalfa. Freezing tolerance in plants is regulated by a complex network of genes [27]. Further, the multiple QTL for FT and WH [19] with low to moderate  $R^2$  indicated that genomic selection (GS) could be a viable option to improve these traits. GS is becoming an effective method for the rapid selection of superior genotypes for cold hardiness improvement in different crops [28, 29]. Therefore, we also suggest using GS in alfalfa to capture

maximum genetic variability present in the germplasms. The GS model for winter-hardiness selection can be better trained using indoor phenotyping of the training population. Potential of application of GS on alfalfa has been described [30, 31].

Based on a comparative genomics approach, the favorable QTL identified in this experiment on chromosome 6 (FT-d4, FT-d5) most likely represents a genic region harboring the important freezing tolerance related gene *CBF* (c-repeat binding factor). This is because a set of *CBF* genes are positioned on the long arm of chromosome six of the alfalfa closest relative *M. truncatula* [32] whose reference was used to assign chromosomes of the linkage groups used for mapping [19]. Expression analysis of *CBF* related genes from *M. truncatula* in alfalfa indicated that the genes (*MsCBFI-17* and *MsCBFI-18*) are potential functional homologs of *CBF3* [33]. The *CBF* homologs and gene group were located not only on *M. truncatula* chromosome 6, but also on chromosome 5 [33, 34] on which we also detected several QTL associated with freezing sensitivity and a favorable QTL (BR-d2) (Table 2). Therefore, the genomic regions of chromosome 6 and 5 where we detected QTL are crucial for alfalfa freezing tolerance improvement. As we observed several freezing sensitivity related QTL, the genomic regions may also harbor cold regulated (*COR*) genes that influence the expression of *CBF*. Hence, the *CBF* transcription factors in alfalfa comprise potential candidate gene families for freezing tolerance improvement and consequently better field winter hardiness.

## Conclusions

In this study, we reported an indoor approach for rapid screening of alfalfa freezing tolerance (FT) which is useful for accelerating breeding for winter hardiness (WH) in the field. Since FT and WH are moderately correlated, we suggest the indoor selection of alfalfa germplasms before taking them to the field for better winter survival selection. We identified 20 QTL for FT related traits in the population tested. The most favorable QTL were located in the proximity on chromosome six, where the crucial cold tolerance gene family (*CBF*) has been reported for the alfalfa closest relative *Medicago truncatula*. The validation of these QTL in various genetic backgrounds will be warranted before adopting them in MAS to ensure the loci are stable and repeatable across populations and environments. However, some of the favorable QTL that explained 10% or more of the phenotypic variance could be tested directly in Marker-assisted selection (MAS).

## Methods

### Plant Materials

The plant materials used in this experiment include two alfalfa parent cultivars, their F1 progenies and winter survival checks. The commercial certified seeds of alfalfa cultivar CW 1010 and 3010 were obtained from Alforex Seeds (Woodland, CA, USA) and BrettYoung (Winnipeg, Manitoba, Canada), respectively. The CW 1010 has the fall dormancy level (FD) ten and the cultivar 3010 has FD level two. Information about these varieties is found in NAFA variety ratings



(<https://www.alfalfa.org/pdf/2015%20NAFA%20Variety%20Leaflet.pdf>) and the details about the varieties can also be searched in the database (<https://www.alfalfa.org/varietyratings.php>). The check varieties were obtained from The National Plant Germplasm System (NPGS). We screened alfalfa pseudo-testcross F<sub>1</sub> [3010 (♂) x CW 1010 (♀)] population for freezing temperature response and mapped the associated quantitative trait loci (QTL) using genotyping by sequencing (GBS). The maternal parent '3010' is a dormant type alfalfa cultivar with relatively higher winter survival whereas CW 1010 is non-dormant and cold-sensitive. The mapping population development and field experiment were described previously [19, 35, 36]. Briefly, the population was grown in two locations, Blairsville and Watkinsville research farms of the University of Georgia (UGA) using three clonal replications in a randomized complete block design (RCBD). This study focuses on freeze testing of alfalfa clones of the mapping population using a walk-in freezer (ESPEC North America, Inc.). Twenty clones per genotype were generated by stem-cuttings of 184 F<sub>1</sub> progenies and parents. The clones were grown under the normal growing conditions and management in the greenhouse. Prior to cold acclimation, twenty clones were divided into two sets, one for control and the other is the treatment group. Together with the experimental mapping population, we also grew the alfalfa winter survival (WS) check cultivars recommended by NAAIC to optimize the freezing test. The checks include the cultivars ZG 9830 (WS = 1), 5262 (WS = 2), WL325HQ (WS = 3), G-2852 (WS = 4), Archer (WS = 5), and Cuf 101 (WS = 6), where WS '1' and '6' indicate extremely winter-hardy and non-winter-hardy, respectively [6]

## Indoor Phenotyping

For the check cultivars, we tested seeded plants while for the population, we tested vegetative clones. The check seeds were sown in 14 cm tall (Ray Leach, SC10) cone-tainers filled with farm soil and 5 cm depth of the Fafard germ mix (Fafard, MA) at the top of the cone. The Cecil sandy loam type farm soil falls under soil class; fine, kaolinitic, thermic Typic Kanhapludults as described previously [37]. The seeded cones were placed in RL98 trays and the seedlings were grown in the greenhouse for 6-8 weeks. Then, the plants were transferred to an acclimation chamber at a temperature of 4 °C for 3 weeks. The chamber was adjusted to 8/16 (light/dark) hours dark period, and 70% relative humidity (RH). The plants were watered weekly, and fertilized with Hoagland's nutrient solution once during the acclimation period. The plants in treatment and control sets were randomized so that both sets have uniform phenotypic distributions. Various combinations of cold temperatures and times of exposure were tested to optimize the freezing trial until significant differences (chi-square test with  $P = 0.95$ ) was observed between samples of the checks 5262 (WS=2) and G-2852 (WS = 4), as described previously [6]. After the freezing test, the plants were moved to normal greenhouse conditions. The top portion of the plants in both the control and the treatment groups were then clipped leaving two nodes above the crown to allow regrowth. After two weeks from treatment, the data was recorded as survival percentage, regrowth height, and visual rating based on freezing tolerance on a scale of 1-5, with 1 being the most freezing tolerant and 5 being the most freezing sensitive. The criterion used for freezing tolerance rating had a slight modification in the bases used for winter survival rating [6]. We scored 1 for genotypes with no injury and uniform regrowth whereas 5 was given to heavily injured barely survived genotypes. We also recorded the

biomass of tested samples after three weeks of the freezing test. Four traits, visual rating based freezing tolerance (FT), percentage % survival (PS), treatment vs. control regrowth ratio (RR), and biomass ratio (BR) were recorded.

### **Relationship Between FT and WH**

Phenotypic correlation between FT traits from indoor screening and WH scores from the field was computed using the PROC CORR procedure in SAS 9.4 (SAS Institute Inc.). In the 2016/2017 winter, Georgia experienced mild weather but early spring frost that occurred in the first week of March caused severe damage in the new regrowth of the winter. Therefore, we compared the FT with WH field data obtained for the season 2016/2017 from both locations and combined environments (Table 1).

### **Genetic Linkage and QTL Mapping**

The information on GBS library preparation, Illumina sequencing, data processing, and marker identification was provided in a previous report [19]. Briefly, the single nucleotide polymorphism (SNP) markers identified using GBS were filtered for single-dose allele (SDA) loci polymorphic within a parent (Aaaa x aaaa) as described [38]. Then, we constructed 32 linkage groups for 8 chromosomes and each of four homologs separately for the maternal and paternal parent. The 32 linkage maps specific to maternal parent retained 1837 SDA SNPs, whereas paternal linkage maps retained a total of 1377 SNPs. The chromosome of corresponding linkage groups was assigned based on the alignment of marker tag sequences on *M. truncatula* reference [19]. We mapped the freezing tolerance related traits on the linkage maps using Windows QTL Cartographer version 2.5. The QTL mapping was conducted with the composite interval mapping (CIM) method [39]. The LOD score specific to the trait was computed using 1000 permutations at a genome-wide threshold of  $P \leq 0.05$  [19]. The genotypic means of all four traits were used as trait values for QTL mapping.

### **Developing Breeding Materials**

The freezing tolerant genotypes we selected in the study were deemed as source germplasms for winter-hardy alfalfa cultivar development. To begin the breeding and selection process, we transplanted the freeze-tolerant F1 plants in the greenhouse and performed polycrossing among the full sibs using bee mediated pollination. The F2 seeds were bulk harvested and germinated in the greenhouse. The seedlings were then screened for freezing tolerant genotypes using a constant freezer environment. The survival half-sibs were then transplanted in bigger containers and intermated. Then F2:3 seeds were harvested separately for each parent and germinated.

## **List Of Abbreviations**

BR = Biomass ratio

GBS = Genotyping by sequencing

PS = Percentage survival

RR = Regrowth ratio

SNP = Single nucleotide polymorphisms

FT = Freezing tolerance

WH = Winter hardiness

MAS = Marker-assisted selection

CBF = C-repeat-binding factor

## **Declarations**

### **Ethics approval and consent to participate**

Not applicable

### **Consent for publication**

Not applicable

### **Availability of data and materials**

The raw reads from genotyping-by-sequencing can be found in the NCBI SRA under the following link <https://www.ncbi.nlm.nih.gov/sra/SRP150116>

### **Competing interests**

The authors declare that they have no competing interests.

### **Funding**

This work was supported by the USDA National Institute of Food and Agriculture, Hatch/Evans-Allen/McIntire Stennis project [1008718]. The fund was used for experimental set up and genotyping. Partial funding was provided by the UGA Cultivar Development and Research Program for the indoor freeze screening and data generation. The funding was used to buy research materials and equipments.

### **Authors' Contributions**

LA did phenotypic data collection, genotyping, data analysis, and manuscript preparation, SOM involved in phenotyping, clone preparation, and manuscript revision, OML assisted in protocol design and experiment accomplishment, AMM designed the experiment, reviewed data analysis, and wrote the manuscript. All authors read and approved the submitted version of the article.

## Acknowledgment

We would like to thank Mr. Jonathan Markham for their assistance in this research with planting and field data collection. We also like to thank Ms. Kathryn Prado and Mrs. Muna Sharma for their assistance in clonal propagules preparation.

## References

1. Leep, R., J. Andresen, and P. Jeranyama, *Fall Dormancy and Snow Depth Effects on Winterkill of Alfalfa*. Vol. 93. 2001.
2. Bélanger, G., et al., *Winter damage to perennial forage crops in eastern Canada: Causes, mitigation, and prediction*. Vol. 86. 2006.
3. <http://www.canr.msu.edu/uploads/resources/pdfs/e2310.pdf>.
4. Castonguay, Y., et al., *An indoor screening method for improvement of freezing tolerance in alfalfa*. *Crop science*, 2009. **49**(3): p. 809-818.
5. Lindstrom, O. and M. Dirr, *Acclimation and low-temperature tolerance of eight woody taxa*. *HortScience*, 1989. **24**(5): p. 818-820.
6. <https://www.naaic.org/stdtests/wintersurvivalnew.pdf>.
7. Castonguay, Y., et al., *Molecular physiology and breeding at the crossroads of cold hardiness improvement*. Vol. 147. 2012.
8. Adhikari, L., M. Mohseni-Moghadam, and A. Missaoui, *Allelopathic effects of cereal rye on weed suppression and forage yield in Alfalfa*. *American Journal of Plant Sciences*, 2018. **9**(4): p. 685-700.
9. Brouwer, D.J., S.H. Duke, and T.C. Osborn, *Mapping Genetic Factors Associated with Winter Hardiness, Fall Growth, and Freezing Injury in Autotetraploid Alfalfa Research support provided by a Hatch grant from the College of Ag. and Life Sci., Univ. of Wisconsin-Madison*. *Crop Science*, 2000. **40**(5): p. 1387-1396.
10. Lindstrom, O.M., *Transplant date influences cold hardiness of Leyland cypress following transplanting into the field*. *HortScience*, 1992. **27**(3): p. 217-219.
11. Peixoto, M.d.M. and R.F. Sage, *Improved experimental protocols to evaluate cold tolerance thresholds in Miscanthus and switchgrass rhizomes*. *Gcb Bioenergy*, 2016. **8**(2): p. 257-268.
12. Mishra, A., et al., *Chlorophyll fluorescence emission as a reporter on cold tolerance in Arabidopsis thaliana accessions*. *Plant signaling & behavior*, 2011. **6**(2): p. 301-310.
13. Castonguay, Y., et al., *Cold-Induced Biochemical and Molecular Changes in Alfalfa Populations Selectively Improved for Freezing Tolerance*. *Crop Science*, 2011. **51**(5): p. 2132-2144.
14. Adkins, J.A., M.A. Dirr, and O.M. Lindstrom, *Cold hardiness potential of ten Hydrangea taxa*. *Journal of Environmental Horticulture*, 2002. **20**(3): p. 171-174.
15. Kimball, J.A., et al., *Assessing freeze-tolerance in St. Augustinegrass: temperature response and evaluation methods*. *Euphytica*, 2017. **213**(5): p. 110.

16. Miura, K. and T. Furumoto, *Cold signaling and cold response in plants*. International journal of molecular sciences, 2013. **14**(3): p. 5312-5337.
17. Hinch, D.K. and E. Zuther, *Plant Cold Acclimation*. 2014: p. 6.
18. Miller, A.K., G. Galiba, and J. Dubcovsky, *A cluster of 11 CBF transcription factors is located at the frost tolerance locus Fr-Am2 in Triticum monococcum*. Molecular Genetics and Genomics, 2006. **275**(2): p. 193-203.
19. Adhikari, L., et al., *Dissecting Key Adaptation Traits in the Polyploid Perennial Medicago sativa Using GBS-SNP Mapping*. Frontiers in Plant Science, 2018. **9**(934).
20. Li, X., et al., *Mapping fall dormancy and winter injury in tetraploid alfalfa*. Crop Science, 2015. **55**(5): p. 1995-2011.
21. Kalberer, S.R., M. Wisniewski, and R. Arora, *Deacclimation and reacclimation of cold-hardy plants: Current understanding and emerging concepts*. Plant Science, 2006. **171**(1): p. 3-16.
22. Pikaard, C.S. and O. Mittelsten Scheid, *Epigenetic regulation in plants*. Cold Spring Harbor perspectives in biology. **6**(12): p. a019315-a019315.
23. Dubé, M.-P., et al., *Characterization of two novel cold-inducible K3 dehydrin genes from alfalfa (Medicago sativa spp. sativa L.)*. Theoretical and Applied Genetics, 2013. **126**(3): p. 823-835.
24. <https://extension.umaine.edu/publications/2272e/>.
25. Jones, F.R., *Winter Injury and Longevity in Unselected Clones from Four Wilt-Resistant Varieties of Alfalfa 1*. Agronomy Journal, 1945. **37**(10): p. 828-838.
26. Castonguay, Y., et al., *Differential accumulation of carbohydrates in alfalfa cultivars of contrasting winterhardiness*. Crop Science, 1995. **35**(2): p. 509-516.
27. McKhann, H.I., et al., *Natural variation in CBF gene sequence, gene expression and freezing tolerance in the Versailles core collection of Arabidopsis thaliana*. BMC Plant Biology, 2008. **8**(1): p. 105.
28. Michel, S., et al., *Improving and Maintaining Winter Hardiness and Frost Tolerance in Bread Wheat by Genomic Selection*. Frontiers in Plant Science, 2019. **10**(1195).
29. Beil, C.T., et al., *Genomic selection for winter survival ability among a diverse collection of facultative and winter wheat genotypes*. Molecular Breeding, 2019. **39**(2): p. 29.
30. Jia, C., et al., *Genomic Prediction for 25 Agronomic and Quality Traits in Alfalfa (Medicago sativa)*. Frontiers in plant science, 2018. **9**: p. 1220-1220.
31. Biazzi, E., et al., *Genome-Wide Association Mapping and Genomic Selection for Alfalfa (Medicago sativa) Forage Quality Traits*. PLoS One, 2017. **12**(1): p. e0169234.
32. Tayeh, N., et al., *A tandem array of CBF/DREB1 genes is located in a major freezing tolerance QTL region on Medicago truncatula chromosome 6*. BMC Genomics, 2013. **14**(1): p. 814.
33. Kanchupati, P., et al., *The CBF-Like Gene Family in Alfalfa: Expression Analyses and Identification of Potential Functional Homologs of Arabidopsis CBF3*. Crop Science, 2017. **57**(4): p. 2051-2063.
34. Shu, Y., et al., *Transcriptome sequencing analysis of alfalfa reveals CBF genes potentially playing important roles in response to freezing stress*. Genetics and molecular biology, 2017. **40**(4): p. 824-

833.

35. Adhikari, L., S.O. Makaju, and A.M. Missaoui, *QTL mapping of flowering time and biomass yield in tetraploid alfalfa (Medicago sativa L.)*. BMC Plant Biology, 2019. **19**(1): p. 359.
36. Adhikari, L. and A.M. Missaoui, *Quantitative trait loci mapping of leaf rust resistance in tetraploid alfalfa*. Physiological and Molecular Plant Pathology, 2019. **106**: p. 238-245.
37. Adhikari, L. and A.M. Missaoui, *Nodulation response to molybdenum supplementation in alfalfa and its correlation with root and shoot growth in low pH soil*. Journal of Plant Nutrition, 2017. **40**(16): p. 2290-2302.
38. Li, X., et al., *A saturated genetic linkage map of autotetraploid alfalfa (Medicago sativa L.) developed using genotyping-by-sequencing is highly syntenous with the Medicago truncatula genome*. G3 (Bethesda, Md.), 2014. **4**(10): p. 1971-1979.
39. Wang, S., C. Basten, and Z. Zeng, *Windows QTL cartographer version 2.5*. Statistical genetics. North Carolina State University, Raleigh, 2005.

## Tables

Table 1

Correlations between different variables from indoor freezing tolerance test and winter hardiness data recorded under field conditions.

	PS	RR	BR	FT	WH017JPC	WH-JPC	WH017BVL	WH-BVL
PS		0.55 <sup>***</sup>	0.40 <sup>**</sup>	-0.91 <sup>***</sup>	-0.33 <sup>**</sup>	-0.26 <sup>*</sup>	-0.23 <sup>ns</sup>	-0.19 <sup>ns</sup>
RR			0.78 <sup>***</sup>	-0.65 <sup>***</sup>	0.19 <sup>ns</sup>	-0.25 <sup>*</sup>	-0.22 <sup>ns</sup>	-0.25 <sup>ns</sup>
BR				-0.46 <sup>***</sup>	-0.25 <sup>*</sup>	-0.28 <sup>*</sup>	-0.20 <sup>ns</sup>	-0.13 <sup>ns</sup>
FT					0.36 <sup>**</sup>	0.25 <sup>*</sup>	0.23 <sup>*</sup>	0.24 <sup>*</sup>
* P < 0.05, **P < 0.01, *** P < 0.001, <sup>ns</sup> non significant								
WH017JPC = Genotypic LS mean for WH score recorded in 2017 at UGA JPC farm								
WH-JPC = Genotypic LS mean for WH score recorded for overall (3 years) at UGA JPC farm								
WH017BVL = Genotypic LS mean for WH score recorded in 2017 at UGA Blairsville farm								
WH-BVL = Genotypic LS mean for WH score recorded for overall (3 years) at UGA Blairsville farm								

Table 2

QTL for the traits related to cold temperature tolerance in an F1 pseudo-testcross (3010 × CW 1010) population. The following QTL were detected on the genetic map constructed for female parent (3010). Allele direction '-' and '+' means the loci have negative and positive effects, respectively on the trait of interests.

Trait	Parent	QTL code	Chr.	Peak Marker	Peak LOD	R <sup>2</sup>	Allele dir.	LSI (cM)	Flanking Markers
FT (visual rating based)	3010	FT-d1	3C	TP49015	6.2	0.19	-	3.3–6.2	TP45927 - TP70292
		FT-d2	3C	TP49370	5.8	0.18	-	9.0–10.9	TP78263 - TP75378
		FT-d3	4B	TP65152	5.1	0.17	-	85.3–90.2	TP65152 - TP15305
		FT-d4	6B	TP52704	4.0	0.12	+	73.6–75.2	TP77043 - TP80446
		FT-d5	6D	TP4357	3.2	0.10	+	0–3	TP4357 - TP36795
	CW1010	FT-n1	2A	TP48213	3.3	0.11	+	49.9–52.9	TP48213 - TP87635
		FT-n2	5B	TP50569	5.4	0.24	-	69.6–70.9	TP61799 - TP52871
		FT-n3	5B	TP8562	6.7	0.29	-	72.6–74.5	TP47547 - TP81049
		FT-n4	5B	TP80460	6.4	0.28	-	78.1–81.5	TP47971 - TP56384
% survival PS	3010	PS-d	8B	TP55382	3.4	0.10	+	84.2–87.1	TP3723 - TP54987
	CW1010	PS-n1	5B	TP8562	4.5	0.15	-	73.1–73.8	TP8562 - TP81049
		PS-n2	5B	TP80460	4.3	0.14	-	78.1–79.4	TP47971 - TP56384
		PS-n3	7A	TP13294	4.3	0.13	-	76.6–81	TP32322 - TP13294
		PS-n4	8A	TP8423	3.0	0.07	-	11.5–12	TP34845 - TP9964
regrowth ratio (RR)	3010	RR-d1	3C	TP49015	3.4	0.12	-	4.1–6.2	TP45927 - TP70292
		RR-d2	3C	TP18855	3.09	0.11	-	10.9–14.8	TP75378 - TP55943

Trait	Parent	QTL code	Chr.	Peak Marker	Peak LOD	R <sup>2</sup>	Allele dir.	LSI (cM)	Flanking Markers
	CW1010	RR-n1	4D	TP66640	3.5	0.13	-	37.8–41.9*	TP36603 - TP65276
		RR-n2	8D	TP2543	3.1	0.11	-	44.2–46.0**	TP2543 - TP88682
biomass ratio (BR)	3010	BR-d1	2B	TP49524	5.1	0.18	+	20.6–22.5 <sup>§</sup>	TP78533 - TP43844
		BR-d2	5D	TP61232	3.4	0.12	+	23.6–28.3	TP66670 - TP70116

## Figures



**Figure 1**

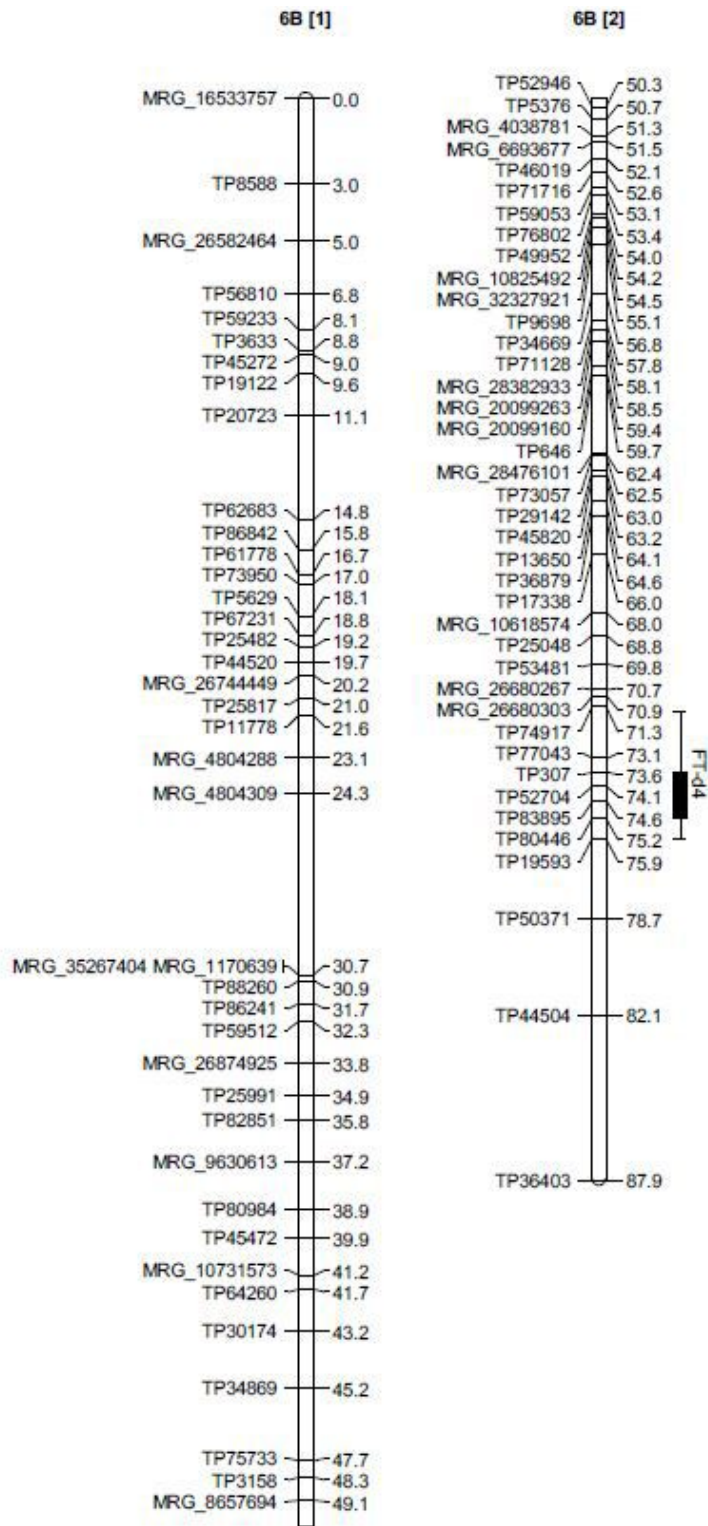
Regrowth pattern of indoor freeze tested alfalfa plants from the treatment group (left) and the control group (right) after two weeks of treatment. Some genotypes in the treatment group had good regrowth and some were dead.





**Figure 2**

Winter frost tolerant (left) and sensitive (right) progenies from a QTL mapping population developed from a cross between a cold-tolerant alfalfa cultivar (3010) and a non-dormant cold-sensitive cultivar (CW 1010). The image was taken after frost occurrence in the first week of March 2017 at Watkinsville, GA. Before the frost, the winter was mild and the alfalfa gained sufficient regrowth.



**Figure 3**

Linkage map of homolog 6B for the maternal parent (3010) with a QTL (FT-d4) for freezing tolerance. Chromosome 6 is the potential region for CBF gene families in alfalfa relative *M. truncatula* L.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryFileFileS.docx](#)