# GENETIC ANALYSIS OF FROST TOLERANCE IN RAPESEED/CANOLA (*BRASSICA NAPUS* L.)

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**Title**

Genetic analysis of frost tolerance in rapeseed/canola (*Brassica napus* L.)

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

**DOCTOR OF PHILOSOPHY**

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## ABSTRACT

Frost can be detrimental to canola (*Brassica napus* L.) production. Depending on the severity, the entire field can be killed. Having frost tolerance in canola would benefit growers by allowing them to plant early, utilize early season moisture, and avoid high heat during flowering. However, frost tolerance in canola has not been well studied. A protocol was developed that determined 14 day old seedlings should be acclimated at 4°C for 7 days before being exposed to overnight frost (-4°C) in a small freezing chamber. However, when a larger chamber was used for freezing, the protocol was optimized to -8°C instead. A greenhouse study was conducted on a diverse collection of 231 genotypes and genome-wide association scan (GWAS) was conducted to identify potential genes that were related to frost tolerance or abiotic stress tolerance. Thirty-eight significant single nucleotide polymorphism (SNP) markers were selected based on 10,000 bootstraps and 0.1 percent tail of the empirical distribution. The markers were located on chromosomes A01, A02, A03, A04, A07, A08, A09, A10, C03, C05, C06, C07, and C09. Stepwise regression highlighted a QTL located on chromosomes A02. Another GWAS was done on 147 canola germplasm lines phenotyped under natural conditions. Thirty-eight significant SNPs identified from this study were located on chromosomes A05, A07, A09, C01, C02, C03, C04, C05, C06, C07, and C09. Stepwise regression identified a QTL located on chromosome C04. A protocol was developed to measure the freezing induced electrolyte leakage from leaves of rapeseed/canola. A total of 157 germplasm lines were evaluated for freezing induced (-12°C for 2 h) electrolyte leakage. Thirty-six significant SNPs located on chromosomes A01, A02, A03, A04, A05, A06, A07, A08, A09, A10, C01, C02, C04, C05, C06, C07, and C09 were identified. Stepwise regression identified 10 QTL located on chromosomes A01, A02, A04, A06, A07, C02, C05, C07, C09, and one that could not be assigned. All GWAS studies identified

potential genes of interest that were related to frost tolerance, abiotic stress, and transcription factors. Both the greenhouse and field studies indentified transcription factor dehydration-responsive element-binding (DREB) proteins that play a role in cold tolerance.

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# CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

## 1.1. History of canola

Rapeseed (*Brassica napus* L.) oil was first used as fuel in lamps and cultivated by ancient civilizations in Asia and the Mediterranean (Colton and Potter, 1999). It has been grown in Europe since the 13$^{th}$ century. Rapeseed oil was also used as a high-quality lubricant in steam engines. During the Second World War, rapeseed oil was heavily used as a lubricant for a large number of steam engines used by naval and merchant ships. Therefore, rapeseed production was increased significantly during the World War. Later rapeseed oil was starting to be used for cooking oil, but due to its perceived low quality it was not well accepted. Canola was developed at the University of Manitoba, Canada in 1974 by Dr. Keith Downey and Dr. Baldur Stefansson (Brown *et al*., 2008). Canola was bred from rapeseed (*B. napus*) by lowering the amounts of erucic acid in oil and glucosinolates in seed meal. Consumption of rapeseed oil with high erucic acid is harmful for humans, has relatively low digestibility, and has been associated with health problems (Beare *et al*., 1963). In the livestock and poultry feed industries, glucosinolate content in rapeseed meal reduces the feed quality and adversely affects the nutritional value and palatability when used as feed (Hansen *et al.,* 1997).

The first low erucic acid content rapeseed cultivar 'Oro' was released in 1966. Later, a Polish cultivar, 'Bronowski' was identified with low glucosinolate content in the seed meal. Both Oro and Bronowski were used in the breeding program and the cultivar 'Tower' was released in 1974 at the University of Manitoba and was the first cultivar that had both low erucic acid content in seed oil and low glucosinolate content in the seed meal (Stefansson and Kondra, 1975). 'Canola' is a registered trademarked term of the Canadian Canola Association that is derived from *Can*adian *O*il *L*ow *A*cid. The crop canola is also known as double low rapeseed or oilseed rape.

The United States Food and Drug Administration (FDA) granted Generally Recognized as Safe (GRAS) to canola oil in 1985 (Brown *et al*., 2008). Cultivars that contain less than 2% erucic acid in seed oil and less than 30 μmol $g^{-1}$ of glucosinolates in the seed meal were granted GRAS status. In the U.S.A., growers began cultivation of canola in 1991. Crop production increased as consumer demand increased in the country and worldwide.

**1.2. Evolution of canola**

Rapeseed/canola (*Brassica napus* L.) is an amphidiploid (AACC) species and generated through natural hybridization between two diploid species, *B. rapa* (AA) and *B. oleracea* (CC). The "Triangle of U" (U, 1935; Raymer, 2002) explained the relatedness among *Brassica* species (Fig. 1.1). It has been speculated that *B. napus* species may have been better adapted by multiple hybridizations between *B. rapa* and *B. oleracea* (Raymer, 2002). With over 400 years of domestication, *B. napus* is one of the most economically important edible oilseed crops grown in the world (Gomez-Campo and Prakash, 1999).

The family that contains the *Brassica* genus is the *Brassicaceae*, also known as the *Cruciferae* or Mustard family. This family consists of 338 genera and 3709 species (Cheng *et al*., 2014). Glucosinolates (a large group of Sulphur-containing glucosides) are produced by the *Brassicaceae* family. These compounds can help to defend the plant against microorganisms and animals. However, they should not be ingested in large quantities by humans or other animals which is toxic for their growth and development.

*Brassica rapa,* one of the progenitor species of *B. napus*, originated in the highlands near the Mediterranean Sea (Tsunoda, 1980). From there, it spread to many areas including Scandinavia, Eastern Europe, and Germany (Nishi, 1980; Rakow, 2004). It moved into China as an agricultural species through either Western Asia or Mongolia (Rakow, 2004). It then spread to

Japan through either China or Siberia. Currently, *B. rapa* is cultivated in India, Sweden, Finland, and Canada as an oilseed crop.

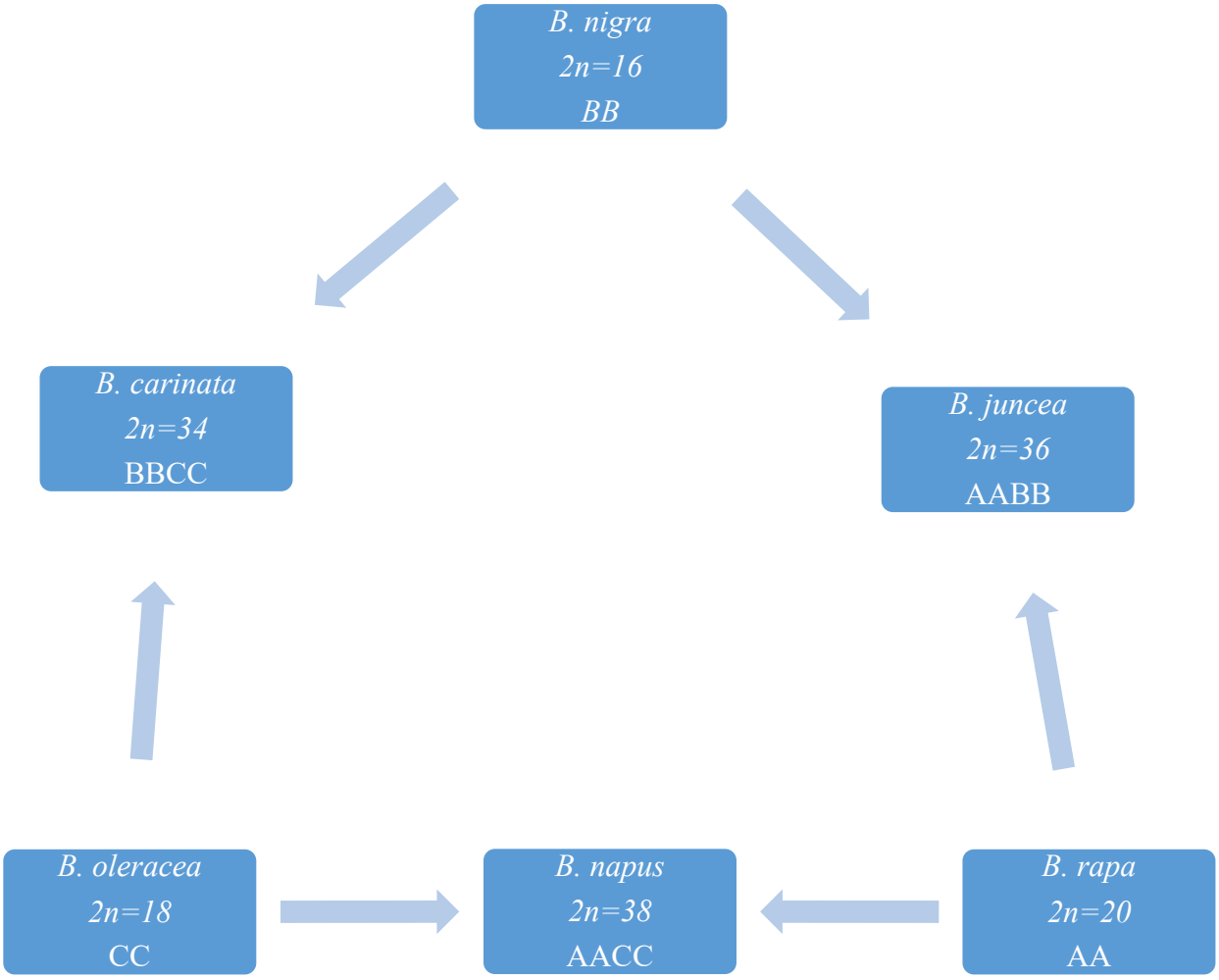


Fig. 1.1. "The triangle of U" showing genomic relationships among *Brassica*s. Adapted from U (1935).

The second progenitor species of *B. napus* is *B. oleracea* which has been found on coastal southern and western Europe (Snogerup, 1980; Rakow, 2004). Six distinct phenotypes of *B. oleracea* include kales, collards, cabbages, kohlrabi, inflorescence kales (cauliflower and broccoli), and Chinese kale have been identified.

Wild *B. napus* may not exist as it is difficult to find (Hinata and Prakash, 1984; Gupta and Pratap, 2007). If it does exist, it would most likely be found in the Mediterranean or European regions, where *B. rapa* and *B. oleracea* overlapped. Many spontaneous hybridizations may have produced *B. napus* (Olsson, 1960; Gupta and Pratap, 2007). These spontaneous hybridizations could have occurred when the two species were grown side by side (Gupta and Pratap, 2007). Different *B. napus* germplasm could have been developed from different interspecific hybridizations.

**1.3. Growth habits and cultivation of rapeseed/canola**

Rapeseed/canola germplasm are mostly classified into three types, winter-, semi-winter, and spring-type. Winter-type is fall-seeded, withstands low temperature with snow cover for vernalization to induce flowering in spring, and is harvested in the summer. Winter canola planting dates can be difficult to determine (Brown *et al*., 2008). Seedlings need sufficient growth to survive over the winter. Cold acclimation is required for the plants to survive. Planting dates may vary slightly from year to year to ensure enough moisture, vernalization time, and plant growth will occur before winter. Spring canola is planted in the spring and is harvested during the summer. Planting date depends on the location and the environment such as soil moisture and temperature (Brown *et al*. 2008). Canola is sensitive to herbicide carryover and care must be taken to avoid herbicide toxicity. Canola should be planted in crop rotations and the recommend planting rotation is once every four years (Brown *et al*. 2008). Semi-winter canola does not require the same vernalization as winter canola. However, without vernalization, it will take longer time to flower and may not complete its life cycle before frost.

### 1.4. Rapeseed/Canola (*B. napus*) genome

Rapeseed/canola has 19 chromosomes, of which 10 are from its progenitor species *B. rapa* and the other 9 of them are from its other progenitor species *B. oleracea*. The genome size of *B. napus* is about 1,130 Mb (Chalhoub *et al*., 2014). The subgenome sizes are consistent with the parent genomes of *B. rapa* and *B. oleracea*. The subgenome C (525.8 Mb) of canola is larger than the subgenome A (314.2 Mb) and the genome assembly contains 34.8% transposable elements (TEs). Based on RNA-sequencing (35.5 Gb), it is estimated that the *B. napus* genome contains about 101,040 gene models. The ab initio gene prediction, protein and EST alignments, and transposon masking are also used to estimate the gene models. Many of the gene models (91,167) matched with the *B. rapa* and *B. oleracea* predicted proteomes. It was found that genes tended to be found in higher amounts in distal euchromatin and not near the centromeric heterochromatin.

### 1.5. Importance of canola

Canola is an important oilseed crop all over the world. It is the second largest source of vegetable oil in the world after soybean (Foreign Agricultural Service, 2016). Canola oil is used in many products, including salad dressings, margarine, and is used in frying and baking. Canola oil contains low levels of saturated fats, high levels of monounsaturated, and moderate levels of polyunsaturated fats (Oomah and Mazza, 1999). Monounsaturated fatty acids include oleic acid while polyunsaturated fatty acids include linoleic and linolenic acids (Fig. 1.2). These characteristics make canola oil a healthier alternative for human consumption. The essential fatty acids in canola oil can be linked with blood clotting, immune response, and lower cholesterol. Canola is the second largest protein meal produced in the world and can be utilized as a livestock feed. The goal of breeding is to develop varieties that are higher in monounsaturated fatty acids

and lower in polyunsaturated fatty acids which can be used in products that are considered to be free of *trans*-fats.

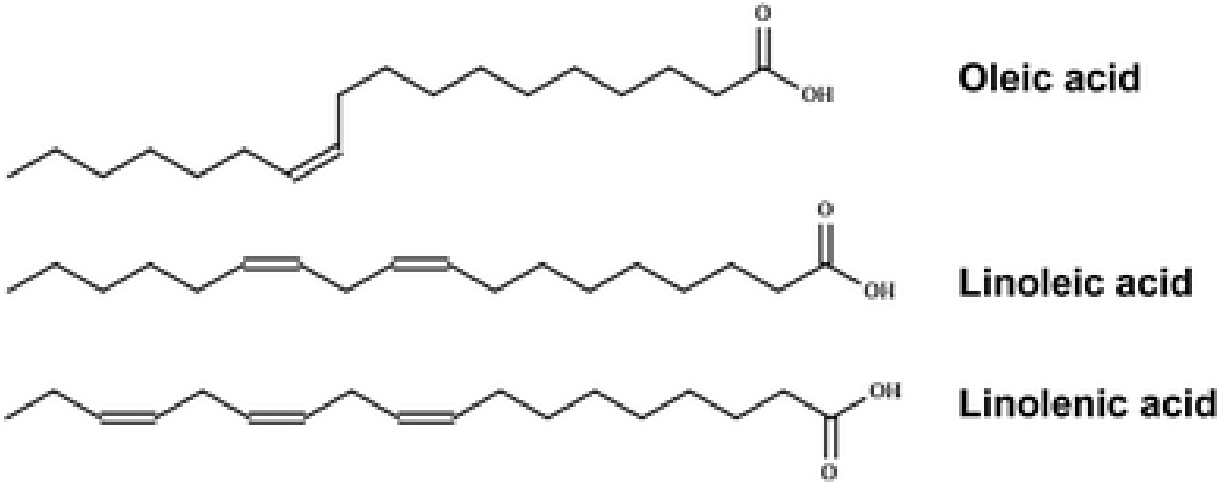


Fig. 1.2. Structure of oleic, linoleic, and linolenic acids that are monounsaturated and polyunsaturated fatty acids found in oils. (Image adapted from: Nah *et al*., 2013).

**1.6. Canola production**

The area planted in the U.S. has fluctuated from year to year (Table 1.1) (USDA-ERS, 2015). North Dakota is the leader of canola acreage with about 84% of U.S. canola planted in this area. The planting area in North Dakota is mostly concentrated near the Canadian border, from the northeast to northwest and the north central tier of the state (82% of ND canola planting area). Minnesota, Kansas, Oklahoma, and the Pacific Northwest also grow canola. Canola is typically planted in a rotation with small grains in the Northern Plains (USDA-ERS, 2016).

The world production of canola is concentrated in areas away from the equator (USDA-ERS, 2016) that tend to be drier and have a shorter growing season. The top five producing countries for rapeseed are China, Canada, India, Germany, and France (data from 1993-2014). Production of rapeseed broke into world production regions and now North America produces just under one quarter of all rapeseed in the world, while Europe and Asia make up the majority of rapeseed production (Fig. 1.3).

Table 1.1. Canola area (hectares) planted in the USA between 2010 and 2016.

| Year | Area planted ('000 hectares) |
|---|---|
| 2016 | 694 |
| 2015 | 719 |
| 2014 | 694 |
| 2013 | 546 |
| 2012 | 710 |
| 2011 | 430 |
| 2010 | 586 |

*Data from NASS, 2016.

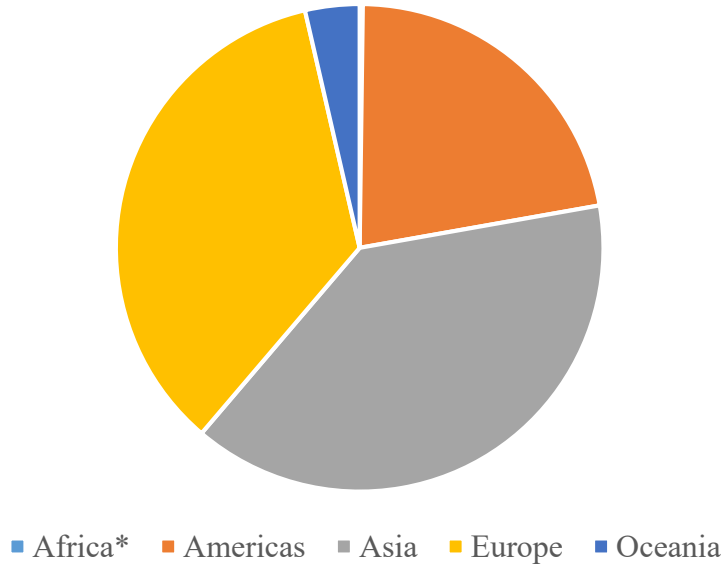


Fig. 1.3. Rapeseed production by region.
Data from FAOSTAT, 2016.
*The percentage for African production is too small to appear on the graph.

The major nutrients needed for canola production that are limited in soils, are nitrogen (N), phosphorus (P), potassium (K), and sulfur (S). Sulfur requirements are higher in canola than many other crops. Recommendations from the US Canola Association is one pound of S for each 100 pounds per acre of seed yield (Brown *et al*., 2008). Sulfur tends to leach out of the soil, so

lower amounts can be used especially in areas with a lower expected yield while a higher amount can be used in areas that tend to yield higher.

Canola tends to shatter; therefore, growers have a small window to harvest before seed loss occurs (USDA-ERS, 2016). Canola can be swathed or cut and allowed to finish ripening and dry down before being harvested. This helps to eliminate the pod shattering and seed loss that can occur when the plants are standing. Desiccator chemicals can be used to speed up the dry down process of the crop. Generally, desiccator is applied when 80-90% of seeds in the pod are turning from green to brown. Combining should occur within 14 days of swathing. The timing for harvesting is important to reduce the yield loss due to shatter, whether desiccation is used or not.

**1.7. Diseases affecting canola**

Many diseases affect canola such as, blackleg (*Leptosphaeria maculans* and *Leptosphaeria biglobosa*), sclerotinia stem rot (*Sclerotinia sclerotiorum*), clubroot (*Plasmodiophora brassicae*), verticillium wilt, and *Rhizoctonia solani*. Canola diseases can greatly reduce yield and can persist in the soil for many years. Blackleg is a fungal pathogen which could be very destructive to canola fields. Tillage and crop rotation can help growers to reduce the pathogen infection (Guo *et al*., 2005). Crop rotations must be long enough and tillage must be sufficient to break down infected stubble and increase decomposition. Blackleg can survive for many years in crop residue and infected stubble (Brown *et al*., 2008). Spores can travel in the wind up to three miles and infect other fields. Blackleg symptoms can be found on the leaves, stems, or pods (Brown *et al*., 2008). Leaf spots or lesions can spread spores. Irregular or round shaped lesions are usually found on the leaf. These spots are typically white or light tan in the center. Infection is usually more likely to occur when the canopy is wet and the

temperature is between 21°C to 24°C. Spread of the disease is more common when the temperature is high (above 30°C) or low (below 10°C). Stem cankers, black spots, or girdling are the symptoms found on the stems. Lodging can occur due to stem infections because the stems are weakened by the fungus. The leaf and stem infections can produce spores that can infect pods. The infected pods can infect the seeds where the disease can survive. Infected seed that is planted in non-infected areas has caused the disease to spread.

Sclerotinia is caused by the fungus *Sclerotinia sclerotiorum* (Hind-Lanoiselet and Lewington, 2004). Besides canola, this disease may affect many other broadleaf plants, such as soybeans, peas, beans, and sunflowers, and many others. Yield losses can be considerable depending on disease severity. Crop rotation is important to minimize infection (Brown *et al*., 2008). Rotations of at least three years between host species is the best way to avoid soil-bourne disease inoculum. Sclerotia, the overwintering structures of the fungus, in the soil and on the residue, can be a source of new inoculum. Sclerotinia infection generally occurs during humid or wet conditions. A major symptom of sclerotinia is the appearance of a white fuzzy mold on the plants (Brown *et al*., 2008).

Another disease that can affect canola is clubroot (*P. brassicae*). Clubroot is a serious soil-bourne disease (Tewari *et al*., 2004). The disease prefers acidic soils for infection. Galls on the roots is a major symptom of the disease and can significantly reduce yield. In Alberta, about 30% yield loss has been observed in fields that had 94% of plants infected (Tewari *et al*., 2004). Crop rotation is important in reducing the disease incidence. Enough time must be left between host species to help eliminate the pathogen.

### 1.8. Frost tolerance

The frost-free date in North Dakota varies from the northern to the southern region significantly. This date may vary from year to year in North Dakota. Typically, frost in North Dakota happens overnight and may last for a short time or can last for a longer period.

Cavalier County is considered the canola capital of North Dakota, which is at 48°47′43″N/97°37′24″W with an elevation of 270 meters (NDAWN, 2016). The average minimum temperature is 7°C and 8°C in May and June, respectively, from 2006 – 2016 (NDAWN, 2016) (Table 1.2). While this is not below freezing, it is still considered cold. Canola can survive below these temperatures, but damage may occur in colder temperatures. The lowest minimum temperature recorded in Langdon, which is in Cavalier county is 1°C and 3°C in May 2010 and June 2013, respectively. The plants are generally small at this point and it has been found that canola is more susceptible to frost at the cotyledon stage than at the three to four leaf stages, which makes canola most susceptible early in the spring when the risk of frost is higher. The severity of the injury depends on many factors, including moisture, growth stage, and duration of the temperature. Being able to plant earlier and not worry about frost damage is a strong desire for the growers. Planting earlier also has other benefits, such as the crop can avoid high temperatures during flowering, plants can utilize early season moisture, and can compete better with the early season weeds.

Frost is typically seen on the leaves and can severely injure the plant depending on the duration and severity of the temperature. Wilting and bleaching of leaves or even plant death can occur from the frost damage. Bleaching occurs due to phyto-oxidation of the pigments in the leaves (Wilson, 1997). Cell death can occur when the membrane is physically disrupted by ice in the cell. This can cause the cell to burst which is lethal. Ice in the extracellular space can cause

adhesions of the cell walls and membranes that can cause cell rupture (Olien and Smith, 1977; Thomashow, 1999). Wilting is caused by the loss of water in the cells. Freezing resistance is a complex trait and difficult to introgress the trait into cultivated lines. Damage due to frost can be seen on the plants for a long time after the actual frost occurs. The colder the temperature, the longer the damage will be seen on the plants. Sometimes, plants cannot recover and are killed by frost damage. Thinner stands due to dead plants can affect canola yield (Ananga *et al*., 2012).

Germplasm screening for frost tolerance is an important research objective which can be tested in the field and in the greenhouse. Field testing requires land, labor, and most importantly suitable freezing conditions for seedling screening. Frost may not happen when the plants are in the active growing stage. However, artificial freezing simulating conditions can be created in a controlled environment such as in a greenhouse and in a plant growth chamber. Testing frost tolerance in controlled conditions is faster, easy, and can avoid many factors seen in the field.

**1.9. Physiology of frost tolerance**

Cold temperatures can cause physiological changes in the cell. Phase changes in the cell membrane from a liquid to a gel can occur when the temperature starts lowering. Gel in the cell membrane can start to be observed at about 10°C in castor bean (*Ricinus communis* L.) (Wade *et al*., 1974). Other crops can have the phase change starting at about 15°C (Lyons, Raison, and Steponkus, 1979).

Different mechanisms are viewed when the plants experience chilling stress versus freezing stress. Chilling stress can occur at temperatures above those that induce ice formation. Oxidative damage in the chloroplasts and mitochondria can be caused by reactive oxygen species (ROS) production when the temperatures are above freezing and as low as -3°C to -4°C.

Mitochondrial oxidation can be reduced under chilling temperatures and can lead to injury (Lyons and Raison, 1970).

Table 1.2. Monthly minimum, maximum, and average temperatures in Langdon (Cavalier county), North Dakota in May and June from 2006 through 2016.

| Year | Month | Maximum temperature (°C) | Minimum temperature (°C) | Average temperature (°C) |
|---|---|---|---|---|
| 2006 | May | 18 | 6 | 12 |
| 2007 | May | 24 | 12 | 18 |
| 2008 | May | 18 | 6 | 12 |
| 2009 | May | 23 | 12 | 18 |
| 2010 | May | 16 | 1 | 9 |
| 2011 | May | 21 | 9 | 15 |
| 2012 | May | 16 | 2 | 8 |
| 2013 | May | 22 | 9 | 16 |
| 2014 | May | 16 | 6 | 11 |
| 2015 | May | 21 | 11 | 16 |
| 2016 | May | 15 | 5 | 10 |
| | Average | 19 | 7 | 13 |
| 2006 | June | 22 | 11 | 17 |
| 2007 | June | 18 | 6 | 12 |
| 2008 | June | 23 | 11 | 17 |
| 2009 | June | 16 | 4 | 11 |
| 2010 | June | 23 | 12 | 17 |
| 2011 | June | 17 | 5 | 11 |
| 2012 | June | 22 | 11E* | 16E |
| 2013 | June | 17 | 3 | 11 |
| 2014 | June | 23 | 12 | 17 |
| 2015 | June | 19 | 8 | 14 |
| 2016 | June | 23 | 9 | 16 |
| | Average | 20 | 8 | 14 |

Source: NDAWN, 2016.
*E = estimated value

Long-term moderate freezing temperature or short-term low freezing temperature (-8°C to -10°C) may cause severe cell dehydration and the cell can collapse. Osmoprotectants can mimic water or reduce the water potential which is essentially reduce the tendency of water to exit the cell (McNeil *et al*., 1999). The extracellular water potential drops due to ice formation.

The amount and type of damage depends on the temperature and the rate of freezing and the rate of thawing. Ice crystals form and punch the cell when freezing is rapid. Mucilage (anti-freeze) proteins can be exported outside of the cell and can break up or slow down the ice formation (Goldstein and Nobel, 1991). When the temperature is -12°C or below, a phenomenon called energy of adhesion occurs. This happens when the ice adheres to the cell and tears it as the cell dehydrates and collapses away from the cell wall. Mucilage and osmoprotectants are good for winter freezing and in some cases for long-term freezing, respectively.

A phase change inside the phospholipid bilayer is the lamellar to hexagonal-II (hex-II) transition (Quinn, 1985). The polar heads of the lipid molecules essentially reorient towards each other when the dehydration is intense enough to result in loss of the lipid bilayer. This lipid conformation change is known as the hex-II and causes plant death. Likewise, severe dehydration that is not severe enough to result in hex II lipid conformation changes may still cause the hydrophilic heads of the lipid bilayers to reorient and fold back on itself to form multilamellar folds in the membrane.

Cold acclimation can affect the cells respond to freezing temperatures. Unacclimated cells react differently to cold temperatures than acclimated cells (Steponkus, 1984). Acclimated cells have blebs that form when the water leaves the cell. Thus, the membrane retains its surface area and when water reenters, the cell membrane can easily expand to its original size and shape. Unacclimated cell membranes fold in and can stick to themselves or form intra cellular vesicles. When the water rushes back in, the cell can pop if the vesicles cannot be reincorporated quickly enough into the cell membrane or if the folds cannot disassociate readily. Some plants, like many fruit tree species, avoid both stresses by producing molecules that allow the available water to supercool. Some species have been shown to supercool to as cold as -40°C. However, if

supercooling is lost and ice nucleation occurs, ice crystal growth is often exceedingly energetic and almost always results in significant cellular damage.

Cold acclimation occurs through changes in gene expression. Signals, possibly generated directly or indirectly by lipid phase changes- though the exact temperature sensing mechanisms are unknown, initiate changes in gene expression that result in cold acclimation. However, it is known that the sumoylation of ICE1 can occur when the temperature drops. ICE1 binds to and induces the *CBF* promotor resulting in expression this class of transcription factors. These CBF transcription factors and other transcription factors such as abscisic acid responsive element binding factors bind to and activate activates the *COR* genes (Chinnusamy *et al*., 2003).

The phospholipid bilayer needs water to maintain its shape and function. Water is needed for the phospholipid bilayer, but during cold stress the water go away. Some *COR* gene encoded proteins can mimic the water. Osmoprotectants such as proline and sugars can also mimic the water in the phospholipid bilayer (McNeil *et al*., 1999). These will protect the plants from some forms of freezing damage that result from cellular dehydration caused by intracellular water that can be lost as the water moves from inside the cell to the growing crystals of extracellular ice.

**1.10. Electrolyte leakage**

Multiple types of tests can be used to test frost or freezing tolerance in plants. One of those tests is electrolyte leakage which is a result of membrane damage. Electrolyte leakage can occur when plants are exposed to freezing temperatures and the membranes are damaged or broken. It can be used to estimate the water stress induced cell membrane damage in plants (Bajji *et al*., 2001). Electrolyte leakage measurements have been found to be correlated with many other physiological and biochemical stress related functions. This method can be used to identify resistant cultivars to certain stresses (Leopold *et al*., 1981; Stevanoic *et al*., 1997; Bajji *et al*.,

2001). This is a quick test that can be conducted in a laboratory with a small amount of leaf tissue (Murray *et al*., 1989).

**1.11. Genome-wide association scan**

Genome-wide association scan (GWAS) is used for many mapping projects. Linkage disequilibrium (LD) is the measurement of the degree of non-random association between traits and alleles at different loci which is considered in GWAS studies (Zhu *et al*., 2008). Genome-wide association scan can identify common alleles in a diverse population with historical recombination events. The alleles identified for certain traits can be used for introgression of genes into cultivated lines.

Linkage disequilibrium is the correlation between the values of alleles at two loci (Flint-Garcia *et al*., 2003). LD can be caused by a shared history and recombination within the population. However, a population will be in linkage equilibrium if a large random-mating population with independently segregating loci that does not have any selection, mutation, or migration (Falconer and Mackey, 1996; Flint-Garcia *et al*., 2003). Different crops have different values for LD which can vary from longer stretches to shorter stretches. In *Arabidopsis,* the stretches of LD have been found to be greater than 50 cM (Nordborg *et al*., 2002: Kraakman *et al*., 2004). However, this was found in populations that were inbred and only had a few genetic parents. A common measure of LD is $r^2$ which is used to quantify and compare LD. This is the squared correlation coefficient between pairs of biallelic markers (Pritchard and Przeworski, 2001; Kraakman *et al*., 2004). The $r^2$ value is calculated between all pairs of loci and is plotted against either genetic distance (cM) or physical distance (kb). This determines the LD decay of a population. Barley, an inbred, has higher LD than sugar beet and corn.

Linkage and LD can be confused, but they are different concepts. Linkage refers to the inheritance of loci that have a physical connection or are located close to each other on a chromosome, while LD is the correlation between alleles in a population (Flint-Garcia *et al*. 2003).

Usually, the germplasm used for GWAS have a large recombination history that originated from multiple rounds of recombination throughout the germplasm history. Germplasm improvement and trait security can be enhanced by GWAS. This is accomplished by identifying QTL in natural populations and diverse individuals (Zhu *et al*., 2008). Linkage mapping, in comparison, only looks at alleles from two parents (Remington *et al*., 2001; Kraakman *et al*., 2004).

Genome-wide association scan (GWAS) is a powerful method, has high resolution, and is good for complex traits (Yu and Buckler, 2006; Sun *et al*., 2014). It is an alternative to traditional biparental mapping or QTL mapping (Sun *et al*., 2014). GWAS examines genetic variation in the whole genome to find association signals for quantitative traits (Risch and Merikangas, 1996; Zhu *et al*., 2008). The repeatability of the experiment is increased by association mapping (AM), where the repeatability in linkage mapping is not always possible. The populations must be in the same linkage phase and be polymorphic for repeatability in linkage mapping, which is not always the case. The goal of AM is to identify consistent marker-QTL associations across all populations now and in the future (Lamkey *et al*., 2013).

Phenotyping and genotyping are the two main components in AM study. Due to strong competition among commercial companies, the cost of genotyping is getting cheaper. However, the cost of phenotyping remains expensive, because it is a longer process that requires multiple replicates and locations or years for better phenotyping. Accurate phenotyping can be difficult in

some situations as genotype by environment interactions can play a role in the performance of specific trait for various genotypes.

Genome-wide association scan analysis may need to account for population structure and/or relatedness (Ghavami *et al*., 2011). Different populations require different analysis. Population structure or relatedness can change the associations found between the phenotype and genotype. Stich *et al*. (2008) looked at various methods for AM that accounted for structure, relatedness, or both to find what works better for different populations. Populations that have structure, relatedness, or both will have false-positives if the population is not dealt with correctly. Many association studies test multiple models to find the best model. The models typically include a model that does not account for anything, models that account for structure, models that account for relatedness, and models that account for both structure and relatedness. The best model is chosen and used for further analysis and false-positive associations can be eliminated by using the correct model.

Genome-wide association scan studies have been conducted in many crops including canola (*Brassica napus* L.), soybean (*Glycine max* L.), wheat (*Triticum aestivum* L.), and many others. The studies evaluated yield, agronomy, seed quality, and disease resistant related traits. GWAS studies have been conducted in *B. napus* using 49 genetically diverse winter-type germplasm for seed phenolic compounds (Rezaeizad *et al*., 2011), 89 winter-type accessions for 6 seed quality traits (Gajardo *et al*., 2015), 139 spring-, semi-winter- and winter-type germplasm for blackleg disease (Rahman *et al*., 2016), 143 spring-, semi-winter- and winter-type accessions on branch angle (Liu *et al*., 2016), 248 winter-type germplasm for seed germination and early vigor (Hatzig *et al*., 2015), 405 accessions of winter oilseed, winter fodder, swede, semi-winter, spring, spring fodder, and vegetable types for agronomic and seed quality traits (Körber *et al*.,

2016), and 540 accessions of winter-, semi-winter-, spring- and vegetable-type for fatty acid profile (Qu *et al*., 2017). Many others studies have also been conducted and published in peer-reviewed journals.

Genome-wide association study of frost tolerance has not been conducted before in rapeseed/canola. However, this study was conducted in other crops. Visioni *et al*. (2013) conducted GWAS for frost tolerance in barley and identified two significant markers linked to the trait. Revilla *et al*. (2016) evaluated frost tolerance in maize (*Zea mays* L.) inbreds and identified a few QTL associated with the cold tolerant trait. Three frost tolerant QTL have been identified in barley significantly associated with previously known cold tolerant areas (Visioni *et al*., 2013). Strigens *et al*. (2013) conducted a chilling tolerance study in maize, and 19 highly significant association signals have been identified. Another GWAS was conducted to evaluate the frost tolerance in winter faba bean (*Vicia faba* L.) (Sallam *et al*., 2016). The freezing tolerance genes identified in faba bean were also identified in other crops such as cereals and legumes (Maqbool *et al*., 2009; Link *et al*., 2010; Sallam *et al*., 2016). Genes associated with frost tolerance that are identified in multiple crops can be useful for practical plant breeding research and can be used for future research.

**1.12. Objectives**

The objectives of this research are:

1. To develop a protocol for frost tolerance screening in controlled conditions.
2. To identify markers/QTL associated with freezing tolerance in *B. napus* under controlled conditions.
3. To identify markers/QTL associated with freezing tolerance in *B. napus* under field conditions.

4. To develop a protocol for electrolyte leakage measurement from leaf tissue.

5. To identify genomic region associated with electrolyte leakage of *B. napus*.

### 1.13. Literature cited


Ananga, A.O., E.C. Cebert, J.W. Ochieng, S. Kumar, D. Kambiranda, H. Vasanthaiah, V. Tsolova, Z. Senwo, K. Konan, and F.A. Anike. p. 1-32. *In* U.G. Akpan (ed.) Oilseeds. InTech, Rijeka, Croatia.

Bajji, M., J.-M. Kinet, and S. Lutts. 2001. The use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in Durum wheat. Plant Growth Regulation. 00:1-10.

Beare, J.L., J.A. Campbell, C.G. Youngs, and B.M. Craig. 1963. Effects of saturated fat in rats fed rapeseed oil. Can. J. of Biochem. And Phys. 41:605-612.

Brown, J., J.B. Davis, M. Lauver, and D. Wysocki. 2008. US Canola Association Canola Growers' Manual.

Chalhoub, B., F. Denoeud, S. Liu, I.A.P Parkin, H. Tang, X. Wang, J. Chiquet, H. Belcram, C. Tong, B. Samans, M. Correa, C. Da Silva, J. Just, C. Falentin, C.S. Koh, I. Le Clainche, M. Bernard, P. Bento, B. Noel, K. Labadie, A. Alberti, M. Charles, D. Arnaud, H. Guo, C. Daviaud, S. Alamery, K. Jabbari, M. Zhao, P.P. Edger, H. Chelaifa, D. Tack, G. Lassalle, I. Mestiri, N. Schnel, M.-C. Le Paslier, G. Fan, V. Renault, P.E. Bayer, A.A. Golicz, S. Manoli, T.-H. Lee, V.H.D. Thi, S. Chalabi, Q. Hu, C. Fan, R. Tollenacre, Y. Lu, C. Battail, J. Shen, C.H.D. Sidebottom, X. Wang, A. Canaguier, A. Chauveau, A. Berard, G. Deniot, M. Guan, Z. Liu, F. Sun, Y.P. Lim, E. Lyons, C.D. Town, I. Bancroft, X. Wang, J. Meng, J. Ma, J.C. Pires, G.J. King, D. Brunel, R. Delourme, M. Renard, J.-M. Aury, K.L. Adams, J. Batley, R.J. Snowden, J. Tost, D. Edwards, Y. Zhou, W. Hua, A.G. Sharpe, A.H. Paterson, C. Guan, P. Wincker. 2014. Early allopolyploid evolution in the post-Neolithic *Brassica napus* oilseed genome. Science. 345(6199):950-953.

Cheng, F., J. Wu, and X. Wang. 2014. Genome triplication drove the diversification of *Brassica* plants. Hort. Res.1. doi:10.1038/hortes.2014.24.

Chinnusamy, V., M. Ohta, S. Kanrar, B.H. Lee, X. Hong, M. Agarwal, and J.-K. Zhu. 2003. ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. Genes and Devel. 17:1043-1054.

Colton, B., and Potter T. 1999. History. Chapter 1. *In*: Canola in Australia: The first thirty years. P.A. Salisbury, T. Potter, G. McDonald, AG Green, eds. p. 1-4.

Falconer and Mackey. 1996. Introduction to Quantitative Genetics. Pearson Prentice Hall. New York.

Flint-Garcia, S.A., J.M. Thornsberry, and E.S. Buckler IV. 2003. Structure of Linkage disequilibrium in plants. Annu. Rev. Plant. Biol. 54:357-74.

(FAOSTAT) Food and Agriculture Organization of the United Nations. 2017. Accessed: http://www.fao.org/faostat/en/#home (24 April 2017).

Gajardo, H.A., B. Wittkop, B. Soto-Cerda, E.E. Higgins, I.A.P. Parkin, R.J. Snowdon, M.L. Federico, and F.L. Iniguez-Luy. 2015. Association mapping of seed quality traits in *Brassica napus* L. using GWAS and candidate QTL approaches. Mol. Breeding. 35:143.

Ghavami, F., E.M. Elias, S. Mamidi, O. Ansari, M. Sargolzaei, T. Adhikari, M. Mergoum, and S.F. Kianian. 2011. Mixed model association mapping for Fusarium head blight resistance in Tunisian-derived Durum wheat populations. Genes Genomes Genetics. 1:209-218.

Goldstein, G. and P.S. Nobel. 1991. Changes in osmotic pressure and mucilage during low temperature acclimation of *Opuntia ficus-indica*. Plant Physiol. 97:954-961.

Gomez-Campo, C., and Prakash, S. 1999. Origin and domestication. *In* C. Gomez-Champo (ed.) Biology of Brassica cenospecies. Elsevier Publishers, Amsterdam, Netherlands.

Guo, X.W., W.G.D. Fernando, and M. Entz. 2005. Effects of crop rotation and tillage on blackleg disease of canola. Can. J. Plant Pathol. 27:53-57.

Gupta, S.K. and A. Pratap. 2007. History, Origin, and Evolution. Advances in Botanical Res. 45:1-20.

Hanson, M., A.M. Laustsen, C.E. Olsen, L. Poll, and H. Sorensen.1997. Chemical and sensory quality of broccoli (*Brassica oleracea* L. var *Italica*). J. of Food Quality. 20:441-459.

Hatzig, S.V., M. Frisch, F. Breuer, N. Nesi, S. Ducournau, M.-H. Wagner, G. Leckband, A. Abbadi, and R.J. Snowdon. 2015. Genome-wide association mapping unravels the genetic control of seed germination and vigor in *Brassica napus*. Front. Plant Sci. 6(221):1-13. doi: 10.3389/fpls.2015.00221.

Hinata, K. and S. Prakash. 1984. Ethnobotany and evolutionary origin of Indian oleiferous Brassicae. Indian J. of Genet. 44:102-112.

Hind-Lanoiselet, T, and F. Lewington. 2004. Canola concepts: managing sclerotinia. http://www.australianoilseeds.com/__data/assets/pdf_file/0011/4430/sclerotinia_in_canola.pdf (accessed 18 July 2016).

Kraakman, A.T.W., R.E. Niks, P.M. Van den Berg, P. Stam, and F.A.Van Eeuwijk. 2004. Linkage disequilibrium mapping of yield and yield stability in modern spring barley cultivars. Genetics 168: 435-446.

Körber, N., A. Bus, J. Li, I.A.P. Parkin, B. Wittkop, R.J. Snowdon, and B. Stich. 2016. Agronomic and seed quality traits dissected by genome-wide association mapping in *Brassica napus*. Frontiers in Plant Sci. 7:386. doi:10.3389/fpls.2016.00386.

Lamkey, C.M., T.C. Helms, and R.J. Goos. 2013. Marker-assisted versus phenotypic selection for iron-deficiency chlorosis in soybean. Euphytica: doi: 10.1007/s10681-013-0961-8.

Leopold, A.C., M.E. Musgrave, and K.M. Williams. 1981. Solute leakage resulting from leaf dessication. Plant Physiol. 68:1222-1225.

Link, W., C. Balko, and F.L. Stoddard. 2010. Winter hardiness in faba bean: physiology and breeding. F. Crop Res. 115:287-296. doi:10.1016/j.fcr.2008.08.004.

Liu, J., W. Wang, D. Mei, H. Wang, L. Fu, D. Liu, Y. Li, and Q. Hu. 2016. Characterizing variation of branch angle and genome-wide association mapping in rapeseed (*Brassica napus* L.). Front Plant Sci 7:21.

Lyons, J.M. and J.K. Raison. 1970. Oxidative activity of mitochondria isolated from plant tissues sensitive and resistant to chilling injury. Plant Physiol. 45:386-389.

Lyons, J.M., J.K. Raison, and P.L. Steponkus. 1979. The Plant Membrane in Response to Low Temperature: An overview. Pg. 1-24. *In* Low Temperature Stress in Crop Plants: the role of the membrane. Lyons, J.M., D. Graham, and J.K. Raison. Academic Press, New York.

Maqbool, A., S. Shafiq, and L. Lake. 2009. Radiant frost tolerance in pulse crops- a review. Euphytica. 172:1-12. doi:10.1007/s10681-009-0031-4.

McNeil, S.D., M.L. Nuccio, and A.D. Hanson. 1999. Betaines and related osmoprotectants. Targets for metabolic engineering of stress resistance. Plant Physiol. 120:945-949.

Murray, M.B., J.N. Cape, and D. Fowler. 1989. Quantification of frost damage in plant tissues by rates of electrolyte leakage. New Phytol. 113: 307-311.

Nah, T., S.H. Kessler, K.E. Daumit, J.H. Kroll, S.R. Leone, K.R. Wilson. 2013. OH-initiated oxidation of sub-micron unsaturated fatty acid particles. Phys. Chem. Chem. Phys. 15:18649-18663.

Nishi, S. 1980. Differentiation of *Brassica* crops in Asia and the breeding of "Hakuran", a newly synthesized leafy vegetable. p.133-150. *In* S. Tsunoda, K. Hinata, and C. Gomez-Chapo (eds.) *Brassica* crops and wild allies, biology and breeding. Japan Sci. Soc. Press, Tokyo, Japan.

Nordborg, M., J.O. Borevitz, J. Bergelson, and C.C. Berry, J. Chory, J. Hagenblad, M. Kreitman, J.N. Maloof, T. Noyes, P.J. Oefner, E.A. Stahl, and D. Weigel. 2002. The extent of linkage disequilibrium in *Arabidopsis thaliana*. Nature Genet. 30:190-193.

North Dakota Agricultural Weather Network (NDAWN). 2016. Accessed: https://ndawn.ndsu.nodak.edu/station-info.html?station=34 (3 Aug. 2016).

Olien, C.R. and M.N. Smith. 1977. Ice adhesions in relation to freeze stress. Plant Physiol. 60:499-503.

Olsson, G. 1960. Species crosses within the genus Brassica II. Artificial *Brassica napus* L. *Hereditas*. 46:351-386.

Oomah, B.D. and G. Mazza. 1999. Health benefits of phytochemicals from selected Canadian crops. Trends in Food Sci. and Technol. 10:193-198.

Pritchard, J.K. and M. Przeworski. 2001.Linkage disequilibrium in humans: models and data. Am. J. Hum. Genet. 69:1-14.

Qu, C., L. Jia, F. Fu, H. Zhao, K. Lu, L. Wei, X. Xu, Y. Liang, S. Li, R. Wang, and J. Li. 2017. Genome-wide association mapping and identification of candidate for fatty acid composition in *Brassica napus* L. using SNP markers. BMC Genomics. 18:232. doi:10.1186/s12864-017-3607-8.

Quinn, P.J. 1985. A lipid-phase separation model of low-temperature damage to biological membranes. Cryobiology. 22:128-146.

Rahman, M., S. Mamidi, L. del Rio, A. Ross, M.M. Kadir, M.M. Rahaman, and M. Arifuzzaman. 2016. Association mapping in *Brassica napus* (L.) accessions identifies a major QTL for blackleg disease resistance on chromosome A01. Mol. Breeding. 36:90. doi:10.1007/s11032-016-0513-8.

Rakow, G.F.W. 2004. Species, origin and economic importance of Brassica. *In* by E.C. Pua and C.J. Doulas (eds.) Brassica. Springer, Berlin.

Raymer, P.L. 2002. Canola: An emerging oilseed crop. p. 122-126. *In* J. Janick and A. Whipkey (eds.) Trends in new crops and new uses. ASHS Press, Alexandria, VA.

Remington, D.L., J.M. Thornsberry, Y. Matsuoka, L.M. Wilson, S.R. Whitt, J. Doebley, S. Kresovich, M.M. Goodman, I.V. Buckler. 2001. Structure of linkage disequilibrium and phenotypic associations in the maize genome. Proc. Natl. Acad. Sci. U.S.A. 98: 11479-11484.

Revilla, P., V.M. Rodríguez, A. Ordás, R. Rincent, A. Charcosset, C. Giauffret, A.E. Melchinger, C.-C. Schön, E. Bauer, T. Altmann, D. Brunel, J. Moreno-González, L. Campo, M. Ouzunova, A. Alvarez, J.I.R. de Galarreta, J. Laborde, and R.A. Malvar. 2016. Association mapping for cold tolerance in two large maize inbred panels. BMC Plant Bio. 16:127. doi:10.1186/s12870-016-0816-2.

Rezaeizad, A., B. Wittkop, R. Snowdon, M. Hasan, V. Mohammadi, A. Zali, and W. Friedt. 2011. Identification of QTLs for phenolic compounds in oilseed rape (*Brassica napus* L.) by association mapping using SSR markers. Euphytica. 177(3):335-342.

Risch, N. and K. Merikangas. 1996. The future of genetic studies of complex human diseases. Science 273:1516-1517.

Sallam, A., M. Arbaoui, M. El-Esawi, N. Abshire, and R. Martsch. 2016. Identification and verification of QTL associated with frost tolerance using linkage mapping and GWAS in winter faba bean. Frontiers in Plant Sci. 7:1098. doi: 10.3389/fpls.2016.01098.

Snogerup, S. 1980. The wild forms of the *Brassica oleracea* group (2n=18) and their possible relations to the cultivated ones. p. 121-132. *In* S. Tsunoda, K. Hinata, and C. Gomez-Campo (eds.) *Brassica* crops and wild allies, biology and breeding. Japan Sci. Soc. Press, Tokyo, Japan.

Stefansson, B.R. and Z.P. Kondra. 1975.Tower Summer Rape. Can. J. Plant Sci. 55:343-344.

Steponkus, P.L. 1984. Role of the plasma membrane in freezing injury and cold acclimation. Annu. Rev. Plant Physiol. 35:543-584.

Stevanoic, B., J. Sinzar, and O. Gilsic. 1997. Electrolyte leakage differences between poikilohydrous and homoiohydrous species of Gesneriaceae. Biol. Plant. 40:299-303.

Stich, B., J. Möhring, H.-P. Piepo, M. Heckenberger, E.S. Buckler, and A.E. Melchinger. 2008. Comparison of Mixed-model approaches for association mapping. Genetics. 178:1745-1754.

Strigens, A., N.M Freitag, X. Gilbert, C. Grieder, C. Riedelsheimer, T.A. Schrag, R. Messmer, and A.E. Melchinger. 2013. Association mapping for chilling tolerance in elite flint and dent maize inbred lines evaluated in growth chamber and field experiments. Plant, Cell, and Env. 36:1871-1887.

Tewari, J.P., S.E. Strelkov, D. Orchard, M. Hartman, R.M. Lange, and T.K. Turkington. 2004. Identification of clubroot of crucifers on canola (*Brassica napus*) in Alberta. Disease report.

Thomashow, M.F. 1999. Plant cold acclimation: Freezing Tolerance Genes and Regulatory Mechanisms. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50:571–99.

Tsunoda, S. 1980. Eco-physiology of wild and cultivated forms in *Brassica* and allied genera. p. 109-120. *In* S. Tsunoda, K. Hinata, and C. Gomez-Champo (eds.) *Brassica* crops and wild allies, biology and breeding. Japan Sci. Soc. Press, Tokyo, Japan.

U, N. 1935. Genome analysis in *Brassica* with special reference to the experimental formation of *Brassica napus* and peculiar mode of fertilization. Japanese J. Botany. 7:389-452.

(USDA) United States Department of Agriculture. 2015. Economic Research Service (ERS). Oil crops yearbook. Beltsville, MD. http://www.ers.usda.gov/data-products/oil-crops-yearbook.aspx.

(USDA) United States Department of Agriculture. 2016. Foreign Agricultural Service. Accessed: https://www.fas.usda.gov/ (24 April 2017).

(USDA) United States Department of Agriculture. 2017. National Agricultural Statistics Service. Accessed: https://www.nass.usda.gov/ (24 April 2017).

(USDA) United States Department of Agriculture. Updated 2016. Economic Research Service (ERS). Soybeans and oil crops. Accessed: http://www.ers.usda.gov/topics/crops/soybeans-oil-crops/canola.aspx#canolaprod (16 May 2016).

van Swaau, A.C., E. Jacobsen, and W.J. Feenstra. 1985. Effect of cold hardening, wilting, and exogenously applied proline on leaf proline content and frost tolerance of several genotypes of *Solanum*. Physiol. Plant. 64:230-236.

Visioni, A., A. Tondelli, E. Francia, A. Pswarayi, M. Malosetti, J. Russell, W. Thomas, R. Waugh, N. Pecchiono, I. Romagosa, and J. Comadran. 2013. Genome-wide association mapping of frost tolerance in barley (*Hordeum vulgare* L.). BMC Genomics. 14:424.

Wade, N.L., R.W. Breidenbach, J.M. Lyons, and A.L. Keith. 1974. Temperature-induced phase changes in the membranes of glyoxysomes, mitochondria, and proplastids from germinating castor bean endosperm. Plant Physiol. 54:320-323.

Wilson, J.M. 1997. Mechanisms of chilling resistance in plants. p. 111-122. *In* Basra, A.S. and R.K. Basra (ed.) Mechanisms of environmental stress resistance in plants. Harwood Academic Publishers, Amsterdam, The Netherlands.

Yu, J.M. and E.S. Buckler. 2006. Genetic association mapping and genome organization of maize. Curr. Opin. Biotech. 17:155-160.

Zhu, C., G. Michael, S.B. Edward, and J. Yu. 2008. Status and prospects of association mapping in plants. The Plant Genome 1: 5-20.

# CHAPTER 2. DEVELOPMENT OF A PROTOCOL FOR FROST-TOLERANCE EVALUATION IN RAPESEED/CANOLA (*BRASSICA NAPUS* L.)

## 2.1. Abstract

Spring frost can severely damage or even kill rapeseed/canola (*Brassica napus* L.) seedlings. A protocol for large scale screening of rapeseed germplasm under frost-simulating conditions has not yet been developed. Accordingly, the present study was conducted to develop a protocol for screening rapeseed germplasm under artificial frost-simulation conditions in a plant growth chamber and in a greenhouse. Nine rapeseed varieties, including three commercial hybrids, three spring types, and three winter types were used. Cold acclimation at 4°C was applied for 0, 7, or 14 days to 14 day old seedlings. The seedlings were treated with four freezing temperatures (−4°C, −8°C, −12°C, or −16°C). The length of the freezing period was 16 h, including the ramping of temperature down from 4°C and up from the respective freezing temperature to 4°C. Plants were allowed to recover at 4°C for 24 h before they were moved back to the greenhouse. Frost damage was scored on a 0–5 scale, where 0 denotes completely dead and 5 denotes no damage. Seedling survival from the freezing treatment increased from the non-acclimation to the cold acclimation treatment. However, no significant differences ($P < 0.05$) were found between 7 and 14 days of acclimation. Frost treatment at −4°C resulted in significant differences in seedling damage relative to the other three temperatures, with the −16°C treatment resulting in the highest overall seedling damage. Significant differences were found between the spring type and the other two types (hybrid and winter). However, no significant differences were found between the hybrid and winter types. The suggested protocol for the assessment of frost tolerance is acclimation of two-week old seedlings for 7 days at 4°C followed by frost treatment at −4°C for 16 h.

**Keywords:** *Brassica napus*, frost, protocol

## 2.2. Introduction

Rapeseed/canola (*Brassica napus* L.) is an important crop for the U.S. state of North Dakota (ND), which produces about 84% of the U.S. crop. It is grown primarily in the northeast and north central parts of the state. Canola is considered to be a healthy oil for human consumption compared to other vegetable oils because of favorable combinations of the essential fatty acids in seeds (Oomah and Mazza, 1999).

Frost susceptibility is an abiotic stress that impairs plant growth and crop production (Chinnusamy *et al.*, 2007). Frost at the seedling stage of rapeseed can be harmful and may destroy the whole crop. The frost-free date in North Dakota is generally considered to be in May, but the date can vary from northern to southern regions of the state and from year to year. Given that canola is grown in the northern part of the state, the frost-free period tends to start later. The average air temperatures for Langdon, ND in April and May are 4ºC and 11ºC, respectively (NDAWN, 2014). However, the minimum temperatures during the same time period are –2ºC and 4ºC, respectively. The severity of frost injury depends on moisture conditions, plant growth stage, cold severity, duration of cold temperatures, and other factors.

Canola seedlings are not affected by a light spring frost that causes leaf wilting but not browning. Frost damage can be seen on leaves and symptoms can include wilting, bleaching, or in extreme cases, plant death. Bleaching occurs due to phyto-oxidation of pigments in leaves (Wilson, 1997). Wilting is caused by a loss of water from cells. Resistance to chilling by frost is complex and may be difficult to incorporate. Canola growers usually look for blackened cotyledons and/or leaves as an indicator of frost damage necessitating replanting. It is necessary to wait 5–10 days to confirm whether the plants are recovering by generating green shoots at the growing point of apical meristems in the center of the frozen leaf rosette. Canola is more susceptible to frost at the cotyledon stage than at the three- to four-leaf stage. When early spring-

seeded canola is exposed to cold temperatures, the defense mechanism allows the plant to withstand cold temperature via gradual hardening of plant tissue. Slow-growing seedlings are harder and less susceptible to cold than rapidly growing seedlings. In spring canola, the process of unhardening the plants to initiate active growth is rapid (Sovero, 1993). Usually, winter type canola is capable of hardening faster, can tolerate cold temperatures for a longer time, and is unhardened slower, reducing frost damage (Ananga *et al*., 2012). However, variation in frost hardiness is also available within winter- and spring-type germplasm.

Identifying frost tolerance in canola would be beneficial for growers, especially in North Dakota, but also in other places where early planting poses the threat of frost damage. Screens for frost tolerance in canola using artificial growing conditions have not been established. Field testing of frost tolerance relies heavily on weather conditions each year and these cannot be predicted. Thus, screening for frost tolerance under controlled environmental conditions may help to identify frost-tolerant germplasm and can also be performed multiple times in a year, increasing screening capacity over that by field testing.

Canola displays different growth habits. The winter type is grown mainly in Western Europe and part of the USA. Vernalization is required for flowering of winter-type rapeseed. The spring type is grown in Canada, USA, Australia, India, Eastern Europe, and other countries. China grows mainly a semi-winter type. Due to its severe winters, North Dakota grows only spring-type canola.

This study aimed to identify a protocol for screening frost tolerance in canola under artificial frost-simulation conditions.

## 2.3. Materials and methods

### 2.3.1. Plant materials

Nine canola varieties chosen from two growth types, were planted in a randomized complete block design (RCBD) with three replicates and eight plants per line per replicate were grown in the greenhouse for 14 days at 20ºC. The photoperiod was 16 h of light and 8 h of dark and the average humidity was 47%. The varieties grown included three commercial hybrids (DKL 70-07, Pioneer 45H26, and Sprinter), three spring lines (NDSU 15-1000, Hi-Q, and Kanada), and three winter lines (Fashion, ARC 2180-1, and Galileo). The hybrids are commercial varieties commonly grown in North Dakota and were chosen for this reason. The winter and spring type varieties are commonly used in the North Dakota State University canola breeding program. The varieties represented two growth habit types and are commonly used in the breeding program, therefore, we chose these winter- and spring-type varieties for this study.

### 2.3.2. Experimental design

After 14 days of growth, plants were moved to the plant growth chamber for acclimation at 4ºC with a 12-h photoperiod provided by GE Ecolux F32T8 SP35 Eco (32 W T8) style bulbs (General Electric Company). Three acclimation times (0, 7, and 14 days) were used. A total of 162 seedlings (9 varieties × 6 seedlings/variety × 3 acclimation times) per replication per freezing treatment were used. Seedlings were fertilized with 20-20-20 water-soluble fertilizer prior to cold acclimation.

An ESPEC BTU-433 freezing chamber (ESPEC North America, Inc.) was used for frost simulation. Four freezing temperatures were tested: –4ºC, –8ºC, –12ºC, and –16ºC. The total time for frost simulation was 16 h, including the lowering and raising of the temperature from and to 4ºC, along with holding at the minimum temperature. Sixteen h of treatment was chosen, based on overnight freezing temperatures in North Dakota.

In the –4ºC treatment, the temperature started at 4ºC and was lowered at –2ºC $h^{-1}$ over 4 h to reach the treatment temperature. The seedlings were kept at –4ºC for 8 h. The temperature was raised again to 4ºC at a rate of 2ºC $h^{-1}$, requiring another 4 h. In the –8ºC treatment, the temperature started at 4ºC and was lowered at –2ºC $h^{-1}$ for 6 h to reach the treatment temperature. The seedlings were kept at –8ºC for 4 h. The temperature was raised again to 4ºC at 2ºC $h^{-1}$ over another 6 h. In the –12ºC treatment, the temperature started at 4ºC and was lowered at –3ºC $h^{-1}$ over 5.33 h to reach the treatment temperature. The seedlings were kept at –12ºC for 5.34 h and the temperature was again raised to 4ºC at 3ºC $h^{-1}$ over another 5.33 h. Finally, in the –16ºC treatment, the temperature started at 4ºC and was lowered at –3ºC $h^{-1}$ for 6.66 h to reach the treatment temperature. The seedlings were kept at –16ºC for 2.67 h. The temperature was again raised to 4ºC at 3ºC $h^{-1}$ over another 6.66 h (Table 2.1).

Table 2.1. Treatment times in the freezing chamber.

| Treatment (ºC) | Starting temp (ºC) | Temp ramp-down rate (ºC $h^{-1}$) | Time required to reach the treatment temp (h) | Treatment length (h) | Temp ramp-up rate (ºC $h^{-1}$) | Time required to reach at 4 ºC (h) |
|---|---|---|---|---|---|---|
| –4 | 4 | –2 | 4 .00 | 8 .00 | +2 | 4 .00 |
| –8 | 4 | –2 | 6.00 | 4.00 | +2 | 6 .00 |
| –12 | 4 | –3 | 5.33 | 5.34 | +3 | 5.33 |
| –16 | 4 | –3 | 6.66 | 2.67 | +3 | 6.66 |

After frost simulation, seedlings were placed in the growth chamber at 4ºC for 24 h before being moved back to the greenhouse for scoring seedling damage and evaluations. Scoring was performed every three days starting three days after the frost treatment. Each plant was scored individually using a 0 to 5 scale, where 0 denoted dead, 5 denoted no damage, and scores of 1–4 were based on visual estimation of frost damage. A 1 had a little green, typically the growing point but usually would not continue to grow. A 4 had minor damage to the

cotyledons. Score of 2 and 3 had various degrees of damage to the leaves and cotyledons. Notes on general plant color were also taken. The experiment was replicated three times. A total of 1944 seedlings (9 varieties × 6 plants/variety × 3 acclimations × 4 frost treatments × 3 replications) were scored in the greenhouse and in the growth chamber.

### 2.3.3. Statistical analysis

The means of seedling damage from all plants within growth habits (hybrid, spring-type, and winter-type) were used. SAS 9.3 (SAS Institute Inc., USA) was used to calculate the analysis of variance (ANOVA). The analysis was performed for an RCBD and run as a split-split-plot arrangement where A was temperature, B was acclimation time, and C was genotype. LSDs were calculated for significant factors. All data were combined with SAS to conduct this calculation (e.g. N = 36, which is four temperatures × three acclimation times × three genotypes).

## 2.4. Results

The ANOVA indicated that all three factors were significant (Table 2.2). Some of the interactions were also highly significant. These interactions included temperature x time and temperature x genotype. The ANOVA was calculated using all the data from the experiment. LSDs were calculated for the individual factors (A, B, and C). Genotypes showed different reactions across different temperatures and acclimation times.

The means of seedling damage for the frost-simulating temperatures were significantly different (Table 2.3). The warmest temperature (-4°C) had the highest overall mean (3.1963), corresponding to the lowest seedling damage, and the coldest temperature (-16°C) has the lowest overall mean (1.4271), corresponding to the highest seedling damage. Different temperatures affected the canola differently. The coldest temperatures (-12°C and -16°C) caused bleaching

and seedling death, whereas the warmest temperature (-4°C) did not cause as much damage and some seedlings showed no damage.

Table 2.2. ANOVA from plants scored 3 days after frost simulation.

| Source | DF | Sum of Squares | Mean Square | F Value | P-value |
|---|---|---|---|---|---|
| rep | 2 | 4.240 | 2.120 | 91.46 | .0001*** |
| A | 3 | 48.260 | 16.087 | 694.02 | .0001*** |
| rep*A | 6 | 4.437 | 0.739 | 31.90 | .0001*** |
| B | 2 | 38.229 | 19.114 | 824.65 | .0001*** |
| A*B | 6 | 8.220 | 1.370 | 59.11 | .0001*** |
| rep*A*B | 16 | 10.700 | 0.669 | 28.85 | .0001*** |
| C | 2 | 0.245 | 0.122 | 5.28 | 0.0085** |
| A*C | 6 | 0.659 | 0.110 | 4.74 | 0.0007*** |
| B*C | 4 | 0.130 | 0.033 | 1.40 | 0.2466ns |
| A*B*C | 12 | 0.289 | 0.024 | 1.04 | 0.4294ns |

A = freezing temperature; B = acclimation time; C = rapeseed variety
ns, not significant; **, $p < 0.01$; ***, $p < 0.001$

Table 2.3. Effect of different freezing temperatures on seedling damage using $\alpha = 0.05$ and scored 3 days after the frost treatment.

| Temperature (°C) | Mean (seedling damage score) | t grouping* | N |
|---|---|---|---|
| -4 | 3.20 | A | 27 |
| -8 | 2.17 | B | 27 |
| -12 | 1.74 | BC | 27 |
| -16 | 1.43 | C | 27 |

*Means accompanied by the same letter are not significantly different.
LSD = 0.5727

Cold acclimation time had an effect between 0 and 7 days, but no significant differences were observed between acclimation for 7 and 14 days (Table 2.4). Seven or 14 days of acclimation did not change the overall survival of the genotypes. Thus, the optimum acclimation that should be used is 7 days in the growth chamber.

Genotype differences in response to frost were observed. Frost damage to spring genotypes was significantly different from that of hybrid and winter genotypes (Table 2.5). The

observed differences between the genotypes were somewhat expected. The genotypes used in this study were chosen based on spring and winter types.

Table 2.4. Effect on seedling damage of different lengths of frost acclimation periods using α = 0.05 and scored 3 days after frost treatment.

| Acclimation time (days) | Mean (seedling damage score) | t grouping* | N |
|---|---|---|---|
| 14 | 2.71 | A | 36 |
| 7 | 2.37 | A | 36 |
| 0 | 1.31 | B | 36 |

*Means accompanied by the same letter are not significantly different.
LSD = 0.4186

Table 2.5. Response of genotypes on seedling damage using α = 0.05 and scored 3 days after the frost treatment.

| Genotype | Mean (seedling damage score) | t grouping* | N |
|---|---|---|---|
| Hybrid | 2.18 | A | 36 |
| Winter | 2.15 | A | 36 |
| Spring | 2.07 | B | 36 |

*Means accompanied by the same letter are not significantly different.
LSD = 0.0722

Visual differences could be detected on the plants after frost. The plants that underwent frost simulation tended to be darker green and wilted, whereas the control was lighter green and stood upright (Fig. 2.1). The initial frost damage did not imply later visual damage, as plants could still recover the appearance of a healthy non-frost exposed plant.

Scoring of plants was performed in such a way as to avoid bias as much as possible. A score of 5 indicated that the plant had grown past the initial shock and was showing no sign of damage (Fig. 2.2). A score of 0 indicated that the plant was completely dead (Fig. 2.2). Dead plants were usually white in color, a phenotype called bleaching that can occur when cells are ruptured by freezing. A score of 1 meant that the plant was almost dead, a score of 4 meant that the plant had very little damage, and scores of 2 and 3 were estimated on the basis of level of

seedling damage after respective treatments. Visually, differences appeared between acclimation, temperature, and genotype. These differences were confirmed by analysis.


Fig. 2.1. Plants exposed to frost (left) compared with the control plants (right).

**2.5. Discussion**

Frost tolerance of cultivars would allow growers to plant canola earlier with less concern about damage to the crop. Early-planted canola, which usually flowers early and can thereby avoid high temperatures during flowering, could use early-season moisture and better compete with warm-season weeds, resulting in higher seed yield. In North Dakota, early-planted canola often suffers frost damage that severely affects the crop stand. For this reason, it is important for canola growers to have genetically frost-tolerant canola varieties with rapid-germination capacity

that can grow well at low temperature and tolerate early spring freezing and thawing, thereby overcoming problems posed by early spring planting (Ananga et al., 2012).

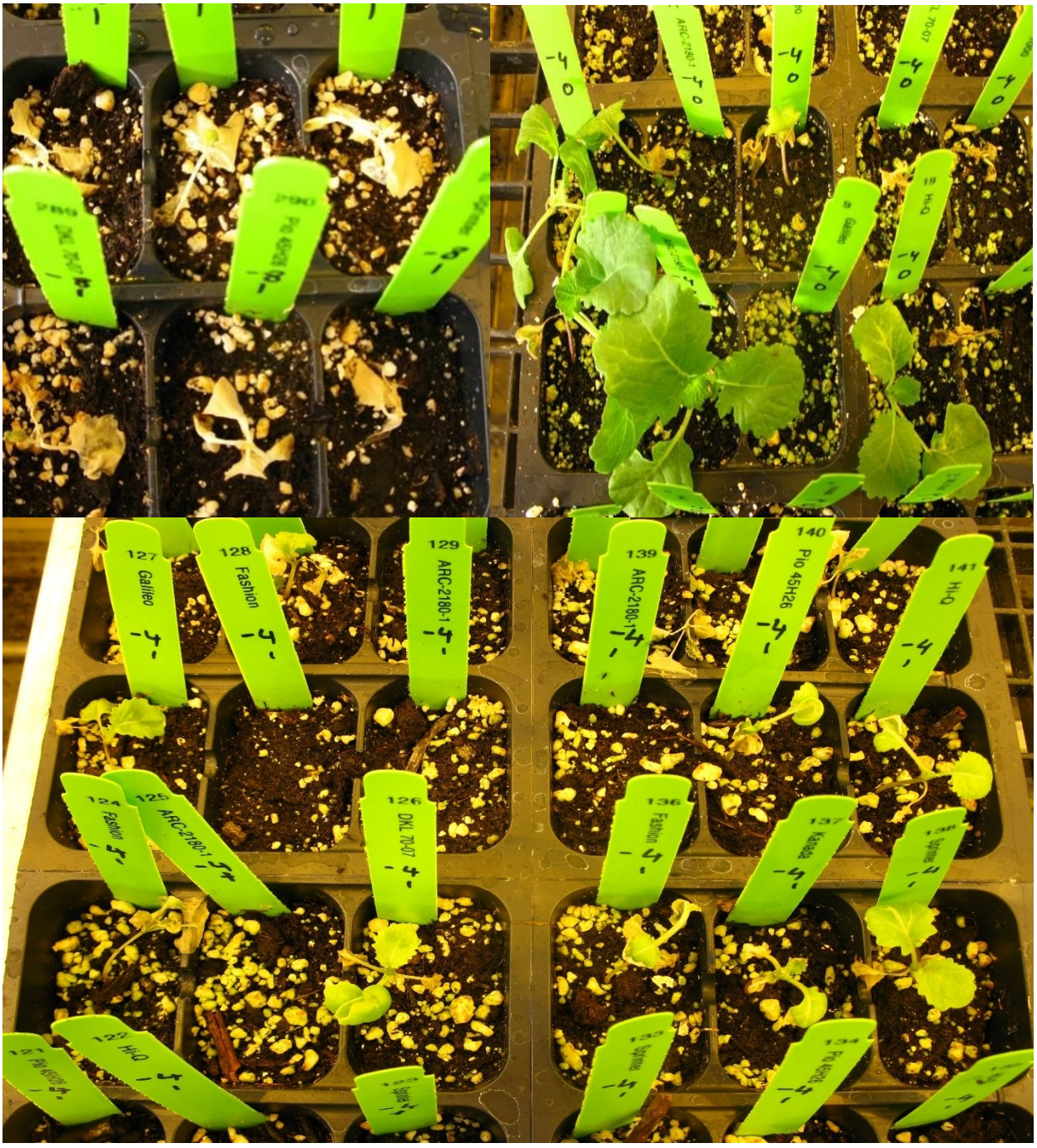


Fig. 2.2. A score of 0; the plant is completely dead and plants are bleached (top left). A score of 5 showing no damage (top right). Scores of 1 – 4 are pictured in the bottom image. A score of 4 has some damage to the cotyledons and a score of 1 is mostly dead. Scores of 2 to 3 have varying amounts of damage to the cotyledons and leaves.

We have developed a protocol for frost treatment in a controlled environment, where the cold acclimation and the freezing temperature can be maintained as required. The naturally acclimated plants tend to experience temperature changes that are more variable. Plants grown in controlled environments are not exposed to this variation. Although naturally acclimated plants may be exposed to cooler temperatures, they are also exposed to varying temperatures, which may affect their frost tolerance. To get the variation of response of seedlings to freezing temperature, it is best to use a controlled environment where the optimum freezing temperature can be simulated to screen germplasm for frost-tolerant cultivar development. Moreover, because we do not have control over the field sites where seedlings often exhibit either complete survival or complete frost kill, it is difficult to screen germplasm under natural conditions. A strong correlation between field survival and growth chamber studies has been reported (Salgado and Rife, 1996).

Plant growth stage is an important criterion for screening frost tolerant germplasm. Plants are more susceptible to freezing at the cotyledon stage. We accordingly used 14 day old seedlings, which were expected to show the most variation in response to different freezing temperatures.

Various methods have been used to screen freezing tolerance in plants, such as plant tissue water content (Brule-Babel and Fowler, 1988), ion leakage from cold-stressed plant cells (Teutonico *et al*., 1993), and changes in luminescence (Brzostowicz and Barcikowska, 1987). A laboratory freezing tolerance screening at the meristem regrowth stage was performed to assess the viability of damaged seedlings (Andrews and Morrison, 1992). These authors used various freezing temperatures to determine whether the plant tissues were alive or dead after exposure to cold temperature. Evaluation of freezing-tolerant plants is usually performed by placing plant

tissue in distilled water and measuring the electrical conductivity of the resulting liquid solution (Murray *et al*., 1989; Madakadze *et al*., 2003). Higher electrolyte loss is an indication of tissue damage by freezing. Another method, chlorophyll fluorescence, can also be used to screen plants for freezing tolerance (Ehlert and Hincha, 1981). However, none of these studies were conducted on seedlings under frost-simulating conditions. We have developed a new method for screening a large number of germplasm entries at the 14 day seedling stage under frost-simulating conditions in a plant growth chamber.

Cold acclimation is one step that is necessary for frost tolerance. Cold acclimation is the process of introducing the plant to cool temperatures to improve their survival at freezing temperatures. Usually, plants are naturally acclimated before exposure to natural freezing temperatures. For this reason, we acclimated seedlings before freezing treatment in the plant growth chamber. Many species show increased frost tolerance when cold acclimation is applied before frost exposure (Steponkus, 1978; Hume and Jackson, 1981). The optimum acclimation procedure must also be used for artificial conditions (Steponkus, 1978). The optimum acclimation procedure can be tested and determined before frost tolerance studies are initiated. Different species may have different optimum acclimation procedures, but a starting point should be established. For this reason, we used different lengths of time for acclimation before frost treatment. Cold acclimation activates cold-induced genes associated with several physiological and biochemical alterations in the plants to protect cell membranes against freezing-induced injury (Thomashow, 1999). Cold acclimation and freezing showed a strong positive correlation in frost tolerance in both winter- and spring-type rapeseed (Rapacz and Markowski, 1999). Our study showed a significant difference between acclimation and non-acclimation to cold temperature before exposure to freezing temperature.

Another factor that may affect frost tolerance is water content in leaves and stems. Higher tissue water content has been shown to be associated with lower hardiness and cold tolerance in plants (Olien, 1967; Metcalf *et al*., 1970; Svec and Hodges, 1972; Pomeroy *et al.*, 1975; Fowler and Carles, 1979; Stout, 1980; Swenson and Murray, 1983). High water content in tissues could decrease the survivability; however, drought-stressed plants should also have decreased survivability. In artificial conditions, the amount of water received by plants can be controlled and a frost tolerance study can be conducted. This is not the case in the field.

The genetic composition of plants plays a vital role in cold tolerance. The aim of the study was to develop a protocol to identify frost-tolerant germplasm in a wide collection of accessions for use in breeding programs. Freezing tolerance of wheat is a genetically complex trait and complementary gene action may be involved in freezing-tolerance genetics (Skinner and Mackey, 2009). However, it may be possible to develop genetically tolerant germplasm for growers (Skinner and Mackey, 2009). Winter survival of barley has been studied and complex inheritance is suspected (Ennus *et al.,* 1962; Skinner and Garland-Campbell, 2008). Different combinations of genes could control winter hardiness in different varieties and winter hardiness is controlled by both recessive and dominant genes (Rhode and Pulham, 1960; Ennus *et al.,* 1962). Accordingly, in this study, representatives of different growth habit types including winter-type, spring-type, and hybrid cultivars were used to reveal genetic variability in the germplasm.

**2.6. Conclusion**

We have developed a protocol for frost tolerance evaluation under controlled environmental conditions. In this protocol, seedlings were grown for 14 days in the greenhouse, acclimated at 4ºC for seven days, exposed to frost at –4ºC, allowed to recover at 4ºC for 24 h, and scored in the greenhouse for frost damage three days after treatment.

## 2.7. Literature cited


Ananga, A.O., E. Cebert, J.W. Ochieng, S. Kumar, D. Kambiranda, H. Vasanthaiah, V. Tsolova, Z. Senwo, K. Konan, and F.N. Anike. 2010. Prospects for transgenic and molecular breeding for cold tolerance in canola (*Brassica napus* L.). pp. 1–32. *In* U.G. Akpan (ed). Oilseeds. InTech, Croatia.

Andrews, J. and J. Morrison. 1992. Freezing and ice tolerance tests for Winter Brassica. Agron. J. 84:960–962.

Brule-Babel, A.L. and D.B. Fowler. Genetic control of cold hardiness and vernalization requirement in winter wheat. 1988. Crop Sci. 28:879–884.

Brzostowicz, A. and V. Barcikowska. Possibility of frost resistance testing of *Brassica napus* with the help of delayed luminescence intensity. 1987. Cruciferae Newsletter. 12:27.

Chinnusamy, V., J.H. Zhu, and J.K. Zhu. 2007. Cold stress regulation of gene expression in plants. Trends Plant Sci. 12:444–451.

Ehlert, B., and D.K. Hincha. 2008. Chlorophyll fluorescence imaging accurately quantifies freezing damage and cold acclimation responses in Arabidopsis leaves. Plant Methods 4:1.

Eunus, A.M., L.P.V. Johnson, and R. Aksel. 1962. Inheritance of winter hardiness in an eighteen-parent diallel cross of barley. Can. J. Genet. Cytol. 4:356–376.

Fowler, D.B. and R.J. Carles. 1979. Growth, development, and cold tolerance of fall-acclimated cereal grains. Crop Sci. 19:915–922.

Hume, D.J. and A.K.H. Jackson. 1981. Frost tolerance in soybeans. Crop Sci. 21:689–692.

Madakadze, C., K. Stewart, R. Madakadze, an D. Smith. 2003. Base temperature for seedling growth and their correlation with chilling sensitivity for warm-season grasses. Crop Sci. 43:874–878.

Metcalf, E.L., C.E. Cress, C.R. Olien, and E.H. Everson. 1970. Relationship between crown moisture content and killing temperature for three wheat and three barley cultivars. Crop Sci. 10:362–365.

Murray, B., J. Cape, and D. Fowler. 1989. Quantification of frost damage in plant tissue by rates of electrolyte leakage. New Phytol. 113:307–311.

(NDAWN) North Dakota Agricultural Weather Network. 2014. Available at: http://ndawn.ndsu.nodak.edu/get-table.html?station=34 (Accessed 21 November 2014) North Dakota State Univ., Fargo.

Olien, C.R. 1967. Freezing stresses and survival. Annu. Rev. Plant Physiol. 18:387–408.

Oomah B.D and G. Mazza. 1999. Health benefits of phytochemicals from selected Canadian crops. Trends Food Sci. Technol. 10:93–198.

Pomeroy, M.K., C.J. Andrews, and G. Fedak. 1975. Cold hardening and dehydrating responses in winter wheat and winter barley. Can. J. Plant Sci. 55:529–535.

Rapacz, M. and F. Markowski. 1999. Winter hardiness, frost resistance and vernalization requirement of European winter oilseed rape (*Brassica napus* var. oleifera) cultivars within the last 20 years. Crop Sci. 183:243–253.

Rhode, C.R. and C.F. Pulham. 1960. Genetic studies of winter hardiness in barley. Univ. Nebraska Coll. Agric. Exp. Stat. Res. Bull. 193:18.

Salgado, J.P. and C.L. Rife. 1996. Selection for cold hardiness in oilseed rape (*Brassica napus*), Cruciferae Newsletter, 18:92–93.

Skinner, D.Z. and B. Mackey. 2009. Freezing tolerance of winter wheat plants frozen in saturated soil. Field Crops Res. 113:335–341.

Skinner, D.Z. and K.A. Garland-Campbell. 2008. Evidence of a major factor conditioning freezing sensitivity in winter wheat. Plant Breed. 127:228–234.

Sovero, M. 1993. Rapeseed, a new oilseed crop for the United States. pp. 302–307. *In* J. Janick, and J.E. Simon (eds). New Crops. Wiley, New York.

Steponkus, P.L. 1978. Cold hardiness and freezing injury of agronomic crops. pp. 51-98. *In* N.C. Brady (ed.). Advances in Agronomy. Academic Press, New York.

Stout, D.G. 1980. Alfalfa water status and cold hardiness as influenced by cold acclimation and freezing stress. Plant Cell Environ. 3:237–241.

Svec, L.V. and H.F. Hodges. 1972. Cold hardening and morphology of barley seedlings in controlled and natural environments. Can. J. Plant Sci. 52:955–963.

Swensen, J.B. and G.A. Murray. 1983. Cold acclimation of field peas in a controlled Environment. Crop Sci. 23:27–30.

Teutonico, A., P. Palta, and C. Osborn. 1993. In vitro freezing tolerance in relation to winter survival of rapeseed cultivars. Crop Sci. 3:103–107.

Thomashow, M.F. 1999. Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50:571–599.

Wilson, J.M. 1997. Mechanisms of chilling resistance in plants. pp. 111-122. *In* A.S. Basra and R.K. Basra (eds). Mechanisms of environmental stress resistance in plants. Harwood Academic Publishers, Amsterdam.

# CHAPTER 3. PHENOTYPING AND GENOME WIDE ASSOCIATION SCAN FOR FROST TOLERANT LINES IN RAPESEED/CANOLA (*BRASSICA NAPUS* L.) IN ARTIFICIAL CONDITIONS

## 3.1. Abstract

Rapeseed/canola (*Brassica napus* L.) seedlings can be easily damaged by frost which can rupture the plant membranes and kills the plant. Different methods can be used to evaluate the frost damage. A protocol was developed to screen rapeseed/canola germplasm under artificial freezing simulation in a plant growth chamber. A genome-wide association scan was conducted using 231 diverged rapeseed/canola germplasm to find the genomic region controlling the freezing tolerance traits. The genotypes were spring, winter, and semi-winter and were obtained from 21 countries. A total of 37,699 single nucleotide polymorphism (SNP) markers were identified using genotyping-by-sequencing. No growth type clusters were identified in the three mixed population clusters. One QTL was identified that explains about 3% of the phenotypic variation and was located on chromosome A02. Eight freezing tolerance/abiotic stress tolerance genes have been identified in this study.

**Keywords:** *Brassica napus*, frost, artificial conditions, genome-wide association scan

## 3.2. Introduction

Rapeseed/canola (*Brassica napus* L.) is the second most important oilseed crop in the world (Foreign Agricultural Service, USDA). Rapeseed evolved through spontaneous interspecific hybridization followed by genome duplication, chromosome fission, fusion, and rearrangements between two diploid species (Lagercrantz and Lydiate, 1996; Lagercrantz, 1998; U, 1935). It is considered an amphidiploid (AACC, 2n=4x=38) and is the hybrid of *B. rapa* L. (AA, 2n=2x=20) and *B. oleracea* L. (CC, 2n=2x=18). The genome size of *B. napus* is consistent

with the genome sizes of *B. rapa* and *B. oleracea*, and is about 1,130 Mb (Chalhoub *et al*., 2014).

Three growth habit types are found in canola: spring, winter, and semi-winter. Spring canola is typically grown in North Dakota (ND) as the winter canola is not hardy enough to survive the harsh winters experienced in ND. Spring canola is planted in the spring and harvested during the same growing season, and has a yield of about 1.5 ton $ha^{-1}$ (Rakow, 2007). China produces semi-winter rapeseed while Western Europe grows mostly the winter rapeseed. Winter rapeseed is a winter annual (planted in the fall and harvested the following growing season). Winter rapeseed has a higher yield potential than the spring types. The yield for winter types can be greater than 3.5-ton $ha^{-1}$ (Rakow, 2007). Spring canola/rapeseed is important to ND as it produces about 84% of U.S. canola (USDA-ERS, 2015). Cold or freezing temperature is a concern for growers in ND and Canada because of the potential threat of a frost shortly after planting. Crop production and plant growth can be severely affected by freezing temperatures and the whole crop can be destroyed (Chinnusamy *et al*., 2007).

Frost tolerance would allow growers to plant earlier and utilize early season moisture, compete with early weeds, and avoid heat during flowering time which can reduce seed yield. Generally, May is considered as frost-free in ND, but frost can happen later as well. Canola is more susceptible to frost at the cotyledon stage than the three to four leaf stages, which makes canola most susceptible in the early spring when the risk of frost is higher. The severity of the injury depends on many factors, including moisture, growth stage, and duration of the temperature. Frost damage can have different symptoms such as plant death, bleaching, or wilting of the plant. Wilting is caused by the loss of water in the leaves. Bleaching is due to the phyto-oxidation of leaf pigments (Wilson, 1997). Plant cells can be ruptured during freezing due

to ice formation, thus the plant can be killed. Artificial freezing simulating conditions can be used to evaluate canola germplasm for frost tolerance year-round and is not dependent on outside environmental conditions.

Genome-wide association scan (GWAS) is a method that is more powerful than the traditional QTL mapping and is often used for quantitative traits (Rafalski, 2002; Yu and Buckler, 2006; Waugh *et al*., 2009; Visioni *et al*., 2013; Sun *et al*., 2014). GWAS examines genetic variation throughout the whole genome to find association signals for quantitative traits (Risch and Merikangas, 1996; Zhu *et al*., 2008). An important concept of GWAS is linkage disequilibrium which is the non-random association between traits and alleles at different loci (Zhu *et al*., 2008). A diverse population with many historical recombinations is often used for GWAS and common trait-linked alleles can be identified from the population.

The objective of the study was to screen a wide collection of *B. napus* germplasm lines for frost tolerance under artificial conditions and to find the genomic regions that controls the frost tolerant traits.

### 3.3. Materials and methods

#### 3.3.1. Plant materials

A diversity panel, consisting of 231 *B. napus* genotypes were used for association analysis in this study. The germplasm was obtained from the Germplasm Resources Information Network (GRIN) (http://www.ars-grin.gov/npgs/searchgrin.html), were originated/obtained from 21 countries on 4 continents, and consisted of three growth habit types (spring, winter, and semi-winter) (Table A1). Ninety-five spring, 95 winter, and 41 semi-winter germplasm were included. All lines were self-pollinated in the greenhouse for 4-5 generations before being used in this study.

### 3.3.2. Experimental design

A randomized complete block design was used with three replications. Each replication had six plants per genotype. The plants were allowed to grow for 14 days in a greenhouse at 20°C with a 16 h photoperiod provided by natural sunlight supplemented with 400 W HPS PL 2000 lights (P.L. Light Systems Inc.). The plants were fertilized using 20-20-20 liquid fertilizer. After 14 days, the seedlings were moved to a plant growth chamber (BioCold line of Environmental Rooms, Innovative Laboratory Systems Inc.) for cold acclimation at 4°C with a 12 h photoperiod for seven days. Light was provided by GE Ecolux F32T8 SP35 Eco (32 W T8) style bulbs (General Electric Company). After 7 days of acclimation, artificial freezing conditions were created in the plant growth chamber where the temperature started at 4°C and was lowered to reach the treatment temperature (−8°C). The seedlings were kept at −8°C for 8.0 h, and the temperature was raised to 4°C. The treated seedlings were kept in the chamber at 4°C for 24 h, and then were brought back to the greenhouse for seedling damage scoring. The scoring was conducted at three, six, and nine days after the freezing treatment. Each plant was scored according to Fiebelkorn and Rahman (2016), where 0 = dead, 5 = no damage, and scores of 1-4 were based on visual damage estimation. The same experiment was repeated two times. The first scoring at three days after freezing treatment was used for genome wide association scan study. Typically, the scores did not change significantly after the first three days, so it was determined that the first score would be adequate for futher analysis.

### 3.3.3. Statistical analysis

Data was analyzed using SAS ® 9.3 (SAS Institute Inc., USA). Medians were used to calculate the one-way analysis of variance (ANOVA) using non-parametric methods. The estimated relative effect and the 95% confidence interval were also calculated using SAS ® 9.3

(SAS Institute Inc., USA). Heritability on an entry mean basis was calculated. Data normality was tested using the Shapiro-Wilk normality test using R 3.2.4 (The R Foundation).

### 3.3.4. DNA extraction and SNP identification

Qiagen DNeasy kit (Qiagen, CA, US) was used to extract the DNA. Genotyping-by-sequencing (GBS) was conducted at the Institute of Genome Diversity (IGD) at Cornell University. The genome sequencing was done on a GAII sequencer. The GBS libraries were prepared and analyzed according to Elshire *et al*. (2011). The enzyme used for digestion was *Ape*KI and 96 unique barcodes were used to create the GBS libraries. A total of 42,575 SNPs were obtained for a collection of 366 genotypes. Markers with minor allele frequency of less than 5% were removed from the analysis. Alignment was conducted using bwa-mem (Li, 2013) of the GBS data. Multi sample SNP calling was conducted using VarScan (Koboldt *et al*., 2012) and further imputation of the SNPs was conducted using FastPHASE 1.3 (Scheet and Stephens, 2006) using default settings.

### 3.3.5. Population structure

A subset of 3,135 markers were selected that were disbursed randomly across the 19 linkage groups. The number of genetic clusters/subpopulations (K) was calculated using STRUCTURE 2.3.4 (Pritchard Lab, Stanford University). The burn-in period and MCMC (Markov Chain Monte Carlo) were 100,000 and 200,000, respectively. The K-values ranged from 1 – 10 and each K value was averaged across three iterations. The natural log probability [LnP(D)] and delta K (ΔK) (Evanno *et al*., 2005) were used to find the optimal K – value. Delta K is found based on the rate of change of LnP(D) between the K – values.

### 3.3.6. Markers and minor allele frequency

A total of 42,575 SNPs were obtained for the 231 germplasm. Genome-wide association scan (GWAS) was conducted using TASSEL 5.0 (Bradbury *et al*., 2007). Markers were removed if they had less than 5% minor allele frequency, and finally 37,699 were used for the analysis.

### 3.3.7. Linkage disequilibrium (LD) decay

The partial squared allele frequency correlation coefficient ($r^2$) between the pairs of biallelic markers were used to estimate LD decay (Pritchard and Przeworski, 2001; Kraakman *et al*., 2004). The $r^2$ used in this study was 0.2. LD decay was calculated for each subgenome (A and C) and each chromosome (A01-C09) using TASSEL 5.0 (Bradbury *et al*., 2007) and R 3.2.4 (The R Foundation).

### 3.3.8. Model selection

Principal component analysis was used to control the population structure (Price *et al*., 2006). TASSEL 5.0 (Bradbury *et al*., 2007) was used to estimate the principle components (PCs). Cumulative variation that explained 25% and 50% were used in the regression model. To account for relatedness between genotypes, an identify by state matrix was calculated in TASSEL (Zhao *et al*., 2007). Six models, naïve, $PC_3$, $PC_{29}$, Kinship, $PC_3$+Kinship, and $PC_{29}$+Kinship were used. The best model was used based on the lowest mean square difference (MSD). Observed and expected p-values were used to determine the MSD (Mamidi *et al*., 2011).

### 3.3.9. Association mapping

The significant markers were identified based on the *p*-value of the marker and is within the 0.1 percentile tail of 10,000 bootstraps (Mamidi *et al*., 2014; Gurung *et al*., 2014). Stepwise regression was conducted on the significant markers to estimate the combined variation ($r^2$) explained by these significant markers as well as to define the major QTL (Mamidi *et al*., 2014;

Gurung *et al*., 2014). A Manhattan plot was calculated using the -$\log_{10}$(p) values using R 3.2.4 (The R Foundation). The observed verses expected -$\log_{10}$(p) values are were plotted using R 3.2.4 (The R Foundation) to make a QQ plot.

### 3.3.10. Candidate gene search

The annotation of the selected markers was determined using the TAIR 10 protein database. The selected markers included a 100 kb region on each side of the marker. The selected regions were blasted with the *Arabidopsis thaliana* proteome to find candidate genes. Chalhoub *et al*. (2014) published gene models and the genome that was the basis for this study. Published literature was used to identify functions related to frost tolerance or abiotic stress responses for the selected annotations.

## 3.4. Results

### 3.4.1. Phenotypic results

A median of thirty-six readings was considered the phenotypic score. A wide variation of seedling damage scores were observed for the plants treated with the freezing temperature. The overall median score varied from 1.0 to 4.0 with a common median of 1.75 (Table A2). The scores were significantly different at the $\alpha = 0.001$ level (Table 3.1). The broad-sense heritability for the greenhouse study was 0.54 and the standard error was 0.065. The Shapiro-Wilk normality test indicates non-normality as the p-value is less than 0.05 (p = 5.788e-11). The number of genotypes that fit each value were plotted using the frequency of the medians for the study (Fig. 3.1).

Table 3.1. ANOVA for the -8°C greenhouse results run using nonparametric statistics.

| Effect | Num DF | Den DF | Chi-Square | F Value | Pr > ChiSq | Pr > F |
|---|---|---|---|---|---|---|
| Genotype | 230 | 1061 | 541.61 | 2.35 | <0.0001*** | <0.0001*** |

***, p < 0.001

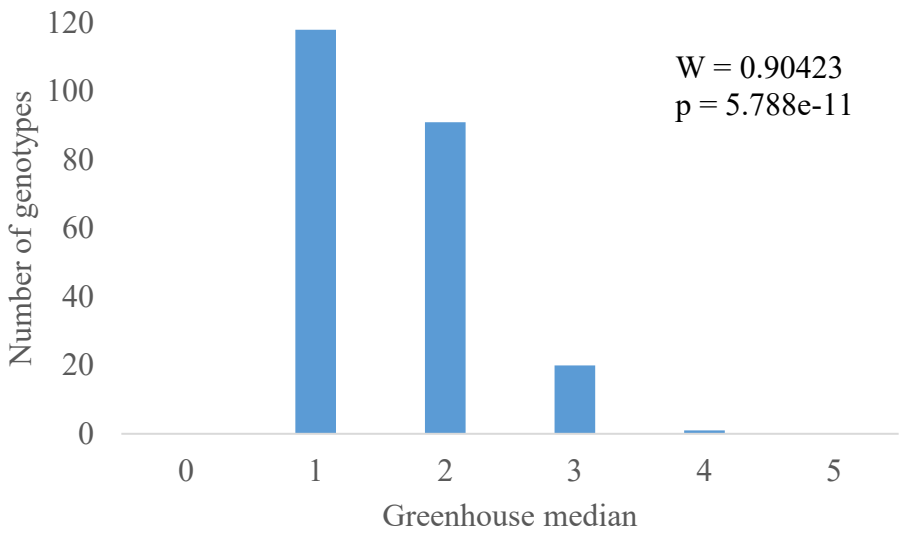


Fig. 3.1. Histogram of the greenhouse medians tested at -8°C. The x-axis is the medians and the y-axis the number of genotypes that fit each median.

### 3.4.2. Estimated relative effect

The estimated relative effect and the confidence interval (95%) was calculated based on the medians (Fig. 3.2 and Table A2.). The relative effect varies between 0 to 1 for the different genotypes. A relative effect closer to 1 means that the genotypes are more tolerant to frost. The confidence interval indicates that 95% of the time the relative effect will be in that range. The data was scored 0 = dead and 5 = no damage, which means that a higher number is more tolerant.

The mean rank of the genotypes was also calculated. A higher mean rank means the genotype performed better while a lower mean rank means the genotype is more susceptible to frost. The overall median is used to calculate the mean rank. The medians are ranked from low to high and the mean of the ranks for each genotype is used.

### 3.4.3. Population structure

A subset of the markers were selected for population structure analysis. Random markers from the 19 linkage groups were selected for a total of 3,135. Based on the LnP(D) and ΔK (Evanno *et al*, 2005), three subpopulations were identified (Fig. 3.3). The LnP(D) calculation did

not show a clear plateau, but the ΔK calculation indicated a peak at K = 3, or three subpopulations.

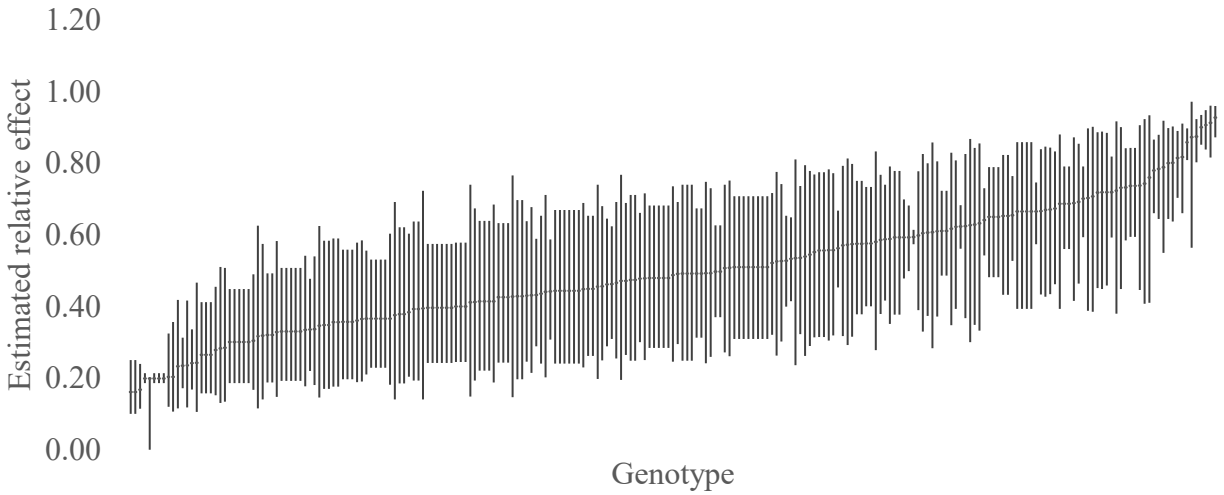


Fig. 3.2. Estimated relative effect and 95% confidence interval for the medians from the greenhouse study. Genotypes toward the right side of the graph are closer to 1 and considered more frost tolerant while the genotypes on the left side are closer to 0 and considered less frost tolerant.

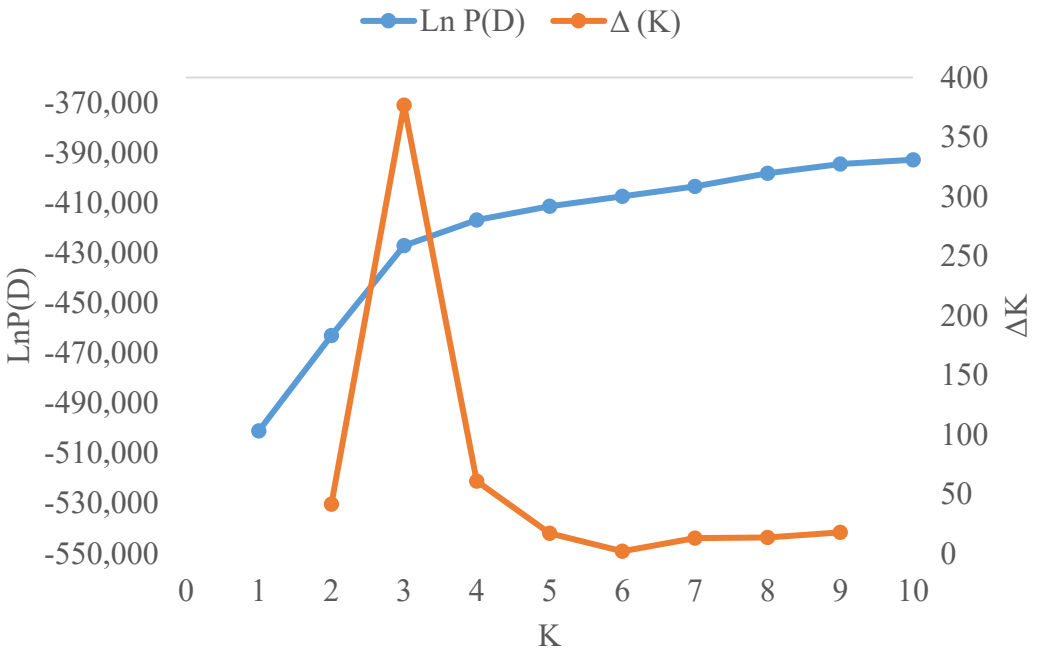


Fig. 3.3. The natural log probability [LnP(D)] and delta K (ΔK) for each value of K averaged over three iterations with 100,000 and 200,000 burn-in and MCMC, respectively. The values of K = 1 – 10, where the K = 3 is the number of subpopulations chosen in the population.

### 3.4.4. Linkage disequilibrium (LD) decay

The LD decay was calculated in the A and C genome as well as individual chromosomes (A01-C09). The partial squared allele frequency correlation coefficient ($r^2$) is commonly used as a measure for LD mapping, and to quantify and compare LD. In this study $r^2$=0.2 was used as the cutoff. The physical distance (kb) for LD decay in the A genome was about 29 kb (Fig. 3.4) and in the C genome was about 158 kb (Fig.3.5). LD decay of each of the 19 chromosomes was calculated using the same cutoff ($r^2$=0.2). The A genome had shorter distances in kb for LD decay than the C genome (Fig. 3.6). The LD decay in the A genome ranged from 18-52 kb (Fig. A1-A10) while the C genome ranged from 61-364 kb (Fig. A11-A19).

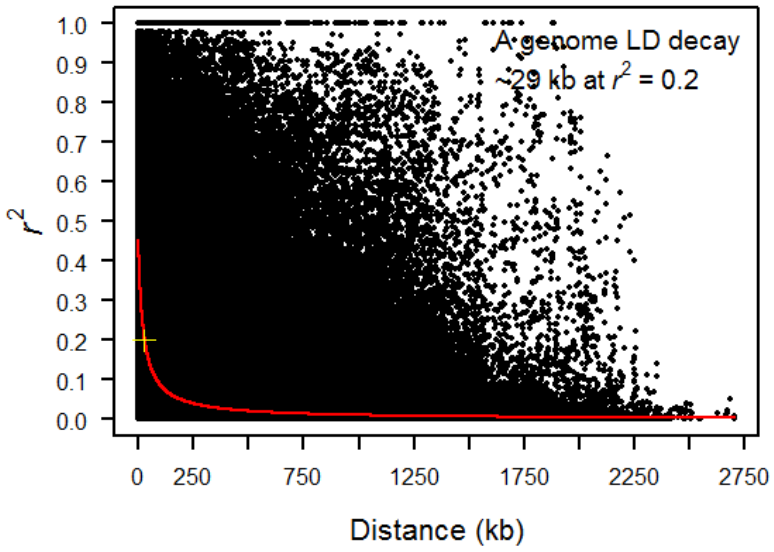


Fig. 3.4. LD decay for the A genome. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2$ = 0.2 is about 29 kb.

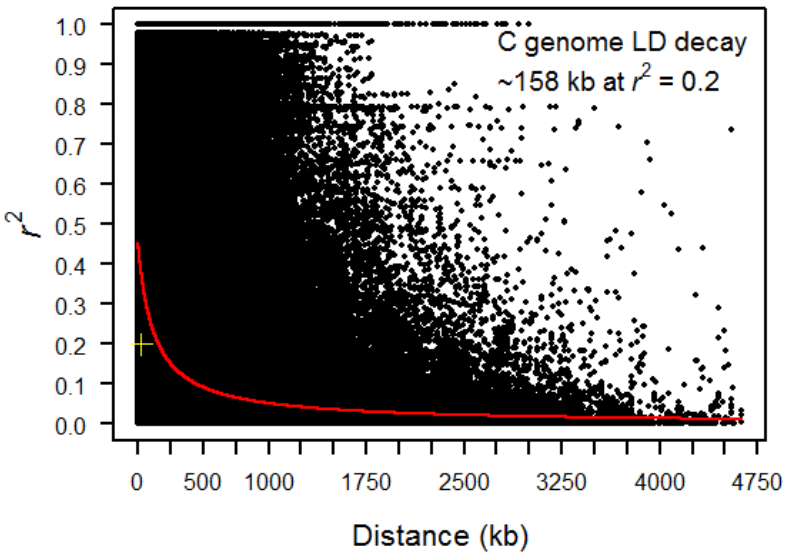


Fig. 3.5. LD decay for the C genome. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 158 kb.

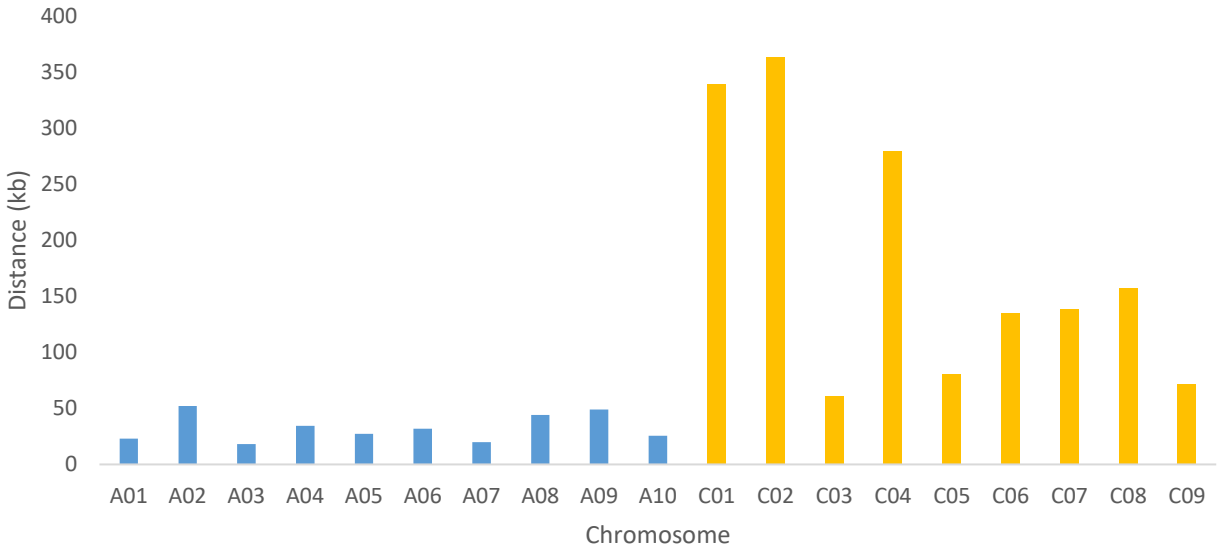


Fig. 3.6. Linkage disequilibrium (LD) decay for 19 chromosomes in canola. The x-axis is the chromosome and the y-axis the physical distance (kb) for the chromosomes at a threshold of $r^2$=0.2.

### 3.4.5. Markers, minor allele frequency, and genome-wide association scan

The total number of SNPs were filtered for minor allele frequencies (<5%) which left a total of 37,699 SNPs. Cumulative variation of 25% and 50% were accounted for using $PC_3$ and $PC_{29}$. These were used to control population structure in the model. The first two PCs, $PC_1$ and $PC_2$ explained about 13% and 9%, respectively. The PCs clustered into groups (Fig. 3.7). The clusters were random and not by growth habit.

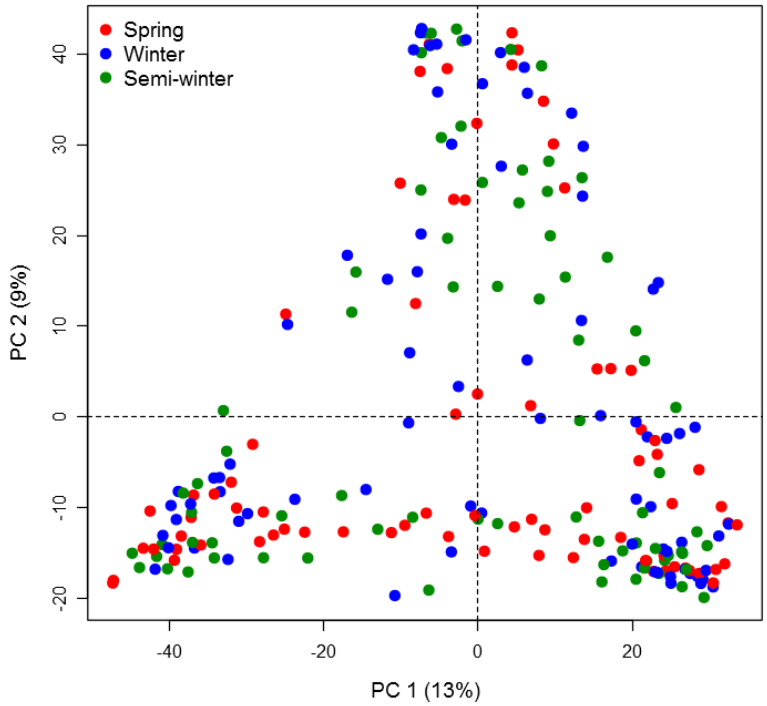


Fig. 3.7. PCA graph showing the distribution of the first two principle components using the medians from the greenhouse study. $PC_1$ explains 13% of the variation and $PC_2$ explains 9% of the variation. $PC_1$ is on the x-axis and $PC_2$ is on the y-axis.

The $PC_{29}$+Kinship (where 50% of the variation is explained and relatedness) model was selected as the best model based on the MSD calculation. Based on 10,000 bootstraps and the 0.1% tail of the empirical distribution, 38 markers were selected and considered significant (Table 3.2). The cutoff p-value was p = 0.001146. One SNP was selected using stepwise

regression on chromosome A02 (21.5 Mbp) and about 3% of the phenotypic variation is explained by this SNP.

A Manhattan plot was calculated using the -$\log_{10}$(p) values (Fig. 3.8). The significant -$\log_{10}$(p) = 2.94 and the significant markers are above the dashed blue line. Markers present on Ann_rand, Cnn_rand, and Unn_rand were removed from the analysis because they were not assigned to any chromosome based on the published *B. napus* genome (Chalhoub *et al*., 2014).

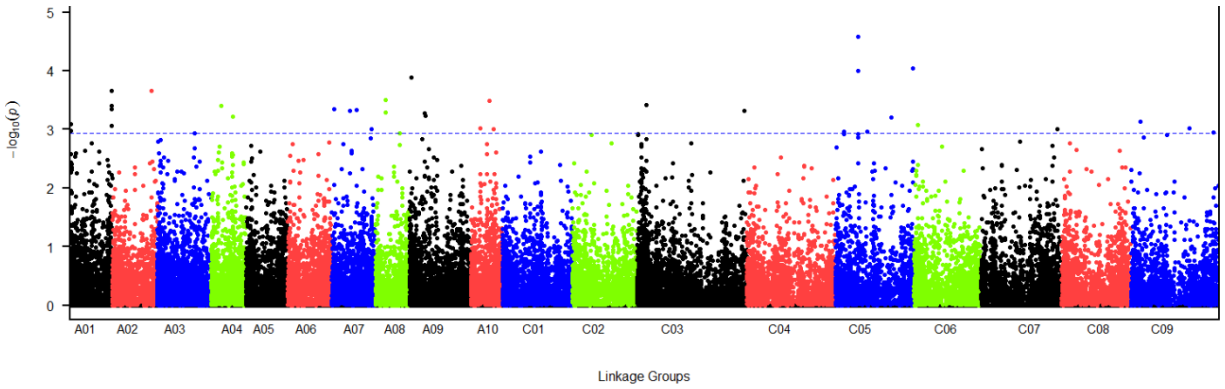


Fig. 3.8. Manhattan plot for the greenhouse study tested at -8°C. The x-axis is the chromosomes and the y-axis is the -$\log_{10}$(p). The dashed horizontal line is at 2.94. The significant markers are above the line. The GWAS model is Kinship+PC29.

The QQ plot indicates the fitness of the model based on observed and expected -$\log_{10}$(p) (Fig. 3.9). The SNP identified during stepwise regression (chrA0221498544) is highlighted in green and labeled on the plot.

A 100 kb region on each side of the significant marker (chrA02_21498544) selected using stepwise regression was blasted with the *Arabidopsis* proteome (TAIR 10). Genes that were annotated were subjected to a literature search to identify functions and genes that were related to frost or abiotic stress (Table 3.3). The functions ranged from ABA degradation,

involvement in DREB2A ubiquitination, to involvement in cell wall metabolism and is repressed under stress conditions.

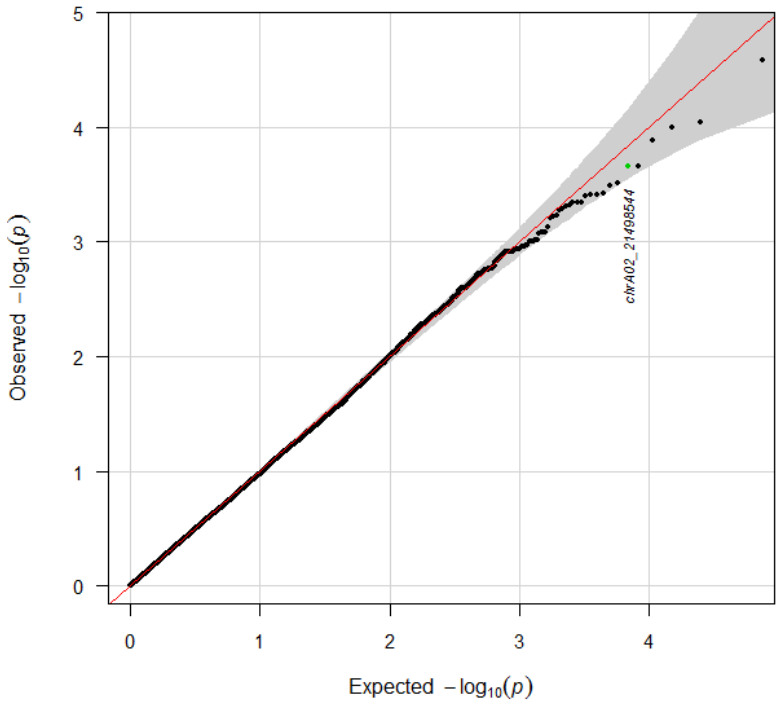


Fig. 3.9. QQ plot showing the distribution of the -$\log_{10}$(p) for the observed and expected p-values. The x-axis is the expected and the y-axis is the observed p-values.

Table 3.2. Significant markers for the greenhouse medians and tested at -8°C and selected based on the 0.1% tail of the Empirical distribution. The reference allele, alternate alleles, and the heterozygote had the means calculated for each marker.

| Marker | Chromosome | Position | Reference allele/ Alternate allele | Reference allele | | Alternate allele | | Heterozygote (H) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Obs | Mean | Obs | Mean | Obs | Mean |
| chrA01_22991233 | A01 | 22991233 | G/A | 74 | 1.70 | 61 | 1.71 | 95 | 1.85 |
| chrA01_22991236 | A01 | 22991236 | C/T | 79 | 1.71 | 58 | 1.71 | 93 | 1.84 |
| chrA01_22991254 | A01 | 22991254 | G/A | 73 | 1.68 | 62 | 1.72 | 95 | 1.86 |
| chrA01_22991258 | A01 | 22991258 | T/C | 75 | 1.69 | 60 | 1.72 | 95 | 1.85 |
| chrA01_576522 | A01 | 576522 | G/A | 140 | 1.81 | 39 | 1.76 | 51 | 1.65 |
| chrA01_576531 | A01 | 576531 | C/T | 83 | 1.76 | 54 | 1.78 | 93 | 1.76 |
| chrA02_21498544* | A02 | 21498544 | A/T | 194 | 1.72 | 17 | 2.10 | 19 | 1.91 |
| chrA03_20587761 | A03 | 20587761 | C/T | 140 | 1.78 | 38 | 1.86 | 52 | 1.65 |
| chrA04_11707842 | A04 | 11707842 | T/G | 97 | 1.83 | 54 | 1.72 | 79 | 1.71 |
| chrA04_5650012 | A04 | 5650012 | G/A | 191 | 1.73 | 19 | 1.79 | 20 | 2.05 |
| chrA07_10110972 | A07 | 10110972 | G/A | 130 | 1.74 | 40 | 1.86 | 60 | 1.75 |
| chrA07_13505270 | A07 | 13505270 | G/A | 190 | 1.71 | 19 | 1.95 | 21 | 2.07 |
| chrA07_21672105 | A07 | 21672105 | G/A | 170 | 1.77 | 28 | 1.74 | 32 | 1.74 |
| chrA07_rand_1125045 | A07_rand | 1125045 | A/G | 192 | 1.72 | 16 | 1.66 | 22 | 2.24 |
| chrA08_5411182 | A08 | 5411182 | G/A | 88 | 1.81 | 52 | 1.76 | 90 | 1.72 |
| chrA08_5411221 | A08 | 5411221 | C/A | 88 | 1.81 | 53 | 1.75 | 89 | 1.73 |
| chrA09_8152488 | A09 | 8152488 | C/A | 152 | 1.79 | 35 | 1.83 | 43 | 1.62 |
| chrA09_8867241 | A09 | 8867241 | T/G | 83 | 1.82 | 61 | 1.70 | 86 | 1.76 |
| chrA09_rand_1053698 | A09_rand | 1053698 | A/C | 102 | 1.80 | 44 | 1.74 | 84 | 1.73 |
| chrA10_12339004 | A10 | 12339004 | C/T | 106 | 1.76 | 53 | 1.88 | 71 | 1.68 |

Obs = observations
*Marker identified through stepwise regression
na = not calculated

Table 3.2. Significant markers for the greenhouse medians and tested at -8°C and selected based on the 0.1% tail of the Empirical distribution (continued). The reference allele, alternate alleles, and the heterozygote had the means calculated for each marker.

| Marker | Chromosome | Position | Reference allele/ Alternate allele | Reference allele | | Alternate allele | | Heterozygote (H) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Obs | Mean | Obs | Mean | Obs | Mean |
| chrA10_4985855 | A10 | 4985855 | A/C | 149 | 1.79 | 34 | 1.85 | 47 | 1.62 |
| chrAnn_rand_4644594 | Ann_rand | 4644594 | C/T | 127 | 1.69 | 47 | 1.80 | 56 | 1.91 |
| chrC03_4962575 | C03 | 4962575 | A/C | 100 | 1.80 | 58 | 1.67 | 72 | 1.79 |
| chrC03_58989086 | C03 | 58989086 | C/A | 155 | 1.83 | 35 | 1.65 | 40 | 1.63 |
| chrC05_12310312 | C05 | 12310312 | A/C | 150 | 1.82 | 34 | 1.71 | 46 | 1.63 |
| chrC05_12310366 | C05 | 12310366 | T/A | 148 | 1.81 | 35 | 1.74 | 47 | 1.63 |
| chrC05_17437784 | C05 | 17437784 | C/A | 142 | 1.71 | 43 | 1.85 | 45 | 1.86 |
| chrC05_31062008 | C05 | 31062008 | C/T | 93 | 1.74 | 56 | 1.78 | 81 | 1.78 |
| chrC05_42883306 | C05 | 42883306 | T/A | 93 | 1.77 | 47 | 1.83 | 90 | 1.73 |
| chrC05_4660254 | C05 | 4660254 | A/T | 166 | 1.74 | 30 | 1.78 | 34 | 1.87 |
| chrC06_2107349 | C06 | 2107349 | A/T | 184 | 1.71 | 20 | 1.80 | 26 | 2.09 |
| chrC07_42313730 | C07 | 42313730 | A/C | 147 | 1.72 | 42 | 1.84 | 41 | 1.84 |
| chrC09_31942271 | C09 | 31942271 | C/T | 125 | 1.79 | 49 | 1.75 | 56 | 1.73 |
| chrC09_45401975 | C09 | 45401975 | A/C | 98 | 1.70 | 52 | 1.75 | 80 | 1.85 |
| chrC09_4883671 | C09 | 4883671 | G/A | 120 | 1.69 | 53 | 1.82 | 57 | 1.88 |
| chrCnn_rand_73744305 | Cnn_rand | 73744305 | T/C | 122 | 1.71 | 48 | 1.78 | 60 | 1.87 |
| chrCnn_rand_80593610 | Cnn_rand | 80593610 | T/C | 150 | 1.72 | 39 | 1.79 | 41 | 1.91 |

Obs = observations
*Marker identified through stepwise regression
na = not calculated

### 3.5. Discussion

Frost tolerance is an important trait for canola and for any other crop in North Dakota. Canola is typically grown in the northern part of the state and in Canada where spring frost is a concern. Frost tolerance can be a difficult trait to study in natural conditions due to unpredictability of the environmental conditions. Therefore, germplasm screening under simulated freezing conditions allowed us to reliably control the temperature, humidity, light, and moisture in the plant growth chamber. Strigens *et al.* (2013) conducted chilling experiments in both the field and growth chamber and reported that the growth chamber study was reliable to overcome the fluctuating temperature experienced in the field. Earlier, we have developed a protocol to screen canola germplasm using an ESPEC BTU-433 freezing chamber (4 cubic foot) (ESPEC North America, Inc.) to conduct the frost simulation. The suggested protocol for the freezing tolerant screening is cold acclimation of 14 day old seedlings for 7 days at 4°C followed by frost treatment at −4°C for 8 h (Fiebelkorn and Rahman, 2016). This protocol did not work well in the current larger plant growth chamber (960 cubic foot). Therefore, we used the frost treatment at −8°C for 8 h for better seedling damage scores. This is logical that the smaller chamber has better temperature control over the larger chamber. We used 14 day old seedlings for the freezing tolerant screening. At this stage the seedlings are more susceptible to freezing temperature and expected to observe the most variation of seedling damage score in response to different freezing temperatures. We have repeated the experiments both at −4°C and −8°C treatments. Repeated experiments help to correctly phenotype the germplasm which is the most important parameter for the association study.

### 3.5.1. Genome-wide association scan

Genome-wide association scan (GWAS) is a powerful tool to identify marker-trait associations and has been used for many mapping projects (Li *et al*., 2011; Jia *et al*., 2012). This method aims to identify common alleles in a diverse population. Traditional QTL mapping can also be used to identify trait-linked markers. However, GWAS is a population-based screening method which is based on LD in crop species to identify trait-marker relationships that significantly increased the power to localize the QTL (Kraakman *et al*., 2004; Meuwissen and Goddard, 2000). GWAS utilizes a diverse population which has wider genetic variability generated over many rounds of historical recombination (Hanson *et al*., 2001; Kraakman *et al*., 2006). In this study, we used a wide collection of diverged germplasm accessions for GWAS. It was a large collection of 231 lines. There is no clear consensus on the number of genotypes to be used for GWAS. However, GWAS studies have been conducted in *B. napus* using 49 genetically diverse winter-type germplasm for seed phenolic compounds (Rezaeizad *et al*., 2011), 89 winter-type accessions for 6 seed quality traits (Gajardo *et al*., 2015), 139 spring-, semi-winter- and winter-type germplasm for blackleg disease (Rahman *et al*., 2016), 143 spring-, semi-winter- and winter-type accessions for branch angle (Liu *et al*., 2016), 248 winter-type germplasm for seed germination and early vigor (Hatzig *et al*., 2015), 405 accessions of winter oilseed, winter fodder, swede, semi-winter, spring, spring fodder, and vegetable types for agronomic and seed quality traits (Körber *et al*., 2016), and 540 accessions of winter-, semi-winter-, spring- and vegetable-type for fatty acid profiles (Qu *et al*., 2017).

A moderate broad-sense heritability (54%) was identified for the trait. This indicates that the progeny could inherit the trait from the parents. Frost tolerance is believed to be controlled by a large number of genes, which is also indicated by the heritability estimate.

The population structure analysis indicated that three clusters were present in the population. The individuals in the subpopulation did not show any geographical or growth habit type distribution in respect to the origin or growth types. Previous studies that have analyzed structure in *B. napu*s include Hasan *et al*. (2008), Bus *et al*. (2011), Qian *et al*. (2014), Raman *et al*. (2014), and Li *et al*. (2014). These studies did not find geographic or growth habit clusters in the population. Our study agreed with the findings of the previous studies in respect to the number of clusters and growth habit types.

False-positives can be observed due to confounding effects during the analysis. False-positives are often seen in populations that have structure or relatedness. Models can be used that will account for structure, relatedness, or both (Pritchard *et al*., 2000a, 2000b; Falush *et al*., 2003; Yu *et al*., 2006). The six models tested for this study were naïve, $PC_3$, $PC_{29}$, Kinship, $PC_3$+Kinship, and $PC_{29}$+Kinship. The PC models were used to account for structure, kinship was used for relatedness, and PC+Kinship was used for both structure and relatedness. The best model based on the lowest MSD was $PC_{29}$+Kinship which accounts for both structure and relatedness. The empirical distribution of p-values that was suggested by Mamidi *et al*. (2014) was used to select significant markers. QTL peaks for marker assisted selection (MAS) were identified through stepwise regression. Stepwise regression eliminated markers that had a minor effect on the phenotype. One significant marker located on chromosome A02 has been identified from this study.

The reference genome paper by Chalhoub *et al*. (2014) were unable to assign all scaffolds to pseudochromosomes. Nineteen pseudochromosomes were named according to the subgenome (chrA01-chrA10 and chrC01-chrC09). Scaffolds that were mapped but had an unknown orientation were named random based on the subgenome (A01_random-A10_random and

C01_random-C09_random). Other scaffolds were unmapped but could be assigned to a subgenome and were called Ann_random and Cnn_random. Other scaffolds could not be mapped or assigned to a subgenome and were called Unn_random. This study abbreviated random to rand.

The germplasm accessions used in this study had low LD (Michalak *et al.*, unpublished). Therefore, we took 100 kb sequence from each side of the significant marker for the candidate gene search (Chalhoub *et al.*, 2014). NCBI-BLAST was used to identify genes associated with frost tolerance or abiotic stress. The 200 kb section was compared with *Arabidopsis thaliana*. Eight potential genes were identified as being related to abiotic stress or frost tolerance. All of the potential genes were identified on chromosome A02 as that is the only significant marker chosen through stepwise regression. The first gene of interest was annotated as HXXXD-type acyl-transferase family protein and the function of this gene is involved in cell wall metabolism and is repressed under stress conditions (Zhu *et al*., 2013). Two genes were annotated as cytochrome P450 family proteins and the function was found to be involved in ABA degradation during dehydration stress and seed imbibition (Shinozaki and Yamaguchi-Shinozaki, 2007). Two genes were annotated as expansin and the function is to loosen cell walls and hydrogen bonding disruption (Lu *et al*., 2013). The expression is increased under abiotic stress. Two genes were identified as MYB transcription factors or MYB family transcription factors. The function of the MYB family transcription factor is a transcription factor that is involved with abiotic stress response (Hong *et al*., 2013). The function of the MYB transcription factor is involved in biotic and abiotic stress response (Dubos *et al*., 2010). The last gene was annotated as E3 ubiquitin-protein ligase and the function of this gene was found to be involved in DREB2A ubiquitination mediation (Qin *et al*., 2008). The DREB (dehydration responsive) genes are involved with

dehydration response which can happen during cold stress. DREB/CBF (C repeat-binding proteins) factors have been linked to many abiotic stresses, including low temperature stress (Liu *et al*., 1998; Nakashima *et al*., 2000; Gao *et al*., 2002; Qin *et al*., 2004; Li *et al*., 2005; Vogel *et al*., 2005; Oh *et al*., 2007; Qin *et al*., 2007; Gutha and Reddy, 2008; Morran *et al*., 2011).

Frost tolerance is a complex trait that is controlled by many genes. It is important for the breeding program to know and understand the trait. Knowing the genes associated with frost tolerance and understanding whether they are positively or negatively related to stress tolerance would also benefit the breeder. Incorporating the positively related genes and minimizing the negatively related genes would help to increase frost tolerance of canola.

### 3.6. Conclusion

Frost tolerance studies in the field can be difficult because temperatures are hard to predict. Artificial conditions or greenhouse studies are better able to regulate the temperatures and therefore, have a more consistent screen. Field and greenhouse studies have been found to be correlated so greenhouse studies can be used to help predict frost tolerance. This study identified eight potential genes that are associated with frost tolerance or abiotic stress. A large population was screened to identify these genes. Further research to utilize and introgress the genes into commercial cultivars is needed.

Table 3.3. Gene functions for candidate genes identified from the frost study conducted at -8°C in the greenhouse.

| Brassica gene model | Marker | Gene start (bp) | Gene end (bp) | Marker distance from gene (bp) | A. thaliana equivalent | Gene annotation | Function | Reference |
|---|---|---|---|---|---|---|---|---|
| BnaA02g29330D | chrA02_21498544 | 21,453,042 | 21,455,195 | 43,349 | AT5G67150 | HXXXD-type acyl-transferase family protein | Involved in cell wall metabolism and repressed under stress conditions | Zhu *et al*. (2013) |
| BnaA02g29380D | chrA02_21498544 | 21,478,746 | 21,480,637 | 17,907 | ARALYDRAFT_484649 | Cytochrome P450 family protein | Involved in ABA degradation during seed imbibition and dehydration stress | Shinozaki and Yamaguchi-Shinozaki (2007) |
| BnaA02g29390D | chrA02_21498544 | 21,480,796 | 21,483,364 | 15,180 | ARALYDRAFT_484649 | | | |

Table 3.3. Gene functions for candidate genes identified from the frost study conducted at -8°C in the greenhouse (continued).

| Brassica gene model | Marker | Gene start (bp) | Gene end (bp) | Marker distance from gene (bp) | A. thaliana equivalent | Gene annotation | Function | Reference |
|---|---|---|---|---|---|---|---|---|
| BnaA02g29440D | chrA02_21498544 | 21,517,952 | 21,518,817 | 19,408 | AT3G11600 | E3 ubiquitin-protein ligase | Involved in DREB2A ubiquitination mediation | Qin *et al*. (2008) |
| BnaA02g29490D | chrA02_21498544 | 21,541,176 | 21,542,959 | 42,632 | AAM64792 | MYB family transcription factor | Transcription factor involved in responses to abiotic stesses | Hong *et al*. (2013) |

Table 3.3. Gene functions for candidate genes identified from the frost study conducted at -8°C in the greenhouse (continued).

| Brassica gene model | Marker | Gene start (bp) | Gene end (bp) | Marker distance from gene (bp) | A. thaliana equivalent | Gene annotation | Function | Reference |
|---|---|---|---|---|---|---|---|---|
| BnaA02g29510D<br>BnaA02g29640D | chrA02_21498544<br>chrA02_21498544 | 21,546,621,591,970 | 21,549,27121,593,790 | 48,12893,426 | AT2G03090<br>AT3G29030 | Expansin | Loosens cell walls and disrupts hydrogen bonds when expression is increased under abiotic stress | Lu *et al*. (2013) |
| BnaA02g29620D | chrA02_21498544 | 21,590,163 | 21,591,088 | 91,619 | AT3G29020 | MYB transcription factor | Involved in responses to biotic and abiotic stresses | Dubos *et al*. (2010) |

### 3.7. Literature cited


Bradbury, P.J., Z. Zhang, D.E. Kroon, T.M. Casstevens, Y. Ramdoss, and E.S. Buckler. 2007. TASSEL: software for association mapping of complex traits in diverse samples. Bioinformatics. 23(19):2633-2635. doi: https://doi.org/10.1093/bioinformatics/btm308.

Bus, A., N. Korber, R.J. Snowdon, and B. Stich. 2011. Patterns of molecular variation in a species wide germplasm set of *Brassica napus*. Theor Appl Genet. 123: 1413–1423.

Chalhoub, B., F. Denoeud, S. Liu, I.A.P Parkin, H. Tang, X. Wang, J. Chiquet, H. Belcram, C. Tong, B. Samans, M. Correa, C. Da Silva, J. Just, C. Falentin, C.S. Koh, I. Le Clainche, M. Bernard, P. Bento, B. Noel, K. Labadie, A. Alberti, M. Charles, D. Arnaud, H. Guo, C. Daviaud, S. Alamery, K. Jabbari, M. Zhao, P.P. Edger, H. Chelaifa, D. Tack, G. Lassalle, I. Mestiri, N. Schnel, M.-C. Le Paslier, G. Fan, V. Renault, P.E. Bayer, A.A. Golicz, S. Manoli, T.-H. Lee, V.H.D. Thi, S. Chalabi, Q. Hu, C. Fan, R. Tollenacre, Y. Lu, C. Battail, J. Shen, C.H.D. Sidebottom, X. Wang, A. Canaguier, A. Chauveau, A. Berard, G. Deniot, M. Guan, Z. Liu, F. Sun, Y.P. Lim, E. Lyons, C.D. Town, I. Bancroft, X. Wang, J. Meng, J. Ma, J.C. Pires, G.J. King, D. Brunel, R. Delourme, M. Renard, J.-M. Aury, K.L. Adams, J. Batley, R.J. Snowden, J. Tost, D. Edwards, Y. Zhou, W. Hua, A.G. Sharpe, A.H. Paterson, C. Guan, P. Wincker. 2014. Early allopolyploid evolution in the post-Neolithic *Brassica napus* oilseed genome. Science. 345(6199):950-953.

Chinnusamy, V., J. Zhu, and J.-K. Zhu. 2007. Cold stress regulation of gene expression in plants. Trends in Plant Sci. 12(10):444-451.

Dubos, C., R. Stracke, E. Grotewold, B. Weisshaar, C. Martin, and L. Lepiniec. 2010. MYB transcription factors in Arabidopsis. Trends in Plant Sci. 15(10):573-581. doi:10.1016/j.tplants.2010.06.005.

Elshire, R.J., J.C. Glaubitz, J.A. Poland, K. Kawamoto, E.S. Buckler, and S.E. Mitchell. 2011. A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. PLOS ONE. 6(5):e19379.

Evanno, G., S. Regnaut, and J. Goudet. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology. 14:2611-2620.

Falush, D., M. Stephens, and J.K. Pritchard. 2003. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics 164:1567-1587.

Fiebelkorn, D. and M. Rahman. 2016. Development of a protocol for frost-tolerance evaluation in rapeseed/canola (*Brassica napus* L.). The Crop J. 4:147-152.

Gajardo, H.A., B. Wittkop, B. Soto-Cerda, E.E. Higgins, I.A.P. Parkin, R.J. Snowdon, M.L. Federico, and F.L. Iniguez-Luy. 2015. Association mapping of seed quality traits in *Brassica napus* L. using GWAS and candidate QTL approaches. Mol. Breeding. 35:143.

Gao, M.J., G. Allard, L. Byass, A.M. Flanagan, and J. Singh. 2002. Regulation and characterization of four CBF transcription factors from *Brassica napus*. Plant Mol. Biol. 49:459-471.

Gutha, L.R. and A.R. Reddy. 2008. Rice DREB1B promoter shows distinct stress-specific responses, and the overexpression of cDNA in tobacco confers improved abiotic and biotic stress tolerance. Plant Mol. Biol. 68:533-555.

Gurung, S., S. Mamidi, J.M. Bonman, M. Xiong, G. Brown-Guedira, and T.B. Adhikari. 2014. Genome-wide association study reveals novel quantitative trait loci associated with resistance to multiple leaf spot diseases of spring wheat. Plos One 9(9):e108179.

Hansen, M., T. Kraft, S. Ganestam, T. Säll, and N.-O. Nilsson. 2001. Linkage disequilibrium mapping of the bolting gene in sea beet using AFLP markers. *Genetical Research*. 77(1):61–66.

Hasan, M., W. Friedt, J. Pons-Kuhnemann, N.M. Freitag, K. Link K, and R.J. Snowdon. 2008. Association of gene-linked SSR markers to seed glucosinolate content in oilseed rape (*Brassica napus ssp.napus*). Theor Appl Genet. 116: 1035–1049.

Hatzig, S. V., Frisch, M., Breuer, F., Nesi, N., Ducournau, S., Wagner, M.-H., Leckband, G., Abbadi, A., Snowdon, R. J. 2015. Genome-wide association mapping unravels the genetic control of seed germination and vigor in *Brassica napus*. Frontiers in Plant Science, 6. doi: 10.3389/fpls.2015.00221

Hong, S.-Y., L.V. Roze, and J.E. Linz. 2013. Oxidative stress-related transcription factors in the regulation of secondary metabolism. Toxins. 5(4):683-702. doi:10.3390/toxins5040683.

Jia, L., W. Yan, C. Zhu, H.A. Agrama, A. Jackson, K. Yeater, X. Li, B. Huang, B. Hu, A. McClung, and D. Wu. 2012. Allelic analysis of sheath blight resistance with association mapping in rice. Plos One. 7(3):e32703.

Koboldt, D.C., Q. Zhang, D.E. Larson, D. Shen, M.D. McLellan, L. Lin, C.A. Miller, E.R. Mardis, L. Ding, and R.K. Wilson. 2012. VarScan 2: Somatic mutation and copy number alteration discovery in cancer by exome sequencing. Genome Res. 22:568-576.

Körber, N., A. Bus, J. Li, I.S.P. Parkin, B. Wittkop, R.J. Snowdon, and B. Stich. 2016. Agronomic and seed quality traits dissected by genome-wide association mapping in *Brassica napus*. Front. Plant Sci. 7:386. doi:https://doi.org/10.3389/fpls.2016.00386.

Kraakman, A.T.W., F. Martínez, B. Mussiraliev, F.A. van Eeuwijk, and R.E. Niks. 2006. Linkage disequilibrium mapping of morphological, resistance, and other agronomically relevant traits in modern spring barley cultivars. *Molecular Breeding*. 17(1):41–58.

Kraakman, A.T.W., R.E. Niks, P.M.M.M. Van den Berg, P. Stam, and F.A. Van Eeuwijk. 2004. Linkage disequilibrium mapping of yield and yield stability in modern spring barley cultivars. Genetics. 168:435-446.

Lagercrantz, U. 1998. Comparative mapping between *Arabidopsis thaliana* and *Brassica nigra* indicates that brassica genomes have evolved through extensive genome replication accompanied by chromosome fusions and frequent rearrangements. Genetics. 150:1217-1228.

Lagercrantz, U. and D.J. Lydiate. 1996. Comparative genome mapping in Brassica. Genetics. 144:1903-1910.

Li, F., B. Chen, K. Xu, J. Wu, W. Song, I. Bancroft, A.L. Harper, M. Trick, S. Liu, G. Gao, N. Wang, G. Yan, J. Qiao, J. Li, H. Li, X. Xiao, T. Zhang, and X. Wu. 2014. Genome-wide association study dissects the genetic architecture of seed weight and seed quality in rapeseed (*Brassica napus* L.). DNA Res. 21: 355–367.

Li, H. 2013. Aligning sequence reads, clone sequences, and assembly contigs with BWA-MEM. arXiv:1303.3997 http://arxiv.org/abs/1303.3997.

Li, X.P., A.G. Tian, G.Z. Luo, Z.Z. Gong, J.S. Zhang, and S.Y. Chen. 2005. Soybean DRE-binding transcription factors that are responsive to abiotic stresses. Theor. Appl. Genet. 110:1355-1362.

Li, X., W. Yan, H. Agrama, L. Jia, X. Shen, A. Jackson, K. Moldenhauer, K. Yeater, A. McClung , and D. Wu. 2011. Mapping QTLs for improving grain yield using the USDA rice minicore collection. Planta 234:347–361.

Liu, J., W. Wang, D. Mei, H. Wang, L. Fu, D. Liu, Y. Li, and Q. Hu. 2016. Characterizing variation of branch angle and genome-wide association mapping in rapeseed (*Brassica napus* L.). Front Plant Sci 7:21.

Liu, Q., M. Kasuga, Y. Sakuma, H. Abe, S. Miura, K. Yamaguchi-Shinozaki, and K. Shinozaki. 1998. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively in *Arabidopsis*. Plant Cell. 10:1391-1406.

Lu, P., M. Kang, X. Jiang, F. Dai, J. Gao, and C. Zhang. 2013. *RhEXPA4*, a rose expansin gene, modulates leaf growth and confers drought and salt tolerance to *Arabidopsis.* Planta. 237:1547-1559. doi:10.1007/s00425-013-1867-3.

Mamidi, S., R.K. Lee, R.J. Goos, and P.E. McClean. 2014. Genome-wide association studies identifies seven major regions responsible for iron deficiency chlorosis in soybean (*Glycine max*). Plos One 9(9):e107469.

Mamidi, S., S. Chikara, R.J. Goos, D.L. Hyten, D. Annam, S.M. Moghaddam, R.K. Lee, P.B. Cregan, and P.E. McClean. 2011. Genome-wide association analysis identifies candidate genes associated with iron deficiency chlorosis in soybean. Plant Genome 4:154–164.

Meuwissenk, T. and M. Goddard. 2000. Fine mapping of quantitative trait loci using linkage disequilibria with closely linked marker loci. Genetics 155(1):421–430.

Michalak de Jiménez, M., S. Mamidi, P.E. McClean. and M. Rahman. (Unpublished). Low level of linkage disequilibrium and population structure in a core collection of *Brassica napus* discovered using SNPs derived from GBS.

Morran, S., O. Eini, T. Pyvovarenko, B. Parent, R. Singh, A. Ismagul, S. Eilby, N. Shirley, P. Langridge, and S. Lopato. 2011. Improvement of stress tolerance of wheat and barley by modulation of expression of DREB/CBF factors. Plant Biotech. J. 9:230-249.

Nakashima, K., Z.K. Shinwari, Y. Sakuma, M. Seki, S. Miura, K. Shinozaki, and K. Yamaguchi-Shinozaki. 2000. Organization and expression of two *Arabidopsis* DREB2 genes encoding DRE-binding proteins involved in dehydration- and high-salinity-responsive gene expression. Plant Mol. Biol. 42:657-665.

Oh, S.J., C.W. Kwon, D.W. Choi, S.I. Song, and J.K. Kim. 2007. Expression of barley HvCBF4 enhances tolerance to abiotic stress in transgenic rice. Plant Biotechnol. J. 5:646-656.

Price, A.L., N.J. Patterson, R.M. Plenge, M.E. Weinblatt, N.A. Shadick, and D. Reich. 2006. Principal components analysis corrects for stratification in genome-wide association studies. Nature Genet. 38(8):904-909.

Pritchard, J.K. and M. Przeworski. 2001.Linkage disequilibrium in humans: models and data. Am. J. Hum. Genet. 69:1-14.

Pritchard, J.K., M. Stephens, and P. Donnelly. 2000a. Inference of population structure using multilocus genotype data. Genetics 155:945-959.

Pritchard, J.K., M. Stephens, N.A. Rosenberg, and P. Donnelly. 2000b. Association mapping in structured populations. Am. J. Hum. Genet. 676:170-181.

Qian, L., W. Qian, and R.J. Snowdon. 2014. Sub-genomic selection patterns as a signature of breeding in the allopolyploid *Brassica napus* genome. BMC Genomics. 15:1170. doi:10.1186/1471-2164-15-1170.

Qin, F., Y. Sakuma, J. Li, Q. Liu, Y.Q. Li, K. Shinozaki, and K.Y. Yamaguchi-Shinozaki. 2004. Cloning and functional analysis of a novel DREB1/CBF transcription factor involved in cold-responsive gene expression in *Zea mays* L. Plant Cell Physiol. 45:1042-1052.

Qin, F., Y. Sakuma, L.-S. Phan Tran, K. Maruyama, S. Kidokoro, Y. Fujita, M. Fujita, T. Umezawa , Y. Sawano, K.-I. Miyazono, M. Tanokura, K. Shinozaki, and K. Yamaguchi-Shinozaki. 2008. *Arabidopsis* DREB2A-interacting proteins function as RING E3 ligases and negatively regulate plant drought stress–responsive gene expression. The Plant Cell. 20(6):1693-1707. doi: http://dx.doi.org/10.1105/tpc.107.057380.

Qin, Q.L., J.G. Liu, Z. Zhang, R.H. Peng, A.S. Xiong, Q.H. Yao, and J.M. Chen. 2007. Isolation, optimization, and functional analysis of the cDNA encoding transcription factor OsDREB1B in *Oryza sativa* L. Mol. Breed. 19:329-340.

Qu, C., L. Jia, F. Fu, H. Zhao, K. Lu, L. Wei, X. Xu, Y. Liang, S. Li, R. Wang, and J. Li. 2017. Genome-wide association mapping and Identification of candidate genes for fatty acid composition in *Brassica napus* L. using SNP markers. BMC Genomics 18:232.

Rafalski, A. 2002. Applications of single nucleotide polymorphisms in crop genetics. Curr. Opin. Plant Biol. 5:94-100.

Rahman, M., S. Mamidi, L. del Rio, A. Ross, M.M Kadir, M.M. Rahaman, and M. Arifuzzaman. 2016. Association mapping in *Brassica napus* (L.) accessions identifies a major QTL for blackleg disease resistance on chromosome A01. Mol. Breeding. 36:90. doi:10.1007/s11032-016-0513-8.

Rakow, G. 2007. Rapeseed genetics and breeding research for sustainable oilseed production. Proceedings of the 12th International Rapeseed Congress 1:2072-210.

Raman, H., J. Dalton-Morgan, S. Diffey, R. Raman, S. Alamery, D. Edwards, and J. Batley. 2014. SNP markers-based map construction and genome-wide linkage analysis in *Brassica napus*. Plant Biotech J. 12: 851–860.

Rezaeizad, A., B. Wittkop, R. Snowdon, M. Hasan, V. Mohammadi, A. Zali, and W. Friedt. 2011. Identification of QTLs for phenolic compounds in oilseed rape (Brassica napus L.) by association mapping using SSR markers. Euphytica 177:335–342.

Risch, N. and K. Merikangas. 1996. The future of genetic studies of complex human diseases. Science 273:1516-1517.

Scheet, P. and M. Stephens. 2006. A fast and flexible statistical model for large-scale population genotype data: applications to inferring missing genotypes and haplotypic phase. Am J. Human Genet. 78:629-644.

Shinozaki, K. and K. Yamaguchi-Shinozaki. 2007. Gene networks involved in drought stress response and tolerance. J. Exp. Bot. 58(2):221-227. doi: https://doi.org/10.1093/jxb/erl164.

Strigens, A., N.M. Freitag, X. Gilbert, C. Grieder, C. Riedelsheimer, T.A. Schrag, R. Messmer, and A.E. Melchinger. 2013. Association mapping for chilling tolerance in elite flint and dent maize inbred lines evaluated in growth chamber and field experiments. Plant, Cell, and Env. 36:1871-1887.

Sun, J., N. Guo, J. Lei, L. Li, G. Hu, and H. Xing. 2014. Association mapping for partial resistance to *Phytophthora sojae* in soybean (*Glycine max* L.). J. of Genetics. 2:355-363.

U, N. 1935. Genome analysis in *Brassica* with special reference to the experimental formation of *Brassica napus* and peculiar mode of fertilization. Japanese J. Botany. 7:389-452.

(USDA) United States Department of Agriculture. 2015. Economic Research Service (ERS). Oil crops yearbook. Beltsville, MD. http://www.ers.usda.gov/data-products/oil-crops-yearbook.aspx.

(USDA) United States Department of Agriculture. Updated 2016. Foreign Agricultural Service. Accessed: https://www.fas.usda.gov (26 March 2017).

Visioni, A. A. Tondelli, E. Francia, A. Pswarayi, M. Malosetti, J. Russell, W. Thomas, R. Waugh, N. Pecchioni, I. Romagosa, and J. Comadran. 2013.Denome-wide association mapping of frost tolerance in barley (*Hordeum vulgare* L.). BMC Genomics. 14:424-437. doi: http://www.biomedcentral.com/1471-2164/14/424.

Vogel, J.T., D.G. Zarka, H.A. Van Buskirk, S.G. Fowler, and M.F. Thomashow. 2005. Roles of CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of *Arabidopsis*. Plant J. 41:195-211.

Waugh, R., J.L. Jannink, K. Muller, and L. Ramsay. 2009. The emergence of whole genome association scans in barley. Curr. Opin. Plant Biol. 12:1-5.

Wilson, J.M, 1997. Mechanisms of chilling resistance in plants. pp. 111-122. *In* A.S. Basra and R.K. Basra (eds). Mechanisms of environmental stress resistance in plants.

Yu, J.M. and E.S. Buckler. 2006. Genetic association mapping and genome organization of maize. Curr. Opin. Biotech. 17:155-160.

Yu, J., G. Pressoir, W.H. Briggs, I.V. Bi, M. Yamasaki, J.F. Doebley, M.D. McMullen, B.S. Gaut, D.M. Nielsen, J.B. Holland, S. Kresovich, and E.S. Buckler. 2006. A unified mixed-model method for association mapping that accounts for multiple levels of relatedness. Nat. Genet. 38:203–208.

Zhao, K., M.J. Aranzana, S. Kim, C. Lister, C. Shindo, C. Tang, C. Toomajian, H. Zheng, C. Dean, P. Marjoram, and M. Nordborg. 2007. An Arabidopsis example of association mapping in structured samples. PLoS Genetics 3:e4. doi: http://dx.doi.org/10.1371/journal.pgen.0030004.

Zhu, J., J.C. Jeong, Y. Zhu, I. Sokolchik, S. Miyazaki, J.-K. Zhu, P.M. Hasegawa, H.J. Bohnert, H. Shi, D.-J. Yun, and R.A. Bressan. 2008. Involvement of *Arabidopsis* HOS15 in histone deacetylation and cold tolerance. PNAS.105(12):4945–4950.

Zhu, Y.-N., D.-Q. Shi , M.-B. Ruan, L.-L. Zhang, Z.-H. Meng, J. Liu, and W.-C. Yang. 2013. Transcriptome analysis reveals crosstalk of responsive genes to multiple abiotic stresses in Cotton (*Gossypium hirsutum* L.). PLOS ONW. 8(11):1-13. doi: http://dx.doi.org/10.1371/journal.pone.0080218.

# CHAPTER 4. PHENOTYPING AND GENOME WIDE ASSOCIATION SCAN FOR FROST TOLERANT LINES IN RAPESEED/CANOLA (*BRASSICA NAPUS* L.) UNDER NATURAL CONDITIONS

## 4.1. Abstract

Late frost is a concern for producers, especially in the northern canola growing regions, such as North Dakota and Canada. Plants can be killed due to their cells rupturing when exposed to frost. There are different ways to evaluate frost tolerance, including field evaluations. Due to environmental variation, it is difficult to get ideal conditions for field evaluations. In this study, seedlings were grown in the greenhouse for 14 days at 20°C and cold acclimated for 7 days at 4°C before the seedlings were placed outside to be exposed to frost. After a night of frost exposure, seedlings were scored for damage. Genome-wide association scan (GWAS) was conducted on 147 spring, winter, and semi-winter germplasm lines obtained from 15 countries. A total of 37,111 single nucleotide polymorphism (SNP) markers were used for the analysis. Three mixed populations with no growth type or geographic patterns were identified. One QTL was identified as being associated with frost tolerance of canola. This QTL explained about 5% of the phenotypic variation and was located on chromosome C04. Seven potential genes related to frost tolerance and abiotic stress tolerance were identified.

**Keywords:** *Brassica napus*, frost tolerance, field, genome-wide association scan

## 4.2. Introduction

Rapeseed/canola (*Brassica napus*, AACC, 2n = 4x = 38) is an important crop worldwide. It is the second largest source of oil in the world (Foreign Agricultural Service, 2016). Canola evolved from two diploid species *B. rapa* L. (AA, 2n = 2x= 20) and *B. oleracea* L. (CC, 2n = 2x = 18) (Lagercrantz and Lydiate 1996; Lagercrantz 1998; U 1935). The genome size is consistent with the genome sizes of *B. oleracea* and *B. rapa* at about 1,130 Mb (Chalhoub *et al*., 2014).

According to growth habit, there are three types of canola; winter, spring, and semi-winter. North Dakota grows mainly the spring type, as the winters are too harsh for survival. Winter canola is mainly grown in Western Europe and seed yield can be 3.5 ton $ha^{-1}$ (Rakow, 2007). Winter canola requires vernalization to induce flowering and is therefore planted in the fall and harvested the following spring/summer. Semi-winter canola is grown in China, while the U.S. and Canada mainly produce spring canola (planted and harvested during the same growing season). Spring canola has a lower seed yield of 1.5 ton $ha^{-1}$. About 10% of the U.S. canola is winter type and is located mainly in Kansas and Oklahoma. North Dakota grows about 85% of U.S. canola.

Many factors affect canola production, such as disease, insects, flooding, drought, heat stress, and frost. Late frost is a big concern for spring planting of canola, but is not a major concern for in the later growing season. Generally, May is considered as the frost-free period for North Dakota, but a later frost can happen. Canola is more susceptible to frost at the seedling stage. Depending on the severity, frost damage can cause wilting, bleaching, or death of the plant. Bleaching occurs due to phyto-oxidation of pigments in the leaves (Wilson, 1997) and wilting is caused by the loss of water in the leaves. Frost damage can be viewed after it occurs, but determining survival can take longer. Damage evaluations should be taken multiple times before determining survival. Frost damage will be worse at colder temperatures and less damage will occur at milder temperatures. Tolerance to frost would benefit growers as they would be able to plant earlier in the spring and not be concerned about crop damage. Planting earlier in the spring would help to utilize early season moisture and to avoid heat during flowering.

Genome-wide association scan (GWAS) is a powerful method used in quantitative traits (Rafalski, 2002; Yu and Buckler, 2006; Waugh *et al*., 2009; Visioni *et al*., 2013; Sun *et al*.,

2014). This method is more powerful than traditional QTL mapping. Linkage disequilibrium (LD) is an important concept of GWAS. Linkage disequilibrium is the measurement of the degree of non-random associations between traits and alleles at different loci (Zhu *et al*., 2008). Common alleles in a diverse population with recombination can be identified through GWAS.

The objective of this study was to screen a wide collection of germplasm accessions under natural freezing conditions in the field, and to find the genomic region controlling the freezing tolerant traits.

## 4.3. Materials and methods

### 4.3.1. Plant materials

A total of 153 germplasm accessions were used for freezing tolerance study under field condition including checks. The germplasm, minus checks, were obtained from the Germplasm Resources Information Network (GRIN) (http://www.ars-grin.gov/npgs/searchgrin.html) originated/obtained from 15 countries and included three growth habit types (spring, winter, and semi-winter) (Table A3). Sixty-nine were spring growth type, 50 were winter growth type, and 29 were semi-winter growth type. Before being used in this study, all accessions were self-pollinated in the greenhouse for 4 to 5 generations.

### 4.3.2. Experimental design

A total of eight experiments were conducted in the field of which 3 were in spring 2014, 1 in fall 2014, 1 in spring 2015, and 3 in fall 2015. The spring 2014 planting was seeding in pots in the field, while the rest of the experiments had the seeding done in the greenhouse and the seedlings were brought outside for freezing treatment. The experiments were planted multiple times to synchronize with the desired field freezing temperature. In each experiment, all germplasm accessions were planted in the greenhouse in a randomized complete block design

with two replications, and three plants per germplasm per replication. Plants were allowed to grow for 14 days in the greenhouse at 20°C with a 16 h photoperiod. Natural sunlight was supplemented with 400 W HPS PL 2000 lights (P.L. Light Systems Inc.). The seedlings were fertilized with 20-20-20 liquid fertilizer. After 14 days, the seedlings were moved to a vernalization chamber for cold acclimation. The chamber was set at 4°C with a 12 h photoperiod for at least seven days. The light was provided by GE Ecolux F32T8 SP35 Eco (32 W T8) style bulbs (General Electric Company). Based on predicted outside temperature of -4°C to -6°C, the individual sets were brought outside for natural freezing shock. After overnight outside freezing, a damage score was conducted three, six, and nine days after the shock. Each plant was scored individually using a 0 to 5 scale according to Fiebelkorn and Rahman (2016), where 0 denoted dead, 5 denoted no damage, and scores of 1–4 were based on visual estimation of freezing damage.

### 4.3.3. Statistical analysis

A one-way analysis of variance was calculated using SAS® 9.3 (SAS Institute Inc., Cary, NC, USA, 2012). The analysis was conducted using the medians. Nonparametric statistics were run in SAS® 9.3 (SAS Institute Inc., Cary, NC, USA, 2012) to calculate the relative effect and the 95% confidence interval. Data normality was tested using R 3.2.4 (The R Foundation). The Shapiro-Wilk test was used to test normality. Broad-sense heritability was calculated.

### 4.3.4. DNA extraction and SNP identification

Qiagen DNeasy kit (Qiagen, CA, US) was used to extract the DNA. Genotyping-by-sequencing (GBS) was conducted at the Institute of Genome Diversity (IGD) at Cornell University. GBS libraries were prepared and analyzed according to Elshire *et al*. (2011). The enzyme *Ape*KI was used for digestion and library was created with 96 unique barcodes. Illumina

GAII sequencer was used to sequence the genotypes. The marker data comes from a subset of SNPs obtained from 366 genotypes with a total of 42,575 SNPs obtained. Alignment of the GBS data was done using bwa-mem (Li, 2013) and VarScan (Koboldt *et al*., 2012) was used to perform multi sample SNP calling. FastPHASE 1.3 (Scheet and Stephens, 2006) was used to further impute the SNPs using the default settings. Markers with less than 5% minor allele frequency were removed.

**4.3.5. Population structure**

The number of subpopulations in the germplasm was determined by using a random subset of 3,135 SNPs that were randomly distributed across the 19 linkage groups. STRUCTURE 2.3.4 (Pritchard Lab, Stanford University) was used to perform the analysis. Three iterations were run using K - values of 1 to 10. Each iteration had a burn-in period of 100,000 iterations and 200,000 iterations of MCMC (Markov Chain Monte Carlo). The optimal K - value was determined by calculating and plotting the natural log probability [LnP(D)] of the data and the delta K (ΔK) statistic. The ΔK is based on the rate of change of LnP(D) between the K - values (Evanno *et al*., 2005).

**4.3.6. Markers and minor allele frequency**

A total of 42,575 markers were obtained from the 147 genotypes that were used for genome-wide association scan (GWAS). Markers were removed if they had less than 5% minor allele frequency which left 37,111 markers to be used for GWAS. TASSEL 5.0 (Bradbury *et al*., 2007) was used for the GWAS analysis.

**4.3.7. Linkage disequilibrium (LD) decay**

Linkage disequilibrium (LD) decay was calculated for each subgenome (A and C) and each chromosome (A01-C09) using TASSEL 5.0 (Bradbury *et al*., 2007) and R 3.2.4 (The R

Foundation). The partial squared allele frequency correlation coefficient ($r^2$) was used to estimate LD (Pritchard and Przeworski, 2001; Kraakman *et al*., 2004). The coefficient ($r^2$) is between pairs of biallelic markers. An $r^2 = 0.2$ was used for this study.

### 4.3.8. Model selection

Population structure was controlled by a principal component analysis (Price *et* al., 2006). Principle components (PCs) were estimated using TASSEL 5.0 (Bradbury *et al*., 2007). Principal components that explained 25% ($PC_3$) and 50% ($PC_{22}$) of the variation was used in the regression model to control for population structure. An identity by state matrix was estimated in TASSEL 5.0 (Bradbury *et al.*, 2007) to account for relatedness between genotypes (Zhao *et al*., 2007). Six models were used to determine the model with the lowest mean square difference (MSD) which was determined to be the best model for further analysis. The models were naïve, $PC_3$, $PC_{22}$, Kinship, $PC_3$+Kinship, and $PC_{22}$+Kinship. The MSD is determined by using the observed and expected p-values (Mamidi *et al*., 2011).

### 4.3.9. Association mapping

Significant markers were determined based on the p-value and is the 0.1 percentile tail of 10,000 bootstraps (Mamidi *et al*., 2014; Gurung *et al*., 2014). The significant markers were run through stepwise regression to define the major QTL and to estimate the combined variation ($r^2$) that was explained by the significant markers (Mamidi *et al*., 2014; Gurung *et al*., 2014). R 3.2.4 (The R Foundation) was used to plot the $-\log_{10}(p)$ values on a Manhattan plot. A QQ plot was constructed in R 3.2.4 (The R Foundation) using the oberseved and expected $-\log_{10}(p)$ values.

### 4.3.10. Candidate gene search

The TAIR 10 protein database was used to determine the annotation of the selected region of the significant markers. A 100 kb region sequence on both sides were blasted to find

the candidate genes. Gene models are based on the genome and gene models published by Chalhoub *et al*. (2014). Genes of interest had their functions identified by searching published literature to find functions associated with frost and abiotic stress tolerance.

## 4.4. Results

### 4.4.1. Field experiments

In the spring of 2014, three experiments were planted outside on 10 April, 25 April, and 10 May. The seeds did not germinate until after the last frost and the plants were not affected. In October 2014 three sets were set outside on October 30. The temperature reached -10°C (NDAWN, 2017) (Supplementary Table A4) which was too cold and killed all plants. In March 2015, two sets were placed outside on 20 March. The temperature reached -9.7°C (NDAWN, 2017) (Table A4). Again, this was too cold and all plants were killed. In the fall of 2015, three experiments were set outside and two were used. The first set was placed outside on 16 October when the low temperature overnight was -3.3°C (NDAWN, 2017) (Fig. 4.1 and Table A4) which gave an adequate screen. On 5 November, a second set was placed outside when the minimum temperature was above 0 (NDAWN, 2017) (Table A4) which did not cause damage to the plants. On 12 November, a third set was placed outside and the minimum temperature was -4.5°C (NDAWN, 2017) (Fig. 4.2. and Table A4). This data was kept as the temperature was adequate for the screen.

### 4.4.2. Phenotypic results

The medians of the readings were calculated and used as the phenotypic score. The scores ranged from 1 to 5. The medians for the field had some variations, but were not significant (Table 4.1). The overall medians for the genotypes ranged from 1 to 5 with a common median of 3.5 (Table A5). The broad-sense heritability for the field study was 0.078. The

frequency of the medians for the field study were plotted based on the number of genotypes that fit each value (Fig. 4.3). The Shapiro-Wilk normality test indicated non-normality as the p-value is less than 0.05 (p = 8.72e-6).

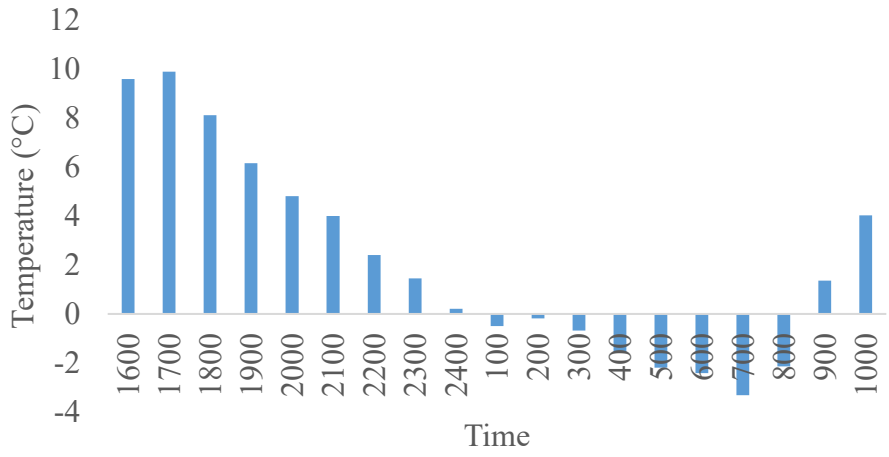


Fig. 4.1. Temperatures of the field screening from 15-16 October 2015. Plants were placed outside at 4:00 pm (1600 hours) and brought back inside at 10:00 am (1000 hours). Data obtained from NDAWN, 2017.

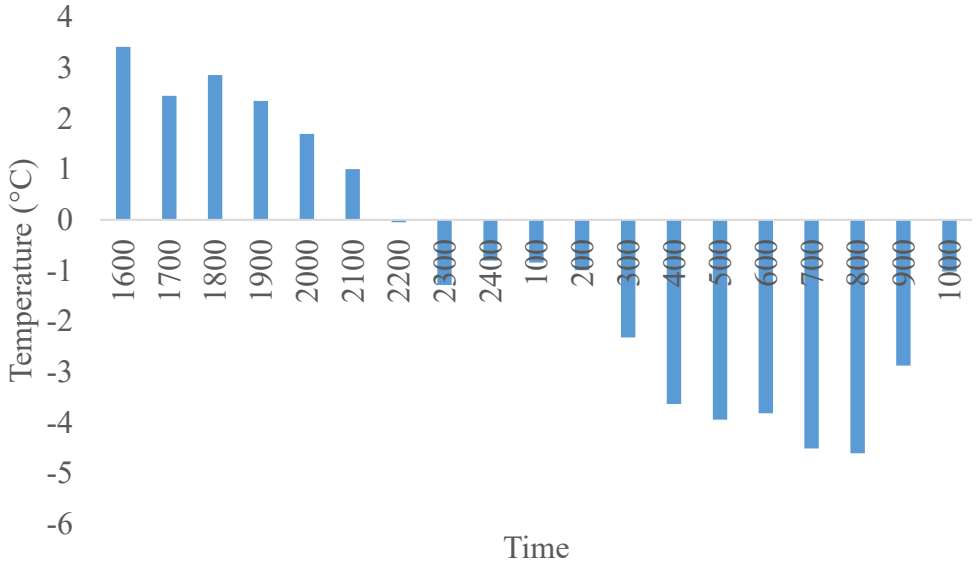


Fig. 4.2. Temperatures of the field screening from 12-13 November 2015. Plants were placed outside at 4:00 pm (1600 hours) and brought back inside at 10:00 am (1000 hours). Data obtained from NDAWN, 2017.

Table 4.1. ANOVA for the field experiment using nonparametric statistics.

| Effect | Num DF | Den DF | Chi-Square | F Value | Pr > ChiSq | Pr > F |
|---|---|---|---|---|---|---|
| Genotype | 149 | 152 | 171.98 | 1.15 | 0.0957$^{ns}$ | 0.1898$^{ns}$ |

ns, not significant

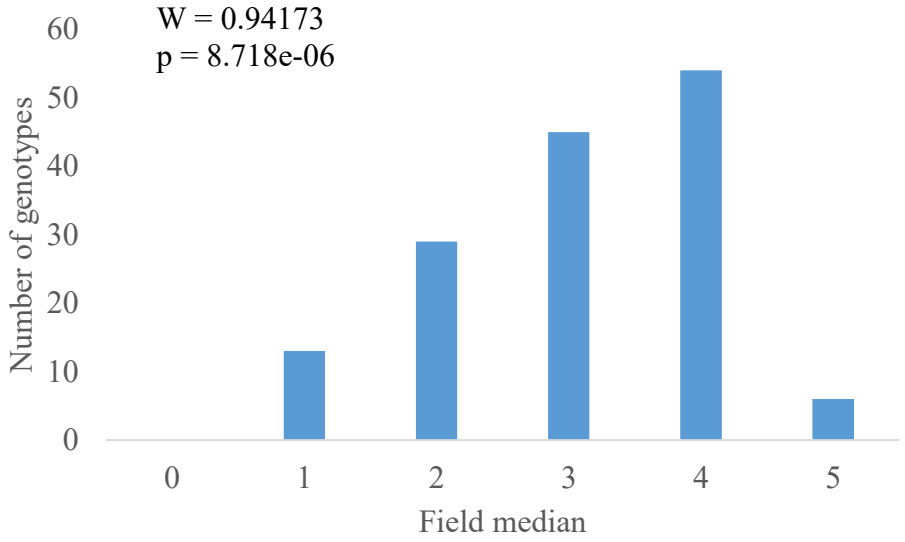


Fig. 4.3. Normality of the median data from the field. The x-axis is the medians and the y-axis is the frequency.

### 4.4.3. Estimated relative effect

The estimated relative effect was calculated along with the 95% confidence interval (Fig. 4.4 and Table A5). The relative effect ranges from 0 to 1, where genotypes closer to 1 are better. The plants were scored where a 5 = no damage and a 0 = dead which means that higher numbers have better survival and therefore, the relative effect should be higher. The confidence interval indicates that 95% of the time the genotype will fall in that range. The confidence intervals were very wide in this study which may have been due to environmental variation.

The mean rank was also calculated (Table A5). The mean rank is higher when the genotype performed better under frost and was more tolerant. Susceptible genotypes had a lower rank as the medians were typically lower. The mean rank is calculated by ranking all the medians

from low to high and taking the mean of the ranks. Five commercial checks were used in the field study. The commercial checks had variation among them and some of the tested lines performed better than the checks. The median, mean rank, and estimated relative effect was higher in some of the tested lines which indicates that these lines have better frost tolerance than the commercial hybrids that were tested in this study.

### 4.4.4. Population structure

A subset of 3,135 markers selected randomly from the 19 linkage groups were used for analysis of population structure. The genotypes could be assigned into three subpopulations based on the LnP(D) and ΔK (Fig. 4.5). LnP(D) and ΔK were described by Evanno *et al*. (2005) and were used to calculate the number of subpopulations. The ΔK shows a clear peak at K = 3.

### 4.4.5. Linkage disequilibrium (LD) decay

The A and C genomes and each of the 19 chromosomes (A01-C09) had LD decay calculated. Pritchard and Przeworski (2001) and Kraakman *et al*. (2004) determined that the coefficient, $r^2$, is the squared correlation coefficient between pairs of biallelic markers and is commonly used as a measure for LD mapping. The cutoff for this study was $r^2 = 0.2$. The physical distance (kb) for the A genome was about 37 kb (Fig. 4.6) while the C genome was about 201 kb (Fig. 4.7). The same $r^2 = 0.2$ was used for the individual chromosome calculation. The chromosomes in the A genome has shorter LD decay distances (kb) than the C genome chromosomes (Fig. 4.8). The chromosomes in the A genome ranged from 24-69 kb (Fig. A20-A29) while the C genome ranged from 78-511 kb (Fig. A30-A38).

### 4.4.6. Markers, minor allele frequency, and genome-wide association scan

After filtering and removing minor allele frequencies (<5%) a total of 37,111 markers were identified. To account for cumulative variation of 25% and 50%, 3 and 22 PCs,

respectively, were used. These PCs were used to control population structure in the model. The first PC ($PC_1$) explains about 15% of the variation and the second PC ($PC_2$) explains about 9% of the variation. The first two PCs clustered the genotypes into groups (Fig. 4.9). The groups were random and not by growth type.

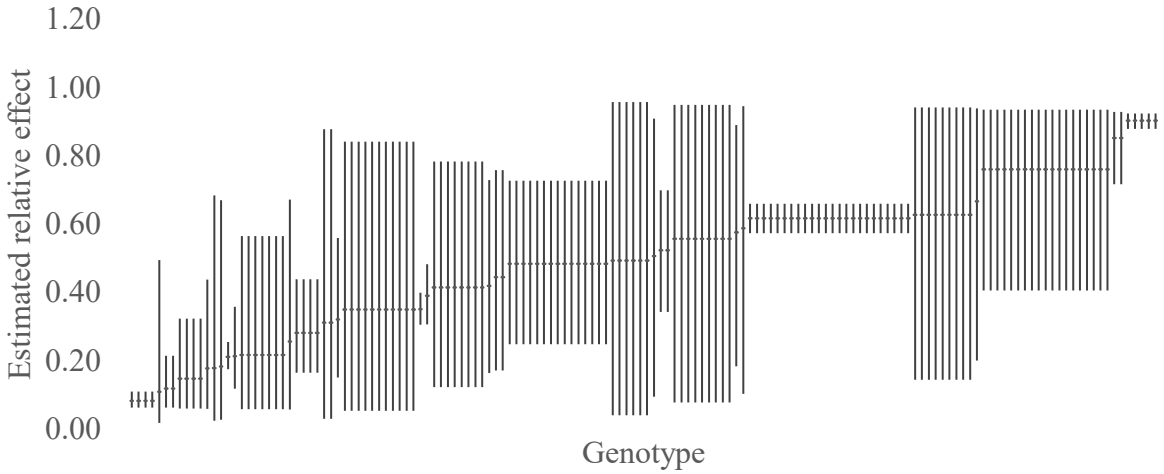


Fig. 4.4. Estimated relative effect and 95% confidence interval for the medians from the field study. Genotypes on the right side of the graph are considered more tolerant while the left side is more susceptible. The estimated relative effect is the dark black spot in on the graph and the confidence interval is the bar.

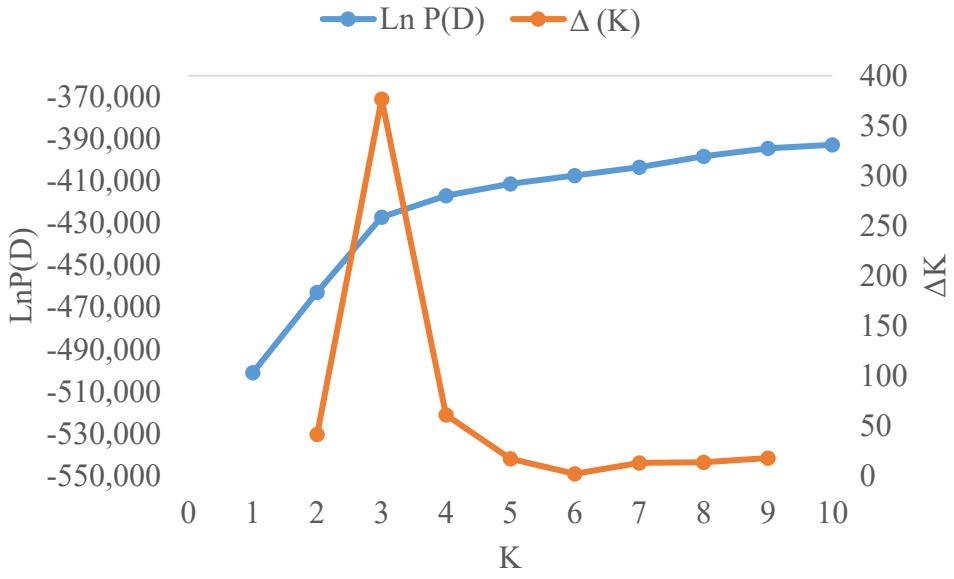


Fig. 4.5. The natural log probability [LnP(D)] and delta K (ΔK) for each value of K averaged over three iterations with 100,000 and 200,000 burn-in and MCMC, respectively. The values of K=1-10 were tested, where the K=3 is the number of subpopulations chosen in the population.

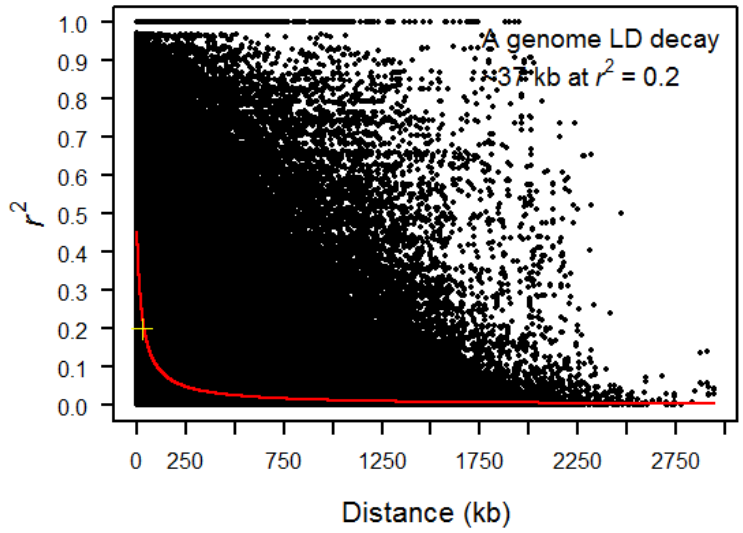


Fig. 4.6. LD decay for the A genome. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2$ = 0.2 is about 37 kb.

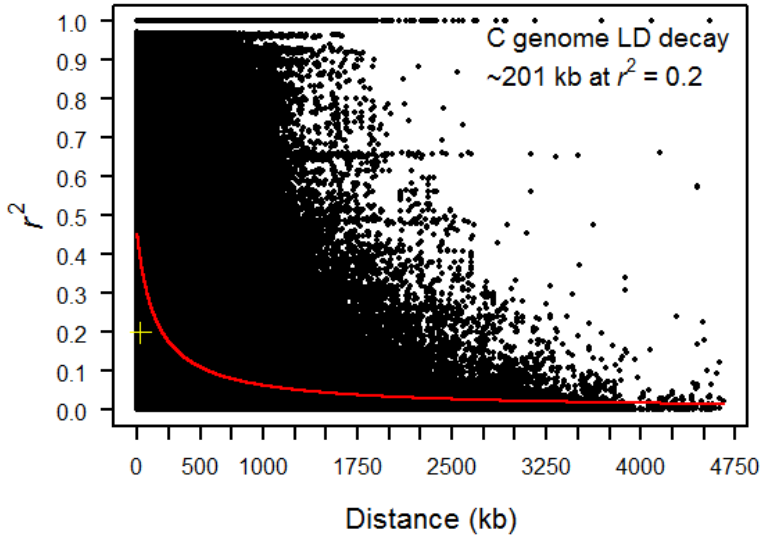


Fig. 4.7. LD decay for the C genome. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2$ = 0.2 is about 201 kb.

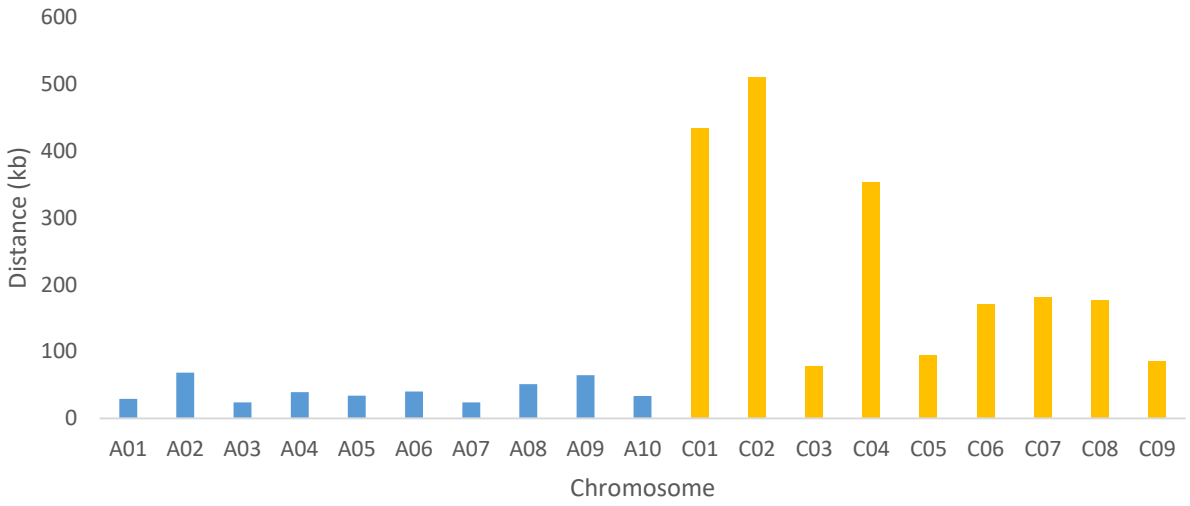


Fig. 4.8. LD decay for 19 chromosomes in canola. The x-axis is the chromosome and the y-axis the physical distance (kb) for the chromosomes at a threshold of $r^2$=0.2.

Of the six models tested, the $PC_3$ model was identified as the best model based on the mean square difference (MSD). Thirty-eight markers were considered significant based on 10,000 bootstraps and the 0.1% tail of the empirical distribution (Table 4.2). The p-value cutoff is p = 0.001526. Stepwise regression selected one marker on chromosome C04 (38.920 Mbp). The phenotypic variation explained by this marker is 5%.

The $-\log_{10}(p)$ values were plotted in a Manhattan plot (Fig. 4.10). The significant markers are above the dashed blue line ($-\log_{10}(p) = 2.82$). Not all the 38 markers are present above the line because the markers present on Ann_rand, Cnn_rand, and Unn_rand were removed for the analysis as they were not assigned to any chromosome based on the *B. napus* reference genome (Chalhoub *et al*., 2014).

The QQ plot based on the observed and expected $-\log_{10}(p)$ indicated the fitness of the model (Fig. 4.11). The top SNP (chrC04_38919755) is listed on the graph. The most significant SNPs vary from the line.

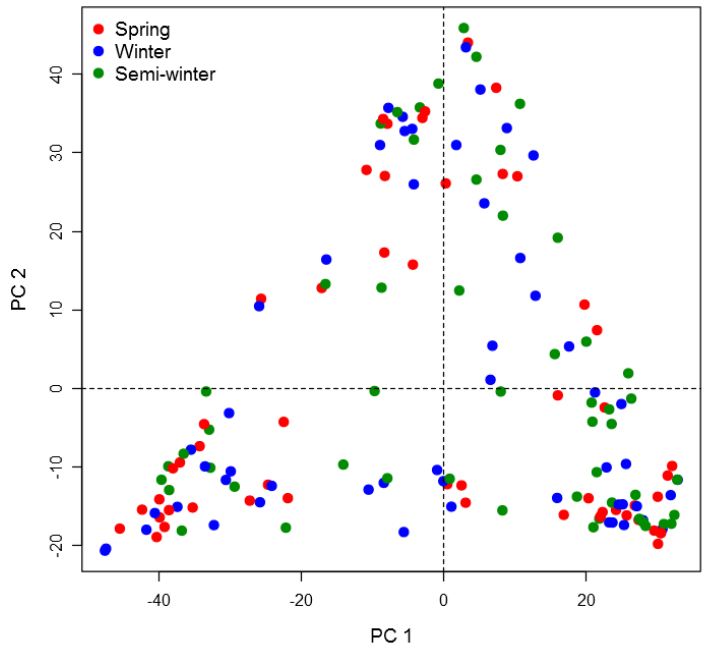


Fig. 4.9. PCA analysis for the medians from the field study on frost tolerance. $PC_1$ and $PC_2$ are evaluated against each other to evaluate population groupings. $PC_1$ explains about 15% of the variation and $PC_2$ explains about 9% of the variation. $PC_1$ is on the x-axis and $PC_2$ is on the y-axis.

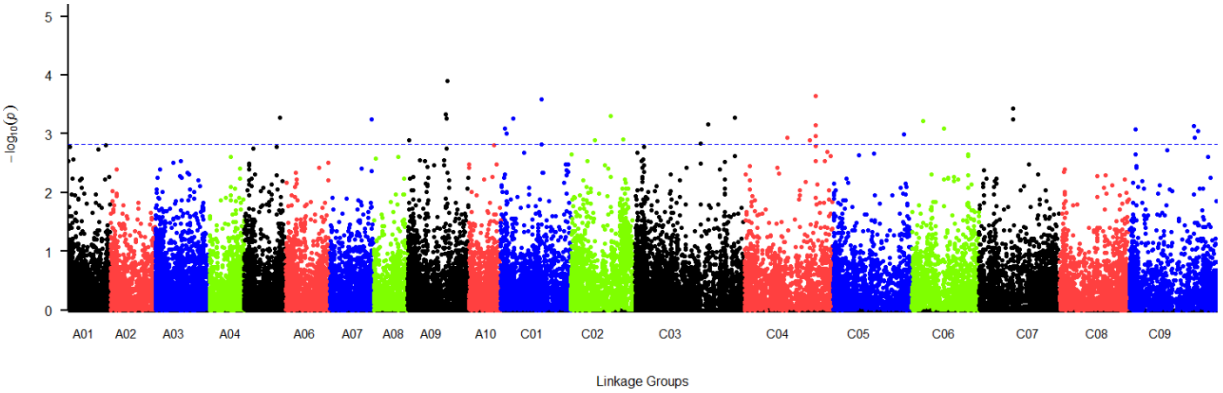


Fig. 4.10. Manhattan plot for the field study. The x-axis s the chromosomes and the y-axis is the $-\log_{10}(p)$. The values above the horizontal dashed line are the significant SNPs chosen through bootstrapping and based on the Empirical distribution. The dashed line is at 2.82. The GWAS model is $PC_3$.

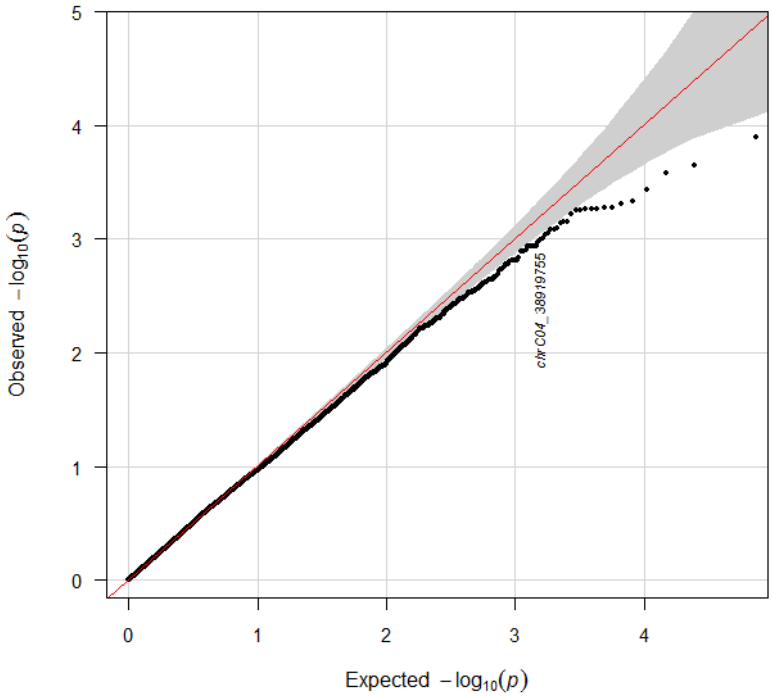


Fig. 4.11. QQ plot of the field medians from the $PC_3$ model. The x-axis is the expected -$\log_{10}$(p) and the y-axis is the observed -$\log_{10}$(p).

The significant marker along with a 100 kb region on each side that was selected through stepwise regression was run through BLAST and compared with the *Arabidopsis thaliana* proteome (TAIR 10). Annotated genes underwent a literature search to determine functions that were related to frost tolerance or abiotic stress. The candidate genes identified are genes associated with frost tolerance or abiotic stress tolerance/response (Table 4.3). Some of the functions of candidate genes include protein aggregation dissolution, negative regulator early in ABA signal transduction, involvement in abiotic stress-inducible gene regulation, and encodes a dehydration-responsive element-binding (DREB) superfamily.

Table 4.2. Significant markers for the field study that were selected based on the 0.1% tail of the Empirical distribution. The reference allele, alternate alleles, and the heterozygote had the means calculated for each marker.

| Marker | Chromosome | Position | Reference allele/Alternate allele | Reference allele | | Alternate allele | | Heterozygote (H) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Obs | Mean | Obs | Mean | Obs | Mean |
| chrA05_19818897 | A05 | 19818897 | A/T | 108 | 3.32 | 19 | 3.55 | 20 | 3.28 |
| chrA07_22705786 | A07 | 22705786 | T/C | 61 | 3.28 | 33 | 3.23 | 53 | 3.49 |
| chrA09_20760335 | A09 | 20760335 | G/C | 67 | 3.47 | 36 | 3.44 | 44 | 3.07 |
| chrA09_21506510 | A09 | 21506510 | T/C | 114 | 3.41 | 15 | 3.57 | 18 | 2.76 |
| chrA09_21667604 | A09 | 21667604 | G/A | 109 | 3.35 | 19 | 3.30 | 19 | 3.38 |
| chrA09_527522 | A09 | 527522 | G/T | 120 | 3.37 | 14 | 2.96 | 13 | 3.50 |
| chrAnn_rand_17804038 | Ann_rand | 17804038 | C/T | 117 | 3.42 | 12 | 3.38 | 18 | 2.82 |
| chrC01_2250328 | C01 | 2250328 | C/T | 118 | 3.38 | 12 | 3.92 | 17 | 2.69 |
| chrC01_22692192 | C01 | 22692192 | C/T | 102 | 3.21 | 22 | 3.48 | 23 | 3.82 |
| chrC01_22692197 | C01 | 22692197 | T/G | 102 | 3.21 | 22 | 3.48 | 23 | 3.82 |
| chrC01_22692215 | C01 | 22692215 | C/T | 102 | 3.21 | 22 | 3.48 | 23 | 3.82 |
| chrC01_22692221 | C01 | 22692221 | T/C | 103 | 3.22 | 22 | 3.48 | 22 | 3.78 |
| chrC01_3340581 | C01 | 3340581 | G/T | 125 | 3.35 | 10 | 3.18 | 12 | 3.44 |
| chrC01_6790091 | C01 | 6790091 | T/C | 82 | 3.27 | 29 | 3.47 | 36 | 3.41 |
| chrC02_13271272 | C02 | 13271272 | A/G | 110 | 3.42 | 17 | 3.10 | 20 | 3.16 |
| chrC02_22121079 | C02 | 22121079 | A/G | 108 | 3.47 | 18 | 2.86 | 21 | 3.14 |
| chrC02_28801735 | C02 | 28801735 | T/C | 72 | 3.30 | 35 | 3.22 | 40 | 3.54 |
| chrC03_36281982 | C03 | 36281982 | C/T | 77 | 3.22 | 34 | 3.30 | 36 | 3.66 |
| chrC03_40219443 | C03 | 40219443 | G/A | 73 | 3.32 | 37 | 3.29 | 37 | 3.46 |
| chrC03_55129272 | C03 | 55129272 | C/A | 86 | 3.38 | 26 | 3.24 | 35 | 3.33 |
| chrC03_55129282 | C03 | 55129282 | G/A | 86 | 3.37 | 26 | 3.30 | 35 | 3.33 |

Obs = observations, SD = standard deviation
*Marker identified during stepwise regression
na = not calculated

Table 4.2. Significant markers for the field study that were selected based on the 0.1% tail of the Empirical distribution (continued). The reference allele, alternate alleles, and the heterozygote had the means calculated for each marker.

| Marker | Chromosome | Position | Reference allele/Alternate allele | Reference allele | | Alternate allele | | Heterozygote (H) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Obs | Mean | Obs | Mean | Obs | Mean |
| chrC04_23256454 | C04 | 23256454 | G/A | 98 | 3.42 | 23 | 3.27 | 26 | 3.13 |
| chrC04_35956367 | C04 | 35956367 | G/A | 96 | 3.21 | 26 | 3.40 | 25 | 3.80 |
| chrC04_38919755* | C04 | 38919755 | A/T | 83 | 3.58 | 26 | 3.10 | 38 | 3.00 |
| chrC04_38919768 | C04 | 38919768 | C/G | 79 | 3.57 | 28 | 3.09 | 40 | 3.08 |
| chrC04_38919797 | C04 | 38919797 | A/T | 84 | 3.56 | 26 | 3.10 | 37 | 3.03 |
| chrC05_38816924 | C05 | 38816924 | T/A | 115 | 3.42 | 15 | 3.42 | 17 | 2.81 |
| chrC06_17935061 | C06 | 17935061 | A/G | 81 | 3.25 | 33 | 3.67 | 33 | 3.25 |
| chrC06_6479434 | C06 | 6479434 | G/C | 93 | 3.35 | 25 | 3.22 | 29 | 3.43 |
| chrC07_18646925 | C07 | 18646925 | T/C | 67 | 3.19 | 33 | 3.44 | 47 | 3.50 |
| chrC07_18793743 | C07 | 18793743 | A/C | 77 | 3.52 | 24 | 3.38 | 46 | 3.04 |
| chrC09_35631039 | C09 | 35631039 | C/T | 107 | 3.31 | 19 | 3.33 | 21 | 3.56 |
| chrC09_35884498 | C09 | 35884498 | A/G | 116 | 3.26 | 14 | 3.32 | 17 | 3.96 |
| chrC09_35884500 | C09 | 35884500 | C/G | 116 | 3.26 | 14 | 3.32 | 17 | 3.96 |
| chrC09_35884504 | C09 | 35884504 | C/T | 116 | 3.26 | 14 | 3.32 | 17 | 3.96 |
| chrC09_35884552 | C09 | 35884552 | C/G | 116 | 3.26 | 14 | 3.32 | 17 | 3.96 |
| chrC09_38011140 | C09 | 38011140 | A/G | 65 | 3.32 | 39 | 3.18 | 43 | 3.54 |
| chrC09_rand_3334214 | C09_rand | 3334214 | T/C | 83 | 3.37 | 32 | 3.12 | 32 | 3.50 |

Obs = observations, SD = standard deviation
*Marker identified during stepwise regression
na = not calculated

## 4.5. Discussion

Frost tolerance is important in any crop, but especially canola in North Dakota. Canola is typically grown in the northern part of the state where late frosts can be more of a concern. Frost tolerance is a difficult trait as it is quantitative and temperature predictions can be difficult. Natural or field conditions can be used to study frost tolerance, but the temperature needed to adequately screen may not be obtainable. Artificial or greenhouse conditions can be a more effective way to obtain the adequate temperature. Greenhouse conditions can be controlled and monitored based on the desired temperature, whereas field conditions will vary depending on the day.

Frost tolerance in canola would benefit the growers. First, frost tolerance would allow for an earlier planting date, which would help to plant to utilize early season moisture. Second, the seedlings could better compete with early season weeds. Third, earlier planted crops can avoid high heat during flowering. Yield can be increased when high temperatures are avoided during flowering.

This study followed the general guidelines laid out by Fiebelkorn and Rahman (2016). The plants were grown and acclimated in controlled conditions. The difference came during frost exposure when the plants were placed outside in the field overnight instead of being exposed to frost in a growth chamber. The differences in the environment could be seen after frost exposure and was based on how the lines performed.

Variation in the field may not have been visible between genotypes because the temperature needed for frost is difficult to obtain in the field. Even though visible variation was present, the ANOVA did not show significance. The temperature needed for adequate frost was hard to obtain in the field as often the predicted temperature was either too warm or too cold. The greenhouse experiments showed significant variation. Strigens *et al*. (2013) conducted

chilling experiments in both the growth chamber and field using maize. The conclusion was that greenhouse or growth chamber experiments have better control of the environment which causes less variation. Lower variation within genotypes indicates more significant variation between genotypes. Greenhouse studies can be used to more accurately predict genetic differences than field studies because of the environmental variation.

### 4.5.1. Genome-wide association scan

Genome wide association scan (GWAS) is used for many mapping projects (Li *et al.*, 2011; Jia *et al.*, 2012). This method aims to identify common alleles in a diverse population. GWAS brings advantages over traditional QTL mapping, which is another method to identify trait-linked markers. Some advantages of GWAS include wider genetic variability of germplasm, historical recombination events can be utilized, and biparental populations do not need to be developed which saves time and money (Hansen *et al*., 2001; Kraakman *et al*., 2006). This study used 147 genotypes that had three different growth habits (spring, winter, and semi-winter) and were obtained from 15 countries. The genotypes were obtained from a wide collection of environments and had diverse genetic backgrounds. A clear consensus on the number of germplasm needed for GWAS has not been established. A larger number of germplasm typically encompass more recombination historically which is better for GWAS (Hong and Park, 2012). Many GWAS studies in *B.* napus have been conducted. Rezaeizad *et* al. (2011) used 49 diverse winter germplasm lines for seed phenolic compounds, six seed quality traits were evaluated on 89 winter lines (Gajardo *et* al., 2015), blackleg disease was evaluated on 139 spring, winter, and semi-winter lines (Rahman *et al*., 2016), branch angle was evaluated on 143 spring-, semi-winter- and winter-type accessions (Liu *et al*., 2016), seed germination and early vigor was evaluated using 248 winter-type lines (Hatzig *et al*., 2015), agronomic and seed quality traits

used 405 accessions of winter oilseed, winter fodder, swede, semi-winter, spring, spring fodder, and vegetable types (Körber *et al*., 2016), and 540 accessions of winter-, semi-winter-, spring- and vegetable-type were evaluated for fatty acid profile (Qu *et al*., 2017). This is a small of number of published GWAS papers.

The broad-sense heritability was calculated at 7% does not indicate that the progeny will inherit the trait from the parents. This suggests that frost tolerance could be controlled by many genes that each have a small effect. The technique used was GBS which allows for a large number of markers that are distributed across the genome (Elshire *et al*., 2011; Poland and Rife, 2012). By using the large number of SNPs, the whole genome should be covered.

Based on the structure analysis, three distinct clusters were identified in the population. This agrees with previous studies in *B. napus* (Hasan *et al*., 2008; Bus *et al*., 2011; Qian *et al*., 2014; Raman *et al*., 2014; Li *et al*., 2014). The three clusters were not associated with growth habit, but were considered mixed clusters. Bus *et al.* (2011), Qian *et al*. (2014), and Raman *et al*. (2014) all reported that growth habits made up the different clusters. However, mixed clusters were reported for *B. napus* in studies by Li *et al*. (2014) and Wang *et al*. (2014).

Confounding effects can cause false-positive associations between the marker and trait. These false-positive associations are often seen if structure is present in the population. Approaches can be used to correct for population structure, relatedness, or both (Pritchard *et al*., 2000a, 2000b; Falush *et al*., 2003; Yu *et al*., 2006). Six models were tested that included structure, relatedness, and both. The six models used were naïve, $PC_3$, $PC_{22}$, Kinship, $PC_3$+Kinship, and $PC_{22}$+Kinship. The models that included PCs were used to account for structure, kinship was used to account for relatedness, and PC+Kinship models were used to account for structure and relatedness. The best model based on the lowest MSD was the $PC_3$

model, which accounts for structure. The significant markers were selected based on the empirical distribution of p-values that was suggested by Mamidi *et al*. (2014). Stepwise regression was used to identify QTL peaks for marker assisted selection (MAS) (Mamidi *et al*., 2014). Stepwise regression was used to select markers that had a major effect and eliminate some markers that have a minor effect on the phenotype. Only one marker was selected for this study and it was located on chromosome C04.

The reference genome paper by Chalhoub *et al*. (2014) identified scaffolds that were not able to be orientated onto the map. Pseudochromosomes were named based on the sub-genome (A or C) they belonged to. Nineteen of these were named (chrA01_random - chrA10_random and chrC01_random - chrC09_random). Other scaffolds were unmapped, but assigned to a sub-genome and were called chrAnn_random and chrCnn_random. Other scaffolds, chrUnn_random, could not be mapped or assigned to a sub-genome. For this study, random was abbreviated to rand.

The germplasm accessions used in this study has low LD (Michalak *et al*., unpublished). Therefore, a 100 kb sequence on each side of the marker was selected for further analysis (Chalhoub *et al*., 2014). To identify genes that are associated with frost tolerance, the significant marker and the surrounding region was run through NCBI-BLAST with the *Arabidopsis thaliana* proteome. Seven potential genes were identified all of which were on chromosome C04. One of the identified genes was annotated as a heat shock protein and the function is involvement in protein aggregate dissolution (Hong and Vierling, 2000). Two genes were identified for the field that had a function that negatively regulates early ABA signal transduction (Nishimura *et al*., 2009). The annotation for this was protein phosphatase 2C family protein. Three of the genes were found to be involved with abiotic stress response or involved in abiotic stress inducible

gene regulation. The annotation for the gene involved with abiotic stress response was ubiquitin conjugating enzyme (Zhou *et al*., 2010). Two genes were involved in abiotic stress-inducible gene regulation (Stockinger *et al*., 1997; Jaglo-Ottosen *et al*., 1998; Kasuga *et al*., 1999; Gilmour *et al*., 2000; Haake *et al*., 2002; Zhang *et al*., 2005) and was annotated as AP2 domain transcription factor-like. The last gene of interest from the field study was annotated as an integras-type DNA-binding superfamily protein and has a function that encodes a DREB subfamily (Renak *et al*., 2012). DREB genes are dehydration responsive genes that have been linked to cold tolerance in previous studies. Many DREB/CBF (C repeat-binding proteins) factors have been linked to other abiotic stress including cold (Liu *et al*., 1998; Nakashima *et al*., 2000; Gao *et al*., 2002; Qin *et al*., 2004; Li *et al*., 2005; Vogel *et al*., 2005; Oh *et al*., 2007; Qin *et al*., 2007; Gutha and Reddy, 2008; Morran *et al*., 2011).

Knowing and understanding the genes involved in frost tolerance or other abiotic stresses are important for the breeding program. Knowing whether the genes are positively or negatively related to frost is also important when utilizing the genes in a breeding program. However, more research is needed to validate the genes and the roles they may play on frost tolerance and the usefulness in MAS for the breeding program.

**4.6. Conclusion**

Field studies on frost tolerance can be difficult as an adequate freezing temperature is needed for the study. It can be difficult in natural conditions to obtain the correct temperature without causing damage to other crops. Also, adequate temperatures are not always present after germination which further decreases damage to the plants. Germination must occur for the study to work as well. Greenhouse studies should be used to help decrease the environmental variation. This study identified genes that were involved in abiotic stress response, especially cold

tolerance. After analysis, possible genes were identified that were related to frost tolerance. A large population was screened to identify these genes. The information from this study will help to utilize frost tolerance and have the genetic background necessary for plants to survive the cold. Further research is needed to utilize these genes and introgress them into commercial cultivars.

Table 4.3. Candidate genes and their functions associated with cold stress.

| Brassica gene model | Marker | Gene start | Gene end | Marker distance from gene | A. thaliana equivalent | Gene annotation | Function | Reference |
|---|---|---|---|---|---|---|---|---|
| BnaC04g37500D | chrC04_38919755 | 38,842,969 | 38,843,793 | 75,962 | AT2g25030 | HSP100/ClpB | Involved in protein aggregate dissolution | Hong and Vierling (2000) |
| BnaC04g37510D | chrC04_38919755 | 38,845,252 | 38,847,432 | 72,323 | AT2G25070 | Protein phosphatase 2C family protein | Negative regulator early in ABA signal transduction | Nishimura *et al*. (2009) |
| BnaC04g37520D | chrC04_38919755 | 38,849,667 | 38,851,871 | 67,884 | AT2G25070 | | | |
| BnaC04g37650D | chrC04_38919755 | 38,933,580 | 38,935,207 | 13,825 | AT5G56150 | Ubiquitin-conjugating enzyme | Involved in abiotic stress response | Zhou *et al*. (2010) |

Table 4.3. Candidate genes and their functions associated with cold stress (continued).

| Brassica gene model | Marker | Gene start | Gene end | Marker distance from gene | A. thaliana equivalent | Gene annotation | Function | Reference |
|---|---|---|---|---|---|---|---|---|
| BnaC04g37670D | chrC04_38919755 | 38,952,054 | 38,952,550 | 32,299 | At5g67000 | AP2 domain transcription factor-like | Involved in abiotic stress-inducible gene regulation | Stockinger *et al*. (1997); Jaglo-Ottosen *et al*. (1998); Kasuga *et al*. (1999); Gilmour *et al*. (2000); Haake *et al*. (2002); Zhang *et al*. (2005) |
| BnaC04g37680D | chrC04_38919755 | 38,962,892 | 38,963,394 | 43,137 | At5g67000 | | | |
| BnaC04g37680D | chrC04_38919755 | 38,962,892 | 38,963,394 | 43,137 | AT5G67000 | Integrase-type DNA-binding superfamily protein | Encodes DREB subfamily | Renak *et al*. (2012) |

## 4.7. Literature cited


Bradbury, P.J., Z. Zhang, D.E. Kroon, T.M. Casstevens, Y. Ramdoss, and E.S. Buckler. 2007. TASSEL: software for association mapping of complex traits in diverse samples. Bioinformatics. 23(19):2633-2635. doi: https://doi.org/10.1093/bioinformatics/btm308.

Bus, A., N. Körber, R.J. Snowdon, and B. Stich. 2011. Patterns of molecular variation in a species wide germplasm set of *Brassica napus*. Theor Appl Genet. 123: 1413–1423.

Chalhoub, B., F. Denoeud, S. Liu, I.A.P Parkin, H. Tang, X. Wang, J. Chiquet, H. Belcram, C. Tong, B. Samans, M. Correa, C. Da Silva, J. Just, C. Falentin, C.S. Koh, I. Le Clainche, M. Bernard, P. Bento, B. Noel, K. Labadie, A. Alberti, M. Charles, D. Arnaud, H. Guo, C. Daviaud, S. Alamery, K. Jabbari, M. Zhao, P.P. Edger, H. Chelaifa, D. Tack, G. Lassalle, I. Mestiri, N. Schnel, M.-C. Le Paslier, G. Fan, V. Renault, P.E. Bayer, A.A. Golicz, S. Manoli, T.-H. Lee, V.H.D. Thi, S. Chalabi, Q. Hu, C. Fan, R. Tollenacre, Y. Lu, C. Battail, J. Shen, C.H.D. Sidebottom, X. Wang, A. Canaguier, A. Chauveau, A. Berard, G. Deniot, M. Guan, Z. Liu, F. Sun, Y.P. Lim, E. Lyons, C.D. Town, I. Bancroft, X. Wang, J. Meng, J. Ma, J.C. Pires, G.J. King, D. Brunel, R. Delourme, M. Renard, J.-M. Aury, K.L. Adams, J. Batley, R.J. Snowden, J. Tost, D. Edwards, Y. Zhou, W. Hua, A.G. Sharpe, A.H. Paterson, C. Guan, P. Wincker. 2014. Early allopolyploid evolution in the post-Neolithic *Brassica napus* oilseed genome. Science. 345(6199):950-953.

Elshire, R.J., J.C. Glaubitz, J.A. Poland, K. Kawamoto, E.S. Buckler, and S.E. Mitchell. 2011. A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. PLOS ONE. 6(5):e19379.

Evanno, G., S. Regnaut, and J. Goudet. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology. 14: 2611–2620.

Falush, D., M. Stephens, and J.K. Pritchard. 2003. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics 164:1567-1587.

Fiebelkorn D. and M. Rahman. 2016. Development of a protocol for frost-tolerance evaluation in rapeseed/canola (*Brassica napus* L.). The Crop Journal 4:147-152.

Gajardo, H.A., B. Wittkop, B. Soto-Cerda, E.E. Higgins, I.A.P. Parkin, R.J. Snowdon, M.L. Federico, and F.L. Iniguez-Luy. 2015. Association mapping of seed quality traits in *Brassica napus* L. using GWAS and candidate QTL approaches. Mol. Breeding. 35:143.

Gao, M.J., G. Allard, L. Byass, A.M. Flanagan, and J. Singh. 2002. Regulation and characterization of four CBF transcription factors from *Brassica napus*. Plant Mol. Biol. 49:459-471.

Gilmour, S.J., A.M. Sebolt, M.P. Salazar, J.D. Everard, and M.F. Thomashow. 2000. Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiol. 124:1854–1865.

Gurung, S., S. Mamidi, J.M. Bonman, M. Xiong, G. Brown-Guedira, and T.B. Adhikari. 2014. Genome-wide association study reveals novel quantitative trait loci associated with resistance to multiple leaf spot diseases of spring wheat. Plos One 9(9):e108179.

Gutha, L.R. and A.R. Reddy. 2008. Rice DREB1B promoter shows distinct stress-specific responses, and the overexpression of cDNA in tobacco confers improved abiotic and biotic stress tolerance. Plant Mol. Biol. 68:533-555.

Haake, V., D. Cook, J.L. Riechmann, O. Pineda, M.F. Thomashow, and J.Z. Zhang. 2002. Transcription factor CBF4 is a regulator of drought adaptation in Arabidopsis. Plant Physiol. 130:639–648.

Hansen, M., T. Kraft, S. Ganestam, T. Säll, and N.-O. Nilsson. 2001. Linkage disequilibrium mapping of the bolting gene in sea beet using AFLP markers. *Genetical Research*. 77(1):61–66.

Hasan, M., W. Friedt, J. Pons-Kuhnemann, N.M. Freitag, K. Link K, and R.J. Snowdon. 2008. Association of gene-linked SSR markers to seed glucosinolate content in oilseed rape (*Brassica napus ssp.napus*). Theor Appl Genet. 116: 1035–1049.

Hatzig, S.V., M. Frisch, F. Breuer, N. Nesi, S. Ducournau, M.-H. Wagner, G. Leckband, A. Abbadi, and R.J. Snowdon. 2015. Genome-wide association mapping unravels the genetic control of seed germination and vigor in *Brassica napus*. Frontiers in Plant Science, 6. doi: 10.3389/fpls.2015.00221.

Hong, E.P. and J.W. Park. 2012. Sample size and statistical power calculation in genetic association studies. Genom. Inform. 10:117–122.

Hong, S.-W. and E. Vierling. 2000. Mutants of *Arabidopsis thaliana* defective in the acquisition of tolerance to high temperature stress. PNAS. 97(8):4392-4397.

Jaglo-Ottosen, K.R., S.J. Gilmour, D.G. Zarka, O. Schabenberger, and M.F. Thomashow. 1998. Arabidopsis CBF1 overexpression induces COR genes and enhances freezing tolerance. Sci. 280:104–106.

Jia, L., W. Yan, C. Zhu, H.A. Agrama, A. Jackson, K. Yeater, X. Li, B. Huang, B. Hu, A. McClung, and D. Wu. 2012. Allelic analysis of sheath blight resistance with association mapping in rice. Plos One. 7(3):e32703.

Kasuga, M., Q. Liu, S. Miura, K. Yamaguchi-Shinozaki, and K. Shinozaki. 1999. Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nat. Biotechnol. 17:287–291.

Koboldt, D.C., Q. Zhang, D.E. Larson, D. Shen, M.D. McLellan, L. Lin, C.A. Miller, E.R. Mardis, L. Ding, and R.K. Wilson. 2012. VarScan 2: Somatic mutation and copy number alteration discovery in cancer by exome sequencing. Genome Res. 22:568-576.

Körber, N., A. Bus, J. Li, I.S.P. Parkin, B. Wittkop, R.J. Snowdon, and B. Stich. 2016. Agronomic and seed quality traits dissected by genome-wide association mapping in *Brassica napus*. Front. Plant Sci. 7:386. doi:https://doi.org/10.3389/fpls.2016.00386.

Kraakman, A.T.W., F. Martínez, B. Mussiraliev, F.A. van Eeuwijk, and R.E. Niks. 2006. Linkage disequilibrium mapping of morphological, resistance, and other agronomically relevant traits in modern spring barley cultivars. *Molecular Breeding*. 17(1):41–58.

Kraakman, A.T.W., R.E. Niks, P.M.M.M. Van den Berg, P. Stam, and F.A. Van Eeuwijk. 2004. Linkage disequilibrium mapping of yield and yield stability in modern spring barley cultivars. Genetics. 168:435-446.

Lagercrantz, U. 1998. Comparative mapping between *Arabidopsis thaliana* and *Brassica nigra* indicates that brassica genomes have evolved through extensive genome replication accompanied by chromosome fusions and frequent rearrangements. Genetics. 150:1217-1228.

Lagercrantz, U. and D.J. Lydiate. 1996. Comparative genome mapping in Brassica. Genetics. 144:1903-1910.

Li, F., B. Chen, K. Xu, J. Wu, W. Song, I. Bancroft, A.L. Harper, M. Trick, S. Liu, G. Gao, N. Wang, G. Yan, J. Qiao, J. Li, H. Li, X. Xiao, T. Zhang, and X. Wu. 2014. Genome-wide association study dissects the genetic architecture of seed weight and seed quality in rapeseed (*Brassica napus* L.). DNA Res. 21: 355–367.

Li, H. 2013. Aligning sequence reads, clone sequences, and assembly contigs with BWA-MEM. arXiv:1303.3997 http://arxiv.org/abs/1303.3997.

Li, X.P., A.G. Tian, G.Z. Luo, Z.Z. Gong, J.S. Zhang, and S.Y. Chen. 2005. Soybean DRE-binding transcription factors that are responsive to abiotic stresses. Theor. Appl. Genet. 110:1355-1362.

Li, X., W. Yan, H. Agrama, L. Jia, X. Shen, A. Jackson, K. Moldenhauer, K. Yeater, A. McClung , and D. Wu. 2011. Mapping QTLs for improving grain yield using the USDA rice minicore collection. Planta 234:347–361.

Liu, J., W. Wang, D. Mei, H. Wang, L. Fu, D. Liu, Y. Li, and Q. Hu. 2016. Characterizing variation of branch angle and genome-wide association mapping in rapeseed (*Brassica napus* L.). Front Plant Sci 7:21.

Liu, Q., M. Kasuga, Y. Sakuma, H. Abe, S. Miura, K. Yamaguchi-Shinozaki, and K. Shinozaki. 1998. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively in *Arabidopsis*. Plant Cell. 10:1391-1406.

Mamidi, S., R.K. Lee, R.J. Goos, and P.E. McClean. 2014. Genome-wide association studies identifies seven major regions responsible for iron deficiency chlorosis in soybean (*Glycine max*). Plos One 9(9):e107469.

Mamidi, S., S. Chikara, R.J. Goos, D.L. Hyten, D. Annam, S.M. Moghaddam, R.K. Lee, P.B. Cregan, and P.E. McClean. 2011. Genome-wide association analysis identifies candidate genes associated with iron deficiency chlorosis in soybean. Plant Genome 4:154–164.

Michalak de Jiménez, M., S. Mamidi, P.E. McClean. and M. Rahman. (Unpublished). Low level of linkage disequilibrium and population structure in a core collection of *Brassica napus* discovered using SNPs derived from GBS.

Morran, S., O. Eini, T. Pyvovarenko, B. Parent, R. Singh, A. Ismagul, S. Eilby, N. Shirley, P. Langridge, and S. Lopato. 2011. Improvement of stress tolerance of wheat and barley by modulation of expression of DREB/CBF factors. Plant Biotech. J. 9:230-249.

Nakashima, K., Z.K. Shinwari, Y. Sakuma, M. Seki, S. Miura, K. Shinozaki, and K. Yamaguchi-Shinozaki. 2000. Organization and expression of two *Arabidopsis* DREB2 genes encoding DRE-binding proteins involved in dehydration- and high-salinity-responsive gene expression. Plant Mol. Biol. 42:657-665.

Nishimura, N., A. Sarkeshik, K. Nito, S.-Y. Park, A. Wang, P.C. Carvalho, S. Lee, D.F. Caddell, S.R. Cutler, J. Chory, J.R. Yates, and J.I. Schroeder. 2010. PYR/PYL/RCAR family members are major in-vivo ABI1 protein phosphatase 2C-interacting proteins in Arabidopsis. The Plant J. 61:290–299. doi:10.1111/j.1365-313X.2009.04054.x.

Oh, S.J., C.W. Kwon, D.W. Choi, S.I. Song, and J.K. Kim. 2007. Expression of barley HvCBF4 enhances tolerance to abiotic stress in transgenic rice. Plant Biotechnol. J. 5:646-656.

Poland, J.A. and T.W. Rife. 2012. Genotyping-by-sequencing for plant breeding and genetics. The Plant Genome. 5(3):92-102.

Price, A.L., N.J. Patterson, R.M. Plenge, M.E. Weinblatt, N.A. Shadick, and D. Reich. 2006. Principal components analysis corrects for stratification in genome-wide association studies. Nature Genet. 38(8):904-909.

Pritchard, J.K. and M. Przeworski. 2001. Linkage disequilibrium in humans: models and data. Am. J. Hum. Genet. 69:1-14.

Pritchard, J.K., M. Stephens, and P. Donnelly. 2000a. Inference of population structure using multilocus genotype data. Genetics 155:945-959.

Pritchard, J.K., M. Stephens, N.A. Rosenberg, and P. Donnelly. 2000b. Association mapping in structured populations. Am. J. Hum. Genet. 676:170-181.

Qian, L., W. Qian, and R.J. Snowdon. 2014. Sub-genomic selection patterns as a signature of breeding in the allopolyploid *Brassica napus* genome. BMC Genomics. 15:1170. doi:10.1186/1471-2164-15-1170.

Qin, F., Y. Sakuma, J. Li, Q. Liu, Y.Q. Li, K. Shinozaki, and K.Y. Yamaguchi-Shinozaki. 2004. Cloning and functional analysis of a novel DREB1/CBF transcription factor involved in cold-responsive gene expression in *Zea mays* L. Plant Cell Physiol. 45:1042-1052.

Qin, Q.L., J.G. Liu, Z. Zhang, R.H. Peng, A.S. Xiong, Q.H. Yao, and J.M. Chen. 2007. Isolation, optimization, and functional analysis of the cDNA encoding transcription factor OsDREB1B in *Oryza sativa* L. Mol. Breed. 19:329-340.

Qu, C., L. Jia, F. Fu, H. Zhao, K. Lu, L. Wei, X. Xu, Y. Liang, S. Li, R. Wang, and J. Li. 2017. Genome-wide association mapping and Identification of candidate genes for fatty acid composition in *Brassica napus* L. using SNP markers. BMC Genomics 18:232.

Rafalski, A. 2002. Applications of single nucleotide polymorphisms in crop genetics. Curr. Opin. Plant Biol. 5:94-100.

Rahman, M., S. Mamidi, L. del Rio, A. Ross, M.M Kadir, M.M. Rahaman, and M. Arifuzzaman. 2016. Association mapping in *Brassica napus* (L.) accessions identifies a major QTL for blackleg disease resistance on chromosome A01. Mol. Breeding. 36:90. doi:10.1007/s11032-016-0513-8.

Rakow, G. 2007. Rapeseed genetics and breeding research for sustainable oilseed production. Proceedings of the 12th International Rapeseed Congress 1:2072-210.

Raman, H., J. Dalton-Morgan, S. Diffey, R. Raman, S. Alamery, D. Edwards, and J. Batley. 2014. SNP markers-based map construction and genome-wide linkage analysis in *Brassica napus*. Plant Biotech J. 12: 851–860.

Reňák, D., N. Dupl'áková, and D. Honys. 2012. Wide-scale screening of T-DNA lines for transcription factor genes affecting male gametophyte development in *Arabidopsis*. Sex Plant Reprod. 25:39-60. doi:10.1007/s00497-011-0178-8.

Rezaeizad, A., B. Wittkop, R. Snowdon, M. Hasan, V. Mohammadi, A. Zali, and W. Friedt. 2011. Identification of QTLs for phenolic compounds in oilseed rape (Brassica napus L.) by association mapping using SSR markers. Euphytica 177:335–342.

Scheet, P. and M. Stephens. 2006. A fast and flexible statistical model for large-scale population genotype data: applications to inferring missing genotypes and haplotypic phase. Am J. Human Genet. 78:629-644.

Stockinger, E.J., S.J. Gilmour, and M.F. Thomashow. 1997. Arabidopsis thaliana CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proc. Natl Acad. Sci. USA. 94:1035–1040.

Strigens, A., N.M. Freitag, X. Gilbert, C. Grieder, C. Riedelsheimer, T.A. Schrag, R. Messmer, and A.E. Melchinger. 2013. Association mapping for chilling tolerance in elite flint and dent maize inbred lines evaluated in growth chamber and field experiments. Plant, Cell, and Env. 36:1871-1887.

Sun, J., N. Guo, J. Lei, L. Li, G. Hu, and H. Xing. 2014. Association mapping for partial resistance to *Phytophthora sojae* in soybean (*Glycine max* L.). J. of Genetics. 2:355-363.

U, N. 1935. Genome analysis in *Brassica* with special reference to the experimental formation of *Brassica napus* and peculiar mode of fertilization. Japanese J. Botany. 7:389-452.

(USDA) United States Department of Agriculture. Updated 2016. Foreign Agricultural Service. Accessed: https://www.fas.usda.gov (26 March 2017).

Visioni, A., A. Tondelli, E. Francia, A. Pswarayi, M. Malosetti, J. Russell, W. Thomas, R. Waugh, N. Pecchioni, I. Romagosa, and J. Comadran. 2013.Denome-wide association mapping of frost tolerance in barley (*Hordeum vulgare* L.). BMC Genomics. 14:424-437. doi: http://www.biomedcentral.com/1471-2164/14/424.

Vogel, J.T., D.G. Zarka, H.A. Van Buskirk, S.G. Fowler, and M.F. Thomashow. 2005. Roles of CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of *Arabidopsis*. Plant J. 41:195-211.

Wang, N., F. Li, B. Chen, K. Xu, G. Yan, J. Qiao, J. Li, G. Gao, I. Bancroft, J. Meng, G.J. King, and X. Wu. 2014. Genome-wide investigation of genetic changes during modern breeding of *Brassica napus*. Theor Appl Genet. 127:1817–1829.

Waugh, R., J.L. Jannink, K. Muller, and L. Ramsay. 2009. The emergence of whole genome association scans in barley. Curr. Opin. Plant Biol. 12:1-5.

Wilson, J.M. 1997. Mechanisms of chilling resistance in plants. pp. 111-122. *In* A.S. Basra and R.K. Basra (eds). Mechanisms of environmental stress resistance in plants. Harwood Academic Publishers, Amsterdam.

Yu, J.M. and E.S. Buckler. 2006. Genetic association mapping and genome organization of maize. Curr. Opin. Biotech. 17:155-160.

Yu, J., G. Pressoir, W.H. Briggs, I.V. Bi, M. Yamasaki, J.F. Doebley, M.D. McMullen, B.S. Gaut, D.M. Nielsen, J.B. Holland, S. Kresovich, and E.S. Buckler. 2006. A unified mixed-model method for association mapping that accounts for multiple levels of relatedness. Nat. Genet. 38:203–208.

Zhang, J.-Y., C.D. Broeckling, E.B. Blancaflor, M.K. Sledge, L.W. Sumner, and Z.-Y. Wang. 2005. Overexpression of WXP1, a putative *Medicago truncatula* AP2 domain-containing transcription factor gene, increases cuticular wax accumulation and enhances drought tolerance in transgenic alfalfa (*Medicago sativa*). The Plant J. 42:689–707.

Zhao, K., M.J. Aranzana, S. Kim, C. Lister, C. Shindo, C. Tang, C. Toomajian, H. Zheng, C. Dean, P. Marjoram, and M. Nordborg. 2007. An Arabidopsis example of association mapping in structured samples. PLoS Genetics 3:e4. doi: http://dx.doi.org/10.1371/journal.pgen.0030004.

Zhou, GA., R.Z. Chang, and L.J. Qiu. 2010. Overexpression of soybean ubiquitin-conjugating enzyme gene GmUBC2 confers enhanced drought and salt tolerance through modulating abiotic stress-responsive gene expression in Arabidopsis. Plant Mol. Biol. 72:357-367. doi:10.1007/s11103-009-9575-x.

Zhu, J., J.C. Jeong, Y. Zhu, I. Sokolchik, S. Miyazaki, J.-K. Zhu, P.M. Hasegawa, H.J. Bohnert, H. Shi, D.-J. Yun, and R.A. Bressan. 2008. Involvement of *Arabidopsis* HOS15 in histone deacetylation and cold tolerance. PNAS.105(12):4945–4950.

# CHAPTER 5. PROTOCOL OPTIMIZATION FOR ELECTROLYTE LEAKAGE IN RAPESEED/CANOLA *(BRASSICA NAPUS* L.)

## 5.1. Abstract

Development of freezing tolerant germplasm is one of the important breeding objectives in the rapeseed/canola breeding program. Various methods can be used to screen germplasm for freezing tolerance. Electrolyte leakage measurement is one of the methods used for freezing induced tissue damage scoring of plants. A protocol is developed in this study to measure the electrolyte leakage for a large scale rapeseed germplasm screening under simulated freezing conditions. Nine rapeseed varieties, including three commercial hybrids, three spring types, and three winter types were used for this study. The seedlings were grown in a greenhouse for 14 days and cold acclimation at 4ºC was applied for 7 days in a plant growth chamber. The leaf samples were treated with four freezing temperatures (–4ºC, –8ºC, –12ºC, and –16ºC) for two hours each in a freezing water bath containing a 1:1 ratio of water and ethylene glycol. Electrolyte leakage was measured at two different times, the first measurement was two hours after freezing treatment, and the second measurement was taken after overnight freezing (-20ºC) and thawing. Lethal temperature 50 (LT50) was calculated using the percent leakage. Differences were present between genotypes and temperature. We did not find growth habit type (winter, spring) electrolyte leakage difference among the germplasm. The suggested protocol for the electrolyte leakage study is 7 days of acclimation for 14 day old seedlings followed by freezing treatment at –12ºC for 2 hours.

**Key words**: *Brassica napus*, protocol, electrolyte leakage.

## 5.2. Introduction

Rapeseed/canola (*Brassica napus* L.) is the second most important oilseed crop worldwide. Based on growing season, rapeseed is classified as spring, winter, and semi-winter types (Ferreira *et al*., 1995). The spring type is planted in the spring, flowers without vernalization in the summer, and is harvested at the end of summer. The winter type is planted in the fall, covered with snow (vernalization) during the winter to induce flowering the following spring, and harvested in the summer. The semi-winter types are intermediate of winter and spring types. They do not have frost hardiness like winter canola, therefore, they are cultivated in the moderate winter temperature region like Central China (Wang *et al*., 2011).

North Dakota grows about 84% of U.S. canola and the majority of the crop is planted across the northeast and north central tier of the state. The crop growing area of North Dakota may be exposed to freezing temperature in late spring or early fall. Freezing temperatures during the early growing stage of rapeseed negatively affects plant growth and crop production that may destroy the whole crop (Chinnusamy *et al*., 2007). Typically, frost is seen on the leaves, which may cause cellular dysfunction followed by wilting and bleaching of leaves that leads to plant death. Various factors such as moisture conditions, plant growth stage, cold severity, and duration of cold temperature are associated with the severity of frost damage. Canola seedlings are moderately tolerant to frost damage and can withstand a light spring frost that may cause leaves to wilt only and may survive over time.

Frost tolerance in canola is an important trait that has not been studied in depth. Multiple types of tests have been conducted to assess freezing/frost tolerance such as electrolyte leakage (Madakadze *et al*., 2003), protoplasmic streaming (Graham and Patterson, 1982), chlorophyll fluorescence (Hetherington *et al.*, 1989), and many others. Electrolyte leakage can occur when plants are exposed to freezing temperatures and the membranes are damaged or broken.

Osterhaut (1922) conducted the first test on electrolyte leakage from freezing and wounding treated plants. Since then these measurements are being used as a test for the stress-induced injury and for scoring of plants for stress tolerance (Blum and Ebercon, 1981; Bajji *et al.*, 2002; Lee and Zhu, 2010).

Electrolyte leakage measurements can help estimate the stress induced cell membrane damage in plants (Bajji *et al*., 2002). This measurement has been found to be correlated with many other physiological and biochemical functions in response to environmental stress. Electrolyte leakage measurements can be used to identify resistant cultivars to certain stresses (Leopold *et al*., 1981; Stevanoic *et al*., 1997; Bajji *et al*., 2002). This measurement has been identified as a good indicator of frost tolerance in crops (McCollum and McDonald, 1991). This is a quick test that can be conducted in a laboratory with a small amount of leaf tissue (Murray *et al*., 1989). Although it is a quick method to screen for freezing tolerance, this method has not been used in rapeseed/canola to study frost tolerance. Therefore, the present study has been conducted to develop a standard protocol to screen breeding population for electrolyte leakage in rapeseed/canola.

## 5.3. Materials and methods

### 5.3.1. Plant materials

Nine canola accessions/varieties including three winter type (Fashion, ARC 2180-1, and Galileo), three spring type open pollinated (NDSU 15-1000, Hi-Q, and Kanada), three spring type commercial hybrids (DKL 70-07, Pioneer 45H26, and Sprinter), were used for this study. The plants were grown in a greenhouse at 20°C with a 16-h photoperiod provided by natural sunlight supplemented with 400 W HPS PL 2000 lights (P.L. Light Systems Inc.).

### 5.3.2. Experimental design

The accessions/varieties were planted using a randomized complete block design. The seeds were sown in 10 cm (diameter) by 15 cm (depth) pots filled with Sunshine-Mix-1 (Sun Gro Horticulture) and watered every day. The Sunshine-Mix media was supplemented with 20-20-20 liquid fertilizer after 7 days. The seedlings were grown for 14 days in the greenhouse conditions and then transferred to a vernalization chamber for cold acclimation at a temperature of 4°C with a 12-h photoperiod provided by GE Ecolux F32T8 SP35 Eco (32 W T8) style bulbs (General Electric Company). After 7 days of cold acclimation in the vernalization chamber, the leaves from each germplasm were collected for electrolyte leakage testing. Four replications were used with four plants per accession per replication. A total of 576 seedlings (9 varieties × 4 plants/variety × 4 temperatures × 4 replications) were used for the study.

### 5.3.3. Electrolyte leakage

The electrolyte leakage test was conducted according to Lee and Zhu (2010) with minor modifications. Protocol modifications were made based on personal communication with Dr. David Horvath, USDA, Fargo, ND. A freezing water bath (Thermo Scientific, Isotemp Circulators/Bath 4100) with a lowest freezing temperature capacity of -16°C was used for this study. In the water bath, a 1:1 ratio of water and ethylene glycol was used. Four different freezing temperatures (-4°C, -8°C, -12°C, and -16°C) were used to find out the best freezing temperature at which the most electrolyte leakage occurs in canola leaves. Electrolyte leakage was measured in ppm. Leaf samples were placed into 15 mL test tubes with 100 µL of $dH_2O$. The test tubes were placed in a circulating freezing water bath with respective freezing temperature (-4°C, -8°C, -12°C, or -16°C) and held for two hours. The test tubes were removed from the water bath and allowed to thaw slowly over ice. The samples were then transferred to a

50 mL test tube containing 25 mL of $dH_2O$. The test tubes were placed on a shaker for two hours. The electrolyte leakage was measured using a conductivity meter (Primo, Hanna Instruments). The first conductivity reading was taken after two hours of shaking on a shaker.

After taking the first conductivity reading, the test tubes with leaf samples were placed in a freezer (-20°C) overnight. The samples were taken out of the freezer and placed on the table at room temperature and allowed to thaw. The second conductivity reading was taken on the samples when they reached at room temperature. The percent electrolyte leakage was calculated by dividing the first measurement by the second measurement.

### 5.3.4. Statistical analysis

A one-way analysis of variance was calculated using SAS® 9.3 (SAS Institute Inc., USA). An RCBD with a split-plot arrangement was used for this analysis, where A was temperature, and B was genotype.

## 5.4. Results

### 5.4.1. Percent leakage and lethal temperature 50 (LT50)

The percent leakage (measurement 1/measurement 2)*100 varied among the genotypes with regard to temperature. -4°C had the lowest percent leakage while -12°C or -16°C had the highest percent leakage (Fig. 5.1 a-i).

The ANOVA for the percent electrolyte leakage indicates that most of the factors including replication and temperature were significant (Table 5.1). The replication x temperature interaction was also significant. However, temperature x genotype and genotype were not significant. LSDs were calculated for A (freezing temperature) and B (genotype).

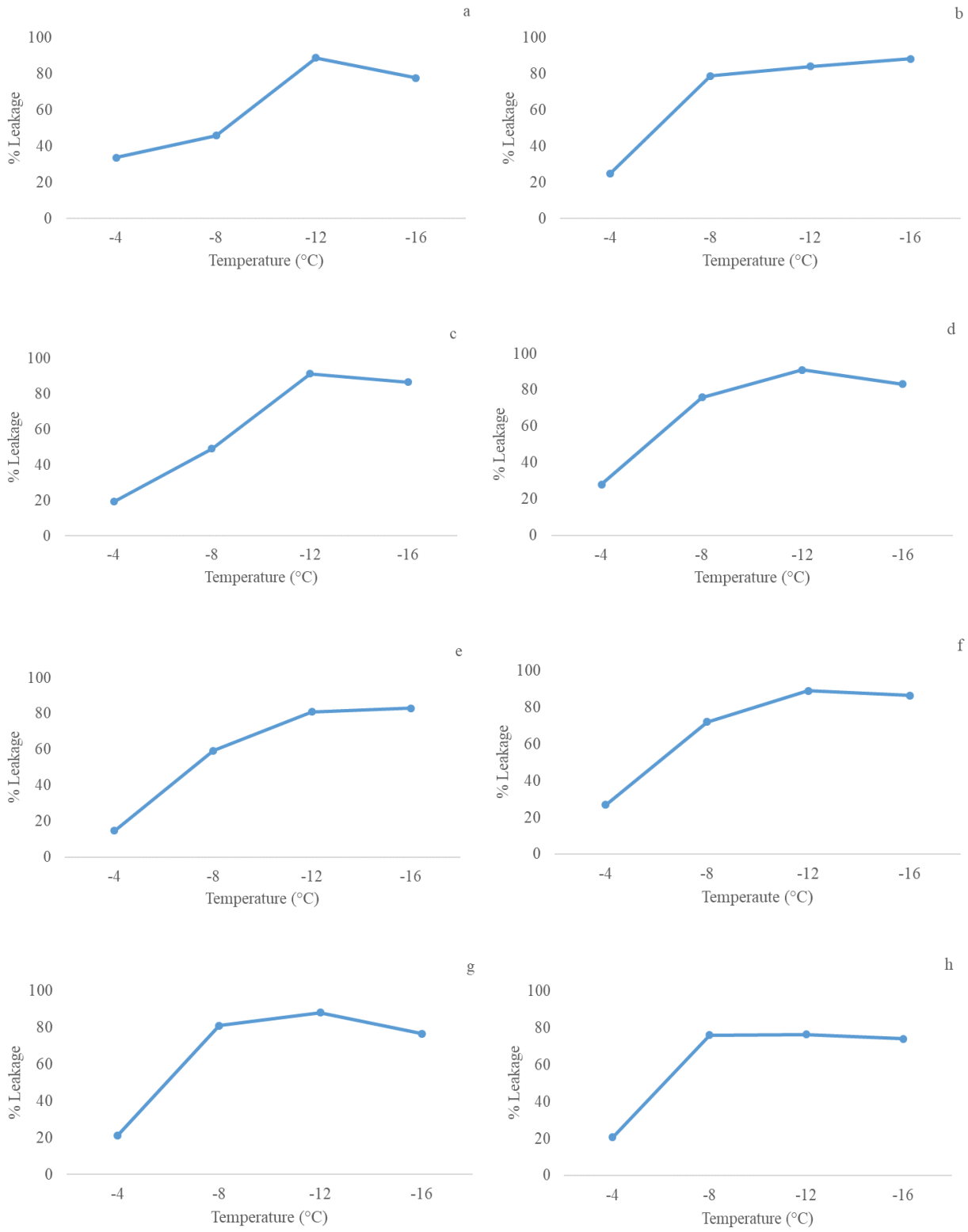


Fig. 5.1. The % Leakage for the nine genotypes tested at -4°C, -8°C, -12°C, and -16°C. The genotypre are a) DKL 70-07, b) NDSU 15-1000, c) Sprinter, d) Pioneer 45H26, e) Hi-Q, f) Kanada, g) Fashion, h) ARC 2180-1, and i) Galileo.

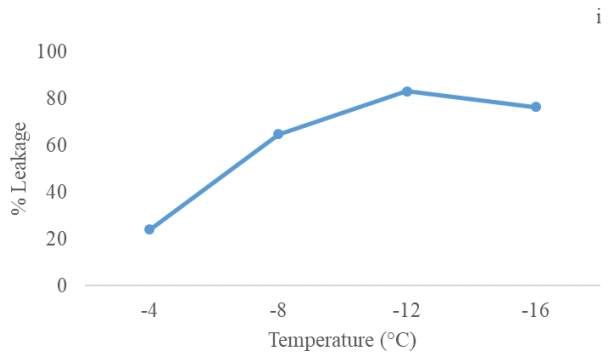


Fig. 5.1. The % Leakage for the nine genotypes tested at -4°C, -8°C, -12°C, and -16°C. The genotypre are a) DKL 70-07, b) NDSU 15-1000, c) Sprinter, d) Pioneer 45H26, e) Hi-Q, f) Kanada, g) Fashion, h) ARC 2180-1, and i) Galileo (continued).

Table 5.1. ANOVA for the percent electrolyte leakage study. The first measurement was divided by the second measurement.

| Source | DF | Anova SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| rep | 3 | 4847.1690 | 1615.7230 | 11.53 | <.0001*** |
| A | 3 | 86684.3849 | 28894.7950 | 206.26 | <.0001*** |
| rep*A | 9 | 4242.9215 | 471.4357 | 3.37 | 0.0013** |
| B | 8 | 1948.0515 | 243.5064 | 1.74 | 0.0993ns |
| A*B | 24 | 6120.1336 | 255.0056 | 1.82 | 0.0218ns |

Note: A = freezing temperature; B = genotype; ns = not significant; **= p < 0.01; ***= p < 0.001.

The percent leakage of the two coldest temperatures (-12°C and -16°C) (Table 5.2) did not show any significant difference among them. The two warmest temperatures (-4°C and -8°C) were significantly different from each other and from the other temperatures. The LSD was 0.1158 using $\alpha = 0.05$. The percent leakage for three genotypes had significance between genotypes (Table 5.3). The LSD was 0.0831 using $\alpha = 0.05$.

The lethal temperature 50 (LT50) was calculated for each genotype. The LT50 ranged from -5.8 to -8.2 (Table 5.4). This indicates that 50% percent of the plants in each genotype will be killed at these temperatures. The three commercial hybrids (DKL 70-07, Pioneer 45H26, and Sprinter) had LT50s of -8.2°C, -5.8°C, and -8.1°C, respectively. The three spring types (NDSU

15-1000, Hi-Q, and Kanada) had LT50s of -5.8°C, -7.1°C, and -6.0°C, respectively. The winter types Fashion, ARC 2180-1, and Galileo) had LT50 values of -5.9°C, -6.1°C, and -6.6°C, respectively.

Table 5.2. Effect of different freezing temperatures (A) on % leakage using $\alpha = 0.05$.

| Temperature | % Leakage | t Grouping | N |
|---|---|---|---|
| -12 | 85.863 | A | 36 |
| -16 | 81.374 | A | 36 |
| -8 | 67.008 | B | 36 |
| -4 | 23.751 | C | 36 |

Note: *means followed by the same letter are not significantly different; LSD = 0.1158.

Table 5.3. Average percent electrolyte leakage of nine genotypes (B) under all freezing temperatures using $\alpha = 0.05$.

| Genotype | % Leakage | t Grouping | N |
|---|---|---|---|
| NDSU 15-1000 | 69.474 | A | 16 |
| Pioneer 45H26 | 69.097 | A | 16 |
| Kanada | 68.531 | A | 16 |
| Fashion | 66.744 | AB | 16 |
| Galileo | 62.111 | AB | 16 |
| Sprinter | 61.729 | AB | 16 |
| ARC 2180-1 | 61.728 | AB | 16 |
| DKL 70-07 | 61.572 | AB | 16 |
| Hi-Q | 59.504 | B | 16 |

Note: *means followed by the same letter are not significantly different; LSD = 0.0831.

Table 5.4. The lethal temperature 50 (LT50) for each genotype tested.

| Genotype ID | LT50 (°C) |
|---|---|
| DKL 70-07 | -8.2 |
| Pioneer 45H26 | -5.8 |
| Sprinter | -8.1 |
| NDSU 15-1000 | -5.8 |
| Hi-Q | -7.1 |
| Kanada | -6.0 |
| Fashion | -5.9 |
| ARC 2180-1 | -6.1 |
| Galileo | -6.6 |

## 5.5. Discussion

Tolerance to freezing temperature during the early growing season is an important objective in canola breeding programs as well as for the growers who are interested in early planting. Early-planted canola may bring many advantages including access to early-season moisture, better competition with warm-season weeds, avoid high temperatures during flowering, and finally produce higher seed yield. Phenotyping for freezing tolerance is the most important step to develop freezing or frost tolerant germplasm. Various analytical methods have been used to score the germplasm against freezing/frost tolerance. Measurement of electrolyte leakage from the leaf is one of the methods have been used for germplasm screening in a breeding program (Madakadze *et al*., 2003). There is no protocol available for measurement of electrolyte leakage in canola. Therefore, we have attempted to develop a standard protocol to measure electrolyte leakage in a breeding program for frost tolerant germplasm screening.

We have used controlled environments such as the greenhouse and a plant growth chamber to develop the protocol for electrolyte leakage measurements. In the plant growth chamber, the seedlings were grown for 7 days at 4°C for cold acclimation after 14 days of growth in the greenhouse at 20°C. The cold acclimation is based on a natural hardening of seedlings before being exposed to freezing temperature. Thus, canola seedlings can tolerate moderate freezing temperature in nature. However, the naturally acclimated seedlings are exposed to variable cold temperatures, which may affect the rating of freezing tolerance. Therefore, to get a unique rating for germplasm screening for freezing tolerance, it is best to use a controlled environment where the optimum cold acclimation can be applied. We used four different freezing temperature (-4°C, -8°C, -12°C, -16°C) to test the rate of electrolyte leakage. These freezing temperatures have been used for protocol development to screen rapeseed/canola germplasm against freezing damage (Fiebelkorn and Rahman, 2016). A freezing water bath was

used to induce respective freezing temperature. Again, these controlled freezing temperatures gave us an opportunity to minimize other factors related to this screening process. Salgado and Rife (1996) reported a strong correlation between field and growth chamber studies. This report indicated that our controlled environment screening could also be used for actual field frost damage screening purposes.

We have conducted two time periods of electrolyte leakage measurements, the first measurement at two hours after freezing treatment, and the second measurement is after overnight freezing (-20°C) followed by thawing. Percent leakage was calculated by dividing measurement 1 by measurement 2 and multiplying by 100. Significant higher percent leakage (range 67%-85.9%) was observed for colder temperature (-8°C, -12°C, -16°C) compared to -4°C (23.8%). However, there was not significant differences among -12°C and -16°C.

In this study, different growth habits of winter and spring germplasm were used. Winter type germplasms exposed to cold/freezing temperature need a process of vernalization for flowering. The reason of choosing different growth habit types is to test if there any differences available among the growth habit types. The hypothesis was the winter types are more tolerant to freezing temperature and will be tolerant to electrolyte leakage. However, our study identified that the winter types were in the middle with regard to percent leakage. This study was conducted on 14 day old seedlings that had an additional 7 days of acclimation. This finding indicating that irrespective of growth habit types, the young seedlings are susceptible to freezing damage at early growth stage and could be used as an experimental unit for freezing tolerant study.

## 5.6. Conclusion

The plants were grown for 14 days at 20°C and acclimated for 7 days at 4°C. The current study suggests that the freezing tolerance screening could be conducted at -12°C for 2 hours to measure higher electrolyte leakage for germplasm screening. This optimized protocol will be used for future studies in canola that are used in the breeding program.

## 5.7. Literature cited


Bajji, M, J.M. Kine, and S. Lutts. 2002. Osmotic and ionic effects of NaCl on germination, early seedling growth and ion content of *Atriplex halimus* (Chenopodiaceae). Can. J. Bot. 80:297-304.

Blum, A. and A. Ebercon. 1981. Cell membrane stability as a measure of drought and heat tolerance in wheat. Crop Sci. 21: 43-47.

Chinnusamy, V, J. Zhu J, and J.K. Zhu. 2007. Cold stress regulation of gene expression in plants. Trends Plant Sci. 12, 444–451.

Ferreira, M.E., J. Satagopan, B.C. Yandell, R.H. Williams, and T.C. Osborn. 1995. Mapping loci controlling vernalization requirement and flowering time in *Brassica napus*. Theor Appl Genet. 90:727–732.

Fiebelkorn D. and M. Rahman. 2016. Development of a protocol for frost-tolerance evaluation in rapeseed/canola (*Brassica napus* L.). The Crop Journal 4:147-152.

Graham, D. and B.D. Patterson. 1982. Response of plants to low, non-freezing temperatures: Proteins, metabolism, and acclimation. Annu. Rev. Plant Physiol. 84:872–878.

Hetherington, S.E., J. He, and R.M. Smillie. 1989. Photoinhibition at low temperature in chilling-sensitive and -resistant plants. Plant Physiol. 90:1609–1615.

Lee, B.H. and J.K. Zhu. 2010. Phenotypic analysis of *Arabidopsis* mutants: Electrolyte leakage after freezing stress. Cold Spring Harbor Protoc; doi: 10.1101/pdb.prot4970.

Leopold, A.C., M.E. Musgrave, and K.M. Williams. 1981. Solute leakage resulting from leaf desiccation. Plant Physiol. 68: 1222–1225.

Madakadze, I.C., K.A. Stewart, R.M. Madakadze, and D.L. Smith. 2003. Base temperatures for seedling growth and their correlation with chilling sensitivity for warm-season grasses. Crop Sci. 43:874–878.

McCollum, T.G. and R.E. McDonald. 1991. Electrolyte leakage, respiration, and ethylene production as indices of chilling injury in grapefruit. Hort. Sci. 26:1191-1192.

Murray, A.J.S., R.D. Blackwell, and P.J. Lea. 1989. Metabolism of hydroxypyruvate in a mutant of barley lacking NADH-dependent hydroxypyruvate reductase, an important photorespiratory enzyme activity. Plant Physiol. 91 395–400

Osterhaut, W.J.V. 1922. Injury, recovery, and death, in relation to conductivity and permeability. Philadelphia, JB Lippincott. London: J.B. Lippincott Company.

Salgado, J.P. and C.L. Rife. 1996. Selection for cold hardiness in oilseed rape (*Brassica napus*), Cruciferae Newsletter, 18: 92–93.

Stevanovic, B., J. Sinzar, and O. Glisic. 1997. Electrolyte leakage differences between poikilohydrous and homoiohydrous species of Gesneriaceae. Biol. Plant. 40: 299–303.

Wang, N., W. Qian, I. Suppanz, L. Wei, B. Mao, Y. Long, J. Meng, A.E. Müller, and C. Jung. 2011. Flowering time variation in oilseed rape (*Brassica napus* L.) is associated with allelic variation in the *FRIGIDA* homologue *BnaA.FRI.a.* J. Exp. Bot. 62: 5641-5658.

# CHAPTER 6. GENOME-WIDE ASSOCIATION SCAN FOR ELECTROLYTE LEAKAGE IN RAPESEED/CANOLA *(BRASSICA NAPUS* L.)

## 6.1. Abstract

Freezing temperatures/frosts can cause significant damage to plants by rupturing plant cells. Rapeseed/canola (*Brassica napus* L.) is susceptible to freezing temperatures at the early seedling stage. The degree of cell rupture or seedling damage can be evaluated through the measurement of electrolyte leakage. A protocol is developed to measure the electrolyte leakage of canola germplasm under simulated freezing conditions. The suggested protocol for electrolyte leakage measurement includes cold acclimation of 14 day old seedlings for 7 days at 4°C followed by freezing treatment at −12°C for 2 h. With the measured electrolyte leakage, a genome-wide association scan was conducted on 157 winter, semi-winter, and spring types that originated from 15 countries. A total of 37,454 single nucleotide polymorphism (SNP) markers based upon genotyping-by-sequencing were used for analysis. Ten QTL were identified associated with electrolyte leakage of canola seedlings, which together explained 43% of the phenotypic variation. Nine orthologs were associated with environemental stress, some specificially with cold.

**Keywords**: electrolyte leakage, *Brassica napus*, frost, genome-wide association scan

## 6.2. Introduction

Rapeseed/canola (*Brassica napus* L.) is the second most important oilseed crop in the world (Foreign Agricultural Service, USDA, October 2016). It is an amphidiploid (AACC, 2n=4x=38) that evolved through spontaneous interspecific hybridization followed by genome duplication, chromosome fission, fusion and rearrangements between two diploid species *B. rapa* L. (AA, 2n=2x=20) (turnip) and *B. oleracea* L. (CC, 2n=2x=18) (cabbage) (Lagercrantz and

Lydiate, 1996; Lagercrantz, 1998; U, 1935). According to Chalhoub *et al*. (2014) the genome has an estimated size of about 1,130 Mb.

Canola has different growth habits that include spring, winter, and semi-winter. In Western Europe, rapeseed is grown as an annual winter crop (planted in the fall and harvested the following growing season, requiring vernalization to induce flowering) with highest seed yield of about 3.5 ton/ha (Rakow, 2007). In China, it is mostly semi-winter type rapeseed. Canada and the U.S.A. grow spring type rapeseed (planted and harvested in the same growing season) with moderate seed yield of 1.5 ton/ha. North Dakota grows about 84% of US canola. Winter canola lacks sufficient winter hardiness and is not adapted to North Dakota. Therefore, spring canola is a strategic crop of great importance to North Dakota. The majority of the crop is planted across the northeast and north central tier of the state. The crop growing area of North Dakota may be exposed to freezing temperatures in the early spring or early fall. Freezing temperatures during the early growing stage of rapeseed negatively affects plant growth and crop production that may destroy the whole crop (Chinnusamy *et al*., 2007).

Typically, frost is seen on the leaves, which may cause cellular dysfunction followed by wilting and bleaching of leaves and lead to plant death. Various factors such as moisture conditions, plant growth stage, cold severity, and duration of cold temperature affect the severity of frost damage. Canola seedlings are moderately tolerant to frost damage and can withstand a light spring frost that causes wilting of leaves, but can survive over time.

Frost tolerance in canola is an important trait that has not been studied in depth. Many types of tests have been conducted to assess freezing/frost tolerance such as electrolyte leakage (Madakadze *et al*., 2003), protoplasmic streaming (Graham and Patterson, 1982), and chlorophyll fluorescence (Hetherington *et al*., 1989). Measurement of electrolyte leakage is the

common method and occurs when seedlings are exposed to freezing temperatures, by which the membranes are damaged or broken. Osterhaut (1922) conducted the first test on electrolyte leakage from freezing and wounding treated plants. Since then this measurement is being used as a test for the stress-induced injury and for scoring of plants for stress tolerance (Blum and Ebercon, 1981; Bajji *et al.*, 2002; Lee and Zhu, 2010).

The electrolyte leakage measurement can help estimate the stress induced cell membrane damage in plants (Bajji *et al*., 2002). It also can be used to identify resistant cultivars to certain stress conditions (Leopold *et al*., 1981; Stevanoic *et al*., 1997; Bajji *et al*., 2002). Electrolyte leakage measurements have been identified as a good indicator of frost tolerance in crops and correlated with many other physiological and biochemical functions in response to environmental stress (McCollum and McDonald, 1991). This is a quick test that can be conducted in a laboratory with a small amount of leaf tissue (Murray *et al*., 1989). Although it is a quick method to screen for freezing tolerance, this has not been used in rapeseed/canola for a frost tolerance study.

Genome-wide association mapping scan (GWAS) is a population based study that unravels the genetic basis of many traits. It depends upon linkage disequilibrium (LD), the non-random association of alleles at two loci relative to their allelic frequencies. GWAS is a powerful method that can be used to identify genomic regions controlling a quantitative trait in a population (Yu and Buckler, 2006; Sun *et al*., 2014). The objective of this study was to perform a genome-wide association scan to find the genomic regions or QTL controlling electrolyte leakage of rapeseed/canola.

## 6.3. Materials and methods

### 6.3.1. Plant materials

170 *B. napus* accessions obtained from Germplasm Resources Information Network (GRIN) (http://www.ars-grin.gov/npgs/searchgrin.html) were used in this study. Of these 170, lines, 157 were used for GWAS. The genotypes used in the study are a subset of a collection of the *B. napus* lines used for a diversity study (Michalak *et al*., unpublished). The germplasm accessions represented a wide origin and different growth habit types including 77 spring, 54 winter, and 26 semi-winter types. Based on the origin of the accessions, 65 accessions were originated in North America, while the remaining 92 accessions were from Asia, Eastern Europe, and Western Europe (Table A6). All the accessions were self-pollinated for 4 to 5 generations to develop pure lines before being used in this study. The germplasm lines were grown in the greenhouse.

### 6.3.2. Experimental design

The germplasm lines were planted in a randomized complete block design with three replications. Four individual seedlings of each accession were grown in each replication. The plants were grown for 14 days at 20°C and 20-20-20 liquid fertilizer was applied after 7 days. The greenhouse had a 16 h photoperiod that was natural sunlight supplemented with 400 W HPS PL 2000 lights (P.L. Light Systems Inc.). After 14 days, plants were placed into a growth chamber (BioCold line of Environmental Rooms, Innovative Laboratory Systems Inc.) for 7 days of acclimation at 4°C. The growth chamber had a 12 h photoperiod and light was provided by GE Ecolux F32T8 SP35 Eco (32 W T8) style bulbs (General Electric Company).

### 6.3.3. Electrolyte leakage

The protocol for the electrolyte leakage test was conducted according to Lee and Zhu (2010) and modified based on personal communication with Dr. David Horvath, USDA, Fargo, ND. The protocol has been described in the previous chapter.

Leaves were placed into 100 µL of $dH_2O$ in a 15 mL test tube. Test tubes were placed into a freezing water bath (Thermo Scientific, Isotemp Circulators/Bath 4100) that contained a 1:1 ratio of water and ethylene glycol that was set at -12°C. Leaves were in the water bath for two hours before being allowed to thaw. Leaves were then placed in 25 mL of $dH_2O$ in a 50 mL test tube and then placed on a shaker for two hours. A conductivity meter (Primo, Hanna Instruments) was used to take the electrolyte leakage measurement. Leaves were frozen overnight at -20°C. Leaves were allowed to thaw and a second measurement was taken. Percent leakage was calculated by dividing the first measurement by the second measurement and multiplied by 100.

### 6.3.4. Statistical analysis

The means, standard deviations, and analysis of variance (ANOVA) were generated using SAS 9.3 (SAS Institute Inc., Cary, NC, USA 2012). The Shapiro-Wilk test was used to test the data normality and plotted on the histogram that was calculated using R 3.2.4 (The R Foundation).

### 6.3.5. DNA extraction and SNP identification

DNA was extracted using Qiagen DNeasy kit (Qiagen, CA, US). Genotyping-by-Sequencing (GBS) was obtained from the Institute of Genome Diversity (IGD) at Cornell University. GBS libraries were prepared and analyzed according to Elshire *et al*. (2011). The enzyme *Ape*KI was used for digestion and library was created with 96 unique barcodes. Illumina

GAII sequencer was used to sequence the genotypes. This marker data is a subset of SNPs obtained for 366 genotypes. A total of 42,575 SNPs were obtained for the entire collection. The quality cleaned GBS data was aligned using bwa-mem (Li, 2013) and multi sample SNP calling was performed using VarScan (Koboldt *et al*., 2012). The SNPs were further imputed using the default setting in FastPHASE 1.3 (Scheet and Stephens, 2006). Markers with less than 5% minor allele frequency for this set of 157 genotypes were removed for the GWAS analysis.

### 6.3.6. Population structure

A subset of 3,135 SNP markers randomly selected from 19 chromsomes were included for STRUCTURE 2.3.4 (Pritchard Lab, Stanford University) to determine the number of possible genetic clusters/subpopulations (K). The burn-in period and the number of MCMC (Markov Chain Monte Carlo) replications were 100,000 and 200,000, respectively. An admixture model was used with K-values ranging from 1-10. Each K-value was averaged across three iterations to calculate the natural log probability [LnP(D)] and delta K (ΔK) (Evanno *et al*., 2005). These values were used to find the K-value (number of subpopulations) in the population.

### 6.3.7. Markers and minor allele frequency

A total of 42,575 SNPs were obtained for the germplasm and markers with a minor allele frequency of less than 5% were removed which left 37,454 markers. TASSEL 5.0 (Bradbury *et al*., 2007) was used to conduct GWAS.

### 6.3.8. Linkage disequilibrium (LD) decay

LD decay was calculated for each subgenome and each individual chromosome using TASSEL 5.0 (Bradbury *et al*., 2007) and R 3.2.4 (The R Foundation). LD decay was estimated using the partial squared allele frequency correlation coefficient ($r^2$). This is between pairs of biallelic markers (Pritchard and Przeworski, 2001; Kraakman *et al*., 2004).

### 6.3.9. Model selection

Principal components (PCs) were estimated in TASSEL 5.0 (Bradbury *et al*., 2007). PCs that account for 25% ($PC_3$) and 50% ($PC_{24}$) of the cumulative variation were used in the regression model to control population structure (Price *et al*., 2006). An identity by state matrix was also estimated in TASSEL to account for relatedness between the genotypes (Zhao *et al*., 2007). Six regression models were used to determine the best model for further analysis. The models include naïve, $PC_3$, $PC_{24}$, Kinship, $PC_3$+Kinship, and $PC_{24}$+Kinship. The best model was selected based on the least Mean Square Difference (MSD) between the observed and expected *p*-values (Mamidi *et al*., 2011).

### 6.3.10. Association mapping

The most significant markers were determined based on the 0.1 percentile tail of an empirical distribution obtained from 10,000 bootstraps (Mamidi *et al*., 2014). Stepwise regression on the significant markers was conducted to minimize the number of markers for QTL determination, estimating allelic combinations, and candidate gene search as described in Mamidi *et al*. (2014) and Gurung *et al*. (2014). Manhattan and QQ plots were calculated using the $-\log_{10}(p)$ values and plotted using R 3.2.4 (The R Foundation).

### 6.3.11. Candidate gene search

Markers selected in stepwise regression had the annotation determined using the TAIR 10 protein database. A 100 kb region on each side of the markers was selected for BLAST. The BLAST analysis compared the selected regions with the *Arabidopsis thaliana* proteome to find candidate genes. Published gene models (Chalhoub *et al*., 2014) were used to identify functions related to frost tolerance or abiotic stress responses.

## 6.4. Results

### 6.4.1. Phenotypic results

The ANOVA of the electrolyte leakage measurement indicated that the genotypes were highly significant, but rep was not significant (Table 6.1). The significance indicated between genotypes is expected as a large number of genotypes were tested and they had diverse backgrounds.

Table 6.1. ANOVA for the percent electrolyte leakage measurement.

| Source | DF | Sum of Squares | Mean Square | Error DF | F Value | Pr > F |
|---|---|---|---|---|---|---|
| Genotype | 169 | 57204.00 | 338.48 | 353 | 5.88 | <.0001*** |
| Rep | 2 | 74.52 | 37.26 | 353 | 0.65 | 0.5240ns |
| Residual | 353 | 20314.00 | 5754.54 | . | . | . |

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

The frequency of the mean values of percent electrolyte leakage were plotted based on the number of genotypes that fit at each value (Fig. 6.1). The Shapiro-Wilk normality test for electrolyte leakage have a p-value greater than 0.05 (p=0.06774) which indicates a normal distribution of data. The values are between and 41% and 97%.

### 6.4.2. Population structure

A subset of 3,135 random markers from the 19 linkage groups were selected for population structure analysis. Based on LnP(D) and Delta K, the 157 genotypes could be assigned into three subpopulations (Fig. 6.2) where the number of individuals in a subpopulation varied. Evanno *et al*. (2005) described the LnP(D) and ΔK which were used to calculate the number of subpopulations. The ΔK value showed a clear peak at K=3.

### 6.4.3. Linkage disequilibrium (LD) decay

LD decay was calculated for all 19 chromosomes (A01-C09) and for the A and C subgenomes. A commonly used cutoff is $r^2$ and for this study $r^2 = 0.2$ was used. This measure is used to quantify and compare LD and is the partial squared allele frequency correlation coefficient. The physical distance (kb) was used in this study to determine the LD decay. Subgenome A had an LD decay of about 34 kb (Fig. 6.3) while subgenome C was longer and had a decay of about 186 kb (Fig. 6.4). The individual chromosome LD decay was typically lower for A01 - A10 than for C01 - C09 (Fig. 6.5 and Fig. A39-A57). The range of the LD decay was about 22-62 kb for A01 - A10 and was about 76-436 kb for C01 - C09.

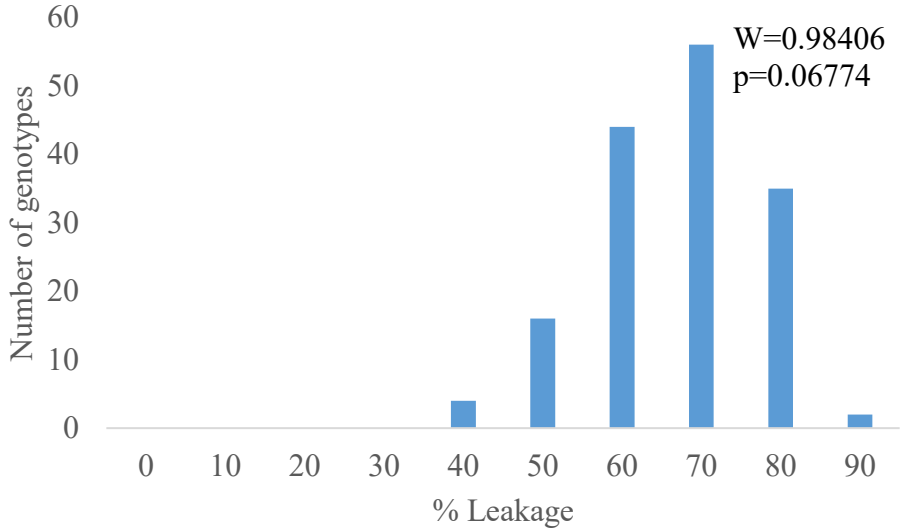


Fig. 6.1. Histogram for % electrolyte leakage tested at -12°C. The x-axis is the medians and the y-axis the number of genotypes that fit each median.

### 6.4.4. Markers, minor allele frequency, and genome-wide association scan

A total of 37,454 markers were used after data filtering and removing the minor allele frequencies (<5%). Three and twenty-four PCs were used that account for a cumulative variation of 25% and 50%, respectively. These PCs were used for controlling population structure in the mixed linear model. $PC_1$ explains about 14% of the cumulative variation and $PC_2$ explains about

9% of the cumulative variation. The first two PCs distributed the genotypes into three groups, which could not be clustered into discrete groups based on growth habit (Fig. 6.6).

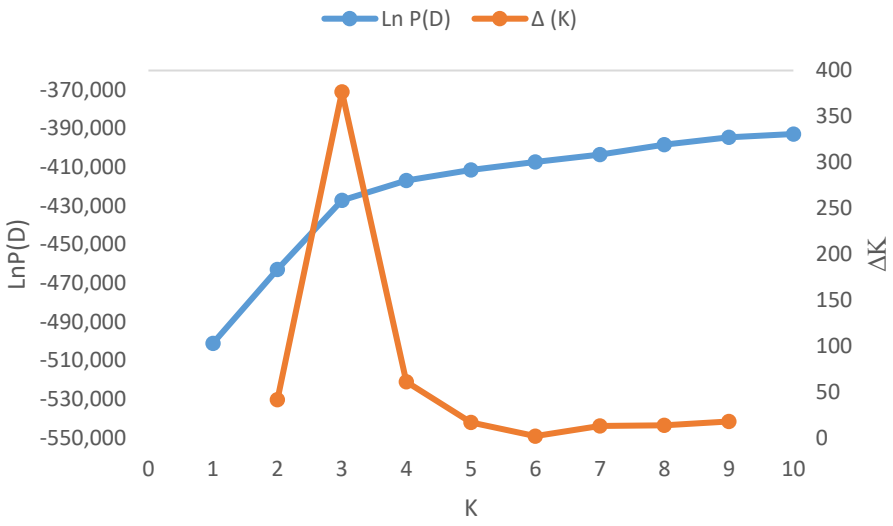


Fig. 6.2. The natural log probability [LnP(D)] and delta K (ΔK) for each value of K averaged over three iterations with 100,000 and 200,000 burn-in and MCMC, respectively. The values of K = 1 – 10, where the K = 3 is the number of subpopulations chosen in the population.

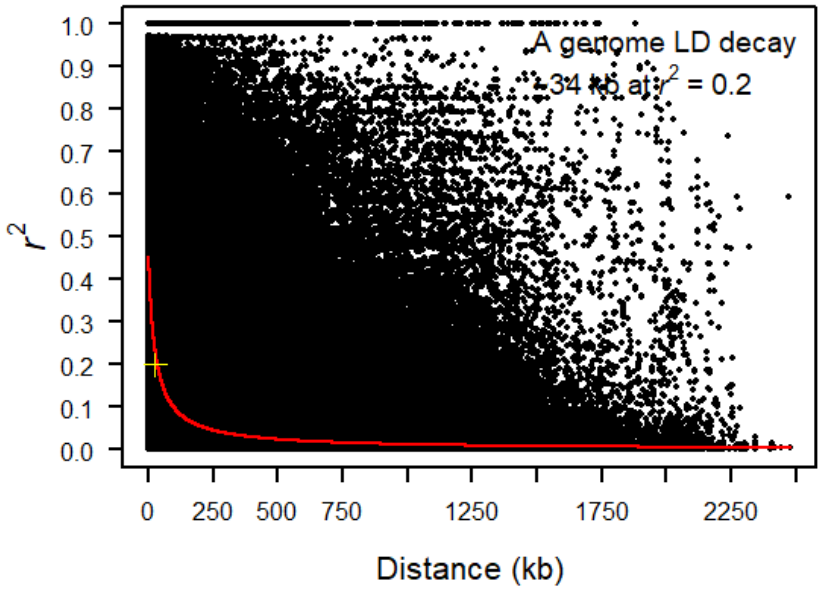


Fig. 6.3. LD decay for the A genome. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 34 kb.

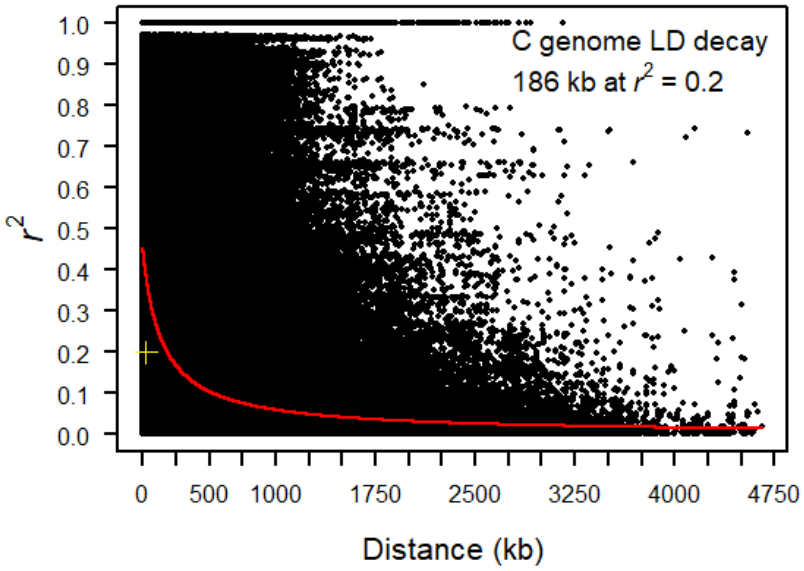


Fig. 6.4. LD decay for the C genome. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 186 kb.

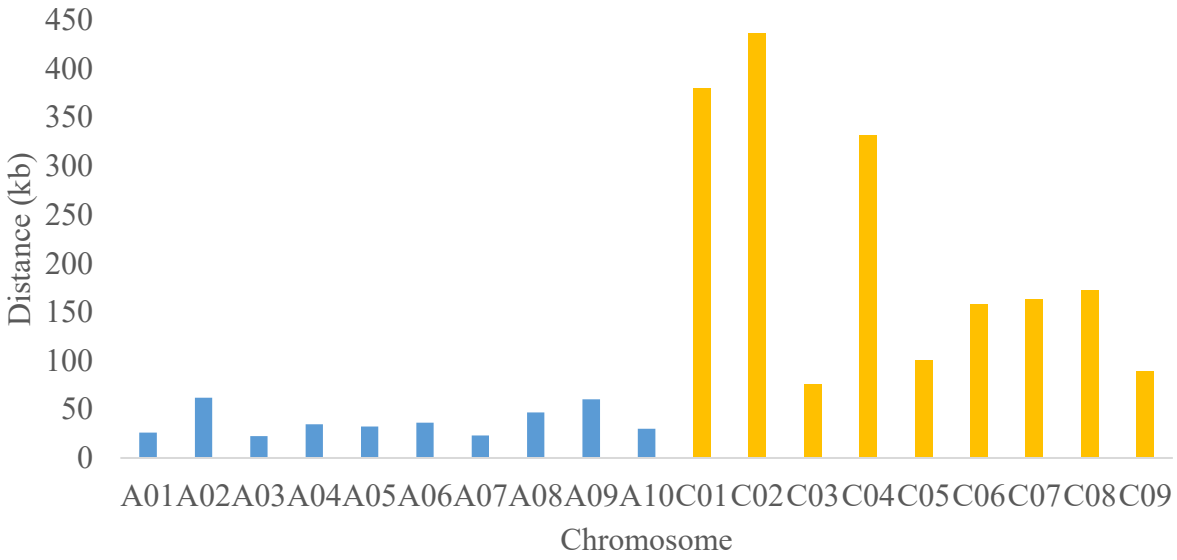


Fig. 6.5. Linkage disequilibrium (LD) decay for 19 chromosomes in canola. The x-axis is the chromosome and the y-axis the physical distance (kb) for the chromosomes at a threshold of $r^2$=0.2.

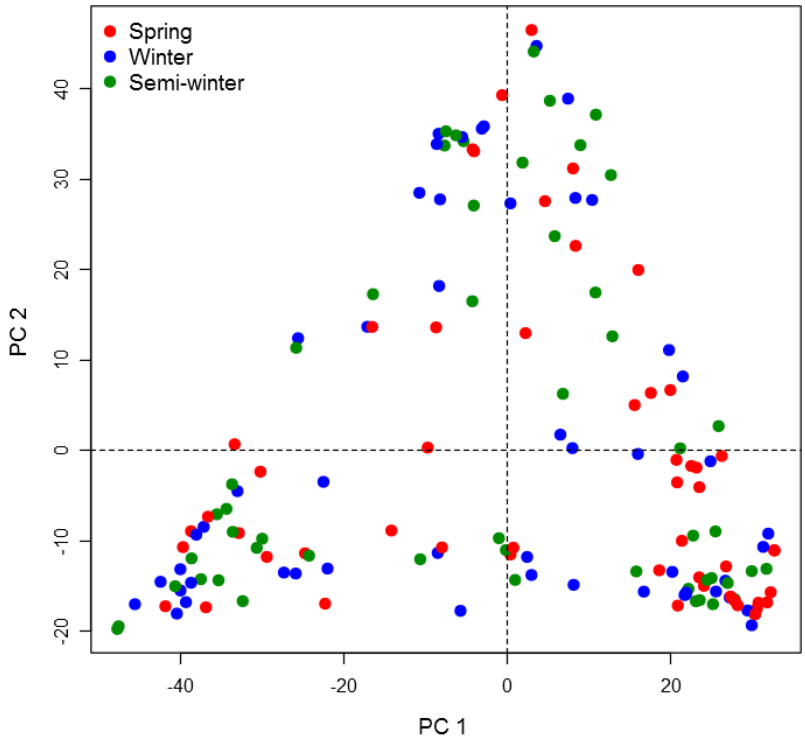


Fig.6.6. PCA graph showing the distribution of the first two principle components for electrolyte leakage. $PC_1$ explained 14% of the variation and $PC_2$ explained 9% of the variation.

Among the six models tested, the $PC_{24}$ model was identified as the best model based on the mean square difference (MSD). Thirty-six markers were significant at the 0.1 percentile tail of the empirical distribution after 10,000 bootstraps were identified ($p < 0.000708$; Table 6.2). Stepwise regression identified 10 QTL regions located on chromosome A01 (19.846 Mbp), A02 (3.690 Mbp), A04 (11.900 Mbp), A06 (21.440 Mbp), A07 (15.780 Mbp), C02 (22.001 Mbp), C05 (42.900 Mbp), C07 (11.985 Mbp), C09 (6.480 (Mbp), and one that could not be assigned to any chromosome based on the *B. napus* reference genome sequence (Chalhoub *et al*., 2014). These QTLs together explained 43% of the total phenotypic variation.

The $-\log_{10}(p)$ values were calculated and plotted in a Manhattan plot (Fig. 6.7). The dashed blue line indicates significant markers and is at $-\log_{10}(p) = 3.15$. SNPs on Ann_rand,

Cnn_rand, and Unn_rand are not present because they could not be assigned to any chromosome (Chalhoub *et al*., 2014).

The QQ plot indicated the fitness of the selected model based on the observed and expected -$\log_{10}$(p) (Fig. 6.8). The 10 SNPs selected through stepwise regression are highlighted and labeled on the graph.

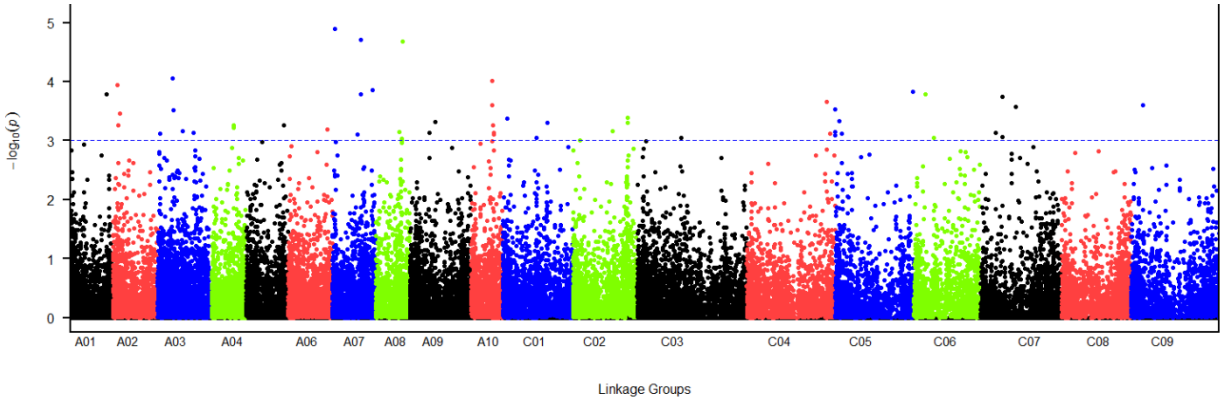


Fig. 6.7**.** Manhattan plot for electrolyte leakage. The x-axis is the chromosomes and the y-axis is the -$\log_{10}$ (p). The horizontal dashed blue line indicates the significant markers, -$\log_{10}$(p) = 3.15. The GWAS model is $PC_{24}$.

Table 6.2. Significant markers for the electrolyte leakage study selected using the 0.1% tail of the Empirical distribution. The reference allele, alternate alleles, and the heterozygote had the means calculated for each marker.

| Marker | Chromosome | Position | Reference allele/Alternate allele | Reference allele | | Alternate allele | | Heterozygote (H) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Obs | Mean | Obs | Mean | Obs | Mean |
| chrA01_19846016* | chrA01 | 19846016 | G/A | 58 | 0.75 | 26 | 0.71 | 73 | 0.69 |
| chrA02_2307152 | chrA02 | 2307152 | C/G | 102 | 0.72 | 8 | 0.79 | 47 | 0.71 |
| chrA02_3086797 | chrA02 | 3086797 | T/C | 92 | 0.71 | 9 | 0.62 | 56 | 0.75 |
| chrA02_3689678* | chrA02 | 3689678 | T/C | 139 | 0.73 | 4 | 0.54 | 14 | 0.68 |
| chrA03_13785957 | chrA03 | 13785957 | C/T | 138 | 0.73 | 12 | 0.64 | 7 | 0.68 |
| chrA03_8326025 | chrA03 | 8326025 | G/C | 124 | 0.71 | 17 | 0.75 | 16 | 0.74 |
| chrA03_8713005 | chrA03 | 8713005 | T/C | 70 | 0.71 | 66 | 0.72 | 21 | 0.75 |
| chrA04_11899018 | chrA04 | 11899018 | G/A | 71 | 0.69 | 62 | 0.76 | 24 | 0.69 |
| chrA04_11899095* | chrA04 | 11899095 | G/C | 62 | 0.76 | 63 | 0.69 | 32 | 0.70 |
| chrA05_20898044 | chrA05 | 20898044 | T/C | 87 | 0.73 | 36 | 0.74 | 34 | 0.66 |
| chrA06_21439204* | chrA06 | 21439204 | A/T | 134 | 0.71 | 11 | 0.80 | 12 | 0.68 |
| chrA07_15727418 | chrA07 | 15727418 | C/T | 111 | 0.73 | 38 | 0.73 | 8 | 0.57 |
| chrA07_15775877* | chrA07 | 15775877 | C/A | 142 | 0.73 | 11 | 0.68 | 4 | 0.47 |
| chrA07_1662683 | chrA07 | 1662683 | C/T | 132 | 0.73 | 17 | 0.71 | 8 | 0.55 |
| chrA07_22137015 | chrA07 | 22137015 | C/T | 82 | 0.72 | 11 | 0.59 | 64 | 0.74 |
| chrA08_13034681 | chrA08 | 13034681 | T/A | 137 | 0.73 | 9 | 0.71 | 11 | 0.61 |
| chrA08_15028373 | chrA08 | 15028373 | A/C | 79 | 0.72 | 63 | 0.74 | 15 | 0.60 |
| chrA09_13997359 | chrA09 | 13997359 | A/T | 138 | 0.72 | 2 | 0.82 | 17 | 0.73 |
| chrA10_11369355 | chrA10 | 11369355 | A/G | 142 | 0.72 | 9 | 0.59 | 6 | 0.78 |
| chrA10_11369370 | chrA10 | 11369370 | G/T | 143 | 0.72 | 7 | 0.56 | 7 | 0.79 |
| chrA10_11872726 | chrA10 | 11872726 | G/C | 145 | 0.72 | 6 | 0.81 | 6 | 0.57 |
| chrC01_24626301 | chrC01 | 24626301 | T/G | 74 | 0.72 | 54 | 0.71 | 29 | 0.73 |

Obs = observations

*Marker identified through stepwise regression

Table 6.2. Significant markers for the electrolyte leakage study selected using the 0.1% tail of the Empirical distribution (continued). The reference allele, alternate alleles, and the heterozygote had the means calculated for each marker.

| Marker | Chromosome | Position | Reference allele/Alternate allele | Reference allele | | Alternate allele | | Heterozygote (H) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Obs | Mean | Obs | Mean | Obs | Mean |
| chrC01_rand_2517210 | chrC01_rand | 2517210 | G/A | 57 | 0.73 | 10 | 0.61 | 90 | 0.72 |
| chrC02_22001286* | chrC02 | 22001286 | C/T | 124 | 0.73 | 4 | 0.50 | 29 | 0.72 |
| chrC02_29995249 | chrC02 | 29995249 | A/G | 104 | 0.73 | 38 | 0.72 | 15 | 0.63 |
| chrC02_29995255 | chrC02 | 29995255 | A/G | 104 | 0.73 | 39 | 0.72 | 14 | 0.62 |
| chrC04_44016709 | chrC04 | 44016709 | C/T | 125 | 0.72 | 3 | 0.46 | 29 | 0.72 |
| chrC05_2379280 | chrC05 | 2379280 | C/T | 87 | 0.71 | 2 | 0.82 | 68 | 0.72 |
| chrC05_42899933* | chrC05 | 42899933 | C/A | 145 | 0.73 | 6 | 0.52 | 6 | 0.70 |
| chrC05_rand_65405 | chrC05_rand | 65405 | T/A | 91 | 0.72 | 3 | 0.50 | 63 | 0.72 |
| chrC06_6408610 | chrC06 | 6408610 | C/A | 107 | 0.72 | 36 | 0.74 | 14 | 0.61 |
| chrC07_11984509* | chrC07 | 11984509 | C/T | 90 | 0.71 | 10 | 0.62 | 57 | 0.74 |
| chrC07_19358948 | chrC07 | 19358948 | T/A | 111 | 0.73 | 5 | 0.80 | 41 | 0.69 |
| chrC09_6480034* | chrC09 | 6480034 | G/A | 86 | 0.71 | 31 | 0.75 | 40 | 0.72 |
| chrCnn_rand_32792537 | chrCnn_rand | 32792537 | C/T | 138 | 0.72 | 3 | 0.71 | 16 | 0.75 |
| chrCnn_rand_48004638* | chrCnn_rand | 48004638 | C/T | 70 | 0.72 | 16 | 0.67 | 71 | 0.73 |

Obs = observations

*Marker identified through stepwise regression

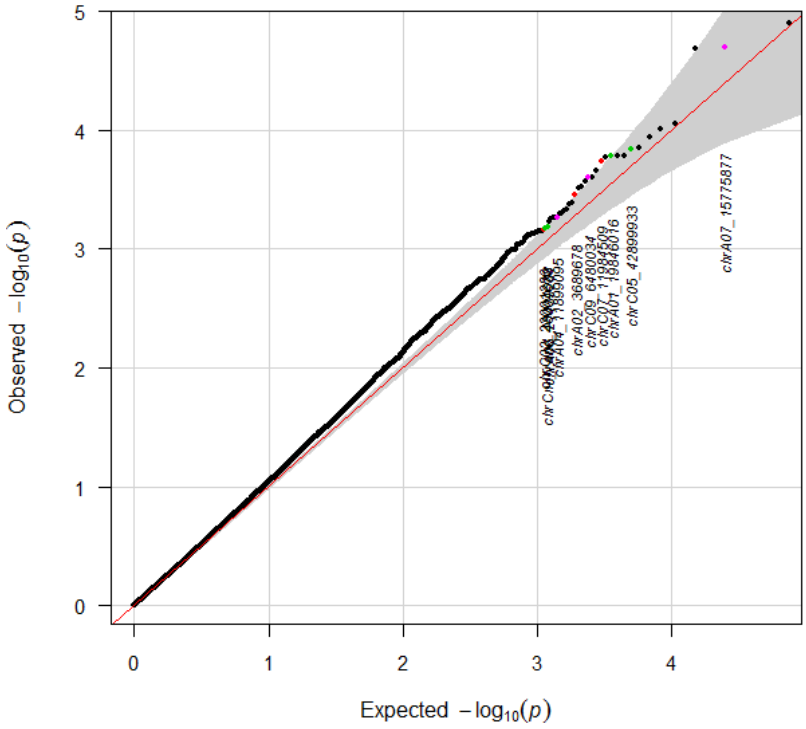


Fig. 6.8. Q-Q Plot: Distribution of the -$\log_{10}$ p-values for the observed and expected p-values. The x axis is the expected p-values and the y axis is the observed p-values.

Genes present within 100 kb on either side of the 10 selected SNPs were used for further analysis. The sequences were blasted with *Arabidopsis thaliana* proteome (TAIR 10). The annotation of the genes was subject to a literature search to identify candidate genes associated with freezing/frost tolerance and genes related to abiotic stress responses (Table 6.3). Potential genes were involved with abiotic stress tolerance and response. One gene was involved in the pentose phosphate pathway which can be associated with some environmental stresses. Others were involved in defense response, response to cold and salinity, and response to oxidative sress.

Table 6.3. Candidate genes associated with electrolyte leakage and cold stress response.

| Brassica gene model | Marker | Gene start | Gene end | Marker distance from gene | A. thaliana equivalent | Gene annotation | Function | Reference |
|---|---|---|---|---|---|---|---|---|
| BnaC05g48310D | chrC05_42899933 | 42,833,794 | 42,835,869 | 64,064 | AT3G02360 | 6-phosphogluconate dehydrogenase family protein | Involved in the pentose phosphate pathway (PPP) which can be associated with some enviornmental stresses | Hou *et al*., (2007) |
| BnaA04g14060D | chrA04_11899095 | 11,864,519 | 11,865,184 | 33,911 | AT2G24040 | Low temperature and salt responsive protein family | Involved in salinity and cold responses | Lin *et al*., (1999) |

Table 6.3. Candidate genes associated with electrolyte leakage and cold stress response (continued).

| Brassica gene model | Marker | Gene start | Gene end | Marker distance from gene | A. thaliana equivalent | Gene annotation | Function | Reference |
|---|---|---|---|---|---|---|---|---|
| BnaA07g20000D | chrA07_15775877 | 15,835,692 | 15,837,496 | 59,815 | AT1G80680 | Suppressor of auxin resistance 3 | Involved in defense response signaling pathway, resistance gene dependent. | Celesnik *et al., (2013)* |
| BnaA07g20020D | chrA07_15775877 | 15,840,209 | 15,841,419 | 64,332 | AT1G80980 | Stress response NST1-like protein | Involved in stress response | Theologis *et al*., (2000) |
| BnaA02g07630D | chrA02_3689678 | 3,639,594 | 3,641,126 | 48,552 | AT5G58390 | Peroxidase superfamily protein | Involved in response to oxidative stress | Hanano *et al*., (2002) |

Table 6.3. Candidate genes associated with electrolyte leakage and cold stress response (continued).

| Brassica gene model | Marker | Gene start | Gene end | Marker distance from gene | A. thaliana equivalent | Gene annotation | Function | Reference |
|---|---|---|---|---|---|---|---|---|
| BnaA02g07880D | chrA02_3689678 | 3,741,574 | 3,743,612 | 51,896 | AT5G58070 | Temperature-induced lipocalin | Involved in response to cold and many other environmental stresses | Hernández-Gras and Boronat (2015) |
| BnaA02g07900D | chrA02_3689678 | 3,746,719 | 3,747,447 | 57,041 | | | | |
| BnaA01g28600D | chrA01_19846016 | 19,885,286 | 19,886,322 | 39,270 | AT3G15670 | Late embryogenesis abundant protein (LEA) family protein | Involved in dessication tolerance | Candat *et al*., (2014) |
| BnaA06g32230D | chrA06_21439204 | 21,525,377 | 21,527,083 | 86,173 | AT3G27280 | Prohibitin 4 | Involved in stress response | Salanoubat *et al*., (2000) |

## 6.5. Discussion

Frost tolerance is important for breeding programs and for crops in North Dakota. Canola is grown in the northern part of the state and into Canada where spring frosts can be a concern. Growers would benefit from being able to plant canola and have frost tolerance in the case of a late frost. Early planting would allow the crop to utilize early season moisture, compete with weeds, and avoid heat during flowering which decreases yield. Various methods have been used to analyze freezing tolerance in crops including field, greenhouse, and electrolyte leakage. Madakadze *et al*. (2003) screened for electrolyte leakage using a single leaf. In grapefruit, electrolyte leakage is a good indicator of frost damage (McCollum and McDonald, 1991). An optimized protocol was developed in the previous study that determined the best temperature to screen canola leaves for electrolyte leakage.

A controlled environment was used to grow the seedlings and to screen the plants. In this study, a cold acclimation (4°C) to the seedling was used. In nature, the plants are exposed to cooler temperatures that allow them to harden and become acclimated to freezing temperatures. This increases the ability of the plant to survive and recover from freezing temperatures. Salgado and Rife (1996) reported a correlation between field and growth chamber studies which indicates that our growth chamber study could provide useful information that could be used in the breeding program.

This study used different growth habit types such as winter, spring, and semi-winter rapeseed/canola germplasm. Different growth types were used as part of the larger collection and to identify genes related to frost tolerance that may not be in spring grown canola. Winter or semi-winter canola could contain the genes necessary for frost tolerance. Differences could also be tested between growth types.

### 6.5.1. Genome-wide association mapping

Gene-tagging or identifying QTL from diversified germplasm collections using linkage disequilibrium (LD)-based association mapping has become a powerful tool to identify marker-trait associations and genes associated with the trait (Li *et al*., 2011, Jia *et al*., 2008). LD refers to the non-random association of alleles at two loci relative to their allelic frequencies. With low levels of LD, association mapping can discover markers close to the gene. In contrast, with longer stretches of LD, the population can be used to discover linked markers at a greater distance from the gene (Rafalski, 2002). Association mapping studies have advantages over traditional biparental based QTL-mapping in many aspects such as use of wider genetic variability of germplasm, utilization of historic recombination events, avoiding the necessity to develop costly and time consuming biparental populations (Kraakman *et al*., 2006; Hansen *et al*., 2001).

Many factors such as germplasm sample size, number of molecular markers, LD patterns, and population history are associated with the success of finding strong markers linked to a trait. There is not a clear consensus on sample size necessary for GWAS studies. However, in general the larger samples will have higher historic recombination and are better fitted for association study (Hong and Park, 2012). Currently, many reports are available on genome-wide association mapping using a wide range of germplasm accession in *B. napus* (Rezaeizad *et al*., 2011; Liu *et al*., 2016; Rahman *et al*., 2016). We used 157 germplasm accessions from three growth habits (winter, semi-winter, and spring) from 15 different countries that include North America, Asia, Western and Eastern Europe. This germplasm panel represents a wide diversity used in this GWAS study.

Using a high number of molecular markers in the GWAS study will give us an opportunity to identify QTLs across the genome. We used GBS technique to get a large number of SNP markers for this study. It has been reported that the GBS technique permits researchers to find a large number of SNPs that are evenly distributed in a mapping population (Elshire *et al*., 2011; Poland and Rife, 2012). Therefore, we obtained a relatively large clean set of SNP markers that covers the whole genome in our study.

False positives in marker-based trait associations can occur due to the confounding effects of population stratification. These spurious associations are often seen if the population exhibits structure or relatedness. To avoid these spurious associations, statistical approaches were used to correct the population structure and relatedness and a combination of both (Yu *et al.*, 2006). We tested six different regression models (naïve, $PC_3$, $PC_{24}$, Kinship, $PC_3$ +Kinship, and $PC_{24}$ +Kinship) that included structure and/or relatedness to remove spurious markers. The naïve model does not account for structure or relatedness, where the PC models account for the structure of the population. The kinship model accounts for the population relatedness. Based on MSD values, we have identified $PC_{24}$ as the best model that accounts for population structure. We have identified the significant markers based on the 0.1% tail of the empirical distribution using p-values suggested by Mamidi *et al.* (2014) and Gurung *et al*. (2014). Stepwise regression was performed to identify the QTL peaks and markers for marker-assisted selection (Mamidi *et al*., 2014). The advantage of using stepwise regression is that this analysis selects the markers that have a major effect in a QTL region, and at the same time remove the QTLs which have minor effects on the phenotype. The selected marker/QTLs from stepwise regression are spread across the genome including chromosomes A01, A02, A04, A06, A07, C02, C05, C07, and C09.

The reference genome paper by Chalhoub *et al*. (2014) identified scaffolds that were not able to be orientated onto the map. Nineteen pseudochromosomes were named according to the subgenome they belonged to (chrA01 - chrA10 and chrC01 - chrC09). Mapped scaffolds that had an unknown orientation were named chrA01_random - chrA10_random and chrC01_random - chrC09_random. Scaffolds that were unmapped but were assigned to a subgenome were named chrAnn_random and chrCnn_random. Some scaffolds could not be mapped or assigned were indicated as chrUnn_random. Random has been abbreviated to rand for this study.

Genes in a one hundred kb sequence on each side (Chalhoub *et al*., 2014) of the selected SNPs were used for the candidate gene search. Due to low LD in the population (Michalak *et al*., unpublished), a longer stretch of the genome sequence was used for the candidate gene search. The selected *B. napus* gene models around the significant markers were used for NCBI-BLAST with *Arabidopsis thaliana* to identify genes that are related to freezing/frost tolerance and abiotic stress. Over 290 hits were observed of which nine potential genes directly or indirectly confer cold tolerance, abiotic stresses, and various physiological activities. It was interesting to see that eight genes were located on A-genome and only one gene was present on the C-genome. This finding indicated that the cold tolerance is mostly controlled by the A genome.

The gene located on the C genome is on chromosome C05. It is 64 kb away from the marker and is involved in the pentose phosphate pathway and may be associated with some environmental stresses (Hou *et al.*, 2007). A marker located 34 kb away from the low temperature and salt responsive gene is on chromosome A04. This gene is involved in salinity and cold responses (Lin *et al*., 1999). Chromosome A07 has two genes that are located 60 and 64 kb, respectively from the marker. The first is involved in defense response signaling pathway and is resistance gene dependent (Celesnik *et al*., 2013). The second gene is involved in stress

response (Theologis *et al.*, 2000). Chromosome A02 had three genes located 49, 52, and 57 kb, respectively away from the marker. The first gene is involved in response to oxidative stress (Hanano *et al*., 2002). The second and third are a temperature-induced lipocalin that is involved in cold response and many other environmenral stresses (Hernádez-Gras and Boronat, 2015). A gene located on chromosome A01 is involved in desiccation toleranance (Candat *et al*., 2014) and is located 39 kb from the marker. The last gene is on chromosome A06 and is located 86 kb away from the marker and is involved in stress response (Salanoubat *et al*., 2000).

Genes that are either positively or negatively related to frost tolerance or abiotic stress are important for a breeding program. Knowing what genes may play a role in tolerance will help make screening of germplasm faster. Marker screenings in the laboratory could provide useful information in a fast and efficient way for germplasm selection. However, more research is needed to validate the role of the genes and gene families for frost tolerance to use in marker-assisted breeding program.

**6.6. Conclusion**

Electrolyte leakage is important in any crop that experiences abiotic stress. The method used for this study was based off a previously studied protocol that was modified to better fit in canola. The leaves were frozen at -12°C in a solution of ethylene glycol and water for 2 hours. The leaves were allowed to thaw and shake for two hours before a measurement was taken. Leaves were then frozen overnight at -20°C before being allowed to thaw when a second measurement was taken. Percent leakage was calculated by dividing the first measurement by the second measurement and multiplying by 100. When GWAS was conducted, genes were identified that may play a role in frost tolerance or electrolyte leakage. Gene functions of the annotated *Arabidopsis* genes were searched using literature to determine if they were related to

frost tolerance or electrolyte leakage. Many genes were identified as being related to abiotic stress.

### 6.7. Literature cited


Bajji, M, J.M. Kine, and S. Lutts. 2002. Osmotic and ionic effects of NaCl on germination, early seedling growth and ion content of *Atriplex halimus* (Chenopodiaceae). Can. J. Bot. 80:297-304.

Blum, A. and A. Ebercon. 1981. Cell membrane stability as a measure of drought and heat tolerance in wheat. Crop Sci. 21: 43-47.

Bradbury, P.J., Z. Zhang, D.E. Kroon, T.M. Casstevens, Y. Ramdoss, and E.S. Buckler. 2007. TASSEL: software for association mapping of complex traits in diverse samples. Bioinformatics. 23(19):2633-2635. doi:https://doi.org/10.1093/bioinformatics/btm308.

Candat, A., G. Paszkiewicz, M. Neveu, R. Gautier, D.C. Logan, M.H. Avelange-Macherel, D. Macherel. 2014. The ubiquitous distribution of late embryogenesis abundant proteins across cell compartments in *Arabidopsis* offers tailored protection against abiotic stress. Plant Cell. 26(7):3148-3166.

Celesnik, H., G.S. Ali, F.M. Robison, A.S.N. Reddy. 2013. *Arabidopsis thaliana* VOZ (Vascular plant One-Zinc finger) transcription factors are required for proper regulation of flowering time. Biol. Open. 4(2):424-431.

Chalhoub, B., F. Denoeud, S. Liu, I.A.P Parkin, H. Tang, X. Wang, J. Chiquet, H. Belcram, C. Tong, B. Samans, M. Correa, C. Da Silva, J. Just, C. Falentin, C.S. Koh, I. Le Clainche, M. Bernard, P. Bento, B. Noel, K. Labadie, A. Alberti, M. Charles, D. Arnaud, H. Guo, C. Daviaud, S. Alamery, K. Jabbari, M. Zhao, P.P. Edger, H. Chelaifa, D. Tack, G. Lassalle, I. Mestiri, N. Schnel, M.-C. Le Paslier, G. Fan, V. Renault, P.E. Bayer, A.A. Golicz, S. Manoli, T.-H. Lee, V.H.D. Thi, S. Chalabi, Q. Hu, C. Fan, R. Tollenacre, Y. Lu, C. Battail, J. Shen, C.H.D. Sidebottom, X. Wang, A. Canaguier, A. Chauveau, A. Berard, G. Deniot, M. Guan, Z. Liu, F. Sun, Y.P. Lim, E. Lyons, C.D. Town, I. Bancroft, X. Wang, J. Meng, J. Ma, J.C. Pires, G.J. King, D. Brunel, R. Delourme, M. Renard, J.-M. Aury, K.L. Adams, J. Batley, R.J. Snowden, J. Tost, D. Edwards, Y. Zhou, W. Hua, A.G. Sharpe, A.H. Paterson, C. Guan, P. Wincker. 2014. Early allopolyploid evolution in the post-Neolithic *Brassica napus* oilseed genome. Science. 345(6199):950-953.

Charron, J.-B.F., G. Breton, M. Badawi, and F. Sarhan. 2002. Molecular and structural analyses of a novel temperature stress-induced lipocalin from wheat and *Arabidopsis*. FEBS Letters. 517:129-132. doi: 10.1016/S0014-5793(02)02606-6.

Chinnusamy, V., J. Zhu, and J.-K. Zhu. 2007. Cold stress regulation of gene expression in plants. Trends in Plant Sci. 12(10):444-451.

Elshire, R.J., J.C. Glaubitz, J.A. Poland, K. Kawamoto, E.S. Buckler, and S.E. Mitchell. 2011. A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. PLOS ONE. 6(5):e19379.

Evanno, G., S. Regnaut, and J. Goudet. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology. 14: 2611–2620.

Graham, D. and B.D. Patterson. 1982. Response of plants to low, non-freezing temperatures: Proteins, metabolism, and acclimation. Annu. Rev. Plant Physiol. 84:872–878.

Gurung, S., S. Mamidi, J.M. Bonman, M. Xiong, G. Brown-Guedira, and T.B. Adhikari. 2014. Genome-wide association study reveals novel quantitative trait loci associated with resistance to multiple leaf spot diseases of spring wheat. Plos One 9(9):e108179.

Hanano, S., M. Amagi, T. Kaneko, C. Kuwata, T. Tsugane, N. Sakurai, Y. Nakamura, D. Shibata, and S. Tabata. 2002. Analysis of gene expression in *Arabidopsis thaliana* by array hybridization with genomic DNA fragments aligned along chromosomal regions. The Plant J. 30(2):247. doi:10.1046/j.1365-313X.2002.01277.x.

Hansen, M., T. Kraft, S. Ganestam, T. Säll, N.-O. Nilsson. 2001. Linkage disequilibrium mapping of the bolting gene in sea beet using AFLP markers. *Genetical Research*. 77(1):61–66.

Hernández-Gras, F. and A. Boronat. 2015. A hydrophobic proline-rich motif is involved in the intracellular targeting of temperature-induced lipocalin. Plant Mol. Biol. 88:301. doi:10.1007/s11103-015-0326-x.

Hetherington, S.E., J. He, and R.M. Smillie. 1989. Photoinhibition at low temperature in chilling-sensitive and -resistant plants. Plant Physiol. 90:1609–1615.

Hong, E.P. and J.W. Park. 2012. Sample size and statistical power calculation in genetic association studies. Genom. Inform. 10:117–122.

Hou, F.-Y., J. Huang, S.-L. Yu, and H.-S, Zhang. 2007. The 6-phosphogluconate dehydrogenase genes are responsive to abiotic stresses in rice. J. of Integrative Plant Bio. 49(5):655.663.

Jia, G., X. Liu, H.A. Owen, D. and Zhao. 2008. Signaling of cell fate determination by the TPD1 small protein and EMS1 receptor kinase. PNAS. 105:1-6.

Koboldt, D.C., Q. Zhang, D.E. Larson, D. Shen, M.D. McLellan, L. Lin, C.A. Miller, E.R. Mardis, L. Ding, and R.K. Wilson. 2012. VarScan 2: Somatic mutation and copy number alteration discovery in cancer by exome sequencing. Genome Res. 22:568-576.

Kraakman, A.T.W., F. Martínez, B. Mussiraliev, F.A. van Eeuwijk, and R.E. Niks. 2006. Linkage disequilibrium mapping of morphological, resistance, and other agronomically relevant traits in modern spring barley cultivars. *Molecular Breeding*. 17(1):41–58.

Kraakman, A.T.W., R.E. Niks, P.M.M.M. Van den Berg, P. Stam, and F.A. Van Eeuwijk. 2004. Linkage disequilibrium mapping of yield and yield stability in modern spring barley cultivars. Genetics. 168:435-446.

Lagercrantz, U. 1998. Comparative mapping between *Arabidopsis thaliana* and *Brassica nigra* indicates that brassica genomes have evolved through extensive genome replication accompanied by chromosome fusions and frequent rearrangements. Genetics. 150:1217-1228.

Lagercrantz, U. and D.J. Lydiate. 1996. Comparative genome mapping in Brassica. Genetics. 144:1903-1910.

Lee, B.-h, and J.-K. Zhu. 2010. Phenotypic analysis of *Arabidopsis* mutants: Electrolyte leakage after freezing stress. Cold Spring Harbor Protoc; doi: 10.1101/pdb.prot4970.

Leopold, A.C., M.E. Musgrave, and K.M. Williams. 1981. Solute leakage resulting from leaf desiccation. Plant Physiol. 68: 1222–1225.

Li, H. 2013. Aligning sequence reads, clone sequences, and assembly contigs with BWA-MEM. arXiv:1303.3997 http://arxiv.org/abs/1303.3997.

Li, X., W. Yan, H. Agrama, L. Jia, X. Shen, A. Jackson, K. Moldenhauer, K. Yeater, A. McClung , and D. Wu. 2011. Mapping QTLs for improving grain yield using the USDA rice minicore collection. Planta 234:347–361.

Lin, X., S. Kaul, S. Rounsley, T.P. Shea, M.I. Benito, C.D. Town, C.Y. Fujii, T. Mason,T, C.L. Bowman, M. Barnstead, T.V. Feldblyum, C.R. Buell, K.A. Ketchum, J. Lee, C.M. Ronning, H.L. Koo, K.S. Moffat, L.A. Cronin, M. Shen, G. Pai, S. Van Aken, L. Umayam, L.J. Tallon, J.E. Gill, M.D. Adams, A.J. Carrera, T.H. Creasy, H.M. Goodman, C.R. Somerville, G.P. Copenhaver, D. Preuss, W.C. Nierman, O. White, J.A. Eisen, S.L. Salzberg, C.M. Fraser, and J.C. Venter. 1999. Sequence and analysis of chromosome 2 of the plant *Arabidopsis thaliana.* Nature. 402(6763):761-768.

Liu, J., W. Wang, D. Mei, H. Wang, L. Fu, D. Liu, Y. Li, and Q. Hu. 2016. Characterizing variation of branch angle and genome-wide association mapping in rapeseed (*Brassica napus* L.). Front Plant Sci 7:21.

Madakadze, C., K. Stewart, R. Madakadze, an D. Smith. 2003. Base temperature for seedling growth and their correlation with chilling sensitivity for warm-season grasses. Crop Sci. 43:874–878.

Mamidi, S., R.K. Lee, R.J. Goos, and P.E. McClean. 2014. Genome-wide association studies identifies seven major regions responsible for iron deficiency chlorosis in soybean (*Glycine max*). Plos One 9(9):e107469.

Mamidi, S., S. Chikara, R.J. Goos, D.L. Hyten, D. Annam, S.M. Moghaddam, R.K. Lee, P.B. Cregan, and P.E. McClean. 2011. Genome-wide association analysis identifies candidate genes associated with iron deficiency chlorosis in soybean. Plant Genome 4:154–164.

McCollum, T.G. and R.E. McDonald. 1991. Electrolyte leakage, respiration, and ethylene production as indices of chilling injury in grapefruit. Hort. Sci. 26:1191-1192.

Michalak de Jiménez, M., S. Mamidi, P.E. McClean. and M. Rahman. (Unpublished). Low level of linkage disequilibrium and population structure in a core collection of *Brassica napus* discovered using SNPs derived from GBS.

Murray, A.J.S., R.D. Blackwell, and P.J. Lea. 1989. Metabolism of hydroxypyruvate in a mutant of barley lacking NADH-dependent hydroxypyruvate reductase, an important photorespiratory enzyme activity. Plant Physiol. 91 395–400

Osterhaut, W.J.V. 1922. Injury, recovery, and death, in relation to conductivity and permeability. Philadelphia, JB Lippincott. London: J.B. Lippincott Company.

Poland, J.A. and T.W. Rife. 2012. Genotyping-by-sequencing for plant breeding and genetics. The Plant Genome. 5(3):92-102.

Price, A.L., N.J. Patterson, R.M. Plenge, M.E. Weinblatt, N.A. Shadick, and D. Reich. 2006. Principal components analysis corrects for stratification in genome-wide association studies. Nature Genet. 38(8):904-909.

Pritchard, J.K. and M. Przeworski. 2001.Linkage disequilibrium in humans: models and data. Am. J. Hum. Genet. 69:1-14.

Rafalski, A. 2002. Applications of single nucleotide polymorphisms in crop genetics. Curr. Opin. Plant Biol. 5:94-100.

Rakow, G. 2007. Rapeseed genetics and breeding research for sustainable oilseed production. Proceedings of the 12$^{th}$ International Rapeseed Congress 1:2072-210.

Rahman, M., S. Mamidi, L. del Rio, A. Ross, M.M Kadir, M.M. Rahaman, and M. Arifuzzaman. 2016. Association mapping in *Brassica napus* (L.) accessions identifies a major QTL for blackleg disease resistance on chromosome A01. Mol. Breeding. 36:90. doi:10.1007/s11032-016-0513-8.

Rezaeizad, A., B. Wittkop, R. Snowdon, M. Hasan, V. Mohammadi, A. Zali, and W. Friedt. 2011. Identification of QTLs for phenolic compounds in oilseed rape (Brassica napus L.) by association mapping using SSR markers. Euphytica 177:335–342.

Salanoubat, M., K. Lemcke, M. Rieger, W. Ansorge, M. Unseld, B. Fartmann, G. Valle, H. Blocker, M. Perez-Alonso, B. Obermaier, M. Delseny, M. Boutry, L.A. Grivell, R. Mache, P. Puigdomenech, V. De Simone, N. Choisne, F. Artiguenave, C. Robert, P. Brottier, P. Wincker, L. Cattolico, J. Weissenbach, W. Saurin, F. Quetier, M. Schafer, S. Muller-Auer, C. Gabel, M. Fuchs, V. Benes, E. Wurmbach, H. Drzonek, H. Erfle, N. Jordan, S. Bangert, R. Wiedelmann, H. Kranz, H. Voss, R. Holland, P. Brandt, G. Nyakatura, A. Vezzi, M. D'Angelo, A. Pallavicini, S. Toppo, B. Simionati, A. Conrad, K. Hornischer, G. Kauer, T.H. Lohnert, G. Nordsiek, J. Reichelt, M. Scharfe, O. Schon, M. Bargues, J. Terol, J. Climent, P. Navarro, C. Collado, A. Perez-Perez, B. Ottenwalder, D. Duchemin, R. Cooke, M. Laudie, C. Berger-Llauro, B. Purnelle, D. Masuy, M. de Haan, A.C. Maarse, J.P. Alcaraz, A. Cottet, E. Casacuberta, A. Monfort, A. Argiriou, M. Flores, R. Liguori, D. Vitale, G. Mannhaupt, D. Haase, H. Schoof, S. Rudd, P. Zaccaria, H.W. Mewes, K.F. Mayer, S. Kaul, C.D. Town, H.L. Koo, L.J. Tallon, J. Jenkins, T. Rooney, M. Rizzo, A. Walts, T. Utterback, C.Y. Fujii, T.P. Shea, T.H. Creasy, B. Haas, R. Maiti, D. Wu, J. Peterson, S. Van Aken, G. Pai, J. Militscher, P. Sellers, J.E. Gill, T.V. Feldblyum, D. Preuss, X. Lin, W.C. Nierman, S.L. Salzberg, O. White, J.C. Venter, C.M. Fraser, T. Kaneko, Y. Nakamura, S. Sato, T. Kato, E. Asamizu, S. Sasamoto, T. Kimura, K. Idesawa, K. Kawashima, Y. Kishida, C. Kiyokawa, M. Kohara, M. Matsumoto, A. Matsuno, A. Muraki, S. Nakayama, N. Nakazaki, S. Shinpo, C. Takeuchi, T. Wada, A. Watanabe, M. Yamada, M. Yasuda, and S. Tabata. 2000. Sequence and analysis of chromosome 3 of the plant *Arabidopsis thaliana*. Nature. 408(6814):820-822.

Scheet, P. and M. Stephens. 2006. A fast and flexible statistical model for large-scale population genotype data: applications to inferring missing genotypes and haplotypic phase. Am J. Human Genet. 78:629-644.

Stevanovic, B., J. Sinzar, and O. Glisic. 1997. Electrolyte leakage differences between poikilohydrous and homoiohydrous species of Gesneriaceae. Biol. Plant. 40: 299–303.

Sun, J., N. Guo, J. Lei, L. Li, G. Hu, and H. Xing. 2014. Association mapping for partial resistance to *Phytophthora sojae* in soybean (*Glycine max* L.). J. of Genetics. 2:355-363.

Theologis, A., J.R. Ecker, C.J. Palm, N.A. Federspiel, S. Kaul, O. White, J. Alonso, H. Altafi, R. Araujo, C.L. Bowman, S.Y. Brooks, E. Buehler, A. Chan, Q. Chao, H. Chen, R.F. Cheuk, C.W. Chin, M.K. Chung, L. Conn, A.B. Conway, A.R. Conway, T.H. Creasy, K. Dewar, P. Dunn, P. Etgu, T.V. Feldblyum, J. Feng, B. Fong, C.Y. Fujii, J.E. Gill, A.D. Goldsmith, B. Haas, N.F. Hansen, B. Hughes, L. Huizar, J.L. Hunter, J. Jenkins, C. Johnson-Hopson, S. Khan, E. Khaykin, C.J. Kim, H.L. Koo, I. Kremenetskaia, D.B. Kurtz, A. Kwan, B. Lam, S. Langin-Hooper, A. Lee, J.M. Lee, C.A. Lenz, J.H. Li, Y. Li, X. Lin, S.X. Liu, Z.A. Liu, J.S. Luros, R. Maiti, A. Marziali, J. Militscher, M. Miranda, M. Nguyen, W.C. Nierman, B.I. Osborne, G. Pai, J. Peterson, P.K. Pham, M. Rizzo, T. Rooney, D. Rowley, H. Sakano, S.L. Salzberg, J.R. Schwartz, P. Shinn, A.M. Southwick, H. Sun, L.J. Tallon, G. Tambunga, M.J. Toriumi, C.D. Town, T. Utterback, S. Van Aken, M. Vaysberg, V.S. Vysotskaia, M. Walker, D. Wu, G. Yu, C.M. Fraser, J.C. Venter, and R.W. Davis. 2000. Sequence and analysis of chromosome 1 of the plant *Arabidopsis thaliana*. Nature. 408(6814):816-820.

U, N. 1935. Genome analysis in *Brassica* with special reference to the experimental formation of *Brassica napus* and peculiar mode of fertilization. Japanese J. Botany. 7:389-452.

(USDA) United States Department of Agriculture. 2016. Foreign Agricultural Service. Accessed: https://www.fas.usda.gov/ (24 April 2017).

Yu, J.M. and E.S. Buckler. 2006. Genetic association mapping and genome organization of maize. Curr. Opin. Biotech. 17:155-160.

Yu, J., G. Pressoir, W.H. Briggs, I.V. Bi, M. Yamasaki, J.F. Doebley, M.D. McMullen, B.S. Gaut, D.M. Nielsen, J.B. Holland, S. Kresovich, and E.S. Buckler. 2006. A unified mixed-model method for association mapping that accounts for multiple levels of relatedness. Nat. Genet. 38:203–208.

Zhao, K., M.J. Aranzana, S. Kim, C. Lister, C. Shindo, C. Tang, C. Toomajian, H. Zheng, C. Dean, P. Marjoram, and M. Nordborg. 2007. An Arabidopsis example of association mapping in structured samples. PLoS Genetics 3:e4. doi: http://dx.doi.org/10.1371/journal.pgen.0030004.

# CHAPTER 7. OVERALL CONCLUSION

A frost tolerance study in rapeseed/canola was conducted in both natural conditions and the greenhouse. In addition, a study on freezing induced electrolyte leakage was also conducted using a freezing water bath. Beofore screening the germplasm panel for freezing tolerance, a protocol was developed to screen the germplasm in the plant growth chamber under artificial frost simulating conditions. Under this protocol, seedlings were grown in a greenhouse for 14 days followed by cold acclimation (4°C) for 7 days and over night freezing treatment (-8°C). A protocol was also developed for the electrolyte leakage measurement, where 14 day old seedlings were cold acclimated for 7 days followed by freezing treatment (-12°C) on leaves in freezing water bath. In the field experiment, the seedlings were grown in a greenhouse for 14 days and cold acclimated (4°C) in a plant growth chamber, and were placed outside when the expected temperature was around -4 to -6°C. Three different growth types of canola (spring, winter, and semi-winter) were used in these studies.

Genome-wide association scan (GWAS) was conducted on each of the three experiments separately. Each of the experiments had around 37,000 SNPs after filtering for minor allele frequency <5%. The greenhouse study identified one QTL on chromosome A02 while the field study also identified one QTL on chromosome C04. The electrolyte leakage study identified 10 QTLs that were located on chromosomes A01, A02, A04, A06, A07, C02, C05, C07, C09, and one that could not be assigned to any chromosome.

All the significant markers selected at the 0.1% tail of the empirical distribution after 10,000 bootstraps for the three GWAS studies were assigned across the 19 chromosomes of *B. napus*. Chromsome C06 has markers for all three traits present, but the electrolyte leakage and natural condition markers are closer to each other than the marker for the greenhouse. Markers

present on chromosome C09 are close for the natural condition and greenhouse studies. The greenhouse and electrolyte leakage traits have markers present on chromosomes A01, A04, A07, and A10. Electrolyte leakage and natural condition markers are present on chromosomes C01, C02, C06, and C07. Chromosomes A07, C06, and C09 have markers for the natural condition and greenhouses studies.

The candidate genes identified here were protein families and transcription factors. All the genes were somehow related to abiotic stress or stress tolerance. Most of the genes were turned on in the presence of abiotic stress. The markers or genes identified here can be used for frost tolerance screening in marker assisted selection (MAS) program.

# CHAPTER 8. FUTURE WORK

Future work to further understand and expand the knowledge of frost tolerance in canola should be conducted. Frost tolerance could still be better understood and the results from these studies can be used to further frost tolerance studies in many ways. The protocol for frost tolerance screening was developed based on small freezing chamber study. The large plant growth chamber did not freeze the same as the small chamber. Therefore, the temperature had to be lowered in the larger freezing chamber to obtain a good freezing induced seedling damage screening. A future study could be conducted to optimize the protocol for the large chamber.

The genome-wide association scan on the greenhouse, field, and electrolyte leakage studies identified many SNP markers. These markers would need to be validated to be used in the breeding program.

The work to be conducted in the future includes:

1. Optimization of the protocol for the larger plant growth chamber.
2. Validation of the SNP markers identified in the natural condition, greenhouse, and electrolyte leakage studies in different populations.
3. Utilization of the markers in marker-assisted selection in breeding program.

## APPENDIX

Table A1. Growth habit and the country that each genotype originated or was obtained from for the -8°C greenhouse study.

| Genotype | Growth habit | Country originated/obtained from |
|---|---|---|
| A-10-999 | Spring | USA |
| A-15-1000 | Spring | Canada |
| A-15-989 | Spring | USA |
| A-16-1013 | Spring | USA |
| A-3-1011 | Spring | USA |
| A-4-1000 | Spring | USA |
| A-7-997 | Spring | USA |
| A-8-1000 | Spring | USA |
| A-9-1013 | Spring | USA |
| A04-72NA | Spring | USA |
| A04-73NA | Spring | USA |
| A04-74NA | Spring | USA |
| A06-19NA | Spring | USA |
| A06-20NA | Spring | USA |
| A07-26NR | Spring | USA |
| A07-28NA | Spring | USA |
| A07-29NI | Spring | USA |
| Aomori | Winter | Japan |
| AR-256 | Winter | Russian Federation |
| AR91004 | Winter | USA |
| ARC-90016 | Winter | USA |
| ARC-97018 | Winter | USA |
| Aviso | Spring | Canada |
| Azuma | Semi-winter | South Korea |
| Azumasho | Semi-winter | South Korea |
| Baraska | Winter | Germany |
| Barkant | Winter | Netherlands |
| Beryl | Winter | Poland |
| Billy | Winter | Sweden |
| Bingo | Spring | USA |
| BNW 161/83 | Winter | Germany |
| BO-63 | Spring | Canada |
| BRA 1168/85 | Winter | Italy |
| Brio | Spring | France |
| Bronowski | Spring | Poland |

Table A1. Growth habit and the country that each genotype originated or was obtained from for the -8°C greenhouse study (continued).

| Genotype | Growth habit | Country originated/obtained from |
|---|---|---|
| Buk Wuk 3 | Spring | South Korea |
| Cathy | Winter | USA |
| Celebra | Spring | Sweden |
| Cescaljarni repka | Semi-winter | South Korea |
| Ceskia Tabor | Spring | Czechoslovakia |
| Chon nam | Semi-winter | South Korea |
| Chun nung 1 | Winter | China |
| Cobra | Winter | Germany |
| Colza | Spring | South Korea |
| Colza 18 Miroc | Semi-winter | South Korea |
| Comet | Spring | Sweden |
| Conquest | Spring | Canada |
| Cougar | Spring | Canada |
| Cresor | Spring | France |
| Crop | Spring | France |
| Crystal | Winter | Sweden |
| Cult | Winter | Canada |
| Czyzowski | Spring | Poland |
| Da vinci | Winter | Canada |
| Dae cho sen | Semi-winter | South Korea |
| Delta | Spring | Sweden |
| Doon major swede | Winter | New Zealand |
| Drakkar | Spring | France |
| Eckendorfer Mali | Semi-winter | South Korea |
| Elena | Winter | Germany |
| Eragi | Winter | Germany |
| Erra | Winter | Germany |
| Evvin | Spring | Russian Federation |
| Expander | Winter | Germany |
| Fashion | Winter | Canada |
| Fertodi | Winter | South Korea |
| Flint | Spring | USA |
| Fonto | Spring | South Korea |
| France 1 | Spring | France |
| Fuji | Spring | South Korea |
| Furax | Winter | France |

Table A1. Growth habit and the country that each genotype originated or was obtained from for the -8°C greenhouse study (continued).

| Genotype | Growth habit | Country originated/obtained from |
|---|---|---|
| G-32327 | Winter | Albania |
| Galant | Spring | USA |
| Galaxy | Spring | Sweden |
| Galileo | Winter | Canada |
| Gebr Dippes | Winter | South Korea |
| Gido | Spring | Germany |
| Girita | Semi-winter | Germany |
| Glacier | Winter | Sweden |
| Global | Spring | Sweden |
| Golden | Spring | Canada |
| Gora | Spring | Germany |
| Goya | Winter | Canada |
| Gulle | Spring | Sweden |
| Gullivar | Spring | Sweden |
| Gundula | Winter | Germany |
| Gylle | Semi-winter | South Korea |
| Helga | Semi-winter | Germany |
| Hi-Q | Spring | Canada |
| Ibiza | Winter | Canada |
| IR-2 | Spring | Hungary |
| Iwao natane | Winter | South Korea |
| Iwawoochi | Winter | South Korea |
| Janetzkis | Spring | South Korea |
| Jasna | Spring | USA |
| Jet Neuf | Winter | Canada |
| Jupiter | Winter | USA |
| Kanada | Spring | Poland |
| Karafuto | Winter | South Korea |
| Kasuya | Winter | South Korea |
| Klinki | Spring | South Korea |
| Korina | Winter | Germany |
| Kosa | Spring | Germany |
| Koubun | Spring | South Korea |
| Kovalevskjj | Spring | Ukraine |
| Kraphhauser | Spring | South Korea |
| Kritmar rape | Spring | South Korea |

Table A1. Growth habit and the country that each genotype originated or was obtained from for the -8°C greenhouse study (continued).

| Genotype | Growth habit | Country originated/obtained from |
|---|---|---|
| KS3579 | Winter | USA |
| KSU-1 | Winter | USA |
| KSU-10 | Winter | USA |
| KSU-2 | Winter | USA |
| KSU-3 | Winter | USA |
| KSU-4 | Winter | USA |
| KSU-5 | Winter | USA |
| KSU-6 | Winter | USA |
| KSU-7 | Winter | USA |
| KSU-8 | Winter | USA |
| KSU-9 | Winter | USA |
| Kuju | Winter | South Korea |
| Kutkowski | Winter | South Korea |
| Ladoga | Winter | Canada |
| Legend | Spring | Sweden |
| Lembkes | Winter | South Korea |
| Lembkes malchower | Winter | South Korea |
| Lesira | Winter | Germany |
| Lester | Winter | Germany |
| Librador | Winter | Germany |
| Licantara | Winter | Germany |
| Lieikoposki | Semi-winter | South Korea |
| Lifura | Spring | South Korea |
| Lindora-00 | Winter | Germany |
| Lindore | Winter | Germany |
| Linglandor | Winter | Germany |
| Linus | Winter | South Korea |
| Lirakotta | Winter | Germany |
| Liratrop | Winter | Germany |
| Lisora | Semi-winter | Germany |
| Lorenze | Winter | Canada |
| Luna | Winter | Germany |
| Major | Semi-winter | France |
| Mar 160059 | Winter | Poland |
| Marinus | Winter | Germany |
| Mar'janovskij | Spring | Ukraine |

Table A1. Growth habit and the country that each genotype originated or was obtained from for the -8°C greenhouse study (continued).

| Genotype | Growth habit | Country originated/obtained from |
|---|---|---|
| Midas | Spring | Canada |
| Miekuro Dane | Spring | South Korea |
| Miochowski | Semi-winter | France |
| Mlochowski | Semi-winter | Poland |
| Murame nadame | Semi-winter | South Korea |
| Mutsumi | Semi-winter | Japan |
| N001-28-246-5-4 | Semi-winter | South Korea |
| Nabo | Semi-winter | South Korea |
| Nilla-1022 | Semi-winter | South Korea |
| NU-41737 | Spring | Turkey |
| NU-51084 | Spring | Sweden |
| Nugget | Semi-winter | South Korea |
| NY-10 | Semi-winter | China |
| NY-20 | Semi-winter | China |
| NY-7 | Semi-winter | China |
| NY-8 | Semi-winter | China |
| Oleifera | Semi-winter | South Korea |
| Oro | Spring | Canada |
| Orpal | Spring | France |
| Panter | Winter | Germany |
| Peace | Spring | Canada |
| Petanova lihonova | Semi-winter | South Korea |
| Polo canola | Spring | USA |
| Premier | Spring | USA |
| Printol | Spring | USA |
| Prota | Spring | Germany |
| Q2 | Spring | Canada |
| Quinta | Winter | Germany |
| R. Creaus | Winter | South Korea |
| Rafal | Winter | France |
| Ramses | Winter | South Korea |
| Rang | Semi-winter | South Korea |
| Rapifera | Winter | South Korea |
| Ratnik | Spring | USA |
| Rebel | Semi-winter | USA |
| Regal | Winter | South Korea |

Table A1. Growth habit and the country that each genotype originated or was obtained from for the -8°C greenhouse study (continued).

| Genotype | Growth habit | Country originated/obtained from |
|---|---|---|
| Regent | Spring | Canada |
| Regina II | Spring | Canada |
| Reston | Spring | USA |
| Rico | Spring | Germany |
| Ridana | Winter | Germany |
| Romeo | Spring | France |
| Rubin | Winter | Germany |
| Rumania 1 | Winter | Romania |
| Russia 5 | Spring | Russian Federation |
| Salamander | Winter | South Korea |
| Santana | Winter | Germany |
| Scherwitz | Winter | South Korea |
| Seoul | Spring | South Korea |
| Sera | Semi-winter | Germany |
| Siberian | Winter | USA |
| Silesia | Winter | Czechoslovakia |
| Silex | Spring | Canada |
| Skrzeszowicki | Winter | Poland |
| Su-weon-chag | Semi-winter | South Korea |
| Sumner | Semi-winter | USA |
| Sunrise | Spring | USA |
| Sval of Karab | Winter | Sweden |
| Sval of Gullen | Spring | South Korea |
| Taichang | Semi-winter | South Korea |
| Taiwan | Spring | Taiwan |
| Takagis-MS | Semi-winter | South Korea |
| Tanka | Semi-winter | South Korea |
| Tanto | Spring | France |
| Titus | Winter | South Korea |
| Todane | Semi-winter | South Korea |
| Tonus | Spring | South Korea |
| Topas | Spring | Sweden |
| Tosharshu | Winter | South Korea |
| Tower | Spring | Canada |
| Turret | Spring | Canada |
| Vanda | Winter | Germany |

Table A1. Growth habit and the country that each genotype originated or was obtained from for the -8°C greenhouse study (continued).

| Genotype | Growth habit | Country originated/obtained from |
|---|---|---|
| Vinnickij 15/59 | Winter | Ukraine |
| Vision | Winter | Canada |
| Vostochno sibirskii | Spring | Russian Federation |
| Wasefuji | Spring | South Korea |
| Weal dong cho | Semi-winter | South Korea |
| Weibulls margo | Semi-winter | South Korea |
| Westar | Spring | Canada |
| Wichita | Winter | USA |
| Wielkopolski | Winter | South Korea |
| Willa | Spring | South Korea |
| Wipol | Semi-winter | Norway |
| Wira | Winter | Germany |
| Yong dang | Semi-winter | South Korea |
| Yonkkaichi kwo | Semi-winter | South Korea |
| Yonkokuban | Winter | South Korea |
| Yudal | Spring | South Korea |

Table A2. Overall median, mean rank, estimated relative effect, and 95% confidence interval for the -8°C greenhouse study.

| | | | | | Confidence interval (95%) for the estimated relative effect | |
|---|---|---|---|---|---|---|
| Genotype | Median | Mean rank | Estimated relative effect | Variance | Lower limit | Upper limit |
| Polo canola | 1.00 | 209.40 | 0.16 | 1.87 | 0.10 | 0.25 |
| Prota | 1.00 | 209.40 | 0.16 | 1.87 | 0.10 | 0.25 |
| Drakkar | 1.00 | 217.58 | 0.17 | 1.31 | 0.11 | 0.24 |
| A07-26NR | 1.00 | 258.50 | 0.20 | 0.06 | 0.19 | 0.21 |
| BO-63 | 1.00 | 258.50 | 0.20 | . | . | . |
| KSU-3 | 1.00 | 258.50 | 0.20 | 0.06 | 0.19 | 0.21 |
| Lifura | 1.00 | 258.50 | 0.20 | 0.06 | 0.19 | 0.21 |
| Turret | 1.00 | 258.50 | 0.20 | 0.06 | 0.19 | 0.21 |
| Azuma | 1.00 | 263.50 | 0.20 | 3.50 | 0.12 | 0.32 |
| Sera | 1.00 | 264.50 | 0.20 | 5.24 | 0.11 | 0.36 |
| Kuju | 1.00 | 302.58 | 0.23 | 7.92 | 0.12 | 0.42 |

. = Not calculated

Table A2. Overall median, mean rank, estimated relative effect, and 95% confidence interval for the -8°C greenhouse study (continued).

| Genotype | Median | Mean rank | Estimated relative effect | Variance | Confidence interval (95%) for the estimated relative effect | |
|---|---|---|---|---|---|---|
| | | | | | Lower limit | Upper limit |
| Luna | 1.00 | 304.42 | 0.24 | 1.67 | 0.17 | 0.31 |
| Marinus | 1.00 | 305.25 | 0.24 | 7.69 | 0.12 | 0.42 |
| IR-2 | 1.00 | 313.60 | 0.24 | 2.39 | 0.17 | 0.34 |
| Cougar | 1.00 | 314.60 | 0.24 | 11.43 | 0.11 | 0.47 |
| A-15-989 | 1.00 | 343.50 | 0.27 | 5.59 | 0.16 | 0.41 |
| Fonto | 1.00 | 343.50 | 0.27 | 5.59 | 0.16 | 0.41 |
| Tanto | 1.00 | 343.50 | 0.27 | 5.59 | 0.16 | 0.41 |
| NY-10 | 1.00 | 360.50 | 0.28 | 8.04 | 0.15 | 0.45 |
| Galant | 1.00 | 366.50 | 0.28 | 13.00 | 0.13 | 0.51 |
| Azumasho | 1.00 | 369.70 | 0.29 | 12.58 | 0.13 | 0.51 |
| A-16-1013 | 1.00 | 389.42 | 0.30 | 6.01 | 0.19 | 0.45 |
| A-9-1013 | 1.00 | 389.42 | 0.30 | 6.01 | 0.19 | 0.45 |
| Goya | 1.00 | 389.42 | 0.30 | 6.01 | 0.19 | 0.45 |
| Kraphhauser | 1.00 | 389.42 | 0.30 | 6.01 | 0.19 | 0.45 |
| Kritmar rape | 1.00 | 389.42 | 0.30 | 6.01 | 0.19 | 0.45 |
| A-15-1000 | 1.25 | 394.42 | 0.30 | 9.28 | 0.17 | 0.49 |
| Kutkowski | 1.00 | 411.00 | 0.32 | 25.83 | 0.12 | 0.63 |
| A-8-1000 | 1.00 | 387.58 | 0.32 | 17.72 | 0.14 | 0.57 |
| Gulle | 1.00 | 415.60 | 0.32 | 8.21 | 0.19 | 0.49 |
| Yong dang | 1.00 | 415.60 | 0.32 | 8.21 | 0.19 | 0.49 |
| Janetzkis | 1.00 | 426.08 | 0.33 | 17.96 | 0.15 | 0.58 |
| Bronowski | 1.00 | 428.50 | 0.33 | 8.91 | 0.19 | 0.51 |
| Crop | 1.00 | 428.50 | 0.33 | 8.91 | 0.19 | 0.51 |
| Mlochowski | 1.00 | 428.50 | 0.33 | 8.91 | 0.19 | 0.51 |
| Regent | 1.00 | 428.50 | 0.33 | 8.91 | 0.19 | 0.51 |
| Vanda | 1.00 | 428.50 | 0.33 | 8.91 | 0.19 | 0.51 |
| Westar | 1.25 | 433.50 | 0.34 | 12.12 | 0.18 | 0.54 |
| Elena | 1.25 | 435.33 | 0.34 | 5.79 | 0.22 | 0.48 |
| A-10-999 | 1.25 | 436.17 | 0.34 | 11.78 | 0.18 | 0.54 |
| Delta | 1.00 | 449.25 | 0.35 | 22.47 | 0.15 | 0.62 |
| BRA 1168/85 | 1.00 | 451.08 | 0.35 | 16.12 | 0.17 | 0.58 |
| Wipol | 1.00 | 451.08 | 0.35 | 16.12 | 0.17 | 0.58 |
| Peace | 1.00 | 461.80 | 0.36 | 16.13 | 0.18 | 0.59 |
| Sumner | 1.00 | 461.80 | 0.36 | 16.13 | 0.18 | 0.59 |
| A06-20NA | 1.00 | 462.50 | 0.36 | 12.04 | 0.20 | 0.56 |

. = Not calculated

Table A2. Overall median, mean rank, estimated relative effect, and 95% confidence interval for the -8°C greenhouse study (continued).

| Genotype | Median | Mean rank | Estimated relative effect | Variance | Confidence interval (95%) for the estimated relative effect | |
|---|---|---|---|---|---|---|
| | | | | | Lower limit | Upper limit |
| Legend | 1.00 | 462.50 | 0.36 | 12.04 | 0.20 | 0.56 |
| Willa | 1.00 | 462.50 | 0.36 | 12.04 | 0.20 | 0.56 |
| Sunrise | 1.00 | 467.00 | 0.36 | 14.08 | 0.19 | 0.58 |
| Buk Wuk 3 | 1.50 | 472.58 | 0.37 | 14.51 | 0.19 | 0.59 |
| Rapifera | 1.25 | 473.83 | 0.37 | 10.87 | 0.21 | 0.56 |
| Baraska | 1.25 | 474.42 | 0.37 | 8.14 | 0.23 | 0.53 |
| Lirakotta | 1.25 | 474.42 | 0.37 | 8.14 | 0.23 | 0.53 |
| Printol | 1.25 | 474.42 | 0.37 | 8.14 | 0.23 | 0.53 |
| Regina-II | 1.25 | 474.42 | 0.37 | 8.14 | 0.23 | 0.53 |
| Tower | 1.25 | 474.67 | 0.37 | 16.85 | 0.18 | 0.60 |
| Ratnik | 1.00 | 487.40 | 0.38 | 32.05 | 0.14 | 0.69 |
| Gido | 1.00 | 490.17 | 0.38 | 18.28 | 0.19 | 0.62 |
| Global | 1.00 | 490.17 | 0.38 | 18.28 | 0.19 | 0.62 |
| Topas | 1.25 | 497.00 | 0.38 | 15.03 | 0.20 | 0.60 |
| Midas | 1.00 | 508.70 | 0.39 | 19.11 | 0.19 | 0.64 |
| NY-8 | 1.00 | 508.70 | 0.39 | 19.11 | 0.19 | 0.64 |
| Miochowski | 1.00 | 511.50 | 0.40 | 37.03 | 0.14 | 0.72 |
| A-4-1000 | 1.50 | 513.50 | 0.40 | 10.03 | 0.24 | 0.58 |
| Aviso | 1.50 | 513.50 | 0.40 | 10.03 | 0.24 | 0.58 |
| Czyzowski | 1.50 | 513.50 | 0.40 | 10.03 | 0.24 | 0.58 |
| Expander | 1.50 | 513.50 | 0.40 | 10.03 | 0.24 | 0.58 |
| KSU-6 | 1.50 | 513.50 | 0.40 | 10.03 | 0.24 | 0.58 |
| Taiwan | 1.50 | 513.50 | 0.40 | 10.03 | 0.24 | 0.58 |
| A07-29NI | 1.50 | 517.60 | 0.40 | 10.06 | 0.25 | 0.58 |
| Cathy | 1.50 | 517.60 | 0.40 | 10.06 | 0.25 | 0.58 |
| Mar 160059 | 1.50 | 517.60 | 0.40 | 10.06 | 0.25 | 0.58 |
| Fuji | 1.00 | 533.60 | 0.41 | 38.71 | 0.15 | 0.74 |
| Eckendorfer Mali | 1.50 | 534.25 | 0.41 | 23.03 | 0.19 | 0.67 |
| A06-19NA | 1.25 | 536.08 | 0.41 | 16.63 | 0.22 | 0.64 |
| Lieikoposki | 1.25 | 536.08 | 0.41 | 16.63 | 0.22 | 0.64 |
| Orpal | 1.25 | 536.08 | 0.41 | 16.63 | 0.22 | 0.64 |
| Oro | 1.00 | 536.50 | 0.41 | 24.96 | 0.19 | 0.69 |
| France 1 | 1.50 | 552.00 | 0.43 | 14.18 | 0.24 | 0.63 |
| Gullivar | 1.50 | 552.00 | 0.43 | 14.18 | 0.24 | 0.63 |
| Wira | 1.50 | 552.00 | 0.43 | 14.18 | 0.24 | 0.63 |

. = Not calculated

Table A2. Overall median, mean rank, estimated relative effect, and 95% confidence interval for the -8°C greenhouse study (continued).

| Genotype | Median | Mean rank | Estimated relative effect | Variance | Confidence interval (95%) for the estimated relative effect | |
|---|---|---|---|---|---|---|
| | | | | | Lower limit | Upper limit |
| Vision | 1.00 | 554.50 | 0.43 | 43.92 | 0.15 | 0.77 |
| Fertodi | 1.00 | 554.90 | 0.43 | 25.39 | 0.20 | 0.70 |
| Major | 1.00 | 554.90 | 0.43 | 25.39 | 0.20 | 0.70 |
| Kasuya | 2.00 | 557.58 | 0.43 | 14.49 | 0.25 | 0.64 |
| Ceskia Tabor | 1.25 | 558.17 | 0.43 | 21.10 | 0.22 | 0.68 |
| Billy | 1.75 | 559.42 | 0.43 | 8.06 | 0.29 | 0.59 |
| Nabo | 1.50 | 563.80 | 0.44 | 16.15 | 0.24 | 0.65 |
| NY-7 | 1.50 | 571.20 | 0.44 | 26.52 | 0.20 | 0.71 |
| Yonkkaichi kwo | 1.50 | 572.70 | 0.44 | 6.93 | 0.31 | 0.59 |
| Doon major swede | 1.50 | 575.17 | 0.44 | 17.77 | 0.24 | 0.67 |
| Iwawoochi | 1.50 | 575.17 | 0.44 | 17.77 | 0.24 | 0.67 |
| Nilla-1022 | 1.50 | 575.17 | 0.44 | 17.77 | 0.24 | 0.67 |
| Seoul | 1.50 | 575.17 | 0.44 | 17.77 | 0.24 | 0.67 |
| Takagis-MS | 1.50 | 575.17 | 0.44 | 17.77 | 0.24 | 0.67 |
| Yonkokuban | 1.50 | 575.17 | 0.44 | 17.77 | 0.24 | 0.67 |
| Yudal | 1.75 | 580.17 | 0.45 | 20.77 | 0.23 | 0.69 |
| Klinki | 1.50 | 582.00 | 0.45 | 14.34 | 0.26 | 0.65 |
| Rebel | 1.50 | 582.00 | 0.45 | 14.34 | 0.26 | 0.65 |
| Barkant | 2.00 | 589.40 | 0.46 | 31.08 | 0.20 | 0.74 |
| Russia-5 | 1.50 | 590.50 | 0.46 | 17.88 | 0.25 | 0.68 |
| Mar'janovskij | 1.75 | 597.92 | 0.46 | 11.66 | 0.29 | 0.65 |
| KSU-2 | 2.00 | 598.50 | 0.46 | 8.94 | 0.31 | 0.62 |
| Lorenze | 1.50 | 604.08 | 0.47 | 18.50 | 0.25 | 0.69 |
| KSU-7 | 1.00 | 610.50 | 0.47 | 35.80 | 0.19 | 0.77 |
| Colza | 2.00 | 610.70 | 0.47 | 17.31 | 0.26 | 0.69 |
| Linus | 1.50 | 613.67 | 0.47 | 21.20 | 0.25 | 0.71 |
| Miekuro Dane | 1.50 | 613.67 | 0.47 | 21.20 | 0.25 | 0.71 |
| Silesia | 1.50 | 618.90 | 0.48 | 12.03 | 0.30 | 0.66 |
| Comet | 2.00 | 619.25 | 0.48 | 21.40 | 0.25 | 0.72 |
| Fashion | 1.75 | 621.08 | 0.48 | 14.93 | 0.28 | 0.68 |
| Karafuto | 1.75 | 621.08 | 0.48 | 14.93 | 0.28 | 0.68 |
| Su-weon-chag | 1.75 | 621.08 | 0.48 | 14.93 | 0.28 | 0.68 |
| Tonus | 1.75 | 621.08 | 0.48 | 14.93 | 0.28 | 0.68 |
| Wichita | 1.75 | 621.08 | 0.48 | 14.93 | 0.28 | 0.68 |
| A-3-1011 | 1.50 | 631.08 | 0.49 | 24.25 | 0.25 | 0.74 |

. = Not calculated

Table A2. Overall median, mean rank, estimated relative effect, and 95% confidence interval for the -8°C greenhouse study (continued).

| Genotype | Median | Mean rank | Estimated relative effect | Variance | Confidence interval (95%) for the estimated relative effect | |
|---|---|---|---|---|---|---|
| | | | | | Lower limit | Upper limit |
| Silex | 1.75 | 636.42 | 0.49 | 14.82 | 0.30 | 0.69 |
| Jet Neuf | 1.50 | 636.83 | 0.49 | 24.37 | 0.25 | 0.74 |
| NU-51084 | 1.50 | 636.83 | 0.49 | 24.37 | 0.25 | 0.74 |
| Ramses | 1.50 | 636.83 | 0.49 | 24.37 | 0.25 | 0.74 |
| Petanova lihonova | 2.00 | 637.00 | 0.49 | 12.07 | 0.31 | 0.67 |
| Q2 | 2.00 | 637.00 | 0.49 | 12.07 | 0.31 | 0.67 |
| Galaxy | 1.50 | 637.80 | 0.49 | 26.19 | 0.24 | 0.75 |
| A-7-997 | 2.00 | 638.50 | 0.49 | 22.05 | 0.26 | 0.73 |
| Hi-Q | 2.00 | 644.42 | 0.50 | 5.78 | 0.37 | 0.63 |
| Linglandor | 2.00 | 644.42 | 0.50 | 5.78 | 0.37 | 0.63 |
| Kovalevskjj | 2.00 | 656.90 | 0.51 | 21.75 | 0.27 | 0.74 |
| Siberian | 2.00 | 657.75 | 0.51 | 24.30 | 0.26 | 0.75 |
| A04-72NA | 2.00 | 740.50 | 0.51 | 15.06 | 0.31 | 0.71 |
| Celebra | 2.00 | 660.17 | 0.51 | 15.06 | 0.31 | 0.71 |
| Flint | 2.00 | 660.17 | 0.51 | 15.06 | 0.31 | 0.71 |
| Gebr Dippes | 2.00 | 660.17 | 0.51 | 15.06 | 0.31 | 0.71 |
| Liratrop | 2.00 | 660.17 | 0.51 | 15.06 | 0.31 | 0.71 |
| Lisora | 2.00 | 660.17 | 0.51 | 15.06 | 0.31 | 0.71 |
| Reston | 2.00 | 660.17 | 0.51 | 15.06 | 0.31 | 0.71 |
| Todane | 2.00 | 660.17 | 0.51 | 15.06 | 0.31 | 0.71 |
| R.Creaus | 2.00 | 675.50 | 0.52 | 14.76 | 0.32 | 0.72 |
| Murame nadame | 2.00 | 680.92 | 0.53 | 27.15 | 0.26 | 0.78 |
| Tanka | 2.00 | 682.25 | 0.53 | 18.68 | 0.30 | 0.74 |
| A07-28NA | 2.00 | 683.50 | 0.53 | 5.64 | 0.40 | 0.65 |
| Scherwitz | 2.00 | 690.33 | 0.53 | 4.81 | 0.41 | 0.65 |
| Iwao natane | 1.50 | 692.10 | 0.54 | 36.02 | 0.24 | 0.81 |
| KSU-1 | 2.00 | 806.25 | 0.54 | 16.34 | 0.32 | 0.74 |
| Vostochno sibirskii | 2.00 | 698.50 | 0.54 | 29.80 | 0.26 | 0.80 |
| Sval of Karab | 2.00 | 704.83 | 0.55 | 24.11 | 0.29 | 0.78 |
| Kosa | 2.00 | 714.50 | 0.55 | 20.25 | 0.31 | 0.77 |
| Cescaljarni repka | 2.00 | 720.75 | 0.56 | 20.83 | 0.31 | 0.78 |
| Golden | 2.00 | 720.75 | 0.56 | 20.83 | 0.31 | 0.78 |
| KSU-5 | 2.00 | 721.25 | 0.56 | 22.80 | 0.31 | 0.78 |
| N001-28-246-5-4 | 2.00 | 721.83 | 0.56 | 20.03 | 0.32 | 0.77 |
| Taichang | 2.00 | 728.83 | 0.56 | 4.02 | 0.45 | 0.67 |
| Gora | 2.00 | 739.25 | 0.57 | 22.49 | 0.32 | 0.79 |

. = Not calculated

Table A2. Overall median, mean rank, estimated relative effect, and 95% confidence interval for the -8°C greenhouse study (continued).

| Genotype | Median | Mean rank | Estimated relative effect | Variance | Confidence interval (95%) for the estimated relative effect | |
|---|---|---|---|---|---|---|
| | | | | | Lower limit | Upper limit |
| Nugget | 1.50 | 741.60 | 0.57 | 28.06 | 0.29 | 0.81 |
| Licantara | 2.00 | 743.92 | 0.58 | 23.22 | 0.32 | 0.80 |
| A04-74NA | 2.00 | 744.58 | 0.58 | 12.89 | 0.38 | 0.75 |
| Ridana | 2.00 | 744.58 | 0.58 | 12.89 | 0.38 | 0.75 |
| Evvin | 2.00 | 745.17 | 0.58 | 10.14 | 0.40 | 0.73 |
| Ladoga | 2.00 | 745.17 | 0.58 | 10.14 | 0.40 | 0.73 |
| Cobra | 2.50 | 751.80 | 0.58 | 32.76 | 0.28 | 0.83 |
| Ibiza | 2.00 | 758.90 | 0.59 | 14.14 | 0.38 | 0.77 |
| Santana | 2.00 | 760.50 | 0.59 | 9.44 | 0.42 | 0.74 |
| Cresor | 2.00 | 761.40 | 0.59 | 18.69 | 0.35 | 0.79 |
| Sval of Gullen | 1.75 | 767.75 | 0.59 | 15.11 | 0.38 | 0.78 |
| Wielkopolski | 2.00 | 767.75 | 0.59 | 15.11 | 0.38 | 0.78 |
| A04-73NA | 2.00 | 767.80 | 0.59 | 4.25 | 0.48 | 0.70 |
| Eragi | 2.00 | 767.92 | 0.59 | 2.86 | 0.50 | 0.68 |
| Helga | 2.00 | 768.50 | 0.59 | 0.14 | 0.57 | 0.61 |
| G-32327 | 2.00 | 775.20 | 0.60 | 13.99 | 0.39 | 0.78 |
| Korina | 2.25 | 782.42 | 0.61 | 24.65 | 0.33 | 0.83 |
| Chun nung 1 | 2.00 | 785.17 | 0.61 | 17.32 | 0.37 | 0.80 |
| Rang | 3.00 | 786.50 | 0.61 | 35.80 | 0.28 | 0.86 |
| Erra | 2.00 | 789.83 | 0.61 | 17.99 | 0.37 | 0.81 |
| Brio | 2.00 | 791.08 | 0.61 | 4.91 | 0.49 | 0.72 |
| Rumania-1 | 2.00 | 791.08 | 0.61 | 4.91 | 0.49 | 0.72 |
| Quinta | 2.50 | 798.83 | 0.62 | 22.97 | 0.35 | 0.83 |
| Da vinci | 2.25 | 806.25 | 0.62 | 16.25 | 0.39 | 0.81 |
| Beryl | 2.00 | 807.00 | 0.62 | 1.25 | 0.56 | 0.68 |
| Skrzeszowicki | 2.00 | 807.60 | 0.62 | 20.44 | 0.37 | 0.83 |
| Rico | 2.50 | 811.60 | 0.63 | 34.24 | 0.30 | 0.87 |
| ARC-97018 | 2.50 | 813.80 | 0.63 | 24.34 | 0.35 | 0.84 |
| Titus | 2.50 | 818.33 | 0.63 | 28.01 | 0.33 | 0.86 |
| Oleifera | 2.00 | 830.17 | 0.64 | 3.01 | 0.54 | 0.73 |
| Conquest | 2.00 | 841.80 | 0.65 | 8.44 | 0.48 | 0.79 |
| Koubun | 2.00 | 841.80 | 0.65 | 8.44 | 0.48 | 0.79 |
| Panter | 2.00 | 841.80 | 0.65 | 8.44 | 0.48 | 0.79 |
| KSU-4 | 2.25 | 845.33 | 0.65 | 14.16 | 0.43 | 0.82 |
| KSU-9 | 2.25 | 845.33 | 0.65 | 14.16 | 0.43 | 0.82 |
| Romeo | 2.00 | 847.58 | 0.66 | 4.88 | 0.53 | 0.76 |

. = Not calculated

Table A2. Overall median, mean rank, estimated relative effect, and 95% confidence interval for the -8°C greenhouse study (continued).

| Genotype | Median | Mean rank | Estimated relative effect | Variance | Confidence interval (95%) for the estimated relative effect | |
|---|---|---|---|---|---|---|
| | | | | | Lower limit | Upper limit |
| Crystal | 2.50 | 860.70 | 0.67 | 20.98 | 0.39 | 0.86 |
| KS3579 | 2.50 | 733.50 | 0.67 | 20.98 | 0.39 | 0.86 |
| Mutsumi | 2.50 | 860.70 | 0.67 | 20.98 | 0.39 | 0.86 |
| NU-41737 | 2.50 | 860.70 | 0.67 | 20.98 | 0.39 | 0.86 |
| Furax | 2.00 | 860.90 | 0.67 | 2.55 | 0.57 | 0.75 |
| Weibulls margo | 2.50 | 862.00 | 0.67 | 15.34 | 0.43 | 0.84 |
| Lembkes | 2.25 | 867.42 | 0.67 | 16.51 | 0.43 | 0.85 |
| Colza 18 Miroc | 2.50 | 868.50 | 0.67 | 15.67 | 0.43 | 0.84 |
| Wasefuji | 2.00 | 872.00 | 0.67 | 12.57 | 0.46 | 0.83 |
| ARC-90016 | 3.00 | 888.50 | 0.69 | 23.06 | 0.39 | 0.88 |
| Girita | 2.00 | 888.70 | 0.69 | 4.59 | 0.56 | 0.79 |
| Gylle | 2.00 | 888.70 | 0.69 | 4.59 | 0.56 | 0.79 |
| BNW 161/83 | 3.00 | 891.08 | 0.69 | 19.82 | 0.42 | 0.87 |
| Regal | 2.00 | 896.10 | 0.69 | 13.97 | 0.46 | 0.85 |
| Lindora-00 | 2.25 | 907.17 | 0.70 | 3.34 | 0.59 | 0.79 |
| Bingo | 3.00 | 909.40 | 0.70 | 25.39 | 0.39 | 0.90 |
| Aomori | 3.00 | 915.00 | 0.71 | 26.13 | 0.39 | 0.90 |
| Lester | 2.75 | 929.08 | 0.72 | 17.56 | 0.45 | 0.89 |
| Weal dong cho | 2.00 | 930.00 | 0.72 | 17.99 | 0.45 | 0.89 |
| Premier | 3.00 | 930.17 | 0.72 | 16.70 | 0.46 | 0.89 |
| Cult | 2.25 | 930.33 | 0.72 | 4.39 | 0.59 | 0.82 |
| Salamander | 3.00 | 935.90 | 0.72 | 28.26 | 0.38 | 0.92 |
| Lesira | 2.75 | 946.50 | 0.73 | 18.90 | 0.45 | 0.90 |
| Dae cho sen | 2.25 | 947.75 | 0.73 | 5.72 | 0.58 | 0.84 |
| Galileo | 2.50 | 953.50 | 0.74 | 5.29 | 0.60 | 0.84 |
| Rafal | 2.50 | 953.50 | 0.74 | 5.29 | 0.60 | 0.84 |
| Glacier | 2.75 | 954.25 | 0.74 | 19.65 | 0.45 | 0.91 |
| Lindore | 3.00 | 961.20 | 0.74 | 25.18 | 0.41 | 0.92 |
| NY-20 | 3.00 | 983.40 | 0.76 | 25.62 | 0.41 | 0.93 |
| Gundula | 2.50 | 1008.90 | 0.78 | 3.56 | 0.66 | 0.87 |
| Kanada | 3.00 | 1015.17 | 0.79 | 4.70 | 0.64 | 0.88 |
| Jasna | 3.00 | 1020.25 | 0.79 | 11.61 | 0.55 | 0.92 |
| KSU-8 | 2.50 | 1035.40 | 0.80 | 5.39 | 0.64 | 0.90 |
| Lembkes malchower | 3.00 | 1037.25 | 0.80 | 5.89 | 0.64 | 0.90 |
| Jupiter | 3.00 | 1053.67 | 0.82 | 2.93 | 0.70 | 0.89 |
| Tosharshu | 3.00 | 1057.60 | 0.82 | 5.18 | 0.66 | 0.91 |

. = Not calculated

Table A2. Overall median, mean rank, estimated relative effect, and 95% confidence interval for the -8°C greenhouse study (continued).

| Genotype | Median | Mean rank | Estimated relative effect | Variance | Confidence interval (95%) for the estimated relative effect | |
|---|---|---|---|---|---|---|
| | | | | | Lower limit | Upper limit |
| Librador | 3.00 | 1110.70 | 0.86 | 0.64 | 0.81 | 0.90 |
| Rubin | 4.00 | 1129.58 | 0.87 | 11.29 | 0.56 | 0.97 |
| Chon nam | 3.00 | 1131.60 | 0.88 | 1.18 | 0.80 | 0.92 |
| Vinnickij 15/59 | 3.00 | 1165.00 | 0.90 | 0.56 | 0.85 | 0.94 |
| AR-256 | 3.00 | 1173.33 | 0.91 | 0.95 | 0.84 | 0.95 |
| KSU-10 | 3.50 | 1181.58 | 0.91 | 1.55 | 0.82 | 0.96 |
| AR91004 | 3.25 | 1200.08 | 0.93 | 0.60 | 0.87 | 0.96 |

. = Not calculated

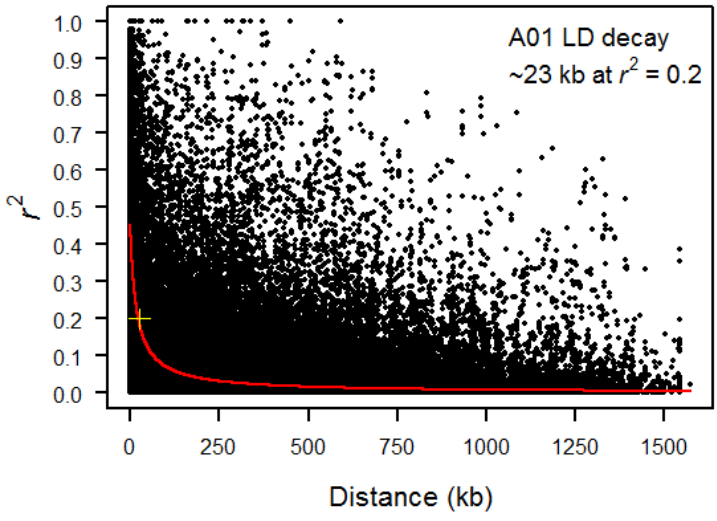


Fig. A1. LD decay for chromosome A01 from the -8°C greenhouse study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 23 kb.

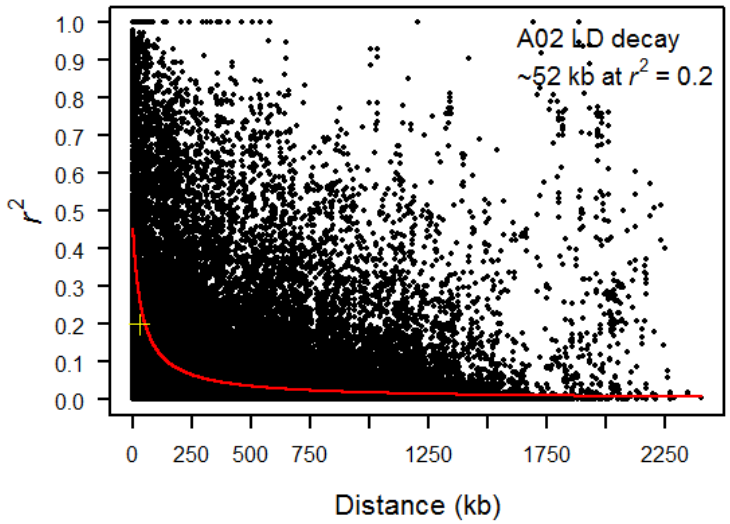


Fig. A2. LD decay for chromosome A02 from the -8°C greenhouse study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 52 kb.

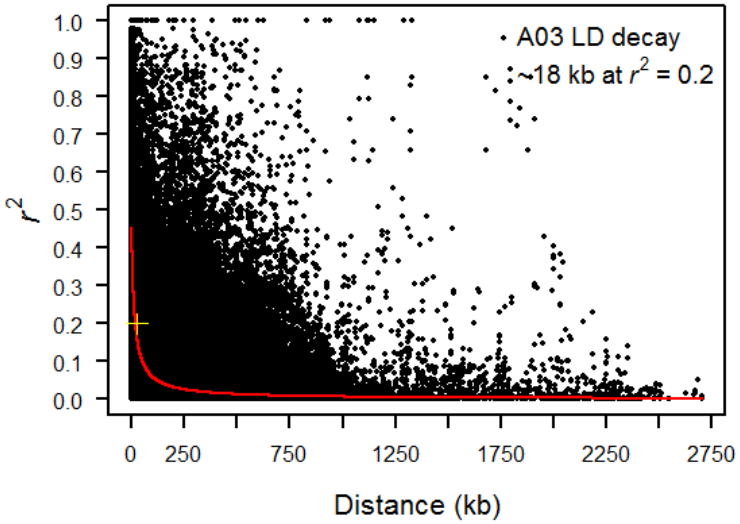


Fig. A3. LD decay for chromosome A03 from the -8°C greenhouse study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 18 kb.

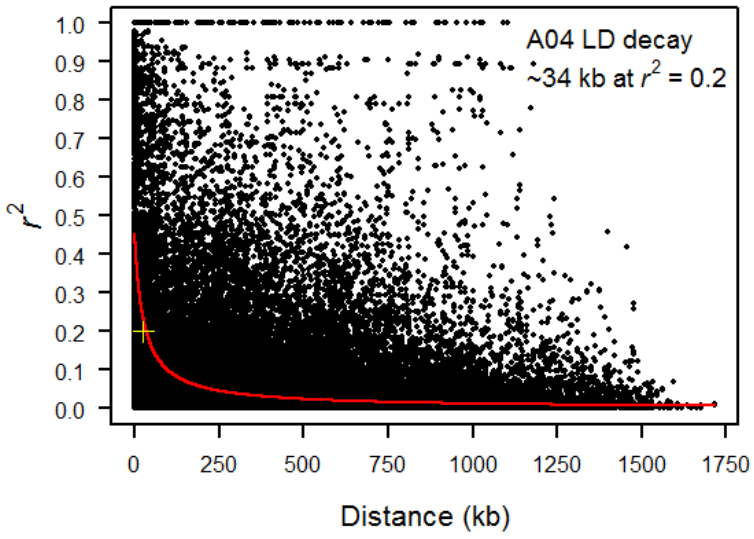


Fig. A4. LD decay for chromosome A04 from the -8°C greenhouse study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 34 kb.

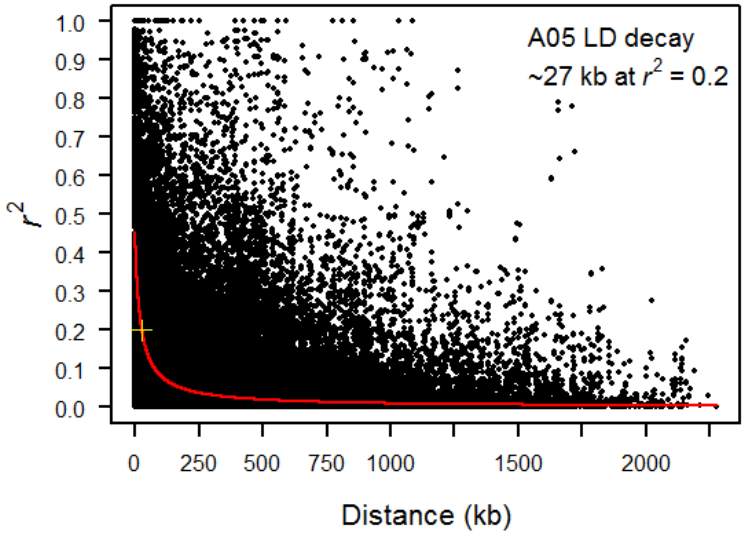


Fig. A5. LD decay for chromosome A05 from the -8°C greenhouse study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 27 kb.

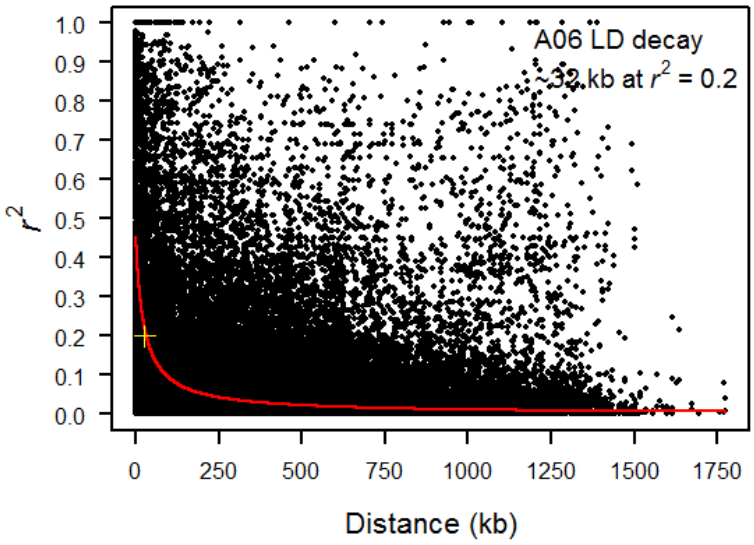


Fig. A6. LD decay for chromosome A06 from the -8°C greenhouse study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 32 kb.

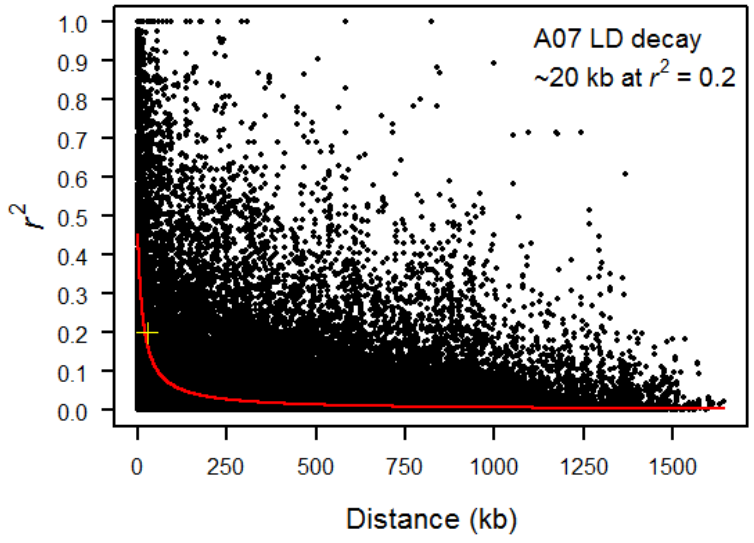


Fig. A7. LD decay for chromosome A07 from the -8°C greenhouse study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 20 kb.

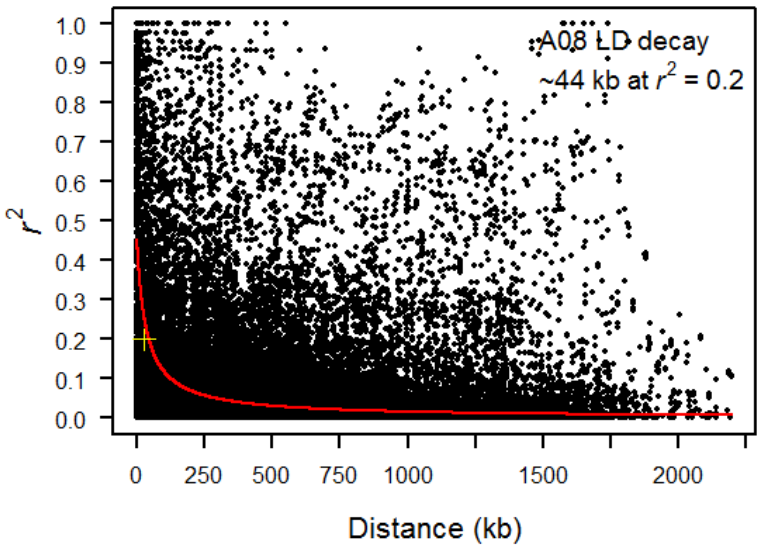


Fig. A8. LD decay for chromosome A08 from the -8°C greenhouse study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 44 kb.

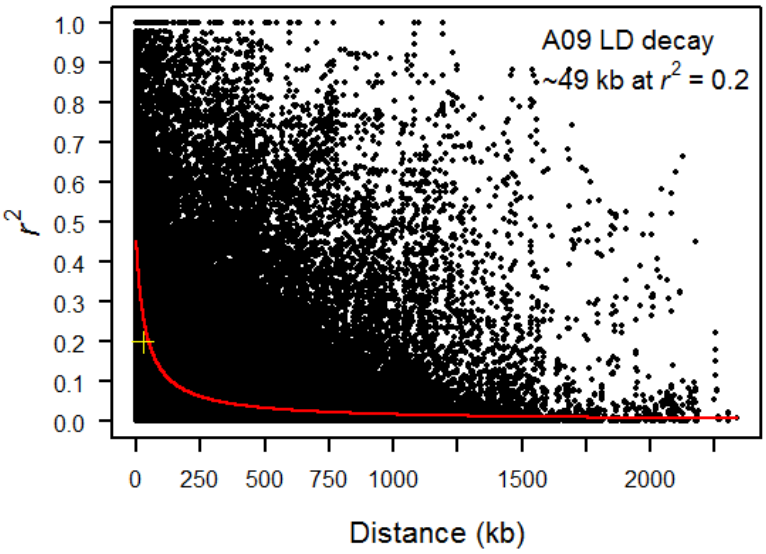


Fig. A9. LD decay for chromosome A09 from the -8°C greenhouse study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 49 kb.

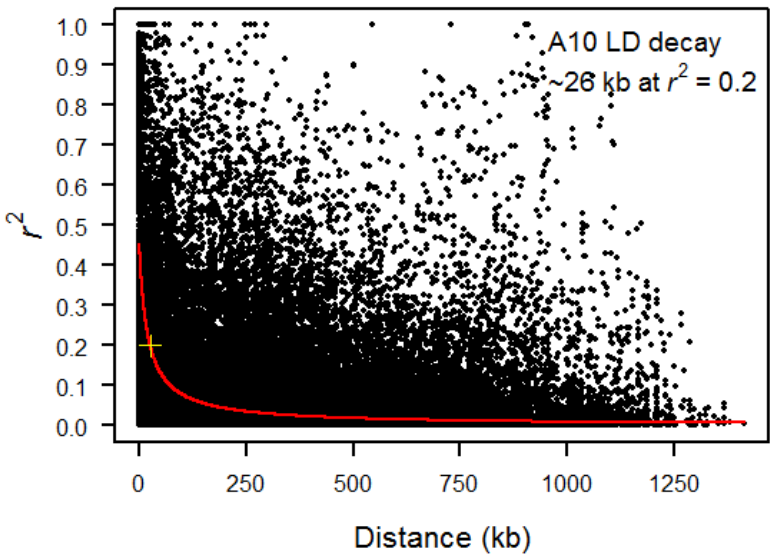


Fig. A10. LD decay for chromosome A10 from the -8°C greenhouse study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 26 kb.

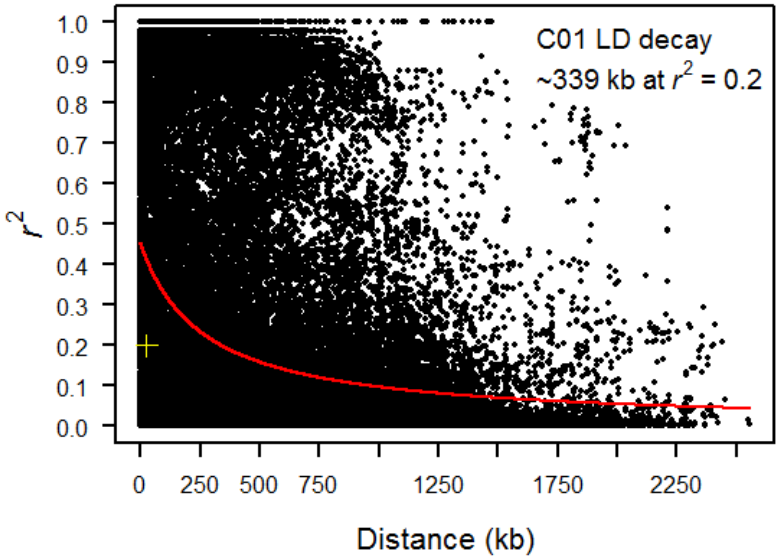


Fig. A11. LD decay for chromosome C01 from the -8°C greenhouse study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 339 kb.

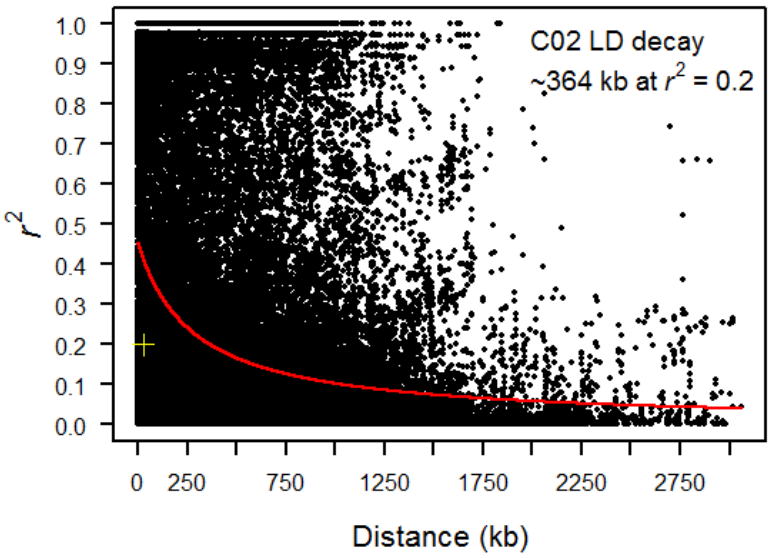


Fig. A12. LD decay for chromosome C02 from the -8°C greenhouse study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 364 kb.

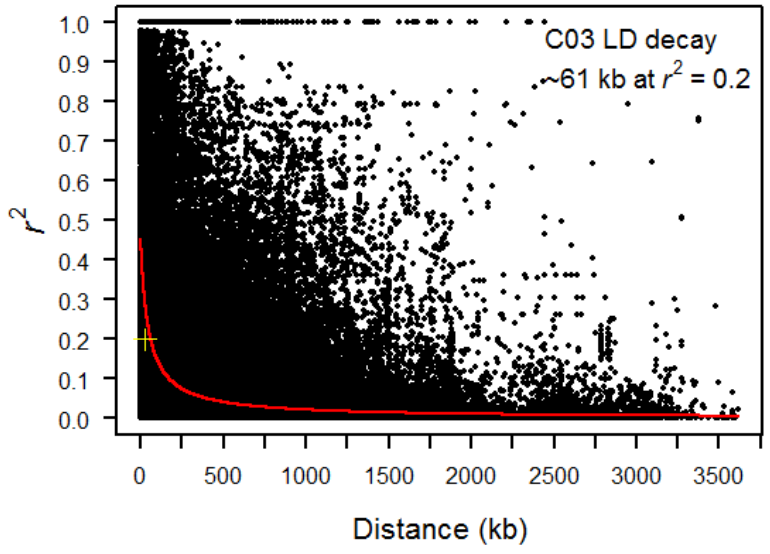


Fig. A13. LD decay for chromosome C03 from the -8°C greenhouse study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 61 kb.

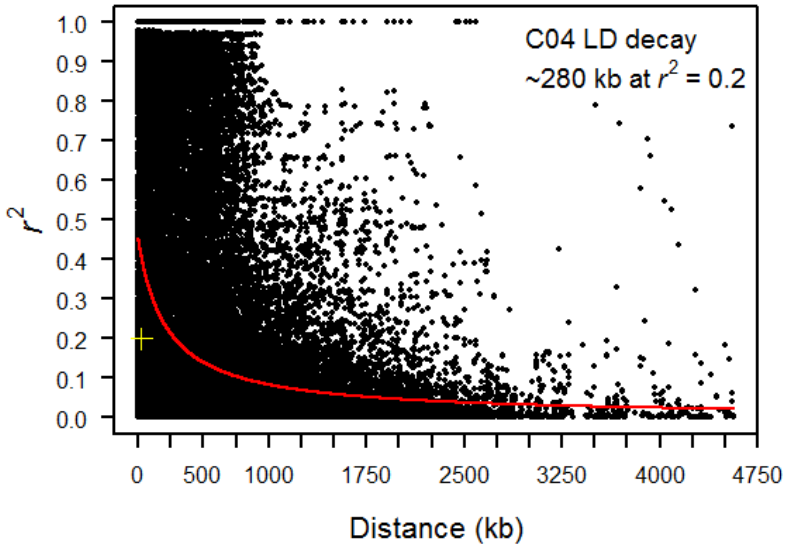


Fig. A14. LD decay for chromosome C04 from the -8°C greenhouse study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 280 kb.

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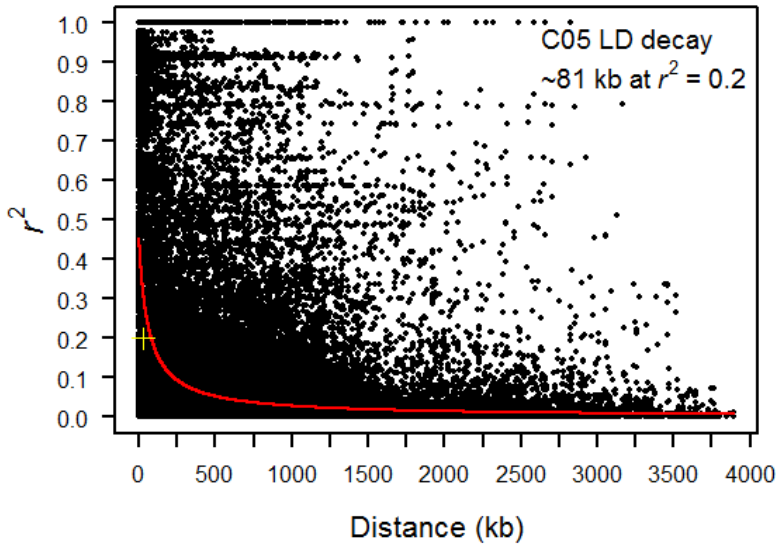


Fig. A15. LD decay for chromosome C05 from the -8°C greenhouse study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 81 kb.

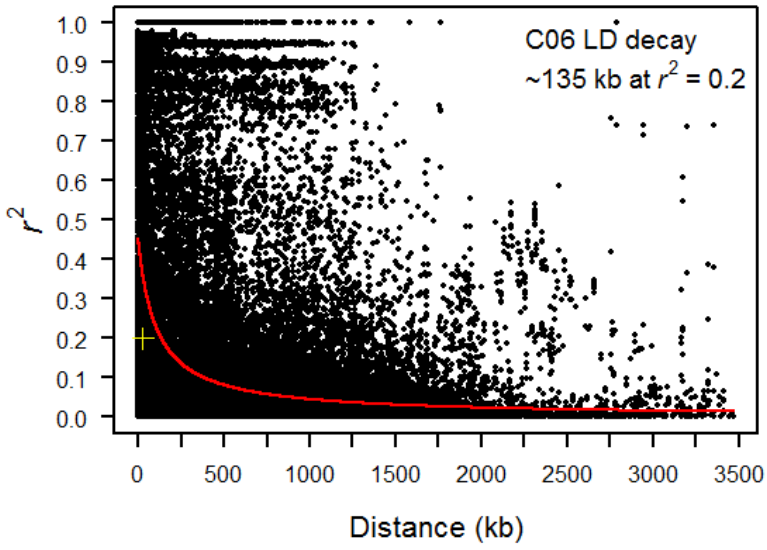


Fig. A16. LD decay for chromosome C06 from the -8°C greenhouse study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 135 kb.

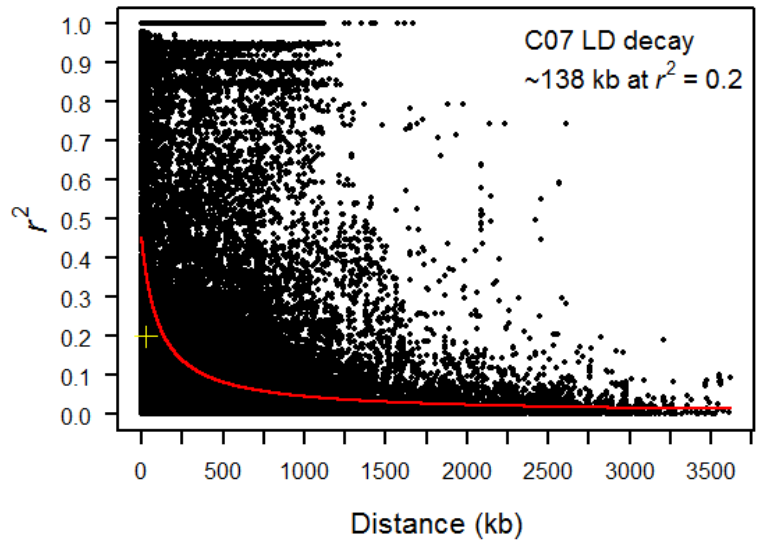


Fig. A17. LD decay for chromosome C07 from the -8°C greenhouse study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 138 kb.

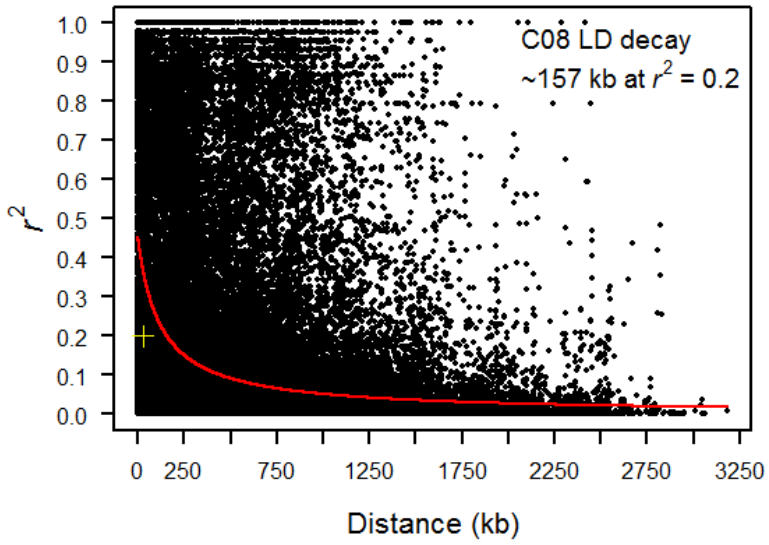


Fig. A18. LD decay for chromosome C08 from the -8°C greenhouse study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 157 kb.

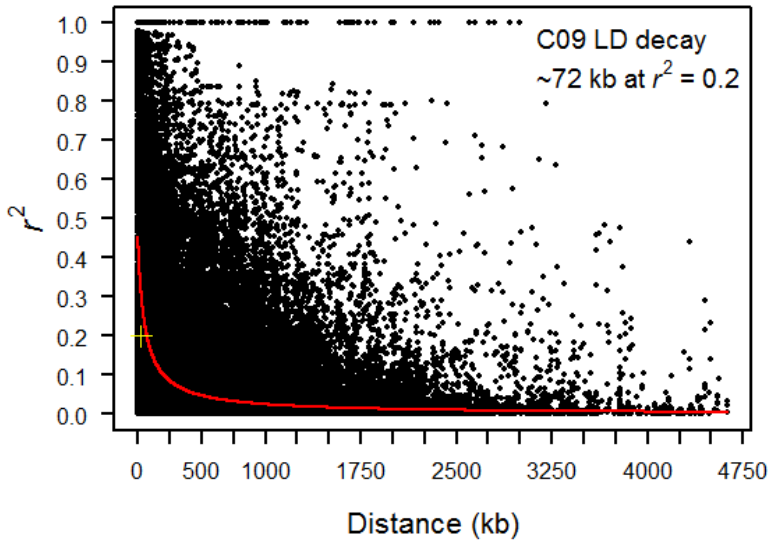


Fig. A19. LD decay for chromosome C09 from the -8°C greenhouse study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 72 kb.

Table A3. Growth habit and the country that each genotype was originated/obtained from for the field study.

| Genotype | Growth habit | Country originated/obtained from |
|---|---|---|
| A-10-999 | Spring | USA |
| A-15-1000 | Spring | Canada |
| A-15-989 | Spring | USA |
| A-16-1013 | Spring | USA |
| A-3-1011 | Spring | USA |
| A-4-1000 | Spring | USA |
| A-7-997 | Spring | USA |
| A-8-1000 | Spring | USA |
| A-9-1013 | Spring | USA |
| A04-72NA | Spring | USA |
| A04-73NA | Spring | USA |
| A04-74NA | Spring | USA |
| A06-19NA | Spring | USA |
| A06-20NA | Spring | USA |
| A07-28NA | Spring | USA |
| A07-29NI | Spring | USA |
| AR91004 | Winter | USA |
| ARC 2180-1 | Winter | USA |
| ARC 90016 | Winter | USA |
| ARC 97018 | Winter | USA |
| ARC 97019 | Winter | USA |
| Aviso | Spring | Canada |
| Azumasho | Semi-winter | South Korea |
| Barkant | Winter | Netherlands |
| Barplina | Winter | South Korea |
| Beryl | Winter | Poland |
| BO 63 | Spring | Canada |
| Brio | Spring | France |
| Bronowski | Spring | Poland |
| Buk Wuk 3 | Spring | South Korea |
| Colza 18 Miroc | Semi-winter | South Korea |
| Comet | Spring | Sweden |
| Conquest | Spring | Canada |
| Corvette | Winter | UK |
| Cougar | Spring | Canada |
| Crop | Spring | France |
| Crystal | Winter | Sweden |
| Cult | Winter | Canada |
| Delta | Spring | Sweden |
| Drakkar | Spring | France |

Table A3. Growth habit and the country that each genotype was originated/obtained from for the field study (continued).

| Genotype | Growth habit | Country originated/obtained from |
|---|---|---|
| Eckendorfer Mali | Semi-winter | South Korea |
| Eragi | Winter | Germany |
| Evvin | Spring | Russian Federation |
| Expander | Winter | Germany |
| Fashion | Winter | Canada |
| Fertodi | Winter | South Korea |
| Fonto | Spring | South Korea |
| France 1 | Spring | France |
| Fuji | Spring | South Korea |
| Galant | Spring | USA |
| Galaxy | Spring | Sweden |
| Galileo | Winter | Canada |
| Girita | Semi-winter | Germany |
| Glacier | Winter | Sweden |
| Global | Spring | Sweden |
| Golden | Spring | Canada |
| Gora | Spring | Germany |
| Gulle | Spring | Sweden |
| Gullivar | Spring | Sweden |
| Helga | Semi-winter | Germany |
| Hi-Q | Spring | Canada |
| Ibiza | Winter | Canada |
| IR 2 | Spring | Hungary |
| Janetzkis | Spring | South Korea |
| Jasna | Spring | USA |
| Jupiter | Winter | USA |
| Kanada | Spring | Poland |
| Klinki | Spring | South Korea |
| Kosa | Spring | Germany |
| Kovalevskjj | Spring | Ukraine |
| KS3579 | Winter | USA |
| KSU-1 | Winter | USA |
| KSU-10 | Winter | USA |
| KSU-2 | Winter | USA |
| KSU-3 | Winter | USA |
| KSU-4 | Winter | USA |
| KSU-6 | Winter | USA |
| KSU-7 | Winter | USA |
| KSU-8 | Winter | USA |
| KSU-9 | Winter | USA |

Table A3. Growth habit and the country that each genotype was originated/obtained from for the field study (continued).

| Genotype | Growth habit | Country originated/obtained from |
|---|---|---|
| Ladoga | Winter | Canada |
| Legend | Spring | Sweden |
| Lembkes | Winter | South Korea |
| Lenora | Winter | South Korea |
| Lester | Winter | Germany |
| Librador | Winter | Germany |
| Licantara | Winter | Germany |
| Lieikoposki | Semi-winter | South Korea |
| Lindora 00 | Winter | Germany |
| Lindore | Winter | Germany |
| Linglandor | Winter | Germany |
| Linus | Winter | South Korea |
| Liratrop | Winter | Germany |
| Lisora | Semi-winter | Germany |
| Lorenze | Winter | Canada |
| Major | Semi-winter | France |
| Mali | Semi-winter | South Korea |
| MAR 160059 | Winter | Poland |
| Mar'janovskij | Spring | Ukraine |
| Midas | Spring | Canada |
| Miekuro Dane | Spring | South Korea |
| Mlochowski | Semi-winter | Poland |
| Murame nadame | Semi-winter | South Korea |
| N001 28-246-5-4 | Semi-winter | South Korea |
| Nilla 1022 | Semi-winter | South Korea |
| Nilla glossy | Semi-winter | South Korea |
| NU 51084 | Spring | Sweden |
| Nugget | Semi-winter | South Korea |
| NY-10 | Semi-winter | China |
| NY-20 | Semi-winter | China |
| NY-7 | Semi-winter | China |
| NY-8 | Semi-winter | China |
| Oleifera | Semi-winter | South Korea |
| Oro | Spring | Canada |
| Peace | Spring | Canada |
| Petanova lihonova | Semi-winter | South Korea |
| Polo Canola | Spring | USA |
| Q2 | Spring | Canada |
| R. Creaus | Winter | South Korea |
| Rafal | Winter | France |

Table A3. Growth habit and the country that each genotype was originated/obtained from for the field study (continued).

| Genotype | Growth habit | Country originated/obtained from |
|---|---|---|
| Rang | Semi-winter | South Korea |
| Ratnik | Spring | USA |
| Rebel | Semi-winter | USA |
| Regal | Winter | South Korea |
| Regent | Spring | Canada |
| Regina II | Spring | Canada |
| Ridana | Winter | Germany |
| Romeo | Spring | France |
| Russia 5 | Spring | Russian Federation |
| Sera | Semi-winter | Germany |
| Silex | Spring | Canada |
| Sunrise | Spring | USA |
| Sval of Gullen | Spring | South Korea |
| Synra | Winter | South Korea |
| Taichang | Semi-winter | South Korea |
| Tanka | Semi-winter | South Korea |
| Tanto | Spring | France |
| Titus | Winter | South Korea |
| Topas | Spring | Sweden |
| Tower | Spring | Canada |
| Turret | Spring | Canada |
| Vision | Winter | Canada |
| Vostochno sibirskii | Spring | Russian Federation |
| Westar | Spring | Canada |
| Wipol | Semi-winter | Norway |
| Wira | Winter | Germany |
| Yong dang | Semi-winter | South Korea |

Table A4. Average air temperature data for the field studies in 2014 and 2015.

| Year | Month | Day | Hour (CST) | Avg. Air Temp. (°C) |
|---|---|---|---|---|
| 2014 | October | 30 | 1600 | 2.1 |
| 2014 | October | 30 | 1700 | 2.0 |
| 2014 | October | 30 | 1800 | 1.2 |
| 2014 | October | 30 | 1900 | -0.4 |
| 2014 | October | 30 | 2000 | -0.7 |
| 2014 | October | 30 | 2100 | -2.2 |
| 2014 | October | 30 | 2200 | -2.5 |
| 2014 | October | 30 | 2300 | -3.5 |
| 2014 | October | 30 | 2400 | -5.1 |
| 2014 | October | 31 | 100 | -6.1 |
| 2014 | October | 31 | 200 | -6.9 |
| 2014 | October | 31 | 300 | -8.0 |
| 2014 | October | 31 | 400 | -8.8 |
| 2014 | October | 31 | 500 | -9.0 |
| 2014 | October | 31 | 600 | -9.5 |
| 2014 | October | 31 | 700 | -10.0 |
| 2014 | October | 31 | 800 | -9.9 |
| 2014 | October | 31 | 900 | -7.4 |
| 2014 | October | 31 | 1000 | -4.2 |
| 2015 | March | 20 | 1600 | -1.0 |
| 2015 | March | 20 | 1700 | -1.1 |
| 2015 | March | 20 | 1800 | -0.9 |
| 2015 | March | 20 | 1900 | -1.3 |
| 2015 | March | 20 | 2000 | -1.1 |
| 2015 | March | 20 | 2100 | -1.9 |
| 2015 | March | 20 | 2200 | -3.5 |
| 2015 | March | 20 | 2300 | -4.6 |
| 2015 | March | 20 | 2400 | -5.1 |
| 2015 | March | 21 | 100 | -6.9 |
| 2015 | March | 21 | 200 | -7.7 |
| 2015 | March | 21 | 300 | -8.3 |
| 2015 | March | 21 | 400 | -9.7 |
| 2015 | March | 21 | 500 | -8.7 |
| 2015 | March | 21 | 600 | -8.3 |
| 2015 | March | 21 | 700 | -8.5 |
| 2015 | March | 21 | 800 | -8.1 |
| 2015 | March | 21 | 900 | -7.0 |
| 2015 | March | 21 | 1000 | -6.1 |
| 2015 | October | 16 | 1600 | 9.6 |
| 2015 | October | 16 | 1700 | 9.9 |
| 2015 | October | 16 | 1800 | 8.1 |

Data from NDAWN (2017).

Table A4. Average air temperature data for the field studies in 2014 and 2015 (continued).

| Year | Month | Day | Hour (CST) | Avg. Air Temp. (°C) |
|---|---|---|---|---|
| 2015 | October | 16 | 1900 | 6.2 |
| 2015 | October | 16 | 2000 | 4.8 |
| 2015 | October | 16 | 2100 | 4.0 |
| 2015 | October | 16 | 2200 | 2.4 |
| 2015 | October | 16 | 2300 | 1.5 |
| 2015 | October | 16 | 2400 | 0.2 |
| 2015 | October | 17 | 100 | -0.5 |
| 2015 | October | 17 | 200 | -0.2 |
| 2015 | October | 17 | 300 | -0.7 |
| 2015 | October | 17 | 400 | -1.6 |
| 2015 | October | 17 | 500 | -2.2 |
| 2015 | October | 17 | 600 | -2.4 |
| 2015 | October | 17 | 700 | -3.3 |
| 2015 | October | 17 | 800 | -2.1 |
| 2015 | October | 17 | 900 | 1.4 |
| 2015 | October | 17 | 1000 | 4.0 |
| 2015 | November | 5 | 1600 | 3.2 |
| 2015 | November | 5 | 1700 | 3.2 |
| 2015 | November | 5 | 1800 | 3.0 |
| 2015 | November | 5 | 1900 | 2.9 |
| 2015 | November | 5 | 2000 | 2.9 |
| 2015 | November | 5 | 2100 | 3.2 |
| 2015 | November | 5 | 2200 | 3.6 |
| 2015 | November | 5 | 2300 | 3.7 |
| 2015 | November | 5 | 2400 | 3.6 |
| 2015 | November | 6 | 100 | 3.5 |
| 2015 | November | 6 | 200 | 3.4 |
| 2015 | November | 6 | 300 | 2.2 |
| 2015 | November | 6 | 400 | 0.8 |
| 2015 | November | 6 | 500 | 1.1 |
| 2015 | November | 6 | 600 | 1.8 |
| 2015 | November | 6 | 700 | 2.6 |
| 2015 | November | 6 | 800 | 2.8 |
| 2015 | November | 6 | 900 | 2.5 |
| 2015 | November | 6 | 1000 | 3.2 |
| 2015 | November | 12 | 1600 | 3.4 |
| 2015 | November | 12 | 1700 | 2.4 |
| 2015 | November | 12 | 1800 | 2.9 |
| 2015 | November | 12 | 1900 | 2.3 |
| 2015 | November | 12 | 2000 | 1.7 |
| 2015 | November | 12 | 2100 | 1.0 |

Data from NDAWN (2017).

Table A4. Average air temperature data for the field studies in 2014 and 2015 (continued).

| Year | Month | Day | Hour (CST) | Avg. Air Temp. (°C) |
|---|---|---|---|---|
| 2015 | November | 12 | 2200 | 0.0 |
| 2015 | November | 12 | 2300 | -1.3 |
| 2015 | November | 12 | 2400 | -0.8 |
| 2015 | November | 13 | 100 | -0.8 |
| 2015 | November | 13 | 200 | -1.0 |
| 2015 | November | 13 | 300 | -2.3 |
| 2015 | November | 13 | 400 | -3.6 |
| 2015 | November | 13 | 500 | -3.9 |
| 2015 | November | 13 | 600 | -3.8 |
| 2015 | November | 13 | 700 | -4.5 |
| 2015 | November | 13 | 800 | -4.6 |
| 2015 | November | 13 | 900 | -2.9 |
| 2015 | November | 13 | 1000 | -1.0 |

Data from NDAWN (2017).

Table A5. Overall median, mean rank, estimated relative effect, and the 95% confidence interval for the genotypes in the field study.

| | | | | | Confidence Interval (95%) for estimated relative effect | |
|---|---|---|---|---|---|---|
| Genotype | Median | Mean rank | Estimated relative effect | Variance | Lower limit | Upper limit |
| Azumasho | 1.00 | 25.50 | 0.08 | 0.04 | 0.06 | 0.11 |
| IR-2 | 1.00 | 25.50 | 0.08 | 0.04 | 0.06 | 0.11 |
| Regina-II | 1.00 | 25.50 | 0.08 | 0.04 | 0.06 | 0.11 |
| Topas | 1.00 | 25.50 | 0.08 | 0.04 | 0.06 | 0.11 |
| Vostochno sibirskii | 1.00 | 33.50 | 0.11 | 3.11 | 0.02 | 0.49 |
| Aviso | 1.25 | 36.25 | 0.12 | 0.42 | 0.06 | 0.21 |
| Sunrise | 1.25 | 36.25 | 0.12 | 0.42 | 0.06 | 0.21 |
| ARC-2180-1 | 1.50 | 45.00 | 0.15 | 1.27 | 0.06 | 0.32 |
| DKL-70-07 | 1.50 | 45.00 | 0.15 | 1.27 | 0.06 | 0.32 |
| Regent | 1.50 | 45.00 | 0.15 | 1.27 | 0.06 | 0.32 |
| Tanka | 1.50 | 45.00 | 0.15 | 1.27 | 0.06 | 0.32 |
| Cougar | 1.75 | 54.25 | 0.18 | 2.73 | 0.06 | 0.44 |
| Miekuro-Dane | 1.50 | 54.50 | 0.18 | 8.83 | 0.02 | 0.68 |
| Lieikoposki | 1.75 | 55.75 | 0.18 | 8.41 | 0.03 | 0.67 |
| A04-74NA | 2.00 | 64.50 | 0.21 | 0.13 | 0.17 | 0.25 |
| A04-72NA | 2.00 | 65.00 | 0.21 | 1.14 | 0.12 | 0.36 |

Table A5. Overall median, mean rank, estimated relative effect, and the 95% confidence interval for the genotypes in the field study (continued).

| Genotype | Median | Mean rank | Estimated relative effect | Variance | Confidence Interval (95%) for estimated relative effect | |
|---|---|---|---|---|---|---|
| | | | | | Lower limit | Upper limit |
| Helga | 2.00 | 66.00 | 0.22 | 5.37 | 0.06 | 0.57 |
| Legend | 2.00 | 66.00 | 0.22 | 5.37 | 0.06 | 0.57 |
| Lembkes | 2.00 | 66.00 | 0.22 | 5.37 | 0.06 | 0.57 |
| Linus | 2.00 | 66.00 | 0.22 | 5.37 | 0.06 | 0.57 |
| NY-7 | 2.00 | 66.00 | 0.22 | 5.37 | 0.06 | 0.57 |
| Russia-5 | 2.00 | 66.00 | 0.22 | 5.37 | 0.06 | 0.57 |
| Westar | 2.00 | 66.00 | 0.22 | 5.37 | 0.06 | 0.57 |
| Corvette | 2.25 | 78.00 | 0.26 | 9.04 | 0.06 | 0.67 |
| A-15-1000 | 2.50 | 85.50 | 0.28 | 1.52 | 0.16 | 0.44 |
| Crop | 2.50 | 85.50 | 0.28 | 1.52 | 0.16 | 0.44 |
| NU 51084 | 2.50 | 85.50 | 0.28 | 1.52 | 0.16 | 0.44 |
| Oro | 2.50 | 85.50 | 0.28 | 1.52 | 0.16 | 0.44 |
| Fertodi | 2.00 | 94.75 | 0.31 | 27.91 | 0.03 | 0.88 |
| Mlochowski | 2.00 | 94.75 | 0.31 | 27.91 | 0.03 | 0.88 |
| Bronowski | 2.75 | 97.50 | 0.32 | 3.63 | 0.15 | 0.56 |
| A-4-1000 | 2.50 | 106.25 | 0.35 | 21.38 | 0.05 | 0.84 |
| A06-20NA | 2.50 | 106.25 | 0.35 | 21.38 | 0.05 | 0.84 |
| AR91004 | 2.50 | 106.25 | 0.35 | 21.38 | 0.05 | 0.84 |
| Brio | 2.50 | 106.25 | 0.35 | 21.38 | 0.05 | 0.84 |
| Kanada | 2.50 | 106.25 | 0.35 | 21.38 | 0.05 | 0.84 |
| Kosa | 2.50 | 106.25 | 0.35 | 21.38 | 0.05 | 0.84 |
| Lisora | 2.50 | 106.25 | 0.35 | 21.38 | 0.05 | 0.84 |
| Murame-nadame | 2.50 | 106.25 | 0.35 | 21.38 | 0.05 | 0.84 |
| Rafal | 2.50 | 106.25 | 0.35 | 21.38 | 0.05 | 0.84 |
| Sera | 2.50 | 106.25 | 0.35 | 21.38 | 0.05 | 0.84 |
| Synra | 2.50 | 106.25 | 0.35 | 21.38 | 0.05 | 0.84 |
| A-9-1013 | 3.00 | 106.50 | 0.35 | 0.17 | 0.31 | 0.40 |
| Q2 | 3.25 | 118.50 | 0.39 | 0.63 | 0.31 | 0.48 |
| Buk Wuk 3 | 3.00 | 125.75 | 0.41 | 12.32 | 0.12 | 0.78 |
| Delta | 3.00 | 125.75 | 0.41 | 12.32 | 0.12 | 0.78 |
| DKL-30-42 | 3.00 | 125.75 | 0.41 | 12.32 | 0.12 | 0.78 |
| DKL-72-40 | 3.00 | 125.75 | 0.41 | 12.32 | 0.12 | 0.78 |
| Lenora | 3.00 | 125.75 | 0.41 | 12.32 | 0.12 | 0.78 |
| Mar'janovskij | 3.00 | 125.75 | 0.41 | 12.32 | 0.12 | 0.78 |
| Silex | 3.00 | 125.75 | 0.41 | 12.32 | 0.12 | 0.78 |
| ARC-97019 | 2.00 | 64.50 | 0.42 | 8.09 | 0.16 | 0.73 |
| BO-63 | 3.25 | 135.00 | 0.45 | 8.91 | 0.17 | 0.76 |

Table A5. Overall median, mean rank, estimated relative effect, and the 95% confidence interval for the genotypes in the field study (continued).

| Genotype | Median | Mean rank | Estimated relative effect | Variance | Confidence Interval (95%) for estimated relative effect | |
|---|---|---|---|---|---|---|
| | | | | | Lower limit | Upper limit |
| Silex | 3.00 | 125.75 | 0.41 | 12.32 | 0.12 | 0.78 |
| ARC-97019 | 2.00 | 64.50 | 0.42 | 8.09 | 0.16 | 0.73 |
| BO-63 | 3.25 | 135.00 | 0.45 | 8.91 | 0.17 | 0.76 |
| KSU-8 | 3.25 | 135.00 | 0.45 | 8.91 | 0.17 | 0.76 |
| A-15-989 | 3.50 | 146.75 | 0.48 | 5.37 | 0.25 | 0.73 |
| A-7-997 | 3.50 | 146.75 | 0.48 | 5.37 | 0.25 | 0.73 |
| Barkant | 3.50 | 146.75 | 0.48 | 5.37 | 0.25 | 0.73 |
| Beryl | 3.50 | 146.75 | 0.48 | 5.37 | 0.25 | 0.73 |
| Galant | 3.50 | 146.75 | 0.48 | 5.37 | 0.25 | 0.73 |
| Galaxy | 3.50 | 146.75 | 0.48 | 5.37 | 0.25 | 0.73 |
| KSU-3 | 3.50 | 146.75 | 0.48 | 5.37 | 0.25 | 0.73 |
| KSU-4 | 3.50 | 146.75 | 0.48 | 5.37 | 0.25 | 0.73 |
| KSU-7 | 3.50 | 146.75 | 0.48 | 5.37 | 0.25 | 0.73 |
| Liratrop | 3.50 | 146.75 | 0.48 | 5.37 | 0.25 | 0.73 |
| Lorenze | 3.50 | 146.75 | 0.48 | 5.37 | 0.25 | 0.73 |
| MAR-160059 | 3.50 | 146.75 | 0.48 | 5.37 | 0.25 | 0.73 |
| NY-8 | 3.50 | 146.75 | 0.48 | 5.37 | 0.25 | 0.73 |
| R.Creaus | 3.50 | 146.75 | 0.48 | 5.37 | 0.25 | 0.73 |
| Romeo | 3.50 | 146.75 | 0.48 | 5.37 | 0.25 | 0.73 |
| Fonto | 3.00 | 149.50 | 0.49 | 50.53 | 0.04 | 0.96 |
| Glacier | 3.00 | 149.50 | 0.49 | 50.53 | 0.04 | 0.96 |
| Ibiza | 3.00 | 149.50 | 0.49 | 50.53 | 0.04 | 0.96 |
| KSU-9 | 3.00 | 149.50 | 0.49 | 50.53 | 0.04 | 0.96 |
| Lester | 3.00 | 149.50 | 0.49 | 50.53 | 0.04 | 0.96 |
| N001-28-246-5-4 | 3.00 | 149.50 | 0.49 | 50.53 | 0.04 | 0.96 |
| Ratnik | 3.25 | 153.50 | 0.51 | 26.02 | 0.09 | 0.91 |
| A06-19NA | 3.75 | 158.75 | 0.52 | 2.72 | 0.34 | 0.70 |
| Drakkar | 3.75 | 158.75 | 0.52 | 2.72 | 0.34 | 0.70 |
| A04-73NA | 3.50 | 169.00 | 0.56 | 35.86 | 0.08 | 0.95 |
| Crystal | 3.50 | 169.00 | 0.56 | 35.86 | 0.08 | 0.95 |
| Golden | 3.50 | 169.00 | 0.56 | 35.86 | 0.08 | 0.95 |
| Hi-Q | 3.50 | 169.00 | 0.56 | 35.86 | 0.08 | 0.95 |
| Kovalevskjj | 3.50 | 169.00 | 0.56 | 35.86 | 0.08 | 0.95 |
| Polo Canola | 3.50 | 169.00 | 0.56 | 35.86 | 0.08 | 0.95 |
| Ridana | 3.50 | 169.00 | 0.56 | 35.86 | 0.08 | 0.95 |
| Sval of Gullen | 3.50 | 169.00 | 0.56 | 35.86 | 0.08 | 0.95 |
| Tanto | 3.50 | 169.00 | 0.56 | 35.86 | 0.08 | 0.95 |

Table A5. Overall median, mean rank, estimated relative effect, and the 95% confidence interval for the genotypes in the field study (continued).

| Genotype | Median | Mean rank | Estimated relative effect | Variance | Confidence Interval (95%) for estimated relative effect | |
|---|---|---|---|---|---|---|
| | | | | | Lower limit | Upper limit |
| Taichang | 3.75 | 174.50 | 0.58 | 15.21 | 0.18 | 0.89 |
| A-3-1011 | 3.75 | 178.25 | 0.59 | 29.80 | 0.10 | 0.95 |
| A-10-999 | 4.00 | 187.00 | 0.62 | 0.14 | 0.57 | 0.66 |
| A-16-1013 | 4.00 | 187.00 | 0.62 | 0.14 | 0.57 | 0.66 |
| A-8-1000 | 4.00 | 187.00 | 0.62 | 0.14 | 0.57 | 0.66 |
| A07-28NA | 4.00 | 187.00 | 0.62 | 0.14 | 0.57 | 0.66 |
| A07-29NI | 4.00 | 187.00 | 0.62 | 0.14 | 0.57 | 0.66 |
| Conquest | 4.00 | 187.00 | 0.62 | 0.14 | 0.57 | 0.66 |
| DKL-38-25 | 4.00 | 187.00 | 0.62 | 0.14 | 0.57 | 0.66 |
| DKL-52-41 | 4.00 | 187.00 | 0.62 | 0.14 | 0.57 | 0.66 |
| Eragi | 4.00 | 187.00 | 0.62 | 0.14 | 0.57 | 0.66 |
| Evvin | 4.00 | 187.00 | 0.62 | 0.14 | 0.57 | 0.66 |
| Expander | 4.00 | 187.00 | 0.62 | 0.14 | 0.57 | 0.66 |
| Galileo | 4.00 | 187.00 | 0.62 | 0.14 | 0.57 | 0.66 |
| Global | 4.00 | 187.00 | 0.62 | 0.14 | 0.57 | 0.66 |
| Jasna | 4.00 | 187.00 | 0.62 | 0.14 | 0.57 | 0.66 |
| KSU-2 | 4.00 | 187.00 | 0.62 | 0.14 | 0.57 | 0.66 |
| Lindore | 4.00 | 187.00 | 0.62 | 0.14 | 0.57 | 0.66 |
| Nilla-1022 | 4.00 | 187.00 | 0.62 | 0.14 | 0.57 | 0.66 |
| NY-20 | 4.00 | 187.00 | 0.62 | 0.14 | 0.57 | 0.66 |
| Peace | 4.00 | 187.00 | 0.62 | 0.14 | 0.57 | 0.66 |
| Rebel | 4.00 | 187.00 | 0.62 | 0.14 | 0.57 | 0.66 |
| Tower | 4.00 | 187.00 | 0.62 | 0.14 | 0.57 | 0.66 |
| Vision | 4.00 | 187.00 | 0.62 | 0.14 | 0.57 | 0.66 |
| Wipol | 4.00 | 187.00 | 0.62 | 0.14 | 0.57 | 0.66 |
| Yong dang | 4.00 | 187.00 | 0.62 | 0.14 | 0.57 | 0.66 |
| Asahi natane | 4.00 | 190.00 | 0.63 | 22.88 | 0.14 | 0.94 |
| Fuji | 4.00 | 190.00 | 0.63 | 22.88 | 0.14 | 0.94 |
| KSU-6 | 4.00 | 190.00 | 0.63 | 22.88 | 0.14 | 0.94 |
| Librador | 4.00 | 190.00 | 0.63 | 22.88 | 0.14 | 0.94 |
| Nugget | 4.00 | 190.00 | 0.63 | 22.88 | 0.14 | 0.94 |
| NY-10 | 4.00 | 190.00 | 0.63 | 22.88 | 0.14 | 0.94 |
| Oleifera | 4.00 | 190.00 | 0.63 | 22.88 | 0.14 | 0.94 |
| Rang | 4.00 | 190.00 | 0.63 | 22.88 | 0.14 | 0.94 |
| Wira | 4.00 | 190.00 | 0.63 | 22.88 | 0.14 | 0.94 |
| Klinki | 4.25 | 202.00 | 0.67 | 16.78 | 0.20 | 0.94 |
| ARC-90016 | 4.50 | 230.25 | 0.76 | 6.13 | 0.41 | 0.94 |

Table A5. Overall median, mean rank, estimated relative effect, and the 95% confidence interval for the genotypes in the field study (continued).

| Genotype | Median | Mean rank | Estimated relative effect | Variance | Confidence Interval (95%) for estimated relative effect | |
|---|---|---|---|---|---|---|
| | | | | | Lower limit | Upper limit |
| Barplina | 4.50 | 230.25 | 0.76 | 6.13 | 0.41 | 0.94 |
| Colza 18 Miroc | 4.50 | 230.25 | 0.76 | 6.13 | 0.41 | 0.94 |
| Comet | 4.50 | 230.25 | 0.76 | 6.13 | 0.41 | 0.94 |
| Cult | 4.50 | 230.25 | 0.76 | 6.13 | 0.41 | 0.94 |
| Eckendorfer Mali | 4.50 | 230.25 | 0.76 | 6.13 | 0.41 | 0.94 |
| France 1 | 4.50 | 230.25 | 0.76 | 6.13 | 0.41 | 0.94 |
| Girita | 4.50 | 230.25 | 0.76 | 6.13 | 0.41 | 0.94 |
| Gora | 4.50 | 230.25 | 0.76 | 6.13 | 0.41 | 0.94 |
| Gulle | 4.50 | 230.25 | 0.76 | 6.13 | 0.41 | 0.94 |
| Janetzkis | 4.50 | 230.25 | 0.76 | 6.13 | 0.41 | 0.94 |
| KS3579 | 4.50 | 230.25 | 0.76 | 6.13 | 0.41 | 0.94 |
| KSU-1 | 4.50 | 230.25 | 0.76 | 6.13 | 0.41 | 0.94 |
| KSU-10 | 4.50 | 230.25 | 0.76 | 6.13 | 0.41 | 0.94 |
| Ladoga | 4.50 | 230.25 | 0.76 | 6.13 | 0.41 | 0.94 |
| Linglandor | 4.50 | 230.25 | 0.76 | 6.13 | 0.41 | 0.94 |
| Midas | 4.50 | 230.25 | 0.76 | 6.13 | 0.41 | 0.94 |
| Regal | 4.50 | 230.25 | 0.76 | 6.13 | 0.41 | 0.94 |
| Titus | 4.50 | 230.25 | 0.76 | 6.13 | 0.41 | 0.94 |
| Licantara | 4.75 | 258.00 | 0.85 | 0.83 | 0.72 | 0.93 |
| Nilla glossy | 4.75 | 258.00 | 0.85 | 0.83 | 0.72 | 0.93 |
| Fashion | 5.00 | 273.50 | 0.90 | 0.04 | 0.88 | 0.92 |
| Gullivar | 5.00 | 273.50 | 0.90 | 0.04 | 0.88 | 0.92 |
| Lindora-00 | 5.00 | 273.50 | 0.90 | 0.04 | 0.88 | 0.92 |
| Major | 5.00 | 273.50 | 0.90 | 0.04 | 0.88 | 0.92 |
| Turret | 5.00 | 273.50 | 0.90 | 0.04 | 0.88 | 0.92 |

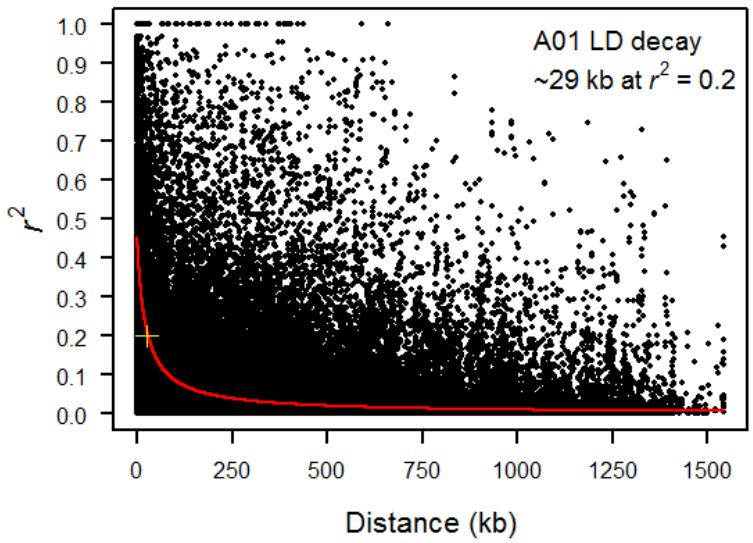


Fig. A20. LD decay for chromosome A01 from the field study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 29 kb.

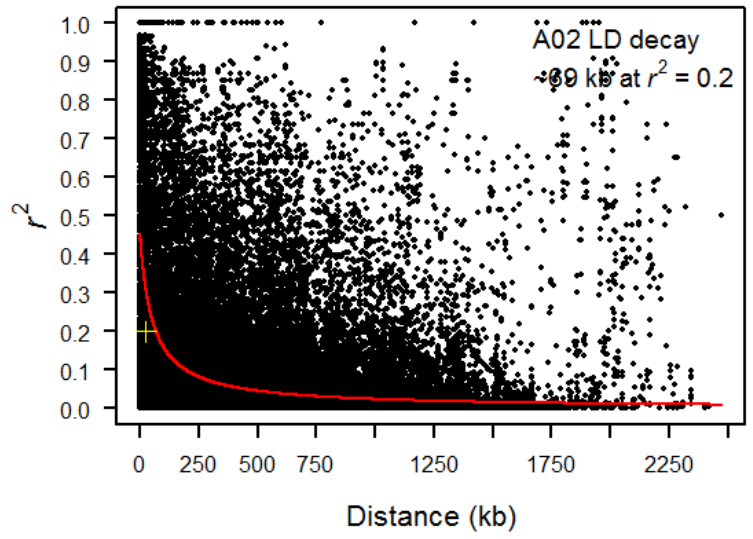


Fig. A21. LD decay for chromosome A02 from the field study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 69 kb.

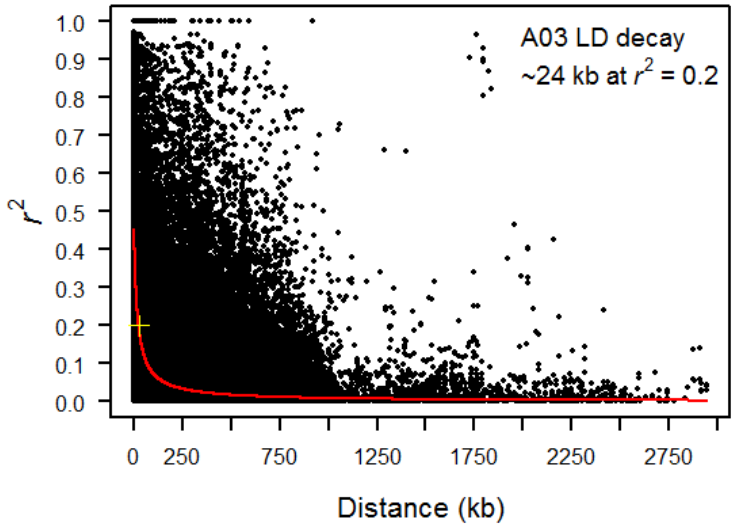


Fig. A22. LD decay for chromosome A03 from the field study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 24 kb.

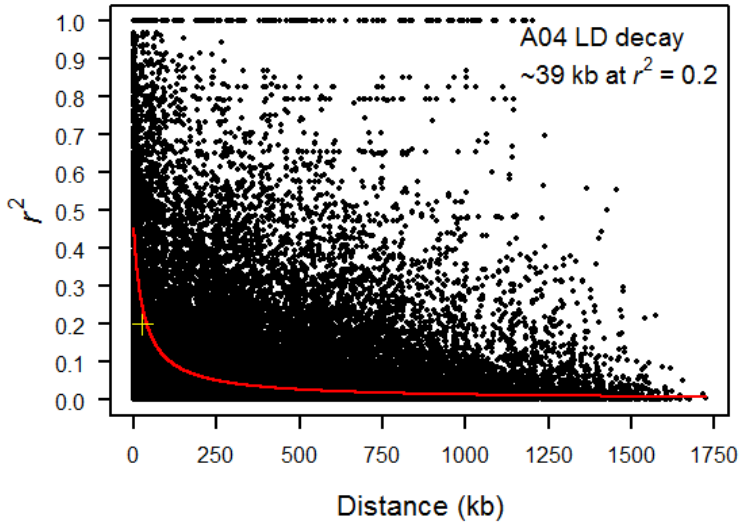


Fig. A23. LD decay for chromosome A04 from the field study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 39 kb.

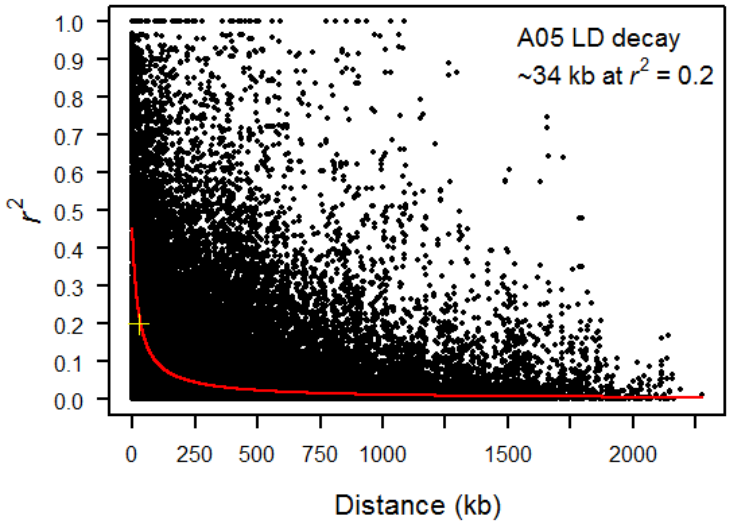


Fig. A24. LD decay for chromosome A05 from the field study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 34 kb.

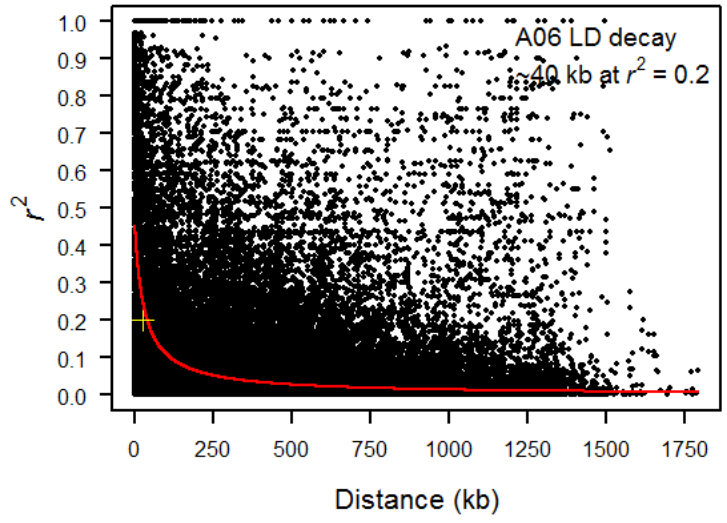


Fig. A25. LD decay for chromosome A06 from the field study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 40 kb.

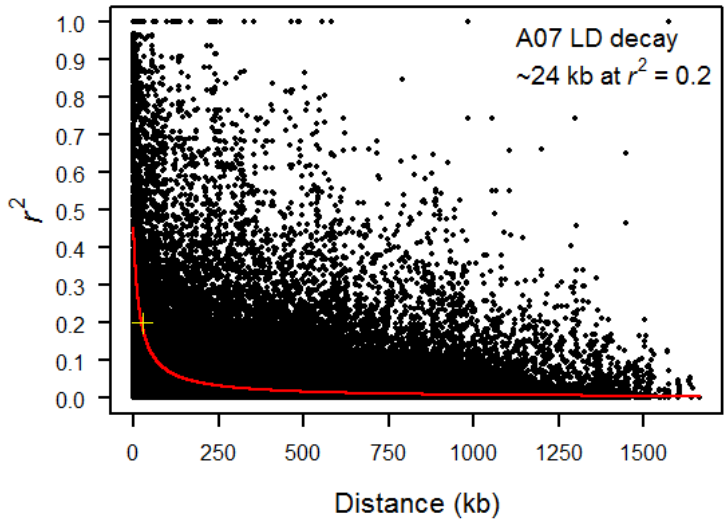


Fig. A26. LD decay for chromosome A07 from the field study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 24 kb.

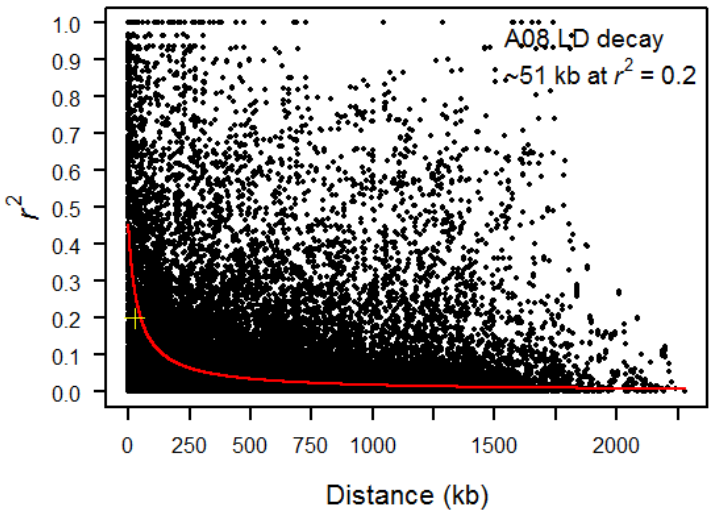


Fig. A27. LD decay for chromosome A08 from the field study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 51 kb.

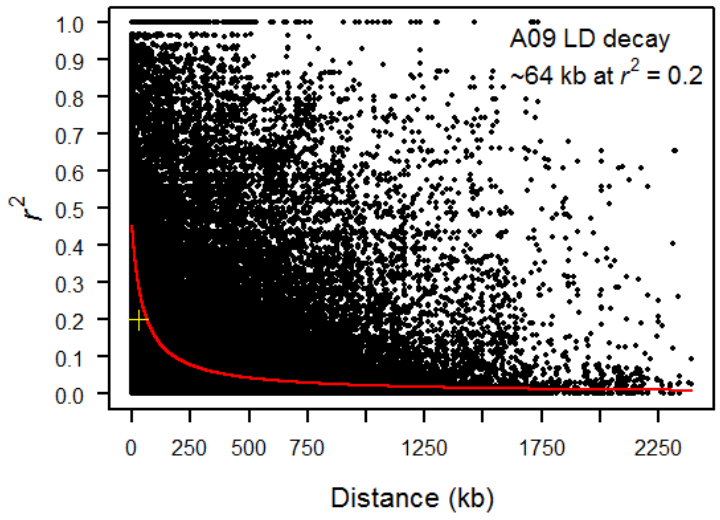


Fig. A28. LD decay for chromosome A09 from the field study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 64 kb.

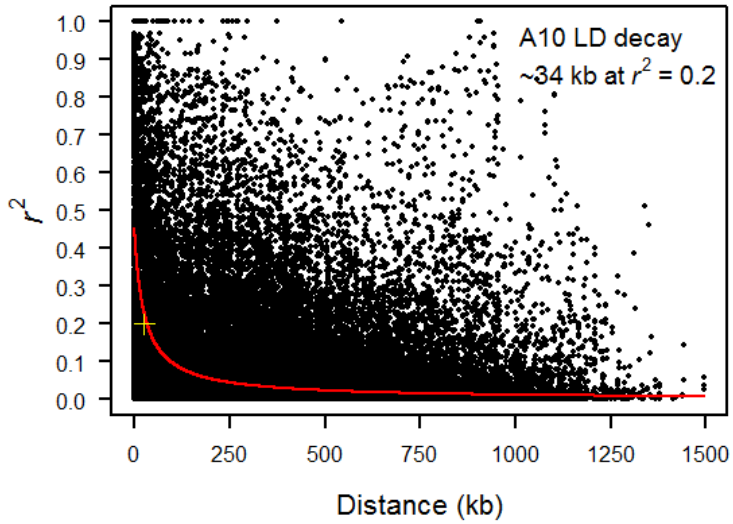


Fig. A29. LD decay for chromosome A10 from the field study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 34 kb.

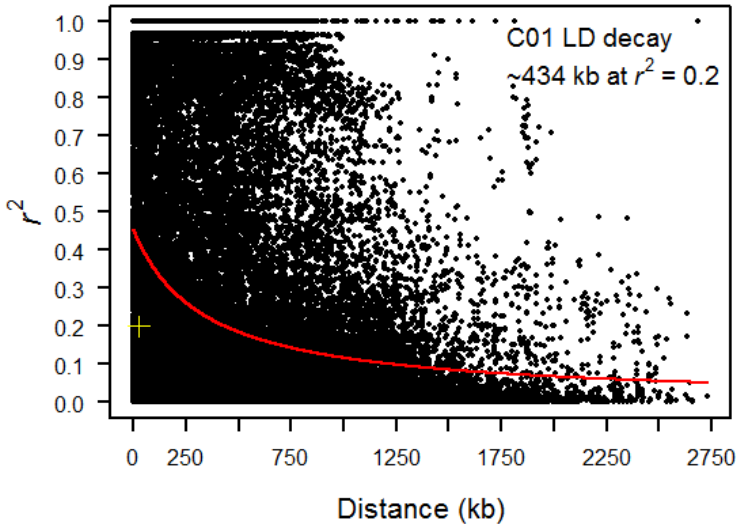


Fig. A30. LD decay for chromosome C01 from the field study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 434 kb.

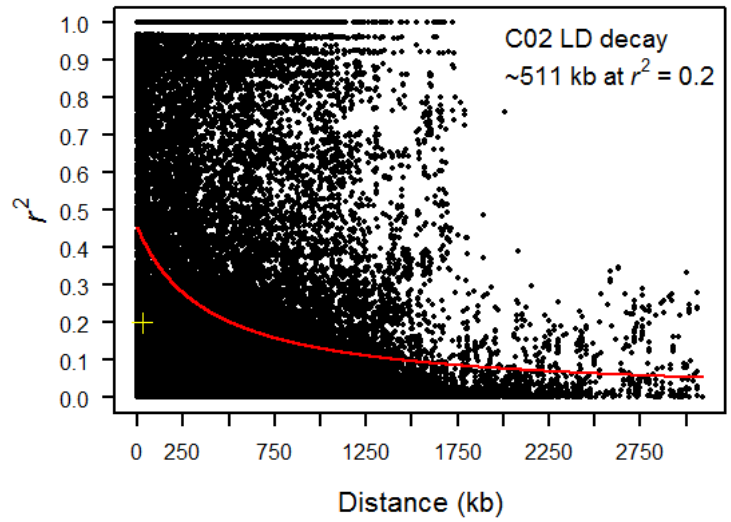


Fig. A31. LD decay for chromosome C02 from the field study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 511 kb.

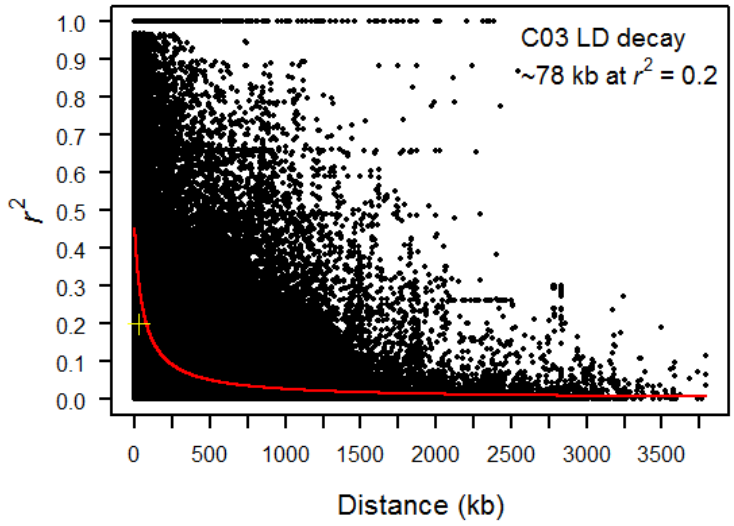


Fig. A32. LD decay for chromosome C03 from the field study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 78 kb.

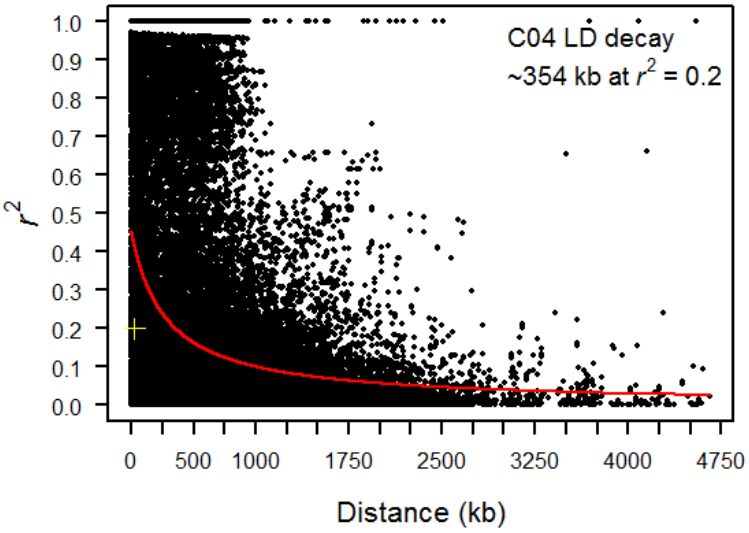


Fig. A33. LD decay for chromosome C04 from the field study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 354 kb.

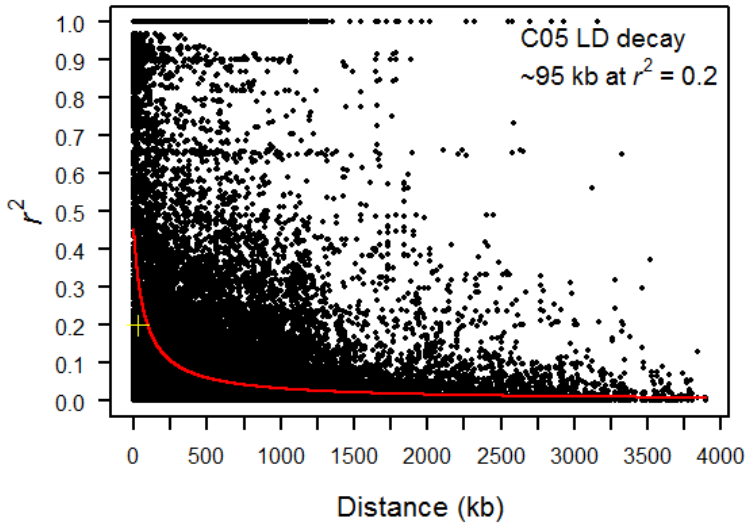


Fig. A34. LD decay for chromosome C05 from the field study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 95 kb.

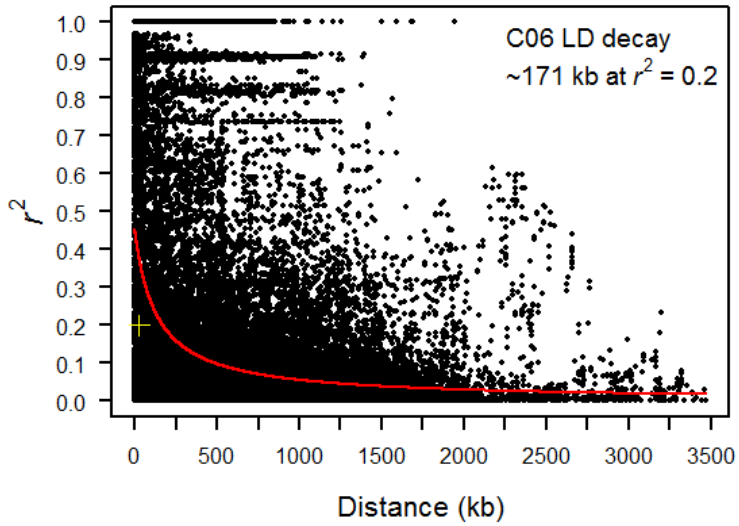


Fig. A35. LD decay for chromosome C06 from the field study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 171 kb.

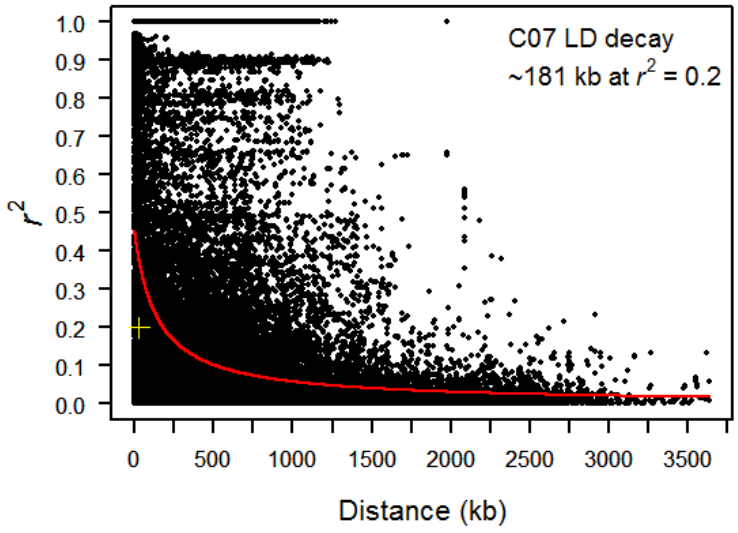


Fig. A36. LD decay for chromosome C07 from the field study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 181 kb.

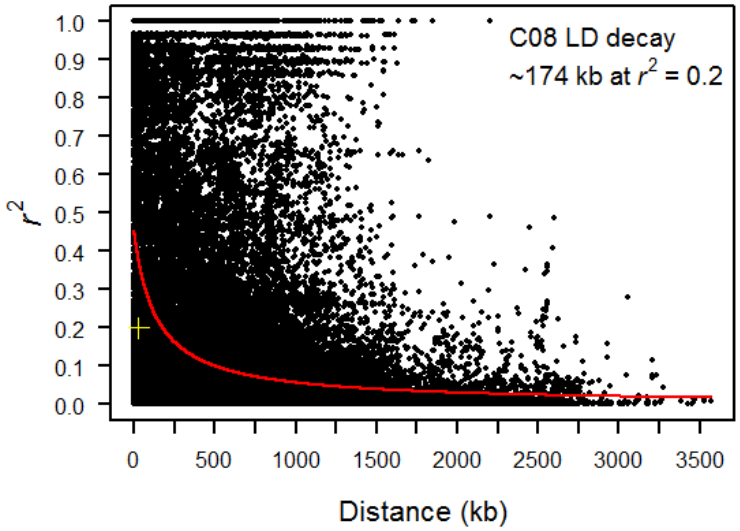


Fig. A37. LD decay for chromosome C08 from the field study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 174 kb.

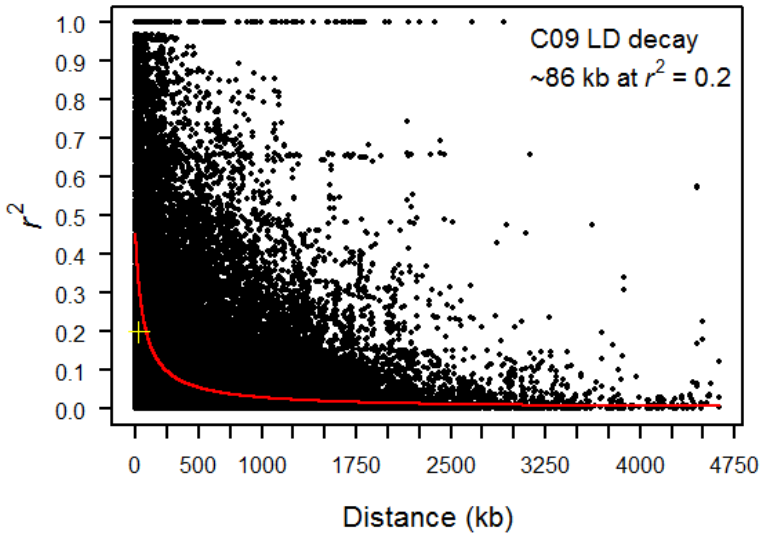


Fig. A38. LD decay for chromosome C09 from the field study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 86 kb.

Table A6. Growth habit and the country obtained/originated from for the electrolyte leakage study.

| Genotype | Growth habit | Country obtained/originated |
|---|---|---|
| A-10-999 | Spring | USA |
| A-15-1000 | Spring | Canada |
| A-15-989 | Spring | USA |
| A-16-1013 | Spring | USA |
| A-3-1011 | Spring | USA |
| A-4-1000 | Spring | USA |
| A-7-997 | Spring | USA |
| A-8-1000 | Spring | USA |
| A-9-1013 | Spring | USA |
| A04-72NA | Spring | USA |
| A04-73NA | Spring | USA |
| A04-74NA | Spring | USA |
| A06-19NA | Spring | USA |
| A06-20NA | Spring | USA |
| A07-28NA | Spring | USA |
| A07-29NI | Spring | USA |
| AR91004 | Winter | USA |
| ARC-2180-1 | Winter | USA |

Table A6. Growth habit and the country obtained/originated from for the electrolyte leakage study (continued).

| Genotype | Growth habit | Country obtained/originated |
|---|---|---|
| ARC-90016 | Winter | USA |
| ARC-97018 | Winter | USA |
| ARC-97019 | Winter | USA |
| Aviso | Spring | Canada |
| Azumasho | Semi-winter | South Korea |
| Barkant | Winter | Netherlands |
| Barplina | Winter | South Korea |
| Beryl | Winter | Poland |
| Bingo | Spring | USA |
| BNW 161/83 | Winter | Germany |
| Brio | Spring | France |
| Bronowski | Spring | Poland |
| Buk Wuk 3 | Spring | South Korea |
| Celebra | Spring | Sweeden |
| Colza 18 Miroc | Semi-winter | South Korea |
| Comet | Spring | Sweden |
| Conquest | Spring | Canada |
| Corvette | Winter | UK |
| Cougar | Spring | Canada |
| Cresor | Spring | France |
| Crop | Spring | France |
| Cult | Winter | Canada |
| Da Vinci | Winter | Canada |
| Dae cho-sen | Semi-winter | South Korea |
| Delta | Spring | Sweden |
| Drakkar | Spring | France |
| Eckendorfer Mali | Semi-winter | South Korea |
| Eragi | Winter | Germany |
| Erra | Winter | Germany |
| Evvin | Spring | Russian Federation |
| Expander | Winter | Germany |
| Fertodi | Winter | South Korea |
| Fonto | Spring | South Korea |
| France 1 | Spring | France |
| Fuji | Spring | South Korea |
| Galant | Spring | USA |
| Galaxy | Spring | Sweden |
| Galileo | Winter | Canada |
| Girita | Semi-winter | Germany |
| Glacier | Winter | Sweden |
| Global | Spring | Sweden |

Table A6. Growth habit and the country obtained/originated from for the electrolyte leakage study (continued).

| Genotype | Growth habit | Country obtained/originated |
|---|---|---|
| Golden | Spring | Canada |
| Gora | Spring | Germany |
| Gulle | Spring | Sweden |
| Gullivar | Spring | Sweden |
| Helga | Semi-winter | Germany |
| Hi-Q | Spring | Canada |
| Ibiza | Winter | Canada |
| IR-2 | Spring | Hungary |
| Iwashiro-natane | Winter | South Korea |
| Janetzkis | Spring | South Korea |
| Jasna | Spring | USA |
| Jet_Neuf | Winter | Canada |
| Jupiter | Winter | USA |
| Kanada | Spring | Poland |
| Klinki | Spring | South Korea |
| Kosa | Spring | Germany |
| Koubun | Spring | South Korea |
| Kovalevskjj | Spring | Ukraine |
| Kritmar rape | Spring | South Korea |
| KS3579 | Winter | USA |
| KSU 1 | Winter | USA |
| KSU 10 | Winter | USA |
| KSU 2 | Winter | USA |
| KSU 3 | Winter | USA |
| KSU 4 | Winter | USA |
| KSU 5 | Winter | USA |
| KSU 6 | Winter | USA |
| KSU 7 | Winter | USA |
| KSU 8 | Winter | USA |
| KSU 9 | Winter | USA |
| Kuju | Winter | South Korea |
| Ladoga | Winter | Canada |
| Legend | Spring | Sweden |
| Lembkes | Winter | South Korea |
| Lester | Winter | Germany |
| Librador | Winter | Germany |
| Licantara | Winter | Germany |
| Lieikoposki | Semi-winter | South Korea |
| Lindora 00 | Winter | Germany |
| Lindore | Winter | Germany |
| Linglandor | Winter | Germany |

Table A6. Growth habit and the country obtained/originated from for the electrolyte leakage study (continued).

| Genotype | Growth habit | Country obtained/originated |
|---|---|---|
| Linus | Winter | South Korea |
| Liratrop | Winter | Germany |
| Lisora | Semi-winter | Germany |
| Lorenze | Winter | Canada |
| Major | Semi-winter | France |
| MAR 160059 | Winter | Poland |
| Mar'janovskij | Spring | Ukraine |
| Midas | Spring | Canada |
| Miekuro Dane | Spring | South Korea |
| Mlochowski | Semi-winter | Poland |
| Murame nadame | Semi-winter | South Korea |
| Mutsumi | Semi-winter | Japan |
| Nilla 1022 | Semi-winter | South Korea |
| NU 51084 | Spring | Sweden |
| Nugget | Semi-winter | South Korea |
| NY-10 | Semi-winter | China |
| NY-7 | Semi-winter | China |
| NY-8 | Semi-winter | China |
| Oleifera | Semi-winter | South Korea |
| Oro | Spring | Canada |
| Peace | Spring | Canada |
| Petanova lihonova | Semi-winter | South Korea |
| Polo Canola | Spring | USA |
| Printol | Spring | USA |
| Prota | Spring | Germany |
| Q2 | Spring | Canada |
| R. Creaus | Winter | South Korea |
| Rafal | Winter | France |
| Rang | Semi-winter | South Korea |
| Ratnik | Spring | USA |
| Rebel | Semi-winter | USA |
| Regal | Winter | South Korea |
| Regent | Spring | Canada |
| Regina II | Spring | Canada |
| Reston | Spring | USA |
| Ridana | Winter | Germany |
| Romeo | Spring | France |
| Russia 5 | Spring | Russian Federation |
| Seoul | Spring | South Korea |
| Sera | Semi-winter | Germany |
| Silex | Spring | Canada |

Table A6. Growth habit and the country obtained/originated from for the electrolyte leakage study (continued).

| Genotype | Growth habit | Country obtained/originated |
|---|---|---|
| Sunrise | Spring | USA |
| Sval of Gullen | Spring | South Korea |
| Synra | Winter | South Korea |
| Taiwan | Spring | Taiwan |
| Tanka | Semi-winter | South Korea |
| Titus | Winter | South Korea |
| Todane | Semi-winter | South Korea |
| Tower | Spring | Canada |
| Turret | Spring | Canada |
| Vision | Winter | Canada |
| Vostochno sibirskii | Spring | Russian Federation |
| Wasefuji | Spring | South Korea |
| Westar | Spring | Canada |
| Wipol | Semi-winter | Norway |
| Wira | Winter | Germany |
| Yong dang | Semi-winter | South Korea |

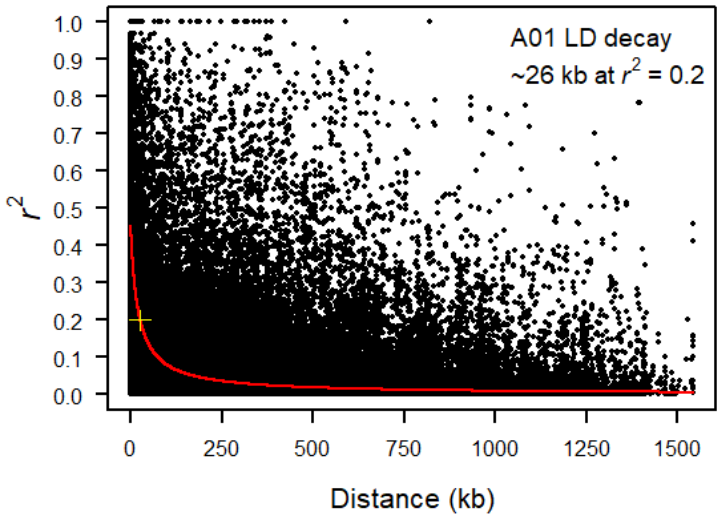


Fig. A39. LD decay for chromosome A01 from the electrolyte leakage study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 26 kb.

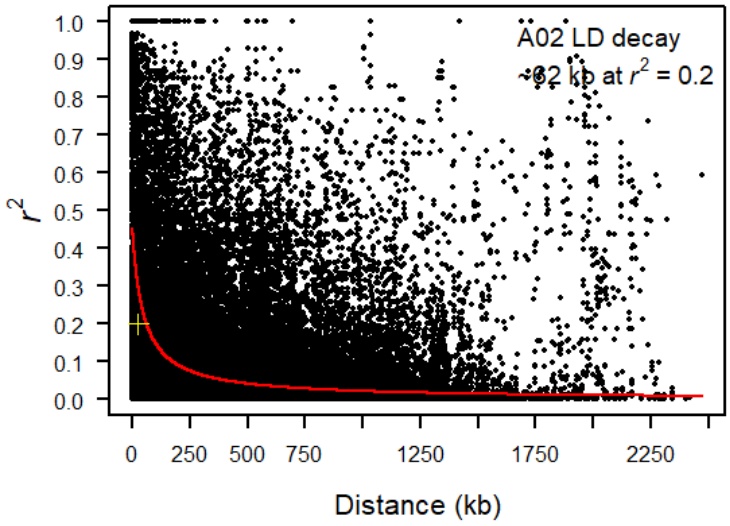


Fig. A40. LD decay for chromosome A02 from the electrolyte leakage study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 62 kb.

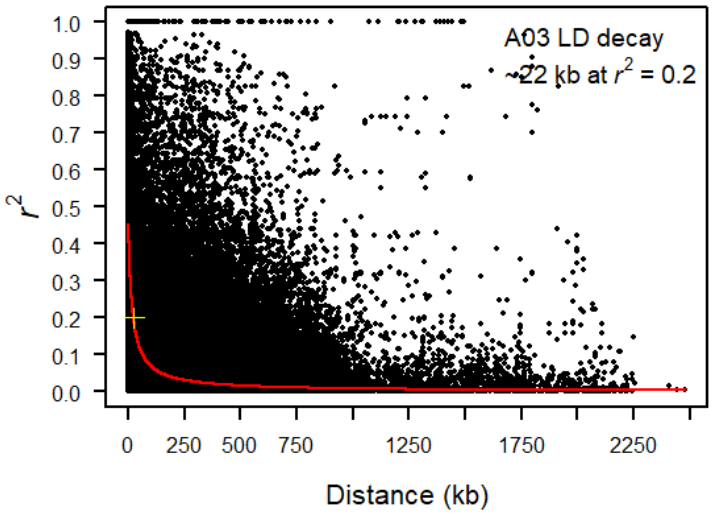


Fig. A41. LD decay for chromosome A03 from the electrolyte leakage study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 22 kb.

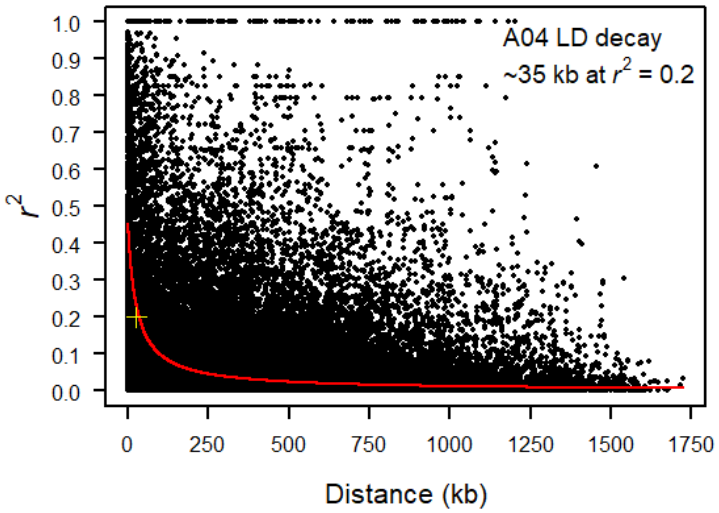


Fig. A42. LD decay for chromosome A04 from the electrolyte leakage study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 35 kb.

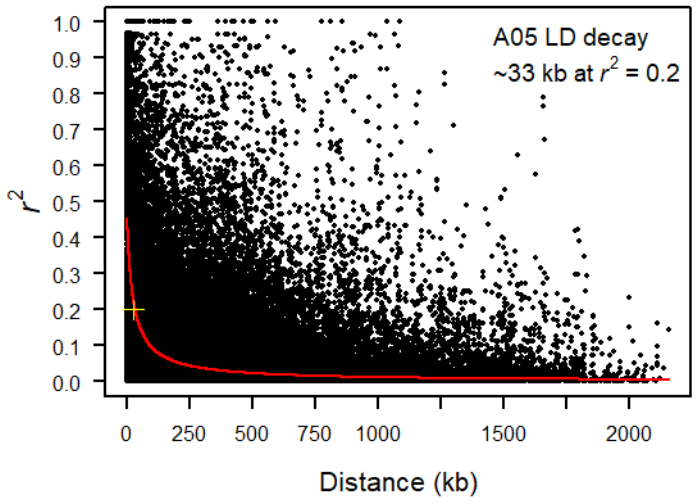


Fig. A43. LD decay for chromosome A05 from the electrolyte leakage study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 33 kb.

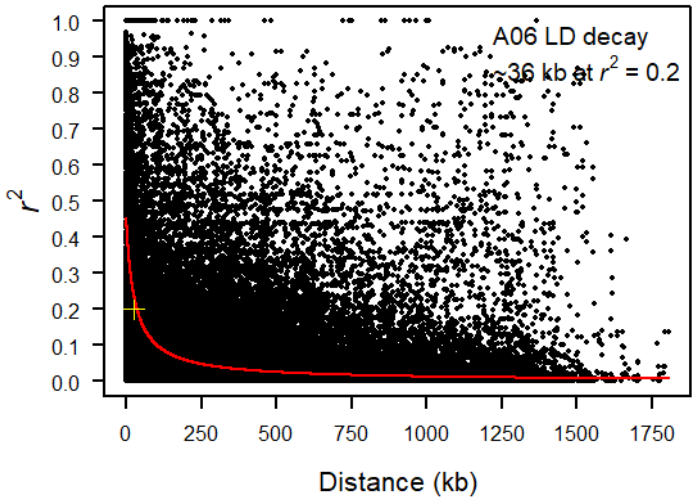


Fig. A44. LD decay for chromosome A06 from the electrolyte leakage study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 36 kb.

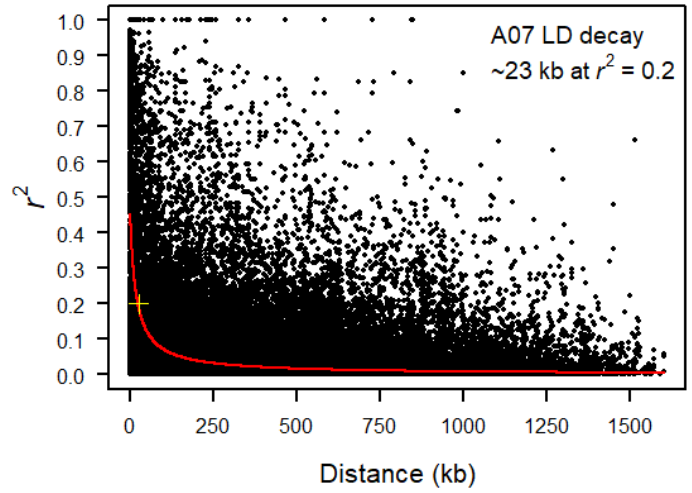


Fig. A45. LD decay for chromosome A07 from the electrolyte leakage study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 23 kb.

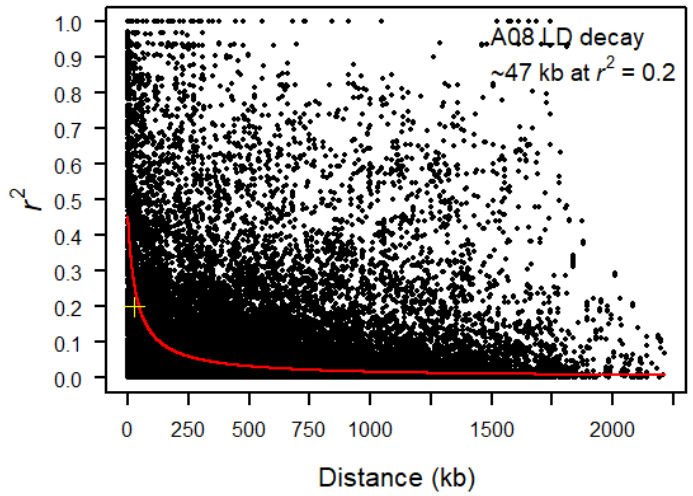


Fig. A46. LD decay for chromosome A08 from the electrolyte leakage study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 47 kb.

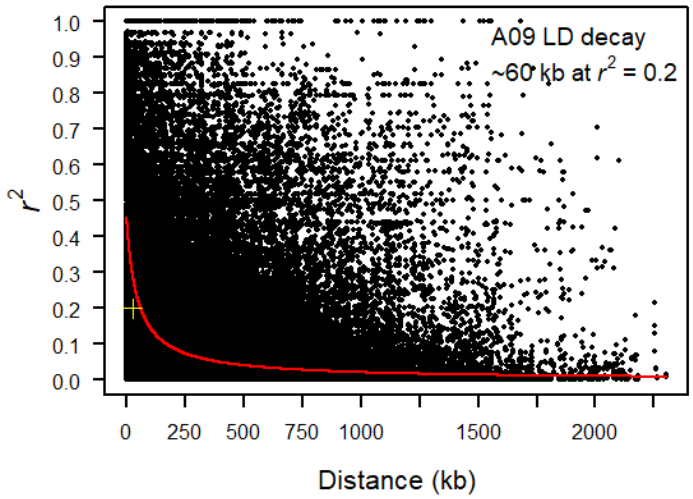


Fig. A47. LD decay for chromosome A09 from the electrolyte leakage study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 60 kb.

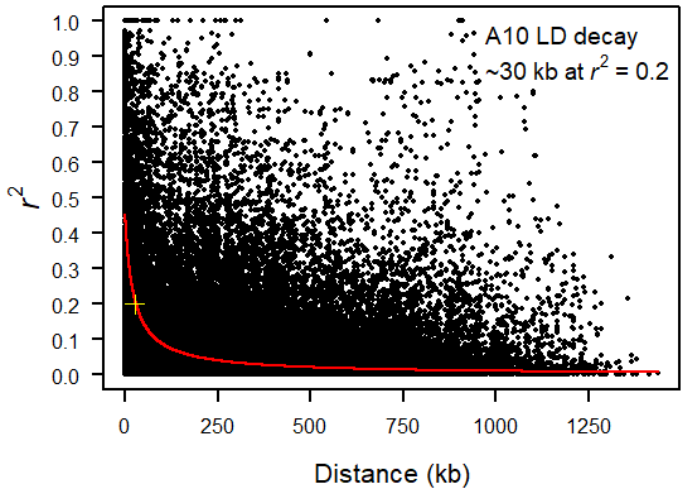


Fig. A48. LD decay for chromosome A10 from the electrolyte leakage study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 30 kb.

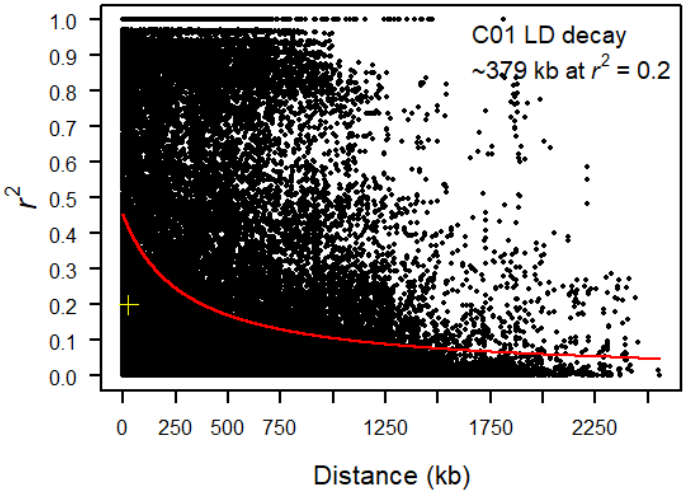


Fig. A49. LD decay for chromosome C01 from the electrolyte leakage study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 379 kb.

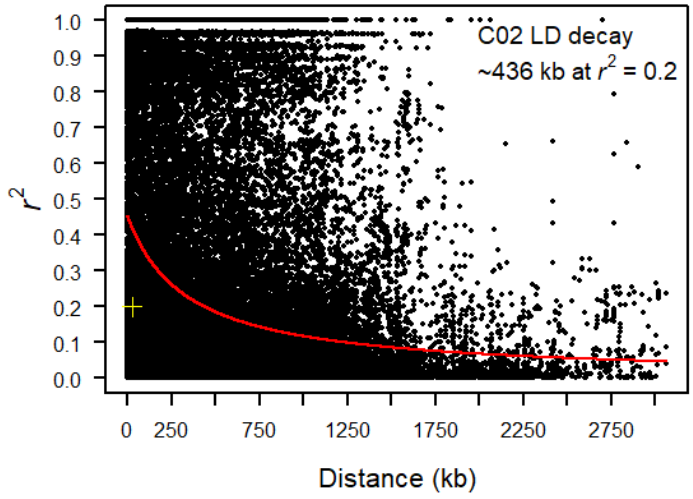


Fig. A50. LD decay for chromosome C02 from the electrolyte leakage study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 436 kb.

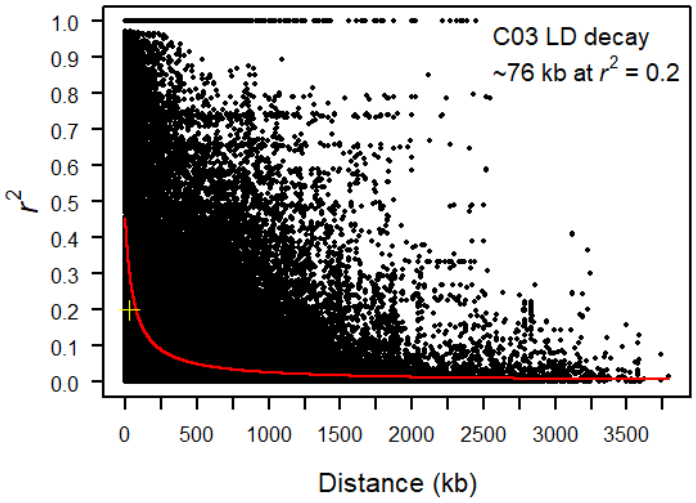


Fig. A51. LD decay for chromosome C03 from the electrolyte leakage study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 76 kb.

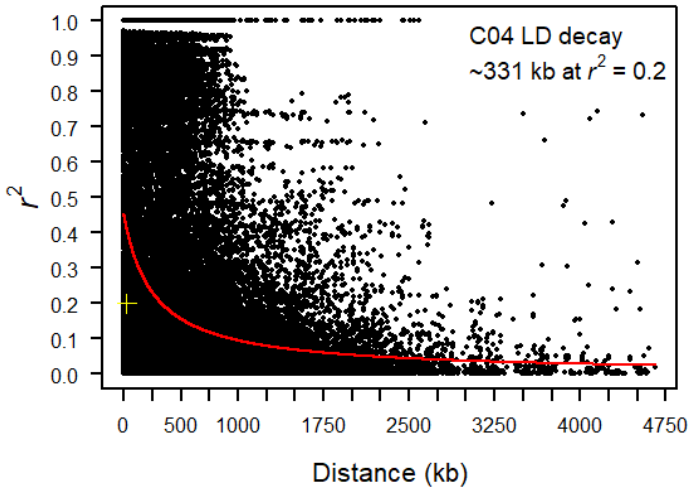


Fig. A52. LD decay for chromosome C04 from the electrolyte leakage study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 331 kb.

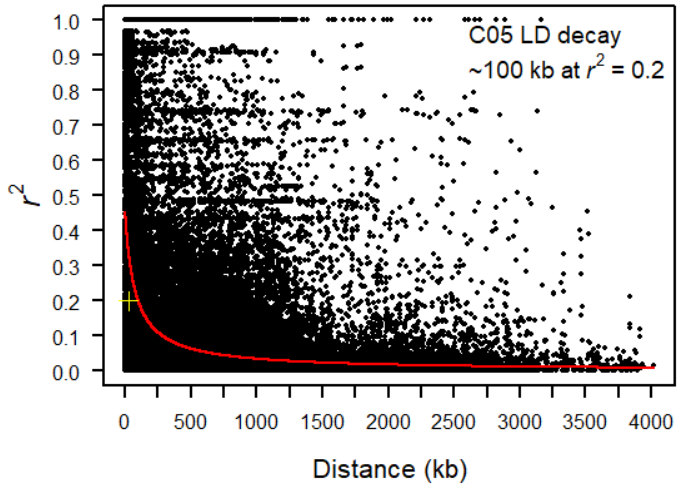


Fig. A53. LD decay for chromosome C05 from the electrolyte leakage study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 100 kb.

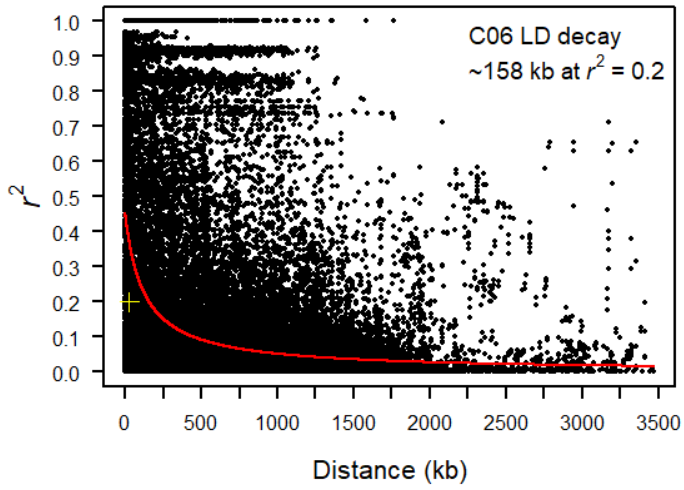


Fig. A54. LD decay for chromosome C06 from the electrolyte leakage study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 158 kb.

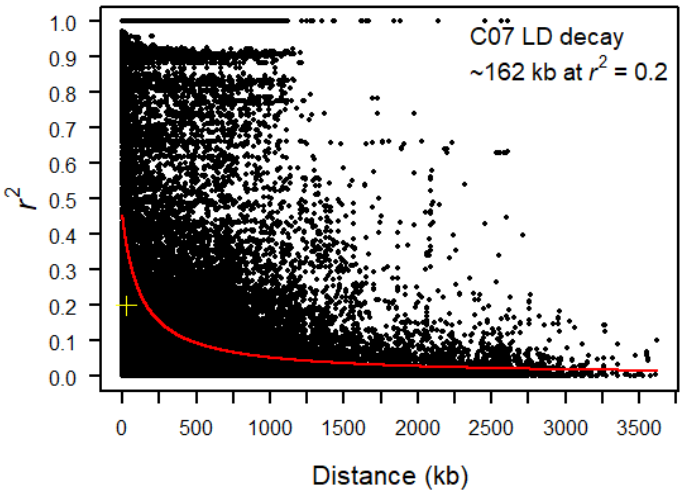


Fig. A55. LD decay for chromosome C07 from the electrolyte leakage study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 162 kb.

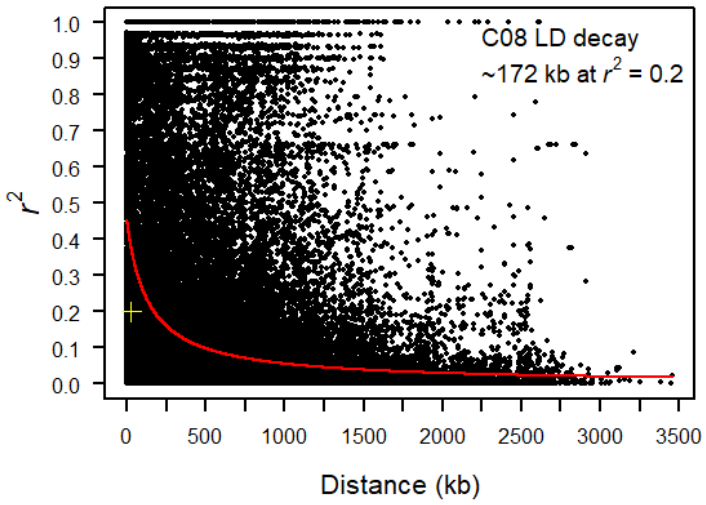


Fig. A56. LD decay for chromosome C08 from the electrolyte leakage study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 172 kb.

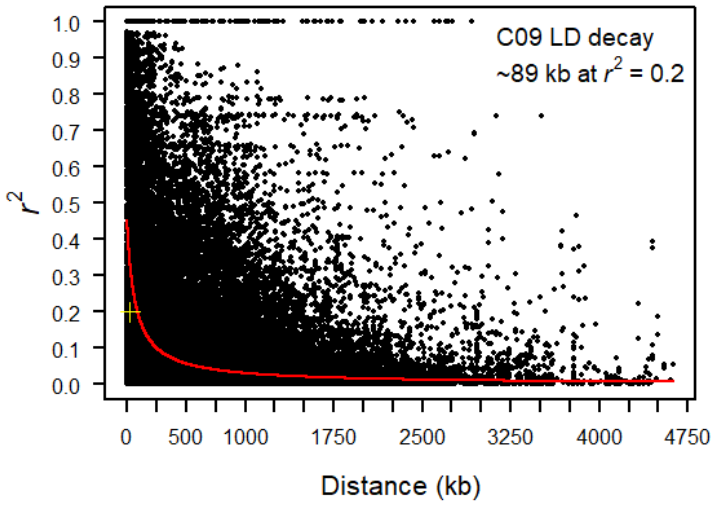


Fig. A57. LD decay for chromosome C09 from the electrolyte leakage study. Distance in kb is on the x-axis and $r^2$ is on the y-axis. The LD decay at $r^2 = 0.2$ is about 89 kb.