STUDY OF FREEZING TOLERANCE AFTER COLD ACCLIMATION FOLLOWED BY DEACCLIMATION IN WINTER AND SPRING CANOLA (*BRASSICA NAPUS* L.)

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Title

Study of Freezing Tolerance After Cold Acclimation Followed by Deacclimation in Winter and Spring Canola (*Brassica napus* L.)

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ABSTRACT

Winter canola/rapeseed (*Brassica napus* L.) produced 20 to 30% greater yield over spring canola. It overwinters to flower in spring. The winter type shows increased freezing tolerance after cold acclimation, which is crucial to survive in winter. However, short duration warm spells can cause canola to lose increased freezing tolerance in a process called “deacclimation” making it again vulnerable to freezing damage. We found canola almost completely deacclimated at 13°C or more for at least two days of deacclimation. However, longer duration of deacclimation (7 & 14 days) showed increased freezing tolerance as compared to 2 & 3 days at 10°C or less. Variation was seen among winter varieties for deacclimation and deacclimation seems unrelated to cold acclimation ability. Previous genome wide association studies implicated *VERNALIZATION INDEPENDENCE 3 (VIP3)* as having a potential role in deacclimation. We demonstrated that mutations in *VIP3* makes Arabidopsis more tolerant to deacclimation.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDIX TABLES

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1. General introduction

Rapeseed, also known as Rape, Oilseed Rape, Rapa, is the yellow flowering members of the family Brassicaceae (mustard family). *Brassica rapa*, *Brassica napus*, and *Brassica juncea* are the major rapeseed species. Canola (*Brassica napus* L.) are the rapeseed species that contains less than 2% erucic acid in seed oil and less than 30 μmol of glucosinolates per gram of seed meal. *Brassica napus* genome ($A_nA_nC_nC_n$) is an important allopolyploidy crop resulting from cross between ancestor of *B. oleracea* and the ancestor of *B. rapa* (Nagaharu, 1935). In 1974, the first low erucic acid and low glucosinolate cultivar "Tower" was developed in Canada (Bell 1982) and Canada is the leader to start cultivating modern canola crop. The word canola comes from "Can" from Canada and "OLA " meaning "Oil, low acid".

Rapeseed provides the second largest source of vegetable oil after soybean (USDA FSA, 2021). In U.S. more than 80% of the canola production occurs in North Dakota U.S. canola acreage and production has increased since its inception in 1988. However, U.S. ranks 10th on world canola importer but the U.S. still holds promise to increase canola acreage and production (U.S. Canola Association, 2022b). Based on growth habit, canola can be divided into three groups, winter, semi-winter, and spring types. Spring and semi-winter type does not require vernalization to start flowering however, winter type requires vernalization. Winter types are usually planted in the fall and flowering during the following spring. Winter canola produced 20 to 30% more seed yield than spring canola type and is mainly planted in Southern Great Plains. Winter canola acreage has been decreasing since 2014 in the Southern Great Plains and farmers are battling weather conditions. Global climate models predict an increase in the mean surface air temperature and the frequency and severity of erratic temperature events (IPCC, 2013). More

unseasonable warm spells could be seen in late winter and early spring followed by frost in the future (Vyse et al., 2019). The rate and timing of deacclimation are therefore key determinants of winter survival, especially during early spring when plants undergo the transition to growth and development. Spring types are the dominant types grown in North Dakota and Canada.

World demand of rapeseed is increasing because it is also used as a hydraulic fluids and biodiesels (Nieschlag & Wolff, 1971). It can also be used as a cover crop to protect soil from erosion during winters (Begna et al., 2017; Holman et al., 2021). Winter canola can also be utilized as a forage crop (Neely et al., 2015).

1.2. History of canola production

Oilseed rapes (*Brassica napus* L.) were recorded as used in India as early as 2000 to 1500 B.C. However, Canada is the leader to start cultivating genetically engineered modern canola crops. Brassica oilseeds growing in North America started when a farmer from Poland brought seeds of *Brassica campestris* to Canada in 1936 (Bell, 1982). That seed was used by the Canada Department of Agriculture before and during World War II for testing across Canada. *B. napus* seeds were obtained from Argentina (Bell, 1982). At first, rapeseed oil was used as an engine lubricant agent. When the demand for rapeseed oil for lubricant declined, Canada remained with an adequate supply of rapeseed. Later it was started to be used as cooking oil (Bell, 1982).

In 1954 the first locally adapted rapeseed variety "Golden" was released at the University of Manitoba. After 1950, scientists were concerned about the negative health effects of two compounds erucic acid and glucosinolate in rapeseed oil and cake, respectively. Chronic exposure to erucic acid in human effect on heart health (Knutsen et al., 2016). Animals cannot digest high concentrations of glucosinolate. Indigested glucosinolate in animals causes reduced palatability, decreased growth, and production (Tripathi and Mishra, 2007). Further research was

focused on reducing erucic acid (Busch et al., 1994). Moreover, two genes, neither dominant over the other control erucic acid content in rapeseed (Downey and Harvey, 1963). The first rapeseed variety “Oro” with low erucic acid was released in 1968. In 1967, canola variety “Bronowski” with low glucosinolate level was discovered and brought to Canada by the polish scientist. In 1974, Baldur Stefansson of the University of Manitoba developed the first low erucic acid and low glucosinolate cultivar “Tower” (Bell 1982).

In 1985, the United States Food and Drug Administration (FDA) granted Generally Recognized as Safe (GRAS) to canola oil highlighting its benefit for heart health. A key factor driving United States (U.S.) demand for canola oil is its potential health benefits (Brown et al., 2008).

1.4. Evolution of canola

Canola (*Brassica napus* L.) is from the *Brassicaceae* family. It is also known as *Cruciferae* or the mustard family. Model plant *Arabidopsis thaliana* also lies in this family. The major rapeseed species include *Brassica rapa, Brassica napus*, and *Brassica juncea*. Figure 1.1 shows that *B. juncea* and *B. napus* are the result of natural hybridization. This figure is called the "triangle of U" after famous Brassica researcher Dr. U N (Nagaharu, 1935).

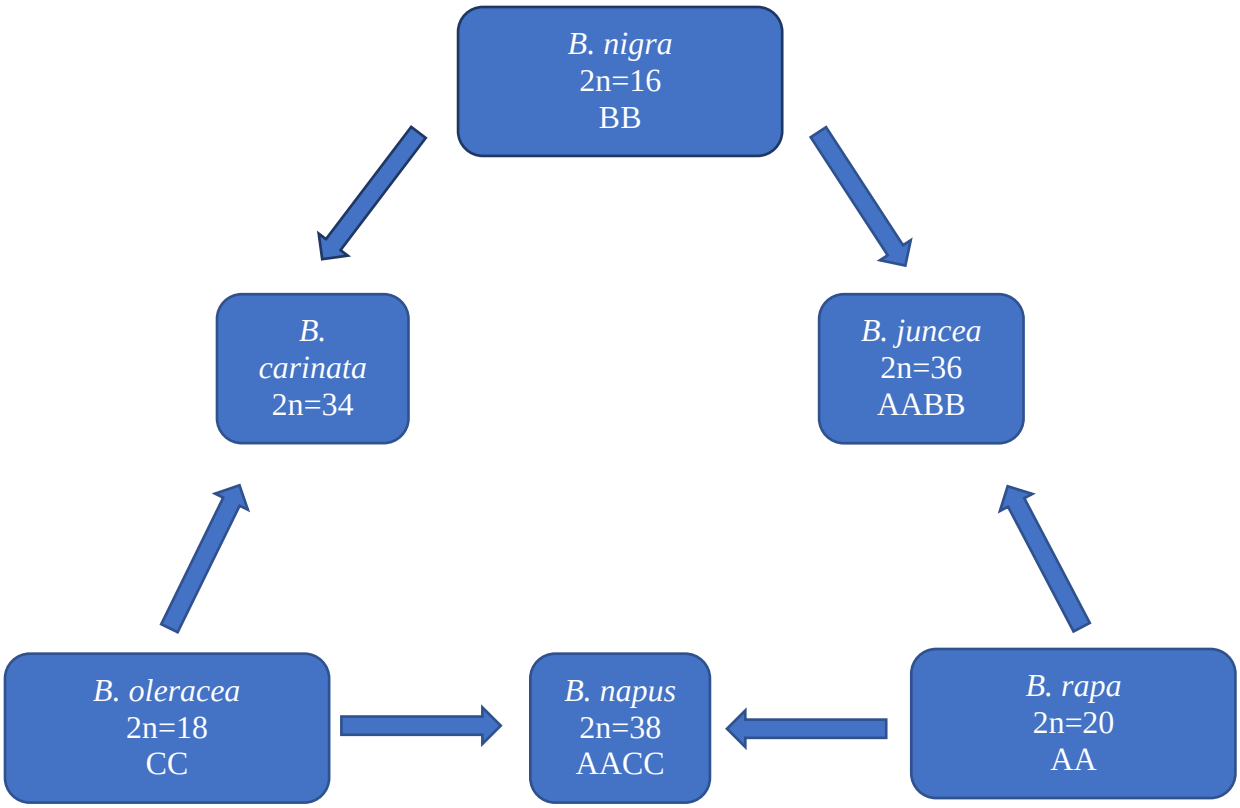

Figure 1.1: "The triangle of U" showing genomic relationships among Brassicas (Nagaharu, 1935).

In this triangle, the diploid Brassica species are *B. nigra* (BB), *B. oleracea* (CC), and *B. rapa* (AA). All other Brassica species are believed to have originated from the natural hybridization of these three species. *B. carinata* (BBCC) from the cross of *B. oleracea* (CC) and *B. nigra* (BB), *B. juncea* (AABB) from the cross of *B. rapa* (AA) and *B. nigra* (BB), and *B. napus* (AACC) from the cross of *B. rapa* (AA) and *B. oleracea* (CC). *Brassica napus* come first in U.S. rapeseed production (Brown et al. 2008).

1.5. Canola genome

Genome duplications and genome mergers are common in Brassicas . Canola has 19 pairs of chromosomes, 9 pairs from *B. oleracea* (C_0C_0) and 10 pairs from *B. rapa* (A_RA_R). The whole genome of *Brassica napus* is 1130 Mb (Chalhoub et al., 2014). The assembled C_n subgenome (528.8Mb) is larger than A_n subgenome (314.2Mb). These subgenomes are consistent with the

relative size of the C_0 and A_R genomes. *Brassica napus* genome contains 34.8% Transposable elements (TEs) with asymmetrical distribution in A_n and C_n subgenome. The same distribution of TE has been found in the progenitor C_0 and A_R genome (Chalhoub et al., 2014; Parkin et al., 2014). The *Brassica napus* genome contains 101040 genes estimated from 35.5 Gb of RNA-Seq data. Out of these 101040 genes 91167 have been matched with *B. oleracea* or *B. rapa* predicted proteomics. These genes are most abundant in distal euchromatin as compared to the centromeric heterochromatin (Chalhoub et al., 2014).

1.6. Importance of canola

Oilseed rape is the second-largest oilseed crop in the world after soybean. Oilseed rape accounts for 13% of the oilseed supply in the world (Eskin and Przybylski, 2003). Oilseed rape contains all the *Brassicaceae* for oil supply but *B. napus* and *B. rapa* are the major ones (Raymer, 2002). Canola is considered a healthy vegetable oil and one of the best oils for heart health. Canola oil has very little saturated fat compared to other cooking oils (Fig. 1.2). It has only 7% saturated fat, the least among all cooking oils. Saturated fat increases the low-density lipoprotein (LDL), a bad cholesterol in the blood which is one of the reasons for coronary heart disease (Micha and Mozaffarian, 2010; Hammad et al., 2016). Canola oil does not contain any trans fats. It also provides linoleic acid (omega -6- fatty acid), alpha-linolenic acid (omega-3-fatty acid), and oleic acid (omega-9-fatty acid). Polyunsaturated fat helps to protect the heart by reducing bad cholesterol and inflammation. It also controls blood pressure (Lin et al., 2013; Watson, 2020). Canola oil has a high smoke point. It can be heated up to 242ºC without developing smoking and trans fatty acids. Therefore, it is ideal for deep frying. Other characteristics are light flavor and smooth texture which makes it a versatile cooking oil. It can

be used as cooking oil for sauteing, stir-frying, grilling, and baking. Moreover, it is used in salad dressings, sauces, and marinades (Watson, 2020).

After the oil is removed from canola seeds, the remaining cake contains a high amount of protein (37 % protein). So, it is used as a high protein livestock supplement. Freitas et al. (2013) highlighted the possible use of canola cake as a substrate to produce proteolytic enzymes by a selected strain of *Aspergillus arizae*.

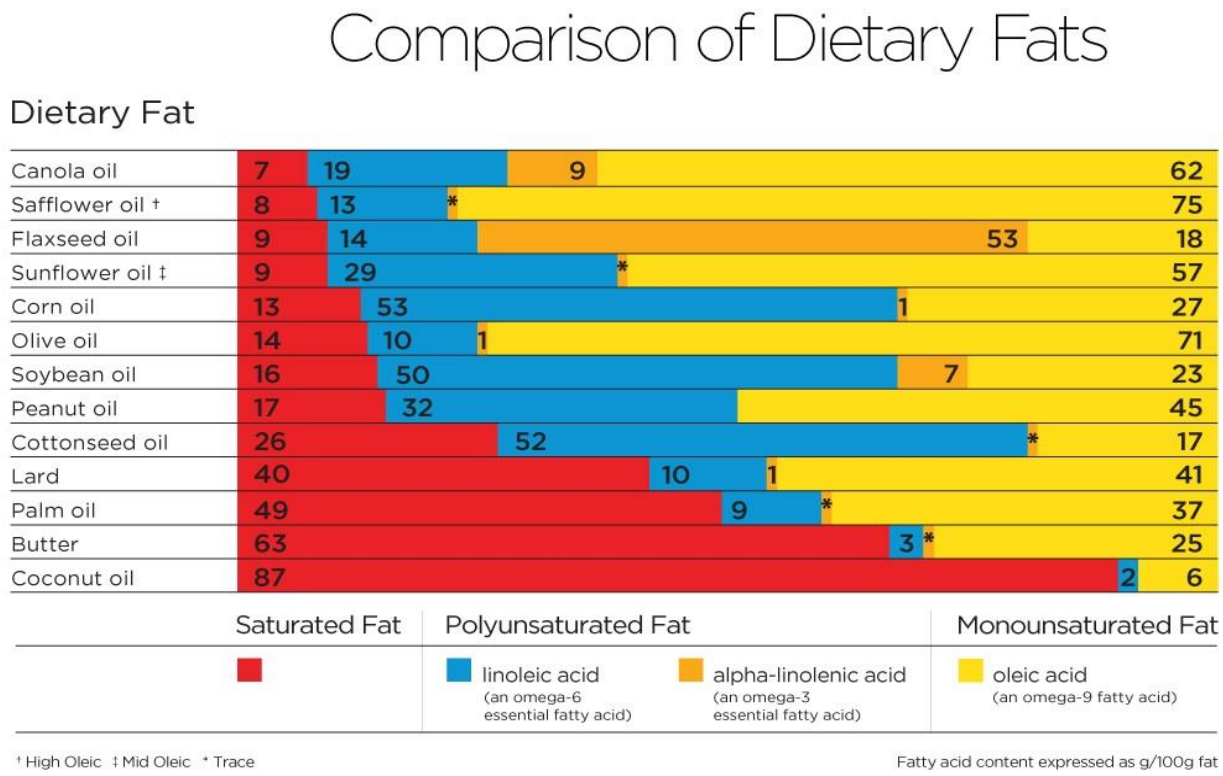

Figure 1.2: Showing dietary fat contains among all cooking oils (the figure adapted from Canola Council of Canada, 2021).

Alternatively, rapeseed oil can be used as a source of biofuels. Currently, a large number of researchers are focusing on the possible use of canola as a biofuel (Ge et al., 2017). Michigan biomass energy program is researching the possible use of rapeseed as a biofuel supported by the Michigan Innovative Farmer's Association. Cultivars that produce more than 45% erucic acid could be utilized as moisturizer for hair and skin, lubricants in continuous steel casting,

formulating lubricants, hydraulic fluids, biodiesel etc. (Nieschlag & Wolff, 1971; Raymer, 2002).

Winter canola has an additional use as a in the Southern Great Plains where mono-cropping is prevalent. Wheat is generally grown followed by fallow in this region. Many of the forage parameters are higher in canola as compared to wheat. It can also be used as a cover crop in winter (Neely et al., 2015; Begna et al., 2017; Holman et al., 2021). It also provides ideal habitat for pollinators. Winter canola provides ground cover through fall, winter, and spring, protecting the soil from erosion. In areas like the Great Plains where winter wheat is the principal crop, winter canola can be used to break the monoculture of wheat. Winter canola is a nonhost crop for soybean cyst nematodes (SCN), so adding the canola to the crop rotation helps to control SCN in soybean and many common weeds and diseases of winter wheat, such as ryegrass and Fusarium head scab (Barrera, 2014).

1.7. Canola production

Rapeseed account for 68.87 million metric tons of product from Fig 1.3 (USDA FSA, 2021). Canada is the single country, producing more than 25% of the world canola production and is the leader in canola export. Other major countries producing canola after Canada are China, India, Germany, and France. Its production has increased sharply after its inception. Every year, approximately 20 million acres of Canadian farmland turns brilliant yellow as canola comes into full bloom. Although the area under canola has remained steady, total production has increased in the last decade because of increases in average yield. Its demand is surging for use as a biofuel. The Canadian canola oil industry has the aim to increase yield to 52 bushels/acre to meet global market demand of 26 million tons by 2025.

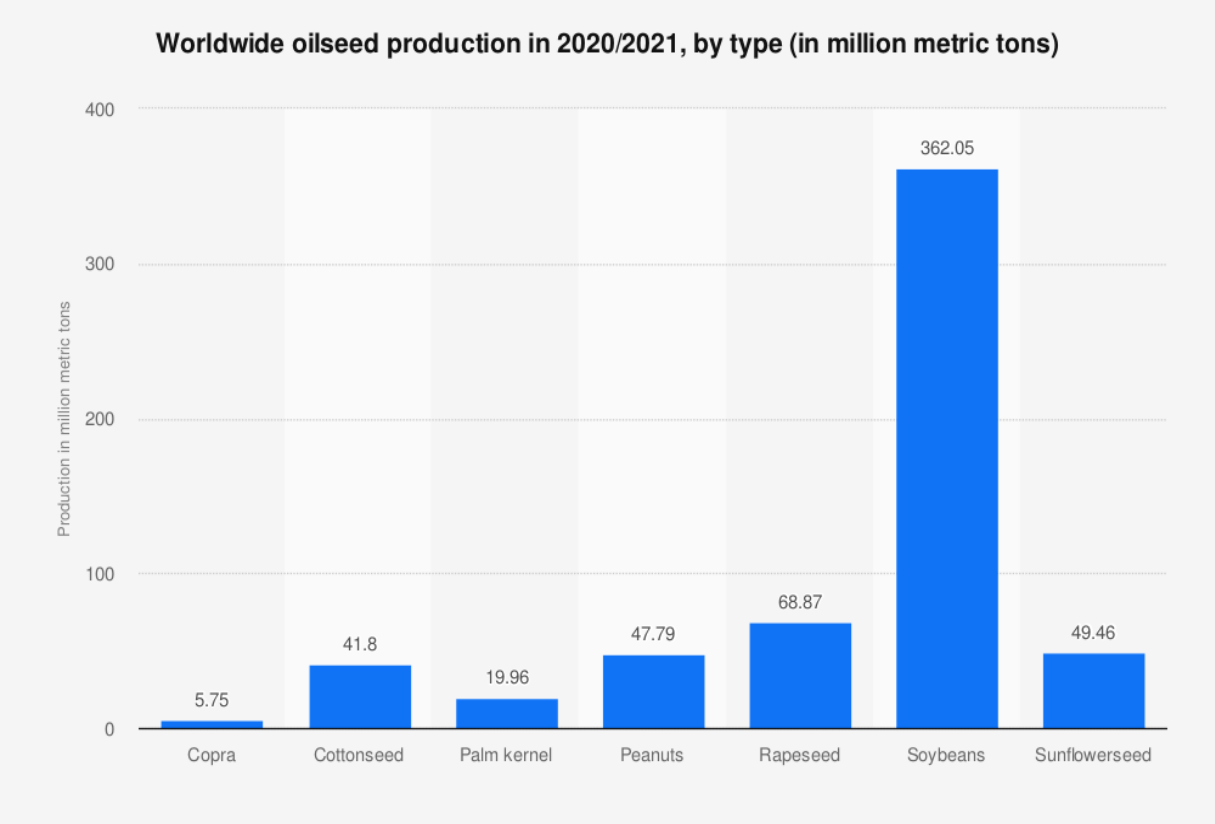

Figure 1.3: Worldwide oilseed production (USDA FSA, 2021).

In the U.S., farmers introduced the canola crop in 1988. U.S. canola acreage and total production have increased dramatically since 1991 (Fig 1.4). As noted above, North Dakota is the leader in U.S. canola production withore than 80% of U.S. canola production. Other key producing states are Oklahoma, Montana, Washington, Minnesota, Kansa, and Idaho. In 2021, the Northern Great Plains (North Dakota and Minnesota) accounted for more than 86% of U.S. canola production. The Pacific Northwest (Montana, Idaho, Washington, and Oregon) ranked second in U.S. for canola production. The Southern Great Plains (Oklahoma, Kansas, and Texas) and mid-South (Virginia, North and South Carolina, Georgia, Tennessee, Kentucky, Alabama, Missouri, Mississippi, and Arkansas) accounted for remaining U.S. canola production (U.S. Canola Association, 2022b).

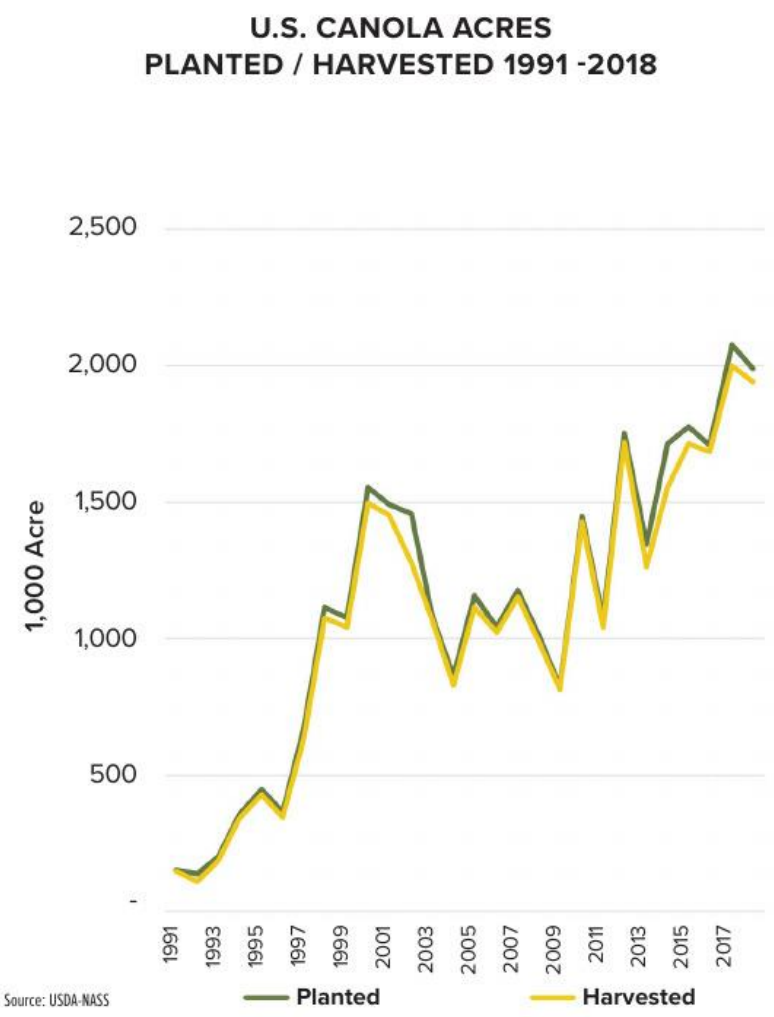

Figure 1.4: U.S. canola production from the year 1991 to 2018 (U.S. Canola Association, 2022b).

1.8. Cold acclimation

Successful canola production depends on the biotic and abiotic stress. Winter survival is key for successful winter canola production. Winter canola and other plants can increase resistance to cold freezing temperatures by the mechanism called cold acclimation (Levitt, 1980). In this process, they acquire increased freezing tolerance by exposing to low non-freezing temperatures. Winter survival of plants like winter canola depends on cold acclimation ability. Cold acclimation generally begins when temperatures drop below 14°C in a short-day length period and cannot occur or occur slowly at temperatures below 0°C (Chinnusamy et al., 2007).

Plants vary greatly in their capacity for surviving freezing temperatures (Jaglo et al., 2001). Herbaceous temperate plants can survive freezing temperatures from -5 to -30º C. Whereas tropical plants are often damaged by low non-freezing temperatures. Freezing tolerance and acclimation abilities are species cultivar -specific (Thomashow, 1999). The maximum freezing tolerance of plants is induced in response to low temperatures below approximately 10°C (Hughes and Dunn, 1996; Thomashow, 1999).

1.9. Physiology of cold acclimation

Freezing temperature causes cell membrane damage (Thomashow, 1999). Freeze-induced membrane damage is the result of severe dehydration in cells (Levitt, 1980; Steponkus, 1984; Steponkus et al., 1988). When the temperature drops below 0ºC, water in intercellular space starts to freeze first because the water inside the cell has a higher freezing point. The chemical potential of ice is less than that of water at a given temperature. This causes the movement of water from inside the cell to the extracellular space. It is noted that, at −10ºC, more than 90% of the osmotically active water typically moves out of the cells (Thomashow, 1999).

Freeze-induced dehydration can cause different membrane damage, including expansion-induced-lysis, lamellar-to hexagonal-II phase transitions, and fracture jump lesions (Thomashow, 1999). Moreover, at freezing temperature production of reactive oxygen species and protein denaturation leads to membrane damage (McKersie and Bowley, 1997). Cold acclimation results in physiological changes such as altered lipid composition stabilize the membrane, preventing freeze-induced damage. Research has shown that cold-acclimation prevents expansion-induced-lysis and the formation of hexagonal II phase lipids in the rye and other plants (Uemura and Steponkus, 1997). Moreover, the accumulation of soluble simple sugars, proline, and stress related proteins (dehydrins) that occur with cold acclimation also seems to contribute to

stabilizing the membrane by providing small polar molecules that can act like water and maintain lipid bilayers (Hauser and Strauss, 1987). It is also believed that certain novel hydrophilic and LEA (late embryogenesis abundant) polypeptides may participate in the stabilization of membranes against freeze-induced injury in a similar manner (Thomashow, 1999).

1.10. Genetics of cold acclimation

Cold acclimation includes the expression of certain cold-induced genes that function to stabilize membranes against freeze-induced injury (Thomashow, 1999). Plasma membrane rigidification raised by a chemical, dimethyl sulfoxide (DMSO) can induce the expression of Cold Regulated Genes (*COR*) (Orvar et al., 2000). Membrane rigidification induces cytosolic $Ca2^{+}$ signatures, and the transient increase in $Ca2^{+}$ regulates *COR* gene expression (Knight et al., 1991).

Studies on *COR* gene expression in Arabidopsis have resulted in the discovery of a family of transcriptional activators, the *CBF/DREB1* proteins, that have a key role in cold acclimation (Thomashow et al., 2001). These transcription factors are induced in response to low non-freezing temperatures, and in turn, activate the expression of hundreds of *COR* genes whose products affect the changes needed for cold acclimation (Zhou et al., 2008). At the post-transcriptional level, low temperature induces nuclear translocation of Trx-h2 from the cytoplasm, and production of CBF expression 1 (ICE1). ICE1 binds to the C- repeat binding factor (CBF) and promotes their expression. Nucleus-localized Trx-h2 reduces all oxidized forms of CBFs polymers (functionally inactive) and changes monomers into a functionally active state. The reduced and active CBF monomers bind to the CRT/DRE motif in some of the promoters of cold-regulated (*COR*) genes and induce their expression (Lee et al., 2021; Kopeć et al., 2022).

In Arabidopsis, plants with mutations at the *ESKIMO1* (*ESK1*) locus accumulate high levels of proline and showed increased freezing tolerance but do not exhibit constitutively increased expression of several cold-regulated genes (*COR6.6*, *COR15a*, *COR47*, and *COR78*). Proline accumulation in *ESK1* mutant plants indicates this gene may mediate freezing tolerance by regulation of transcript levels of genes involved in proline synthesis and degradation (Xin and Browse, 1998).

Differentially expressed genes (DEGs) and differentially accumulated metabolites (DAMs) study in rapeseed (*Brassica napus* L.) shows starch and sucrose metabolism and phenylalanine metabolism were significantly enriched during cold acclimation. Six candidate genes were analyzed on T-DNA insertion Arabidopsis homologous and found all of them differ significantly on the level of freezing tolerance (Raza et al., 2021).

1.11. Deacclimation

When cold-acclimated plants are exposed to warmer temperatures, they lose freezing tolerance acquired during cold acclimation which is called deacclimation (Xin and Browse, 2000). Temperate crops reach a maximum freezing tolerance when fully cold and upon exposure to a warmer temperature in spring, plants lose the freezing tolerance acquired during acclimation by the process of deacclimation and resume growth and development (Xin and Browse, 2000).

Rapacz et al. (2017) found that in common wheat and triticale the rate of deacclimation doesn't depend on cold acclimation ability. In Arabidopsis, which is closely related to canola, during deacclimation, cold acclimation responsive proteins returned to a non-acclimated state while abiotic stress-responsive proteins increase during deacclimation as compared to the non-acclimated state (Miki et al., 2019). In canola, Biochemical responses observed during acclimation were reverted after one week of deacclimation (Horvath et al., 2020b). Plants

respond differently to deacclimation during mid-winter and mid-spring (Wójcik-Jagła et al., 2021). Research suggests that deacclimation rates, like acclimation rates, can be cultivar specific (Trischuk et al., 2014; Juszczak et al., 2016). In *Brassica napus*, the average 24-hour temperature was more important in deacclimation processes than fluctuating day and night temperatures (Rapacz, 2002). Horvath et al. (2020) found several possible genes on freezing tolerance following deacclimation from a Genome Wide Association Study (GWAS) on winter *Brassica napus* varieties. Several studies showed reduction of expression level of *COR* genes and accumulated soluble sugars, proline, and stress proteins such as dehydrins during acclimation also lost after deacclimation (Pagter et al., 2011a; b; Burbulis et al., 2011b; Trischuk et al., 2014). Regulation of oxidative stress by plastid antioxidant system (PAS) gene expression during cold could have an impact on deacclimation in Arabidopsis (Juszczak et al., 2016). Differential gene expression in deacclimation resistant and susceptible varieties has shown the expression difference of oxidative stress (Horvath et al., 2020; Wójcik-Jagła et al., 2021).

1.12. Arabidopsis

Arabidopsis thaliana is a small weed in the *Brassicaceae* family. It is found all over Europe, Asia, and North America, and has been used as a model plant in plant biology research. A short life cycle of 6 weeks and small size makes this plant ideal for genetic analysis (Reeve, 1995). It has five chromosomes with 120 mega base genome size and contains an estimated 20,000 genes (The Arabidopsis Genome Initiative, 2000; Meinke et al., 2022). Research on the Arabidopsis genome has clarified that analysis of plant genomes helps to understand basic principles of biology in living organisms. Arabidopsis communities have developed chemical and insertional mutations, efficient ways to perform crosses, and DNA transplantation. Extensive collection of mutations with diverse phenotypes and molecular markers makes it easy to perform

the genetic study on Arabidopsis. Insertional mutagenesis is created with transferred DNA (T-DNA) from *Agrobacterium tumefaciens* (The Arabidopsis Genome Initiative, 2000).

Genome Wide Association Study (GWAS) on winter *Brassica napus* found several possible genes impacting on deacclimation (Horvath et al., 2020). One such gene *VERNALIZATION INDEPENDENCE 3, (VIP3)* protein is composed of WD motif. An Arabidopsis ortholog of this gene, "AT4G29830" is 1742 bp long. This gene sequence has conserved across multiple unicellular and multicellular organisms. Research showed that this gene involves in flower timing and development in Arabidopsis (TAIR; http://arabidopsis.org).

1.13. Research objectives

Our deacclimation study on winter and spring canola could characterize the temporal and temperature thresholds that initiate deacclimation and mutation analysis could verify the genes impacting deacclimation. This research could ultimately help to better understand deacclimation on physiological and molecular level which could lead to develop better deacclimation resistant cultivars.

The main objectives of this study were,

- To investigate deacclimation at various temperatures and durations
- To screen effect of cold acclimation duration on freezing tolerance among winter varieties
- To analyze variation among winter canola varieties for deacclimation
- To functionally validate putative deacclimation genes by screening T-DNA insertions in such genes in Arabidopsis

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CHAPTER 2: STUDY OF FREEZING TOLERANCE AFTER COLD-ACCLIMATION FOLLOWED BY DEACCLIMATION AT DIFFERENT TEMPERATURE AND DAYS ON WINTER AND SPRING CANOLA (*BRASSICA NAPUS* L.) VARIETIES

2.1. Abstract

Spring rapeseed/canola (*Brassica napus* L.) dominates the U.S. canola production. Farmers can benefit from improved winter canola types in both Southern Great Plains and Northern Great Plains as a seed and forage instead of fallow land in winter. Winter survival is a key metric for successful winter canola production. During winter and early spring, short durations of unseasonably warm weather may occur and indeed, are more likely to occur in the future because of climate change. Such events may cause plants to deacclimate and lose freezing tolerance acquired from cold acclimation. We investigated changes in freezing tolerance of fully acclimated canola varieties with varying levels of freezing tolerance following deacclimation for 1-14 days and at temperatures ranging from 5°C - 15°C. In general, little deacclimation occurred at 7°C, and the majority of the varieties deacclimated quickly (within 2 days) at 10°C and above. However, we did find variation among the varieties for deacclimation rates with several varieties showing poor freezing tolerance both before and after deacclimation, and other varieties showed relatively good freezing tolerance both before and after deacclimation. However, several winter varieties showed changes in their relative freezing tolerance following deacclimation suggesting they had greater resistance to deacclimation than other varieties. The breeder can look at these varieties to develop better cultivars for deacclimation resistance.

Keywords: *Brassica napus*, deacclimation, freezing tolerance

2.2. Introduction

Oilseed rape provides the second-largest amount of oilseed crop in the world after soybean (USDA FSA, 2021). Canola (*Brassica napus* L.) is different from rapeseed because it contains less than 2% erucic acid in seed oil and less than 30 μmol of glucosinolates per gram of seed meal (Brown et al., 2008). Canola oil has only 7% saturated fat, least among all cooking oils, so, it is considered a healthy vegetable oil and one of the best oils for heart health (Micha and Mozaffarian, 2010; Watson, 2020).

World demand for canola oil is high., U.S. canola acreage and total production have increased since 1991. U.S. ranks 10th on world canola importer but the U.S. still holds promise to increase canola acreage and production. Spring canola accounts for the majority of U.S. production. It is grown in the Northern States. Winter canola is grown in the Southern States. Winter canola produces 20 to 30 percent more yield than spring type (Brown et al., 2008). Winter canola is also gaining in popularity in the Northern Great Plains states like Montana as new varieties with strong freezing tolerance ability are developed (Droogsma, 2019). Winter canola can also be used for forage crop. Many of the forage parameters are higher in canola as compared to wheat. It can be used as a cover crop in winter (Neely et al., 2015; Begna et al., 2017; Holman et al., 2021) and it also provides ideal habitat for pollinators. Incorporating winter canola can help to increase U.S. canola production as well as adds multiple benefits.

Successful winter canola production depends on winter survival. Plants show increased resistance to cold freezing temperatures when they are exposed to a low non-freezing temperature for a considerable period, due to a process called “cold acclimation” (Levitt, 1980). Winter survival in canola depends on the cultivar's freezing tolerance, cold acclimation ability, and its interaction with the environment (Stamm, 2021) . Additionally, acclimated plants can lose

freezing tolerance when they are exposed to a warmer temperature, a process known as "deacclimation" (Xin and Browse, 2000). Thus, it is not only cold temperature that determines winter survival but also fluctuating temperatures following acclimation can impact winter kill. However, the effect of fluctuating temperatures and deacclimation during the winter is complicated (Stamm, 2021). Global climate models predict an increase in the mean surface air temperature and the frequency and severity of erratic temperature events (IPCC, 2013; lam, 2018; Vyse et al., 2019).

There have been considerable investigations on the mechanisms of cold-acclimation and subsequent freezing tolerance (Thomashow, 1999). However, very little is known about the mechanisms impacting deacclimation processes (Vyse et al., 2019). Regulation of oxidative stress by plastid antioxidant system (PAS) gene expression during cold could have an impact on deacclimation in Arabidopsis (Juszczak et al., 2016). Several studies suggested different genetic mechanisms controlling deacclimation rather than reversal of cold acclimation (Juszczak et al., 2016; Horvath et al., 2020b; Wójcik-Jagła et al., 2021).

Thus, a better understanding of deacclimation in winter canola is needed at the physiological and molecular levels to provide breeders with tools to select crops with increased deacclimation resistance and to help growers better estimate damage and losses following climatic events that might result in deacclimation of their crops.

This study focuses on freezing tolerance after deacclimation at different times and temperatures on 9 different winter canola cultivars and 2 spring varieties. We investigated both the temporal aspects of deacclimation at various temperatures and the threshold temperatures in which deacclimation is initiated. We also identified canola varieties that showed divergent rates of deacclimation at intermediate threshold temperatures. The data generated should provide

varieties and conditions needed to begin dissecting the genetic mechanisms underlying deacclimation processes.

2.3. Materials and methods

2.3.1. Plant materials

Eleven varieties/lines were chosen from two different growth types, winter, and spring. Nine varieties/lines were winter type (ars229, ars261, ars189, ars269, ars312, ars346, ars228, ars233, and ars246) and 2 were spring type (ars036 and Regina II). An experiment was conducted on randomized complete block design (RCBD) with six replicates and a single plant as the experimental unit. Plants were grown in 800 mL pots and potting soil (Promix from PREMIER HORTICULTURE INC) under greenhouse conditions for 4 weeks (6-8 leaf stage) at ~22°C. The photoperiod was 16 h of light with supplemental halogen lighting as needed. The spring type variety “Regina II" was obtained from Dr. Rahman's lab at North Dakota State University and all others were collected from the United States Department of Agriculture (USDA) sunflower and plant biology research unit and were derived from a primarily winter breeding collection provided by Dr. Michael Stamm, Kansas State University (Horvath et al., 2020a)

2.3.2. Experimental design

After 4 weeks growth under the greenhouse conditions, plants were moved to a cold acclimation chamber at 4-5°C with 12-h photoperiod (with supplemental full spectrum LED lighting—Lumigrow LU50001) for 4 weeks. Plants were fertilized with 20-20-20 NPK mixed fertilizer once a week in a greenhouse and plants were watered as needed. Plants were then moved to a growth chamber for deacclimation at a constant temperature of 5, 7, 10, 13, & 15°C with a 12-hour photoperiod. Plants were deacclimated for 1, 2, 3, 7, & 14 days at each

temperature. To synchronize the freezing time, we transferred 3, 7, & 14 days deacclimation treatment plants on the first day after cold treatment, 2 days on the second day, and 1 day on the third day after cold treatment. There were 5 temperatures × 5 days = 25 different treatments. Plants were assigned randomly on each treatment after cold acclimation. Plants deacclimated at 1, 2, & 3 days of deacclimation, 7 days of deacclimation, and 14 days of deacclimation on each variety were separately grouped for freezing. 5°C deacclimation is essentially the same as cold acclimation. Thus, for the first freezing cycle, 5°C treated plants received close to 1 month of cold-acclimated plants. Likewise, 5 weeks of cold-acclimated plants for the second freezing cycle (7 days of deacclimation) and 6 weeks of cold acclimated plants for the third freezing cycle (14 days of deacclimation). Plants were randomly placed within the freezing chamber. Freezing was initiated at around noon at 15°C, then ramped down to 5°C, over five hours, then ramped down to the freezing temperature (−12°C) over eleven hours (4:00 AM), then held at that temperature for 4 h before ramping back up to 0°C, over 1 hour (h), and then back up to 15°C by noon the following day (lights were turned off at 5:00 PM when plants had reached 5°C and then turned on again at 8:00 AM during the last hour of freezing temperatures). Plants were moved to a greenhouse and were scored for visual damage on a 0–3 scale with 0 being death, 1 having>50% foliar damage but maintaining at least one living meristem, 2 having between 50% and 10% foliar damage, and 3 having 0–10% foliar damage after a one-week recovery period (Figure 2.2). Experiments were repeated for selected 5 winter varieties.

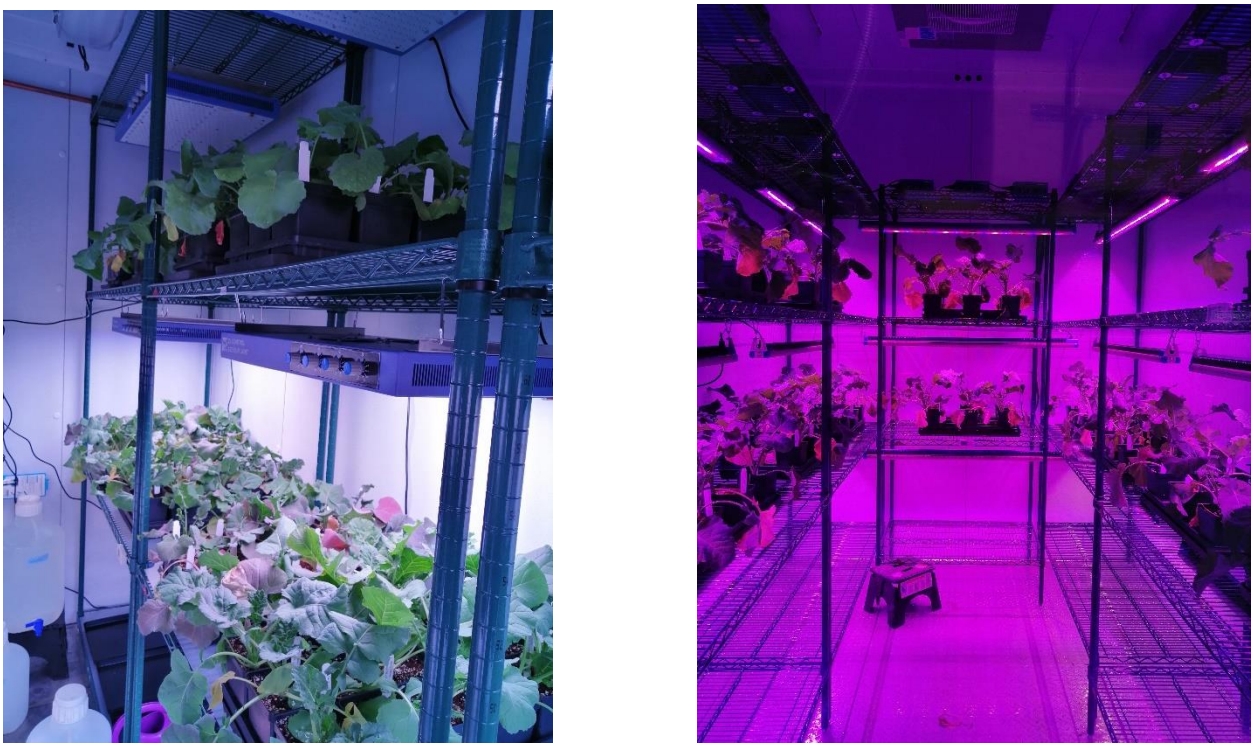

Figure 2.1: Plants in cold acclimation chamber (left) and freezing chamber (right).

Figure 2.2: Plant scored 3, 2, 1, & 0 from left to right.

2.3.3. Statistical analysis

The mean visual score for each combination of days and temperature for every variety was used. Analysis of variance (ANOVA) was performed on R language version 4.0.1 with basic function for RCBD design on factorial arrangement. R package “agricole” was used to test mean separation by The Fisher Least Significant Difference (LSD). Three factors were deacclimation temperature, deacclimation days, and canola varieties. Further analysis was carried out on each

level of factor to see the response of different canola lines. All the repeated experiments were combined for analysis.

2.4. Results

ANOVA results showed that all the three factors, deacclimation days, deacclimation temperature, and canola variety were highly significant. All of the interactions, two-way and three-way were also highly significant (Table 2.1). LSD was calculated on individual factors and two-way interactions. Significant results are discussed from all the analyses.

Table 2.1: ANOVA from deacclimation study on winter canola varieties.

Source	DF	Sum Of Squares	Mean Square	F-Value	P-Value
A	9	340.3	37.8	45.649	< 2E-16***
B	4	67.7	16.9	20.427	6.40E16***
C	4	1345.9	336.5	406.239	< 2E-16***
Rep	5	22.4	4.5	5.418	6.74E05***
A*B	35	155.1	4.4	5.349	< 2E-16***
A*C	36	223.2	6.2	7.484	< 2E-16***
B*C	16	109.9	6.9	8.296	< 2E-16***
Rep*A	45	57	1.3	1.531	0.016*
Rep*B	20	6.6	0.3	0.397	0.992^{ns}
Rep*C	20	23.4	1.2	1.41	0.109^{ns}
A*B*C	140	303.3	2.2	2.616	2.53E16***
Rep*A*B	175	104.5	0.6	0.721	0.996^{ns}
Rep*A*C	180	168	0.9	1.127	0.149^{ns}
Rep*B*C	80	40.6	0.5	0.613	0.997^{ns}
Rep*A*B*C	700	305.6	0.4	0.527	1^{ns}
Residuals	690	571.5	0.8		

A =canola variety; B = deacclimation days; C = deacclimation temperature, Rep = replication
ns, not significant; **, $p < 0.01$; ***, $p < 0.001$

Table 2.2: Mean separation for freezing tolerance at different cold-acclimation period.

Cold acclimation days	Mean (plant survival score)	Grouping*
6 weeks	2.65	a
5 weeks	2.42	a
1 month	1.62	b

*Means accompanied by the same letter are not significantly different.

Table 2.3: Mean of freezing survival among varieties for a different acclimation period.

Variety	1 month	5 weeks	6 weeks
ars346	3.00	3.00	3.00
ars269	3.00	2.50	3.00
ars229	2.50	2.83	2.75
ars233	2.50	2.50	2.83
ars228	2.17	3.00	3.00
ars261	1.83	2.25	2.91
ars189	1.67	2.83	3.00
ars312	1.42	3.00	3.00
ars246	0.42	1.75	3.00
ars036 (spring type)	0.25	1.17	1.00
Regina II	0.00	0.0	0.0

*Mean of 3.00 indicates all plants are survived without any damage

We found five weeks of cold acclimation at 5°C significantly improved freezing tolerance on winter canola varieties but six weeks of cold acclimation did not result in a significant difference in freezing tolerance from the 5 weeks treatment (Table 2.2). Among the winter varieties except *ars246*, five weeks of cold acclimation significantly Improved freezing tolerance over 4 weeks at acclimating temperatures (Table 2.3). Unlike the other varieties, variety *ars246* significantly increased freezing survival between 5 and 6 weeks at acclimating temperatures. For spring varieties, even 6 weeks of cold acclimation is not enough to allow full survival under our freezing conditions.

Table 2.4: Mean separation for deacclimation days.

Days	Mean (plant survival score)	Grouping*
14 days	1.2238095	a
1 day	1.2166667	a
7 days	1.1791667	a
2 days	0.8785714	b
3 days	0.7714286	b

*Mean accompanied by the same letter are not significantly different.

Table 2.5: Deacclimation score mean on temperature and day interaction.

Day/Temperature	5°C		7°C		10°C		13°C		15°C	
1 day	1.62	b	1.93	a	1.18	c	0.49	e	0.87	d
2 days	1.62	b	1.92	a	0.69	c	0.07	d	0.09	d
3 days	1.62	a	1.58	a	0.63	b	0	c	0.02	c
7 days	2.42	a	2.03	b	1.2	c	0.2	d	0.05	d
14 days	2.65	a	2.1	b	1.29	c	0.12	d	0	d

*Mean accompanied by the same letter are not significantly different across rows.
Mean separation on temperature separately on each day of deacclimation (across rows only)

Interestingly, 2 and 3 days of deacclimation were significantly different from 1, 7, and 14 days of deacclimation (Table 2.4). Deacclimation at 7°C was significantly different from 5°C after 1 month of cold acclimation except for 3 days. Deacclimation at 7°C for 3 days was significantly different from 1 and 2 days. At 10°C, 2 and 3 days of deacclimation was significantly different from 1 day. Plants showed much greater damage after 2 days of deacclimation at 10°C than after one day of deacclimation. Plants survival is very low for 13 and 15°C except for 1 day suggesting that one day is insufficient time to complete deacclimation. Surprisingly, on 1 day of deacclimation 15°C showed significantly better survival on deacclimation than 13°C (Table 2.5).

Table 2.6: The difference among varieties at 7 and 14 days of deacclimation.

Variety	Mean (plant survival score)	Grouping*
ars346	1.6000000	a
ars189	1.5333333	a
ars312	1.4444444	a
ars261	1.3583333	a
ars229	1.3500000	a
ars269	1.3500000	ab
ars228	1.2000000	ab
ars233	1.1333333	ab
ars246	0.9833333	b
ars036	0.3500000	c
Regina II	0.0000000	d

*Mean with the same letter are not significantly different

There was no significant difference among winter canola varieties after freezing following 7 and 14 days of deacclimation except *ars246.* Spring varieties were significantly different from winter varieties at all times and temperatures (Table 2.5). Varieties showed significant differences for 2 and 3 days of deacclimation at 10°C.

Table 2.7: Relative rank among varieties for deacclimation resistance at different temperature.

5°C	7°C	10°C	13°C	15°C
ars346	ars346	ars229	ars261	ars229
ars228	ars269	ars261	ars346	ars346
ars269	ars228	ars189	ars229	ars189
ars229	ars229	ars346	ars189	ars261
ars312	ars312	ars312	ars233	ars269
ars233	ars233	ars269	ars228	ars228
ars189	ars189	ars233	ars246	ars246
ars261	ars261	ars228	ars269	ars312
ars246	ars246	ars246	ars312	ars233
ars036(spring type)	ars036	ars036	ars036	ars036
(Regina II)	(Regina II)	(Regina II)	(Regina II)	(Regina II)

*Varieties at top represent the most resistant at each temperature shown and susceptibility to freezing in descending order. Yellow highlighted varieties showed relatively high freezing tolerance under all tested conditions. Pink highlighted varieties showed relatively low freezing tolerance under all tested conditions. Light blue highlighted varieties showed a change in rank indicative of a relatively higher levels of deacclimation resistance and green highlighted varieties showed a change in rank indicative of having a relatively faster deacclimation rate than the other varieties tested.

We ranked the variety with the top average survival score as “1” and then the following varieties were each successively ranked between 1 and 11 in descending order at each combination of deacclimation temperature and day. If varieties had the same mean, they were ranked identically. Finally, Rank 11 was denoted for complete death after freezing. Finally, for each deacclimation temperature, the ranking was averaged from all deacclimation day at that deacclimation temperature. Varieties *ars346* and *ars229* highlighted by yellow color remained among the top four for each deacclimation temperature while *ars228* and *ars269* highlighted by green color significantly dropped in rank after 10°C deacclimation. On the other hand, *ars189* and *ars261* highlighted by blue color showed strong deacclimation resistance at 10°C even though they showed moderate to poor freezing tolerance in their fully acclimated state. Winter

varieties *ars246* and spring varieties, highlighted in pink, *ars036* and *Regina II* remained at the bottom.

2.5. Discussion

In this study, we evaluated freezing tolerance after deacclimation at five different temperatures (5, 7, 10, 13, & 15°C) for 1, 2, 3, 7, & 14 days on each temperature. We tested nine different winter canola varieties (*Brassica napus)* and two spring type. Moreover, freezing tolerance was assessed after 33, 35, and 42 days of cold acclimation. Freezing tolerance following acclimation showed varietal differences. In this study, 5 weeks of cold acclimation at 5°C significantly increased freezing resistance in most of the winter canola varieties we tested. This generally agrees with a previous study that indicated 4 weeks of cold acclimation is sufficient for survival with minimum damage (Ghassemi-Golezani et al., 2008). However, variety *ars246* showed some additional increase in freezing tolerance between 5 and 6 weeks at acclimating temperatures. This suggests that cold acclimation rate is a varietal characteristic. Based on other studies, winter canola required a longer period of cold acclimation if they were cold acclimated at later growth stage of development and spring canola showed better freezing tolerance and acclimation capacity in their early growth stage (Trischuk et al., 2014; Fiebelkorn and Rahman, 2016). In our study, winter varieties showed good freezing survival after 1 month of cold acclimation and at least one spring variety appeared to acclimate to some extent (Table 2.3).

In our study, plants survived the least when deacclimated temperatures were 13°C or higher for longer than 1 day. Interestingly, deacclimation at 7°C for 1 and 2 days significantly improves freezing survival as compared to 33 days of cold acclimation. However, 33 days of cold acclimation followed by deacclimation for 7 days or more at 7°C significantly reduced

freezing tolerance as compared to 33 days of cold acclimation (at 5°C) when all varieties were considered together.

Our study showed that 2 and 3 days of deacclimation at 7°C or above caused a greater loss of freezing tolerance than when plants were allowed to remain at the warmer temperatures for 7 to 14 days at least for temperatures at or below 10°C for most varieties. This result was not consistent with previous observations in rapeseed shoots in vitro (Burbulis et al., 2011b). Our study found plants were more susceptible to freezing after two or three days of deacclimation as compared to 7 days and tolerance when deacclimating at 7°C remains same up to 14 days. Similar observations, deacclimation rapidly in initial days and more resistant at later days was seen in *Lolium* species and timothy (*Phleum pratense L.)* (Gay and Eagles, 1991; Jørgensen et al., 2010). One possible explanation for this observation might be that the trigger for deacclimation might be the temporal increase in temperature rather than the absolute temperature threshold, or that plants might have a chance to regain freezing tolerance when left at minimally acclimating temperatures. Another possibility is that the longer time spent at the higher temperatures allowed the plants to regain or reset their metabolic activity. Stamm (2021) suggested that long-duration deacclimation could increase metabolic activity in green leaf tissue, rejuvenating the overwintering plants. This is also supported in winter barley as mid-winter deacclimation did not upregulate genes for developmental changes (Wójcik-Jagła et al., 2021). However, prolonged deacclimation could be devastating if it happens after the vernalization requirements. After vernalization requirement, plants tend to grow rapidly at warmer temperatures when in their reproductive stage and thus might be expected to be more vulnerable to freezing. Deacclimation followed by elongation growth resulted in greater reduction in freezing tolerance in oilseed rape (Rapacz, 2002; Trischuk et al., 2014) and in perennial ryegrass

(Webster and Ebdon, 2005). In our study, winter varieties showed smaller differences following a longer period of deacclimation (7 and 14 days) as compared to 2 and 3 days at 10°C. Thus, prolonged deacclimation seems to generally improve freezing tolerance and thus winter varieties may be rejuvenated during longer periods of deacclimation at temperatures of 10°C or below.

In our study, we found two groups of winter varieties; one whose freezing tolerance rank remained unchanged, and other groups whose rank changed following deacclimation. Several varieties such as *ars346* and *ars229* showed generally higher freezing tolerance regardless of the deacclimation temperature at least for shorter durations. Other varieties such as *ars246* and the two spring varieties showed poor freezing tolerance even when fully acclimated, and thus showed little change in rank regardless of the deacclimation conditions they experienced. However, several lines such as *ars261* and *ars189* showed an increase in freezing tolerance relative to the other varieties following deacclimation at temperatures as high as 13°C. Likewise, varieties such as *ars269* and *ars228* showed a greater loss of freezing tolerance than others in this study. In some Arabidopsis accessions and in Timothy (*Phleum pratense* L.), the most freezing tolerant varieties showed the greatest deacclimation rates relative to the less freezing tolerant varieties (Jørgensen et al., 2010; Zuther et al., 2015). In our studies this correlation was not true in all cases. Although *ars189* has relatively low freezing tolerance and showed a slower deacclimation rate than the relatively more tolerant *ars228*, highly freezing tolerant varieties such as *ars346* did not show a faster rate of deacclimation than other less freezing tolerant varieties. Our study showed no relation between cold acclimation rate and deacclimation rate and thus is similar with previously described winter wheat and triticale (Rapacz et al., 2017), turfgrass species (Espevig et al., 2014). Our finding showed difference within winter canola varieties for deacclimation resistance at least in some temperature and similar response was seen

in triticale (Rapacz et al., 2017), Arabidopsis (Zuther et al., 2015), Timothy (*Phleum pratense L.)* (Jørgensen et al., 2010), and in winter barley (Wójcik-Jagła et al., 2021).

Actual field conditions could be much different from controlled environments. Plants tend to experience more variable temperature change in field conditions. However, previously strong correlation was found between field condition and controlled environment study (Salgado and Rife, 1996). Moreover, studies in controlled environment could help to accelerate research progress. Some plants appeared to survive freezing even after deacclimation at 13°C or higher and had a little green in the crown tissue even though the rest of the plant was bleached and subsequently died. In field conditions, these plants might revive and produce seeds. Thus, even though these plants had high damage scores in the greenhouse study, the results may have been different under field conditions or if scored by different metrics.

2.6. Conclusion

We analyzed the impact of different temperatures for various days on deacclimation in winter canola varieties. In these studies, deacclimation seems to occur at or above 10°C for as few as 2 days. This data could help canola growers to predict winter kill after short periods of warm spells in field conditions. However, for 7 days or longer plants could revive after damage at no more than 10°C. Growers can use this information to predict the winter kill following unseasonably warm spells in late winter or early spring. Based on our research, 10°C for 2 or 3 days is ideal to screen varieties for deacclimation in a controlled environment. To further understand on physiological and genetic level it would be interesting to compare the physiological responses of varieties such as *ars229* that show consistent and high levels of freezing tolerance following both acclimation and deacclimation to varieties such as and *ars261* or *ars189* that show larger differences in freezing tolerance following deacclimation relative to

their fully acclimated state. It should be noted that although some vernalization is observed after 4 weeks at 5°C, longer periods of acclimation are generally needed to fully vernalize most winter varieties (Zanewich & Rood, 1995). Given that vernalization can impact freezing tolerance in some species (Wójcik-Jagła et al., 2021), it might also be interesting to test deacclimation responses in fully vernalized plants.

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CHAPTER 3: ARABIDOPSIS MUTATIONS SCREENING ON DEACCLIMATION TREATMENT TO FUNCTIONALLY VALIDATE THE ROLE OF A PUTATIVE GENES

3.1. Abstract

Many temperate plants can tolerate freezing temperatures once they are cold acclimated. However, if cold-acclimated plants are exposed to warmer temperatures they can rapidly deacclimate and become vulnerable to freezing damage. Previous studies have focused on freezing tolerance that accumulates during acclimation on a molecular and physiological level but the study on deacclimation at this level is still lacking. Research showed deacclimation as a genetically determined process as opposed to just a physiological reversal of cold acclimation. A previous Genome Wide Association Study (GWAS) on deacclimation in canola identified several potential candidate genes underlying four loci associated with deacclimation resistance. After initial screening of Arabidopsis lines with mutations in two genes, (*VERNALIZATION INDEPENDENCE 3 (VIP 3)* and *PHYTOCHROME KINASE SUBSTRATE 4, (PKS4)*, were selected for further verification. In multiple experiments, mutations in the *VIP3* gene showed significantly higher freezing tolerance than wildtype following 3 days of deacclimation at 23°C. Mutations in *PSK4* resulted in inconsistent differences between the wildtypes in regard to deacclimation resistance. Arabidopsis has seven members of the *VIP* gene family *VIP1* through *VIP7*. These genes govern flower timing by activating MADS-box gene, *FLOWERING LOCUS C (FLC)* but its effect on flower timing seems small. *VIP* genes also have pleiotropic effects and control other developmental processes. We hypothesized that mutations that knock out the expression of this gene will reduce the rate of deacclimation in other Brassica crop species such as canola (*Brassica napus L*), cabbage (*Brassica oleracea*), or camelina (*Camelina sativa*).

Keywords: *VIP3*, deacclimation, canola, mutation

3.2. Introduction

Rapeseed belongs to *Brassicaceae* family which includes *Arabidopsis thaliana*. The major rapeseed species include *Brassica rapa*, *Brassica napus*, and *Brassica juncea* (Nagaharu, 1935). Canola (*Brassica napus* L.) is a variety of rapeseed that contains less than 2% erucic acid in seed oil and less than 30 μmol of glucosinolates per gram of seed meal (Brown et al., 2008). Winter canola requires vernalization in winter months to produce flowers in spring. Spring-type varieties can flower without prior vernalization. Thus, successful winter canola production depends on winter survival.

The primary cause of freezing stress in most temperate crops is cell membrane damage (Thomashow, 1999). Freeze-induced-membrane damage is a result of severe dehydration in cells (Levitt, 1980; Steponkus, 1984; Steponkus et al., 1988). Temperate crops such as canola increase freezing tolerance when they are exposed to a low non-freezing temperature, a process called cold acclimation (Levitt, 1980). Cold acclimation has been shown to result in multiple physiological and genetic changes in plant cells (Levitt, 1980; Steponkus, 1984; Steponkus et al., 1988). Cold acclimation alters plasma membrane composition by accumulation of soluble sugars, proline, stress-related proteins mainly dehydrins (Sasaki, 1996; Wanner and Junttila, 1999; Burbulis et al., 2008, 2011a; Takahashi et al., 2013). Cold regulated genes (*COR*) stabilize the plasma membrane to prevent damage in response to freezing (Thomashow, 1999).

Although many temperate plants including canola can cold acclimate (Levitt, 1980) if given sufficient time at non-freezing temperature, relatively brief periods of warm temperatures can erase the effects of acclimation, leaving the plants vulnerable to again freezing conditions (Xin and Browse, 2000). This phenomenon of deacclimation has significant implications for

winter survival of biennial and perennial species that must survive winter conditions. Frequency of erratic temperature events are increasing as mean surface air temperature is increasing because of global warming (IPCC, 2013; lam, 2018). It is more likely to see short duration of unseasonable warm spells during early winter and freezing events in late spring in future (Vyse et al., 2019).

There is still a lack of knowledge on deacclimation at the molecular and physiological levels. Research indicates that deacclimation induced mainly the reduction of expression level of *COR* genes, accumulated soluble sugars, proline, and stress proteins such as dehydrins (Pagter et al., 2011a; b; Burbulis et al., 2011b; Trischuk et al., 2014). Several studies found variation within same species for deacclimation rate and deacclimation resistant was not correlated with cold acclimation ability or freezing tolerance (Wolf and Cook, 1992; Arora et al., 2004; Espevig et al., 2014; Rapacz et al., 2017; Horvath et al. 2020). One research hypothesized slow deacclimation in Arabidopsis was due to low plastid antioxidant system (PAS) gene expression (Juszczak et al., 2016) .Horvath et al. (2020) combined a GWAS study with RNAseq analysis of a fast and slow deacclimating varieties of canola and purposed several candidate genes controlling deacclimation in canola (*Brassica napus* L.). Transcriptomics analysis has shown expression differences in processes involving oxidative stress, photosynthesis, light-regulated diurnal responses, and growth regulation (Horvath et al., 2020b; Juszczak et al., 2016). During deacclimation, in susceptible winter barley, transcripts associated with stress response, especially oxidoreductase was found to be higher (Wójcik-Jagła et al., 2021).

In this study, we test the hypothesis that mutations in target genes identified by GWAS studies on deacclimation in canola can alter the deacclimation rate in Arabidopsis.

3.3. Materials and methods

3.3.1. Plant materials

Ten Arabidopsis Orthologues of the potential target genes were selected from the canola GWAS study as described in (Horvath et al., 2020) (Table 3.1). Two different T-DNA insertion Arabidopsis homologous was ordered from each 10 Arabidopsis orthologues from the Arabidopsis Information Resource (TAIR; http://arabidopsis.org) (Garcia-Hernandez et al., 2002; Poole, 2005; Reiser & Rhee, 2005). Initially, 20 different Arabidopsis mutations with wild type were analyzed and we narrowed down to 4 mutations with wild type for further confirmation.

Table 3.1: Arabidopsis mutant with gene.

Arabidopsis gene	Probable gene function	Mutation 1	Mutation 2
AT3G19980	FLOWER-SPECIFIC, PHYTOCHROME-ASSOCIATED PROTEIN PHOSPHATASE 3	cs16218	salk150743
AT4G29830	VERNALIZATION INDEPENDENCE 3	cs804895	salk060207
AT5G57240	OSBP(oxysterol binding protein)-related protein 4C	cs824381	SALK138661
AT5G04220	SYNAPTOTAGMIN 3	cs873609	SALK005585
AT5G04160	UUAT1	cs875740	salk023619
AT1G14800	Nucleic acid-binding, OB-fold-like protein	salk000271	salk066035
AT1G31280	AGO2, ARGONAUTE 2, ATAGO2	salk003380	salk201709c
AT1G52730	Transducin/WD40 repeat-like superfamily protein	salk085488	salk104464
AT5G04190	PHYTOCHROME KINASE SUBSTRATE 4, PKS4	SALK086002	SALK206187c
AT4G29840	METHIONINE OVER-ACCUMULATOR 2	SALK081561	SALK081563

3.3.2. Experimental design

To grow Arabidopsis, 0.01 gm (around 150 seeds) was mixed with 0.1% agar solution. Seeds in agar solution was pipetted in pots containing growing medium (Promix from PREMIER

HORTICULTURE INC). Fine vermiculite was applied in thin layer after irrigating the soil. All pots were wrapped with cling film and placed at 5°C for 3 days for stratification. After stratification, plants were moved to growing chamber at 23°C. After 4 days in growing chamber, small hole was made in the cling film with the help of blade to allow seedlings to begin acclimating to low humidity. After 3 days the wrapped plastic was removed. Three treatments independently were applied after 3 weeks of growth from the date of planting. Those were cold-acclimation (3 weeks at 4-5°C with 12-h photoperiod) followed by deacclimation at 23°C (12-h photoperiod) for 3 days, cold-acclimation only, without cold-acclimation and deacclimation (control). For each treatment, we had 3 to 6 replicates for each genotype by treatment. We synchronized planting so that we could freeze all at the same time following the protocol described in Table 3.2. For synchronization, we plant for cold acclimation 3 days after planting for deacclimation, and planting for control was carried out after 3 weeks of cold-acclimation planting. Plants were randomized in a freezing chamber and were moved to the greenhouse after freezing. Visual scoring was done after one week of freezing. For visual scoring, we used a scale of 0 -10. "0" means complete death none of a single Arabidopsis plant survived and "10" means complete survived. The whole experiment was repeated once.

Figure 3.1: Arabidopsis plants in cold acclimation chamber.

Table 3.2: Freezing protocol.

Time	Temperature	Lights
12:00 PM	15.0°C	on
5:00 PM	15.0°C	off
9:00 PM	5.0°C	off
4:00 AM	-13.0°C	off
8:00 AM	-13.0°C	on
9:00 AM	0.0°C	on
12:00 PM	15°C	on

3.3.3. Statistical analysis

Analysis of variance (ANOVA) was performed on R language version 4.0.1 with basic function for RCBD design with 6 replications. R package “agricole" was used to test mean separation by LSD. A separate analysis was carried out for each treatment. The repeated experiment was analyzed separately.

3.4. Results

From preliminary experiment of 10 gene set, two gene were selected based on the consistency for deacclimation and freezing resistance in the preliminary studies (data not shown). Two lines, salk060207 and cs804895 are independent knock-out mutations of the Arabidopsis gene “AT4G29830” which encodes the *VERNALIZATION INDEPENDENCE 3 (VIP3)* protein, also known as the *ARABIDOPSIS THALIANA HOMOLOG OF YEAST SKI8, (SKI8)*, and lines Salk086002 and Salk206187c that contain knock-out mutations of AT5G04190 which encodes the gene *PHYTOCHROME KINASE SUBSTRATE 4*, (*PKS4*). More rigorous experiments with these two genes showed that lines with mutations in *VIP3* consistently showed statistically higher deacclimation resistance than the wild type. Lines containing mutations in *PSK4* were not consistently more resistant to deacclimation than wild type (Tables 3.3 - 3.6).

Table 3.3: ANOVA of deacclimation on mutations (First experiment).

Source	DF	Sums of Squares	Mean Square	F-Value	P-Value
Replication	5	5.60	1.12	1.31	0.2986^{ns}
mutation	4	60.53	15.13	17.73	2.33E-06***
Residuals	20	17.07	0.85		

ns, not significant; **, $p < 0.01$; ***, $p < 0.001$

Table 3.4: Mean separation on deacclimation for different mutations (First experiment).

Mutation gene	Mutations	Mean of survival score	Grouping*
VIP3	salk060207	9.0	a
VIP3	cs804895	8.33	a
PSK4	Salk086002	8.0	a
Wild type	Wild Type	6.67	b
PSK4	Salk206187c	5.00	c

*Means accompanied by the same letter are not significantly different.

Table 3.5: ANOVA of deacclimation on mutations (Second experiment).

Source	DF	Sums of Squares	Mean Square	F-Value	P-Value
Replication	5	7.87	1.57	0.94	0.477^{ns}
mutation	4	113.33	28.33	16.93	3.31E-06***
Residuals	20	33.47	1.67		

ns, not significant; **, $p < 0.01$; ***, $p < 0.001$

Table 3.6: Mean separation on deacclimation for different mutations (Second experiment).

Mutation gene	Mutations	Mean of survival score	Grouping*
VIP3	salk060207	7.0	a
VIP3	cs804895	4.67	b
PSK4	Salk206187c	2.67	c
Wild type	Wild Type	2.33	c
PSK4	Salk086002	1.67	c

*****Means accompanied by the same letter are not significantly different.

Overall, means are higher in the first experiment than in the second experiment. However, the *VIP3* mutant showed a similar response in both experiments.

Table 3.7: ANOVA of acclimation on mutations (First experiment).

Source	DF	Sums of Squares	Mean Square	F-Value	P-Value
Replication	5	1.578e-29	3.155e-30	1	0.443^{ns}
mutation	4	1.262e-29	3.155e-30	1	0.441^{ns}
Residuals	20	6.311e-29	3.155e-30		

ns, not significant; **, $p < 0.01$; ***, $p < 0.001$

Table 3.8: ANOVA of acclimation on mutations (Second experiment).

Source	DF	Sums of Squares	Mean Square	F-Value	P-Value
Replication	5	4.300	0.8600	1.084	0.399^{ns}
mutation	4	8.133	2.0333	2.563	0.070^{ns}
Residuals	20	15.867	0.7933		

ns, not significant; **, $p < 0.01$; ***, $p < 0.001$

Table 3.9: ANOVA of non-acclimation on mutations (First experiment).

Source	DF	Sums of Squares	Mean Square	F-Value	P-Value
Replication	5	27.10	5.420	5.685	0.00202**
mutation	4	12.13	3.033	3.182	0.03552 *
Residuals	20	19.07	0.953		

ns, not significant; **, $p < 0.01$; ***, $p < 0.001$

Table 3.10: Mean separation on non-acclimation for different mutations (First experiment).

Mutation gene	Mutations	Mean of survival score	Grouping*
VIP3	salk060207	2.333333	a
PSK4	Salk086002	1.666667	ab
VIP3	cs804895	1.000000	b
Wild type	Wild Type	1.000000	b
PSK4	Salk206187c	0.500000	b

*Means accompanied by the same letter are not significantly different.

Table 3.11: ANOVA of non-acclimation on mutations (Second experiment).

Source	DF	Sums of Squares	Mean Square	F-Value	P-Value
Replication	5	36.30	7.260	3.736	0.01499*
mutation	4	44.33	11.083	5.703	0.00313**
Residuals	20	38.87	1.943		

ns, not significant; **, $p < 0.01$; ***, $p < 0.001$

Table 3.12: Mean separation on non-acclimation for different mutations (Second experiment).

Mutation gene	Mutations	Mean of survival score	Grouping*
PSK4	Salk206187c	4.000000	a
PSK4	Salk086002	3.166667	ab
VIP3	cs804895	3.000000	ab
VIP3	salk060207	1.833333	bc
Wild type	Wild Type	0.500000	c

*Means accompanied by the same letter are not significantly different.

Results were not significantly different after cold acclimation treatment (Table 3.7 & 3.8). None of the ecotypes showed any damage. Lastly, the damage was prominent on all ecotypes after freezing without cold acclimation and results were not consistently different among ecotypes (Table 3.9, 3.10, 3.11, 3.12).

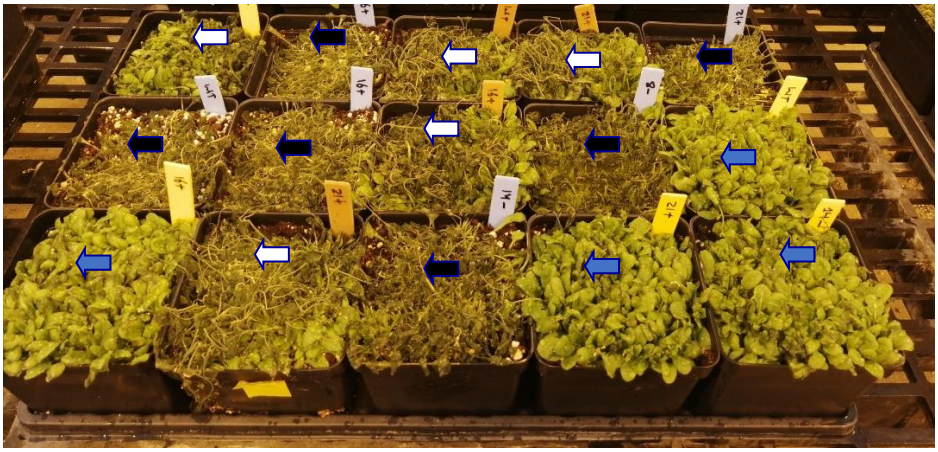

Figure 3.2: Arabidopsis plants after freezing followed by one week of recovery period.
*Pots pointed by blue arrow indicates cold acclimation treatment, while white arrow points deacclimation treatment, and black arrow shows control treatment.

3.5. Discussion

Temperate crops can increase freezing tolerance after cold acclimation. Cold acclimation ability is crucial for winter survival. However, in after fully cold acclimated plants, short duration of warm spells can cause plants to deacclimate and lose freezing tolerance (Xin and Browse, 2000). If temperature again falls to freezing rapidly after deacclimation, plants could be killed (Horvath et al., 2020b). Understanding of deacclimation is of equal importance to develop better freezing tolerance cultivars. In this study we evaluated the freezing tolerance of Arabidopsis lines with mutations in genes identified as having a potential role in deacclimation from a GWAS study on deacclimation in canola (Horvath et al 2020). From this, we identified 1 gene *VIP3* in which two independent T-DNA insertion mutations consistently displayed higher deacclimation resistance than wild type.

VIP3 encodes the protein consisting of WD motif, which is thought to involve in protein complex formation (Zhang et al., 2003; van Nocker and Ludwig, 2003). *VIP3* gene is member of polymerase II-associated factor 1 (Paf1c) complex which involves in histone modification (Park et al., 2010; Jensen et al., 2016) and component of cytoplasmic Super killer (SKI) complex which is thought to be involved in exosome-mediated RNA decay (Dorcey et al., 2012). *VIP3* protein sequence is also known as Arabidopsis SKI8 homologous. SK18 is member of SKI complex has found to involve multiple roles apart from its cytoplasmic role in nuclear yeast (Dorcey et al., 2012). IN Arabidopsis, *VIP3* controls the flowering timing and development (Oh et al., 2004). Seven different *VERNALIZATION INDEPENDENCE* (*VIP*) gene classes (*VIP1, VIP2, …., VIP7*) have been identified in Arabidopsis (Zhang et al., 2003; Zhang & van Nocker, 2002). *VIP* genes show pleiotropic effects including repression of flowering by activating MADS-box gene *FLOWERING LOCUS C* (*FLC*) (Oh et al., 2004).

Although there is no obvious function for *VIP3* on deacclimation, *VIP3* gene may make plants more susceptible to deacclimation through pathways other than flower timing. To further understand this mutant, targets of *VIP3* gene during deacclimation can be identified by looking at expression differences in mutant lines and wild type. Evaluating the over expression of *VIP3* mutants during deacclimation conditions could help identify the function of *VIP3* in deacclimation. Moreover, Evaluating the effect of *VIP3* knockouts among deacclimation susceptible and resistant cultivars could lead to a better understanding of these genes on deacclimation.

3.6. Conclusion

Our mutation analysis showed the *VIP3* gene contributes to deacclimation. This could lead on understanding deacclimation processes in physiological and molecular levels in plants, which in turn breeder can develop better deacclimation resistant canola and possibly other crops cultivars.

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4. OVERALL CONCLUSION

We performed a deacclimation study on rapeseed/canola germplasm (*Brassica napus* L.). Our study showed variation in deacclimation response among winter lines. Cold acclimation rate also differs among winter lines. Four weeks of cold acclimation was sufficient for full acclimation in some lines. However, five weeks and even six weeks of cold acclimation significantly improve the freezing tolerance in some winter lines and also in one spring line. All the lines were deacclimated after more than 2 days of deacclimation at 10°C. Interestingly, longer days of deacclimation (7 and 14 days) affected less than shorter days of deacclimation (2 and 3 days) at or below 10°C. We found no relation between deacclimation rate and cold acclimation rate. Some freezing tolerant winter lines were deacclimated faster but some deacclimated slower. Some winter lines those were poor in freezing tolerance showed a strong deacclimation resistant at or below 10°C. Our, mutation analysis showed that the mutation on gene *VERNALIZATION INDEPENDENCE 3 (VIP3)* significantly increased the deacclimation compared to wild type in both experiments. This gene has shown to be involved in flower timing and development in Arabidopsis, but we could not pinpoint any mechanism for the deacclimation role.

5. FUTURE WORK

There has not been lots of study on deacclimation. Very little is known about deacclimation in the genetic and molecular level. Further understanding on deacclimation is needed. Our work evaluates deacclimation before the vernalization requirement. It would be worthful to evaluate deacclimation after the vernalization requirement because plants tend to grow rapidly after vernalization for the flowering stage. Elongation growth makes plants more susceptible for freezing damage. More study is needed to figure out the role of *VERNALIZATION INDEPENDENCE 3 (VIP3)* gene. Differential study between *VIP3* mutant and wild type in Arabidopsis could help to understand the mechanism of *VIP3* gene. Gene editing technique such as CRISPR/Cas9 can be used to edit *VIP3* gene in canola (*Brassica napus* L.) to make canola more tolerant to deacclimation.

APPENDIX

Table A1: Deacclimation score mean at 1 day.

Variety	Mean (plant survival score)	Grouping*
ars269	2.541667	a
ars261	2.416667	a
ars346	2.375000	a
ars229	2.291667	a
ars228	2.000000	ab
ars233	1.583333	bc
ars246	1.291667	c
ars312	1.104167	c
ars236	1.062500	c
Ars036	1.062500	c
ars189	1.041667	c
Regina ll	0.000000	d

*Mean with same letter are not significantly different

Table A2: Deacclimation score mean at 5 and 7°C for 2 and 3 days.

Variety	Mean (plant survival score)	Grouping*
ars229	2.8333333	a
ars261	1.1250000	b
Ars269	0.9166667	bc
ars228	0.5000000	bcd
Ars346	0.5000000	bcd
Ars246	0.4583333	cd
Ars189	0.4166667	cd
ars312	0.3333333	cd
ars233	0.2500000	cd
Ars036	0.0000000	d
Regina ll	0.0000000	d

*Mean with same letter are not significantly different

Table A3: Deacclimation score at 10°C for 2 and 3 days.

Variety	Mean (plant survival score)	Grouping*
ars261	2.233333	a
ars229	1.933333	ab
Ars346	1.766667	bc
ars228	1.733333	bc
Ars269	1.633333	bc
Ars189	1.400000	c
Ars233	1.400000	c
ars312	0.850000	d
Ars246	0.450000	e
Ars036	0.050000	f
Regina ll	0.000000	f

*Mean with same letter are not significantly different

Table A4: Deacclimation score at 13 and 15°C for 2 and 3 days.

Variety	Mean (plant survival score)	Grouping*
ars229	0.20833333	a
Ars189	0.12500000	ab
Ars261	0.12500000	ab
Ars346	0.08333333	ab
Ars036	0.00000000	b
Ars228	0.00000000	b
Ars233	0.00000000	b
Ars246	0.00000000	b
ars269	0.00000000	b
Ars312	0.00000000	b
Regina ll	0.00000000	b

*Mean with same letter are not significantly different