THESIS

PHENOTYPIC CHANGES AND DNA METHYLATION STATUS IN CRYOPRESERVED SEEDS OF RYE (SECALE CEREALE L.)

Submitted by

Jie Lu

Department of Soil and Crop Sciences

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Summer 2017

Master's Committee:

Advisor: Patrick Byrne

Stephanie Greene
Anireddy Reddy

ABSTRACT

PHENOTYPIC CHANGES AND DNA METHYLATION STATUS IN CRYOPRESERVED SEEDS OF RYE (SECALE CEREALE L.)

Conserving genetic diversity is one of the major tasks for seed banks worldwide. At present, there are two long-term preservation methods in the USDA-ARS National Laboratory of Genetic Resources Preservation (NLGRP) : storage in a -18 ℃ vault (conventional storage) and storage in a liquid nitrogen vapor phase ranging from -135 ºC to -180 ℃ (cryopreservation). Cryopreservation is considered to be the best method for long-term storage of vegetatively propagated plants and sometimes can be effective for recalcitrant seeds, but its value for orthodox seeds has not been thoroughly tested.

Rye (Secale cereale L.) is a diploid allogamous plant species with 2n=14, a relatively high degree of gametophytic self-incompatibility and orthodox seeds. It has been an important crop in Europe and one of its major uses nowadays is in wheat breeding. In order to test the effects of long-term cryopreservation of orthodox seeds, 40 rye accessions (20 with spring habit and 20 with winter habit) stored for 25 years under both conventional storage and cryogenic conditions were evaluated.

In our research, field and seedling evaluation and DNA methylation experiments were conducted. Winter rye seeds were planted in October 2014 and spring rye seeds were planted in April 2015 at CSU's Agricultural Research Education and Development Center (ARDEC) near Fort Collins, CO. Seedling evaluation was conducted at NLGRP in 2014-2015. A methylation sensitive amplified fragment length polymorphism (metAFLP) technique was used to evaluate

DNA methylation of two accessions of the 40 total accessions used in the field experiment. This experiment was conducted in the summer of 2016.

In the field evaluation, only spike length in the winter trial was significantly different ($P=0.045$) between storage treatments. Spikes of plants grown from conventionally stored seeds were slightly longer than those from cryopreserved seeds. Seedlings from cryopreserved samples had significantly higher normal germination percentage ($P<0.0001$) and lower abnormal germination percentage ($P<0.01$) than those stored under conventional conditions. In addition, root length in the winter trial and average root diameter in both trials showed significant differences ($P<0.05$) between the two storage treatments. Seedlings from cryopreserved seeds had longer roots and smaller root diameter than seedlings from conventionally stored seeds in the winter trial and seedlings from conventionally stored seeds had smaller root diameter than seedlings from cryopreserved seeds in the spring trial. No other significant differences between storage methods were detected. Our results indicated that cryopreservation has only minimal affects on phenotypic variation and may preserve seed for a longer period than conventional storage in orthodox cereal seeds.

In the metAFLP experiment, only 5 of 311 loci in accession V/108 and 3 of 308 loci in accession Omka showed unadjusted methylation status differences between the two storage treatments at the $P=0.05$ significance level. However, after false discovery rate (FDR) adjustment, no differences in methylation were detected between storage treatments on an individual locus basis.

To my knowledge, this study was the first evaluation of long-term cryopreservation versus conventional storage in orthodox seeds. The results indicated that cryopreserved seeds had increased viability; plants grown from cryopreserved seeds has only minimal

phenotypic differences; and no epigenetic differences were detected compared to conventionally stored seeds. Therefore, based on the results of this study, cryopreservation is an appropriate method for long- term storage of rye seeds.

ACKNOWLEDGEMENTS

Several people have supported me and contributed their intelligence and effort in this research project. I would like to thank my advisor, Patrick Byrne, for his patience in advising me in this project and I really appreciate his feedback and time. I also thank the other committee members, Drs. Stephanie Greene and Anireddy Reddy, for their efforts in this project. Many thanks to the Byrne lab members for helping me in the field trial and providing guidance for the molecular experiment design. Thanks to the USDA NLGRP for financially supporting this project and helping in the seedling experiment. Also, thanks to the Department of Soil and Crop Sciences for providing support for me to finish the project in various ways. In addition, I thank my family and my friends for their support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

Literature review

Conservation of genetic diversity

Genetic diversity of crops is the result of their evolution over millennia in different growing environments and selection for adaptation to husbandry practices. Genetic diversity is important for crops to ensure their abilities to adapt and survive environmental change (Iriondo et al., 2008). At first, germplasm collection focused on landraces of crops, which are varieties resulting from gradual evolution of crop populations within a specific environment, over a long period of time, due to both farmer selection and natural selection. After the potential value of landraces as gene donors was appreciated by plant breeders in the early 20th century, more collections of plant materials were made by plant breeders and explorers in different regions (Peres, 2016). The expansion of plant genetic resources conservation happened at the same time as modernization and intensification of agricultural practices in many parts of the world, the Green Revolution of the 1960s and 1970s (Peres, 2016). Seed banks were expanded as an ex situ conservation strategy to protect agricultural diversity against genetic erosion (Peres, 2016). At present, through coordinated global effort, there are over 1,300 genebanks and germplasm collections with approximately 7.4 million accessions worldwide (Engelmann and Engels, 2002; Yu et al., 2016).

There are two basic conservation strategies for plant genetic resources, in situ and ex situ. In situ conservation is to conserve, maintain and recover viable population of species in their own natural habitats. Ex situ conservation is to conserve biodiversity outside their habitats (Engelmann and Engels, 2002). Ex situ strategies are subdivided into more specific technologies,

including seed storage, in vitro storage, DNA storage, pollen storage, field genebank and botanic garden conservation for ex situ (Engelmann and Engels, 2002). Storage of seeds under low temperature and low humidity is the most widely practiced method for ex situ conservation, and 90% of 6.1 million accessions were conserved as seeds as ofthe mid-1990s (FAO, 1996). However, gene bank conservation faces two major challenges. First, the cost of conservation techniques needs to be as low as possible due to the large number of accessions. Secondly, because of easier management for conservation of orthodox seeds, they are overrepresented in gene banks, while recalcitrant seeds and vegetatively propagated species are underrepresented (Engelmann and Engels, 2002). In order to protect genetic biodiversity, the U.S. Department of Agriculture, Agricultural Research Service (USDA-ARS) has established the National Plant Germplasm System (NPGS) to coordinate federal, state, and private organizations and research units in ex situ preservation of germplasm collections of crops and their wild relatives. NPGS has 17 gene banks located throughout the United States. These gene banks have curators to manage different collections including acquiring, maintaining, evaluating and distributing germplasm. These are also called active collections. The USDA-ARS National Laboratory for Genetic Resource Preservation (NLGRP) in Fort Collins holds the base collection, which means the security back up collection that accessions are duplicated at two locations. As of 2017, there were over 576,945 accessions of more than 15,116 species maintained in the NPGS and most of the seed collection is safely backed up at NLGRP (GRIN-Global Web. Retrieved March 14, 2017, from https://npgsweb.ars- grin.gov/gringlobal/query/summary.aspx).

In order to archive data from NPGS collection, a centralized database, the Germplasm Resources Information Network (GRIN), was developed in the late 1970s. From the 1980s, the GRIN database has been regularly used to input collection and germplasm data by scientists and

users. The GRIN database includes records for passport, taxonomic, inventory and evaluation data. Both conventional preservation and cryopreservation are used to preserve accessions at the NLGRP ("Country Report", 2011).

Storage methods

For long-term storage in NLGRP, the standard preservation method stores materials in a -18 ^{o}C vault at a low moisture content, 3 to 7% of fresh weight basis (Engelmann and Engels, 2002). The cryopreservation method preserves material in liquid nitrogen vapor phase ranging from -135 ^{o}C to -180 ^{o}C (Walter et al., 2004).

Standard preservation method

Seed longevity, the total time period for seed to remain viable, is an important trait in ecology, agronomy and the economy. In their review paper, Sano et al. (2015) reported that the mobility of molecules and viscosity of cytoplasm were highly correlated with seed longevity over a wide range of temperatures and water content. Based on Genebank Standards, provided by FAO/IPGRI in 1994, the recommended condition for long-term germplasm storage is to conserve material at -18 ^{o}C or below with 3 to 7% moisture depending on species. At NLGRP, the temperature for conventional storage was reduced to -18 ^{o}C in 1977 (Walters et al., 2004). This method extends viability of stored seeds for a relatively long time period, possibly centuries (Zevallos, et al., 2014). Walter et al. (2004) have estimated that under -18 ^{o}C condition, the seed of lettuce *(Lactuca sativa*) is estimated to survive around 150 years on average for a group of 43 lettuce accessions.

Cryopreservation

Cryopreservation is the use of very low temperature to preserve living cells and tissues. Cryopreservation is considered to be the best method for long-term storage of vegetatively

propagated plants and recalcitrant seeds. Plant tissue was first cryopreserved in the 1970's (Takagi and Engelmann, 2000), and the feasibility of the cryopreservation method was first evaluated at NLGRP in 1977 (Walters et al., 2004). Because of the much lower temperature of the cryopreservation method, it has been hypothesized that cryopreserved material can be preserved for an even longer time than conventional storage, perhaps thousands of years (Zevallos et al., 2014). Controlled freezing, vitrification, dehydration-encapsulation, dormant bud preservation and combinations of these methods are the main techniques used for conserving clonal propagules under cryo-conditions (Reed, 2004). The first concern about this long-term storage method is whether the physiological stage of plant material before liquid nitrogen storage has an effect on the quality of cryopreservation. Castillo et al. (2010) showed that compared to traditionally propagated Rubus plants, those propagated in tissue culture showed increased vigor after cryopreservation. The second concern is the physical and biochemical stability of cryopreserved plant material (Reed, 2004). Also, thermal-stress can cause serious damage to stored materials. Although slow cooling process can reduce the chance of thermal damage from non-uniform temperature distribution and cryoprotectants protect material by changing the icy microstructure, fractures can still happen randomly in preserved materials (Reed, 2004). Before long-term storage, the formation of glass in the cells can prevent structural damage (Walter s, 2014). Three types of relaxations (i.e., α, β, γ), which refer to the formation of glass in material science, were found in seed transition. After α relaxation, the void volume is large enough for molecules passing each other in diffusive motion. Then with further drying and cooling, molecules compress and small molecules have no space to rotate. Chemical reactions are expected to be different between fluid material and glass material because of the requirement of different types of molecular motions. Even within the glass, some chemical activity changes

could still be expected as void volume changes to limit or relax different types of molecular motion (Walter s, 2014).

Phenotypic traits

There have been several research studies to explore how liquid nitrogen treatment affects plants. In the study by Zevallos et al. (2014), the authors subjected a single accession of tomato *(Solanum lycopersicum* Mill.) seeds to a liquid nitrogen treatment for 2 weeks. They then conducted a field experiment, measured morphological traits, and harvested plants after maturity. They found no statistically significant phenotypic differences in traits that included fruit mass, leaf position, and length of the longest stem, among others, between cryo-treated and noncryo-treated seeds. However, there were changes in DNA sequence after exposure to liquid nitrogen based on simple sequence repeat (SSR) marker evaluation that may potentially affect other phenotypic traits. In a study on one accession of common bean (*Phaseolus vulgaris*) seeds, Cejas et al. (2013) did not find statistically significant differences in either phenotypic traits, such as percentage of seed germination at 10 days, plant height at harvest, and number of seeds per pod at harvest, or molecular results, based on SSR markers. However, because of different agronomic conditions for seed production and different storage methods used in different gene banks, Cejas et al. (2013) suggested that plant material be tested under cryopreserved conditions before using this method routinely.

Rodirigues et al. (2014) used a single accession each of 10 species of *Bromeliaceae* to investigate phenotypic characteristics of seeds and seedlings, including seed water content, 1000-seed mass, germination, and lipid content. They found that none of the species showed physical damage to seeds or seedling plants after cryopreservation. After 365 days of cryopreservation, there was a higher normal germination percentage than in fresh seeds at 5% and 7% water

content. Also, there was a lower percentage of abnormal seedlings at 3% water content after 180 days cryopreservation. Zaidi et al. (2010) found that there were no significant differences in germination percentage due to germination time or liquid nitrogen treatment period for three different seed coat types of *Tuberaria macrosepala* seeds after cryopreservation compared to non-treated seeds. Interestingly, Castillo et al. (2010) found significant morphological differences, e.g., for leaflet number, fruit traits and seed traits, between screen house mother Rubus plants and more than 12 years (12yr to 15 yr) cryopreserved *Rubus* plants. Zeliang et al. (2010) tried to develop a cryopreservation protocol for calli of the rice wild relative *Oryza rufipogon* Griff. Among panicles of plants regenerated from cryopreserved calli, cryoprotectant-exposed calli and callus-derived plants, there were no significant differences for agronomic traits. However, physical characters (awn length, seed breadth and seed girth) showed significant differences between the seeds from seed-derived control plants and all in vitro culture-derived cryopreserved plants.

Perullo et al. (2015) found that cryopreserved seeds showed a higher germination rate than non-cryopreserved seeds for *Helonias bullata*. These seeds are classified as intermediate seeds, sharing characters of orthodox and recalcitrant seeds. Salomao (2002) studied seeds of 66 tropical species and found no significant difference for germination rate between cryopreservation and control treatments in 51 species. However, there were differences in germination rate observed between cryopreserved and control seeds of the remaining 15 species, which led the authors to conclude that liquid nitrogen removes physical dormancy. Similar results were provided by Pirondini and Sgarbi (2014), who found the effect of cryopreservation on germination percentage varied among different species of orchid seeds.

DNA methylation

One of the most important issues of long-term storage is whether the genetic stability of the stored seed population is maintained. Some research shows that there is a correlation between the reduction of aged seed viability during storage and an increase of chromosomal aberrations (Puchalski et al., n. d. and references therein). DNA methylation is also a possible changes because of seed aging and stressed environments. DNA methylation occurs when a methyl group (CH3) covalently binds to the C-5 position of a cytosine of DNA through the action of DNA methyltransferases. In plants, DNA methylation is mostly considered to be heritable (Jin et al., 2011). Zhang et al. (2010) reviewed the evidence for DNA methylation as an epigenetic factor involved in regulating transcription, DNA replication and repair and cell differentiation. In the review by Chan et al. (2005), cytosine-5 methylation was stated to be the main form of methylation in eukaryotic gene silencing. In animals, the methylation modification levels are usually from 3% to 8%, while the levels in plants range from 6% to 30% (Zhang et al., 2010). In animal genomes, the methyl groups attach only to CG dinucleotides, but in plants, methylation also occurs at CHG and asymmetric CHH sites (H indicating any base other than G) (Chan et al., 2005). There are two distinct and complementary enzymatic activities for DNA methylation, catalyzed by “de novo” and “maintenance” DNA methyltransferases. Both types of enzymes function together with DNA demethylases, histone-modifying enzymes, chromatin remodeling factors, and RNA interference machinery to regulate cytosine methylation in plants (Zhang et al., 2010). Although the manner in which cytosine methylation controls developmental patterning in plants is still unclear, there is some evidence that cytosine methylation can affect more than one trait and can affect many genes involved in plant development (Zhang et al., 2010). In mammalian embryos, there is systematic reprogramming of epigenetic status in every individual

during normal preimplantation development and germ cell development (Reik et al., 2001). Although scientists have found significant reduction in cytosine methylation in the endosperm, a tissue in which differentiation has terminated and which is inconsequential to heredity, there is still no clear evidence of reprogramming epigenetic status during development in plants (Zhang et al., 2010). Several research studies show that methylation in plants is highly stable and can be inherited through multiple generations (Zhang et al., 2007a; Zhao et al., 2007). However, Teixeira et al. (2009) found that cytosine methylation and gene silencing can be very dynamic through plant development.

Unlike animals, most plants are not mobile and cannot move from one place to another when facing a stressful environment. Therefore, plants have a higher probability of being exposed to a stressed environment. New genetic variation is one way for plants to contribute to phenotypic variation in stressful environments; however, the rate of environmental changes is faster than new gene mutation or recombination can keep up with. An additional adaptation mechanism is the epigenotype, adding another level of complexity to the system (Grativol et al., 2011). DNA methylation may directly regulate gene expression, which plays a key role in acclimation; additionally, transposons are also regulated by methylation and play an important role in plant response to stress (Grativol et al., 2011).

There have been some attempts to examine the methylation rate after exposure to liquid nitrogen. In previous research, studies have shown methylation changes in shoot tips or in vitro shoot cultured material after cryo-treatment. Kaczmarczyk et al. (2010) found that both long-term in vitro culture and cryopreservation may contribute to epigenetic change. In their research, on potato (*Solanum tuberosum*) they found consistent hypomethylation signal changes detected by methylation-sensitive amplified polymorphism (MSAP) were 0.6% (3 of 469 bands) and

consistent hypermethylation signal changes were 0.2% (1 of 469 bands) when comparing cryopreservation with in vitro treatments at room temperature for a seven-year period (Kaczmarczyk et al., 2010). Mikula et al. (2011) used an embryogenic cell suspension culture method to suspend cells of *Gentiana cruciata* and then cryopreserved the cells. They found that cryopreserved cells had a higher regeneration rate than those that were cultured in vitro without cryo-treatment. They also found non-significant differences in methylation by methylation sensitive amplified fragment length polymorphism (metAFLP) markers. Johnston et al. (2009) used in vitro shoot culture of a *Ribes ciliatum* accession to detect molecular changes after cryopreservation. They found a trend for demethylation in low cryotolerant genotypes during the acclimation treatment and in successive stages of the encapsulation dehydration. However, for the most tolerant species, methylation occurred during acclimation. Maki et al. (2015) also detected slight methylation rate changes by using MSAP in wasabi *(Wasabia japonica*) plants. In their research, the shoot material cryopreserved for 10 years had a 5.5% change in methylation rate (both hypo- and hyper- methylation), while material cryopreserved for 2 hours only had a 0.12% change in methylation. Therefore, they proposed that methylation changes might accumulate under long-term cryopreservation in shoots. Heringer et al. (2013) found that PVS3 vitrification solution could induce an increase in methylation rate for somatic embryos of peach palm (*Bactris gaspipaes*) when incubated and cryopreserved in liquid nitrogen. In addition, after 24 weeks of recovery at 25 oC in the dark, there was a decreased trend for methylation rate compared to non-liquid nitrogen treated embryos.

Sisunandar et al. (2010) used zygotic embryos of four different cultivars of the coconut palm (*Cocos nucifera* L.) to evaluate methylation changes after cryopreservation by using high performance liquid chromatography (HPLC). Three of the four cultivars did not show any

differences between non-cryopreserved seedlings and cryopreserved seedlings at P=0.05, whereas one of the cultivars showed a higher global DNA methylation rate in non-cryopreserved seedlings. The author proposed that the methylation changes might be caused by dehydration during the cryopreservation process. Hao et al. (2002) found that after cryopreservation, strawberry (genus *Fragaria*) shoot-tips had significant changes in methylation levels based on the MSAP method. They found one demethylation site and two sites changed from full methylation to hemimethylation. In addition, Hao's group (2001) found that cryopreservation induced a decrease in methylation level in apple (species not provided) shoot tips.

DNA methylation detection methods

There are several methods to detect DNA methylation changes. The most common methods are AFLP-based methylation sensitive platforms, HPLC, and whole genome bisulfite sequencing (WGBS). HPLC analysis has demonstrated that the global level of cytosine methylation across plant species is variable (Alonso et al., 2016). In addition, HPLC also can be used to evaluate the magnitude of variation within or among populations (Alonso et al., 2016). However, this method cannot differentiate between coding and non-coding DNA sequences and cannot detect subtle differences in the methylation groups of individual genes (Johnston et al., 2005). WGBS is the newest method for methylation analysis. The general idea of this method is to convert unmethylated cytosine to uracil. Then after PCR amplification and sequencing with sodium bisulfite treatment, the methylated cytosines can be identified (Schmitz and Zhang, 2011). Both of these methods are suitable for methylation analysis. However, because facilities and funding needed for those methods are beyond the reach of some laboratories, AFLP-based methods are also used to detect methylation changes.

AFLP is a technique based on selectively amplifying restriction fragments from enzyme digested genomic DNA (Vos et al., 1995). The original use for AFLP was to construct high-density linkage maps for application in positional cloning of genes and molecular breeding. AFLP have several advantages over other molecular markers. First of all, the AFLP method does not require prior sequence information, which means the start-up cost is relatively low. Furthermore, this method is suitable for automation and is highly multiplexed, which allows development of high-density markers in organisms lacking a genomic platform. However, AFLP also has some limitations. It is hard for AFLP to detect differences between samples with relatively low sequence homology (less than 90%), because few common bands may be shared by two samples at the subspecies level (Vuylsteke et al., 2007). Another limitation is that DNA samples with little sequence variation may be hard to distinguish by AFLP despite a large number of fragments evaluated for polymorphisms (Vuylsteke et al., 2007). Balancing advantages and limitations, AFLP is still a useful method to detect methylation changes.

In order to detect methylation patterns, the AFLP method develops pairs of isoschizomers, which have different sensitivity in recognizing the site of methylation (Bednarek et al., 2007). MSAP is one of the major AFLP based platforms used in detecting methylation changes. In this platform, HpaII and MspI are isoschizomers acting as frequent cutters with different cytosine methylation sensitivity (5'-CCGG-3'). HpaII shows sensitivity to methylation of the internal cytosine of both strands while MspI is sensitive to methylation of the external cytosine in either strand. EcoRI is the endonuclease and the rear cutter in this case (Perez-Figueroa, 2013). MSAP has been used in several methylation studies (Maki et al., 2015; Peredo et al., 2009; Hao et al., 2002; Kaczmarczyk et al., 2010). However, MSAP can only restrict specific sites (5'-CCGG-3') and evaluate only some methylation types.

The metAFLP method can correct some of the problems of MSAP by using the *Acc65I/MseI* and *KpnI/Mse*I AFLP platform. *Acc65I* and *KpnI* enzymes can cut at the same site. The difference is that the activity of Acc65I enzyme is blocked at CpG methylation sites, while *KpnI* is not sensitive to any type of methylation (Machczynska et al, 2013; Bednarek et al., 2007). MetAFLP has been applied in barley (Hordeum vulgare) to analyze tissue culture induced variation at both sequence and methylation levels (Bednarek et al., 2007). The metAFLP method also has been used in Gentiana pannonica to detect the tissue culture induced variation for methylation level changes (Fiuk et al., 2010). In addition, Mikula et al. (2011) proved that metAFLP is a valuable tool for detecting methylation changes in tissue culture induced variation in Gentiana cruciata. In addition to detecting tissue culture induced variation, the metAFLP method has also been used to analyze epigenetic variation in population studies. Abratowska et al. (2012) used metAFLP to analyze the methylation changes between metallicolous and non-metallicolous populations of *Armeria maritime*, and Chwedorzewska and Bednarek (2012) studied morphological dissimilarities in *Deschampsia antarctica* populations by using metAFLP to determine if the dissimilarities are caused by methylation changes or mutation.

Rye

Rye is a member of the grass family in the tribe *Triticeae* and is closely related to wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) (Schlegel, 2014). Rye diverged from wheat about 3.5 to 4 million years ago and shows a relatively close relationship with wheat based on a 37 kb segment of chloroplast sequence containing both protein-coding and non-coding regions (Middleton et al., 2014). It is a diploid allogamous plant species with 2n=14 and has a relatively high degree of gametophytic self-incompatibility (Geiger and Miedaner, 2009). The main regions of rye diversity are Turkey, Lebanon, Syria, Iran, Iraq and Afghanistan, where rye is an

important crop, and also a weed within barley and wheat fields (Geiger and Miedaner, 2009). The first cultivation of rye was around the Caspian Sea at about 3000 to 4000 BC. The world production of rye is around 13 million tons, mostly in Russia, Poland and Germany. In the United States in 2010, the average rye production was about 189,000 tons, mostly in South Dakota, Georgia, Nebraska, North Dakota and Minnesota (Schlegel, 2014). Most of the rye grown has a winter habit, and spring rye is grown in extremely cold areas with snow cover lasting more than three months. Compared to other small grain crops, rye has the best winter hardiness and the highest tolerance to drought, salt, and aluminium stress (Geiger and Miedaner, 2009). Because of high yield potential, hybrid rye is even competitive with triticale (×*Triticosecale*) and wheat on better soil (Geiger and Miedaner, 2009). The major uses of rye are to make rye bread, feed animals, and produce alcohol (Schlegel, 2014). In addition, the most important use for rye in wheat breeding is that rye can translocate the short arm of chromosome 1 (1RS) to wheat, which has benefited from genes for resistance to pathogens and insects and yield improvement (Berzonsky and Francki, 1999). Another use of rye in crop breeding is to produce triticale, by crossing rye with common or durum wheat (*T. aestivum* or *T. durum*) and then typically backcrossing with wheat several times to incorporate the desirable agronomic traits (Francois, 2015). In the NLGRP, 1,861 S. *cereale* subsp. *cereale* accessions are currently safely backed up (GRIN, Retrieved April 14, 2015, from http://www.ars-grin.gov/cgi-bin/npgs/html/taxon.pl?33443).

Goals and objectives

The overall goal of this project was to compare the phenotypic and epigenetic characters of rye (*Secale cereale subsp. cereale*) after 25 years of storage by conventional preservation and cryopreservation. Specific objectives are as follows:

- To determine if there are phenotypic differences between plants grown from conventionally stored rye seed compared to plants from cryopreserved seed during the entire life cycle.
- To determine whether the effect of preservation treatments differs in different soil moisture environments.
- To determine if changes in DNA methylation rate are associated with preservation methods.

Chapter 2

Phenotypic differences between cryopreserved and conventionally stored seeds of rye (*Secale cereale* L.)

Summary

Conserving genetic diversity is one of the major tasks of seed banks worldwide. At present, there are two long-term preservation methods used in the USDA-ARS National Laboratory for Genetic Resources Preservation (NLGRP): -18 ℃, called conventional vault storage, and storage in a liquid nitrogen vapor phase ranging from -135 ℃ to -180 ℃ (cryopreservation). Cryopreservation is considered to be the best method for long-term storage of vegetatively propagated plants and recalcitrant seeds, but its value for orthodox seeds has not been thoroughly tested. A major concern is the physical and biochemical stability of cryopreserved plant material. Therefore, we conducted a field trial experiment and a seedling lab experiment in Fort Collins, CO in 2014-2015 with 20 accessions of winter rye (*Secale cereale*) and 20 accessions of spring rye seeds. Individual seed lots had been split, and half stored using conventional storage conditions and the other half stored cryogenically for 25 years. In the field experiment, plants grown from seed of each storage treatment were evaluated for qualitative traits including straw, glume and awn colors, awn type, and glume pubescence, and for the quantitative traits lodging, plant height, heading date, anthesis date, spike length, and spike weight. In the lab experiment, seedlings were evaluated for total root length, average root diameter, total projected root area, total root surface area, total root volume, total dry weight, shoot dry weight, normal germination percentage and abnormal germination percentage. In the field study, spike length of

cryopreserved winter accessions was slightly less ($P<0.05$) than the same accessions stored conventionally. Seedlings from cryopreserved samples had significantly higher normal germination percentage ($P<0.0001$) and lower abnormal germination percentage ($P<0.01$) than those stored under conventional conditions. In addition, root length in the winter rye type and average root diameter for both winter and spring types differed ($P<0.05$) between the two storage treatments. No other significant differences between storage methods were detected. Our results indicated that cryopreservation has only minimal effects on phenotypic variation and may preserve seed in longer period in orthodox cereal seeds.

Introduction

The evolution of crops over millennia based on different growing environments, husbandry practices, and cultural preferences has resulted in a vast array of genetic diversity in most crop species. Collecting diverse germplasm for use in crop improvement began in the early 20th century, notably through the work of the Russian botanist Nikolai Vavilov (Peres, 2016). Germplasm conservation efforts were intensified in the 1960s and 1970s through the establishment or expansion of 'seed banks' (Peres, 2016). Nowadays, with global effort, there are over 1,300 gene banks and germplasm collections with approximately 7.4 million plant accessions collected worldwide (Yu et al., 2016; Engelmann and Engels, 2002). In the U.S., the U.S. Department of Agriculture, Agricultural Research Service (USDA-ARS) has established the National Plant Germplasm System (NPGS) to coordinate federal, state, and private organizations and research units in *ex situ* preservation of germplasm collections of crops and their wild relatives. Currently the NPGS holds over 536,000 accessions of more than 13,000 species (Bretting et al., 2011).

Seed longevity, the total time period in which a seed retains viability, is a valuable trait in ecology, agriculture, and the global economy. Based on Genebank Standards, provided by FAO/IPGRI in 1994, the recommended condition for long-term plant germplasm storage is to conserve material at -18 ^{o}C or below with 3 to 7% moisture depending on species.

Another method to conserve plant germplasm is cryopreservation, the storage of biological material ranging from -135 ^{o}C to -180 ^{o}C in the vapor phase of liquid nitrogen (Walter et al., 2004). Plant tissue was first cryopreserved in the 1970's (Takagi and Engelmann, 2000). Cryopreservation is considered to be the best method for long-term storage of vegetatively propagated plants and recalcitrant seeds (Takagi and Engelmann, 2000). However, there are two main concerns with this method. The first concern is whether the physiological stage of plant material before storage in liquid nitrogen has an effect on the quality of cryopreservation. The second concern is the physical and biochemical stability of cryopreserved plant material (Zevallos et al., 2014). Different plant materials have shown different results. Plants grown from cryopreserved wild tomato (*Solanum lycopersicum*), common bean (*Phaseolus vulgaris*) and *Tuberaria macrosepala* seeds did not show any differences for several phenotypes compared to control groups (Zevallos et al., 2014; Cejas et al., 2013; Zaidi et al., 2010). However, other researchers have found differences for some phenotypic characters. Castillo et al. (2010) found no morphological differences between cryopreserved *Rubus* plants and control plants in a greenhouse, but cryopreserved plants were more vigorous than control plants in a field study, with larger seeds, fruits and leaves. In addition to *Rubus* plants, seeds of *Helonias bullata*, some orchids, and several wild tropical species showed germination differences between cryopreserved and non-cryopreserved conditions (Perullo et al., 2015; Salomao, 2002; Pironfini and Sgarbi, 2014). The differences

in those studies were in both directions, which cryopreserved seeds showing both higher and lower germination.

To my knowledge, no studies have been reported on the effects of long-term cryopreservation on seeds of grain crops, which account for most of the world's food supply nor have any studies compared the difference between the two storage regimes after 25 years. Rye (*Secale cereale*) is a member of the grass family (*Poaceae*) in the tribe *Triticeae* and is closely related to wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*). It is a diploid allogamous plant species with 2n=14 and a relatively high degree of gametophytic self-incompatibility (Geiger and Miedaner, 2009). Therefore, it is an outcrossing species with a high degree of heterogeneity within cultivars and landraces. It is an important crop in Europe and the Middle East.

The overall goal of this project was to determine the effects of long-term cryopreservation of seeds on phenotypic traits of the resulting plants. Specific goals were to

(1) Compare phenotypic traits of rye plants grown from cryopreserved and conventionally stored seeds, under well-watered and limited moisture field conditions.

(2) Compare germination and phenotypic traits of rye seedlings grown from cryopreserved and conventionally stored seeds.

Materials and methods

Plant materials

In this research, the subset of rye germplasm (*Secale cereale* subsp. *cereale*) was originally received by NLGRP from the Botanical Garden of the Polish Academy of Sciences in 1988 with seed initial moisture ranging from 4.5 to 6.5% and initial viability ranging from 99 to 88 (Appendix Table 1). Twenty rye accessions with winter growth habit

and 20 rye accessions with spring growth habit were used in this study. Accession names, Plant Introduction numbers, origins, and type of germplasm are listed in Table 2.1. Samples from the same seed lot of each accession were cryopreserved in liquid nitrogen vapor for 25 years (Dec. 1988 to 2014), or stored at -18°C for the same length of time (Seed in Base, PVP, or Plant Registration Collections. (n.d.). Retrieved April 14, 2015, from http://www.ars.usda.gov/Services/docs.htm?docid=23146). Thus, there was a total of 40 entries (accession x preservation method combination) for the winter rye trial and 40 entries for the spring rye trial.

Field experiment

Experimental design and trial management

To evaluate the effects of different preservation treatments, rye accessions, and soil moisture environments, field trials were conducted at the CSU Agricultural Research Education and Development Center (ARDEC) near Fort Collins, CO (latitude 40.652°, longitude -104.999°, and elevation 1549 m). For each growth habit, two trials were grown, one designated for irrigation and one for rainfed conditions, in adjacent locations of the same field. Trials were designed as generalized randomized experiments with two replications and with each entry represented twice in each replication. Entries were grown in paired plots of the two treatment methods of each accession, so that the cryopreserved and standard entries of the same accession were always side- by-side. Each plot consisted of two rows 66 cm long and spaced 30 cm apart, in which a total of 22 seeds were manually planted approximately 3 cm deep and 13 cm apart. The winter trials were planted September 29 and October 1, 2014 and the spring trials were planted April 13, 2015. Huskie® brand herbicide (a.i. pyrasulfotole, bromoxynil octanoate and bromoxynil heptanoate) was applied in both winter and spring trials to control broadleaved weeds. For the winter rye trial, there

was no opportunity to impose differential moisture treatments because of heavy rainfall during April and May 2015. For the spring trial, a drip irrigation system was installed in the irrigated treatment. Irrigation was applied on June 25, July 1 and July 16, 2015. On each date, approximately 25 mm water was applied.

Phenotypic evaluation

Based on the International Board for Plant Genetic Resources (1985) crop descriptors, awn color, awn type, glume color, glume pubescence and straw color were the qualitative traits used to describe natural characters of rye plants under field conditions (Table 2.2). Those traits were uniform for each accession and therefore, were recorded for each plot rather than for individual plants. Plant height, anthesis date, heading date, spike weight, spike length and lodging were the quantitative traits evaluated (Table 2.3). We collected plant height, anthesis date and heading date data by recording trait values from five competitive plants in each plot. Plant height was measured from the base of the plant to the tip of the tallest spike, excluding awns. Heading date and anthesis date were recorded as the number of days from January 1 in winter trials and the number of days from planting dates for spring trials on which the first spike was completely emerged above the flag leaf and the first anthers were visible, respectively. For the spike traits, we collected three spikes on each of five plants for a total of 15 in each plot after plant maturity was reached in the winter trials. However, because of heterogeneity for phenology in the spring accessions, we randomly collected 5 to 15 mature spikes for each plot based on availability. After collecting spikes, we measured spike length (excluding awns) and weighed the whole group of spikes for each plot. This weight was divided by the spike number to obtain the average weight per spike per plot.

Data analysis

Data were analyzed with the software SAS University Edition (SAS Institute, Cary, NC) and Excel (Microsoft Corp., Redmond, WA). For field traits measured on an individual plant basis (anthesis date, plant height, spike length, and spike weight), the mean, variance, and range for each plot were calculated in Excel. To determine if there were differences due to storage treatment or accession, we conducted analysis of variance with the SAS "GLIMMIX" procedure, using plot mean data. According to the SAS/STAT 9.3 User's Guide (SAS Institute, 2011), "The GLIMMIX procedure fits and analyzes generalized linear mixed models (GLMM), models with random effects for data that can be non-normally distributed." Accessions were treated as a random variable in our model since the 40 accessions were randomly selected from a set of accessions whose seed was increased at the Botanical Garden of the Polish Academy of Sciences in Poland in 1988. Replication was also considered a random variable and storage treatment a fixed variable. In the spring rye trial, when comparing differences between two moisture levels, moisture level was treated as a fixed variable. Covariance parameter estimates were obtained to estimate the value of the random factors (accession and interaction between accession and storage treatment). A Type III F-test was used to determine the significance of the fixed effect (storage method). Then "estimate" statements were used to compare accessions and storage methods within accessions. The "Covtest" statement was used to determine if there was an interaction between storage method and accession based on a Chi-square test and best linear unbiased prediction (BLUP) were calculated.

Seedling experiment

Seedling experimental method

To investigate possible phenotypic differences at the seedling stage between the two preservation treatments, methods of the Association for Official Seed Analysts (AOSA, 2014) were followed. One hundred seeds of each entry (4 replications of 25 seeds each) were allowed to germinate for 7 days at 20 ^{O}C in a germination chamber. At the end of the 7-day germination period, normal germinated seed numbers and abnormal germinated seed numbers (as described in AOSA, 1994) were counted and germination percentages were calculated. After collecting germination data, three replications of eight seedlings each were selected for digital scanning from the normal germinated seedlings. Prior to scanning, roots were manually spread out in a thin layer of water. Using the 'root grayscale' scan settings on an EPSON scanner dialog box four seedlings at a time were scanned, but each seedling plant was analyzed separately. After scanning, roots were separated from shoots, placed in a coin envelope, and dried in a 53^{o}C oven for at least 4 days. The dried material was then weighed. Not all 40 accessions were used for the dry weight measurements. The number of accessions used for seedling weight was 14 for both storage treatments. The scanned images were analyzed with WinRhizo software (Regent Instruments Inc., 2015) for total root length, total projected root area, total surface root area, average root diameter and root volume.

Phenotypic evaluation

In addition to germination percentage, the following traits were evaluated for seedlings: shoot dry weight, root dry weight, total dry weight, root diameter, root length, root surface area, root projected area and root volume. The details of those traits are listed in Table 2.5. In order to collect dry weight for shoots and roots, total dry weights were first recorded and then

dry root tissue was carefully removed from the envelopes and shoot tissue was weighed. Other root traits were recorded or calculated by using WinRhizo software.

Seedling data analysis

Data was analyzed in SAS University Version, using a PROC MIXED statement. Accession was treated as a random variable while storage treatment was treated as a fixed variable. Replication was nested within accession. The Type III F-test was used to determine the significance of the fixed effect (storage treatments). Then the "lsmeans" statement was used to obtain the least squares means values and standard error of different traits for each storage treatment.

Results and discussion

In the winter rye field study, emergence was spotty but there was a minimum of 3 plants per plot and average plant number per plot was 12.5. Plants overwintered well and grew vigorously in spring. When rye first emerged from the ground, a few albino seedlings and seedlings with bright pink pigment bands on their leaves in both storage treatments were observed in the field; however most of these recovered to a normal appearance within two weeks. Because heavy rainfall prevented the imposition of two irrigation treatments, we combined data for both treatments into a single analysis with four replications.

In the spring rye set, germplasm used in our experiment showed a high level of variation, especially for anthesis date and heading date within each plot. Some accessions were possibly misclassified as spring type because flowering was extremely delayed in some members of this set, indicative of winter types that received insufficient vernalization time. In addition to collecting quantitative traits on individual plants, several qualitative traits were also recorded through the growing season on a plot basis (Appendix Table 2).

There are some minor differences between two storage treatments in the field for these qualitative traits, mainly because of variation within plots.

In the analysis of variance for quantitative field traits, only spike length showed a statistically significant difference (P=0.045) due to storage treatment in the winter rye set (Table 2.7). However, the value of the difference between the two storage treatments was minor (0.18 ± 0.087 cm), with a slightly longer mean spike length in the conventionally stored treatment. As the spike weight of the storage treatments did not differ significantly (P=0.35), from a practical perspective, the difference in spike length in the winter rye set is likely not functionally important. Even though the overall effect on spike length was significant, for individual winter accessions, no significant differences between storage treatments were found for that trait. The interaction of storage and accession was not significant for any traits.

In the spring rye set, no statistically significant difference was detected for any trait between storage treatments or for the storage treatments and accession interaction. When comparing differences between the two irrigation treatments, there were significant differences (P<0.05) for days to anthesis, plant height, and lodging (Table 2.6). Plants in the irrigated treatment had taller plants, more days to anthesis, and higher lodging rates than those in the rainfed treatment. The BLUPs were calculated for each accession in the field experiment and are listed in Appendix Tables 2 and 3.

After germinating 100 seeds per accession from each storage treatment, cryopreserved seeds had 91% normal germination, while seeds from the standard storage method had 88% normal germination, a difference that was significant at P<0.0001 (Table 2.9). In addition to normal germination percentage, adjusted germination percentage also showed higher

germination in cryopreserved seeds at 98.8%, while germination percentage for conventional method is 95.7% (Appendix Table 8). The abnormal germination percentage also differed between the two storage treatments: 4.4% for cryopreserved seeds and 5.8% for conventionally stored seeds (P=0.0014). Mean values were calculated for each accession by different storage treatments (Appendix Table 6). Although the difference between two storage treatments is small, the normal and abnormal germination percentages are irreversible traits and may accumulate over time. Therefore, our germination results indicated that cryopreservation performs better than the conventional storage method for cereal seed, with higher normal germination percentage and lower abnormal germination percentage after 25 years.

Analysis of variance for the WinRhizo seedling data (Table 2.8) revealed that average root diameter differed significantly between the two storage treatments in both spring habit rye (P=0.05) and the winter habit rye set (P=0.007). Seedlings from cryopreserved seeds showed higher average root diameter (0.36 ± 0.0032 mm) in the spring rye set, but lower average root diameter in the winter rye set (0.36 ± 0.0035 mm) than seedlings from conventionally stored seeds (0.35 ± 0.0033 mm; 0.37 ± 0.0035 mm in the spring and winter sets, respectively). In addition, the difference in root length in winter rye due to storage method was significant (P=0.019). However, the difference was small (79.52 ± 1.77cm for cryopreservation and 76 ± 1.77cm for conventional method). Least squares mean values were calculated for each accession by different storage methods (Appendix Table 5).

Galdiano et al. (2013) compared seedling characters developed from the mature seeds of the orchid *Oncidium flexuosum* that were either cryopreserved or non-treated. Their research showed no difference for root length or for several other characters between the two

treatments. However, Cejas et al. (2013) found the root phenolic content of *Phaseolus vulgaris* seedlings was reduced significantly after seeds were immersed in liquid nitrogen for two weeks. Given these varied results, one cannot simply conclude that cryopreservation results in more or fewer changes than conventional storage method. Testing before using this method is necessary for each species.

Previous research also tested seed germination percentage after immersion in liquid nitrogen. Merritt et al. (2014) reported that some orchid species were found with higher germination rates after ultra-low temperature (i.e., -70 ^{O}C to -196 ^{O}C) storage than those stored at higher temperature (i.e., -20 ^{O}C, 4 ^{O}C) over time periods from 12 months to 5 years. Zevallos et al. (2013) also found significantly higher germination rates after five days with liquid nitrogen exposure compared to room temperature for wild *Solanum lycopersicum* seeds. Coelho et al. (2012) found no significant differences or lower germination percentage for 30-day cryopreserved *Thymus lotocephalus* seeds compared to seeds without liquid nitrogen immersion. They also mentioned other research studies showing no negative effects in germination for cryopreserved seed of several wild species, including *Drosophyllum lusitanicum, Tuberaria macrosepala*, *Halimium,* and *Helianthemum* species (Gonçalves and Romano, 2009; Zaidi et al, 2010; Pérez-García and González-Benito, 2008). Cejas et al. (2012) also found no significant germination difference between cryopreserved seeds and control seeds for *Phaseolus vulgaris*. Based on the above-mentioned studies, the effect of cryopreservation apparently depends on the species and the cryopreservation methods used. However, all of those studies evaluated relatively short-term cryopreservation (two weeks to five years). To our knowledge no other research has compared cryopreservation with the conventional storage method over a period as long as the 25 years in this study.

Conclusion

Our study demonstrated that cryopreservation for long-term storage of rye seeds has generally favorable results. Cryopreserved seeds had better normal germination, less abnormal germination, and no significant phenotypic differences compared to conventional storage for most traits in both field and seedling experiments. When phenotypic differences between the two storage treatments for field-grown plants were significant (e.g., spike length in winter rye), they were very small and likely did not represent functional differences. The mechanism responsible for this difference is unknown and would need to be investigated in further studies. Previous studies based on other species have shown different results for the effects of cryopreservation. Therefore, before cryopreservation is routinely employed for a given species, testing of that material is advisable.

Tables

Table 2.1 Accessions of spring rye germplasm used in this study[a]

Accession	Inventory #	Accession Name	Country	Type
PI 239578	105132	I-22.144	Brazil	Breedin g
PI 240675	105138	Centeno de La	Uruguay	Unknown
PI 241283	105144	42	Brazil	Unknown
PI 241292	105153	51	Brazil	Unknown
PI 241578	105156	Centeno 54	Brazil	Cultivar
PI 272333	105228	Fleischmann	Hungary	Cultivar
PI 323356	105324	Florida Black	Spain	Unknown
PI 323358	105326	Elbon	United States	Cultivar
PI 323383	105347	Synthetic II	Spain	Unknown
PI 362395	105469	82	Serbia	Landrace
PI 378233	105524	8/72	Macedonia	Landrace
PI 392064	105573	Carojurz	Germany	Unknown
PI 415371	105643	V/78	Macedonia	Landrace
PI 415375	105646	V/82	Macedonia	Landrace
PI 415376	105647	V/83	Macedonia	Landrace
PI 415378	105649	V/85	Macedonia	Landrace
PI 415385	105656	V/92	Macedonia	Landrace
PI 415386	105657	V/93	Macedonia	Landrace
PI 415401	232556	V/108	Macedonia	Landrace
PI 420545	105698	V/140	Macedonia	Landrace

[a] Information obtained from GRIN (http://www.ars-grin.gov/)

Table 2.2 Accessions of winter rye germplasm used in this study[a]

Accession	Inventory #	Accession Name	Country	Type
PI 254808	105181	Chryzanth Hanserrogge	Austria	Unknown
PI 254809	105182	Edelhofer	Austria	Unknown
PI 254811	105184	Karntner	Austria	Cultivar
PI 254812	105185	Kefermarkter	Austria	Unknown
PI 254816	105189	Oberkarntner	Austria	Unknown
PI 254821	105194	Schlagler	Austria	Cultivar
PI 256026	105198	Line 28	Spain	Unknown
PI 265473	105212	Visa	Finland	Unknown
PI 272336	105231	Kiszvardaj	Hungary	Unknown
PI 280836	105240	Omka	Russian Federatio	Cultivar
PI 280837	105241	Volzanka	Russian Federatio	Cultivar
PI 283974	105248	CPI 24368	Poland	Unknown
PI 289814	105253	153	Iran	Landrace
PI 290419	105256	Lovaszpatonai	Hungary	Cultivar
PI 290428	105265	Zenit	Czechoslovakia	Cultivar
PI 290436	105270	Zeelandskie	Hungary	Unknown
PI 290454	105287	Stupicke S II	Czechoslovakia	Cultivar
PI 290455	105288	Bernburger Tetraroggen	Hungary	Unknown
PI 323362	105330	Elbon	United States	Cultivar
PI 362400	105473	87	Serbia	Landrace

[a] Information obtained from GRIN (http://www.ars-grin.gov/)

Table 2.3 Qualitative traits evaluated in the field experiment[b] at ARDEC, 2014-2015

Awn color, Glume color: plot awn/ glume colors at maturity	
Code	Definition
1	Black
1A	Black and white
1B	Black and brown
2	Blue
3	Brown
3A	Brown and white
4	Grey
5	Purple
6	Red
7	Tan
8	White/amber
9	Yellow
Awn type: Type and extent of the awns	
Code	Definition
1	Awned
3	Awnletted
5	Apicallyawnletted
9	Awnless
Glume pubescence: Type and extent of glume pubescence	
Code	Definition
1	Absent
3	Edge only
5	Short (fine)
9	Long, readily visible
Straw color: Color of straw at maturity.	
Cod	Definition
1	Black
1A	Black and white
1B	Black and brown
2	Blue
3	Brown
3A	Brown and white
4	Grey
5	Purple
6	Red
7	Tan
8	White/amber

9	Yellow
Mix	Row mixed for color

[b] Information obtained from International Board for Plant Genetic Resources (1985)

Table 2.4 Quantitative traits evaluated in the field experiment[a] at ARDEC, 2014- 2015

Traits	**Definition**
Days to headin	Number of days from planting (spring rye) or Jan. 1 (winter rye) to the date when the first head (spike) of a plant emerges completely above the flag leaf
Days to	Number of days from planting (spring rye) or Jan. 1 (winter rye) to the date the appearance of anthers
Spike lengt	Average length of spikes from 5 plants (cm)
Spike weigh	Average weight per spike at maturity (g)
Lodging	Rated on a 1 to 9 scale where 1 = No lodging, 9 = All plants flat
Plant height	Average height of plants at maturity, from the ground to top of the spike, excluding awns (cm)

[a] Information obtained from International Board for Plant Genetic Resources (1985)

Table 2.5 Traits evaluated in the seedling experiment. The first five traits were obtained from WinRhizo[a] analysis of scanned images.

Abbreviations	**Descriptions**
Length	Total root length (cm)
ProArea	Total projected root area (cm^2)
SurfArea	Total root surface area (cm^2)
AvgDiam	Average root diameter (mm)
Root volume	Total root volume (cm^3)
Normal germination	Percentage of normally germinated seeds after seven days, expressed as a percent (%)
Abnormal germination	Percentage of abnormally germinated seeds after seven days, expressed as a percent (%)
Dry weight	Total weight of shoot and root after drying (g)
Shoot dry weight	Weight of shoot after drying (g)
Root dry weight	Weight of root after drying (g)

[a] Regent Instruments Inc., Canada

Table 2.6 Mean values ±SE of quantitative field traits for spring irrigated and rainfed trials evaluated in Fort Collins, CO in 2014-2015

Trait	Irrigated	Rainfed	Differences[a]	*p*b
Days to anthesis	76.17±0.77	78.36±0.78	-2.19±0.57	0.0002
Plant height (cm)	111.56±2.30	104.62±2.29	6.90±1.43	<0.0001
Spike length (cm)	12.05±0.17	12.16±0.17	-0.11±0.14	0.44
Spike weight (g)	1.16±0.05	1.13±0.05	0.03±0.04	0.49
Lodging	2.29±0.14	1.97±0.14	0.32±0.12	0.01

[a] Mean of irrigated treatment – mean of rainfed treatment

[b] Significance of difference between two irrigation treatments

Table 2.7 Mean values ±SE of quantitative field traits for cryopreserved and conventionally stored (-18 ^{O}C) rye seeds evaluated in Fort Collins, CO in 2014-2015

Trait	Trial	Cryopreser-vation	-18 ^{O}C	Difference[a]	*P*[b]
Days to anthesis	Winter	153.27±0.55	153.39±0.55	-0.13±0.14	0.37
	Spring irrigated	76.03±0.88	76.06±0.88	-0.027±0.45	0.95
	Spring rainfed	78.93±1.13	78.38±1.11	0.55±0.87	0.53
Plant height (cm)	Winter	172.22±0.54	172.73±0.54	-0.51±0.25	0.43
	Spring irrigated	111.00±3.13	112.35±3.16	-1.35±1.94	0.49
	Spring rainfed	104.96±2.20	103.85±2.20	1.11±1.42	0.44
Spike length (cm)	Winter	12.27±0.26	12.44±0.26	-0.18±0.087	0.045
	Spring irrigated	11.91±0.21	12.11±0.21	-0.20±0.19	0.29
	Spring rainfed	12.22±0.24	12.17±0.24	0.050±0.16	0.76
Spike weight (g)	Winter	1.76±0.058	1.78±0.058	-0.029±0.031	0.35
	Spring irrigated	1.14±0.060	1.19±0.060	-0.052±0.052	0.32
	Spring rainfed	1.15±0.061	1.13±0.061	0.016±0.054	0.78
Lodging	Winter	4.67±0.52	4.81±0.52	-0.14±0.10	0.18
	Spring irrigated	2.37±0.26	2.21±0.26	0.16±0.19	0.41
	Spring rainfed	2.07±0.17	1.91±0.17	0.15±0.13	0.25

[a] Mean of cryopreservation – mean of -18 ^{O}C

[b] Significance of difference between two storage methods

Table 2.8 Mean values ±SE of seedling root traits for cryopreserved and conventionally stored (-18 ºC) rye seeds evaluated in Fort Collins, CO in 2014-2015

Traits	Trials	Cryopreservation	-18 ºC	Difference[a]	P[b]
Root length (cm)	Spring	83.31±1.48	86.09±1.56	-2.77±1.82	0.13
	Winter	79.52±1.77	76±1.77	3.64±1.51	0.019
Project area (cm^2)	Spring	3.00±0.049	3.02±0.052	-0.038±0.063	0.55
	Winter	2.83±0.047	2.783±0.047	0.043±0.058	0.46
Surface area (cm^2)	Spring	9.38±0.15	9.5±0.16	-0.12±0.20	0.55
	Winter	8.88±0.20	8.74±0.20	0.14±0.18	0.46
Average diameter (mm)	Spring	0.36±0.0032	0.35±0.0033	0.006±0.0030	0.05
	Winter	0.36±0.0035	0.37±0.0035	-0.011±0.0040	0.007
Root volume (cm^3)	Spring	0.085±0.0016	0.085±0.0017	0.00059±0.0020	0.77
	Winter	0.08±0.0020	0.081±0.0020	-0.00092±0.0020	0.64

[a] Mean of cryopreservation – mean of -18 ^{O}C

[b] Significance of difference between two storage methods

Table 2.9 Mean values of germination percentage (n=40) and dry weight for cryopreserved and conventionally stored (-18 ^{o}C) rye seeds evaluated in Fort Collins, CO in 2014-2015

Traits	Cryopreserv-ation	-18 ^{o}C	Difference[a]	*P*[b]
Normal germination (%)	91.00±0.63	88.00±0.63	3.10±0.71	<0.0001
Abnormal germination (%)	4.40±0.39	5.80±0.39	-1.40±0.44	0.0014
Seedling dry weight (mg)	10.23±0.21	10.60±0.22	-0.38±0.28	0.19
Shoot dry weight (mg)	6.82±0.17	7.13±0.17	-0.31±0.19	0.11
Root dry weight (mg)	3.34±0.11	3.34±0.11	-0.03±0.15	0.85

[a] Mean of cryopreservation – mean of -18 ^{O}C

[b] significance of difference between two storage methods

Figures

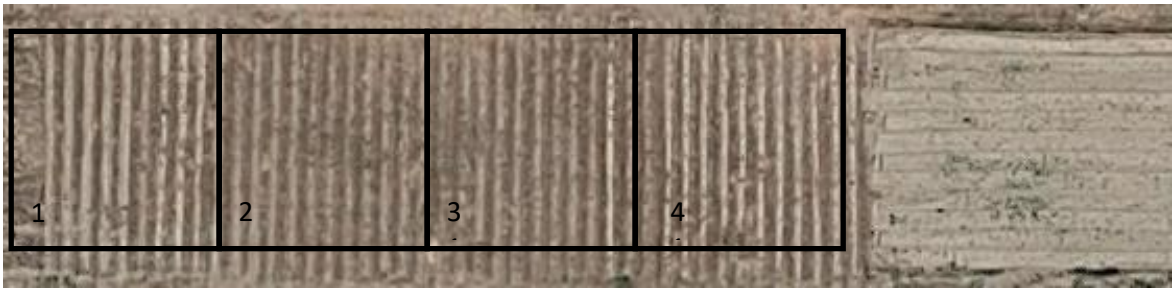


Figure 2.1 Layout of the field experiment in ARDEC. The square areas labeled 1 and 2 are planted with winter rye and those labeled 3 and 4 are planted with spring rye. Area 1 and 3 were intended for irrigation and areas 2 and 4 were designated to be rainfed. (Figure is taken from Google maps on June 15th 2015)

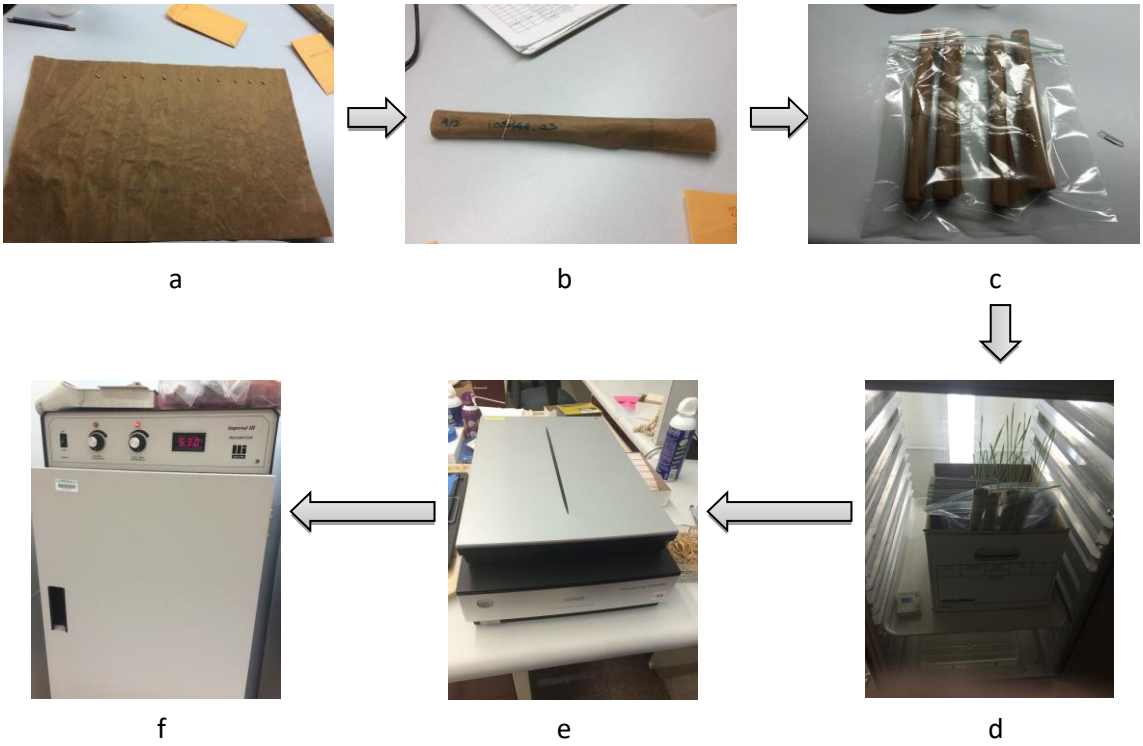


Figure 2.2 Method for seedling experiment at NLGRP. a) Set seeds on germination paper. b) Roll germination paper up tightly. c) Place rolls in Ziploc bags. d) 7-day period germination. e) Scan the seedlings and analyze the pictures. f) Put roots and shoots in oven

Chapter 3

DNA methylation differences between cryopreserved and conventionally stored seeds of rye (*Secale cereale* L.)

Summary

Genetic stability is a concern for long-term cryopreservation of plant germplasm. Methylation changes in plant DNA are known to occur under conditions of environmental stress. Previous research has shown changes in methylation status after long-term cryopreservation of shoot tips, but to my knowledge there is no evidence of methylation changes for cryopreserved orthodox seeds. Therefore, the main objective of this study was to compare DNA methylation patterns in cryopreserved and conventionally stored seeds of rye (*Secale cereale* L.). We used a methylation sensitive amplified fragment length polymorphism (metAFLP) method to detect methylation patterns for one spring rye accession (V/108) and one winter rye accession (Omka). Six to 10 individual plants were evaluated for each accession under each storage condition. A total of 311 scorable bands were observed for the two accessions. Multiple Fisher's exact tests were used to compare methylation patterns between the two storage treatments. Fisher's exact tests were made on a locus-by-locus basis to test the association of frequencies of methylation status across individuals between storage treatments for each accession. Benjamini and Hochberg false discovery rate (FDR) adjusted *P*-values were used to control for multiple testing. Methylation status between storage treatments was significantly different at $P = 0.05$ (unadjusted) for 5 of 311 loci in the spring accession V/108 and for 3 of 308 loci in the winter accession Omka. However, there were no significant differences for any loci in either

accession after FDR adjustment. Therefore, both cryopreservation and conventional storage methods are appropriate methods for long-term storage of rye seeds based on this epigenetic study.

Introduction

Cryopreservation (storage in liquid nitrogen vapor phase) is considered an effective method for long-term storage of plant germplasm. Cryopreservation can be advantageous for vegetatively propagated plants and recalcitrant seeds to avoid frequent regeneration or re-culturing (Takagi and Engelmann, 2000). This method is also believed to extend the viability period for orthodox seeds (Walters, 2014). However, genetic stability of cryopreserved samples has been a concern with this method (Reed, 2004).

Plants facing environmental stress cannot move from place to place like animals to escape stress, but have to remain in the same environment their whole lives. Therefore, plants have a high probability of being exposed to some degree of environmental stress. New gene combinations and mutations are mechanisms for plants to develop phenotypic variation to deal with stressful environments; however, the rate of environmental change may be faster than the rate at which new gene combinations or mutations arise. Epigenetic changes, including those related to DNA methylation or demethylation, provide additional pathways for phenotypic variation in response to stress (Grativol et al., 2011). Typically plants have a 6 to 30% methylation rate across the whole genome (Zhang et al., 2010). Unlike animals, asymmetric cytosine methylation (CHH with H signifying A, T and C) and CHG methylation occur in plants (Zhang et al., 2010). In addition, CG methylation occurs both in plants and animals.

Previous studies have examined methylation changes after treating different plant tissues with liquid nitrogen. Maki et al. (2015) studied tissue cultured shoot tips of wasabi (*Wasabia japonica*), and detected a 5.5% DNA methylation change (both hyper- and hypo-methylated) after 10 years storage in liquid nitrogen at -150℃ compared to tissue cultures stored at 20±1 ℃ for the same time period. In a long-term study of potato shoot tips by Kaczmarczyk et al. (2011), 93.7% of examined DNA sequence showed stable methylation patterns in both *in vitro* at 22 ℃ and cryopreserved status; 3.4% showed loss of methylation in particular cryopreserved samples due to stochastic events; 0.2% showed consistent hypermethylation; and 0.6% showed consistent hypomethylation. *In vitro* tissue cultures of shoot tissues of *Ribes* (Johnson et al., 2009), *Theobroma cacao* (Adu-Gyamfi et al., 2016), *Arabidopsis thaliana* (Wang and He, 2009), *Citrus* (Hao et al., 2002), strawberry (genus *Fragaria*) (Hao et al., 2002), and *Prunus dulcis* (Channuntapipat et al., 2003) all showed significant changes in methylation status during cryopreservation. However, to my knowledge, there is no published research on epigenetic changes in seeds during long-term storage.

Several methods have been used to evaluate DNA methylation, including methylation sensitive amplified fragment length polymorphism (metAFLP). The AFLP technique is based on selectively amplifying restriction fragments from enzyme digested genomic DNA (Vos et al., 1995). There are several advantages of AFLP technology. First, the AFLP technique does not require prior sequence information, which means the start-up cost is relatively low. Secondly, this method is suitable for automation and is highly multiplexed, which allows development of a high-density set of markers for those organisms lacking in genomic sequence data. However, AFLP also has some limitations. One shortcoming is that it is hard for AFLP to

detect differences between two populations with relatively low homogeneity (less than 90%), because common bands are few. Conversely, detection of polymorphic markers in genomic DNA by AFLP may be difficult with too little sequence variation between samples (Vuylsteke et al., 2007). Nevertheless, compared with other methods of detecting methylation patterns, AFLP is still useful for this type of research. MetAFLP is an AFLP method based on isoschizomers, which have different sensitivity to cytosine-methylated sites. In the study by Machezynska et al. (2014), *KpnI/Acc65I* isoschizomers were used. This set of isoschizomers recognizes the same sites. *Acc65I* is sensitive to CpG methylation so that its activity is blocked when the site is methylated, while *KpnI* is insensitive to all types of methylation. The metAFLP method has been used on *Poa annua* (Chwedorzewska and Bednarek, 2012), barley (*Hordeum vulgare*) (Bednarek et al., 2007), *Gentiana pannonica* (Fiuk et al., 2010), *Gentiana cruciata* (Mikula et al., 2011), *Armeria maritime* (Abratowska et al., 2012), *Deschampsia antarctica* (Chwedorzewska and Bednarek, 2012), triticale (×*Triticosecale*) (Machczynska et al., 2014), and bamboo (*Phyllostachys praecox*) (Lu et al., 2012).

Knowledge of the effects of cryopreservation on DNA methylation status of seeds would be useful for germplasm bank managers in planning their conservation strategies. Therefore, the objective of this project was to compare DNA methylation patterns of two accessions of rye seeds stored for 25 years with cryopreservation and conventional methods.

Materials and methods

Plant materials and DNA extraction

We selected two accessions, one winter habit rye (Omka, PI 280836) and one spring habit rye (V/108, PI 415401), from the 40 rye accessions used in the field trial described in Chapter 2. Each accession had been stored for 25 years using both cryopreservation (-135°C

to -180 ^{O}C) and conventional storage (-18 °C). Because of the phenotypic variation (such as for vernalization requirement) in some accessions, the selection of the accessions used in this methylation experiment was based on uniformity of germination and field data collected in 2015. By selecting more homogeneous accessions we hoped to focus on the differences between the two storage treatments, rather than within-accession variation. Seeds from both storage treatments of the selected accessions were germinated in a greenhouse. Approximately 100 mg of young leaf tissue from 6 to 10 individuals for each storage treatment and accession was collected separately into 2 mL centrifuge tubes, freeze-dried, and ground to a fine powder using a Mini-Beadbeater shaking mill (Biospec Products, Bartlesville, OK, USA). DNA was extracted using DNeasy plant mini kits (Cat. No.69104, Qiagen, Germany), according to the manufacturer's instructions. DNA was quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

metAFLP

We used the metAFLP method (Bednarek et al., 2007) to compare methylation differences between plants grown from cryopreserved and conventionally stored rye seeds of each of the two selected accessions. The basic AFLP protocol described by Vos et al. (1995) was adapted for the metAFLP procedure. The isoschizomers *KpnI* and *Acc65I* (restriction enzymes that recognize the same DNA sequence but differ in methylation sensitivity) each in combination with *MseI* (*KpnI/MseI* or *Acc65I/MseI*) (New England Biolabs, MA, USA), were used for separate double digests of the individual DNA samples. Restriction digests used 500 ng genomic DNA with 5U of each enzyme for 5 hours at 37° C in 400 µL reaction volumes. Samples of restriction digests were analyzed on 1% agarose gels to check for complete digestion. Adaptors (Table 3.1) were ligated in subsequent reactions that included

the adaptors, T4 ligase, and T4 ligase buffer (New England Biolabs, MA, USA) added to the restriction digests. The ligation reactions were incubated at 16 °C for 12 hours. Restriction/ligation reactions were diluted 10-fold and 2 µL aliquots were used as templates for pre-selective polymerase chain reaction (PCR) amplifications with the primers listed in Table 3.1 (Integrated DNA Technologies, Coralville, IA). The PCR using diluted pre-selective amplification templates with 15 selective primers sets (Table 3.2) (Integrated DNA Technologies) generated the AFLP fragments for analysis. PCR products from the selective amplifications were mixed 1:1 with gel loading solution (98% formamide and 10 mM EDTA with trace amounts of bromphenol blue and xylene cyanol FF as tracking dyes), denatured in a thermal cycler at 95 °C, and snap-cooled on ice. Samples were electrophoresed in 38 cm x 50 cm x 0.40 mm denaturing polyacrylamide gels containing 5% acrylamide monomer with a 19:1 ratio of acrylamide:bis- acrylamide, 7.5 M urea, and 1x TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) in a Sequi- Gen GT electrophoresis cell (Bio-Rad Laboratories, Hercules, CA, USA). Gels were run at 80 watts constant power for 2.5 hours. Samples were arranged on the gels so amplifications with the same primers from both *KpnI/MseI* and *Acc65I/MseI* libraries of the same individual appeared on the same gel to facilitate accurate scoring. After electrophoresis, gels were silver-stained following the method of Bassam et al. (1991).

Data analysis

Since the material we used consisted of outcrossing orthodox seeds and every seed is potentially different, comparing a single seed from each storage treatment would not adequately represent the accession. Therefore, in this experiment, individual DNA samples from multiple seedlings of each accession in each storage treatment were used.

Data were analyzed with the software SAS University Edition (SAS Institute, Cary, NC), Excel (Microsoft Corp., Redmond, WA) and JMP (SAS Institute, Cary, NC). For each enzyme-primer combination used on each sample, presence or absence of bands on the gels were recorded. After examining results for a given sample with both enzymes, banding patterns were assigned to four categories (A, B, C, D) (Table 3.3). 'A' represents a sample lacking bands with both *KpnI* and *Acc65I*. 'B' represents a sample lacking a band with *KpnI* but with a band present with *Acc65I*. 'C' is for samples that produced a band with *KpnI*, but not with *Acc65I*. 'D' was assigned to samples producing bands with both enzymes. After categorizing the patterns, we eliminated categories A and B in order to simplify the analysis, because A and B categories do not represent methylation status at the loci as explained later in results and discussion. We conducted multiple Fisher's exact tests in "PROC MULTTEST" of SAS using the Benjamini-Hochberg false discovery rate (FDR) (Benjamini and Hochberg, 1995) to determine the significance of differences between DNA from the two storage treatments.

Results and discussion

In this metAFLP experiment, 311 total scorable bands were detected in both accessions, for an average of 20.7 bands per primer set. Appendix Figure 9 is an example of a silver-stained gel for V/108 with both storage treatments. The frequencies of the four categories of banding patterns in our experiment are shown in Fig. 3.1. Categories A and B combined comprised 7.3% of the total bands detected; 78.3% of the bands were in category D, indicating that the sites were not methylated; and 14.4% were classified in category C, which indicates methylated sites. Plants within an accession were not always uniform for a particular banding pattern category, which is consistent with our understanding of the

variability of these outcrossing accessions. The A category might have arisen from mutation at an enzyme restriction site or as an artifact of PCR amplification. For the B category, when two *KpnI/Acc65I* sites are adjacent to each other with one site methylated and one site not, *KpnI* bands are absent while *Acc65I* bands are present. In other words, *KpnI/MseI* digestion formed a short *KpnI/MseI* fragment and a *KpnI/KpnI* fragment while *Acc65I/MseI* only formed a long unmethylated fragment (Bednarek et al., 2007). Another point is that in our experiment, we did not use ^{32}P labeled selective primers for the *KpnI* and *Acc65I* restriction fragments. Therefore, unlike Bednarek et al. (2007) who were able to avoid scoring *MseI/MseI* bands, all the DNA bands in our study were visualized after silver staining and therefore, *MseI/MseI* bands could not be eliminated.

Multiple Fisher's Exact test results, shown in Figures 3.2 and 3.3, were based on 311 bands for V/108 and 308 bands for Omka between both storage treatments after eliminating A and B categories. Based on the raw *P*-value, five sites showed significant differences ($P<0.05$) between the two storage treatments in V/108 and three sites were significant in Omka. After adjustment with the Benjamini-Hochberg FDR, no significant differences were detected between the two storage treatments ($P=1$). However the sample size in this study was relatively small, with a maximum of 10 seeds and as few as 6 seeds from each accession in each storage treatment. Furthermore, there was a relatively high level of variation based on metAFLP bands within each accession, which may have influenced the results. In addition, the number of bands evaluated was limited relative to the large genome size of rye (8.1 Gb) (Martis et al., 2013).

No previous studies have reported results of methylation status for long-term storage of orthodox seeds, while there have been several studies of long-term cryopreserved shoot

tips. Unlike previous shoot tip or cell culture results, no statistical methylation differences were detected in our study of orthodox seeds by Fisher's exact tests on an individual locus basis. In order to confirm these results, different methylation detection method, more plants per accession, and a larger number of accessions may be needed, as individual accessions may vary in their response to liquid nitrogen. Because rye is closely related to wheat and their seed morphology is similar, our results may also apply to long-term conservation of wheat and its wild relatives.

Conclusion

Based on results of this study, there is little evidence of differences in DNA methylation status between the two methods for long-term storage of rye seeds. Therefore, we conclude that cryopreservation and conventional storage methods both protect the genetic integrity of the accessions to the same degree. These results suggest there is no reason to choose one method over the other based on this epigenetic study.

Tables

Table 3.1 Pre-amplification primers (Pre) and adaptors (Adptr) used in this study

Names	**Sequences**
PreMseI PreAcc65I-Kpn1	5'-GAT GAG TCC TGA GTA AC-3' 5'-GCA TGC GTA CAG TAC C-3'
Adptr1-Kpn1 Adptr2-Kpn1	5'-CTC GTAGCA TGC GTA CAG TAC-3' 3'-CAT CGT ACG CAT GT-5'
Adptr1-Mse Adptr2-Mse	5'-TA CTC AGG ACT C ATC-3 3'-GAC GAT GAG TCC TGA G-5'
Adptr1-Acc65I Adptr2-Acc65I	5'-CTC GTA GCA TGC GTA CA-3' 3'-CAT CGT ACG CAT GTC ATG-5'

Table 3.2 Selective primer sets used in this study

Primers	Sequences (5' to 3')
MCAA CpG-ACG	GAT G AG TCC TG A GTA AC A A C AT GCG TAC AGT
MCAA CpG-GCA	GAT G AG TCC TG A GTA AC A A C AT GCG TAC AGT
MCAG CpG-GAC	GAT G AG TCC TG A GTA AC A G C AT GCG TAC AGT
MCAG CpG-GCA	GAT G AG TCC TG A GTA AC A G C AT GCG TAC AGT
MCAG CpXpG-AGC	GAT G AG TCC TG A GTA AC A G ACT
MCAG CpXpG-TGC	GAT G AG TCC TG A GTA AC A G C AT GCG TAC AGT
MCAT CpXpG-ATG	GAT G AG TCC TG A GTA AC A T GAT GCG TAC AGT
MCAT CpXpG-AGG	GAT G AG TCC TG A GTA AC A T C AT GCG TAC AGT
MCT CpG-GGC	GAT G AG TCC TG A GTA ACT
MCT CpXpG-(A/T)GG	GAT G AG TCC TG A GTA ACT
MCT CpXpG-AGA	GAT G AG TCC TG A GTA ACT
MCT CpXpG-ATT	GAT G AG TCC TG A GTA ACT
MCT CpXpG-TTG	GAT G AG TCC TG A GTA ACT
MCT CpXpX-TAA	GAT GAG TCC TG A GTA ACT
MCTG CpG-TCG	GAT G AG TCC TG A GTA ACT G C AT GCG TAC AGT

Table 3.3 Interpretation of the four banding pattern categories used in the experiment ("-" represents absent bands, "+" represents present bands on AFLP gel)

Categories	***KpnI***	***Acc65I***	**Status**
A	-	-	Variation or mutation within accessions
B	-	+	Two adjacent acc65I/KpnI sites. One is methylated and another one is not.
C	+	-	Methylated sites
D	+	+	Unmethylated sites

Figures

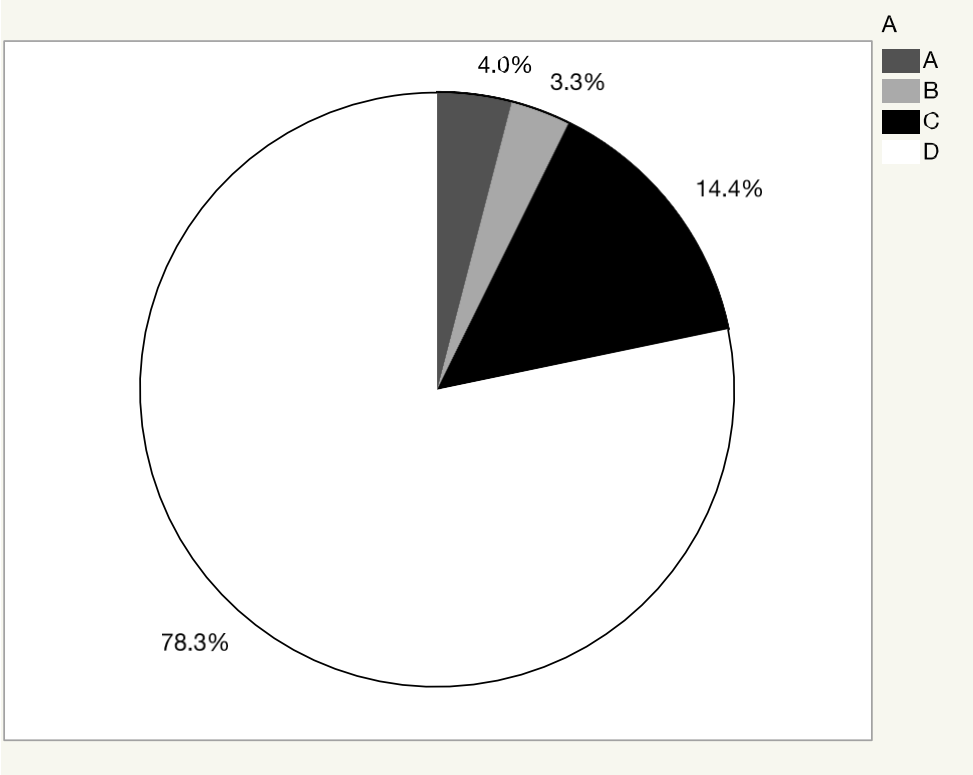


Figure 3.1 Frequency of different categories of methylation patterns combined over all bands. The meaning of the categories is described in Table 3.3.

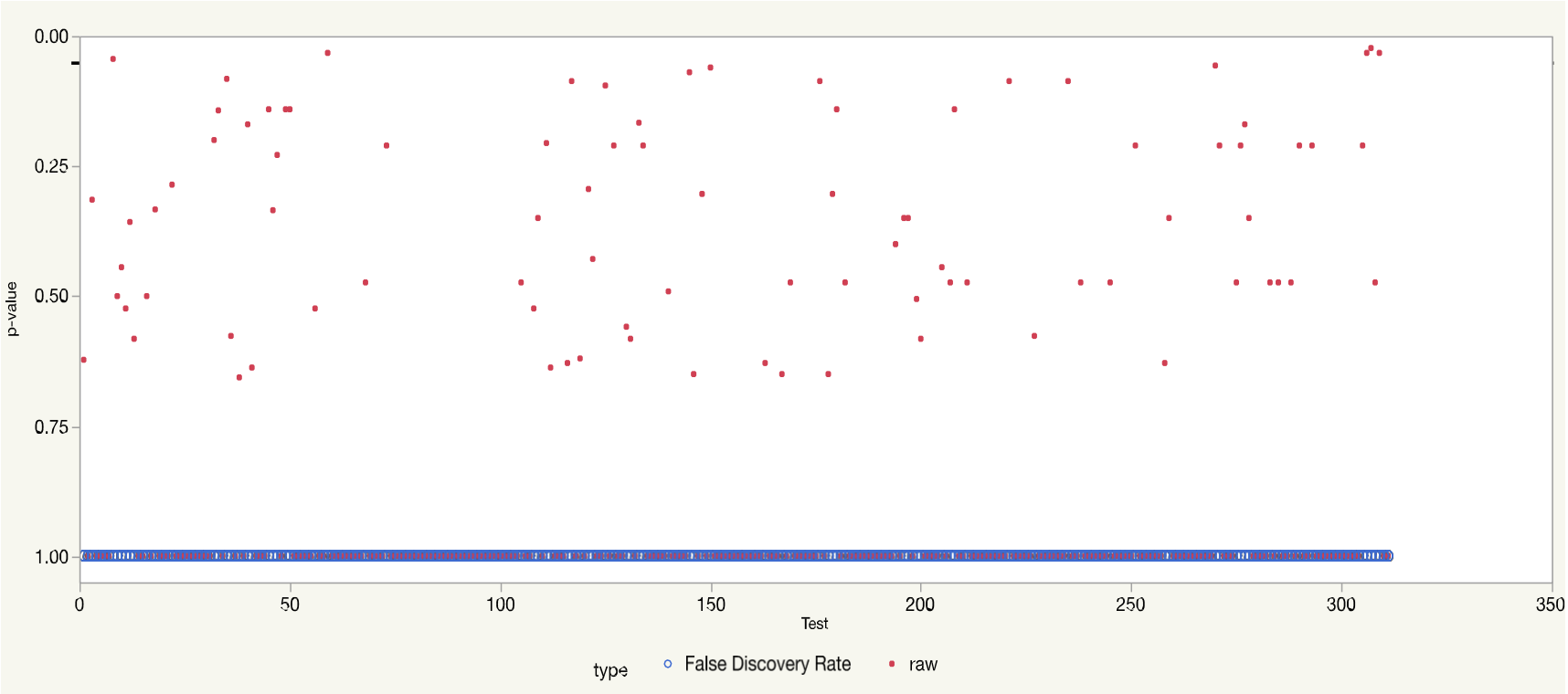


Figure 3.2 Raw and adjusted *P*-values for the comparison of 311 bands between two storage treatments for spring rye accession V/108.

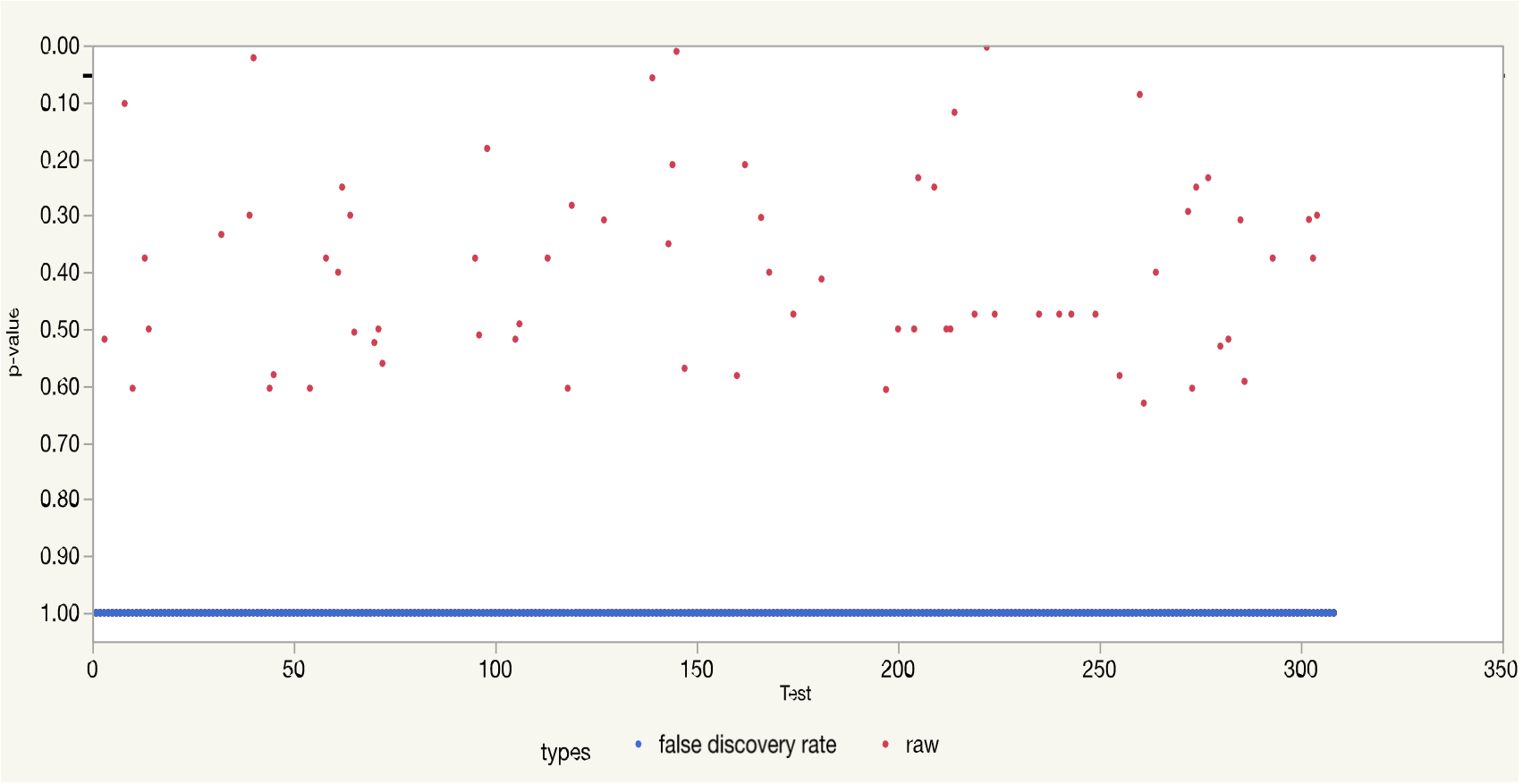


Figure 3.3 Raw and adjusted *P*-values for the comparison of 308 bands between two storage treatments for winter rye accession Omka

References

Abratowska, A., Wąsowicz, P., Bednarek, P. T., Telka, J., & Wierzbicka, M. (2012).Morphological and genetic distinctiveness of metallicolous and non-metallicolous populations of *Armeria maritima* s.l. (Plumbaginaceae) in Poland. *Plant Biology (Stuttgart, Germany), 14*(4), 586-595. doi:10.1111/j.1438-8677.2011.00536.x

Alonso, C., Perez, R., Bazaga, P., Medrano, M., & Herrera, C. M. (2016). MSAP markers and global cytosine methylation in plants: a literature survey and comparative analysis for a wild-growing species. *Molecular Ecology Resources, 16*(1), 80-90. doi:10.1111/1755-0998.12426

Association for Official Seed Analysts (AOSA). (2014). Rules for Testing Seeds Volume 1. Principles and Procedures. AOSA, Washington DC, USA

Association for Official Seed Analysts (AOSA). (1994). Rules for Testing Seeds Volume 4. Seedling evaluation. AOSA, Washington DC, USA

Adu-Gyamfi, R., Wetten, A., & Marcelino Rodríguez López, C. (2016). Effect of Cryopreservation and Post-Cryopreservation Somatic Embryogenesis on the Epigenetic Fidelity of Cocoa (*Theobroma cacao* L.). *Plos One, 11*(7), e0158857. doi:10.1371/journal.pone.0158857

Bassam, B. J., Caetano-Anollés, G., & Gresshoff, P. M. (1991). Fast and sensitive silver staining of DNA in polyacrylamide gels. *Analytical Biochemistry*, *196*(1), 80-83.

Bednarek, P. T., Orłowska, R., Koebner, R. D., & Zimny, J. (2007). Quantification of the tissue-culture induced variation in barley (*Hordeum vulgare* L.). *BMC Plant Biology*, *7*10.

Berzonsky, W. A., & Francki, M. G. (1999). Biochemical, molecular, and cytogenetic technologies for characterizing 1RS in wheat: A review. *Euphytica*, *108*(1), 1-19.

Bretting, P.K., Stoner, A.K., Widrlechner, M.P., & Williams, K.A. (2011) Country Report on the State of Genetic Resources for Food and Agriculture. United State of America. Retrieved from http://www.fao.org/filead mi n/templates/agphome/documents/PGR/SoW2/country_reports/a mericas/US.pdf

Benjamini, Y. and Hochberg, Y. (1995). Controlling the false discovery rate:a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society, Series B 57, 289-300.

Chan, S.W.L., Henderson, I.R., & Jacobsen, S.E. (2005). Gardening the genome: DNA methylation in *Arabidopsis thaliana*. *Nat. Rev. Genet*. 6: 351-360.

Channuntapipat, C., Sedgley, M., & Collins, G. (2003). Changes in methylation and structure of DNA from almond tissues during in vitro culture and cryopreservation. *Journal Of The American Society For Horticultural Science*, *128*(6), 890-897.

Chen, T., and Li, E. (2004). Structure and function of eukaryotic DNA methyltransferases. *Curr. Top. Dev. Biol*. 60:55-89.

Carena, M. J. (2009). *Cereals.* New York: Springer.

Castillo, N. F., Bassil, N. V., Wada, S., & Reed, B. M. (2010). Genetic stability of cryopreserved shoot tips of *Rubus* germplasm. *In Vitro Cellular & Developmental Biology - Plant*, *46*(3), 246-256. doi:10.1007/s11627-009-9265-z

Cejas, I., Méndez, R., Villalobos, A., Palau, F., Aragón, C., Engelmann, F., Carputo, D., Aversano, R., & Lorenzo, J. C. (2013). Phenotypic and molecular characterization of *Phaseolus vulgaris* plants from non-cryopreserved and cryopreserved seeds. *American Journal Of Plant Sciences*, *4*(4), 844-849. doi:10.4236/ajps.2013.44103.

Chwedorzewska, K. J., & Bednarek, P. T. (2011). Genetic and epigenetic studies on populations of *Deschampsia antarctica* Desv. from contrasting environments on King George Island. *Polish Polar Research*, *32*(1), 15-26. doi:10.2478/v10183-011-0005-9

Chwedorzewska, K. J., & Bednarek, P. T. (2012). Genetic and epigenetic variation in a cosmopolitan grass Poa annua from Antarctic and Polish populations. *Polish Polar Research*, *33*(1), 63-80. doi:10.2478/v10183-012-0004-5

Coelho, N., Gonçalves, S., González-Benito, M. E., & Romano, A. (2012). Germination and cryopreservation tolerance of seeds from the rare aromatic species *Thymus lotocephalu*s. *Scientia Horticulturae*, *145*84-86. doi:10.1016/j.scienta.2012.07.031

Engelmann, F., & Engels, J. M. (2002). Technologies and strategies for ex situ conservation. *Managing plant genetic resources* (pp. 89-104). New York: *CABI Pub*. doi:10.1079/9780851995229.0089

Eudes, F. (2015). *Triticale.* Springer. Available from https://ezproxy2.library.colostate.edu/login?url=http://dx.doi.org/10.1007/978-3-319-22551-7

Fiuk, A., Bednarek, P. T., & Rybczyñski, J. J. (2010). Flow Cytometry, HPLC -RP, and metAFLP Analyses to Assess Genetic Variability in Somatic Embryo-Derived Plantlets of *Gentiana pannonica* Scop. *Plant Molecular Biology Reporter*, *28*(3), 413-420. doi:10.1007/s11105- 009-0167-3

FAO (1996). Report on the State of the World's Plant Genetic Resources for Food and Agriculture. Food and Agriculture Organization of the United Nations, Rome, Italy.

Galdiano, R. J., Vendra me, W. A., & de Macedo Lemos, E. G. (2013). Cryopreservation, early seedling development, and genetic stability of *Oncidium flexuosum* Sims[electronic resource]. *Plant Cell, Tissue And Organ Culture*, *114*(1), 139-148.

Gehring, M., & Henikoff, S. (2007). DNA methylation dynamics in plant genomes. *Biochim. Biophys. Acta* 1769: 276-286.

Gehring, M., Bubb, K.L., & Henikoff, S. (2009). Extensive demethylation of repetitive elements during seed development underlies gene imprinting. *Science* 324: 1447-1451.

Geiger, H.H. and Miedaner T. (2009). Rye Breeding. In M. J. Carena (Ed.), *Cereals (P.157-181)* New York: Springer.

Genebank Standards. 1994. Food and Agriculture Organization of the United Nations, Rome, International Plant Genetic Resources Institute, Rome.

Grativol, C., Hemerly, A. S., & Ferreira, P. G. (2012). Genetic and epigenetic regulation of stress responses in natural plant populations. *Biochimica Et Biophysica Acta*, *1819*(2), 176-185. doi:10.1016/j.bbagrm.2011.08.010

Gonçalves, S., & Romano, A. (2009). Cryopreservation of seeds from the endangered insectivorous plant *Drosophyllum lusitanicum*. *Seed Science And Technology*, *37*(2), 485-490.

International Board for Plant Genetic Resources. (1985). *Descriptors for rye and triticale*. Rome: IBPGR Secretariat.

Hao, Y., You, C., & Deng, X. (2002). Analysis of ploidy and the patterns of amplified fragment length polymorphism and methylation sensitive amplified polymorphism in strawberry plants recovered from cryopreservation. *Cryo Letters*, *23*(1), 37-46.

Hao, Y., You, C., & Deng, X. (2002). Effects of cryopreservation on developmental competency, cytological and molecular stability of citrus callus. *Cryo Letters*, *23*(1), 27-35.

Hao, Y., Liu, Q., & Deng, X. (2001). Effect of cryopreservation on apple genetic resources at morphological, chromosomal, and molecular levels. *Cryobiology*, *43*(1), 46-53.

Heringer, A. S., Ree, J. F., Guerra, M. P., Vieira, L. N., Steinmacher, D. A., & Fraga, H. F. (2013). Global DNA methylation profiles of somatic embryos of peach palm (*Bactris gasipaes* Kunth) are influenced by cryoprotectants and droplet-vitrification cryopreservation [electronic resource]. *Plant Cell, Tissue And Organ Culture*, *114*(3), 365-372.

Iriondo, J. M. , I., Maxted, N., & Dulloo, M.. C.A.B. International. (2008). Conserving plant genetic diversity in protected areas: Population management of crop wild relatives (Ebook Library (EBL)). Wallingford, Oxfordshire, UK ; Cambridge, MA: CABI.

Jin, B., Li, Y., & Robertson, K. D. (2011). DNA Methylation: Superior or Subordinate in the Epigenetic Hierarchy? *Genes &Cancer*, *2*(6), 607–617. http://doi.org/10.1177/1947601910393957

Johnston, J. W., Harding, K., Bremner, D. H., Souch, G., Green, J., Lynch, P. T., Grout, B., & Benson, E. E. (2005). HPLC analysis of plant DNA methylation: a study of critical methodological factors. *Plant Physiology and Biochemistry: PPB / Société Française De Physiologie Végétale*, *43*(9), 844-853.

Johnston, J. W., Benson, E. E., & Harding, K. (2009). Cryopreservation induces temporal DNA methylation epigenetic changes and differential transcriptional activity in Ribes germplasm. *Plant Physiology &Biochemistry*, *47*(2), 123-131. doi:10.1016/j.plaphy.2008.10.008

Kaczmarczyk, A., Houben, A., Keller, E. J., & Mette, M. F. (2010). Influence of cryopreservation on the cytosine methylation state of potato genomic DNA. *Cryoletters*, *31*(5), 380-391.

Lu, Y., Wu, Z., Lu, W., Jia, Q., Wang, D., & Li, H. (2012). A comparison on DNA methylation levels in bamboo at five developmental stages [electronic resource]. *Journal Of Forestry Research Ecosystem Management*, *23*(1), 157-159.

Machczyńska, J., Orłowska, R., Zimny, J., & Bednarek, P. (2014). Extended metAFLP approach in studies of tissue culture induced variation (TCIV) in triticale. *Molecular Breeding*, *34*(3), 845-854. doi:10.1007/s11032-014-0079-2

Martis, M. M., Zhou, R., Haseneyer, G., Schmutzer, T., Vrána, J., Kubaláková, M., & ... Stein, N. (2013). Reticulate Evolution of the Rye Genome. *Plant Cell*, *25*(10), 3685-3698. doi:10.1105/tpc.113.114553

Maki, S., Hirai, Y., Niino, T., & Matsumoto, T. (2015). Assessment of molecular genetic stability between long-term cryopreserved and tissue cultured wasabi (*Wasabia japonica*) plants. *Cryo Letters*, *36*(5), 318-324.

Mikuła, A., Tomiczak, K., & Rybczyński, J. J. (2011). Cryopreservation enhances embryogenic capacity of *Gentiana cruciata* (L.) suspension culture and maintains (epi)genetic uniformity of regenerants. *Plant Cell Reports*, *30*(4), 565-574. doi:10.1007/s00299-010-0970-1

Merritt, D., Hay, F., Swarts, N., Sommerville, K., Dixon, K., & Editor: Patrick S. Herendeen. (2014). Ex situ Conservation and Cryopreservation of Orchid Germplasm. *International Journal of Plant Sciences, 175*(1), 46-58. doi:10.1086/673370

Middleton, C. P., Senerchia, N., Stein, N., Akhunov, E. D., Keller, B., Wicker, T., & Kilian, B. (2014). Sequencing of Chloroplast Genomes from Wheat, Barley, Rye and Their Relatives Provides a Detailed Insight into the Evolution of the Triticeae Tribe. *Plos ONE*, *9*(3), 1-12. doi:10.1371/journal.pone.0085761

Peres, S. (2016). Saving the gene pool for the future: Seed banks as archives. *Studies in History & Philosophy Of Biological & Biomedical Sciences, 55,* 96-104. doi:10.1016/j.shpsc.2015.09.002

Pérez-Figueroa, A. (2013).MSAP: a tool for the statistical analysis of methylation-sensitive amplified polymorphism data. *Molecular Ecology Resources*, *13*(3), 522-527. doi:10.1111/1755-0998.12064

Peredo, E. L., Arroyo-Garcia, R., Reed, B. M., & Angeles Revilla, M. (2009). Genetic stability of in vitro conserved germplasm of *Humulus lupulus* L. *Agricultural and Food Science*, *18*(2, Sp. Iss. SI), 144-151.

Perullo, N., Determann, R., Cruse-Sanders, J., & Pullman, G. (2015). Seed cryopreservation and micropropagation of the critically endangered species swamp pink *(Helonias bullata* L.). *In Vitro Cellular & Developmental Biology Plant*, *51*(3), 284-293. doi:10.1007/s11627-015-9679-8

Pirondini, A., & Sgarbi, E. (2014). Effect of cryopreservation on in vitro seed germination and protocorm growth of Mediterranean orchids. *Cryoletters*, *35*(4), 327-335.

Puchalski, J., Niedzielski, M., & Eberhart, S. A. (n. d.) Genetic shift in rye accessions in relation to long-term seed storage.

Rodrigues, A. P., Forzza, R. C., & Andrade, A. S. (2014). Physiological characteristics underpinning successful cryopreservation of endemic and endangered species of Bromeliaceae from the Brazilian Atlantic Forest. *Botanical Journal of the Linnean Society*, *176*(4), 567-578. doi:10.1111/boj.12219

Reed, B. M., (2004). Shoot-tip cryopreservation manual. National Clonal Germplasm Repository- Corvallis. Corvallis, OR, USA.

Reik, W., Dean, W., and Walter, J. (2001). Epigenetic reprogramming in mammalian development. Science 293: 1089-1093.

Sano, N., Rajjou, L., North, H. M., Debeaujon, I., Marion-Poll, A., & Seo, M. (2016). Staying Alive: Molecular Aspects of Seed Longevity. *Plant & Cell Physiology*, *57*(4), 660-674. doi:10.1093/pcp/pcv186

Salomao, Antonieta Nassif. (2002). Tropical seed species' responses to liquid nitrogen exposure. *Braz. J. Plant Physiol.* [online]. vol.14, n.2, pp.133-138. ISSN 1677- 9452. http://dx.doi.org/10.1590/S1677-04202002000200008.

Schlegel, R. H. J. (2014). Rye: Genetics, breeding, and cultivation. Boca Raton, FL: *CRC Press*. Available from http://www.CSU.eblib.com/patron/FullRecord.aspx?p=1375580

Schmitz, R. J., & Zhang, X. (2011). High-throughput approaches for plant epigenomic studies. *Current Opinion In Plant Biology*, *14*(2), 130-136. doi:10.1016/j.pbi.2011.03.010

Sisunandar, Sopade, P. A., Samosir, Y. M., Rival, A., & Adkins, S. W. (2010). Dehydration improves cryopreservation of coconut (*Cocos nucifera* L.). *Cryobiology*, *61*(3), 289-296. doi:10.1016/j.cryobiol.2010.09.007

Sisunandar, Rival, A., Turquay, P., Samosir, Y., & Adkins, S. W. (2010). Cryopreservation of coconut (*Cocos nucifera* L.) zygotic embryos does not induce morphological, cytological or molecular changes in recovered seedlings. *Planta*, *232*(2), 435-447. doi:10.1007/s00425-010-1186-x

Takagi, H., & Engelmann, F. (2000). Cryopreservation of tropical plant germplasm: current research progress and application / F. Engelmann and H. Takagi,(eds). In, *JIRCAS International agriculture series; no. 8* Tsukuba, Japan: Japan International Research Center for Agricultural Sciences; Rome: International Plant Genetic Resources Institute, c2000.

Teixeira, F. K., Heredia, F., Sarazin, A., Roudier, F., Boccara, M., Ciaudo, C., Cruaud, C., Poulain, J., Berdasco, M., Fraga, M. F., Voinnert, O., Wincker, P., Esteller, M., & Colot, V. (2009). ARole for RNAi in the Selective Correction of DNA Methylation Defects. *Science*, *323*(5921), 1600-1604.

Vos, P., Hoger s, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Friters, A., Pot, J., Paleman, J., Kuiper, M., & Zabeau M., (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research*, *23*(21), 4407-4414.

Vuylsteke, M., Peleman, J. D., & van Eijk, M. T. (2007). AFLP technology for DNA fingerprinting. *Nature Protocols*, *2*(6), 1387-1398.

Walters, C., Wheeler, L., & Stanwood, P. C. (2004). Longevity of cryogenically stored seeds. *Cryobiology*, *48*(3), 229-244.

Walters. C. (2014). Extreme biology: Probing life at low water contents and temperatures. *Acta Horticulturae*, 1039, 49-56.

Wang, Z., & He, Y. (2009). Effect of cryopreservation on the development and DNA methylation patterns of Arabidopsis thaliana. *Life Science Journal-Acta Zhengzhou University Overseas Edition*, *6*(1), 55-60.

Yu, X., Li, X., Guo, T., Zhu, C., Wu, Y., Mitchell, S. E., Roozeboom, K. L, Wang, D., Wang, M., Pederson, G. A., Tesso, T. T., Schnable, P. S., Bernardo, R. & Yu, J. (2016). Genomic prediction contributing to a promising global strategy to turbocharge gene banks. *Nature Plants*, *2*, 16150. Retrieved from http://dx.doi.org/10.1038/nplants.2016.150

Zhao, X., Chai, Y., & Liu, B.(2007). Epigenetic inheritance and variation of DNA methylation level and pattern in maize intra-specific hybrids. *Plant Sci*.172: 930-938.

Zaidi, C. A., Gonzalez-Benito, M. E., & Perez-Garcia, F. (2010). Morphological and physiological seed heterogeneity in the Mediterranean annual plant *Tuberaria macrosepala (Cistaceae)*. *Plant Species Biology*, *25*(2), 149-157. doi:10.1111/j.1442-1984.2010.00279.x

Zeliang, P., Pattanayak, A., Iangrai, B., Khongwir, E., & Sarma, B. (2010). Fertile plant regeneration from cryopreserved calli of *Oryza rufipogon* Griff. and assessment of variation in the progeny of regenerated plants. *Plant Cell Reports*, *29*(12), 1423-1433. doi:10.1007/s00299-010-0932-7

Zevallos, B., Cejas, I., Engelmann, F., Carputo, D., Aversano, R., Scarano, M. T., Yanes, E., et al. (2014). Phenotypic and molecular characterization of plants regenerated from non-cryopreserved and cryopreserved wild *Solanum lycopersicum mill*. Seeds. *Cryo letters*, *35*(3), 216-2

Zhang, M., Kimatu, J. N., Xu, K., & Liu, B. (2010). DNA cytosine methylation in plant development. *Journal of Genetics And Genomics, 37*(1), 1-12. doi:10.1016/S1673-8527(09)60020-5

Zhang, M.S., Yan, H.Y., Zhao, N., Lin, X.Y., Pang, J.S., Xu, K.Z., Liu, L.X., & Liu, B. (2007a). Endosperm-specific hypomethylation, and meiotic inheritance and variation of DNA methylation level and pattern in sorghum (*Sorghum bicolor* L.) inter-strain hybrids. *Theor. Appl. Genet*. 115:195-207.

Appendix tables

Appendix Table 1 Initial normal germination percentage and initial seed moisture percentage measured in August to September 1988.

Inventory #	Control (%)	LN2[a] (%)	Difference (%)	Moisture (%)
105132	96	94	-2	6.24
105138	.	.	.	.
105144	90	92	2	6.25
105153	95	93	-2	6.14
105156	94	92	-2	4.89
105228	88	92	4	5.36
105324	93	96	3	6.33
105326	88	87	-1	5.2
105347	89	91	2	5.6
105469	92	96	4	4.91
105524	82	88	6	6.38
105573	.	.	.	.
105643	94	95	1	5.7
105646	92	94	2	5.76
105647	95	93	-2	5.69
105649	96	94	-2	5.9
105656	92	91	-1	5.41
105657	90	91	1	5.34
232556	96	91	-5	5.04
105698	91	90	-1	4.69
105181	99	95	-4	5.81
105182	98	96	-2	6.76
105184	94	97	3	5.8
105185	98	96	-2	5.76
105189	90	96	6	6.59
105194	96	99	3	6.05
105198	96	91	-5	4.87
105212	96	94	-2	6.38
105231	93	96	3	5.6
105240	.	.	.	.
105241	85	90	5	5.49
105248	94	94	0	5.89
105253	96	97	1	6.12

105256	89	94	5	6.45
105265	.	.	.	.
105270	92	89	-3	4.8
105287	92	95	3	6.08
105288	91	88	-3	6.14
105330	88	91	3	5.14
105473	85	87	2	5.16

[a] LN2 represents for liquid nitrogen

Appendix Table 2 Qualitative traits evaluated of 20 spring habit and 20 winter habit rye accessions stored under cryopreservation and conventional conditions, and evaluated at ARDEC in 2014 – 2015. Codes are defined in Table 2.3

Habit	ID	Storage[a]	Glume pubscence	Glume color	Awn color	Awn type	Straw color	Note
Spring	105347	Cryo.	3	8	8	1	7	Non-uniform jointing
Spring	105573	Conv.	.	.	.	.	.	No jointing
Spring	105573	Cryo.	.	.	.	.	.	No jointing
Spring	105132	Conv.	.	.	.	.	.	No jointing
Spring	105132	Cryo.	3	8	8	1	7	Non-uniform jointing
Spring	105138	Conv.	3	8	8	1	7	
Spring	105138	Cryo.	3	8	8	1	7	
Spring	105144	Conv.	3	8	8	1	7&6	
Spring	105144	Cryo.	3	8	8	1	7	
Spring	105153	Conv.	3	7	7	1	7	Non-uniform jointing
Spring	105153	Cryo.	3	8	8	1	7&6	
Spring	105156	Conv.	3	7	7	1	7&5	
Spring	105156	Cryo.	3	7	7	1	7	
Spring	105228	Conv.	3	8	8	1	7&6	
Spring	105228	Cryo.	3	8	8	1	7&6	Non-uniform jointing
Spring	105324	Conv.	3	8	8	1	7	
Spring	105324	Cryo.	3	8	8	1	7	
Spring	105326	Conv.	3	8&7	8&7	1	7	Non-uniform jointing
Spring	105326	Cryo.	3	8	8	1	7	Non-uniform jointing
Spring	105347	Conv.	3	8	8	1	7	Non-uniform jointing
Spring	105469	Conv.	3	7&8	7&8	1	7&5	
Spring	105469	Cryo.	3	7	7	1	7&5	
Spring	105524	Conv.	3	8	8	1	7&5	

Spring	105524	Cryo.	3	8	8	1	7&5	
Spring	105643	Conv.	3	8	8	1	7	
Spring	105643	Cryo.	3	8	8	1	7&5	
Spring	105646	Conv.	3	8	8	1	7	
Spring	105646	Cryo.	3	8	8	1	7&5	
Spring	105647	Conv.	3	8	8	1	7	
Spring	105647	Cryo.	3	8	8	1	7	
Spring	105649	Conv.	3	8	8	1	7	
Spring	105649	Cryo.	3	8	8	1	7	
Spring	105656	Conv.	3	8	8	1	7	
Spring	105656	Cryo.	3	8	7	1	7&6	
Spring	105657	Conv.	3	8	8	1	7&5	Non-uniform jointing
Spring	105657	Cryo.	3	8	8	1	7	
Spring	105698	Conv.	3	7	7	1	7&6	
Spring	105698	Cryo.	3	7	7	1	7&6	
Spring	232556	Conv.	5	8	8	1	7&6	
Spring	232556	Cryo.	3	8	8	1	7&6	
Winter	105181	Conv.	3	8	8	1	9	
Winter	105181	Cryo.	3	8	8	1	9	
Winter	105182	Conv.	3	8	8	1	9&5	
Winter	105182	Cryo.	3	8	8	1	9	
Winter	105184	Conv.	3	8	8	1	9&6	
Winter	105184	Cryo.	3	8	8	1	9&5	
Winter	105185	Conv.	3	8	8	1	9	
Winter	105185	Cryo.	3	8	8	1	9	
Winter	105189	Conv.	3	8	8	1	9	
Winter	105189	Cryo.	3	8	8	1	9	
Winter	105194	Conv.	3	8	8	1	9	

Winter	105194	Cryo.	3	8	8	1	9&5	
Winter	105198	Conv.	3	8	8	1	9	
Winter	105198	Cryo.	3	8	8	1	9	
Winter	105212	Conv.	3	8	8	1	9&5	
Winter	105212	Cryo.	3	8	8	1	9	
Winter	105473	Conv.	3	8	8	1	9&5	
Winter	105473	Cryo.	3	8	8	1	9	
Winter	105231	Conv.	3	8	8	1	9	
Winter	105231	Cryo.	3	8	8	1	9	
Winter	105240	Conv.	3	8	8	1	9	
Winter	105240	Cryo.	3	8	8	1	9&5	
Winter	105241	Conv.	3	8	8	1	9	
Winter	105241	Cryo.	3	8	8	1	9	
Winter	105248	Conv.	3	8	8	1	9	
Winter	105248	Cryo.	3	8	8	1	9	
Winter	105253	Conv.	3	8	8	1	9	
Winter	105253	Cryo.	3	8	8	1	9	
Winter	105256	Conv.	3	8	8	1	9&5	
Winter	105256	Cryo.	3	8	8	1	9	
Winter	105265	Conv.	3	8	8	1	9&5	
Winter	105265	Cryo.	3	8	8	1	9	
Winter	105270	Conv.	3	8	8	1	9&5	
Winter	105270	Cryo.	3	8	8	1	9&5	
Winter	105287	Conv.	3	8	8	1	9	
Winter	105287	Cryo.	3	8	8	1	9&5	
Winter	105288	Conv.	3	8	8	1	9	
Winter	105288	Cryo.	3	8	8	1	9	

Winter	105330	Conv.	3	8	8	1	9&5	
Winter	105330	Cryo.	3	8	8	1	9	

[a] Cryo. represents cryopreservation and Conv. represents conventional storage method

Appendix Table 3 Best linear unbiased predictions (BLUPs)±SE for five quantitative traits of 20 winter habit rye accessions grown at Fort Collins, CO in 2014 – 2015. Means are combined over storage treatments.

Accession	**Days to anthesis**	**Height** (cm)	**Spike weight** (g)	**Spike length** (cm)	**Lodging** (1 - 9)[a]
105181	151.12±0.61	174.95±2.05	1.57±0.10	11.23±0.28	4.80±0.63
105182	152.91±0.61	172.51±2.05	1.96±0.10	12.13±0.28	3.89±0.63
105184	153.19±0.61	179.45±2.05	1.88±0.10	13.23±0.28	5.18±0.63
105185	150.55±0.61	170.42±2.05	1.74±0.10	12.53±0.28	3.89±0.63
105189	153.81±0.61	168.34±2.05	1.88±0.10	12.09±0.28	4.86±0.63
105194	153.90±0.61	175.45±2.05	2.03±0.10	13.00±0.28	3.62±0.63
105198	153.02±0.61	167.20±2.05	1.63±0.10	11.72±0.28	5.77±0.63
105212	153.74±0.61	172.16±2.05	1.73±0.10	13.06±0.28	4.16±0.63
105473	155.28±0.61	171.05±2.05	1.75±0.10	11.84±0.28	5.28±0.63
105231	154.00±0.61	175.27±2.05	2.03±0.10	11.47±0.28	4.05±0.63
105240	153.75±0.61	174.97±2.05	1.51±0.10	12.86±0.28	4.91±0.63
105241	153.28±0.61	174.89±2.05	1.77±0.10	12.91±0.28	4.69±0.63
105248	154.49±0.61	169.69±2.05	1.61±0.10	13.26±0.28	6.94±0.63
105253	153.08±0.61	169.22±2.05	1.71±0.10	11.75±0.28	3.94±0.63
105256	150.38±0.61	166.7±2.05	1.53±0.10	10.89±0.28	5.34±0.63
105265	152.25±0.61	176.69±2.05	2.12±0.10	12.33±0.28	3.41±0.63
105270	154.15±0.61	164.77±2.05	1.48±0.10	12.29±0.28	6.62±0.63
105287	153.5±0.61	180.48±2.05	2.19±0.10	12.54±0.28	3.94±0.63
105288	153.83±0.61	168.66±2.05	1.66±0.10	12.65±0.28	6.3±0.63
105330	156.37±0.61	176.58±2.05	1.61±0.10	13.32±0.28	3.14±0.63

[a] 1= no lodging, 9= all plants flat

Appendix Table 4 Best linear unbiased predictions (BLUPs)±SE for five quantitative traits of 20 spring habit rye accessions grown at Fort Collins, CO in 2015. Means are combined over storage treatments.

Acc.[a]	**Irrigation treatments**	**Days to anthesis**	**Height** (cm)	**Spike weight** (g)	**Spike length** (cm)	**Lodging** (1- 9)[b]
105132	Irrigated	74.31±1.56	105.12±4.89	1.11±0.14	13.36±0.43	1.51±0.47
105138	Irrigated	77.26±1.56	106.03±4.89	1.29±0.12	11.47±0.36	1.80±0.46
105144	Irrigated	77.63±1.39	109.23±4.89	1.41±0.12	12.52±0.36	1.95±0.46
105153	Irrigated	77.49±1.63	107.93±4.89	1.16±0.12	10.79±0.36	1.72±0.46
105156	Irrigated	76.36±1.41	109.78±4.89	1.39±0.12	12.56±0.36	1.95±0.46
105228	Irrigated	72.93±1.39	109.94±5.25	1.11±0.14	11.66±0.43	1.41±0.46
105324	Irrigated	80.87±1.37	112.74±4.89	1.48±0.12	12.65±0.36	2.42±0.46
105326	Irrigated	77.17±1.41	104.78±5.00	1.32±0.13	11.40±0.40	1.99±0.48
105347	Irrigated	77.99±2.07	110.52±5.51	0.97±0.16	11.99±0.56	2.17±0.56
105469	Irrigated	76.25±1.37	114.01±4.89	1.06±0.12	11.50±0.36	2.19±0.46
105524	Irrigated	74.95±1.35	115.36±4.89	1.25±0.12	12.58±0.36	2.97±0.46
105573	Irrigated	76.02±1.75	112.28±6.14	1.16±0.20	12.01±0.72	2.29±0.67
105643	Irrigated	78.12±1.35	112.82±4.89	1.06±0.12	12.56±0.36	2.58±0.46
105646	Irrigated	74.47±1.35	120.09±4.89	1.00±0.12	11.70±0.36	2.50±0.46
105647	Irrigated	73.32±1.35	114.26±4.89	1.05±0.12	11.66±0.36	2.73±0.46
105649	Irrigated	73.66±1.35	115.81±5.22	1.07±0.12	11.63±0.36	2.89±0.46
105656	Irrigated	76.44±1.35	115.42±5.37	1.09±0.12	12.33±0.36	3.12±0.46
105657	Irrigated	77.98±1.42	106.14±5.13	1.03±0.12	11.90±0.38	2.58±0.46
105698	Irrigated	73.75±1.35	114.92±4.89	1.12±0.12	12.05±0.36	2.58±0.46
232556	Irrigated	73.87±1.35	116.35±5.00	1.14±0.12	11.98±0.36	2.42±0.46
105132	Rainfed	84.44±2.69	89.88±3.45	0.87±0.12	12.20±0.36	2.00±0.29
105138	Rainfed	82.86±1.85	94.65±3.45	1.24±0.12	11.68±0.34	1.86±0.29
105144	Rainfed	77.51±1.87	104.11±3.45	1.40±0.12	12.50±0.34	1.77±0.29
105153	Rainfed	79.62±1.87	100.49±3.61	1.38±0.13	12.40±0.38	1.78±0.29
105156	Rainfed	78.22±1.67	100.64±3.45	1.40±0.12	11.70±0.34	2.04±0.29
105228	Rainfed	78.79±3.17	103.14±3.80	1.00±0.13	13.73±0.38	1.85±0.29
105324	Rainfed	79.83±1.95	105.68±3.61	1.16±0.12	12.68±0.36	1.90±0.29
105326	Rainfed	84.16±1.87	90.64±3.80	1.23±0.13	12.48±0.38	1.93±0.29
105347	Rainfed	78.66±4.07	100.77±5.48	1.37±0.18	11.72±0.61	1.99±0.29
105469	Rainfed	82.82±1.75	103.52±3.45	1.01±0.12	11.82±0.34	2.08±0.29
105524	Rainfed	75.95±1.75	106.70±3.45	1.24±0.12	12.44±0.34	1.90±0.29
105573	Rainfed	80.05±3.17	97.93±5.48	0.99±0.16	13.16±0.53	1.91±0.29
105643	Rainfed	80.70±1.75	111.36±3.45	0.96±0.12	11.94±0.34	2.17±0.29

105646	Rainfed	74.25±1.67	115.77±3.45	0.97±0.12	11.90±0.34	2.26±0.29
105647	Rainfed	73.46±1.67	114.06±3.45	1.07±0.12	11.39±0.34	1.99±0.29
105649	Rainfed	75.87±1.67	112.72±3.45	1.05±0.12	11.27±0.34	2.13±0.29
105656	Rainfed	77.56±1.87	104.76±3.45	0.96±0.12	12.11±0.34	2.14±0.29
105657	Rainfed	80.20±1.83	104.92±3.45	1.07±0.12	12.20±0.34	2.08±0.29
105698	Rainfed	73.75±1.67	111.29±3.45	1.16±0.12	12.04±0.34	1.99±0.29
232556	Rainfed	74.44±1.67	115.17±3.45	1.22±0.12	12.50±0.34	1.99±0.29

[a] accessions

[b] 1= no lodging, 9 = all plants flat

Appendix Table 5 Least squares means of root characters of 20 winter and 20 spring rye accessions evaluated in 2014 to 2015 with WinRhizo software[a]

Habit	Accession	Storage[b]	Root length	Projected area	Surface area	Diameter	Root volume
Winter	105181	Cryo.	72.27±2.42	2.68±0.098	8.43±0.31	0.37±0.01	0.079±0.005
Winter	105182	Cryo.	81.92±5.06	2.95±0.21	9.25±0.65	0.36±0.00	0.084±0.007
Winter	105184	Cryo.	83.42±3.77	2.75±0.13	8.63±0.42	0.33±0.00	0.072±0.004
Winter	105185	Cryo.	68.73±5.29	2.74±0.20	8.59±0.62	0.40±0.00	0.086±0.006
Winter	105189	Cryo.	86.51±2.25	2.99±0.10	9.38±0.31	0.34±0.01	0.081±0.004
Winter	105194	Cryo.	80.94±2.87	3.00±0.13	9.43±0.40	0.37±0.01	0.088±0.005
Winter	105198	Cryo.	80.81±2.83	2.93±0.074	9.21±0.23	0.36±0.01	0.084±0.004
Winter	105212	Cryo.	79.07±4.80	2.95±0.13	9.26±0.42	0.37±0.01	0.087±0.005
Winter	105228	Cryo.	86.64±2.28	3.23±0.12	10.15±0.37	0.37±0.00	0.095±0.004
Winter	105231	Cryo.	100.49±7.81	3.19±0.13	10.02±0.41	0.32±0.01	0.081±0.001
Winter	105240	Cryo.	80.85±4.30	2.9±0.14	9.12±0.44	0.36±0.00	0.082±0.004
Winter	105241	Cryo.	81.85±2.63	2.89±0.09	9.08±0.27	0.35±0.01	0.081±0.004
Winter	105248	Cryo.	72.76±2.77	2.48±0.12	7.81±0.38	0.34±0.01	0.067±0.004
Winter	105253	Cryo.	69.40±1.28	2.61±0.09	8.19±0.28	0.37±0.01	0.077±0.004
Winter	105265	Cryo.	81.15±4.77	3.26±0.19	10.23±0.59	0.40±0.00	0.100±0.006
Winter	105270	Cryo.	81.91±5.44	2.96±0.15	9.30±0.46	0.36±0.01	0.085±0.004
Winter	105287	Cryo.	78.57±4.11	2.75±0.15	8.65±0.46	0.35±0.00	0.076±0.004
Winter	105288	Cryo.	76.50±3.79	2.65±0.13	8.32±0.39	0.35±0.00	0.072±0.003
Winter	105330	Cryo.	78.84±1.15	2.71±0.02	8.50±0.08	0.35±0.01	0.074±0.002
Winter	105473	Cryo.	67.68±3.29	1.92±0.32	6.03±1.01	0.28±0.05	0.046±0.015
Winter	105181	Conv.	74.83±5.73	2.71±0.23	8.52±0.73	0.36±0.01	0.078±0.007
Winter	105182	Conv.	82.28±8.97	2.88±0.29	9.06±0.90	0.35±0.02	0.081±0.009
Winter	105184	Conv.	76.91±2.82	2.60±0.11	8.18±0.35	0.34±0.00	0.069±0.003
Winter	105185	Conv.	64.46±5.20	2.62±0.19	8.23±0.61	0.41±0.00	0.084±0.006
Winter	105189	Conv.	77.40±2.17	2.79±0.16	8.75±0.52	0.36±0.01	0.079±0.007
Winter	105194	Conv.	82.75±4.55	3.09±0.15	9.71±0.48	0.38±0.00	0.091±0.004
Winter	105198	Conv.	72.82±7.81	2.60±0.27	8.18±0.85	0.36±0.01	0.074±0.008
Winter	105212	Conv.	77.68±6.88	2.92±0.27	9.19±0.83	0.38±0.00	0.087±0.008

Winter	105228	Conv.	80.29±3.30	3.11±0.12	9.76±0.38	0.39±0.01	0.095±0.004
Winter	105231	Conv.	93.02±8.20	3.22±0.27	10.10±0.84	0.35±0.00	0.088±0.007
Winter	105240	Conv.	73.18±3.49	2.66±0.12	8.36±0.37	0.36±0.01	0.076±0.005
Winter	105241	Conv.	77.53±3.66	2.87±0.18	9.00±0.55	0.37±0.01	0.084±0.007
Winter	105248	Conv.	63.75±0.38	2.40±0.05	7.53±0.16	0.38±0.01	0.071±0.003
Winter	105253	Conv.	66.95±3.64	2.40±0.19	7.53±0.58	0.36±0.01	0.068±0.007
Winter	105265	Conv.	79.53±2.40	3.09±0.08	9.70±0.26	0.39±0.00	0.095±0.002
Winter	105270	Conv.	70.9±6.47	2.73±0.27	8.57±0.85	0.38±0.01	0.083±0.009
Winter	105287	Conv.	70.99±3.81	2.54±0.11	7.97±0.36	0.36±0.01	0.072±0.003
Winter	105288	Conv.	80.95±4.94	2.82±0.15	8.85±0.49	0.35±0.00	0.078±0.004
Winter	105330	Conv.	83.65±3.17	3.07±0.10	9.63±0.31	0.36±0.01	0.089±0.003
Winter	105473	Conv.	67.72±5.31	2.52±0.13	7.90±0.40	0.37±0.01	0.074±0.002
Spring	105132	Cryo.	64.64±3.17	2.50±0.09	7.84±0.28	0.39±0.02	0.077±0.005
Spring	105138	Cryo.	74.93±4.69	2.66±0.19	8.37±0.59	0.36±0.01	0.075±0.006
Spring	105144	Cryo.	81.62±2.09	2.89±0.09	9.09±0.28	0.36±0.01	0.082±0.004
Spring	105153	Cryo.	91.14±3.32	3.11±0.11	9.78±0.35	0.34±0.00	0.084±0.004
Spring	105156	Cryo.	80.21±5.89	2.79±0.15	8.75±0.49	0.35±0.01	0.076±0.004
Spring	105169	Cryo.	80.00±5.78	3.12±0.18	9.82±0.57	0.39±0.01	0.097±0.005
Spring	105228	Cryo.	95.45±3.21	3.44±0.04	10.82±0.13	0.36±0.01	0.099±0.001
Spring	105324	Cryo.	78.06±4.04	2.77±0.12	8.70±0.38	0.35±0.00	0.078±0.003
Spring	105326	Cryo.	89.47±4.03	3.26±0.18	10.23±0.58	0.37±0.01	0.095±0.007
Spring	105347	Cryo.	89.29±8.43	2.80±0.39	8.80±1.23	0.31±0.02	0.070±0.013
Spring	105469	Cryo.	69.30±3.47	2.75±0.04	8.63±0.13	0.40±0.02	0.087±0.003
Spring	105524	Cryo.	99.79±0.77	3.40±0.09	10.68±0.28	0.34±0.01	0.091±0.004
Spring	105573	Cryo.	78.33±4.78	2.88±0.32	9.04±1.01	0.36±0.02	0.084±0.014
Spring	105643	Cryo.	86.99±1.27	3.02±0.04	9.48±0.14	0.35±0.01	0.083±0.003
Spring	105646	Cryo.	86.09±8.00	3.07±0.32	9.64±1.01	0.36±0.00	0.086±0.010
Spring	105647	Cryo.	84.72±6.02	3.13±0.21	9.83±0.65	0.37±0.01	0.091±0.006
Spring	105649	Cryo.	85.14±2.49	3.03±0.06	9.51±0.20	0.36±0.00	0.085±0.001
Spring	105656	Cryo.	84.97±5.09	2.95±0.17	9.26±0.53	0.35±0.00	0.080±0.005
Spring	105657	Cryo.	84.91±6.72	2.97±0.29	9.33±0.90	0.35±0.01	0.082±0.009

Spring	105698	Cryo.	95.00±3.11	3.43±0.12	10.79±0.37	0.36±0.01	0.098±0.006
Spring	232556	Cryo.	69.58±3.66	2.76±0.16	8.67±0.51	0.40±0.02	0.088±0.006
Spring	105132	Conv.	87.19±4.12	3.11±0.12	9.77±0.39	0.37±0.02	0.090±0.007
Spring	105138	Conv.	74.51±6.81	2.68±0.22	8.43±0.68	0.36±0.02	0.078±0.007
Spring	105144	Conv.	105.98±10.11	3.42±0.30	10.75±0.95	0.32±0.00	0.087±0.007
Spring	105153	Conv.	91.22±2.51	3.12±0.01	9.79±0.04	0.34±0.01	0.085±0.003
Spring	105156	Conv.	89.70±6.11	3.02±0.21	9.48±0.67	0.34±0.00	0.080±0.006
Spring	105228	Conv.	98.01±2.71	3.33±0.04	10.47±0.14	0.34±0.01	0.090±0.001
Spring	105324	Conv.	87.59±2.81	3.11±0.10	9.79±0.31	0.35±0.00	0.088±0.003
Spring	105326	Conv.	94.62±7.30	3.64±0.17	11.43±0.52	0.39±0.02	0.11±0.0051
Spring	105347	Conv.	78.42±7.32	2.50±0.25	7.85±0.78	0.31±0.01	0.063±0.007
Spring	105524	Conv.	91.68±5.66	3.18±0.22	9.98±0.68	0.35±0.00	0.087±0.006
Spring	105573	Conv.	77.75±3.74	3.04±0.14	9.54±0.44	0.39±0.01	0.095±0.005
Spring	105643	Conv.	77.64±5.52	2.68±0.17	8.43±0.52	0.34±0.01	0.074±0.004
Spring	105646	Conv.	93.75±3.89	3.23±0.14	10.16±0.44	0.34±0.01	0.088±0.004
Spring	105647	Conv.	76.62±0.73	2.77±0.03	8.71±0.09	0.36±0.00	0.079±0.002
Spring	105649	Conv.	85.87±9.19	3.01±0.31	9.45±0.97	0.35±0.01	0.083±0.009
Spring	105656	Conv.	77.36±7.28	2.60±0.23	8.18±0.72	0.34±0.01	0.069±0.006
Spring	105657	Conv.	96.29±2.89	3.26±0.07	10.25±0.23	0.34±0.00	0.087±0.0015
Spring	105698	Conv.	98.69±1.34	3.51±0.04	11.04±0.11	0.36±0.01	0.099±0.0030
Spring	232556	Conv.	65.06±0.79	2.41±0.10	7.58±0.31	0.37±0.01	0.071±0.0050

[a] Regent Instruments Inc., Canada

[b] Cryo represents cryopreservation and Conv. represents conventional storage method

Appendix Table 6 Least squares means of germination rates of 20 winter rye and 20 spring rye accessions evaluated in 2014 to 2015

Accession	Storage	Normal germination (%)	Abnormal germination (%)
105132	Conventional	97.0±1.0	3.0±1.0
105138	Conventional	90.0±2.6	4.0±1.6
105144	Conventional	82.0±3.8	9.0±3.0
105153	Conventional	89.0±1.9	2.0±1.2
105156	Conventional	96.0±1.6	2.0±1.2
105181	Conventional	82.0±3.5	8.0±1.6
105182	Conventional	80.0±4.3	11.0±4.4
105184	Conventional	91.0±4.4	6.0±3.5
105185	Conventional	85.0±2.5	9.0±1.0
105189	Conventional	93.0±1.9	4.0±1.6
105194	Conventional	93.0±1.9	4.0±0.0
105198	Conventional	86.0±3.8	6.0±1.2
105212	Conventional	90.0±2.6	2.0±1.2
105228	Conventional	88.0±2.4	5.5±1.3
105231	Conventional	90.0±1.2	5.0±1.0
105240	Conventional	73.0±4.7	16.0±6.3
105241	Conventional	83.0±1.9	7.0±3.0
105248	Conventional	80.0±3.7	13.0±1.9
105253	Conventional	86.0±2.6	9.0±2.5
105256	Conventional	92.0±3.7	1.0±1.0
105265	Conventional	89.0±1.9	4.0±1.6
105270	Conventional	87.0±2.5	6.0±3.8
105287	Conventional	95.0±1.9	1.0±1.0
105288	Conventional	94.0±2.6	4.0±2.8
105324	Conventional	89.0±1.9	7.0±1.9
105326	Conventional	83.0±1.9	9.0±1.0
105330	Conventional	82.0±4.2	13.0±3.4
105347	Conventional	85.0±1.9	11.0±2.5
105469	Conventional	88.0±4.6	7.0±1.9
105473	Conventional	75.0±5.0	12.0±1.6
105524	Conventional	88.0±2.3	2.0±1.2
105573	Conventional	87.0±4.4	5.0±1.0
105643	Conventional	91.0±2.5	2.0±1.2
105646	Conventional	90.0±2.0	3.0±1.0
105647	Conventional	87.0±3.4	8.0±3.3
105649	Conventional	87.0±4.4	7.0±2.5
105656	Conventional	95.0±1.9	1.0±1.0
105657	Conventional	92.0±1.6	2.0±1.2
105698	Conventional	92.0±4.9	3.0±3.0

232556	Conventional	97.0±1.0	1.0±1.0
105132	Cryo.	96.0±1.6	4.0±1.6
105138	Cryo.	92.0±3.3	4.0±1.6
105144	Cryo.	90.0±2.0	4.0±1.6
105153	Cryo.	95.0±1.0	3.0±1.0
105156	Cryo.	94.0±2.6	6.0±2.6
105181	Cryo.	93.0±4.1	2.0±1.2
105182	Cryo.	92.0±3.7	5.0±1.9
105184	Cryo.	99.0±1.0	1.0±1.0
105185	Cryo.	90.0±2.6	5.0±1.0
105189	Cryo.	94.0±2.6	2.0±2.0
105194	Cryo.	98.0±1.2	1.0±1.0
105198	Cryo.	86.0±3.5	10.0±1.1
105212	Cryo.	94.0±2.6	3.0±1.9
105228	Cryo.	90.0±0.8	4.0±1.1
105231	Cryo.	97.0±1.9	1.0±1.0
105240	Cryo.	89.0±4.7	4.0±2.8
105241	Cryo.	90.0±3.5	6.0±2.6
105248	Cryo.	56.0±2.8	10.0±2.0
105253	Cryo.	91.0±3.4	8.0±2.8
105256	Cryo.	96.0±2.8	2.0±1.2
105265	Cryo.	90.0±2.6	7.0±3.0
105270	Cryo.	94.0±2.6	4.0±2.3
105287	Cryo.	95.0±3.8	3.0±1.9
105288	Cryo.	91.0±1.9	7.0±3.0
105324	Cryo.	90.0±3.5	6.0±1.2
105326	Cryo.	82.0±2.6	8.0±1.6
105330	Cryo.	87.0±3.0	11.0±3.8
105347	Cryo.	85.0±3.4	5.0±1.0
105469	Cryo.	92.0±1.6	2.0±2.0
105473	Cryo.	88.0±4.3	8.0±2.3
105524	Cryo.	91.0±1.0	2.0±2.0
105573	Cryo.	93.0±2.5	2.0±1.2
105643	Cryo.	92.0±4.3	3.0±3.0
105646	Cryo.	96.0±2.8	2.0±2.0
105647	Cryo.	85.0±3.0	9.0±2.5
105649	Cryo.	92.0±4.0	3.0±1.9
105656	Cryo.	97.0±1.0	1.0±1.0
105657	Cryo.	94.0±2.6	3.0±1.0
105698	Cryo.	96.0±2.8	2.0±1.2
232556	Cryo.	92.0±3.7	4.0±2.3

Appendix Table 7 Estimates of dry weights of 14 rye accessions with both storage treatments evaluated in 2014 to 2015

Accession	Storage	Total weight (mg)	Shoot weight (mg)	Root weight (mg)
105181	conventional	10.00±0.61	6.80±0.32	3.30±0.30
105182	conventional	9.90±1.40	6.80±1.00	3.10±0.40
105185	conventional	10.00±0.70	7.50±0.42	2.90±0.35
105194	conventional	12.00±0.44	7.90±0.21	3.70±0.24
105198	conventional	9.40±0.99	6.10±0.80	3.30±0.28
105231	conventional	11.00±1.10	8.00±0.94	3.50±0.30
105241	conventional	12.00±0.77	8.20±0.43	4.00±0.34
105248	conventional	9.90±0.62	6.30±0.49	3.50±0.25
105256	conventional	12.00±0.00	7.90±0.00	3.90±0.00
105270	conventional	11.00±0.73	7.80±0.58	3.70±0.14
105287	conventional	9.60±0.34	6.70±0.18	2.90±0.17
105288	conventional	9.20±0.77	6.40±0.51	2.80±0.30
105294	conventional	10.00±0.44	6.40±0.32	4.00±0.12
105330	conventional	11.00±0.47	7.60±0.21	3.50±0.31
105181	cryo	9.70±0.32	6.70±0.26	3.00±0.19
105182	cryo	12.00±0.34	8.00±0.036	3.70±0.35
105185	cryo	10.00±0.65	7.00±0.52	3.10±0.14
105194	cryo	10.00±1.20	7.30±0.36	3.10±0.86
105198	cryo	10.00±0.25	6.50±0.25	4.00±0.02
105231	cryo	12.00±0.44	7.80±0.44	3.80±0.19
105241	cryo	11.00±0.49	7.60±0.25	3.50±0.25
105248	cryo	9.30±0.14	5.90±0.12	3.50±0.03
105256	cryo	9.60±0.17	5.30±0.87	4.30±0.87
105270	cryo	11.00±0.26	7.40±0.13	3.30±0.16
105287	cryo	10.00±0.84	7.40±0.59	3.00±0.26
105288	cryo	9.10±0.47	6.40±0.39	2.70±0.10
105294	cryo	8.80±0.71	5.70±0.35	3.10±0.58
105330	cryo	10.00±0.57	7.30±0.38	2.90±0.19

Appendix Table 8 Mean value of adjusted normal germination for both storage treatments

Traits	**Cryopreservation**	**-18 ^{O}C**	**Difference[a]**	***P*[b]**
Adjusted germination(%)	98.80±0.77	95.74±0.77	3.06±0.80	0.0002

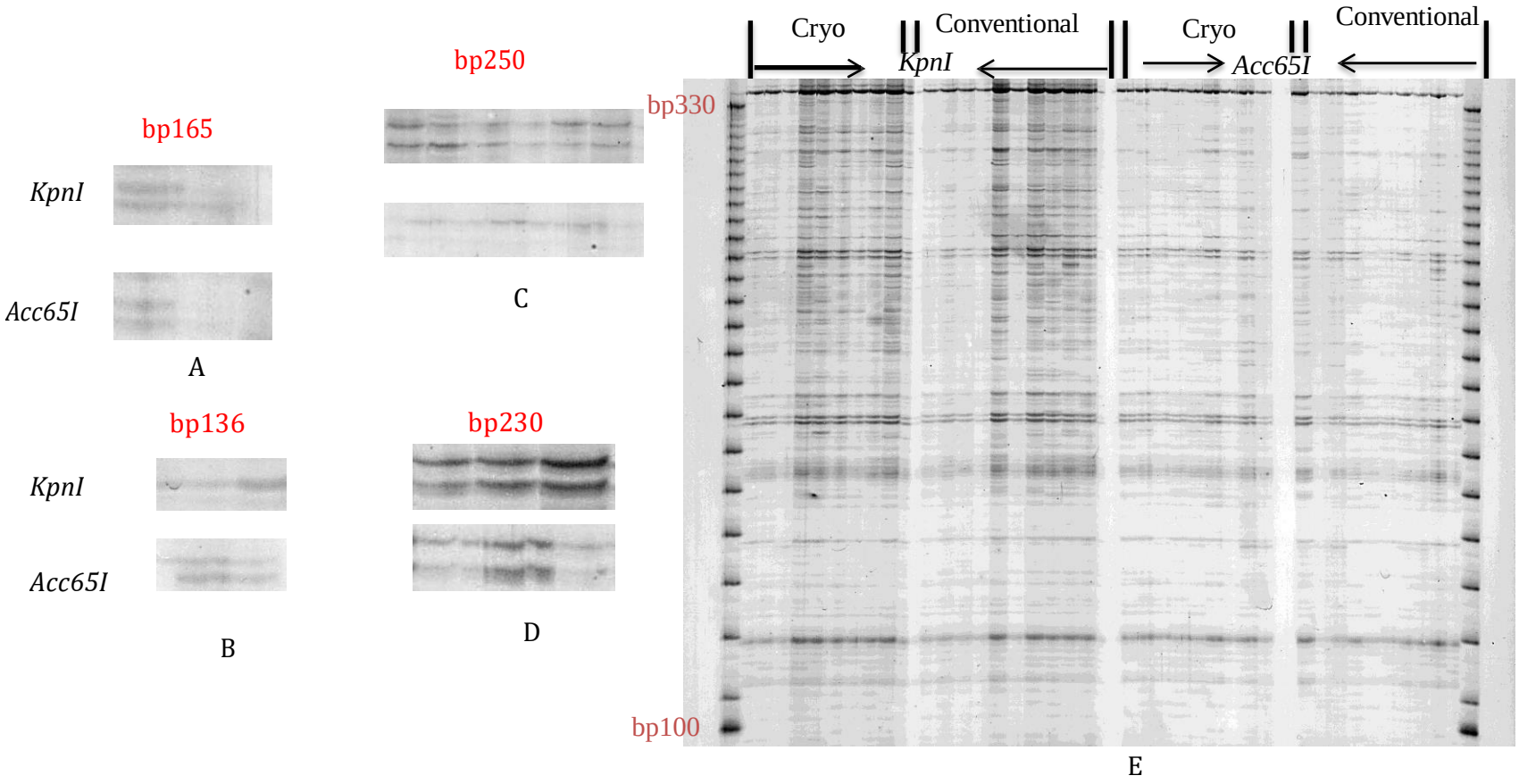
[a] Mean of cryopreservation – mean of -18 ^{O}C

[b] significance of difference between two storage methods

Appendix Table 9 Number of total loci detected in each selective primer set

Primers	**# of loci**
MCAA/CpG-ACG	5
MCAA/CpG-GCA	16
M-CAG/CpG-GAC	19
M-CAG/CpG-GCA	15
MCAG/CpXpG-AGC	8
M-CAG/CpXpG-TGC	14
MCAT/ CpXpG-ATG	18
M-CAT/CpXpG-AGG	26
MCT/CpG-GGC	17
MCT/CpXpG-(A/T)GG	35
MCT/CpXpG-ACA	27
MCT/CpXpG-ATT	46
MCT/CpXpG-TTG	17
MCT/CpXpX-TAA	28
M-CTG/CpG-TCG	20
Total	311

Appendix figures



Appendix Figure 3.1 MetAFLP gel from the primers MCT/CpXpX-TAA for two storage treatments of V/108. A represents no bands cut by both enzymes. B represents no bands cut by *KpnI* but there are bands cut by *Acc65I*. C represents bands cut by *KpnI* and no bands cut by *Acc65I*. D represents bands cut by both enzymes. E is the layout of the gel.