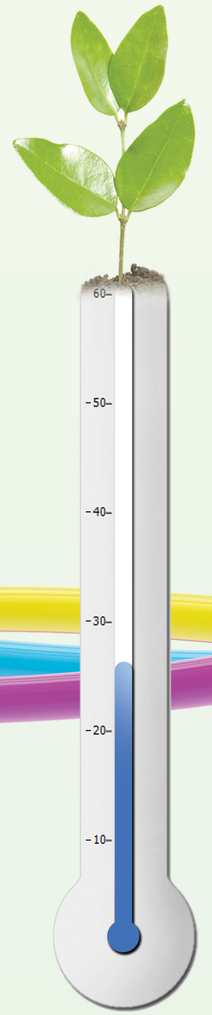
Molecular and genetic basis of freezing tolerance in crucifer species



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Molecular and genetic basis of freezing tolerance in crucifer species

Jae-Yun, Heo

Thesis

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Chapter I. General introduction

Cold stress severely limits the geographic distribution of many overwintering plants. Successful establishment and survival under cold conditions requires diverse adaptive and acclimation strategies at the physiological, cellular and molecular level. Understanding mechanisms underlying different acclimation and adaptation responses to cold stress among plants is important because it can provide insights on how plants react to changing environments. Limited natural variation in cold tolerance has often been observed between crop varieties or genotypes (limiting the potential to improve crop varieties to freezing tolerance); whereas substantial natural variation may exist in related wild species. Genetic analyses, such as QTL mapping in crosses involving wild species, can be used to understand the evolutionary forces acting on quantitative genetic variation and help to explain the geographical distribution of species. Furthermore, advanced molecular biology and bioinformatics tools allow plant biologists and breeders to utilize useful traits found in wild species to improve cold tolerance in crops, either by introgression breeding or in the future with transgenic lines. The Brassicaceae family, including a number of agriculturally and ecologically important overwintering species and crops, is one of most suitable model families for cold tolerance studies. In this thesis natural variation for important cold adaptive strategies such as cold acclimation and deacclimation were studied using wild Brassicaceae species, with a particular focus on *Boechera stricta*.

COLD ACCLIMATION AND GENES REGULATING COLD ACCLIMATION

While many tropical and subtropical plant species exhibit a significantly reduced physiological function when exposed to low temperatures, most temperate plant species can acquire freezing tolerance by prior exposure to non-freezing low temperatures, an adaptive process known as cold acclimation (Guy, 1990). Cold acclimation involves various physiological and biochemical changes (Sanghera et al., 2011). The best-characterized changes include a reduction or cessation of growth, synthesis of cryo-protectant molecules and modification of membrane lipid fluidity (Theocharis et al., 2012).

Membrane systems of cells are sites that are first exposed to low temperature and receive freezing injury in plants (Levitt, 1980). Ice forms in the extracellular spaces in plant cells as temperatures drop below zero. Thus, the cells have a lower vapor pressure than the liquid in the cytoplasm of adjacent cells leading to the withdrawal of water from the cells and

subsequent dehydration of the cytoplasm (Steponkus, 1984). Because these stress factors can cause irreversible damage at the plasma membrane of plants, cold acclimation is believed to enable plants to stabilize membranes against freezing injury. Multiple mechanisms appear to be involved in stabilization of membrane lipids during cold acclimation (Thomashow, 1999). It has been demonstrated that the synthesis of cryo-protectant molecules such as soluble carbohydrates can directly help to protect macromolecules on membrane lipid during freezing and support recovery after freezing (Mahajan and Tuteja, 2005). There is evidence that this synthesis is induced by changes of some genes, many of which have been shown to be regulated by changes in the ratio and types of translatable mRNA in response to cold treatment (Hajela et al., 1990). Subsequently, a number of cold-induced genes have been screened and led to identification of a core-set of cold regulated (COR) genes, including four sub groups: RD (responsive to dehydration), ERD (early responsive to dehydration), LTI (low-temperature induced) and KIN (cold-induced) genes (Thomashow, 1999).

Microarray analysis in Arabidopsis has shown that 655 and 284 of 24,000 genes were up and down-regulated in response to cold treatment, respectively (Lee et al., 2005). Although the functions of many of the cold regulated gene products are still unknown, it has been found that some cold regulated genes have roles in producing important metabolic proteins or cellular protectants, or to regulate genes involving in transducing the cold stress response signal (Yamaguchi-Shinozaki and Shinozaki, 2006). One of the most common categories of genes induced by low temperature encodes LEA (late embryogenesis abundant) proteins (Hundertmark and Hincha, 2008). LEA protein genes are present in all plants, in bacteria and even in nematodes (Bies-Etheve et al., 2008). Plant LEA proteins are induced in response to many stress factors (water, cold, drought or salinity) and are regulated by the stress phytohormone abscisic acid (ABA) (Mahajan and Tuteja, 2005). LEA proteins are generally hydrophilic and intrinsically unstructured, and they remain soluble after drying, freezing, or even boiling (Eriksson and Harryson, 2011). Even though the physiological function of most LEA proteins has not been established, some experiments conducted in vitro demonstrated that they can lead to the stabilization of cellular structures by binding RNA, DNA, water or binding, or activating antioxidants during dehydration (Tunnacliffe and Wise, 2007; Tunnacliffe et al., 2010). The effects of dehydration and freezing on membranes are similar, and there is evidence that the induction of some LEA proteins also contributes to the stabilization of membranes or preserves the activity of enzymes during freezing. Several COR (Cold-Regulated) proteins, that are

considered as hallmarks of freezing stress adaptation in plants, are classified as LEA proteins due to high similarity in sequence (Thomashow, 1999). For instance, the COR15A polypeptide encoded by the COR15A gene is targeted to chloroplasts and is one of most studied LEA-type COR proteins in *Arabidopsis* (Nakayama et al., 2007). Induction of the *COR15A* gene decreases the propensity of membranes to incorporate hexagonal II phase lipids, which is a main reason that membrane damage in response to freezing is reduced (Artus et al., 1996). Furthermore, a study of natural variation in a collection of 54 *Arabidopsis* accessions demonstrated the relative expression of COR15A is positively correlated with the cold-acclimated freezing tolerance (Zuther et al., 2012).

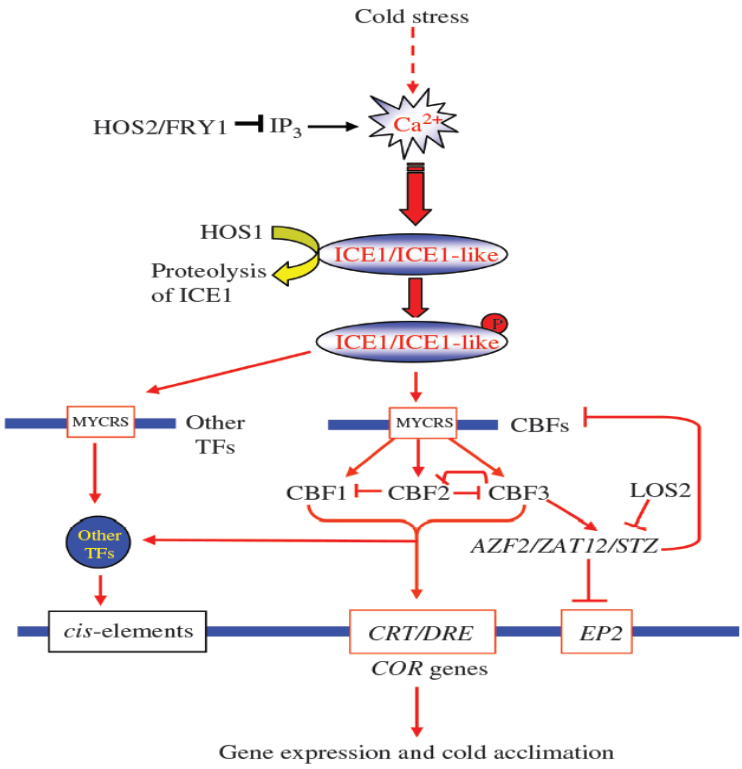

Figure 1. Regulation of transcription factors under cold stress. HOS2, high expression of osmotically responsive genes 2; FRY1, FIERY1, inositol polyphosphate 1-phosphatase; HOS1, high expression of osmotically responsive genes 1, a RING finger E3 ubiquitin ligase; ICE1, inducer of CBF expression 1, a myelocytomatosis (MYC)-type basic helix-loop-helix (bHLH) transcription factor; CBF, C-repeat-binding factor, AP2-type transcription factor; LOS2, low expression of osmotically responsive genes 2, a bi-functional enolase with transcriptional repression activity; AZF2, STZ, and ZAT12, cysteine-2 and histidine-2 type zinc finger transcriptional repressors; EP2, a cis-element originally identified in 5-enolpyruvylshikimate-3-phosphate synthase gene promoter; CRT, C-repeat elements; DRE, dehydration-responsive elements; MYCRS, MYC-type bHLH transcription factor recognition sequences; block arrow, activation; line arrow, induction of expression; line ending with bar, repression (from Chinnusamy et al., 2006).

COLD ACCLIMATION SIGNALLING AND THE CBF REGULATORY PATHWAY

The identity of the plant sensors of low temperature is as yet unknown (Chinnusamy et al., 2006). There is evidence that cold-induced Ca^{2+} in the cytosol is an important second messenger for cold acclimation response, although the underlying mechanism is not yet fully understood (Thomashow, 1999). Cold stress could be sensed by cold-induced changes in the fluid mosaic physical state of the plasma membrane and other changes that cause cold-signature fluxes in cytosolic Ca^{2+} currents (Lecourieux et al., 2006). These Ca^{2+} fluxes also activate kinases or kinase cascades leading to transcription of COR genes (Tahtiharju and Palva, 2001; Townley and Knight, 2002).

A subsequent question is how these signals result in the activation of the cold regulated genes. Many of the Arabidopsis cold-responsive COR genes are regulated by the C-repeat/dehydration-responsive element (CRT/DRE), a cis-acting element containing a CCGAC motif, which activates transcription in response to both low temperature and water deficits (Knight et al. 1999). Studies of COR gene expression in Arabidopsis have resulted in the discovery of a family of transcriptional activators, the CBF/DREB1 proteins (Thomashow, 1999). Three transcriptional activators, CBF1/DREB1B, CBF2/DREB1C and CBF3/DREB1A, belong to the AP2/EREBP family of DNA-binding proteins factors (Buskirk and Thomashow, 2006). The CBF cold acclimation pathway is summarized in Figure 2. It has been shown that the CBF transcription factors can bind to the C-repeat (CRT)/dehydration responsive element (DRE) cis-acting elements contained in numerous downstream genes, thus transmitting the cold signal to the expression of COR genes and their proteins (Maruyama et al., 2004; Xu et al., 2011). For instance, overexpression of CBF1 or CBF3 genes in transgenic Arabidopsis plants led to the constitutive expression of genes with promoters containing the DRE/CRT elements and improved freezing, drought and salt tolerance of non-acclimated Arabidopsis plants (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999). The CBF pathway responding to low temperatures is also found in poplar, wheat, rye and rapeseed, all of which are freezing tolerant (Skinner et al., 2005), and even in tomato and rice which are freezing sensitive (Zhang et al., 2004). The expression patterns of the CBF and COR genes in other freezing tolerant species are similar to those of Arabidopsis and the core regions within the CBF genes are highly conserved (Welling and Palva, 2008). In contrast, freezing-sensitive species such as tomato exhibits a reduced CBF regulon as well as lower induction of other cold-responsive genes (Dubouzet et al., 2003; Zhang et al., 2004). Furthermore, studies of the genetic basis in both monocot and dicot plants have revealed

that major QTL responsible for a large proportion of the variation for freezing tolerance are often associated with CBF gene clusters (Alonso-Blanco et al., 2005; Tayeh et al., 2013). Results reported to date thus provide strong evidence that CBF genes play a major role in configuring the low-temperature transcriptome and in conditioning freezing tolerance.

Although the mechanism whereby the CBF genes are activated by cold temperature is not fully known, the *Inducer of CBF Expression* (*ICE*) was proposed to be a major positive regulator of *CBF3 and CBF1* genes (Chinnusamy et al., 2003). ICE1 encodes a MYC-like bHLH (basic helix-loop-helix) transcription factor binding to element CANNTG in the promoter of CBF3 (Chinnusamy et al., 2003). ICE2, an ICE1 homolog, is also suggested to be involved in cold acclimation, probably by regulating the CBF1 gene (Fursova et al., 2009). ICE1 was found to be negatively- regulated by High expression of Osmotically Responsible genes 1 (HOS1) ending a RING finger-type E3 ligase for ubiquitylation of ICE1 (Lee et al., 2001). The *CBF2* gene is hypothesized to be regulated by members of calmodulin binding transcription activator (CAMTA) family of transcription factors. CAMTA3 binding to conserved DNA motif 2 (CM2) has been identified as a positive regulator of CBF2 expression (Doherty et al., 2009).

Whereas many studies demonstrated that CBF regulatory factors are important for freezing tolerance, Microarray analysis indicated that CBFs regulate only a part of the cold-responsive transcriptome (Vogel et al., 2005). This result points out the possibilities that other novel, CBF independent pathways or other multiple cold gene regulatory networks might contribute to the cold response and acquisition of freezing tolerance (Fowler and Thomashow, 2002). Hence, it is important to identify and analyse more genes controlling freezing tolerance.

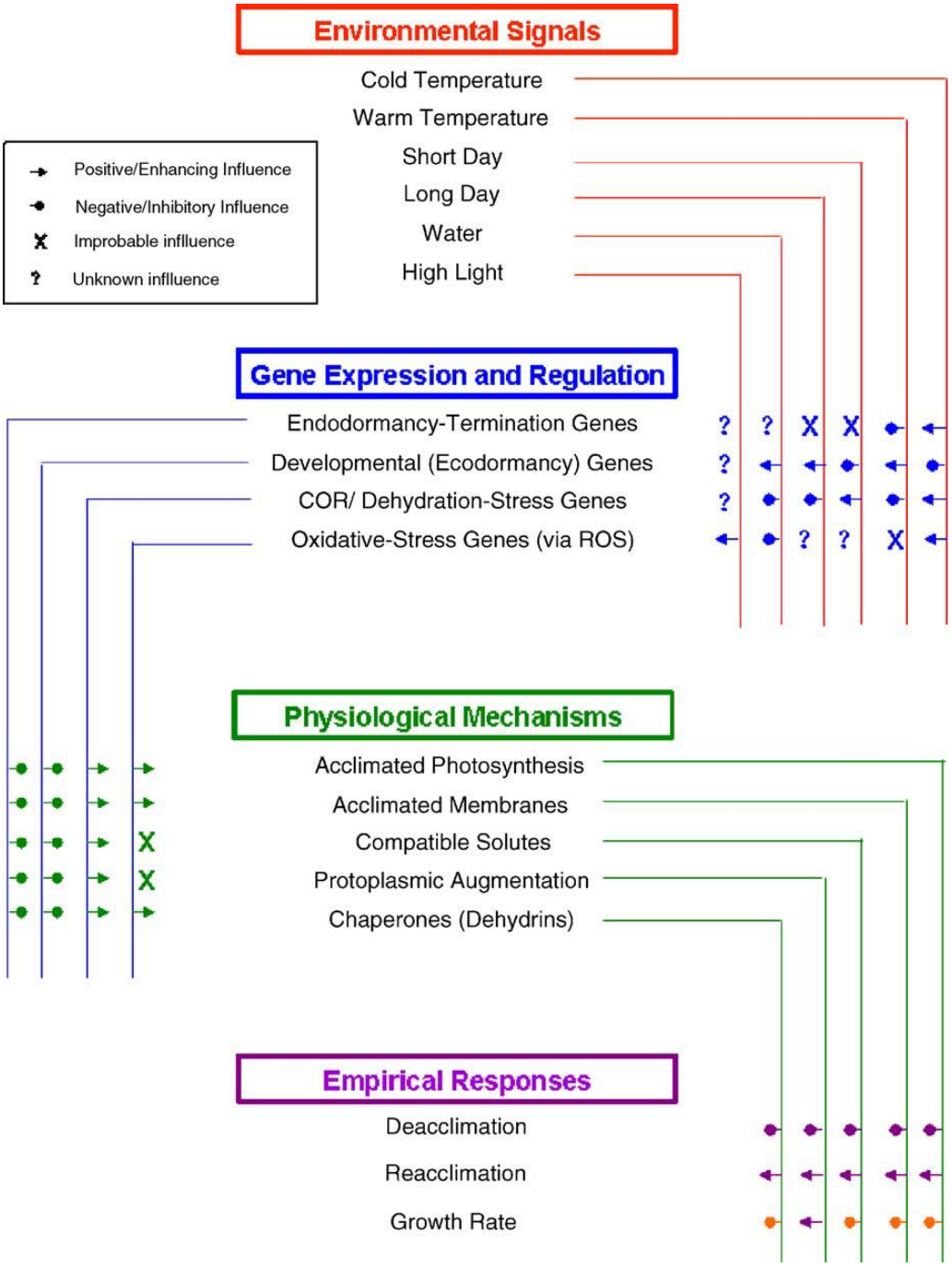

Figure 2. Possible sequence of events underlying transitions in cold hardiness and associated regulatory networks involving four hierarchical levels of control/response. Symbols such as pointed arrows, lollypops, question marks, and cross marks, emanating from the perpendicular lines and pointing towards a particular response indicate a potential cause and effect relationship. Pointed arrows indicate a positive/enhancing influence; lollypops indicate a negative/inhibitory influence; question marks specify that available information is inadequate to propose a relationship; cross marks designate associations that original authors believe to be improbable. The line is the color of the causal factor and the symbol is the color of the potential effect. Orange symbols indicate an indirect effect on plant growth (by causal factors) via competition for energy reserves rather than direct regulation (from Kalberer et al., 2006).

COLD DEACCLIMATION

Resuming plant growth in late winter/early spring is accompanied by a deacclimation response and reduction of freezing tolerance (Kalberer et al., 2006). Global warming is expected to result in more episodes of mild temperatures during winter. If deacclimation progresses in response to warm temperatures, regardless of growth stages, the risk of frost damage during winter may increase due to irregular winter warm spells (Kalberer et al., 2006).

Understanding the mechanisms that regulate deacclimation is therefore important particularly in the light of rising temperatures due to climate change. Surprisingly though, the genetic and molecular basis of deacclimation is not well documented (Figure 2). There, is evidence for natural variation of deacclimation responses and resistance with a genetic basis. Rowland et al. (2005) and Pagter et al. (2011) demonstrated that plant responses to warming temperatures do not occur instantaneously, and that the kinetics of deacclimation is dependent on the genotypes and species. Furthermore, it has been shown that developmental reprogramming in early spring requires *de novo* gene expression. For example, in wheat the *vrn* (vernalization-insensitivity) and *ppd* (photoperiod-insensitivity) genes can have an effect on determining freezing tolerance during deacclimation (Mahfoozi et al., 2001). These results support the need to study natural variation and both physiological and molecular responses using a broad range of genetic resources can be helpful for unravelling and understanding the mechanisms controlling cold deacclimation.

BOCHERA STRICTA AS A MODEL SYSTEM FOR ECOLOGICAL GENOMICS

The genus *Boechera,* a member of *Brassicaceae*, includes 110 species with a broad natural distribution throughout North America, Greenland, and the Russian Far East (Alexander et al., 2013). Based on morphological characters *Boechera* was thought to be originally included within the genus *Arabis,* but karyotypic and molecular analyses made that *Boechera* is considered an independent genus (Koch et al., 2000). In western America, *Boechera* are found in diverse habitats from near sea level to high elevations (Figure 3). Hence, they are exposed to different selective environments and display a wide variation in morphological and physiological traits (Rushworth et al., 2011).

Boechera stricta is one of the best studied *Boechera* species morphologically and molecularly (Schranz et al., 2007). Genetic and molecular analyses indicate that *B. stricta* is predominantly inbreeding (Schranz et al., 2007). Many different *B. stricta* ecotypes have been collected from

natural populations and are available for experimental analysis. The study of population structure in *B. stricta* revealed high levels of genetic diversity for northern and southern population (Song et al., 2006). A large array of genomic tools and resources for comparative genomic studies between *Arabidopsis thaliana* and *B. sticta* have been developed, including a mapping populations for QTL studies and a sequenced genome of one of the parental lines of the mapping populations (Mitchell-olds, unpublished). These resources can accelerate the identification of the molecular determinants of various ecologically important traits (Schranz et al., 2009; Anderson et al., 2011; Prasad et al., 2012; Anderson et al., 2014) as well as to understand the evolutionary forces acting on quantitative genetic variation in the wild, and the history and geographical distribution of ecologically important alleles (Mitchell-Olds, 2001; Mitchell-Olds et al., 2007). Advanced molecular biology and bioinformatics will additionally help to unravel the function of novel genes and provide insights on the evolution of specific gene families controlling ecologically important traits. These advantages make *B. stricta* an excellent system for ecological genomic studies, for example of cold and freezing tolerance.

Figure 3: Species in the genus *Boechera* occupy diverse habitats across the western United States. These habitats include the following: (top row) serpentine (Sierra County, California), sagebrush grassland (Custer County, Idaho) and Chihuahuan desert scrub (Eddy County, New Mexico); (bottom row) subalpine meadow (Ravalli County, Montana), rocky scree (Lemhi County, Idaho) and lava flow (Carrizozo Malpais, Lincoln County, New Mexico) (from Rushworth et al., 2011).

HYPHOTHESIS AND OUTLINE OF THE THESIS

Understanding genetic variation for freezing tolerance is important for unraveling an adaptative strategy of species and for finding out an effective way to improve crop productivity to unfavorable winter environments. The goal of this thesis was to examine natural variation for components of freezing tolerance beyond what has been done using the model organism *Arabidopsis thaliana*; thus, we used a number of related Brassicaceae species (especially *Boechera stricta* (Chapters 2, 3, 4) and "natural" species found in the Netherlands (Chapter 5). Main hypothesis was that we would identify potentially novel and beneficial traits and loci not identified in Arabidopsis (due to experimental approaches, lack of genetic diversity and/or traits not present in this model species). For example, we look at the effects of day-length to freezing tolerance in *B. stricta* (Chapter 2), comparative allelic and expression variation of CBF-loci in *B. stricta* (Chapter 3), freezing surivival and lipid profiling of seedlings *in B. stricta* (Chapter 4) and species-differences in acclimation, deacclimation and flowering time of a number of Dutch Brassicaceae species (Chapter 5).

Many important traits such as resistance to abiotic stresses including freezing and salt tolerance are quantitative traits with a genetic basis. Hence, Quantitative Trait Locus (QTL) mapping approach can be a good methodology to identify genome regions responsible for genotypic differences of freezing tolerance. Previous QTL studies have suggested that CBF (C-repeat binding factor) loci are major regions controlling freezing tolerance of several species (Vágújfalvi et al., 2003; Alonso-Blanco et al., 2005; Båga et al., 2007). Present QTL studies, however, have demonstrated that variation in *CBF* loci does not explain all the quantitative natural variation for freezing tolerance (Gery et al., 2011; Meissner et al., 2013). Parental populations for mapping population of *B. stricta* grows in undisturbed habitats of Western North America with quite differential winter environments and are approximately 1,000 km apart (Schranz et al., 2007). Their unique and different growth environments are expected that QTL analysis of freezing tolerance in *B. stricta* would provide robust examples of evolutionary and ecological adaptation of native species.

In Chapter 2 of this thesis, QTL analysis with a recombinant inbred line (RIL) population of *B. stricta* derived from a cross between two parents from contrasting sites was performed to identify genomic regions controlling freezing tolerance. In order to find out the QTL loci related to freezing tolerance across environments, QTL analyses were done with varying light and temperature regimes, and both in controlled growth-chamber and outdoor

environments This multi-environment (controlled and natural) and multi-trait analysis has rarely been undertaken for a non-model organism, allowing us to identify broad vs. specific QTL regions. In Chapter 3, we characterized the tandemly arranged C-repeat-Binding Factor (CBF)/Dehydration Response Element Binding 1 (DREB1) gene cluster, selected as potential candidate genes by previous QTL analysis in *B. stricta*. We isolated three CBF/DREB1-type genes, compared their sequences and performed phylogenetic analysis with other Brassicaceae CBF/DREB1-type genes. Furthermore, expression patterns of these genes were characterized to investigate if they were differentially regulated and possibly linked to the contrasting freezing tolerances of the two genotypes of *B. stricta*. However, future research is needed to confirm if the gene expression changes are due to trans- or cis-factors. In Chapter 4, we conducted QTL analysis of the *Boechera* seedling freezing tolerance. Because tissues of young plants are often more sensitive to freezing compared to adult plants (Loik and Redar, 2003; Von Meijenfeldt, 2010), we expected that plants may be needed to have differential freezing tolerance strategies at a seedling stage and major loci responsible for freezing tolerance can differ. We also identified a potential candidate gene (DGAT1) related to lipid biosynthesis and examine lipid profiles. We also compare our work in *Boechera* with a DGAT mutatant in Arabidopsis, the first research of the potential link of this gene to freezing tolerance.

In Chapter 5 of this thesis, we tested for natural variation of five selected Brassicaceae species that all have similar plant forms, but have different flowering times, and studied the relationship between cold acclimation in fall and winter, and deacclimation in spring. The Brassicaceae comprises a numerous number of important winter-annual species and crops, and is well known for showing extensive morphological and genetically diversity (Christopher et al., 2005). Early flowering winter annual species likely cope with lower temperature during growth and reproduction compared to late-spring flowering species. We, therefore, framed a hypothesis that strategies and mechanisms to develop or maintain their freezing tolerance can be different among species adapted to these differential growth conditions.

To conclude, the final chapter gives a general discussion of the most main interesting findings from this thesis.

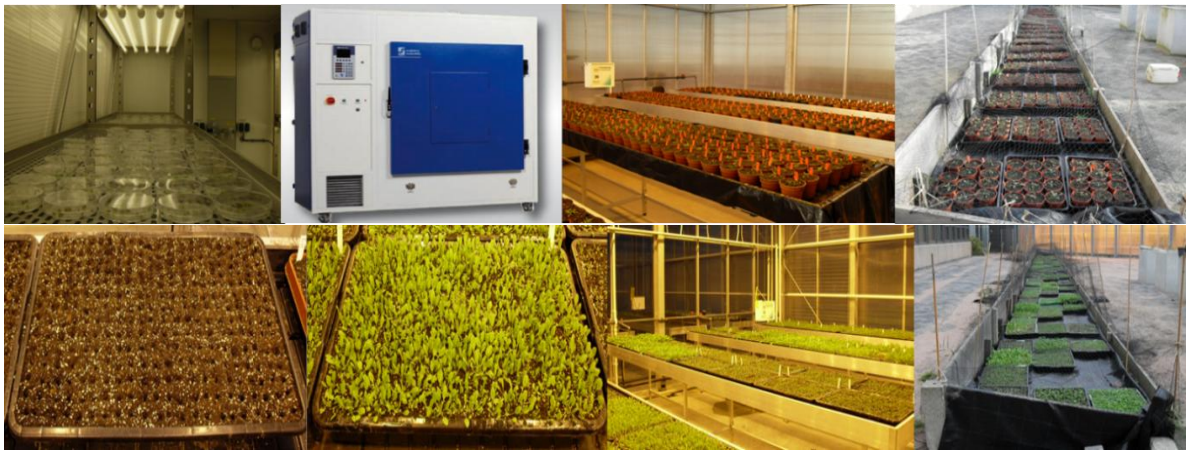

Figure 4. Experimental setup for this thesis.

Chapter 2. Identification of Quantitative Trait Loci and a Candidate Locus for Freezing Tolerance in Controlled and Outdoor Environments in the Overwintering Crucifer *Boechera stricta*

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ABSTRACT

Development of chilling and freezing tolerance is complex and can be affected by photoperiod, temperature and photosynthetic performance; however, there has been limited research on the interaction of these three factors. We evaluated 108 recombinant inbred lines of *Boechera stricta*, derived from a cross between lines originating from Montana and Colorado, under controlled Long-Day (LD), Short-Day (SD) and in an Outdoor Environment (OE). We measured maximum quantum yield of photosystem II, lethal temperature for 50% survival and electrolyte leakage of leaves. Our results revealed significant variation for chilling and freezing tolerance and photosynthetic performance in different environments. Using both single and multi-trait analyses, three main-effect Quantitative Trait Loci (QTL) were identified. QTL on LG3 were SD-specific, whereas QTL on LG4 were found under both LD and SD. Under all conditions, QTL on LG7 were identified, but were particularly predictive for the Outdoor Experiment. The co-localization of photosynthetic performance and freezing tolerance effects supports these traits being co-regulated. Finally, the major QTL on LG7 is syntenic to the Arabidopsis *CBF* locus, known regulators of chilling and freezing responses in *A. thaliana* and other species.

I. INTRODUCTION

Genetic variation for freezing tolerance is important for understanding both adaptation of species to natural environments and for improving crop performance in stressful conditions. Research over the last decades has demonstrated that complex physiological and biochemical changes occur in a wide range of plant species during cold acclimation, including both freezing-tolerant and freezing-sensitive plants (Leinonen, 1996; Hannah et al. 2006; Carvallo et al. 2011). The level of tolerance to freezing in plants is influenced by membrane and cell composition, the accumulation of carbohydrates and adjustments to the photosynthetic apparatus (Sandve et al. 2011). Some low-temperature differentially-regulated genes can be associated with these important changes and can be used for understanding the mechanisms of chilling and freezing tolerance (Thomashow, 2010).

QTL (Quantitative Trait Locus) studied in *Arabidopsis thaiana* and temperate Triticeae species of the grass family have shown QTL regions containing CBF (C-repeat binding factor) genes are associated with their freezing tolerance. Previously, it has been established that three members of the CBF family, CBF1, CBF2 and CBF3, play a key role in the regulation of the transcriptome during cold acclimation (Maruyama et al. 2004; Xu et al. 2011). These CBF genes have been isolated from several herbaceous and woody plant species and different studies have demonstrated their significant role for development of freezing tolerance (Zhang et al. 2004; Skinner et al. 2005; Gamboa et al. 2007; Welling and Palva, 2008; He et al. 2012). CBFs have therefore been used to develop strategies to enhance freezing tolerance in cultivated crops and to understand adaptation to cold environments in native species. However, variation in *CBF* loci does not explain all the quantitative natural variation for freezing tolerance (Gery et al. 2011; Meissner et al. 2013). In addition, the ecological context of *Arabidopsis*, a model species, is often unclear due to their growth in highly disturbed environments.

The genus *Boechera* is closely related to *Arabidopsis* and is being developed as an additional model system to understand plant adaptation. *Boechera stricta* is a genetically tractable, short-lived, overwintering, perennial species. It grows mostly in undisturbed habitats of Western North America, with habitats widely varying in abiotic and biotic conditions. For example, populations can be found across a 2000 m elevation gradient, and this is expected to have an effect on the genetic variation of genes controlling ecologically important traits (Schranz et al. 2009). Previously, a number of genomic resources and a genetic map have been

developed for identifying ecologically relevant QTL in *B. stricta*, allowing extensive comparative analyses with Arabidopsis (Schranz et al. 2007a; Anderson et al. 2011; Anderson et al. 2014). Here, we utilized these resources to study freezing tolerance in *B. stricta* grown under both controlled and outdoor conditions. In addition, photosynthetic performance can be also associated with freezing tolerance because photosynthesis may be a critical factor for freezing or frost tolerance. However, there has been little research conducted on the genetic regulation of freezing tolerance and photosynthetic performance. Hence, we also analyzed photosynthetic performance by measuring the maximum quantum yield of photosystem II.

In this study, we found significant variation on freezing tolerance and photosynthetic performance to freezing stress conditions in *B. stricta* and also observe that photosynthetic performance may be genetically associated with freezing tolerance. Within the QTL analysis, we have identified three major QTLs, which will be useful for clarifying underlying ecologically important questions on freezing tolerance.

II. MATERIALS AND METHODS

PLANT MATERIALS

For all experiments we used 108 selected RIL lines and parental genotypes from a population derived from a cross between two highly inbred lines of *B. stricta* (Graham) Al-Shehbaz. The maternal line 'SAD12' was collected from Colorado (elevation: 2530m) and the paternal line 'LTM' was collected in Montana (elevation: 2390m). The parental sites in Montana and Colorado differ in rainfall, temperature, day length and ecological community (Schranz et al. 2007b). The F_7 RILs have previously been genotyped and used in mapping and QTL experiments (Anderson et al. 2011).

PLANT GROWTH

Seeds were germinated in petri dishes, and then transferred onto pots with the soil mixture of No.1 and No.3 soil (Jongkind Ground BV) in a 1:2 proportion. Plants were established for three to four weeks, and then used to perform three different freezing stress experiments: two controlled climate chamber experiments differing in photoperiod, Long Day (LD) and Short Day (SD), and an Outdoor Environment (OE). Day-lengths in outdoor environment were slightly shorter in Amsterdam than the native field site in Montana.

CONTROLLED LD and SD FREEZING STRESS EXPERIMENTS

RIL and parental lines were grown at 20°C in growth chambers under LD and SD photoperiod regimes. The LD photoperiod was 14 hours light/10 hours darkness, while the SD photoperiod was 10 hours light/14 hours darkness. Details ofExperimental Methods are given below, but are alluded to here in relation to plant growth. One-month-old plants were acclimated to cold by growing at 6°C for 3 weeks. A freezing treatment was done in darkness at -8°C for 24 hours. Ice crysatilization was induced using ice chips. Plants and the soil were totally frozen. Plants were then returned to 6°C. Relative freezing tolerance in RILs and their parental lines was measured before and after 3 weeks of cold acclimation, and the electrolyte leakage was screened immediately after a freezing treatment of 24 hours, and 1 day after they were returned to 6°C to investigate their actual responses to selected freezing temperature. Maximum quantum yield of Photosystem II (Fv/Fm) was measured before and after cold acclimation, and 1 day after freezing treatment, and 1 day after they were returned to 6°C to observe the changes in the photosynthetic performance. To minimum a gradient in damage by harvesting samples, we conducted these experiments dividing into 6 sets, which consisted with 16~20 RILs per group. In addition, plants were randomly placed for the experiment.

OUTDOOR ENVIRONMENT (OE) EXPERIMENT

The freezing stress experiment in Outdoor Environment (OE) was conducted in the winter and early spring of 2011-2012. Responses during and post freezing, and frost damage were evaluated in the same 108 RILs. Plants were germinated and transplanted in mid-October, 2011. Three weeks after transplanting, three individuals/line from 108 RILs plus 3 individuals of each parental line (n= 330 individuals total) were randomized in a research plot area at the University of Amsterdam, The Netherlands and were maintained until the end of experiment. Figure 1 shows the minimum and maximum temperatures during the critical experimental period (January and February). It was a relatively warm early winter. The first sub-zero freezing event occurred on 16th January 2012 with a minimum temperature of -4°C. In February there was a much colder and prolonged period of freezing. The minimum air temperature was -17°C on 4th February 2012, the lowest temperature of the winter. Temperatures remained sub-zero until 13th February 2012. Responses of selected RILs and parental lines to outdoor freezing conditions were screened on both 17th January and 18th February 2012 by measuring Fv/Fm. Plants were frozen during the first and second cold spells, and were not covered with snow.

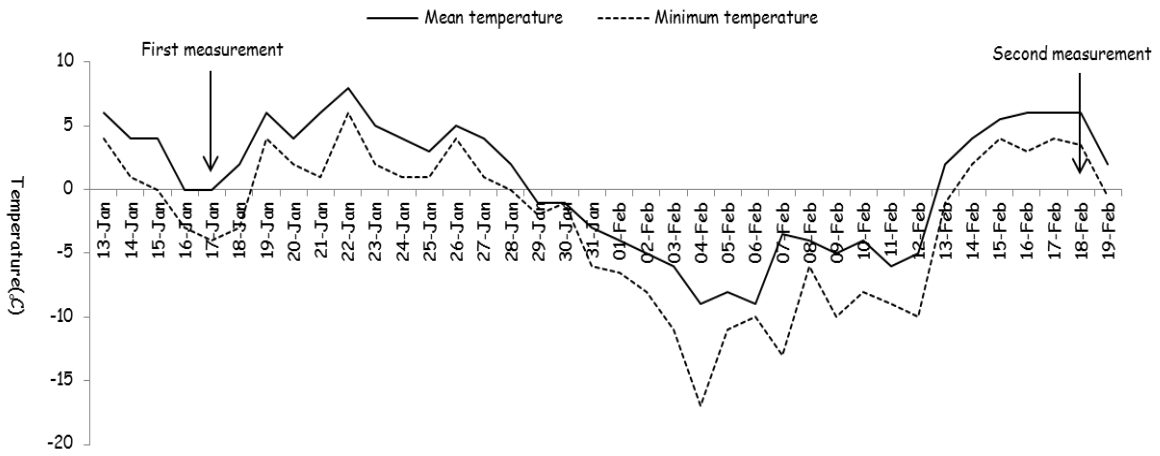

Figure 1. Maximum and Minimum daily temperatures for Outdoor Environment (OE) experienced by *Bochera stricta* Recombinant Inbred Lines (RILs) during January and February of 2012 in Amsterdam, The Netherlands. Responses to freezing-stress were assessed during a mild freezing event (January 18) and after a prolonged severe-freezing event (February 18). Three-week old plants were initially established outdoors in November 2011.

Temperature was measured in the field with use of HOBO® data loggers for outdoor use. The logger was placed at plant height. Overall frost damage from the winter was scored in the middle of March 2012.

TRAIT MEASUREMENTS

MEASUREMENT OF RELATIVE FREEZING TOLERANCE BEFORE AND AFTER COLD ACCLIMATION

Relative plant freezing tolerance was assayed as described previously (Murray et al. 1989), with some modifications. Three uniform leaf disks per plant per line were cut (diameter size of 0.5cm) from non-acclimated or cold-acclimated plants with the use of a leaf disc borer and placed into test tubes containing 100µl de-ionized water. Test tubes were subsequently placed in a completely randomized design in a –1°C Cooling Thermostats containing anti-freeze (Lauda Ecoline RE 312, Lauda Germany) for 1h after which ice crystals were added to nucleate freezing. After an additional 2h of equilibration at –1°C the samples were gradually cooled in increments of –2°C/h. A replicate sample from each genotype was removed every 2 h starting at –3°C until the last samples were removed at –13°C. Once removed the samples were stored on ice until all samples had been collected and left to thaw overnight in the cold room at 4°C. After thawing, all

samples, including the unfrozen controls kept at 4°C (ELunfrozen), were incubated in 1ml de-ionized water with gentle shaking (125 motions/min) at room temperature for 2h. Electrolyte leakage from the leaves was measured using a conductivity meter (B-173 compact conductivity meter, Horiba Scientific). The samples were then placed for 1h in a –80°C freezer, thawed for 30min in a 57°C stove, and shaken gently for an additional 2h before the conductivity of the resulting solution was measured to obtain a value for 100% electrolyte leakage (EL100). The percentage of electrolyte leakage from frozen leaves was calculated according to the equation as described by Webb et al. (1994): %EL = (ELfrozen–ELunfrozen) / (EL100–ELunfrozen) * 100. Response curves were developed for each plant and each LT_{50} was determined based on the sigmoidal response equation by the statistic program R (R Development Core Team. 2008). Two replicate measurements for each photoperiod/temperature combination were performed, and total 5280 individuals were used for this experiment (two temperature conditions x two photoperiods x six time samplings using 6 individuals x two replicates x 108 RILs plus two parental lines ; n= 5280 individual total).A principal component analysis (PCA) of Fv/Fm values under different conditions was also performed to visualize which environmental factors affected the Fv/Fm ratio of RILs most.

MEASUREMENT OF MAXIMUM QUANTUM YIELD OF PHOTOSYSTEM II (Fv/Fm)

Fv/Fm was determined with a PAM-2000 chlorophyll fluorometer system (Heinz Walz, Effeltrich, Germany) using standard instrument settings (e.g. saturating pulse of 12,000 mmol m^{-2} s^{-1} for 0.8s). Fluorescence data were recorded and computed with windows software for PAM fluorometers (2.133 version, Heinz Walz, Effeltrich, Germany). For determination of Fv/Fm, one uniform leaf disc from middle part of the sixth leaf was removed and placed into black 96-well plates containing 200µl de-ionized water. Leaves were dark adapted for 20 min prior to determination of Fv/Fm. In dark-adapted plants, F_0 and also levels of fluorescence measured at a very low photosynthetic photon flux density (PPFD) of, 1 $mmolm^{-2}$ s^{-1} and during a short light-saturating pulse (Fm) were measured and used to estimate the maximum quantum yield of PSII when fully oxidized (Fv/Fm= Fm – F_0/Fm). Three replicate measurements were performed for measuring Fv/Fm, and total 2640 individuals were used for this experiment (four temperature conditions x two photoperiods x three replicates x 108 RIL plus two parental lines ; n= 2640 individual total).

MEASUREMENT OF ELECTROLYTE LEAKAGE DURING AND POST FREEZING

Plant responses during and after freezing were measured by another type of electrolyte leakage assay (Scarpeci et al. 2008), with the protocol slightly modified. Three uniform leaf discs (Ø 0.5cm) from rosette leaves in the same plant being used for measurement of Fv/Fm were sampled with a leaf disc borer and immediately placed into 15ml Greiner tube containing 10ml de-ionized water. The discs were washed for 30min on a shaking platform to remove soil attached during cutting of the disc. Leaf discs were then placed in 12-well plates filled with 3ml of 0.01% Silwet 77 solution and maintained at 25°C for 1hr 30min on a shaking platform. Next, initial leakage was determined by measuring the electrical conductivity of the well plate solution, using a conductivity meter (B-173 compact conductivity meter, Horiba Scientific) with data expressed as $m^{S}cm^{-1}$. The samples were then placed for 2 x 20 sec in microwave (600W) and shaken gently for an additional 4 h before the conductivity of the resulting solution was measured to obtain a value for electrolyte leakage of the heat treated cells. Results were expressed as percentage of total conductivity 'initial leakage / final leakage x 100'. Measurements were carried out in triplicate.

ASSESSMENT OF WINTER SURVIVAL

Winter survival was visually scored on a scale of 0–10 using three individuals among families (0: plant was killed, 10: no apparent frost damage).

QUANTITATIVE TRAIT LOCI ANALYSIS

Single-trait, multi-trait linkage and mixed model multi-environment QTL analyses were performed with GenStat software (15^{th} edition, VSN International, United Kingdom). To normalize results, all QTL analysis was carried out with log10 transformed-adjusted means of each trait. For single-trait QTL analysis, the data was firstly used for the preliminary single environment analysis. Subsequently calculations of the genetic predictors were conducted with a step size of 2cM, and then an initial Genome Scan produced candidate QTL positions by Simple Interval Mapping (SIM). SIM results were then used as cofactors in a subsequent Genome Scan by Composite Interval Mapping (CIM), which was allowed to detect candidate QTLs. The final selection of significant QTL was obtained after a scan with backward elimination of putative QTL. LT_{50}, EL, and Fv/Fm results were subjected to a multi-trait linkage QTL analysis. To arrive at a multi-QTL model, the significant markers, or putative QTL, of the single marker analyses

were used as the starting input set of predictor variables. After completion of this step, genome wide QTL scans by SIM and CIM were performed and then a multi-QTL model after backward selection was fitted to estimate QTL locations and effects (Pastina et al. 2012). For Fv/Fm, mixed model multi-environment QTL analysis was additionally applied. The best suitable variance–covariance model was detected after environment exploratory analysis. Genetic predictors were computed for a step size of 2cM. The genetic model was built using the suggested candidate QTL as main or QTL x Environment (QTLxE) interactions. CIM was used for selecting the final QTL model. QTL significance levels and effects were determined by a final backward selection step at a significance level of 0.05. A significant QTL effect at particular genome positions was associated with a low P value, which was graphically shown on a –log10 scale to resemble the typical LOD profile plot (Pastina et al. 2012). The additive effects, standard errors, high value alleles, the Percentage of Explained Variances (PVE) and positions of the QTLs were estimated and used to determine what traits were affected by the specific QTL.

STATISTICAL ANALYSIS

All further statistical tests were also performed using the GenStat software package (15th edition, VSN International, United Kingdom). To examine the correlation among traits measured across environmental conditions, correlation coefficients (Pearson's) were calculated. A principal component analysis (PCA) of Fv/Fm values under different conditions was also performed to visualize which environmental factors affected the Fv/Fm ratio of RILs most.

III. RESULTS

RELATIVE FREEZING TOLERANCE (RFT) UNDER SD AND LD CONDITIONS

Boechera stricta RILs and parental lines were grown under two photoperiods (SD and LD). The lethal temperature for 50% survival (LT_{50}) was estimated from fitted response curves. The average values, ranges and differences among RILs are presented in Table 1. Non-acclimated RILs and parental lines showed small differences in LT_{50} under either photoperiod conditions. After cold acclimation with SD grown plants, the LT_{50} of the parental lines LTM and SAD12 were –11.3°C and –8.6°C, respectively (delta LTM to SAD12 = -2.7°C). Under LD growth conditions, the LT_{50} of the LTM and SAD12 were –10.4°C and –8.1°C, respectively (delta LTM to SAD = - 2.3°C). LTM shows more freezing tolerance, but also more pronounced difference in

SD relative to LD (delta of LTM SD-LD = -0.9°C; delta of SAD12 SD-LD = -0.5°C). In both light regimes, most RILs had intermediate LT_{50} values to the parents. Similar to results with parental lines, the LD grown RILs on average were less freezing tolerant than SD grown RILs.

Table 1. LT50, Fv/Fm and EL in the RIL population (LTM x SAD12) under control and stressed conditions

	LTM	SAD12	Range in RIL population	Mean in RIL population
LT50(SD_CA)	-11.10	-8.60	-5.10 ~ -14.20	-10.07
LT50(SD_NA)	-6.40	-5.70	-4.60 ~ -7.20	-6.21
Fv/Fm(SD_NA)	0.80	0.80	0.78 ~ 0.81	0.80
Fv/Fm(SD_CA)	0.77	0.77	0.75 ~ 0.79	0.77
Fv/Fm(SD_FT)	0.71	0.65	0.54 ~ 0.77	0.68
EL(SD_FT)	26.90	35.30	17.80 ~ 57.10	28.94
Fv/Fm(SD_1DPF)	0.71	0.58	0.21 ~ 0.77	0.63
EL(SD_1DPF)	24.80	44.60	16.20 ~ 74.10	32.69
LT50(LD_CA)	-10.40	-8.10	-4.70 ~ -14.20	-9.33
LT50(LD_NA)	-6.30	-5.80	-4.40 ~ -7.10	-6.00
Fv/Fm(LD_NA)	0.81	0.80	0.79 ~ 0.82	0.80
Fv/Fm(LD_CA)	0.77	0.76	0.74 ~ 0.78	0.76
Fv/Fm(LD_FT)	0.68	0.60	0.55 ~ 0.75	0.66
EL(LD_FT)	29.10	41.00	20.20 ~ 60.40	32.12
Fv/Fm(LD_1DPF)	0.67	0.42	0.16 ~ 0.77	0.57
EL(LD_1DPF)	28.80	52.50	18.20 ~ 80.10	39.00
Fv/Fm(OE_MF)	0.66	0.57	0.41 ~ 0.72	0.63
Fv/Fm(OE_SF)	0.38	0.37	0.02 ~ 0.64	0.38
OWFD	3.30	4.00	0.307.30	3.32

SD ; short day, LD ; long day, OE : outdoor environment, CA : cold acclimation, FT : freezing treatment, 1DF : 1 day post freezing, MF : mild freezing (Jan., 2012), SF : severe freezing (Feb., 2012)

Table 2. Phenotypic correlations among traits across different environmental conditions

	Control				Cold acclimation				Freezing treatment				1 day post freezing treatment				outdoor environment		
Variable	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3
Control																			
1. LT_{50} (SD)	1																		
2. LT_{50} (LD)	.73**	1																	
3. Fv/Fm (SD)	-.17	-.11	1																
4. Fv/Fm (LD)	-.08	-.09	-.09	1															
Cold acclimation																			
1. LT_{50} (SD)	.67**	.62**	-.10	.01	1														
2. LT_{50} (LD)	.68**	.67**	-.07	.02	.95**	1													
3. Fv/Fm (SD)	-.29**	-.22*	.19*	-.04	-.28**	-.18	1												
4. Fv/Fm (LD)	-.19	-.14	.42**	-.05	-.16	-.12	.26**	1											
Freezing treatment																			
1. EL (SD)	.69**	.65**	-.15	-.01	.86**	.83**	-.33**	-.22*	1										
2. EL (LD)	.68**	.65**	-.14	-.01	.80**	.81**	-.30**	-.19	.85**	1									
3. Fv/Fm (SD)	-.63**	-.60**	.14	-.04	-.91**	-.86**	.29**	.18	-.93**	-.79**	1								
4. Fv/Fm (LD)	-.63**	-.57**	.07	.04	-.77**	-.80**	.26**	.17	-.79**	-.84**	.77**	1							
1 day post freezing treatment																			
1. EL (SD)	.69**	.61**	-.11	-.03	.874**	.86**	-.32**	-.18	.91**	.82**	-.89**	-.77**	1						
2. EL (LD)	.63**	.54**	-.16	-.01	.844**	.86**	-.32**	-.19*	.84**	.84**	-.85**	-.80**	.88**	1					
3. Fv/Fm (SD)	-.66**	-.59**	.10	.07	-.825**	-.80**	.34**	.17	-.87**	-.82**	.83**	.69**	-.95**	-.85**	1				
4. Fv/Fm (LD)	-.62**	-.51**	.17	-.00	-.808**	-.81**	.35**	.19*	-.83**	-.80**	.84**	.78**	-.89**	-.97**	.86**	1			
Outdoor environment																			
1. Overall winter damage	-.08	-.16	-.05	.01	-.06	-.06	.01	-.03	-.01	-.05	.03	-.08	-.03	.05	.05	-.05	1		
2. Fv/Fm (MF)	-.13	-.23*	.11	-.08	-.20*	-.23*	.26**	.14	-.20*	-.21*	.23*	.18	-.21*	-.23*	.20*	-.23*	.40**	1	
3. Fv/Fm (SF)	-.06	-.16	-.05	-.14	-.12	-.12	.21*	.04	-.05	-.11	.10	.06	-.07	-.04	.06	.04	.69**	.56**	1

** means correlation is significant at the 0.01 level (2-tailed) and * means correlation is significant at the 0.05 level (2-tailed).

SD ; short day, LD ; long day, MF : mild freezing (Jan., 2012), SF : severe freezing (Feb., 2012)

ELECTROLYTE LEAKAGE (EL) AND MAXIMUM QUANTUM YIELD OF PHOTOSYSTEM II (Fv/Fm)

We quantified EL of leaves from plants exposed to freezing temperatures. EL increased during or after freezing treatment, and genotypic responses were highly associated with LT50 values. The maximum quantum yield of photosystem II, Fv/Fm, was measured to examine its relationship with cold acclimation ability and actual freezing tolerance. During cold acclimation, Fv/Fm decreased slightly in most of RILs. Although differences on Fv/Fm were detected among RILs, the variation in Fv/Fm was small and no significant correlation between LT_{50} and Fv/Fm was observed. The freezing treatment in darkness for 24 h led to a significant reduction in Fv/Fm. Different responses of the Fv/Fm ratio and EL were observed among RILs and parental lines during and after freezing treatment (Table 1). Interestingly, Fv/Fm was highly correlated with electrolyte leakage assays (Table 2). Plants responded differently to freezing treatment, depending on the photoperiod during cold acclimation. Large variation among RILs was detected 1-day after freezing treatment among experimental time points in both photoperiod conditions (Table 1, see SD_1DFT and LD_1DFT). SAD12, which showed less freezing tolerance, also had lower Fv/Fm than LTM. Similarly, Fv/Fm was negatively affected by freezing, but the decrease of Fv/Fm in freezing tolerant RILs was much less than in freezing sensitive RILs (data not shown).

MAXIMUM QUANTUM YIELD AND WINTER FROST DAMAGE OF OUTDOOR ENVIRONMENT (OE) FREEZING CONDITIONS

In Outdoor Environment (OE) conditions, we measured Fv/Fm to evaluate freezing injury after two freezing periods during the winter of 2011-2012 (Table 1). The first measurement was carried out during a mild freezing event (MF) in mid-January, the first freezing event of the year. Both parental lines had relatively low Fv/Fm values (LTM 0.66, SAD12 0.57), showing both lines suffered from the freezing event. The RILs showed a greater variation in values (0.41-0.72). The second measurement was performed after a prolonged severe freezing (SF) event in mid-February. Significant freezing damage was observed with Fv/Fm values for parental lines and RILs (LTM=0.38, SAD12=0.37, RILs=0.02-0.64). Thus, while the difference between the two parents was minimal in the severe cold period, more variation was observed in the RILs. Interestingly, genotypic responses to two outdoor freezing stress conditions did not correlate well with those under controlled freezing stress conditions (Figure 2). The extreme freezing

events of late winter caused the death of individual plants in some RILs. However, it was sometimes observed that overall winter damage scores for some RILs with low Fv/Fm had less final frost damage due to successful re-growth or recovery of the plants during warm temperatures in early spring.

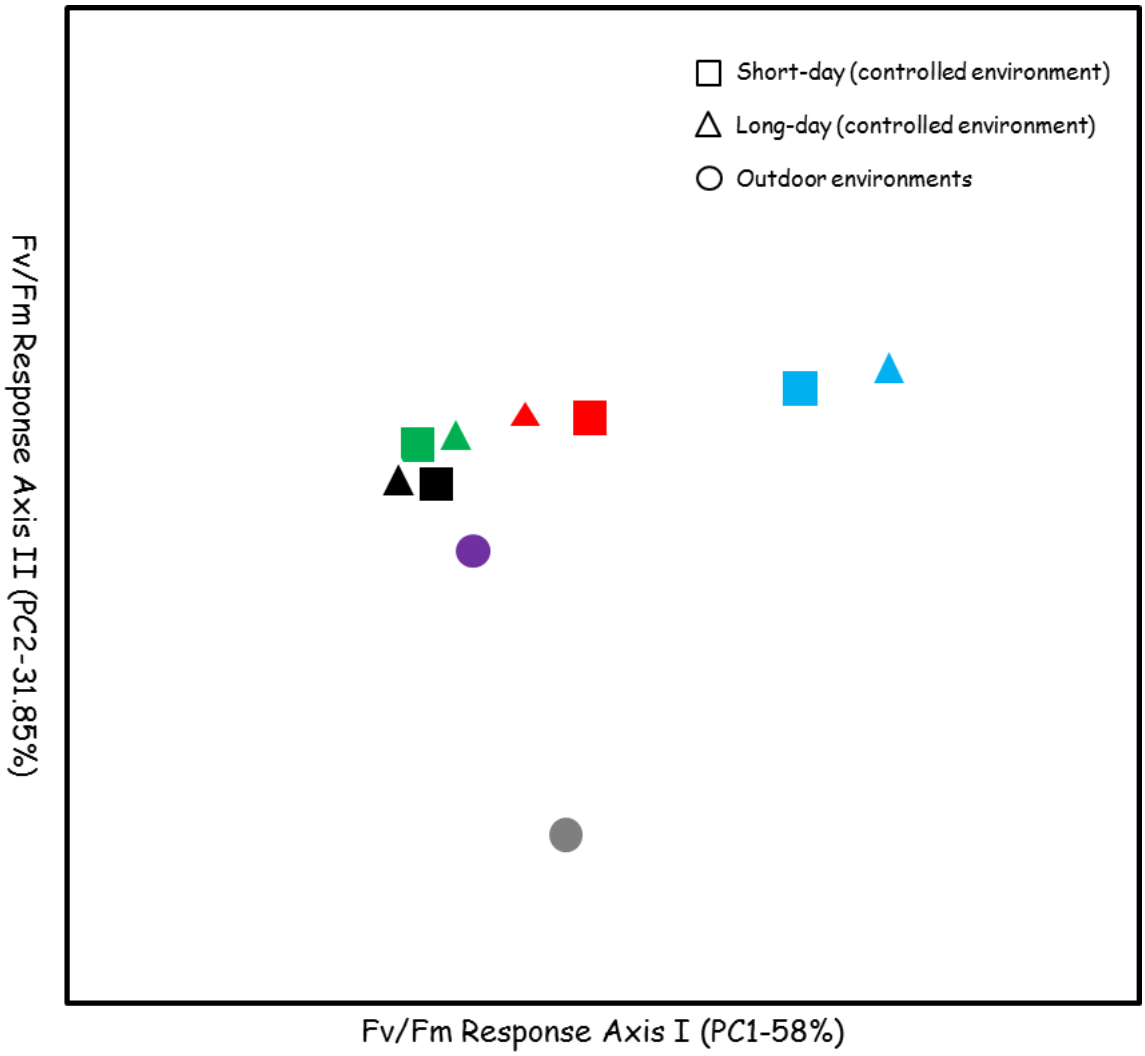

Figure 2. First two principal component axes of Fv/Fm responses of 108 Recombinant Inbred Lines of *Boechera stricta* grown under short-day/controlled environment (squares) long-day/controlled environment (triangles), and outdoor environment (circles) and measured after various temperature regimes. black = non-acclimated at 20°C; green = cold at 6°C for 3 weeks; red = freezing at -8°C for 24 hour;, blue = 1 day post-freezing; purple = outdoor mild-freezing January, 2012; gray = outdoor severe-freezing February, 2012)

QTL ANALYSIS

Complex traits such as freezing tolerance need to be measured for collections of genotypes across multiple environments, because the relative performance of genotypes can change between environments, a well-known phenomenon called genotype by environment interaction. To identify constitutive and inducible QTLs controlling freezing tolerance in *B. stricta* efficiently, we performed multiple trait QTL analysis to test for pleiotropy or close linkage of QTLs, as well as mixed model based multi-environment single trait QTL analysis to detect QTLxEnvironment (QTLxE) interaction loci. Three QTLs were identified by traditional single trait analysis, i.e., on LG 3, 4 and 7 for Fv/Fm, EL and LT50, and the QTL of traits tended to be overlapping (Table 3). Multiple traits QTL analysis revealed that Fv/Fm was genetically associated with freezing tolerance-related traits. Multi-environment single trait QTL analysis using Fv/Fm also identified three QTLs on LG 3, 4 and 7, QTL on LG 7 showed significant QTLxE interactions (Table 4).

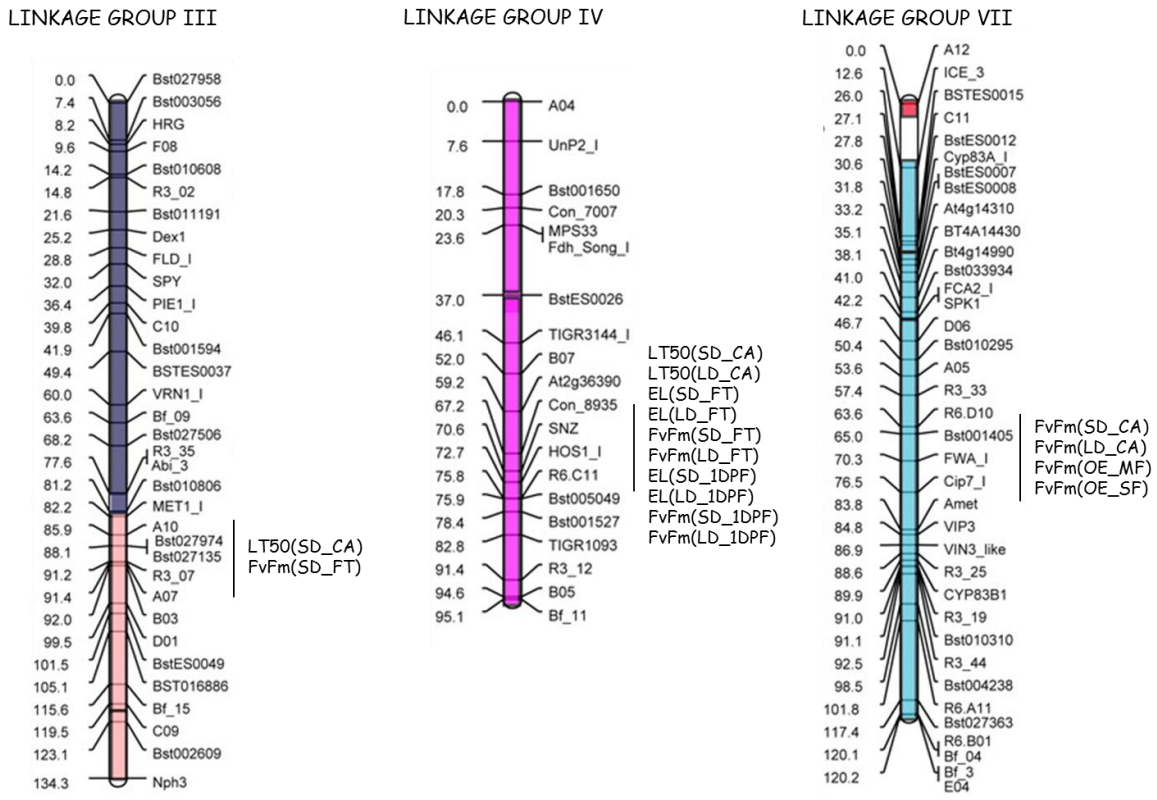

Figure 3. Quantitative Trait Loci (QTL) identified for several traits under variable conditions on the three of the seven Linkage Groups (LG) of this study. SD ; short day, LD ; long day, OE : outdoor environment, CA : cold acclimation, FT : freezing treatment, 1DF : 1 day post freezing, MF : mild freezing (Jan., 2012), SF : severe freezing (Feb., 2012)

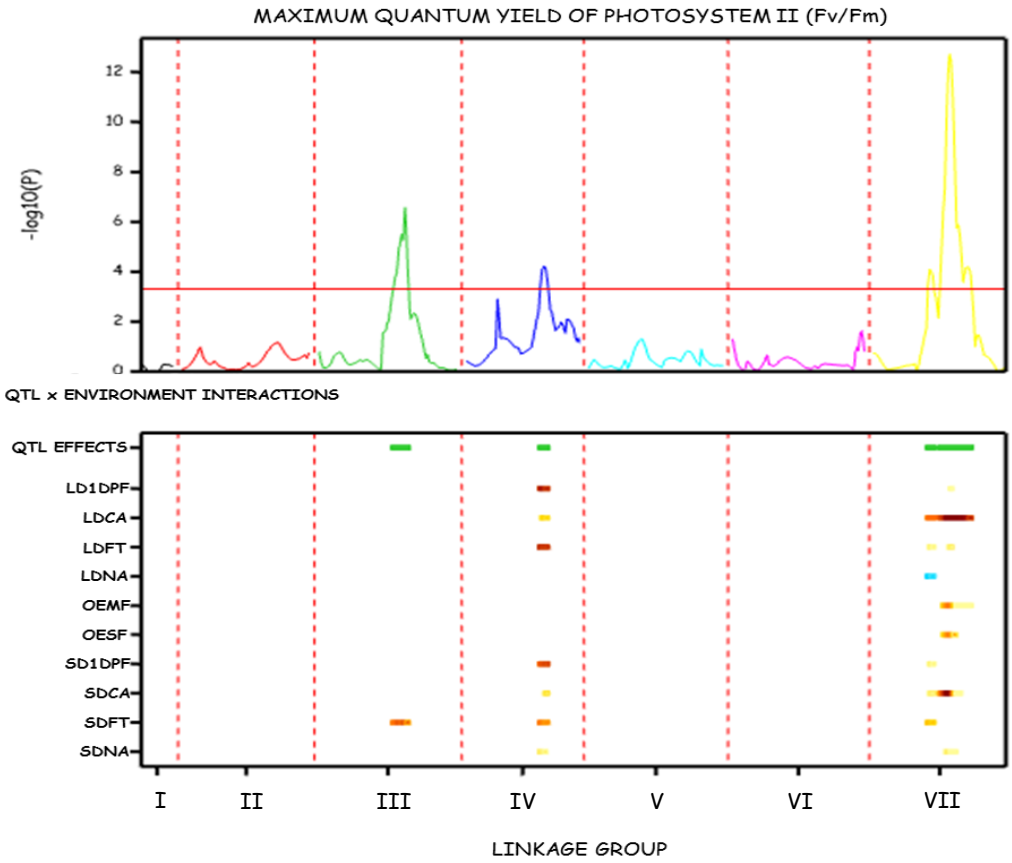

Figure 4. QTL profiles of LTM x SAD12 population, showing chromosomal regions with significant QTLs (upper panel) and their additive effect across environments. SD ; short day, LD ; long day, OE : outdoor environment, CA : cold acclimation, FT : freezing treatment, 1DF : 1 day post freezing, MF : mild freezing (Jan., 2012), SF : severe freezing (Feb., 2012)

SINGLE TRAIT QTL ANALYSIS

For LT_{50} values of non-acclimated RILs in both LD and SD controlled conditions no significant QTLs were identified, likely due to the relative lack of trait variance among RILs. Two LT50 QTL regions for cold acclimated plants were identified, one that was SD specific (LG 3, marker A10) and one common to LD and SD (LG 4, marker At2g36390) (Figure 3 and Table 3). For SD conditions, the accumulated total phenotypic variation explained by the two QTLs was 23.9% (11.7% by LG 3 and 12.2% by LG 4). The single QTL for LT_{50} was found only for long-photoperiod, with -log10(p) = 3.18, and phenotypic variation of this QTL was 11.0%. All superior alleles for freezing tolerance originated from the LTM parent.

For EL and Fv/Fm measurements from different photoperiods and experimental time points, CIM mapping identified a total of 11 QTLs. One putative QTL for Fv/Fm in acclimated

plants grown under SD and LD was identified on linkage group 7. The QTLs on linkage group 7 explained 20.9% and 21.8% for short and long photoperiod, respectively. One QTL for Fv/Fm and EL was detected for the freezing treatment and the 1st day after freezing stress in both photoperiods, co-located on linkage group 4. We also identified other QTLs under different time points. One interesting QTL was identified on linkage group 3 for Fv/Fm at freezing treatment under short photoperiod, overlapping with the locus for LT_{50} under short photoperiod. Under outdoor freezing stress conditions, no putative QTL was observed for overall winter damage. Fv/Fm measurements during winter, however, revealed one QTL region on linkage group 7 that exceeded the significance threshold across stress conditions.

Table 3. Summary of putative QTLs controlling freezing tolerant and photosynthetic performance related traits in the single trait QTL model

Condition	Trait	Linkage group	Locus name	LOD Score	% Expl. Var.	Add.eff	High value allele
Cold acclimation	LT50_SD	3	A10	3.95	11.69	0.03	LTM
	LT50_SD	4	At2g36390	3.94	12.21	0.03	LTM
	LT50_LD	4	At2g36390	3.17	11.01	0.02	LTM
	Fv/Fm_SD	7	Bst001405	5.42	20.95	0.00	LTM
	Fv/Fm_LD	7	Bst001405	5.63	21.76	0.00	LTM
Freezing treatment	EL_SD	4	At2g36390	4.01	14.21	0.04	SAD12
	EL_LD	4	At2g36390	3.12	10.83	0.03	SAD12
	Fv/Fm_SD	3	A10	4.48	13.86	0.01	LTM
	Fv/Fm_SD	4	At2g36390	3.01	8.89	0.01	LTM
	Fv/Fm_LD	4	At2g36390	3.97	14.07	0.01	LTM
1 day post freezing treatment	EL_SD	4	At2g36390	4.90	17.52	0.07	SAD12
	EL_LD	4	At2g36390	5.07	18.16	0.07	SAD12
	Fv/Fm_SD	4	At2g36390	3.48	12.18	0.03	LTM
	Fv/Fm_LD	4	At2g36390	3.93	13.91	0.05	LTM
Outdoor environment	Fv/Fm_MF	7	Bst001405	3.95	15.06	0.01	LTM
	Fv/Fm_SF	7	Bst001405	3.98	15.18	0.09	LTM

SD ; short day, LD ; long day, MF : mild freezing (Jan., 2012), SF : severe freezing (Feb., 2012)

MULTI-TRAIT QTL ANALYSIS AND MULTI-ENVIRONMENT SINGLE TRAIT QTL ANALYSIS

One QTL for EL collocated with a QTL for Fv/Fm from single trait QTL analysis under controlled environments. In order to confirm if freezing tolerant-related traits were associated with photosynthetic performance-related traits, a multi-trait QTL mapping approach was applied, in which variation for several traits was analyzed simultaneously. The results presented in Table S2 showed that most QTLs identified by single-trait analyses were still significant in the multi-trait QTL model. The QTL for EL and Fv/Fm detected on linkage group 4 post freezing was also identified in the multi-trait QTL analysis and the QTL for EL was strongly associated with Fv/Fm. This result suggests that these traits may be co-regulated. Multi-trait QTL analysis revealed exceptions for the short day/cold acclimated (SDCA) and short day/freezing treated (SDFT) conditions. The LT50 QTL on linkage group 3 in SDCA was no longer significant in multi-trait model, reflecting a lack of association with Fv/Fm. By contrast, multivariate analysis detected an additional QTL on linkage group 2 which was not identified with the single-QTL model for SDFT.

Multi-environment, single trait QTL analysis for Fv/Fm was performed to evaluate QTL by environment interaction. Three QTL x E interaction loci were found, on linkage group 3, 4 and 7 (Figure 4 and Table 4). The QTL of linkage group 3 was shown to be highly significant (-log10(p) = 6.57), mainly due to a substantial effect from the specific environment (SDFT). Linkage group 4 contained a QTL (-log10(p) = 4.21), which had relatively high effects on Fv/Fm in controlled environments, but it had little effect in outdoor environments. The most significant QTL x E effect (-log10(p) = 12.45) in the multi-environment QTL model was detected on linkage group 7. Percentage of total phenotypic variation explained was between 0.2 and 22.3%, depending on environmental conditions. This locus had little effect on Fv/Fm in controlled non-acclimated and freezing treated conditions, but it was consistently present in cold or freezing stress conditions, with the superior LTM allele active in the most stressful conditions (Table 4). Our results suggest that QTLs on linkage group 7 would have important roles for freezing tolerance in all environments, while QTLs on linkage group 3 and 4 would be more environment-specific.

Table 4. Summary of putative QTLs controlling freezing tolerant and photosynthetic performance related traits in the multi-trait QTL model

Condition	Linkage group	Locus name	Position	LOD Score	Traits	Add.eff.	High value allele	% Expl. Var.	P	s.e.
SD_CA	4	At2g36390	83.33	4.30	LT50	0.03	LTM	15.50	0.00	0.00
					Fv/Fm	0.00	LTM	2.60	0.07	0.00
	7	Bst001405	83.33	5.99	LT50	0.02	LTM	5.40	0.01	0.00
					Fv/Fm	0.00	LTM	21.70	0.00	0.00
LD_CA	4	At2g36390	83.33	4.58	LT50	0.02	LTM	11.70	0.00	0.00
					Fv/Fm	0.00	LTM	5.60	0.00	0.00
	7	Bst001405	83.33	7.11	LT50	0.01	LTM	4.40	0.03	0.00
					Fv/Fm	0.00	LTM	22.90	0.00	0.00
SD_FT	2	MAF1	121.88	3.82	EL	0.00	LTM	0.00	0.96	0.01
					Fv/Fm	0.00	SAD12	1.00	0.26	0.00
	3	A10	92.74	12.92	EL	0.02	SAD12	3.90	0.03	0.01
					Fv/Fm	0.01	LTM	12.20	0.00	0.00
	4	At2g36390	83.33	3.79	EL	0.04	SAD12	12.80	0.00	0.01
					Fv/Fm	0.01	LTM	8.60	0.00	0.00
LD_FT	4	At2g36390	83.33	3.53	EL	0.03	SAD12	10.80	0.00	0.01
					Fv/Fm	0.01	LTM	14.10	0.00	0.00
SD_1DPF	4	At2g36390	83.33	4.56	EL	0.07	SAD12	17.50	0.00	0.01
					Fv/Fm	0.03	LTM	12.20	0.00	0.01
LD_1DPF	4	At2g36390	83.33	4.76	EL	0.07	SAD12	18.20	0.00	0.01
					Fv/Fm	0.05	LTM	13.90	0.00	0.01

SD ; short day, LD ; long day, CA ; cold acclimation, FT ; freezing treatment, 1DF : 1 day post freezing treatment

Table 5. Descriptive summary results from multi-environment QTL model for Fv/Fm

Linkage group	Locus name	Position	LOD Score	Environment	Add.eff.	High value allele	% Expl. Var.	P	s.e.
3	A10	92.74	6.57	SD_NA	0.00	SAD12	0.10	0.74	0.00
				LD_NA	0.00	SAD12	0.00	0.90	0.00
				SD_CA	0.00	LTM	0.00	0.82	0.00
				LD_CA	0.00	SAD12	0.00	0.93	0.00
				SD_FT	0.02	LTM	12.80	0.00	0.01
				LD_FT	0.00	LTM	0.00	0.98	0.00
				SD_1DPF	0.02	LTM	2.10	0.11	0.01
				LD_1DPF	0.02	LTM	1.00	0.26	0.01
				OE_MF	0.01	LTM	0.60	0.39	0.01
				OE_SF	0.01	LTM	0.90	0.32	0.01
4	At2g36390	83.33	4.21	SD_NA	0.00	LTM	5.00	0.02	0.00
				LD_NA	0.00	LTM	0.00	0.89	0.00
				SD_CA	0.00	LTM	2.60	0.08	0.00
				LD_CA	0.00	LTM	5.40	0.01	0.00
				SD_FT	0.02	LTM	9.40	0.00	0.01
				LD_FT	0.02	LTM	15.20	0.00	0.01
				SD_1DPF	0.04	LTM	13.40	0.00	0.01
				LD_1DPF	0.06	LTM	17.20	0.00	0.01
				OE_MF	0.00	LTM	0.10	0.70	0.01
				OE_SF	0.01	SAD12	0.50	0.45	0.01
7	Bst001405	83.33	12.45	SD_NA	0.00	LTM	4.60	0.04	0.00
				LD_NA	0.00	SAD12	0.20	0.65	0.00
				SD_CA	0.00	LTM	21.40	0.00	0.00
				LD_CA	0.00	LTM	22.30	0.00	0.00
				SD_FT	0.01	LTM	2.50	0.08	0.01
				LD_FT	0.01	LTM	4.40	0.03	0.01
				SD_1DPF	0.02	LTM	3.00	0.07	0.01
				LD_1DPF	0.03	LTM	3.60	0.04	0.01
				OE_MF	0.02	LTM	13.90	0.00	0.01
				OE_SF	0.05	LTM	12.90	0.00	0.01

SD ; short day, LD ; long day, OE : outdoor environment, CA ; cold acclimation, FT ; freezing treatment, 1DF : 1 day post freezing treatment, MF : mild freezing (Jan., 2012), SF : severe freezing (Feb., 2012)

DISCUSSION

Boechera stricta provides an excellent model system to study ecologically important traits such as freezing tolerance. There have often been observed differences in QTLs found under control versus outdoor conditions (Weinig et al. 2002; Martin et al. 2006). For example, a QTL study of flowering time in *B. stricta* identified different loci under different growth conditions (Anderson et al. 2011). Development of freezing tolerance can also be highly influenced by environmental factors such as temperature and photoperiod conditions. Thus, we conducted our QTL study under varying day-length conditions in a controlled environment, and also under outdoor conditions where plants experienced two periods of outdoor freezing stress to provide further information on freezing tolerance (see Table S3 for a summary). We have found significant variation for freezing tolerance and photosynthetic performance to freezing stress conditions in *B. stricta*, and also show that photosynthetic performance-related trait may be genetically associated with freezing tolerance. Below, we discuss our findings and identify a potential candidate locus.

EFFECT OF PHOTOPERIOD ON FREEZING TOLERANCE

Developing freezing tolerance is a complex phenomenon. Freezing tolerance is highly influenced by environmental factors such as duration of cold exposure, conditions of photoperiod, and developmental stage. Transcript profiling studies have shown that hundreds of genes are up-regulated in *A. thaliana* andthey can be differently regulated with responses to environmental conditions in many species (Limin et al. 1997; Skinner et al. 2005; Vagujfalvi et al. 2005; Miller et al. 2006; Baga et al. 2007). Photoperiod condition is an important factors controlling freezing tolerance, but the genetic mechanism is still poorly understood.

In the climate chamber, we determined relative freezing tolerance (LT_{50}) using one-month old RILs and their parents before and after cold acclimation for 3 weeks in different photoperiods. Electrolyte leakage (EL) was also quantified to assess the damage of cellular membranes during and after the freezing treatment. Our genetic analysis indicated that several regulatory factors may influence freezing tolerance in both photoperiods. A total of six LT50- and EL-related QTLs identified across experimental conditions showed intermediate effects. The most significant QTL for freezing tolerance was located on linkage group 4. This QTL was identified across experimental conditions (both LD and SD) and with two types of electrolyte leakage methods, indicating that it may contain genes important for freezing tolerance by

protecting membrane integrity. Another interesting QTL was identified on linkage group 3. Although the effect of this QTL was small, it was detected by LT50 measurements only after cold acclimation grown in the short photoperiod condition. This result indicates that short day can be contributed to the increase of freezing tolerance in B. stricta. It has been reported that freezing tolerance in Gaura coccinea is enhanced with cold treatment when they are exposed to short-day photoperiods (Pietsch et al. 2009). Similarly, cold acclimation ability in barley is enhanced by a short photoperiod condition in combination with low acclimating temperatures, which is associated with a short photoperiod induced delay in the transition from vegetative to reproductive stage (Fowler et al. 2001). In *Arabidopsis*, two accessions also showed different levels of freezing tolerance in response to photoperiods, and phenotypic expression was traced to different QTLs (Alonso-Blanco et al. 2005).Although the role and mechanism for photoperiod on freezing tolerance in herbaceous species is unclear unlike woody plants (Li et al. 2003; Welling and Palva, 2006), this result suggests that that photoperiod conditions affecting freezing tolerance in *B. stricta* can also be under genetic control and lead to differential physiological, metabolic, and transcriptional adjustments during cold acclimation. The molecular mechanism and underlying gene(s) for the SD-specific QTL could not be ellucidated in the current study.

Although remarkable progress has been made in understanding cold acclimation, the molecular mechanisms underlying how plants perceive low temperature and short photoperiod conditions during cold acclimation is still unclear. Further detailed study of the genetic variation present in *B. stricta* can uncover the adaptive mechanism for these differential freezing tolerances and photoperiod responses.

GENETIC RELATIONSHIP BETWEEN PHOTOSYNTHETIC PERFORMANCE AND FREEZING TOLERANCE

The balance between the absorbed energy of light and photo-chemically converted energy used for essential metabolism is critical in photosynthetic organisms (Hüner et al., 1998). Low temperature stress can lead to a reduced rate of photosynthetic electron transport by strongly decreasing CO2 fixation and increase relative excess energy, which can eventually lead to the production of potentially dangerous reactive oxygen species (ROS) and photo-inhibition of photosystem (Baker, 1994). Because production of ROS and occurrence of photo-inhibition may result in the disruptions of cold-induced physiological and biochemical changes necessary for

cellular stability against freezing stress, overwintering species must increase their photosynthetic capacities to regulate such a balance responded to freezing condition during cold acclimation (Hüner et al., 1998). Close relationships between the tolerance to photosynthetic performance and freezing tolerance based on physiological approaches have been reported in many species. Oquist et al. (1993) demonstrated the tolerance to photo-inhibition is related to adjustment of the redox state of primary electron acceptor quinone molecule (QA-) of photo-system II (PSII) reducing ROS induction probability under cold acclimation. Pocock et al. (2001) showed that sensitivity to photo-inhibition was strongly and negatively correlated with freezing tolerance in spring and winter wheat. In addition, molecular research showed that changes in the PSII redox state during cold acclimation affect expression of some important genes involved in freezing tolerance (Ndong et al., 2001; Rapacz et al., 2008). These results suggest that photosynthetic performance and freezing tolerance may be genetically related.

Chlorophyll fluorescence is a quick and inexpensive method widely used for analyzing the status of photosynthetic apparatus and understanding the mechanism by which a range of environmental factors alter photosynthetic activity (Baker, 2008). Among various chlorophyll fluorescence parameters, maximum quantum yield of photosystem II (Fv/Fm) has been applied for evaluating photosynthetic performance because it is the most easily measured and reflects a progressive inactivation of PSII-mediated electron transport. Hence, we screened Fv/Fm to investigate the changes in photosynthetic performance during cold acclimation and freezing stress. Cold acclimation at 6°C caused a slight reduction in Fv/Fm in all RILs, but differences in response in LT_{50} and Fv/Fm were found in some RILs. For instance, some RILs with higher LT50 showed lower Fv/Fm compared to RILs with lower LT50. This result suggests that differences in freezing tolerance among RILs may not be always associated with disparities in Fv/Fm during cold acclimation, thus it should be noted that additional experiments are required to determine if other chlorophyll fluorescence parameters can provide additional information on the functioning of the photosynthetic apparatus during cold acclimation. In our experiment, we have shown that Fv/Fm measured during and after freezing treatment was highly correlated with freezing tolerance related traits such as LT50 and EL. An interesting finding was that the main QTL identified from LT50 and EL was co-located with a QTL for Fv/Fm ratio observed during freezing and post-freezing, suggesting that common genes exist for stabilizing membranes and maintaining photosynthetic performance. This concept is supported by recent research. Dahal et al. (2012) have demonstrated *Brassica napus* BnCBF17 over-expressing line leads to an increase

of freezing tolerance and also has an important role in enhancing photosynthetic performance by increasing energy conversion efficiency during cold acclimation.

OUTDOOR ENVIORMENT QTL AND POTENTIAL CANDIDATE GENE

The genetic basis of research for understanding freezing tolerance is often obstructed by the difficulty in mimicking realistic outdoor environments under controlled conditions and in facing unpredictable environmental events or physiological damages in natural conditions. Significant and high correlations between Fv/Fm and two types of electrolyte leakage assessments were found during freezing and post freezing treatments in controlled environment conditions, indicating that Fv/Fm can be routinely used as a reliable phenotypic assessment for freezing tolerance in *B. stricta* for QTL study. Therefore, this approach was also applied for evaluating freezing tolerance in outdoorconditions. No QTL was detected for overall winter damage, which might be due to high levels of experimental noise under less controlled conditions outdoors. By contrast, data from Fv/Fm measured during winter identified one QTL. Our QTL analyses in more complex freezing conditions and different developmental stages thus identified one genomic region on linkage group 7 with strong effects on freezing tolerance in *B. stricta*.

Data analyses of Fv/Fm also provide the chance to study phenotypic plasticity of related factors. Although the QTL was not identified under controlled freezing conditions, the locus identified in nature environments also has relatively high and consistent effects in controlled stress conditions. This result suggests that it can be a form of phenotypic plasticity that is a key trait affecting fitness of *B. stricta* in variable environments. The locus at linkage group 7 is syntenic with a genomic region in *A. thaliana* that contains DREB1/CBF-type transcription factors. In *A. thaliana*, DREB1/CBF transcription factors have been suggested as a master regulator of morphological, physiological and biochemical adjustments for increasing freezing tolerance (Thomashow, 1999). They play an important role in the protection and stabilization of cellular membranes by inducing cryoprotectant solutes and cryoprotective proteins and, leading to improvement of anti-oxidative mechanisms in *A. thaliana* (Jaglo-Ottosen et al. 1998, Kasuga et al. 1999). These genes are highly conserved in cold adaptable plants, and their importance for freezing tolerance has been reported in a number of species (Zhang et al. 2004; Skinner et al. 2005). Given that in *B. stricta* the LG 7 QTL shows consistent results in both controlled and outdoor conditions, this suggests that DREB1/CBF-type transcription factors may be promising candidates for unraveling molecular mechanism and fitness of freezing

tolerance in this species.

ACKNOWLEDGEMENTS

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Chapter 3. Molecular and evolutionary analysis of three cold regulated *CBF* genes in *Boechera stricta*

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ABSTRACT

Cold acclimation is an important adaptive strategy for overwintering plants growing in temperate and arctic zones to withstand freezing during winter. The signal transduction pathway regulating acclimation responses is well understood in Arabidopsis. The Dehydration Response Element Binding 1 (DREB1)/C-repeat-Binding Factors (CBFs) have been found to play an important role in integrating the activation of multiple components for development of freezing tolerance in Arabidopsis and many other overwintering species. To investigate the possible genotypic and expression differences in *B. stricta* genotypes, we isolated three DREB1/CBF genes and characterized their structure and expression patterns. Our analyses deomonstrated that *Bs*CBF genes contain highly conserved AP2 DNA binding domains that have crucial roles in DNA binding and the activation of cold responsive genes for development of freezing tolerance in *Arabidopsis* CBF genes. In addition, gene expression analysis showed that a genotype difference between LTM and SAD12 is obvious in the levels of CBF genes and cold stress-responsive genes. These results suggest the signal transduction of CBFs genes can be a central pathway in the development of freezing tolerance in *B. stricta*, although future research is needed to see if the gene expression changes are in cis or in trans.

I. INTRODUCTION

Frost is one of the most important environmental factors affecting the geographical distribution of overwintering plant species, and the quality and productivity of many crops. Most temperate plants enhance their freezing tolerance through an adaptive process known as cold acclimation, a response to low but non-freezing temperatures that occurs before freezing (Xin and Browse, 2000). This adaptive process involves various biochemical and physiological changes, including increased levels of solutes, the modification of membrane lipid composition and the accumulation of secondary metabolites (Guy, 1990). The precise regulation of cold acclimation is still unknown, but some genes responding to low temperature can be associated with these important changes (Lee et al., 2001; Fowler and Thomashow, 2002; Chinnusamy et al., 2003; Zhu et al., 2007). Hence, the identification of genes regulated by low temperature can be used for an understanding of mechanism for freezing tolerance.

In *Arabidopsis thaiana* and *Medicago truncatula*, major QTL responsible for a large proportion of the variation for freezing tolerance have been identified and linked to variation in Dehydration Response Element Binding 1 (DREB1)/C-repeat-Binding Factors (CBFs) (Alonso-Blanco et al., 2005; Tayeh et al., 2013). In *Arabidopsis thaliana*, three DREB1/CBFs occur in a tandem array (DREB1B/CBF1, DREB1C/CBF2 and DREB1/CBF3) that are present in the following order within the array: DREB1B/CBF1 -> DREB1/CBF3 -> DREB1C/CBF2). The three CBF genes belong to the AP2/EREBP family of DNA-binding proteins and can bind to the C-repeat (CRT)/dehydration responsive element (DRE) cis-acting elements contained in numerous downstream genes influencing the transmission of cold signals and regulating the expression of related proteins (Maruyama et al., 2004; Xu et al., 2011). Transgenic overexpression of DREB1B/CBF1 and DREB1/CBF3 enhanced cold tolerance by regulating approximately 100 cold-responsive (COR) genes and accumulating sugar and proline (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Kasuga et al., 1999; Gilmour et al., 2000). The DREB1/CBF pathway responding to low temperature was also found in poplar, wheat, rye and *Brassica napus*, all of which are freezing tolerant (Skinner et al., 2005), and even in tomato and rice which are freezing sensitive (Zhang et al., 2004). The expression patterns of the DREB1/CBF and COR genes in other freezing tolerant species were similar to those of *Arabidopsis* and the core regions within the DREB1/CBF genes were highly conserved (Welling and Palva, 2008). In contrast, freezing sensitive species such as tomato exhibited a reduced CBF regulon and induced fewer cold-

responsive genes, which likely contribute to their freezing sensitivity (Dubouzet et al., 2003; Zhang et al., 2004). Hence, DREB1/CBF genes are thought to have a pivotal role in integrating the activation of multiple components for development of freezing tolerance in plants.

Boechera stricta belongs to the family of Brassicaceae and is a genetically tractable short-lived perennial species in mostly undisturbed habitats of the Rocky Mountains. It occurs along a wide elevational gradient and is found in locations varying in abiotic and biotic conditions (Anderson et al., 2012). Recently, the LTM line, one of *B. stricta* genotypes used in this study, has been fully sequenced with the Roche 454 platform by the Department of Energy Joint Genome Institute and with Sanger BAC end-sequences by HudsonAlpha Institute for Biotechnology (Lee et al., 2013). Previously, extensive comparative analyses with *Arabidopsis* have been done for *B. stricta*, providing access to information and techniques from *Arabidopsis* and facilitating molecular genetic studies to understand ecologically important traits (Schranz et al., 2007; Schranz et al., 2009; Rushworth et al., 2011; Prasad et al., 2012). In an attempt to elucidate the genetic determinants of freezing tolerance in two genotypes of *B. stricta*, LTM and SAD12, we reported DREB1/CBF-type genes could be associated with freezing tolerance of *B. stricta* (see chapter 2 of this thesis). In the current study, we isolated DREB1/CBF-type genes, BsCBF1, 2 and 3, and characterized their expression patterns under cold treatment by Polymerase Chain Reaction (PCR) and Real-Time Polymerase Chain Reaction (RT-PCR). Based on the cDNA sequences, we inferred amino acid sequences and analyzed the structure and phylogenetic positions of these three genes. To date, this is the first study of DREB1/CBF-type transcriptional factors in *B. stricta.* Our results could help to enhance the understanding of the evolution for these cold stress-related genes in Brassicaceae.

II. MATERIALS AND METHODS

PLANT MATERIAL AND EXPERIMENTAL CONDITION

Two genotypes of *Boechera stricta*, LTM and SAD12, and one *Arabidopsis thaliana* ecotype, Columbia (Col), were used in this study. Details about plant locations and growth environments for two genotypes of *B. stricta* were previously described in Schranz et al. (2005). All plants were grown on agar plates for the experiment. Seeds were surface sterilized by using 10 % (v/v) bleach solution for 8 min and washed three times with deionized water. The seeds were put on 0.8% agar (Hispanagar, Burgos, Spain) and 0.5X MS media (Duchefa, Haarlem, The Netherlands)

containing 30 mg l^{-1} kanamycin. Plated seeds were kept at 4°C for 7 days before they were transferred to a growth chamber and grown at 20°C under short-day photoperiods (8 h of cool-white fluorescent light, photon flux of 100 µmol m^{-2} s^{-1}). A low temperature treatment was imposed by transferring 18-day-old seedlings to a cold chamber at 4°C under the same light and photoperiodic conditions. Leaves were harvested after 0, 3h, 8h, 12h, 24h and 48h of cold treatment. Harvested leaves were quickly frozen in liquid nitrogen and stored at −80°C until further use.

ISOLATION, SEQUENCE ALIGNMENT AND PHYLOGENETIC ANALYSIS OF CBF GENES IN BOECHERA STRICTA

A draft genome of LTM, sequenced by Department of Energy Joint Genome Institute and HudsonAlpha Institute for Biotechnology, was utilized to isolate CBF 1, 2 and 3 genes from two genotypes of *Boechera stricta*. The scaffold containing BsCBF1, 2 and 3 gene was provided from Mitchell-Olds laboratory at Duke University. Primers for isolating genomic DNAs of CBF1, 2 and 3 from SAD12 were designed using Primer3 software based on the LTM scaffold sequence. Genomic DNAs of LTM and SAD12 genotypes were isolated from DNeasy Plant Mini Kit (Qiagen) and the products were purified using GeneJET PCR Purification Kit according to the manufacturer's instruction (Thermo Scientific). DNA sequencing of the products was done by GATC Biotech, Germany. After comparing the alignment of genomic DNAs between LTM and SAD12, full-length cDNAs of three CBF genes in two genotypes of *B. stricta* were synthesized from RNA of leaves of LTM and SAD12 exposed to cold, from which amino acid sequences were inferred.

The amino acid sequences of *Bs*CBF 1, 2 and 3 were used as query sequences for searching homologue DREB1/CBF genes in Brassicaceae. The survey was conducted against the GenBank (http://www.ncbi.nlm.nih.gov/blast/) or Brassica Database (http://brassicadb.org), and sequences were aligned using the MAFFT program (http://mafft.cbrc.jp/). The initial alignments were improved manually and saved in FASTA or NEXUS formats. FindModel (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html) was used to identify the best base-substitution models for distance analysis and reconstructing gene phylogenies. Bayesian inference method as implemented in MrBayes (v3.1.2) was utilized to construct gene trees and estimate clade support (Ronquist and Huelsenbeck, 2003).

GENE EXPRESSION ANALYSIS OF CBF GENES IN *BOECHERA* AND ARABIDOPSIS

The expression of *CBF* genes in *B. stricta* and *A. thaliana* and during exposure of low temperature was evaluated using reverse transcription-quantitative real-time PCR analysis (RT-PCR). Total RNA was isolated from frozen samples with the RNeasy plant mini kit (Qiagen, Germany) according to the manufacturer's instructions, and treated with RNA-free DNAase I to remove genomic DNA. The quality and concentration were measured using a Nano-drop and then cDNA was synthesized with oligo $d(T)_{18}$ primer and SuperScript® III Reverse Transcriptase (Life Technologies Corporation) from 5 µg of total RNA. Subsequently, the cDNA was utilized to conduct real time PCR using gene-specific primers of *CBF1, 2 and 3* genes in *B. stricta* and *A. thaliana*. Specific primers for *B. stricta* were designed based on the conserved regions within genotypes, whereas for *A. thaliana* they were adapted from earlier studies. In addition, we tested the potential regulatory effects of the three *B. stricta* CBF transcriptional factor genes by analysing the transcript levels of several down-stream, cold stress-responsive genes including COR15A, COR15B, COR47, and COR78, as known hallmarks of freezing stress adaptation in plants (Shinozaki and Yamaguchi-Shinozaki, 1996). Gene specific primers for investigating their gene expressions were generated using the draft genome of LTM. One µl of cDNA template was amplified using the Platinum SYBR Green qPCR supermix-UDG (Invitrogen, the Netherlands) in a 20 µl qPCR reaction according to the manufacturer's protocol. The samples were amplified with PCR as follows: 3min 50°C, 5min 95°C, 40 cycles of 15sec at 95°C followed by 1min 60°C. Melting curve analyses were performed on the PCR products. Actin2 was used as the reference gene to calculate relative expression levels, using the ΔΔCt method (Livak and Schmittgen, 2001). Three RT-PCR runs were performed per genoype/treatment combination.

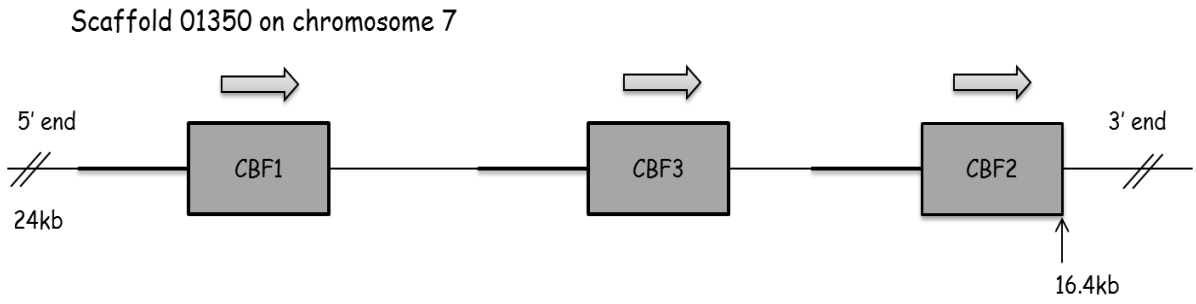

Figure 1: Genomic map of CBFs in *B. stricta*.

III. RESULTS

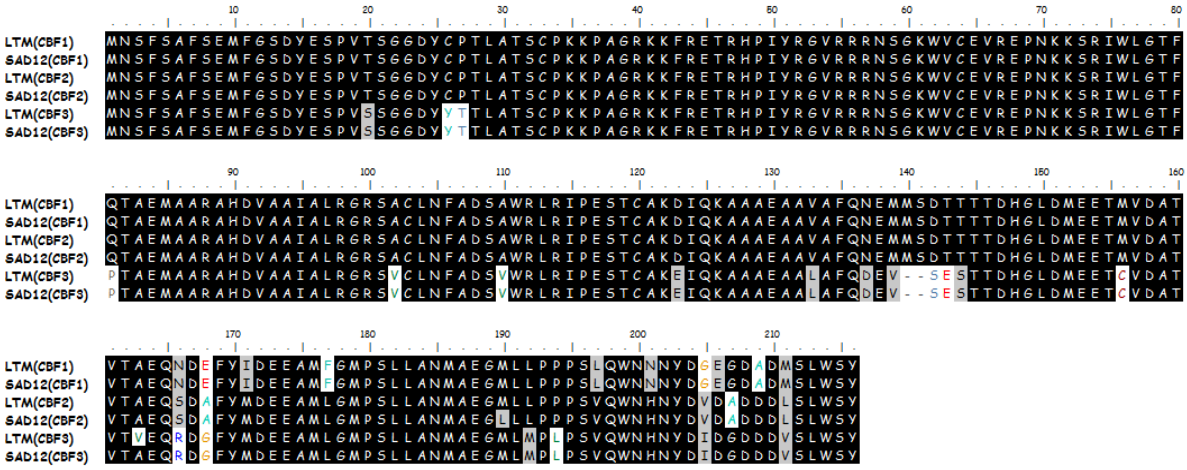

Figure 2. Alignment of the inferred amino acid sequences of CBFs in two genotypes of *B. stricta*.

In order to identify *CBF1, 2* and *3* genes in *B. stricta*, we blasted the draft genome information of LTM against the *Arabidopsis* genome database. The *CBF1, 2* and *3* genes in *B. stricta* are physically organized in a tandem array, as is the case in *A. thaliana* (Figure 1). The complete coding sequences of the three *CBF* genes was inferred from cDNA synthesized from LTM RNA of leaves exposed to cold, using gene specific primers. The full-length cDNAs of *CBF1, 2* and *3* in LTM were 651, 651 and 645 bp, encoding 217, 217 and 215 amino acids, respectively. The cDNA sequence alignment of LTM with *CBF1, 2* and *3* genomic DNA sequences of LTM indicated that the *BsCBF1, 2* and *3* genes included no intron. Specific primer pairs for these genes were also used to amplify the corresponding genes from cold treated leaves of SAD12.

The CBF cDNA amplicons of SAD12 all had the same length as the corresponding LTM. The pairwise sequence alignment of the isolated *CBF1* gene from SAD12 revealed that the *CBF1* gene of SAD12 was identical to LTM. Three SNPs were present in the *CBF2* gene of SAD12 at positions 54, 567 and 615. A variant of C/G and C/T at positions 54 and 615 led to synonymous mutations, whereas a nucleotide transition from A to T at position 567 led to a non-synonymous mutation from methionine to leucine. In SAD12 CBF3, a single T/C variant was observed at position 482, which led to a non-synonymous mutation from valine to alanine (Figure 2). In conclusion, there were only minor differences in CBF genes between two genotypes of *B. stricta.*

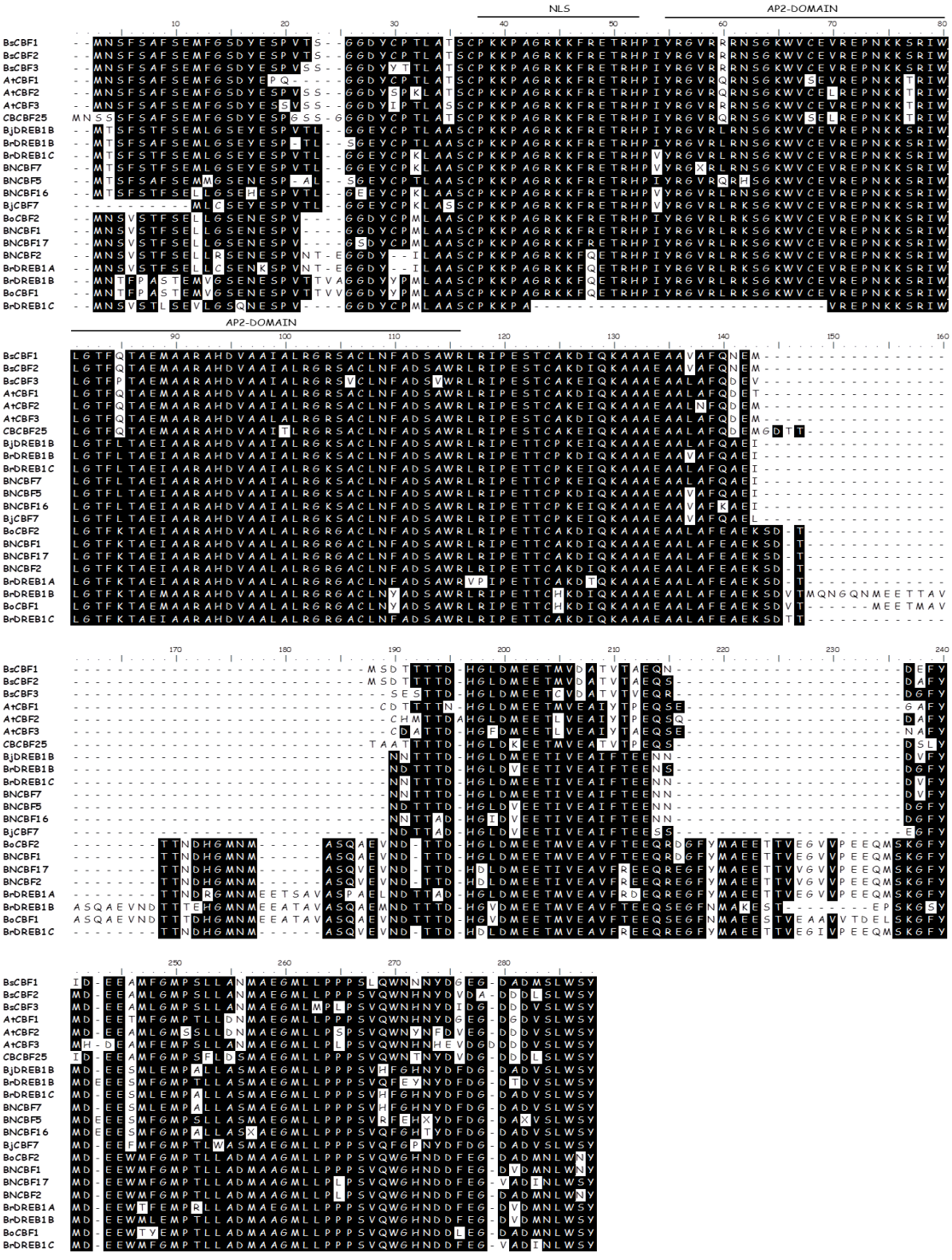

Figure 3. Alignment of the inferred amino acid sequences of *Boechera* CBFs (LTM) with *Brassicaceae* CBFs. *At : Arabidopsis thaliana, BJ : Brassica juncea, BN : Brassica napus, BR : Brassica rapa, BO: Brassica oleracea, Bs : Boechera stricta, CB: Capsella bursa-pastoris.*

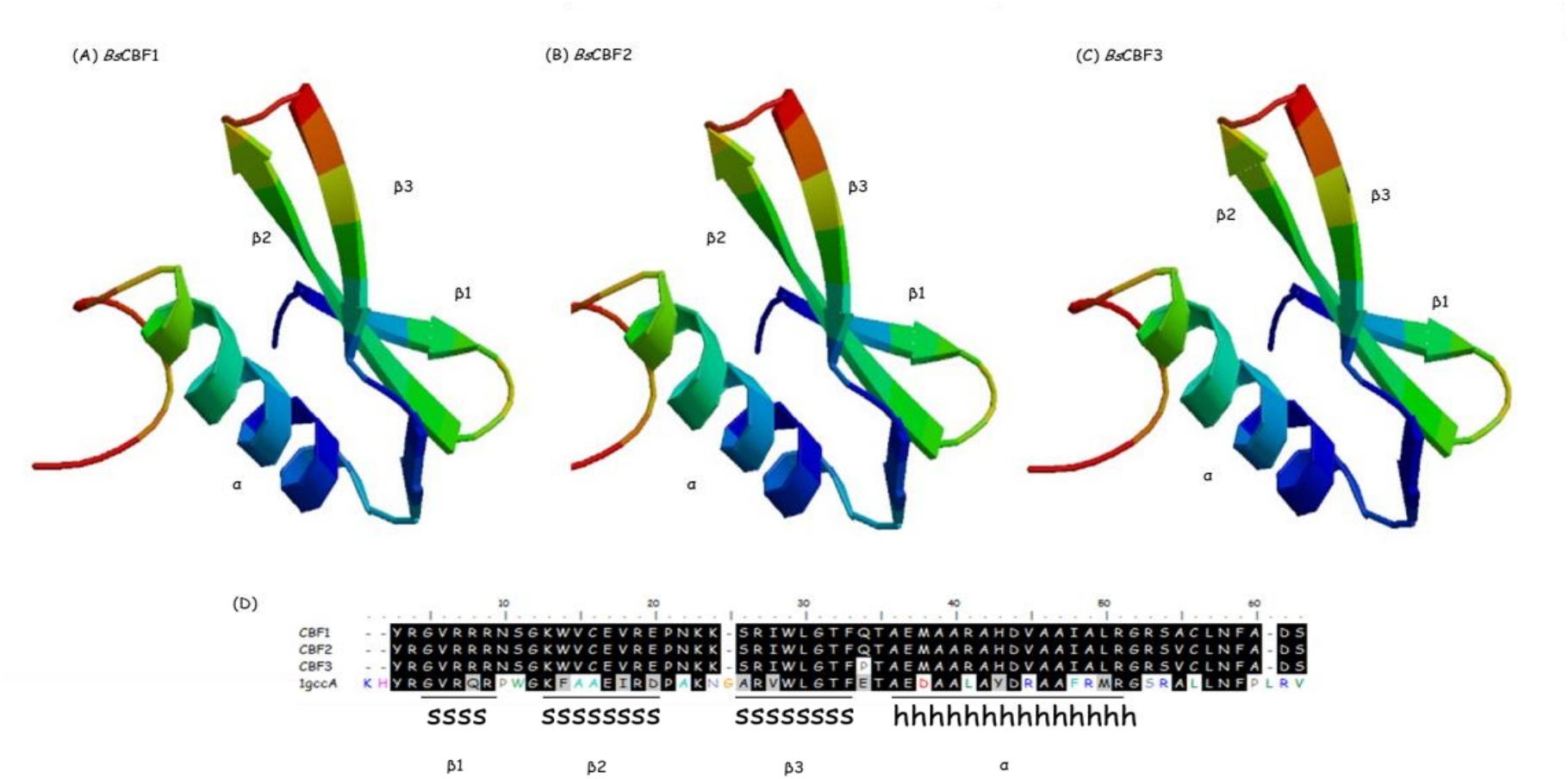

Figure 4. Molecular models of the AP2 DNA binding domain of *Bs*CBF1 (a), 2 (b) and 3 (c) that modeled by SWISS MODEL and alignment analysis (D) of the AP2 DNA binding domain sequence of *At*ERF1 (PDB ID: 1gcc*A*), *Bs*CBF1, *Bs*CBF2 and *Bs*CBF3.

Analyses of the predicted amino acid sequences of *Bs*CBF1, 2 and 3 revealed that they consist of a putative nuclear localization, an AP2 DNA binding domain and a putative acidic activation domain, and have two *CBF* signature sequences, PKKR/PAGR and DSAWR (Figure 3). When compared to the Brassicaceae *DREB1/CBF* amino acid sequences, pairwise amino acid comparison showed AP2 DNA binding domain shared remarkably high degree of sequence identity with *CBFs* in *A. thaliana* (Figure 3). In addition, secondary structure analysis revealed that AP2 DNA binding domains of *Bs*CBF1, 2 and 3 contained three-stranded, antiparallel β-sheets and an a-helix (Figure 4).

To further understand the evolution and origin of DREB1/CBF-type genes isolated from *B. stricta,* phylogenetic relationships were investigated using 19 DREB1/CBF aligned amino acid sequences from various Brassicaceae species (Figure 5). Our results indicated that *Bs*CBF 1, 2 and 3 genes were closely related to *At*CBF 1, 2 and 3 genes, and *Capsella-bursa pastoris* CBF25 gene, although there precise relationship could not be resolved from comparing amino acid sequences.

Our *CBF* gene expression data showed that all *CBF* genes in *B. stricta* were transiently induced by cold treatment and expression kinetics were similar to those of *At*CBF 1, 2, and 3 transcripts (Figures 6 and 7). The *Bs*CBF 1, 2, and 3 transcripts were almost undetectable under control condition, but *Bs*CBF transcripts reached the highest level at 3 h after exposure to low temperature in both genotypesand then showed a gradual decrease toward the 12h time point. Interestingly, expression of the CBF2 and CBF3 gene at 3h and 8h after cold treatment was significantly higher in LTM than in SAD12. Moreover, expression after 24 and 48hr remained higher than initial control levels, especially so for LTM. Previous research showed contrasting regulation of CBF genes resulted in activating the differential expression of downstream target genes. Hence, we further examined the expression patterns of five cold stress-responsive target genes. Under cold condition, the activation of selected cold stress-responsive genes was also observed in two genotypes of *B. stricta* (Figure 8).

Although the expression of all of these genes was detected within 3 h, most of cold stress-responsive genes except for COR47 showed the highest levels in expression after 2 days of cold treatment. The expression levels of COR15B, COR47 and COR78 in the LTM were gradually higher than in the SAD12 during the cold treatments.

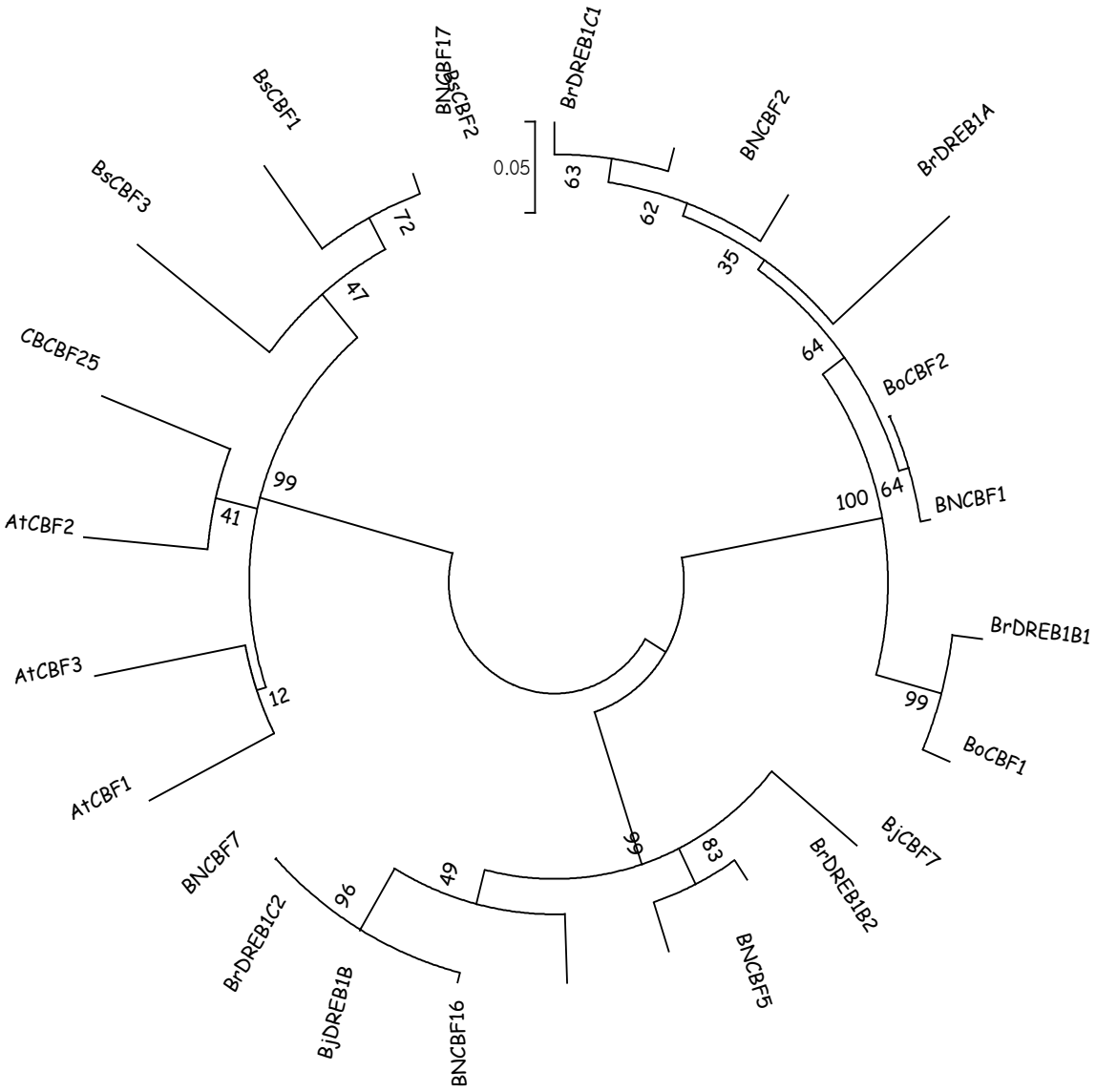

Figure 5. An un-rooted phylogenetic tree of the DBEB1/CBF transcription factors of Brassicaceae. The amino acid sequences of full length of 22 Brassicaceae DREB1/CBF proteins were aligned by MAFFT, and the phylogenetic tree was constructed using MrBayes (v3.1.2). Bootstrap values from 1000 replicates were used to assess the robustness of the trees. Branch lengths indicate genetic distance. The Genbank accession numbers or BRAD gene ID of the different genes used for this analysis are: *AtCBF1*(NM118681), *AtCBF2*(NM118679), *AtCBF3*(NM118680), *BjCBF7*(AY887137), *BjDREB1B*(EU136731), *BnCBF1*(AF370733), *BnCBF2*(AF370734), *BnCBF5*(AF499031), *BnCBF16*(AF499033), *BnCBF17*(AF499034), *BoCBF1*(AF370731), *BoCBF2*(AF370732), *BrDREB1A(*Bra010461), *BrDREB1B1(*Bra010460), *BrDREB1B2(*Bra022770), *BrDREB1C1(*Bra010463), *BrDREB1C2(*Bra028290), CBCBF25(AY491498). At: *Arabidopsis thaliana*, Bj: *Brassica juncea*, Bn: *Brassica napus*, Br: *Brassica rapa*, Bo: *Brassica oleracea*, Bs: *Boechera stricta*, Cb: *Capsella bursa-pastoris*.

DISCUSSION

In the chapter 2 of this thesis, we identified QTLs determining the genotype difference of freezing tolerance in mapping population of *B. stricta* and confirmed a QTL corresponding to a syntenic region containing CBF genes in *A. thaliana* explained a major effect on the genotype difference of freezing tolerance. In *A. thaliana*, the major freezing tolerance QTL was also associated with the three tandem-repeated *CBF* genes. Based on possibility that tandem-repeated CBF genes are involved in the development of freezing tolerance in *B. stricta*, we isolated three DREB1/CBF genes and characterized their structure and expression patterns.

Our Bioinformatics analysis revealed that *Bs*CBF genes have typical motifs of the CBF transcription factors in plants (Medina et al., 2011). We found PKKP/RAGR signature sequences bordering the AP2 DNA binding domain showed 100% identities with those of *At*CBFs. The PKKP/RAGR motif located on immediately upstream of the AP2 DNA binding domain might function as a NLS and has recently turned out to be essential for transcriptional activity of *At*DREB1/CBF proteins (El-Kayal et al., 2006; Canella et al., 2010). We also observed that secondary structure of AP2 DNA binding domain in *Bs*CBFs had three-stranded antiparallel β-sheets connected by loops and an α-helix, participating in interaction with DNA and other transcriptional factors, which resembled it of *At*CBF genes. Our result additionally showed that *Bs*CBF genes contained conserved Valine-14 and Glutamic-19 amino acid residues that play a crucial role in recognition and binding specificity of DRE cis-elements in AP2 domain (Sakuma et al., 2002). Furthermore, our phylogenetic analysis demonstrated *Bs*CBF genes have a closed evolutionary relationship with *At*CBF genes. Several evidences found in bioinformatics analysis strongly imply that *Bs*CBFs may have the same DNA binding specificity as *At*CBFs.

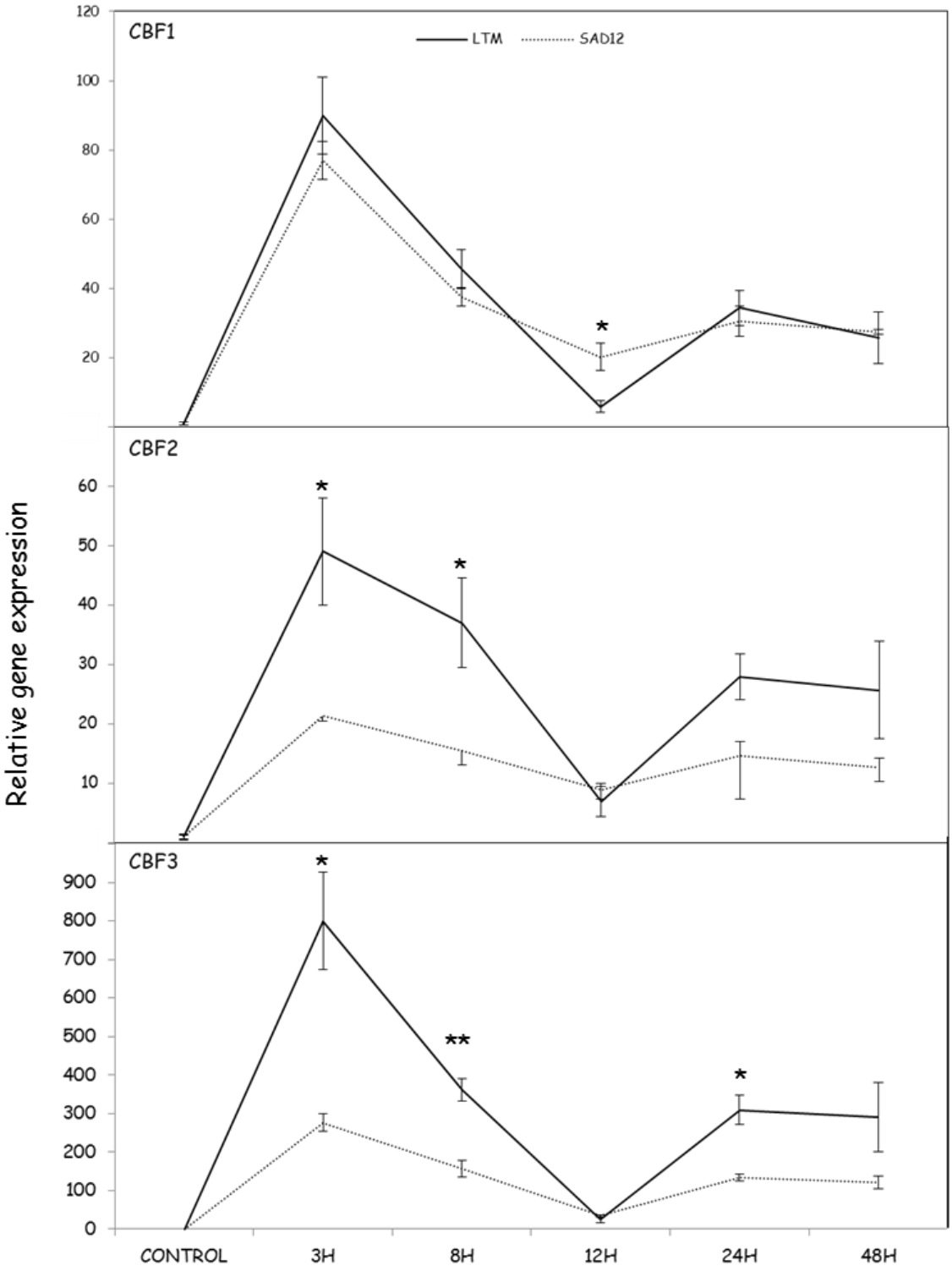

Figure 6. Time course expression profile for *BsCBF* genes in leaves of plants shifted to 4 °C at LTM verse SAD12. Actin 2 was used as the reference gene for two genotypes. Values are expression relative to the control (no cold treatment) for each gene at LTM or SAD12. A star (*P < 0.05 and **P< 0.005) above the error bars at a particular time point indicates a significant difference between LTM and SAD12. It was tested by Student's *t*-test (n=3, error bars indicate SEs).

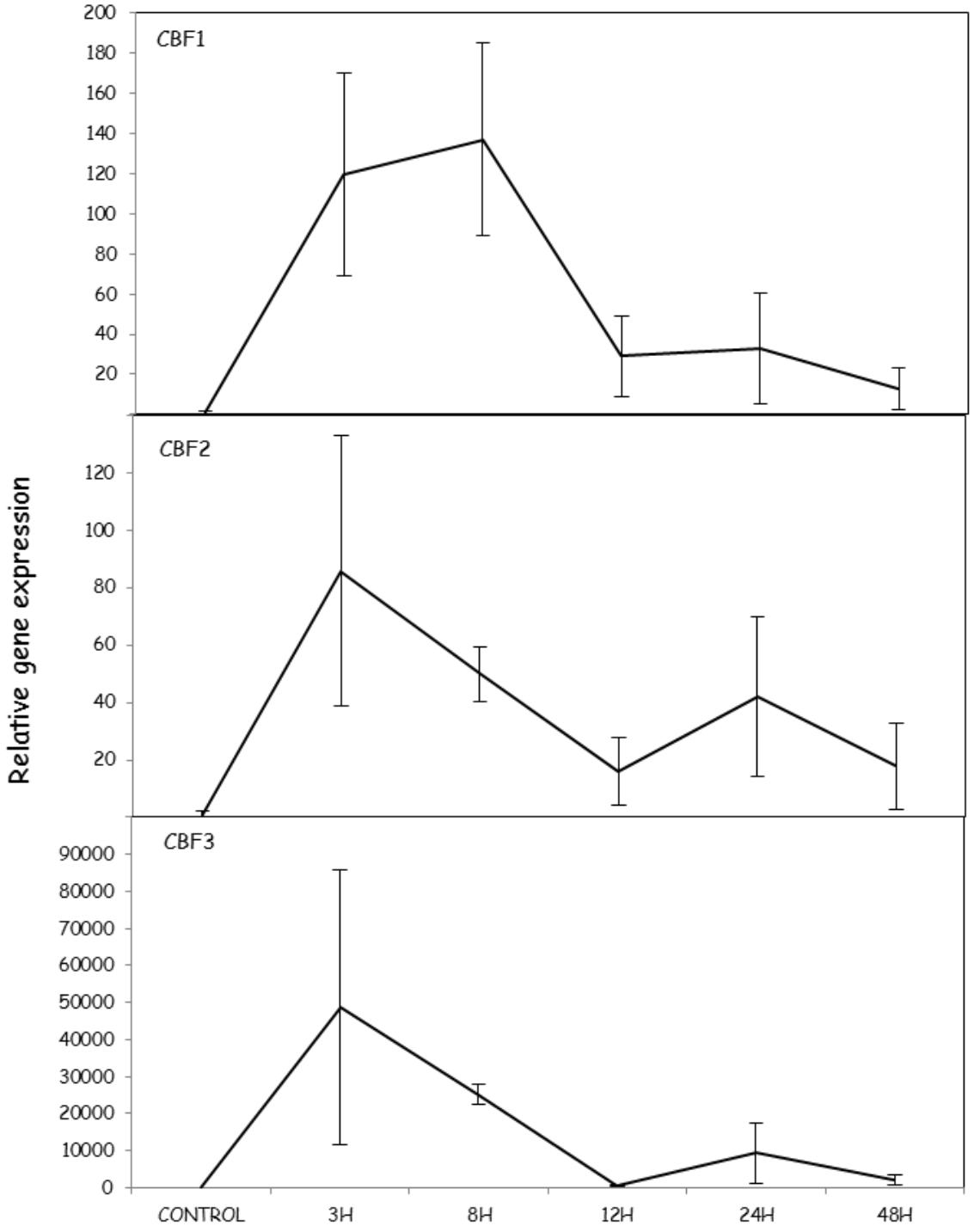

Figure 7. Time course expression profile for *AtCBF* genes in leaves of plants shifted to 4 °C at LTM verse SAD12. Actin 2 was used as the reference gene. Values are expression relative to the control (no cold treatment) for each gene (n=3, error bars indicate SEs).

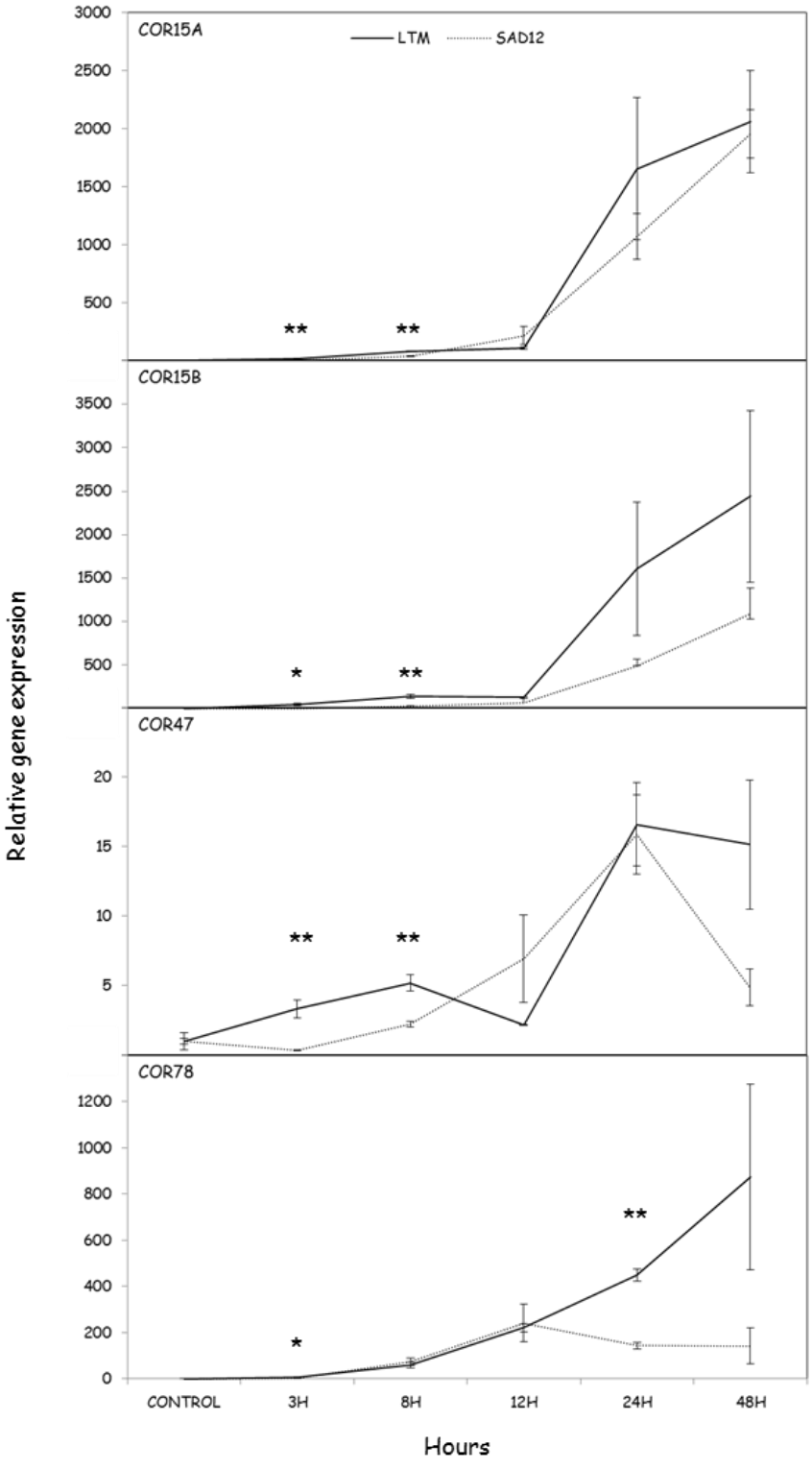

Figure 8. Time course expression profile for *BsCBF* targeted (cold responsive) genes in leaves of plants shifted to 4 °C at LTM verse SAD12. Actin 2 was used as the reference gene for two genotypes. Values are expression relative to the control (no cold treatment) for each gene at LTM or SAD12. A star (*P < 0.05 and **P < 0.005) above the error bars at a particular time point indicates a significant difference between LTM and SAD12. It was tested by Student's *t*-test (n=3, error bars indicate SEs).

In this study, we also examined the relative expression levels of *BsCBF1, 2* and *3* to investigate whether *Bs*CBF genes are induced in response to cold, and if expression levels differ between the two genotypes. As we expected, *Bs*CBF genes had similar expression behaviours with *At*CBFs. In addition, our gene expression study clearly exhibited differential expression at the molecular level in CBF2 and 3 between two genotypes of *B. stricta*, and differences in expression of COR genes existed. Interestingly, expression of these genes was consistently higher in LTM than in SAD12. These results indicated that CBF genes are involved in cold acclimation process in *B. stricta* and can be an important factor underlying differential freezing tolerance in *B. stricta*. Over-expression of *CBF and COR genes results in accumulation of metabolites and enzymes for* sugar metabolism and fatty acid desaturation, components that are essential for winter survival in plants (Cook et al., 2004; Maruyama et al., 2009). Increased levels in expression of CBF and COR genes have already been shown to correlate with enhanced freezing tolerance during cold acclimation in *Arabidopsis* (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Zuther et al., 2012). Similarly, we also observed the LTM developed much higher levels of freezing tolerance than SAD12 during seedling as well as adult stages of cold acclimation from previous studies (Chapter 2 and 4 of this thesis). Hence, differential regulation patterns in two genotype *of Boechera* with contrasting freezing tolerance strongly suggest signal transduction of CBF genes based on genetic background is one of central pathways in the development of freezing tolerance in *B. stricta*.

However, it should be noted that there was significant difference in the expression of CBF 2 and 3 genes, but we only found minor genotypic sequence differences in either CBF2 or CBF3. These results imply that even though DREB1/CBF genes have similar structures, their binding specificity to the CRT/DRE element can be different. Responses leading to different freezing tolerances can thus be due to presence/absence of binding sites in the promoter region (Dubouzet et al., 2003) rather than differences in the CBF proteins. To confirm this it is therefore needed to study promoter regions in the future.

CHAPTER 4. Genetic mapping, gene expression and lipid analysis identify *DGAT1* as a potential candidate gene for the Seedling Koud Intolerance (*SKI*) locus in *Boechera stricta*

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ABSTRACT

Plants cope with freezing by altering gene expression affecting physiological traits and membrane lipid profiles. Freezing stress at the seedling stage is particularly critical since it can reduce physiological function and prevent establishment of overwintering plants. We performed a seedling freezing survival assay in parental and Recombinant Inbred Lines (RILs) of *Boechera stricta*, an Arabidopsis relative. The seedling freezing tolerance trait is quantitatively inherited and QTL mapping identified a single locus, the *Seedling Koud Intolerance* (*SKI*) on Linkage Group (LG) V. Comparative genomic analysis to Arabidopsis allowed us to identify an important gene in lipid biosynthesis, *DGAT1,* as potential candidate gene for *SKI*. To test our hypothesis, we performed gene expression analysis and did lipid profiling in *Boechera* parental lines and in Arabidopsis wild-type and a *DGAT1* mutant line. Significant differences in lipid profiles were found, consistent with our hypothesis. The identification of the genetic and molecular basis of this QTL may provide new perspectives for understanding and improving freezing tolerance.

I. INTRODUCTION

During the winter and late fall, overwintering plants growing in temperate, montane and artic zones must withstand cold and freezing temperatures, and thus have evovled several survival strategies (Yamazaki et al., 2008). Freezing tolerance of overwintering herbaceous plants is tightly connected to the mechanism called extracellular freezing (Uemura and Hausman, 2013). Under freezing conditions, ice generally forms in the extracellular spaces in plant cells that causes both freeze induced dehydration and by the extracellular pressure due to solid ice crystals (Wolfe and Steponkus, 1983). Overwintering plants begin to acclimate themselves from late summer to early autumn when temperatures decline (Guy et al., 2008). This phenomenon, known as cold acclimation, enables plant cells to tolerate mechanical and dehydration stresses of extracellular freezing by adjusting their metabolism and cell structure (Thomashow, 1998). Multiple mechanisms appear to be involved in stabilization of membrane lipids during cold acclimation (Thomashow, 1999). It has been suggested that the increase of poly-unsaturated species of phospholipids, synthesis of cryo-protectant molecules and accumulation of triacylglycerols can directly help to protect macromolecules on membrane lipid during freezing and support recovery after freezing (Uemura and Steponkus, 1989; Mahajan and Tuteja, 2005; Moellering et al., 2010). There have been a multitude of reports that adjustments during cold acclimation are associated with transcriptional changes, but the underlying regulatory mechanisms of these transcriptional changes remain largely unknown (Mahajan and Tuteja, 2005). Current advances in molecular biology, however, provide the opportunities for a better understanding of the molecular basis of abiotic stresses including freezing tolerance in plants (Amudha and Balasubramani, 2011).

Quantitative Trait Locus (QTL) mapping is a useful method to identify chromosomal regions controlling freezing tolerance in plants because the resistance to freezing stress generally exhibit continuous variation (Collins et al., 2008). Potential candidate genes can then be characterized for allelic variation and gene function. For example, QTL studies in *Arabidopsis thaiana, Medicago truncatula* and *Boechera stricta* (see Chapter 2) have identified QTL regions containing DRBE1/CBF genes belonging to the AP2/EREBP family of transcription factors. These genes explain a large proportion of the difference in freezing tolerance (Alonso-Blanco et al., 2005; Tayeh et al., 2013). Previously, it has been established that three members of the CBF family, CBF1, CBF2 and CBF3, play a key role in the regulation of the transcriptome

during cold acclimation (Maruyama et al., 2004; Xu et al., 2011). These CBF/ DRBE1 genes have been isolated from several herbaceous and woody plant species and different studies have demonstrated their significant role for development of freezing tolerance (Zhang et al., 2004; Skinner et al., 2005; Gamboa et al., 2007; Welling and Palva, 2008; He et al., 2012). However, variation in *CBF* loci does not explain all the quantitative natural variation for freezing tolerance. We therefore aim to identify novel cold tolerance loci, especially those associated with seedling survival, using a mapping population of *Boechera stricta*.

Boechera stricta is a genetically tractable short-lived perennial species native to the Rocky Mountains and has become a model ecological genomic study system (Rushworth et al., 2011). *B. stricta* occurs along a broad elevational gradient with varying abiotic and biotic conditions and is locally adapted to some of the ecological differences in these diverse habitats (Anderson et al., 2012). A large array of genomic tools and resources has been developed for comparative genomic studies of *Arabidopsis thaliana* (*A. thaliana*) and *B. stricta*, including a RIL mapping populations for QTL studies (Schranz et al., 2007; Lee et al., 2013). Furthermore, both RIL parental lines (SAD12 from Colorado and LTM from Montana) have recently been sequenced to high-coverage (Mitchell-Olds, unpublished). These resources can accelerate the identification of the molecular determinants of various ecologically important traits in *B. stricta*, including glucosinolate profiles, flowering time, winter-survival and freezing tolerance (Schranz et al., 2009; Anderson et al., 2011; Prasad et al., 2012; Anderson et al., 2013). Our previous genetic studies on cold acclimation and freezing tolerance in adult *B. stricta* identified a major QTL on LG7 across various environmental conditions. This QTL contains a tandem array of DRBE1/CBF-type genes, similar to CBF1, 2 and 3 of *A. thaliana*. At the molecular level, expression and structure of CBF1, 2 and 3 genes in *B. stricta* were highly similar with those of CBF1, 2 and 3 genes in *Arabidopsis* (Chapter 3 of this thesis). The induction of these three *CBF* genes was much higher in LTM than in SAD12 genotypes. A genotypic difference was also found in the expression levels of other cold stress-responsive genes in response to cold treatment. Based on these results, we suggested a tandem array of DRBE1/CBF genes to be an important factor for freezing tolerance in *B. stricta*. However, it should be noted that the expression of the gene/QTL responsible for freezing tolerance could differ among developmental-stages, since freezing tolerance is a complex trait.

During early growth stages of overwintering plants in the autumn, freezing stress affects physiological function and may cause mortality, because thinner tissues of young plants

are more sensitive to freezing compared to adult plants (Loik and Redar, 2003; Von Meijenfeldt, 2010). Here we studied natural variation for seedling freezing tolerance in Recombinant Inbred Lines (RILs) of *B. stricta* after cold acclimation. Our mapping of QTL identified a single locus controlling seedling survival on LG V. Interestingly, earlier QTL studies done with plants in the field also identified this locus, but only in the first season (e.g. early growth) and only at the Colorado site (Anderson et al., 2013). We have found that the locus corresponds to a syntenic region containing diacylglycerol-acyltransferease 1 (DGAT1) gene which is a key mediator of synthesis of triacylglycerols. Triaclylglycerols have recently received the attention of plant stress biologists due to a potential role to protect critical cell structures during freezing stress (Moellering et al., 2010). However, natural variation and mutant studies have not yet been done for the DGAT1 gene. Therefore, we further tested if DGAT1 could be a potential candidate gene for development of freezing tolerance using molecular and lipidomic approaches in two *B. stricta* genotypes and mutant of this gene and wild type in *A. thaliana*.

II. MATERIALS AND METHODS

PLANT MATERIAL AND GROWTH CONDITIONS

For the QTL experiment, we used 108 F_6 RILs derived from a cross between two highly inbred lines of *B. stricta.* The parental lines come from populations in the Rocky Mountains that have experienced little anthropogenic disturbance, with similar vegetation for the last ~3000 years (Anderson et al., 2011). The parental sites in Montana (LTM) and Colorado (CO) differ in both ecological community and abiotic conditions (Schranz et al., 2007). For comparing gene expression and lipid composition we used *Arabidopsis* wild type (ecotype Columbia) and its *DGAT1* mutant called AS11. The AS11 mutant was described previously (Katavic et al., 1995) and was obtained from the Nottingham Arabidopsis stock centre (NASC, UK). All seedlings were grown on agar plates. Surface of seeds was sterilized by washing of 8 min with 50% bleach and a drop of Tween 20, and subsequently sown on plates containing 50% Murashige and Skoog with 0.8% (w/v) Daishin agar (Duchefa Biochemie, Haarlem, The Netherlands). To ensure a homogenous germination, plated *Arabidopsis* and *Boechera* seeds were kept at 4°C for 3 days and 2 weeks, respectively and then transferred to a growth chamber with a 14 h light/10 h darkness irradiance cycle and a 20/16°C temperature regime.

SEEDLING FREEZING TOLERANCE IN *BOECHERA* PARENTAL GENOTYPES

To check for differences in seedling freezing tolerance in two *B. stricta*, we measured the lethal temperature for 50% survival (LT_{50}). Eighteen day-old seedlings were either cold-acclimated at 4°C for 48 hours under a 14h photoperiod (acclimated) or remained in the standard growth conditions (non-acclimated). After the 48 h acclimation period, LT_{50} of the acclimated and non-acclimated seedlings was measured as percentage survival of seedlings after freezing treatments at different temperatures in a tightly-controlled environment cabinet (Snijders Microclima 1000; Snijders Scientific, Tilburg, The Netherlands). The cabinet temperature decreased from 4°C at to –1°C (at a rate of -1.25 degrees per hour) and then was held for one hour at –1°C. Very fine ice chips were applied to all seedlings to prevent super-cooling. The temperature was then further decreased from –1°C to the desired target temperatures (–1, -2, -3, -4, -5, -6, -7, -8, -9, -10 or –11°C) over a 4 h interval. The target temperature was then held for 6 hrs. The seedlings were then thawed for 12h in the dark at 4°C and placed back into a growth chamber set at the standard growth temperature and irradiance conditions. Survival was visually scored 10 days post freezing. The percentage freezing survival per genotype was calculated as: (number of survivors / total number of plants) * 100. LT_{50} was estimated from fitted response curves and calculated by statistic program R (R Development Core Team, 2008, Procedure : http://lukemiller.org/index.php/2010/02/calculating-lt50-median-lethal-temperature-aka-ld50-quickly-in-r/). The fitted response curve was used to select the temperature (-8°C) for further QTL analysis using all RILs.

QTL ANALYSIS IN *BOECHERA* AND SURVIVAL TEST IN *ARABIDOPSIS*

Seedling freezing tolerance of 108 F_6 RILs was accessed at -8°C. Seedlings were grown as above for parental genotypes, with the temperature of cabinet decreasing from –1 to –8°C over a 4 h interval, and then held at –8°C for 6 h. Seedling survival was again scored after 10 days, as described above. This condition was also used to test survival rate in *Arabidopsis* wild type and DGAT mutant seedlings later. For the *Boechera* experiment, 150 seedlings (30 seeds per agar-plate, 5 replicates) for each RIL were prepared. The average seedling survival for RILs was used to map QTLs using a set of 196 mapped markers on the *Boechera* genetic map (Schranz et al., 2007). QTL were detected by Composite Interval Mapping (CIM) with a 2 cM step-size using a 10 cM window and five background cofactors selected via a forward and backward stepwise regression method using WinQTL Cartographer v2.5. A significance threshold value (LOD score)

corresponding to a genome-wide significance of $\alpha = 0.05$ was used for QTL detection and was determined using permutation tests with 1,000 replicates.

ISOLATION AND EXPRESSION PATTERN ANALYSIS OF DGAT1 CANDIDATE GENE IN *BOECHERA* AND *ARABIDOPSIS*

A single QTL was identified and using the established comparative genomic framework between *A. thaliana* and *B. stricta (*Schranz et al., 2007), several candidate genes could be identified. Selection criteria were known cellular and molecular functions using gene ontology (GO) and gene expression patterns during cold treatment in *A. thaliana* using Arabidopsis eFP browser databases. Genomic DNAs in LTM and SAD12 were isolated with DNeasy Plant Mini Kit (Qiagen) and PCR products were purified using GeneJET PCR Purification Kit according to the manufacturer's instructions (Thermo Scientific). A total of five PCR primer-pairs for DGAT1 were designed using the Primer3 software using data from *Boechera* genomic scaffold sequences obtained from Michell-Olds laboratory at Duke University. PCR conditions were optimized for each template-primer system. DNA sequencing was done by GATC Biotech in Germany. Based on obtained nucleotide sequences in two *Boechera* genotypes, coding and peptide sequences of the LTM and SAD12 *DGAT1* alleles were predicted by GeneMark^{TM} (opal.biology.gatech.edu). Predicted coding sequences were utilized to design primers for gene expression. Analysis of RNA samples was done for LTM and SAD12 parental genotypes exposed to 3hr, 8hr, 12hr, 24hr and 48hr of cold treatment. Total RNA was isolated with the RNeasy plant mini kit (Qiagen, Germany) according to the manufacturer's instructions. First, DNA was digested during the preparation by DNase I and the RNA was eluted in RNase-free water. The quality and concentration were measured with a Nano-drop and then cDNA was synthesized with oligo $d(T)_{18}$ primer and SuperScript® III Reverse Transcriptase (Life Technologies Corporation) from 5 µg of total RNA. 1 µl of cDNA template was amplified using the Platinum SYBR Green qPCR supermix-UDG (Invitrogen, the Netherlands) with gene-specific primers for the *DGAT1* gene in *Arabidopsis* and *Boechera* in a 20 µl qPCR reaction according to the manufacturer's protocol. Relative expression values were calculated by the ΔΔCt method (Livak and Schmittgen, 2001) using Actin2 as a control. Gene expression of DGAT1 was analysed in three biological samples for each time point.

ANALYSIS OF POLAR LIPIDS AND TRIACYLGLYCEROLS IN *BOCHERA* AND *ARABIDOPSIS*

Analysis of polar lipids and triacylglycerols for non-acclimated and cold-acclimated leaves of *A. thaliana* and *B. stricta* were performed using three replicate samples per genotype and treatment. To extract lipids from non-acclimated and cold-acclimated leaves of *A. thaliana* and *B. stricta*, leaves were immediately transferred to 3 mL of hot isopropanol with 0.01% butylated hydroxytoluene. After a 15 min incubation at 75 °C, 1.5 mL of chloroform and 0.6 mL of water was added, followed by agitation for 1 h. They were extracted with chloroform/methanol (2 : 1) with 0.01% butylated hydroxytoluene three more times, each time followed by 30 min agitation at room temperature (around 25 °C). The remaining tissue was dried at 90°C overnight and weighed. The extract was washed with 1 mL of 1 M KCl and then 2 mL water. The solvent of the lower phase was evaporated under nitrogen. The dry lipid extract was dissolved in 1 mL of chloroform. The molecular species of polar lipids and triacylglycerols were analysed by ESI triple quadrupole mass spectrometry (API 4000; Applied Biosystems) at Kansas Lipidomics Center in the United States.

TRIACYLGLYCEROL ACCUMULATION TEST BY THIN LAYER CHROMATOGARPHY (TLC) IN *BOECHERA*

We performed a TLC experiment to check if TAGs were accumulated from non-acclimated, cold acclimated and freezing-treated leaves of LTM and SAD12. Each harvested sample was directly transferred to the stop reaction (5% perchloric acid), and then shaken until green tissues turned brownish on a Vortex shaker. Stop reaction was removed and then 400ul of CHCl3/MeOH/HCl (50:100:1 v/v) was added to samples. After 5 min of agitation on the Vortex shaker, 400ul of CHCl3 was added, followed by 200ul of 0.9% NaCl. The lower phase was removed with a Pasteur pipet from the upper phase; CHCl3/MeOH/1M HCl (3:48:47 v/v). The lower phase was transferred to a new tube and then vortexed for 10 sec and centrifuged for 1 min. The dry lipid was finally obtained with 20ul iso-propanol by vacuum centrifugation for 45 min. The dry lipid extract was dissolved in 30ul of CHCl3 and utilized for TLC analysis. Lipids were separated by 80ul developing solvent (acetic acid/petroleum/ethyl ether; 1:80:20 v/v) on TLC developing chamber and TAG was visualized with Iodine staining.

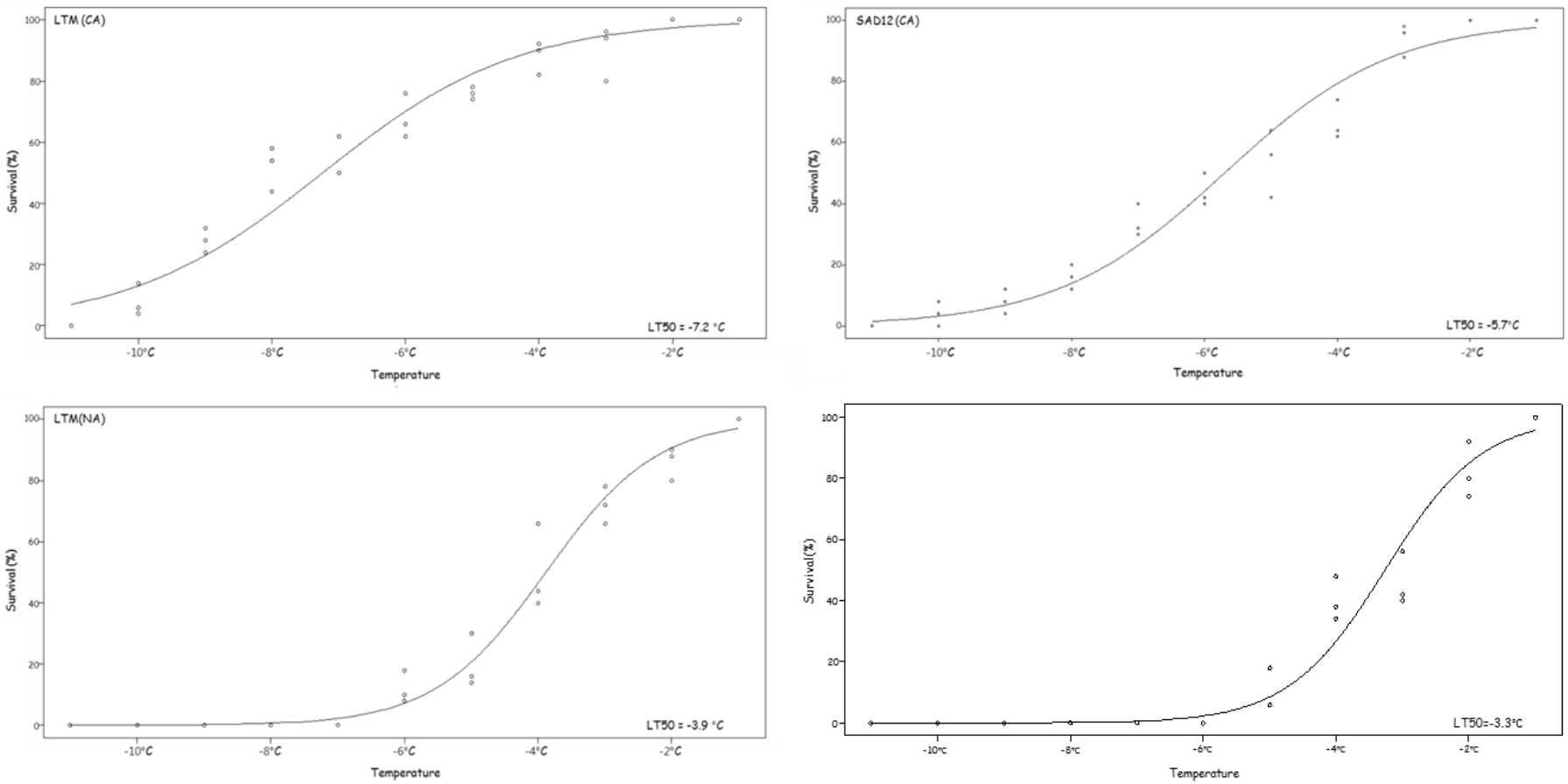

Figure 1: Percentage of seedling survival for *Boechera sticta* genotypes LTM (from Montana) and SAD12 (from Colorado) exposed to a range of freezing temperatures (-1℃ to -11℃ done in -1℃ steps) for both acclimated and non-acclimated seedlings. Results were also used to calculate LT_{50} values (temperature where 50% of the genotypes do not survive). Differences between genotypes are slight for non-acclimated seedlings, but LTM is significantly more freezing tolerant with acclimation (a difference in LT_{50} values of 1.5℃). Maximum differences between genotypes under acclimated conditions was at -8℃ which was used for QTL experiments with RILs.

III. RESULTS

QTL ANALYSIS IDENTIFIES THE *SEEDLING KOUD INTOLERANCE* (*SKI*) LOCUS

Seedling survival and the calculated relative freezing tolerance based on scoring of the seedling as either dead or living prior to and after cold acclimation are shown (Figure 1). Non-acclimated SAD12 and LTM seedlings exposed to freezing temperatures showed similar freezing tolerances. However, there were significant differences between genotypes when testing cold acclimated seedlings with the LTM genotype from Montana being more cold tolerant than the SAD12 genotype from Colorado (Figure 1). The LT_{50} values of cold-acclimated LTM and SAD12 were –7.2°C and –5.7°C, respectively (delta LTM to SAD12 = -1.5°C). The greatest difference in seedling survival for cold-acclimated plants was at –8°C at which 52% of LTM seedlings survived and only 16% of SAD12 survived. When acclimated RIL seedlings were tested at –8°C, we found freezing tolerance to be normally distributed (Figure 2). Using these results, QTL analysis of seedling survival identified a single major QTL on LG V centered on the markers PhyB.I and Bf_20 (Figure 3). We named this QTL the *Seedling Koud Intolerance* (*SKI*) locus (Koud) is the Dutch word for Cold). The maximum LOD-score for *SKI* was 5.67 and explained 22.4% of the total phenotypic variance and had 0.308 of the additive effect of the LTM allele.

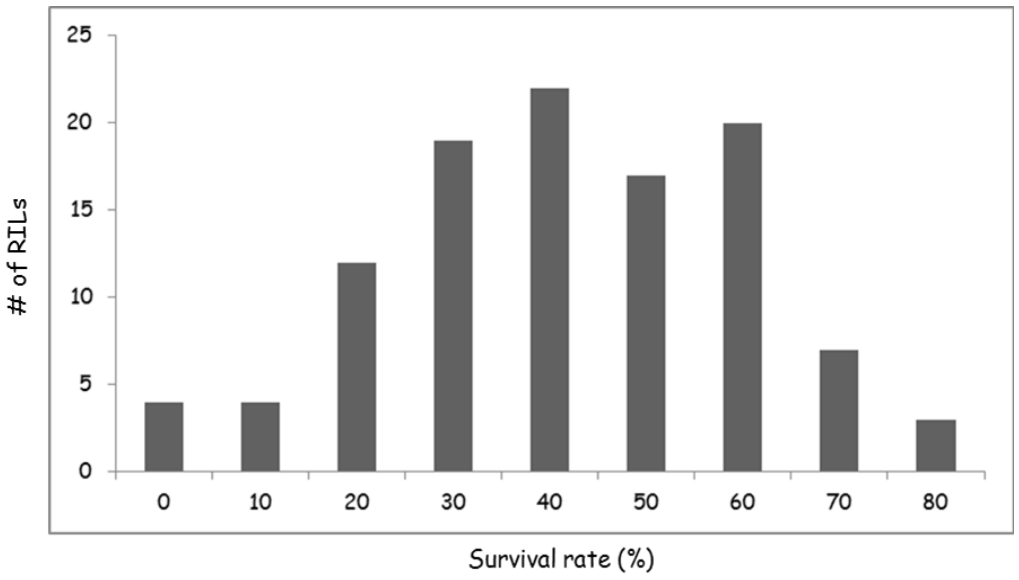

Figure 2. Frequency distribution for seedling survival rate for the 108 F_6 Recombinant Inbred Lines (RILs) of *Boechera stricta*. Acclimated seedlings of RILs were accessed at -8℃. Distribution shows that the trait is normally distributed.

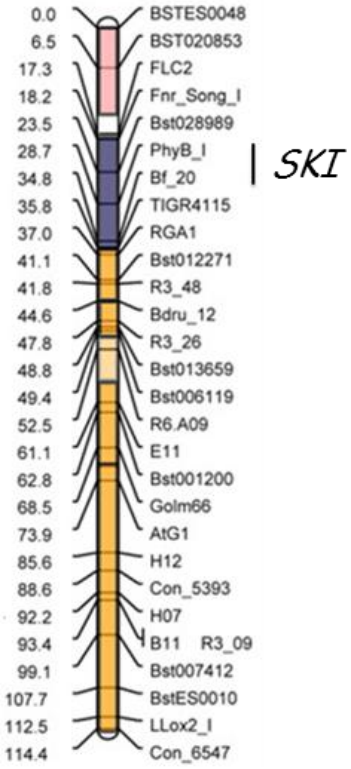

Figure 3: A single major QTL for acclimated seedling survival was detected on LGV of *Boechera stricta* and that we named *SKI* (*Seedling Koud Intolerance*). The *SKI* locus explains 22.4% of the phenotypic variance with the LTM parental allele conferring greater freezing tolerance.

DGAT1 IDENTIFIED AS CANDIDATE GENE UNDERLYING *SKI*

The comparative genomics framework between *B. stricta*, *A. thaliana* and *A. lyrata* and *C. rubella* allowed us to identify potential candidate genes that were found in *A. thaliana* freezing tolerance studies. The one-LOD confidence interval for the *SKI* QTL for seedling survival is 6cM and is contained within genomic block H corresponding to Arabidopsis chromosome 2 approximately from At2g18790 to At2g20050. By GO annotation analysis, three cold responsive genes within this region in Arabidopsis are identified: Diacylglycerol O-acyltransferase 1 (DGAT1, At2g19450), histone binding protein RBBP4 (At2g19540), and an uncharacterized/hypothetical protein (At2g20010). Diacylglycerol O-acyltransferase 1 and histone binding protein RBBP4 are involved in the regulation of triglyceride synthesis and chromatin metabolism, respectively. Analysis of gene expression for the three genes showed that *DGAT1* had the highest transcriptional induction during cold treatment, in particular in the shoot (Figure 4). The function of *DGAT1* has been extensively studied in seeds, but its potential role in freezing tolerance has not yet been studied or identified. However, a relationship between the accumulation of TAGs and freezing tolerance has recently been suggested

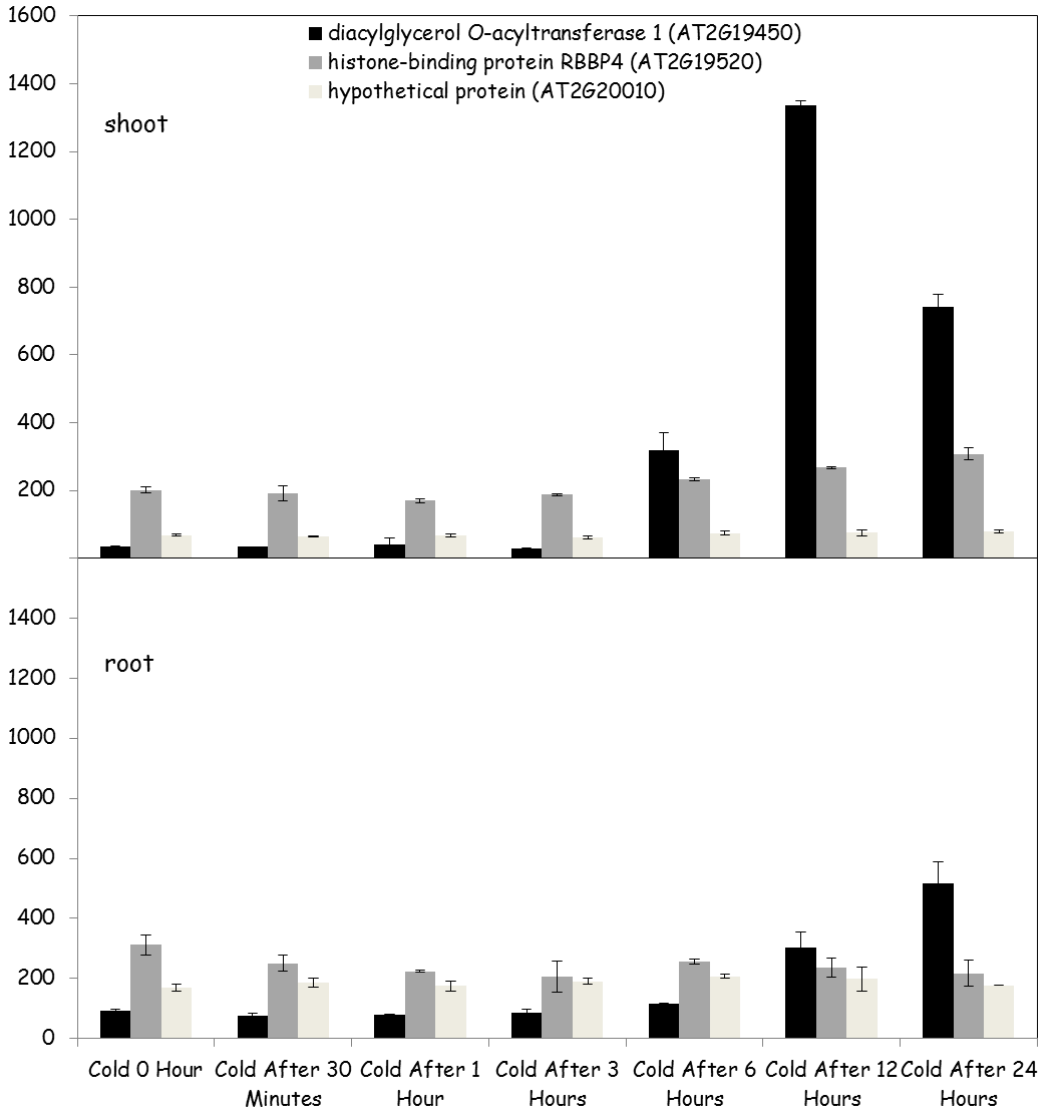

Figure 4. Comparative genomic analysis between Boechera and *Arabidopsis* identified three potential candidate genes associated with Cold-Induction within the 1-LOD confidence interval of the *SKI* locus. The comparison in gene expression profiles under control and cold conditions for the three candidate genes (Diacylglycerol O-acyltransferase 1 (DGAT), histone binding protein RBBP4 and an uncharacterized/hypothetical protein) is shown, with DGAT1 showing a significant induction upon cold treatment.

(Moellering et al., 2010). Thus, we aimed to characterize the function of DGAT1 in both Boechera and Arabidopsis.

SEQUENCE AND EXPRESSION PATTERN ANLYSIS OF DGAT1 IN *BOECHERA*

Comparison of cloned SAD12 and LTM alleles of *DGAT1* identified five SNPs. Only one SNP resulted in a predicted amino acid change in what is a variable region of the protein when compared in more distant species (Data not shown). Significant differences between genotypes

were found in induction of the DGAT1 alleles after cold treatment. The SAD12 allele of DGAT1 was almost undetectable under both non- and cold stress conditions (Figure 5); In contrast, up-regulation of the more freezing tolerant LTM *DGAT1* allele was found in response to cold treatment. To further assess the potential role of *DGAT1* in freezing tolerance, the expression and survival of *Arabidopsis* wild-type and DGAT1 mutant lines were analyzed (Figure 5). Gene expression analysis showed that the *DGAT1* mutant (ASI1) exhibited a consistently lower *DGAT1* expression during cold acclimation compared to wild type. Furthermore, seedling survival Arabidopsis wild -ype seedlings had more freezing tolerance than the mutant (23% of the wild-type seedings survived -8 C freezing and all mutant seedlings were killed). These results suggest that a lower expression of *DGAT1* is correlated with reduced freezing tolerance (directly or indirectly).

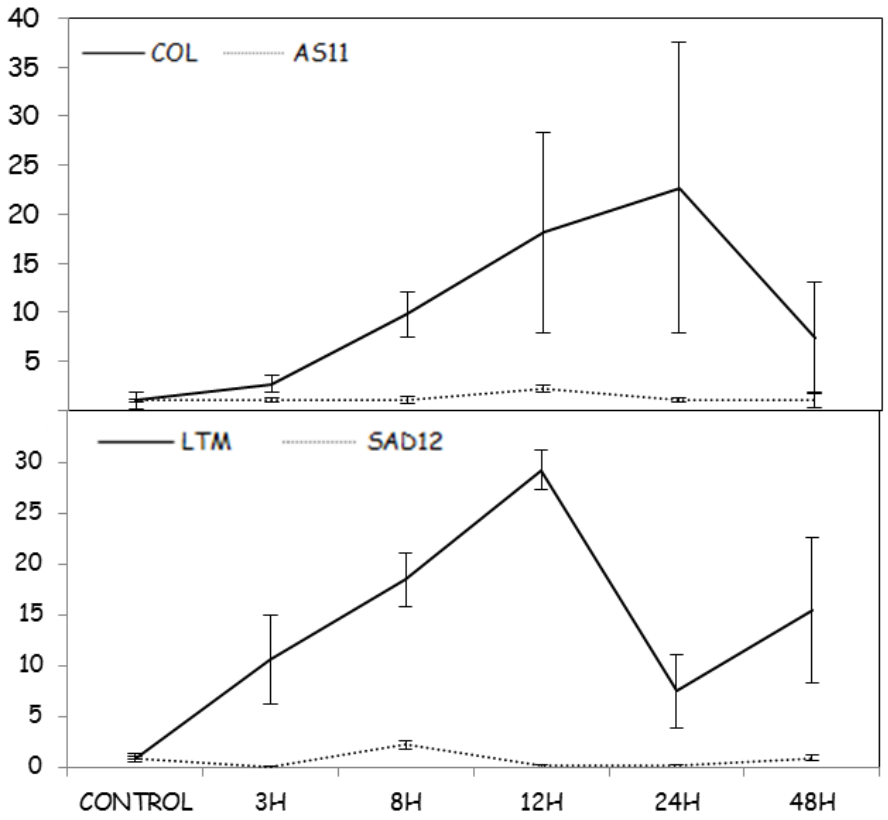

Figure 5: Time course of gene expression for *DGAT1* in leaves of plants shifted to 4 °C of *Arabidopsis* and *Bochera*. Values are expression relative to the control (no cold treatment) for each gene. Actin 2 was used as the reference gene for each species. A star (*P< 0.05 and **P< 0.005) above the error bars at a particular time point indicates a significant difference between LTM and SAD12 or wild type Columbia and DGAT1 mutant. It was tested by Student's *t*-test (n=3, error bars indicate SEs).

Table 1: Lipid composition of the polar lipids isolated from non-acclimated (NA) and cold-acclimated (CA) leaves of *Boechera* and *Arabidopsis*. The results are means and standard errors based on three replicates.

LIPID	LTM(NA)	LTM(CA)	SAD12(NA)	SAD12(CA)	COL(NA)	COL(CA)	DGAT1(NA)	DGAT1(CA)
				mol % of total lipids				
DGDG	15.34±1.60	13.73±0.20	12.85±0.89	12.73±0.45	12.38±1.87	13.48±0.96	14.24±1.18	12.21±0.04*
MGDG	56.27±1.28	50.65±2.83*	52.70±1.65	46.45±1.11**	55.17±3.12	50.75±0.41	58.03±3.07	51.01±1.31*
PG	8.18±0.47	7.65±0.50	8.19±0.13	6.15±0.29**	7.19±0.94	8.44±0.64	6.85±0.50	8.26±0.38*
LysoPG	0.07±0.01	0.09±0.01*	0.08±0.02	0.10±0.01	0.07±0.02	0.04±0.04	0.04±0.02	0.00±0.00*
LysoPC	0.09±0.00	0.09±0.02	0.11±0.01	0.11±0.01	0.08±0.02	0.06±0.01	0.05±0.01	0.08±0.01*
LysoPE	0.08±0.00	0.11±0.02*	0.09±0.00	0.13±0.01**	0.08±0.01	0.09±0.04	0.07±0.01	0.12±0.03**
PC	11.97±0.42	13.14±0.41*	14.99±0.94	14.62±1.10	15.39±1.48	12.61±1.61	12.85±1.29	14.97±0.65*
PE	2.65±0.29	4.03±0.97*	4.45±0.40	4.69±0.29	4.25±0.52	3.98±0.10	2.99±0.61	4.99±0.86*
PI	4.24±0.09	4.17±0.36	5.01±0.47	5.12±0.38	4.80±0.62	4.96±0.51	3.63±0.37	4.95±0.49*
PS	0.03±0.00	0.13±0.05*	0.03±0.00	0.14±0.01**	0.02±0.01	0.22±0.03**	0.11±0.09	0.29±0.06*
PA	1.08±0.82	6.19±1.41**	1.49±0.99	9.73±1.09**	0.56±0.37	5.35±2.43**	1.11±0.92	3.12±0.65*

A star (*P< 0.05 and **P< 0.005) indicates a significant difference between control and cold treated samples. It was tested by Student's *t*-test (n=3).
DGDG: digalactosyldiacylglycerol, MGDG: monogalactosyldiacylglycerol, PG: phosphatidylglycerol, lysoPG: lysophosphatidylglycerol, lysoPC: lysophosphatidylcholine, lysoPE: lysophosphatidylethanolamine, PC: phosphatidylcholine, PE: phosphatidylethanolamine, PI: phosphatidylinositol, PS: phosphatidylserine, PA: phosphatidic acid.

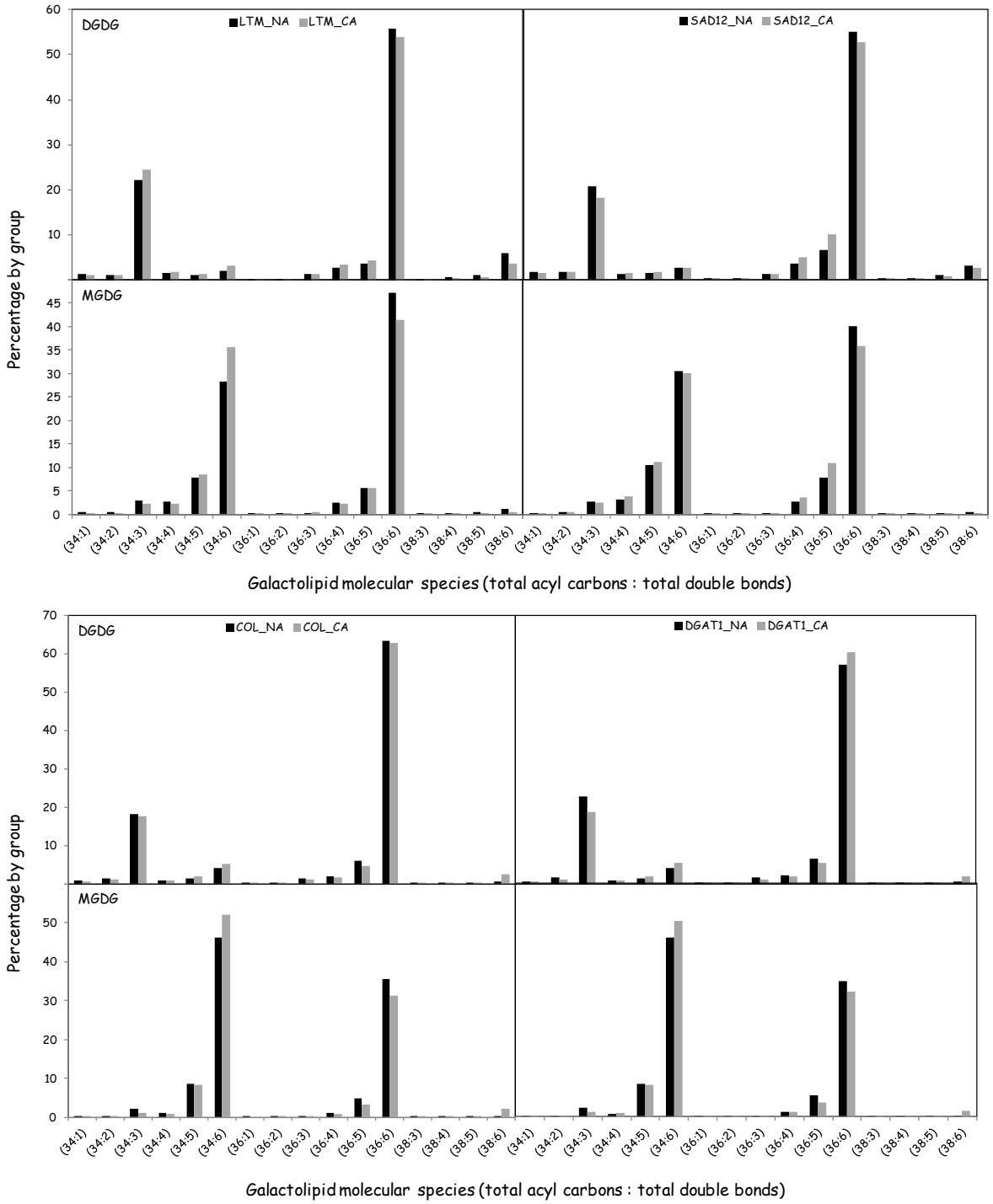

Figure 6. Changes in galatolipid molecular species isolated from non-acclimated (NA) and cold-acclimated (CA) leaves of *Boechera* and *Arabidopsis*.

POLAR LIPID AND TRIACYLGLYCEROL ANALYSES

Based on previous results, we hypothesized that differential regulation of *DGAT1*, one of two major families of DGAT enzymes, might cause differential modification and accumulation of polar lipids and TAGs after cold acclimation. DGAT activity not only mediates cellular fatty acid storage and membrane formation, but also influences fatty acid polyunsaturation and triacylglycerol accumulation (Zhang et al., 2013). Hence, we analysed the molecular species of polar lipids and triacylglycerols in acclimated and non-acclimated plants.

The total amount of galactolipids in *Boechera* leaves remained unchanged (data not shown), but the proportion of galactolipids in total polar lipids after cold acclimation highly decreased from 71.5% to 64.3% and 65.7% to 59.1% in LTM and SAD12, respectively (Table 1). The proportion of monogalactosyldiacylglycerol (MGDG) was significantly reduced in response to the cold in both genotypes. However, LTM accumulated poly-unsaturated MGDG 34:6 following cold acclimation, while SAD12 displayed a reduction for this lipid (Figure 6). Although changes in digalactosyldiacylglycerol (DGDG) were relatively small in both genotypes, LTM had greater DGDG decrease compared to SAD12. A decrease in total proportion of galactolipids resulted in an increase in the proportion of the phospholipids after cold acclimation, which was due primarily to the change of phosphatidic acid (PA). Changes in some phospholipids differed among genotypes. For instance, phosphatidylcholine (PC) which is one of most abundant phospholipids showed decreases in SAD12, but in LTM there was a small increase in PC. Particularly, proportion of several unsaturated PC species including 34:4 and 36:5 increased in LTM, but decreased in SAD12 (Figure 7). Cold acclimation also led to numerous changes in the composition of polar lipids in *Arabidopsis*. Commonly, there was a decrease in MGDG, a predominant galactolipid, and increases in PG, phosphatidylinositol (PI), phosphatidylserine (PS) and PA following cold acclimation (Table 1). Polar lipid analysis, however, showed that polar lipids such as DGDG, PC and PE differed between mutant and wild-type seedlings. Calculation of the values for mol % each lipid molecular species of these lipids showed more clear differences (Figures 7 and 8). For both DGDG and PE, the proportion of 36:6 containing two unsaturated chains increased in wild type seedling, whereas decreased in mutant seedling. In addition, a relatively high increase and low decrease in polyunsaturated PC 36:5 and 36:6 of wild type seedling were observed compared to them of mutant seedling, respectively.

An accumulation in total amount of TAGs was found in *Arabidopsis* wild type and the amount of TAGs either slightly or largely decreased after cold acclimation (Table 2; Supplementary table 1~6). Interestingly, cold acclimated LTM showing up-regulation of DGAT1 during cold acclimation had highly decreased contents in TAGs, but we found by TLC analysis that TAGs in LTM accumulated as a direct response to freezing, but were low in all other cases (Figure 9).

Table 2. Changes in triacylglycerols(TAGs) isolated from non-acclimated (NA) and cold-acclimated (CA) leaves of *Boechera* and *Arabidopsis*.

LIPID	LTM(NA)	LTM(CA)	SAD12 (NA)	SAD12 (CA)	COL(NA)	COL(CA)	DGAT1 (NA)	DGAT1 (CA)
				nmol per mg dry wt				
16:0	0.66±0.21	0.36±0.21	0.15±0.06	0.15±0.09	0.27±0.18	0.30±0.04	0.29±0.10	0.22±0.05
18:0	0.50±0.20	0.23±0.18	0.15±0.06	0.12±0.05	0.29±0.18	0.28±0.05	0.26±0.07	0.22±0.03
18:1	0.32±0.11	0.09±0.06*	0.03±0.01	0.02±0.01	0.04±0.04	0.03±0.01	0.02±0.01	0.02±0.01
18:2	0.48±0.12	0.18±0.10*	0.10±0.03	0.06±0.01	0.09±0.04	0.10±0.05	0.08±0.02	0.07±0.02
18:3	0.60±0.12	0.28±0.15*	0.15±0.05	0.13±0.06	0.19±0.13	0.26±0.03	0.20±0.10	0.18±0.06
20:1	0.05±0.02	0.02±0.02*	0.01±0.00	0.01±0.00	0.02±0.02	0.02±0.00	0.03±0.01	0.02±0.01
Total TAG	2.62±0.74	1.16±0.71*	0.59±0.21	0.48±0.23	0.90±0.58	0.99±0.11	0.88±0.03	0.73±0.17

A star (*P< 0.05) indicates a significant difference between control and cold treated samples (n=3). It was tested by Student's t-test (n=3).

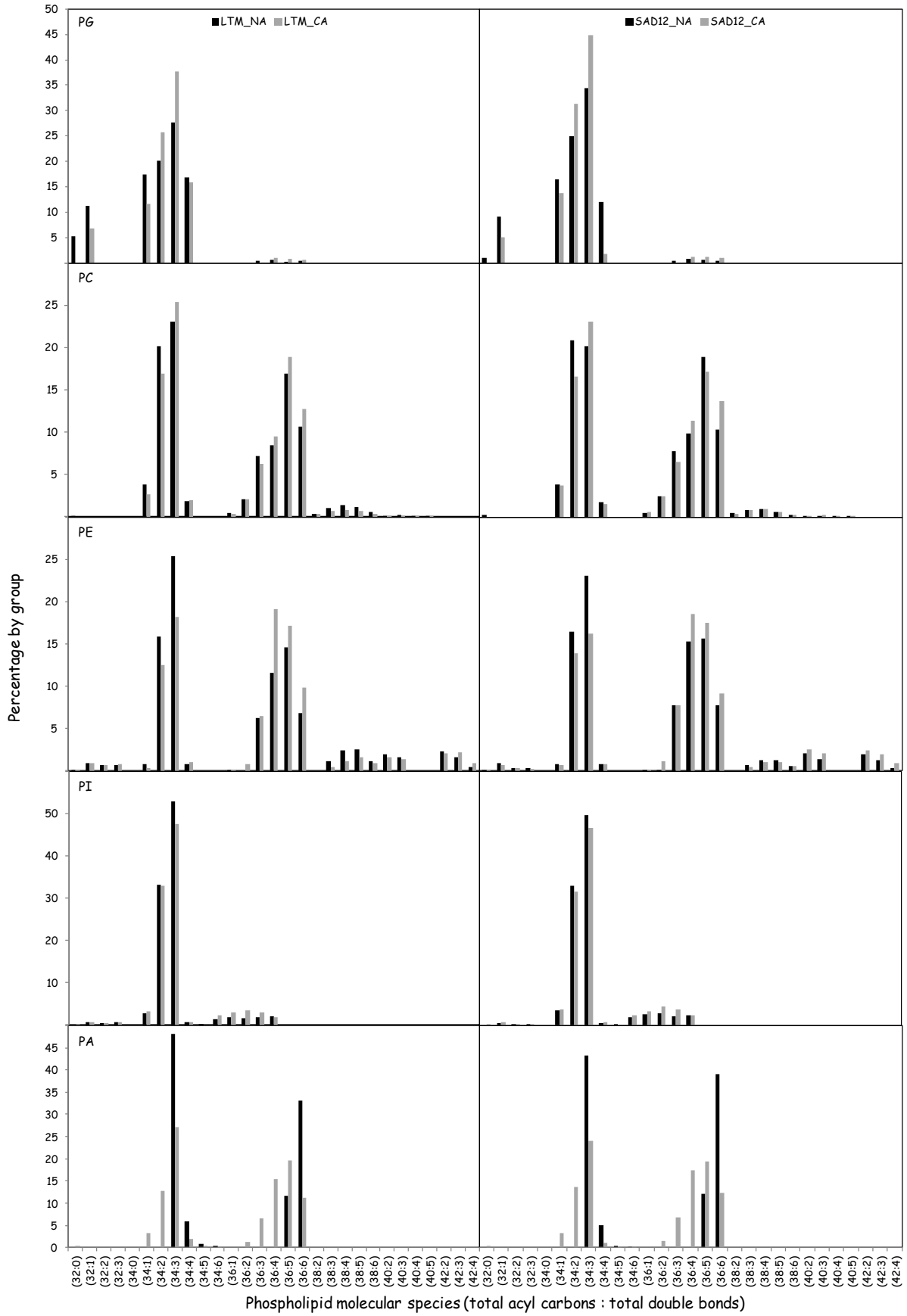

Figure 7. Changes in phospholipid molecular species isolated from non-acclimated (NA) and cold-acclimated (CA) leaves of *Boechera*.

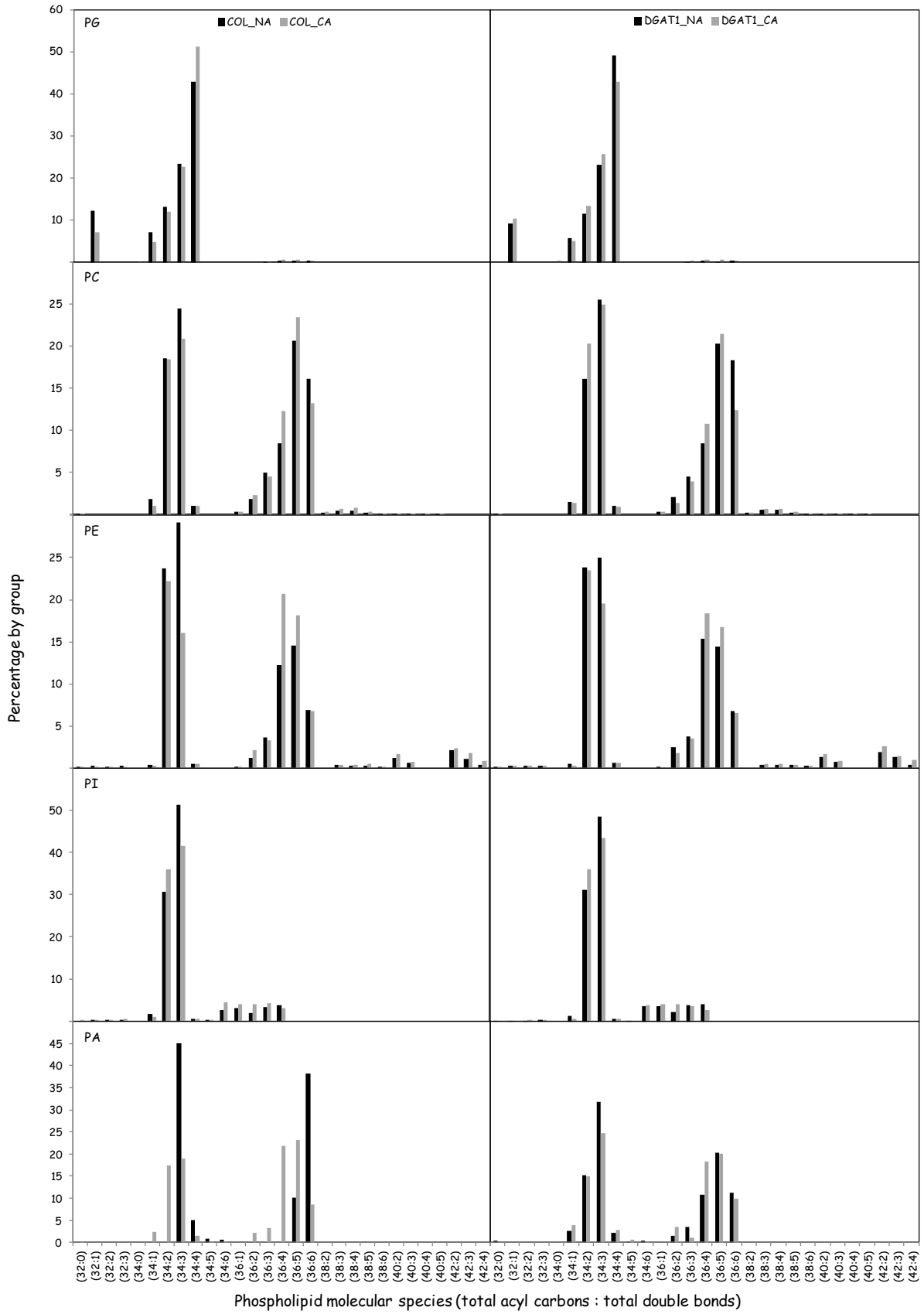

Figure 8. Changes in phospholipid molecular species isolated from non-acclimated (NA) and cold-acclimated (CA) leaves of *Arabidopsi*

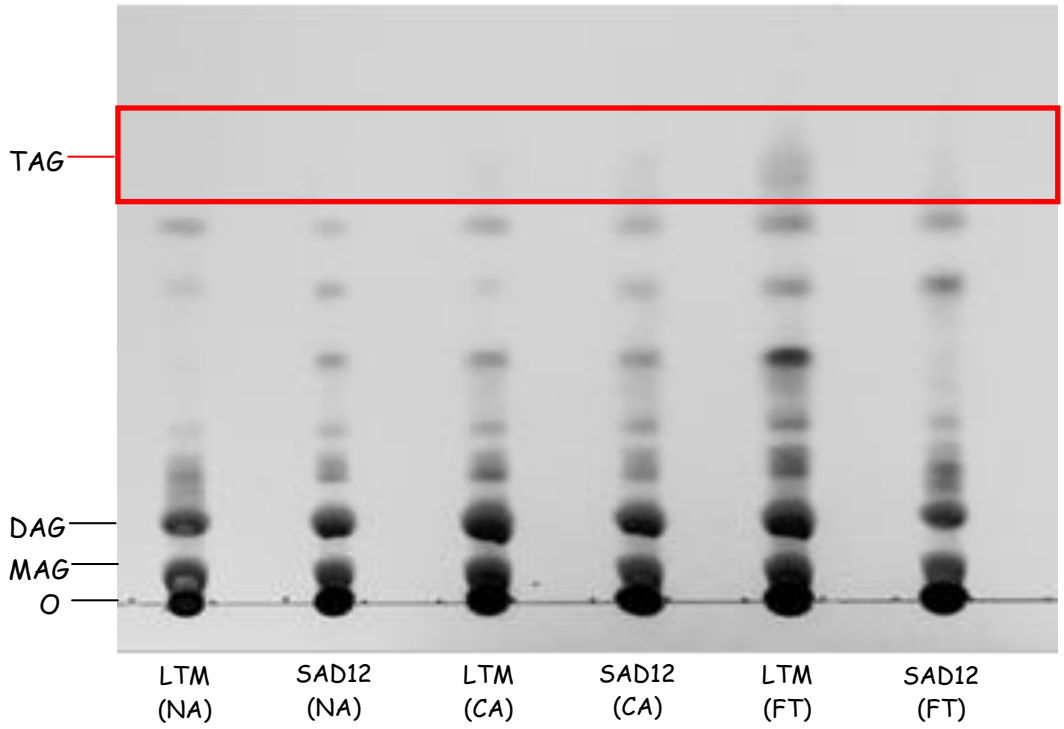

Figure 9. TAG accumulation test by TLC using non-acclimated (NA), cold-acclimated (CA) and freezing treated (FT) leaves of *Boechera*. Lipids were visualized by iodine staining. This result indicates LTM has greater accumulation of TAG under freezing conditions only. TAG, triacylglycerol; DAG, diacylglycerol; MAG, monacylglycerol; O, origin.

DISCUSSION

SKI IS A NOVEL QTL FOR SEEDLING FREEZING TOLERANCE IN *B. STRICTA*

A number of adaptive mechanisms contribute to maintain optimal conditions for growth and development of overwintering plants and protect against cold and freezing stress. The degree of freezing tolerance in overwintering plants depends largely on the developmental stage at which the stress occurs (Beck et al., 2004), and thus different genes according to different developmental-stages could play a role for preventing the damage caused by freezing. Surviving the early growth stage (e.g. as seedlings) determines the persistence of plants in a given habitat (Loik and Redar, 2003; Von Meijenfeldt, 2010). Hence, we set out to identify the genetic determinant of seedling freezing tolerance in *B. stricta*.

Our QTL analysis detected a single significant QTL (*SKI*) explaining more than 24% of the phenotypic variance. *SKI* was not identified in our previous QTL study done with adult plants (Chapter 2 of this thesis). Anderson et al. (2014) did a QTL analysis for several traits in

the original parental environments (Colorado and Montana) to assess patterns of natural selection. In their experiment, several QTLs responsible for winter-survival were identified, mostly LTM alleles contributing to survival at the Montana field site. A single QTL was found in the first year in Colorado. Interestingly, this was the only case an LTM allele was beneficial at the Colorado site. This QTL found in the field overlaps the SKI QTL identified in our controlled greenhouse conditions, again with the LTM allele contributing to a higher freezing tolerance. These results suggest that this locus may play a major role in the adaptive evolution of freezing tolerance in *B. stricta* both in the field and the lab. Furthermore, to our knowledge, *DGAT1* loci have not been previously identified as components of freezing tolerance in other species.

DGAT1 IS A POTENTIAL CANDIDATE GENE FOR FREEZING TOLERANCE

Triacylglycerols (TAGs) are the major storage lipids in seeds, which are important energy reserves in seeds for supporting early seedling growth after germination (Hernandez et al., 2012). These storage lipids have potential value as biofuels, food and feed and there has been much interest in understanding the regulation of their synthesis in seeds (Harwood and Guschina, 2013). Numerous genes controlling freezing tolerance have been identified and characterized in *A. thaliana* in the Brassicaceae family. Comparative genomics within the Brassicaceae, including *B. stricta* of show conserved chromosomal blocks (Schranz, Lysak and Mitchell-Olds; Schranz et al., 2007; Chen and Wang, 2013). Previous comparative genomics study allowed us to identify potential candidate genes in Arabidopsis compared to *B. stricta*. Our *SKI* QTL interval in *Boechera* is collinear on the genomic region that contains acyl-CoA:diacylglycerol acyltransferase 1 (DGAT1) in *Arabidopsis*. In *Arabidopsis,* DGAT1 is the only acyltransferase enzyme that has been confirmed to contribute to triacylglycerol biosynthesis in seeds (Turchetto-Zolet et al., 2011). Although TAGs are typically synthesized during seed maturation, the accumulation of TAGs can also be observed in leaves or other vegetative tissues in response to senescence, cold and freezing stress (Kaup et al., 2002; Moellering et al., 2010; Degenkolbe et al., 2012). It has been shown that up-regulation of *DGAT1* in senescing leaves of *Arabidopsis* and leaf-specific expression of *DGAT1* in transgenic tobacco caused increase of TAGs in their leaves, but the role of *DGAT1* in regulating the synthesis of TAGs in seedlings or developed plants has remained unclear (Kaup et al., 2002; Andrianov et al., 2010). It is also possible that lipids initially stored in the seed are subsequently transferred to the developing seedling. Our analysis of gene expression showed there was an up-regulation of *DGAT1* genes in *Boechera* and

Arabidopsis seedlings during cold treatment, that supports the role of *DGAT1* transcriptional regulation being involved in cold acclimation both species. Furthermore, differential regulation patterns of *DGAT1* alleles in *Boechera* (LTM > SAD12) and *Arabidopsis* (COL-0 > ACI1) support its potential importance in regulating freezing tolerance, particularly by affecting the alternation of lipid composition during cold acclimation.

Lipid profiling results showed several polar lipid changes after cold acclimation in *Boechera* and *Arabidopsis*. A large decrease in the proportion of MGDG, that have a high propensity to form hexagonal II phase, was commonly observed in *Boechera* and *Arabidopsis*. Since the formation of hexagonal II phase is considered as the main cause of membrane injury in plants, the lower propensity of hexagonal II phase after cold acclimation could help to maintain the biological functions of chloroplast membranes against freezing (Uemura et al., 1995). An increase of DGDG with a low propensity to form hexagonal II also contributes to the stabilization of the bilayer membrane and lamellar configuration, but DGDG showed only small changes following cold acclimation in *Boechera* and *Arabidopsis* (Uemura and Steponkus, 1997). Although an increase in DGDG was observed in *Arabidopsis* wild type seedlings, changing degrees in content and proportion of DGDG were not significant, suggesting DGDG was merely active during cold acclimation in *Boechera* and *Arabidopsis*.

In both herbaceous and woody species, cold acclimation leads to incremental change in the contents and proportion of phospholipids, which appear to be associated with increased freezing tolerance by increasing accumulation of TAGs in *Boechera*. Degenkolbe et al. (2012) reported accumulations of TAGs in long-term cold acclimated *Arabidopsis*, which were not directly related to the concomitant increase in freezing tolerance. Amounts of TAGs only account for around 1% of total lipid after cold acclimation, which could means that changes in amounts are too small to show significant differences. In two genotypes of *Boechera* with contrasting freezing tolerance we found some differences in lipid composition, but due to unexpected changing patterns in TAGs that were expected to be associated with increased DGAT1 expression it was difficult to conclude that differential DGAT1 expression affected them and resulted in their distinguishable freezing tolerance.

It has been known that many pathways are involved in lipid modification related to freezing tolerance and there are many complex interactions (Yamaguchi-Shinozaki and Shinozaki, 2006). For these reasons, we could not discard the possibility that other pathways led to these results. However, in *Arabidopsis* we observed wild type seedling showed a higher freezing

tolerance and better preparation against freezing on the lipid modification after cold acclimation compared to those of mutant seedling. Although the degrees on their differences are still small, these results support that DGAT1 can be a potential candidate gene for freezing tolerance.

FUTURE DIRECTIONS

Although our results strongly support *DGAT1* as the candidate gene underlying the *SKI* QTL and changes in freezing tolerance, to prove the relationship between *DGAT1* and freezing tolerance after cold acclimation more research is needed. Additional experiments, therefore, will need to be conducted to characterize its exact role in freezing-tolerance in the future. Degenkolbe et al. (2012) reported TAG contents increased in most of *Arabidopsis* accessions, but very freezing tolerant and sensitive accessions showed similar TAG contents before and after cold acclimation. It was a quite interesting result because LTM with high freezing tolerance did not show increase in TAG contents after cold acclimation. Their and our results might suggest post-transcriptional and translational regulatory mechanisms for *DGAT1* exist, or that the major effect of DGAT1 in freezing tolerance is not in modifying TAG levels, but rather due to other lipid contents affected by more active DGAT1 alleles, such as PC or PE. Upstream molecular mechanisms in plants can differ by regulation of timings and amount of specific stress responses (Mazzucotelli et al., 2008). Hence, it can be assumed that DGAT1 protein in LTM-like plants with more ability to withstand cold might be inactive during mild cold stress conditions. In contrast, their function might be fully activated under more stressful conditions such as freezing, which could lead to some important modifications of lipid or accumulation of TAGs. Our TLC experiment implied that huge amount of TAGs might be accumulated in LTM by freezing treatment. Moellering et al. (2010) reported the freezing treatment resulted in a 7.5 fold increase in TAGs compared to cold acclimation condition, which might contribute to prevent the accumulation of DAG forming non-lamellar phases. Hence, accumulation of TAGs can have a potential role to protect critical cell structures during freezing, but the impact of TAG accumulation on freezing tolerance is still unclear. Further lipid profiling data for freezing-treated samples can help to verify these hypotheses and to characterize their potential role for freezing tolerance more clearly. In addition, we found that *DGAT1* transcript level in SAD12 was much lower than in LTM although *DGAT1* gene was highly conserved between LTM and SAD12. In

this study we, however, did not focus on what causes differential level of transcription of DGAT1 between LTM and SAD12. Promoter region analysis, aiming to detect mutations in binding sites for transcription factors involved in cold tolerance and acclimation, as well as transformation studies will help us to find what leads to the difference.

Supplementary table 1. Changes in TAG 16:0-containing molecular species isolated from non-acclimated (NA) and cold-acclimated (CA) leaves of *Bochera* and *Arabidopsis* [Dry weight (nmol/mg)].

	32:0	32:1	32:2	32:3	34:1	34:2	34:3	34:4	34:5	36:2	36:3	36:4
LTM_NA	0.043	0.001	0.360	0.011	0.006	0.016	0.021	0.010	0.003	0.005	0.007	0.015
LTM_CA	0.005	0.000	0.106	0.003	0.000	0.002	0.004	0.002	0.000	0.000	0.000	0.004
SAD12_NA	0.006	0.000	0.101	0.003	0.000	0.002	0.007	0.001	0.000	0.000	0.000	0.003
SAD12_CA	0.019	0.000	0.194	0.009	0.000	0.013	0.025	0.004	0.001	0.000	0.002	0.011
COL_NA	0.018	0.000	0.212	0.003	0.001	0.004	0.002	0.001	0.000	0.000	0.001	0.002
COL_CA	0.032	0.000	0.221	0.004	0.000	0.004	0.005	0.001	0.000	0.000	0.000	0.004
DGAT1_NA	0.020	0.000	0.237	0.004	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.001
DGAT1_CA	0.013	0.000	0.169	0.002	0.002	0.003	0.002	0.001	0.000	0.000	0.001	0.003
	36:5	36:6	36:7	38:2	38:3	38:4	38:5	38:6	38:7	38:8	38:9	40:2
LTM_NA	0.013	0.022	0.008	0.014	0.015	0.014	0.001	0.004	0.003	0.001	0.002	0.012
LTM_CA	0.006	0.008	0.001	0.001	0.001	0.000	0.000	0.001	0.001	0.000	0.000	0.000
SAD12_NA	0.007	0.009	0.002	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000
SAD12_CA	0.016	0.019	0.003	0.004	0.004	0.004	0.000	0.000	0.000	0.000	0.000	0.004
COL_NA	0.002	0.003	0.004	0.000	0.001	0.000	0.000	0.000	0.001	0.000	0.001	0.000
COL_CA	0.007	0.004	0.005	0.001	0.001	0.001	0.000	0.000	0.002	0.000	0.000	0.000
DGAT1_NA	0.003	0.004	0.004	0.000	0.000	0.000	0.000	0.000	0.002	0.001	0.000	0.000
DGAT1_CA	0.005	0.003	0.002	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000
	40:3	40:4	40:5	40:6	40:7	40:8	40:2	40:3	40:4	40:5	40:6	40:7
LTM_NA	0.022	0.017	0.001	0.001	0.000	0.001	0.001	0.008	0.001	0.001	0.004	0.001
LTM_CA	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.001	0.000
SAD12_NA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000
SAD12_CA	0.008	0.005	0.000	0.000	0.000	0.001	0.000	0.003	0.000	0.001	0.001	0.000
COL_NA	0.000	0.000	0.000	0.001	0.000	0.001	0.000	0.004	0.000	0.001	0.001	0.000
COL_CA	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.003	0.000	0.000	0.001	0.000
DGAT1_NA	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.004	0.000	0.000	0.002	0.000
DGAT1_CA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.005	0.000	0.001	0.002	0.000

Supplementary table 2. Changes in TAG 18:0-containing molecular species isolated from non-acclimated (NA) and cold-acclimated (CA) leaves of *Bochera* and *Arabidopsis* [Dry weight (nmol/mg)].

	30:0	30:1	30:2	30:3	32:2	32:3	32:4	32:5	34:2	34:3
LTM_NA	0.014	0.000	0.013	0.001	0.396	0.004	0.001	0.000	0.007	0.002
LTM_CA	0.002	0.000	0.004	0.000	0.123	0.001	0.000	0.000	0.000	0.001
SAD12_NA	0.005	0.000	0.004	0.000	0.095	0.001	0.000	0.000	0.002	0.000
SAD12_CA	0.009	0.000	0.007	0.001	0.183	0.002	0.001	0.000	0.005	0.002
COL_NA	0.006	0.001	0.009	0.001	0.238	0.002	0.002	0.001	0.003	0.001
COL_CA	0.009	0.002	0.008	0.001	0.231	0.002	0.001	0.000	0.001	0.003
DGAT1_NA	0.008	0.000	0.009	0.001	0.221	0.002	0.001	0.001	0.001	0.000
DGAT1_CA	0.007	0.001	0.006	0.001	0.189	0.002	0.000	0.000	0.000	0.001
	34:4	34:6	34:7	36:2	36:3	36:4	36:5	36:6	36:7	36:8
LTM_NA	0.002	0.001	0.009	0.004	0.001	0.003	0.003	0.004	0.009	0.001
LTM_CA	0.000	0.000	0.003	0.001	0.000	0.001	0.001	0.001	0.003	0.000
SAD12_NA	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.001	0.002	0.000
SAD12_CA	0.001	0.000	0.004	0.000	0.001	0.001	0.001	0.002	0.004	0.001
COL_NA	0.001	0.000	0.006	0.001	0.001	0.000	0.001	0.001	0.005	0.000
COL_CA	0.000	0.000	0.006	0.000	0.002	0.000	0.001	0.001	0.005	0.001
DGAT1_NA	0.000	0.000	0.005	0.000	0.000	0.000	0.000	0.000	0.004	0.001
DGAT1_CA	0.000	0.000	0.004	0.000	0.001	0.001	0.000	0.001	0.004	0.001
	36:9	38:2	38:3	38:4	38:5	38:6	38:7	38:8	40:2	40:4
LTM_NA	0.000	0.004	0.001	0.002	0.000	0.001	0.004	0.001	0.003	0.009
LTM_CA	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.001	0.000
SAD12_NA	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000
SAD12_CA	0.000	0.001	0.002	0.001	0.000	0.000	0.001	0.000	0.000	0.001
COL_NA	0.001	0.001	0.001	0.000	0.000	0.000	0.002	0.001	0.000	0.000
COL_CA	0.000	0.000	0.001	0.000	0.001	0.000	0.002	0.001	0.000	0.000
DGAT1_NA	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.001	0.000	0.000
DGAT1_CA	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000

Supplementary table 3. Changes in TAG 18:1-containing molecular species isolated from non-acclimated (NA) and cold-acclimated (CA) leaves of *Bochera* and *Arabidopsis* [Dry weight (nmol/mg)].

	30:0	30:1	30:2	32:0	32:1	32:2	32:3	32:4	34:1	34:2	34:3
LTM_NA	0.003	0.003	0.002	0.006	0.005	0.009	0.005	0.001	0.004	0.011	0.013
LTM_CA	0.001	0.001	0.001	0.000	0.001	0.002	0.001	0.000	0.000	0.000	0.003
SAD12_NA	0.000	0.001	0.000	0.000	0.001	0.002	0.001	0.000	0.000	0.001	0.003
SAD12_CA	0.000	0.001	0.001	0.004	0.001	0.004	0.001	0.001	0.000	0.006	0.010
COL_NA	0.002	0.001	0.001	0.003	0.001	0.004	0.001	0.001	0.000	0.002	0.001
DGAT1_NA	0.000	0.001	0.001	0.000	0.000	0.004	0.001	0.001	0.000	0.001	0.001
COL_CA	0.000	0.001	0.001	0.000	0.001	0.005	0.001	0.001	0.000	0.002	0.002
DGAT1_CA	0.001	0.001	0.001	0.001	0.000	0.003	0.001	0.001	0.000	0.000	0.004
	34:4	34:5	34:6	36:1	36:2	36:3	36:4	36:5	36:6	36:7	36:8
LTM_NA	0.005	0.001	0.001	0.006	0.005	0.007	0.009	0.008	0.009	0.001	0.001
SAD12_NA	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.002	0.000	0.000
LTM_CA	0.001	0.000	0.000	0.000	0.000	0.000	0.001	0.006	0.006	0.000	0.000
SAD12_CA	0.002	0.001	0.001	0.001	0.000	0.001	0.003	0.006	0.003	0.001	0.001
COL_NA	0.002	0.001	0.001	0.001	0.000	0.000	0.002	0.004	0.002	0.001	0.000
DGAT1_NA	0.000	0.001	0.000	0.000	0.000	0.000	0.001	0.002	0.000	0.001	0.001
COL_CA	0.001	0.001	0.001	0.000	0.000	0.001	0.001	0.003	0.001	0.000	0.002
DGAT1_CA	0.001	0.000	0.001	0.000	0.000	0.000	0.000	0.003	0.001	0.000	0.000
	38:1	38:2	38:3	38:4	38:5	38:7	40:1	40:2	40:3	40:4	40:5
LTM_NA	0.006	0.037	0.018	0.021	0.001	0.001	0.005	0.005	0.033	0.032	0.003
SAD12_NA	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.001	0.001	0.001	0.000
LTM_CA	0.000	0.002	0.001	0.000	0.000	0.000	0.001	0.002	0.001	0.001	0.000
SAD12_CA	0.002	0.009	0.004	0.004	0.000	0.000	0.001	0.011	0.007	0.006	0.000
COL_NA	0.000	0.001	0.001	0.000	0.001	0.001	0.001	0.001	0.000	0.000	0.000
DGAT1_NA	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000
COL_CA	0.000	0.002	0.000	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000
DGAT1_CA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Supplementary table 4. Changes in TAG 18:2-containing molecular species isolated from non-acclimated (NA) and cold-acclimated (CA) leaves of *Bochera* and *Arabidopsis* [Dry weight (nmol/mg)].

	30:0	30:1	32:0	32:1	32:2	32:3	34:0	34:1	34:2	34:3
LTM_NA	0.004	0.003	0.012	0.010	0.007	0.006	0.002	0.011	0.027	0.020
SAD12_NA	0.000	0.001	0.003	0.001	0.001	0.001	0.000	0.000	0.006	0.010
LTM_CA	0.001	0.001	0.004	0.002	0.001	0.001	0.001	0.002	0.010	0.010
SAD12_CA	0.001	0.000	0.010	0.004	0.003	0.002	0.001	0.003	0.015	0.024
COL_NA	0.002	0.002	0.005	0.001	0.002	0.001	0.001	0.002	0.008	0.003
DGAT1_NA	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.000	0.006	0.003
COL_CA	0.001	0.001	0.003	0.001	0.002	0.001	0.001	0.000	0.011	0.007
DGAT1_CA	0.001	0.001	0.001	0.000	0.001	0.001	0.000	0.000	0.005	0.006
	34:4	34:5	36:0	36:1	36:2	36:3	36:4	36:5	36:6	36:7
LTM_NA	0.008	0.002	0.001	0.010	0.015	0.008	0.014	0.018	0.031	0.002
SAD12_NA	0.002	0.000	0.000	0.001	0.000	0.001	0.002	0.003	0.005	0.000
LTM_CA	0.002	0.000	0.000	0.001	0.002	0.003	0.006	0.010	0.017	0.001
SAD12_CA	0.004	0.001	0.000	0.003	0.003	0.004	0.006	0.006	0.009	0.001
COL_NA	0.001	0.000	0.001	0.002	0.002	0.002	0.006	0.006	0.010	0.000
DGAT1_NA	0.001	0.000	0.000	0.001	0.000	0.000	0.004	0.008	0.010	0.000
COL_CA	0.001	0.000	0.002	0.002	0.001	0.001	0.004	0.008	0.007	0.001
DGAT1_CA	0.001	0.000	0.001	0.001	0.000	0.000	0.002	0.005	0.007	0.000
	38:1	38:2	38:3	38:4	38:5	40:0	40:2	40:3	40:4	40:5
LTM_NA	0.000	0.028	0.016	0.020	0.003	0.083	0.035	0.028	0.036	0.004
SAD12_NA	0.000	0.001	0.001	0.000	0.000	0.020	0.001	0.001	0.001	0.000
LTM_CA	0.001	0.001	0.001	0.001	0.000	0.012	0.001	0.001	0.002	0.000
SAD12_CA	0.003	0.008	0.004	0.004	0.000	0.038	0.009	0.005	0.009	0.001
COL_NA	0.000	0.000	0.001	0.000	0.000	0.032	0.000	0.000	0.000	0.000
DGAT1_NA	0.000	0.000	0.000	0.000	0.000	0.038	0.000	0.000	0.000	0.000
COL_CA	0.000	0.000	0.000	0.001	0.001	0.043	0.000	0.000	0.000	0.000
DGAT1_CA	0.000	0.000	0.000	0.000	0.000	0.031	0.000	0.000	0.000	0.000

Supplementary table 5. Changes in TAG 18:3-containing molecular species isolated from non-acclimated (NA) and cold-acclimated (CA) leaves of *Bochera* and *Arabidopsis* [Dry weight (nmol/mg)].

	30:0	32:0	32:1	32:2	34:0	34:1	34:2	34:3	34:4	36:0	36:1	36:2	36:3
LTM_NA	0.002	0.009	0.006	0.006	0.001	0.008	0.011	0.021	0.007	0.002	0.007	0.007	0.009
LTM_CA	0.000	0.004	0.002	0.002	0.000	0.002	0.006	0.001	0.002	0.001	0.000	0.001	0.003
SAD12_NA	0.000	0.004	0.001	0.001	0.000	0.002	0.006	0.011	0.001	0.001	0.000	0.001	0.001
SAD12_CA	0.001	0.011	0.004	0.002	0.001	0.005	0.012	0.021	0.002	0.001	0.002	0.002	0.003
COL_NA	0.001	0.007	0.002	0.002	0.000	0.001	0.002	0.004	0.001	0.002	0.001	0.001	0.002
COL_CA	0.001	0.008	0.002	0.002	0.001	0.002	0.004	0.006	0.001	0.002	0.001	0.001	0.002
DGAT1_NA	0.001	0.006	0.001	0.002	0.000	0.001	0.002	0.004	0.001	0.002	0.000	0.000	0.001
DGAT1_CA	0.000	0.005	0.001	0.002	0.001	0.001	0.004	0.005	0.000	0.002	0.001	0.001	0.001
	36:4	36:5	36:6	38:1	38:2	38:3	38:4	38:5	40:0	40:1	40:2	40:3	40:4
LTM_NA	0.014	0.022	0.027	0.008	0.017	0.013	0.014	0.003	0.313	0.002	0.020	0.018	0.030
LTM_CA	0.007	0.016	0.018	0.000	0.001	0.002	0.001	0.001	0.063	0.001	0.001	0.002	0.002
SAD12_NA	0.003	0.005	0.007	0.000	0.000	0.001	0.000	0.001	0.073	0.000	0.001	0.001	0.001
SAD12_CA	0.006	0.008	0.008	0.002	0.004	0.004	0.003	0.001	0.153	0.001	0.006	0.004	0.007
COL_NA	0.004	0.009	0.007	0.000	0.000	0.001	0.000	0.000	0.135	0.002	0.000	0.000	0.000
COL_CA	0.004	0.007	0.005	0.000	0.000	0.002	0.000	0.001	0.198	0.002	0.000	0.000	0.000
DGAT1_NA	0.003	0.009	0.009	0.000	0.000	0.001	0.000	0.000	0.147	0.002	0.000	0.000	0.000
DGAT1_CA	0.003	0.006	0.005	0.000	0.000	0.000	0.000	0.000	0.141	0.001	0.000	0.000	0.000

Supplementary table 6. Changes in TAG 20:1-containing molecular species isolated from non-acclimated (NA) and cold-acclimated (CA) leaves of *Bochera* and *Arabidopsis* [Dry weight (nmol/mg)].

	28:1	28:2	30:0	30:1	30:2	30:3	30:4	32:1	32:2	32:3	32:4
LTM_NA	0.002	0.001	0.000	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.001
SAD12_NA	0.001	0.000	0.000	0.000	0.000		0.000	0.001	0.000	0.000	0.000
LTM_CA	0.001	0.001	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000
SAD12_CA	0.001	0.001	0.000	0.001	0.000	0.001	0.001	0.001	0.001	0.001	0.000
COL_NA	0.002	0.000	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001
DGAT1_NA	0.002	0.001	0.000	0.001	0.001	0.001	0.001	0.002	0.002	0.001	0.000
COL_CA	0.002	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000
DGAT1_CA	0.001	0.001	0.001	0.001	0.000	0.001	0.001	0.001	0.001	0.002	0.000
	32:5	**32:6**	**34:1**	**34:2**	**34:3**	**34:4**	**34:5**	**34:6**	**34:7**	**34:8**	**36:1**
LTM_NA	0.000	0.002	0.002	0.003	0.003	0.002	0.001	0.000	0.001	0.002	0.001
SAD12_NA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
LTM_CA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
SAD12_CA	0.000	0.001	0.000	0.001	0.001	0.000	0.000	0.001	0.001	0.001	0.000
COL_NA	0.000	0.001	0.000	0.001	0.001	0.001	0.001	0.000	0.001	0.001	0.001
DGAT1_NA	0.001	0.002	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001
COL_CA	0.000	0.001	0.000	0.001	0.000	0.000	0.001	0.000	0.001	0.001	0.001
DGAT1_CA	0.000	0.001	0.000	0.000	0.001	0.000	0.000	0.001	0.001	0.001	0.001
	36:2	**36:3**	**36:4**	**36:5**	**36:6**	**36:7**	**38:2**	**38:3**	**38:4**	**38:5**	**38:6**
LTM_NA	0.001	0.002	0.003	0.001	0.001	0.000	0.002	0.004	0.002	0.000	0.000
SAD12_NA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000
LTM_CA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
SAD12_CA	0.000	0.000	0.001	0.000	0.001	0.000	0.001	0.001	0.001	0.000	0.000
COL_NA	0.000	0.000	0.001	0.000	0.000	0.001	0.000	0.001	0.000	0.000	0.000
DGAT1_NA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
COL_CA	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.001	0.000	0.001	0.001
DGAT1_CA	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000

CHAPTER 5. Natural variation in cold acclimation and deacclimation of five Brassicaceae winter annual species from coastal habitats

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ABSTRACT

In overwintering plants development of tolerance to low temperatures is initiated in autumn and the maximum tolerance is obtained in mid-winter. These processes are reversible by cold de-acclimation. Hence, the ability to acclimate cold, maintain freezing tolerance and to regulate cold de-acclimation is critical for winter survival of overwintering plants. Global warming may cause negative effects on maintaining freezing tolerance and photosynthetic performance of overwintering species during their life cycles, and thus understanding mechanisms to withstand cold stresses will be important. In this study, we screened freezing tolerance and photosynthetic performance of five winter annual species belonging to Brassicaceae growing under outdoor winter environments during their life cycle, and investigated their freezing tolerance in a controlled environment. We demonstrated that the levels of freezing tolerance during life cycles of overwintering plants are presumably under genetic control and vary among species, and photosynthetic performance is highly associated with freezing tolerance.

I. INTRODUCTION

Global climate change will impact ecosystems and agricultural systems in many different ways (et al., 2007; Lobell and Field, 2007). It may lead to increases of average temperatures and extreme temperature fluctuation events, which could have negative impacts on freezing tolerance of overwintering plants (Kalberer et al., 2006). Certain overwintering plants increase their freezing tolerance by a process named cold acclimation, which is affected by their genetic abilities to coordinate physiological, biochemical and molecular responses accompanying temperatures, as well as environmental stimuli (Leinonen, 1996; Thomashow, 1998; Hannah et al., 2006). Processes of cold acclimation differ in the species, but commonly require exposure to cold temperatures (Gusta et al., 2005). In nature, it is initiated with decreased temperature in autumn, and global warming that can cause unfavorable temperature conditions for cold acclimation could lead to lower freezing tolerance of overwintering plants. Global warming may also result in increased risk of frost damage by untimely de-acclimation in winter since de-acclimation can be activated by warm temperature and is a fast process (Rapacz, 2002). In addition, it could bring out shifts in spring flowering times of overwintering plants because temperature can affect the timing of development, both alone and through interactions with other cues such as photoperiod (Ibanez et al., 2010). If loss of freezing tolerance is linked to a developmental shift to the reproductive stage, species could be harmed by premature cold de-acclimation resulting from earlier growth and earlier flowering (Kalberer et al., 2006; Dhillon et al., 2010).

As almost all physiological processes, photosynthesis is strongly affected by temperature changes. In response to cold temperatures, inhibition of photosynthesis (photoinhibition) is commonly observed in plants (Huner et al., 1993). Photoinhibition can cause secondary negative effects such as oxidative stress (Chaves et al., 2009), and hinder successful growth and reproduction of overwintering plants (Huner et al., 1993). If photo-inhibition is highly associated with freezing tolerance somehow, global warming could lead to problems on substantial crop production and plant conservation to climate change. Hence, understanding how overwintering plants maintain freezing tolerance and photosynthetic performance during their life cycles can be important for substantial crop production and plant conservation to climate change, but little progress has been made for it.

The Brassicaceae includes a number of agriculturally and ecologically important winter-

annual species and cultivars, and is characterized by extensive morphological diversity and the ability to adapt to a wide range of habitats and growing environments (Christopher et al., 2005). Hence, it can provide an excellent opportunity to evaluate impacts of sub-zero temperatures on survival and photosynthetic performance of overwintering plants, and to understand ecologically or economically important factors associated with them. For this study, we sampled five representative winter annual species belonging to Brassicaceae from natural habitats in the Netherlands. All form rosettes and have similar plant forms, but have different flowering times. Winter annual species with early spring flowering times most likely have to cope with lower temperature during growth and reproduction. We, therefore, expected that strategies and mechanisms of plants adapted to those conditions may be different compared to late-spring flowering plants.

In this study, we screened freezing tolerance and photosynthetic performance of selected species growing under outdoor environments during their life cycle as well as their freezing tolerance in a controlled environment. Our research questions were 1) Is there variation in freezing tolerance of five winter annual species from same local community? 2) Is timing of cold de-acclimation in spring associated with freezing tolerance in winter, and does it depend on to the flowering time of species?,3) Is photosynthetic performance connected to freezing tolerance?. To our knowledge this is the first study that investigates freezing tolerance over an extended period from fall to spring. This effort is an important step towards understanding ecologically important issues for cold acclimation and cold de-acclimation.

II. MATERIALS AND METHODS

PLANT MATERIALS

The experiment performed using *Arabidopsis thaliana*, *Capsella bursa-pastoris*, *Cochleria danica*, *Draba verna* and *Teesdalia nudicaulis* from coastal region of the Netherlands. In nature, *D. verna* is the earliest flowering species (February), while *A. thaliana*, *C. bursa-pastoris*, *C. danica* and *T. nudicaulis* flower later, in March or April. Individual plants were collected in their native environments and grown in a greenhouse. Selfed seeds from first generation plants (S1) were grown and second generation selfed-seeds (S2) were generated to reduce maternal effects and these seeds were used for this study.

PLANT GROWTH CONDITIONS FOR AN OUTDOOR FREEZING STRESS EXPERIMENT

The outdoor freezing experiment was conducted in the winter and spring of 2010-2011 and plant responses to freezing were evaluated for each species throughout their life-cycle. Prior to sowing, over 6,000 S2 seeds of each species were surface sterilized for 8 minutes with 15% diluted sodium hypochlorite and a drop of Tween 20 and then were stored in 10ml water for 10 days in a dark cold chamber with 4 degree to induce and synchronize the germination of the different species. Seeds of each species were then sown in plastic trays (15 cm x 20 cm x 5 cm) filled with a mixture of one part 'No.1' and two parts 'No.3' (Jongkind Ground BV) (1 : 2) on September 24, 2010. Seedlings of each species and, per species, of approximately equal size were later transplanted into large plastic trays (40 cm x 60 cm x 10 cm) filled with the same soil mixture. Individual plants were planted at equal spacing in a 2.5cm x 2.5cm grid, resulting in 308 plants of each species per tray. We created a total 8 trays per species (eight replicates). Three weeks after sowing the trays were randomly placed in a common garden at the University of Amsterdam in the Netherlands on October 18, 2010 and where they were maintained until the end of experiment. The first census was on November 18, 2010 and subsequent measurements were made at one week intervals thereafter until the end of March, 2011. During the experimental period air temperature was monitored with a HOBO data logger (Onset HOBO Pro H08-032-08, Onset Computer Corporation, MA, USA).

PLANT GROWTH CONDITIONS FOR A CONTROLLED FREEZING STRESS EXPERIMENT

We used seedlings for a controlled freezing stress experiment and they were grown on agar plates. Surface of seeds was sterilized by washing of 8 min with 50% bleach and a drop of Tween 20, and subsequently sown on plates containing 50% Murashige and Skoog with 0.8% (w/v) Daishin agar (Duchefa Biochemie, Haarlem, The Netherlands). To ensure a homogenous germination, plated seeds were kept at 4°C for 3 days and 2 weeks, respectively and then transferred to a growth chamber with a 14 h light/10 h darkness irradiance cycle and a 20/16°C temperature regime.

MEASUREMENT OF FREEZING TOLERANCE IN OUTDOOR AND CONTROLLED FREEZING STRESS EXPERIMENTS

Freezing tolerance is usually measured means of responses to natural freezing event or by controlled freezes in a controlled environment. As each procedure has its advantages and

disadvantages we chose to use both approaches, and measured freezing tolerance under outdoor conditions and in a controlled freezing treatment. The two experiments are described separately.

MEASUREMENT OF FREEZING TOLERANCE FOR AN OUTDOOR FREEZING STRESS EXPERIMENT

Freezing tolerance was measured by electrolyte leakage assay. Significant correlation between electrolyte leakage and resistance to freezing stress has been demonstrated in many plant species (Sutinen et al., 1992). Values of electrolyte leakage were measured at 12:00 and 24:00 on every weekly sampling date. Three uniform leaf discs (Ø 0.5cm) from rosette leaves of each plant were sampled using of a cork-borer and immediately put into 15ml Greiner tube containing 10ml de-ionized water. The discs were washed for 30min on a shaking platform to remove soil attached during cutting of the disc. Leaf discs were then placed in 12 wells plates filled with 3ml of 0.01% Silwet solution and maintained at 25°C for 1hr 30min on a shaking platform. The initial leakage from the disks was determined by measuring the electrical conductivity of the well plate solution, using a conductivity meter; data were expressed as $m^{S}cm^{-1}$. The discs were then irradiated in a microwave oven (600 W) with two 20 sec bursts of radiation and shaken gently for an additional 4hr before the conductivity of the resulting solution was measured to obtain a value for complete leakage. Results were expressed as percentage of total conductivity 'initial leakage / final leakage x 100'. The values are presented as the mean of eight measurements, representing eight replicates.

MEASUREMENT OF FREEZING TOLERANCE IN A CONTROLLED FREEZING STRESS EXPERIMENT

To check for differences in freezing tolerance of five species used for this study in a controlled environment, we measured the lethal temperature for 50% survival (LT_{50}). Eighteen day-old seedlings were either cold-acclimated at 4°C for 48 hours under a 14h photoperiod (acclimated) or remained in the standard growth conditions (non-acclimated). After the 48 h acclimation period, LT_{50} of the acclimated and non-acclimated seedlings was measured as percentage survival of seedlings after freezing treatments at different temperatures in a tightly-controlled environment cabinet (Snijders Microclima 1000; Snijders Scientific, Tilburg, The Netherlands). The cabinet temperature decreased from 4°C at to –1°C (at a rate of -1.25

degrees per hour) and then was held for four hours at –1°C. Very fine ice chips were applied to all seedlings to prevent super-cooling. The temperature was then further decreased from –1°C to the desired target temperatures (–1, -3, -5, -6, -7, -8, -9, -10 or –11°C) over a 6 h interval. The target temperature was held for 8 hrs. The seedlings were thawed for 12h in the dark at 4°C and placed back into a growth chamber set at the standard growth temperature and irradiance conditions. Survival was visually scored by quantifying seedling mortality 10 days post freezing. The percentage freezing survival per genotype was calculated as: (number of survivors / total number of plants) * 100. LT_{50} was estimated from fitted response curves and calculated by the statistic program R (R Development Core Team, 2008, procedure : http://lukemiller.org/index.php/2010/02/calculating-lt50-median-lethal-temperature-aka-ld50-quickly-in-r/).

MEASUREMENT OF MAXIMUM QUANTUM YIELD OF PHOTOSYSTEM II ELECTRON TRANSPORT (Fv/Fm) FOR OUTDOOR EXPERIMENT

Chlorophyll fluorescence is a quick and inexpensive method widely used for analyzing the status of photosynthetic apparatus and understanding the mechanism by which a range of environmental factors alter photosynthetic activity (Baker, 2008). Among various chlorophyll fluorescence parameters, maximum quantum yield of photosystem II (Fv/Fm) has been applied for evaluating photosynthetic performance because it is the most easily measured and reflects a progressive inactivation of PSII-mediated electron transport. Hence, we screened Fv/Fm to investigate the changes in photosynthetic performance. Fv/Fm was determined at 12:00 and 24:00 of the census dates, and was measured using a PAM-2000 chlorophyll fluorimeter system (Heinz Walz, Effeltrich, Germany) using a saturating pulse of 12,000 µmol m^{-2} s^{-1} for 0.8s. Fluorescence data were recorded, and Fv/Fm computed, with the fluorometer software using the equation $Fv/Fm= (Fm – F_0)/Fm$. For determination of Fv/Fm, leaf discs were cut from the middle part of the sixth leaf of the plants used for the measurement of electrolyte leakage. These discs were placed, along with 200 µl de-ionized water, into separate wells in black 96 well plates and dark adapted for 20 min prior to determination of Fv/Fm.

III. RESULTS

ENVIRONMENTAL CONDITIONS IN RESEARCH LOCATION AND FLOWERING TIMES OF FIVE SPECIES IN THE OUTDOOR ENVIRONMENT

The autumn of 2010 started relatively mild with minimum air temperature not falling below 5°C until the middle of November. However, daily temperatures decreased gradually thereafter, following initiation of the study in the middle of November. Twelve days after the start of experiment air temperatures began falling below zero and then remained below zero for 9 consecutive days. Air temperature dropped again to below zero on several occasions in the months of December 2010 and in January and February 2011. Of the 22 weekly measurement days, 5 days had a temperature below zero (Figure 1). The lowest air temperature of -8°C occurred on December 1 and 20, 2010. March was very mild in 2011, without extreme cold periods, and sub-zero temperatures were not recorded. The median flowering time of each species was recorded as the date of the first emerging flower of plants. Among the five species, *A. thaliana* flowered earliest with a flowering date of February 23, 2011. Species to flower second was *D. verna* with a flowering date of February 28, 2011. Third, fourth and fifth were *C. danica*, *C. bursa-pastoris* and *T. nudicaulis*, respectively, with median flowering dates March 14, 16 and 23 in 2011, respectively.

ELECTROLYTE LAKAGE OF PLANTS GROWING OUTDOOR ENVIRONMENTS DURING WINTER AND SPRING

Measurement of electrolyte leakage was employed to quantify the damage of cellular membranes by freezing for this study. Seasonal patterns of freezing tolerance differed according to the temperatures species were exposed to and their growth phase. In November, electrolyte leakage of the leaves remained around 10% for both the midday and midnight measurements of all species (Figure 2). First freezing temperatures in early December caused large increases in leakage and allowed us to observe significant differences on freezing tolerances among species (Figure 3). On the event of midday temperatures below -5°C, values of leakages were 14.4, 14.9, 16.9, 22.8 and 41.8% for *T. nudicaulis*, *D. verna*, *A. thaliana*, *C. bursa-pastoris* and *C. danica*, respectively. The prolonged freezing temperature (-8°C) during the night produced a further increase in leakage, which reached 18.5, 28.6, 32.7 and 46.6 to 52.1% for *D. verna*, *T. nudicaulis*, *C. bursa-pastoris*, *A. thaliana* and *C. danica*, respectively. One week after

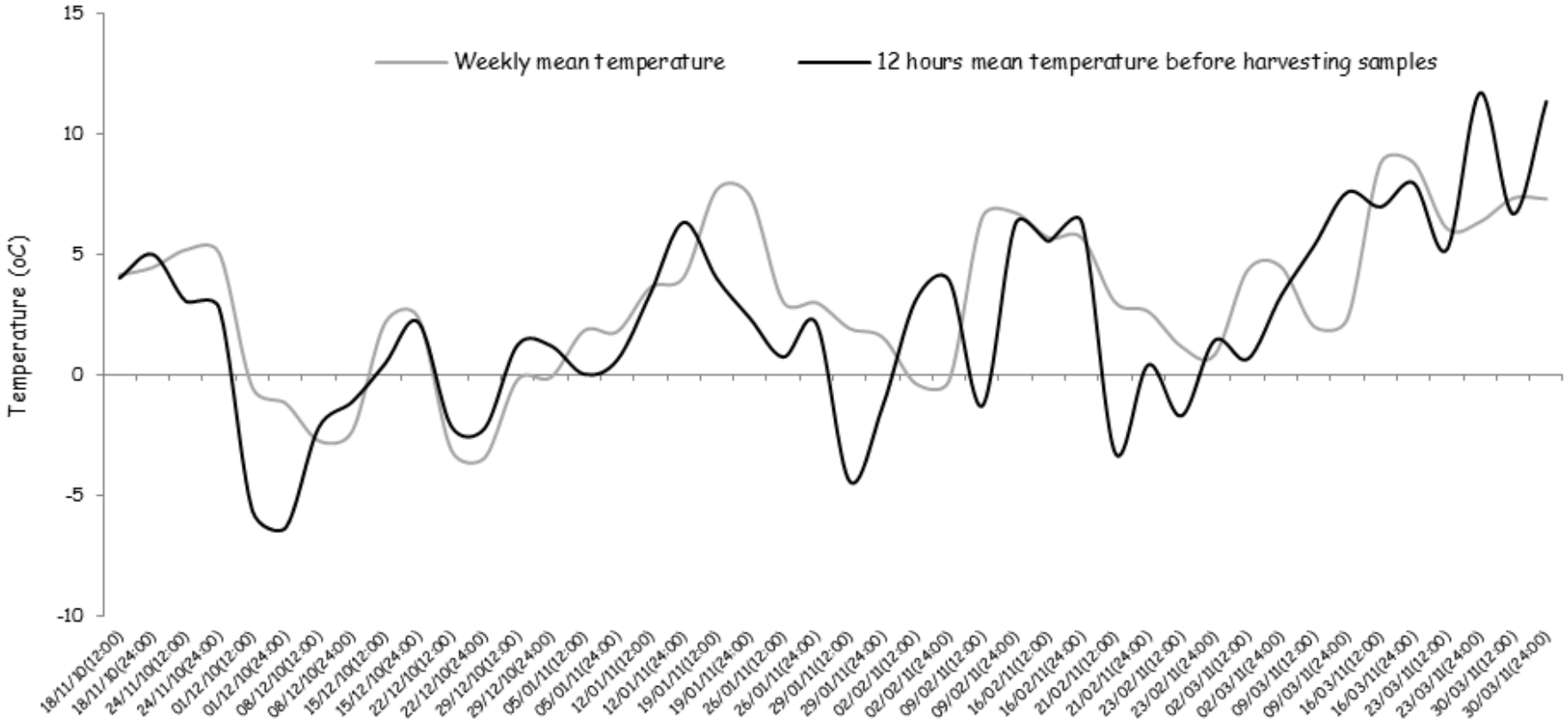

Figure 1. Weekly and 12 hours mean temperature before harvesting samples.

Table 1. Plant responses to similar freezing events in early and late winter

	Fv/Fm (mid-day)			Electrolyte leakage (mid-day)		
	22-12-2010	29-1-2011	23-2-2011	22-12-2010	29-1-2011	23-2-2011
A. thaliana	0.74±0.04	0.74±0.03^{a}	0.53±0.09^{b}	14.90±2.67^{a}	18.46±4.45^{ab}	36.49±11.64^{a}
C. bursa-pastoris	0.73±0.05	0.67±0.09^{a}	0.57±0.07^{ab}	14.19±2.46^{a}	16.15±6.15^{ab}	29.74±5.89^{ab}
C. danica	0.74±0.04	0.56±0.12^{b}	0.59±0.12^{ab}	16.08±2.58^{a}	22.84±7.48^{a}	33.61±4.97^{a}
D. verna	0.75±0.03	0.72±0.04^{a}	0.59±0.08^{ab}	10.58±1.20^{b}	12.06±2.40^{b}	25.42±10.40^{ab}
T. nudicaulis	0.76±0.02	0.69±0.03^{a}	0.66±0.04^{a}	13.11±1.89^{ab}	12.94±3.60^{b}	19.76±5.73^{b}
	Fv/Fm (mid-night)			Electrolyte leakage (mid-night)		
	22-12-2010	29-1-2011	23-2-2011	22-12-2010	29-1-2011	23-2-2011
A. thaliana	0.73±0.04^{b}	0.70±0.04	0.61±0.08	13.00±3.03	15.79±4.40^{a}	30.96±7.13^{a}
C. bursa-pastoris	0.76±0.02^{ab}	0.65±0.06	0.66±0.04	13.04±1.63	15.03±2.29^{ab}	27.95±1.90^{ab}
C. danica	0.75±0.04^{ab}	0.63±0.09	0.62±0.08	12.29±1.41	18.49±3.35^{a}	29.46±7.11^{a}
D. verna	0.77±0.02^{a}	0.72±0.05	0.68±0.08	11.45±1.18	10.39±1.87^{b}	22.06±2.29^{bc}
T. nudicaulis	0.76±0.01^{ab}	0.67±0.10	0.69±0.07	11.19±1.70	13.53±5.07^{ab}	17.25±5.17^{c}

ANOVA post-hoc (Turkey's b test) are indicated on the table, different letters means a significant difference at $P<0.05$. Bars indicate standard errors (n=8).

the first freezing event, levels of leakage had recovered again in all species, but to different extents. In the case of *T. nudicaulis* and *D. verna* leakage had recovered almost to the pre-freeze value of late November and by 15th December it had reached pre-freeze levels (Figure 2). While *C. danica*, *A. thaliana* and *C. bursa-pastoris* also recovered strongly from the December frost period their recovery appeared to be slower than that of *T. nudicaulis*, despite *T. nudicaulis* showing a degree of leakage on 1st December that was only slightly less than *C. bursa-pastoris*. From the middle of December onward, the leakages in all species were maintained below 20% even under subzero conditions throughout winter and minimum leakages in all species were observed during January. During those periods, no diurnal differences of freezing tolerance among species were as evident. The brief cold period by the end of January caused a less extreme but still similar response of species as observed in December, with *D. verna* being the most freezing tolerant species. From the beginning of February, slightly increased leakage in *A. thaliana* was observed and a gradual increase was seen in all species by the end of February. The freezing tolerance as inferred from leakage after the last cold spell, in February was apparently reduced (Table 1). Most plants had started to grow and some already initiated

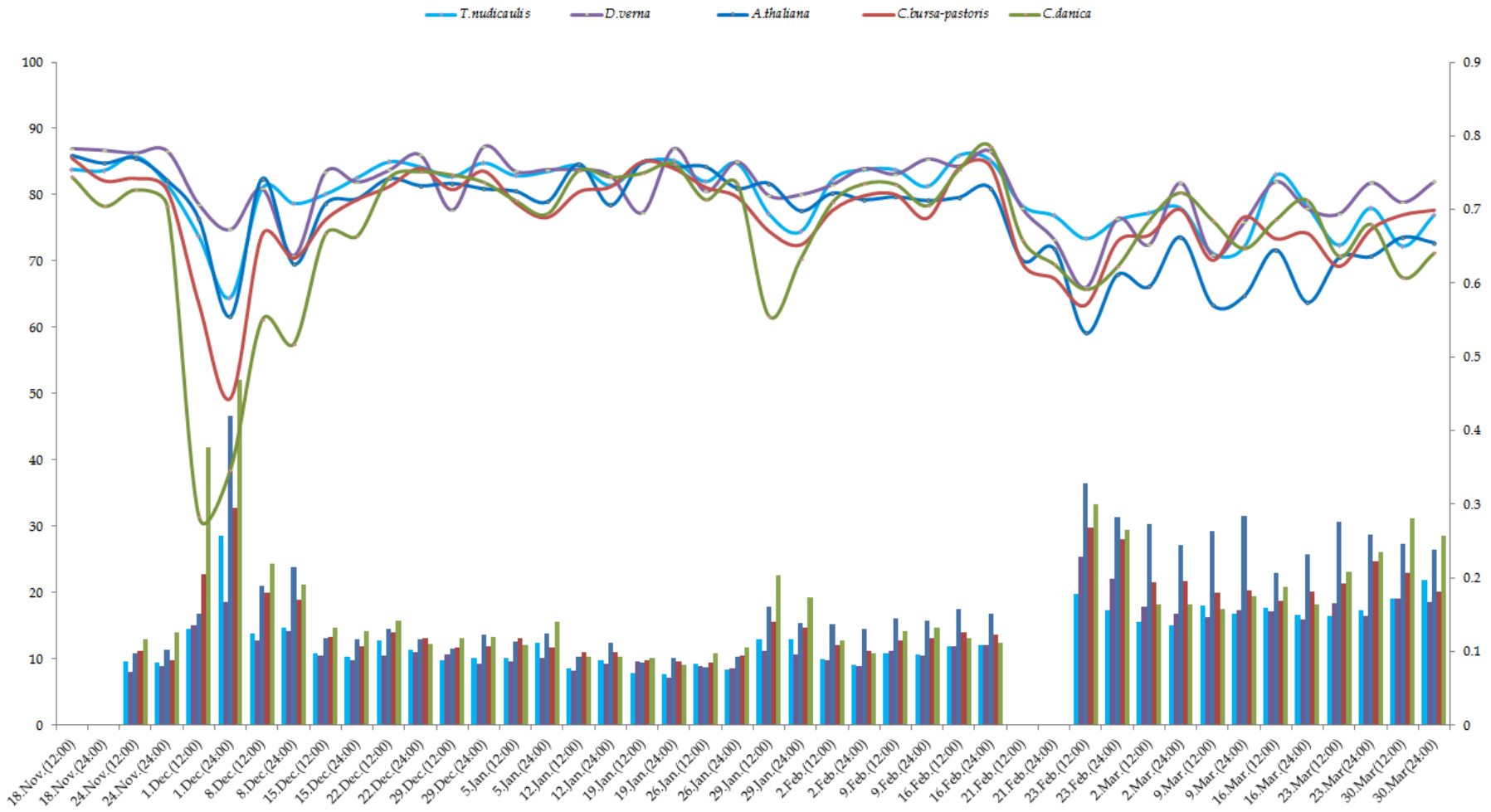

Figure 2. Daily and seasonal changes of electrolyte leakage (bars) and Fv/Fm (lines) during the winter of 2010-2011 in winter annual species.

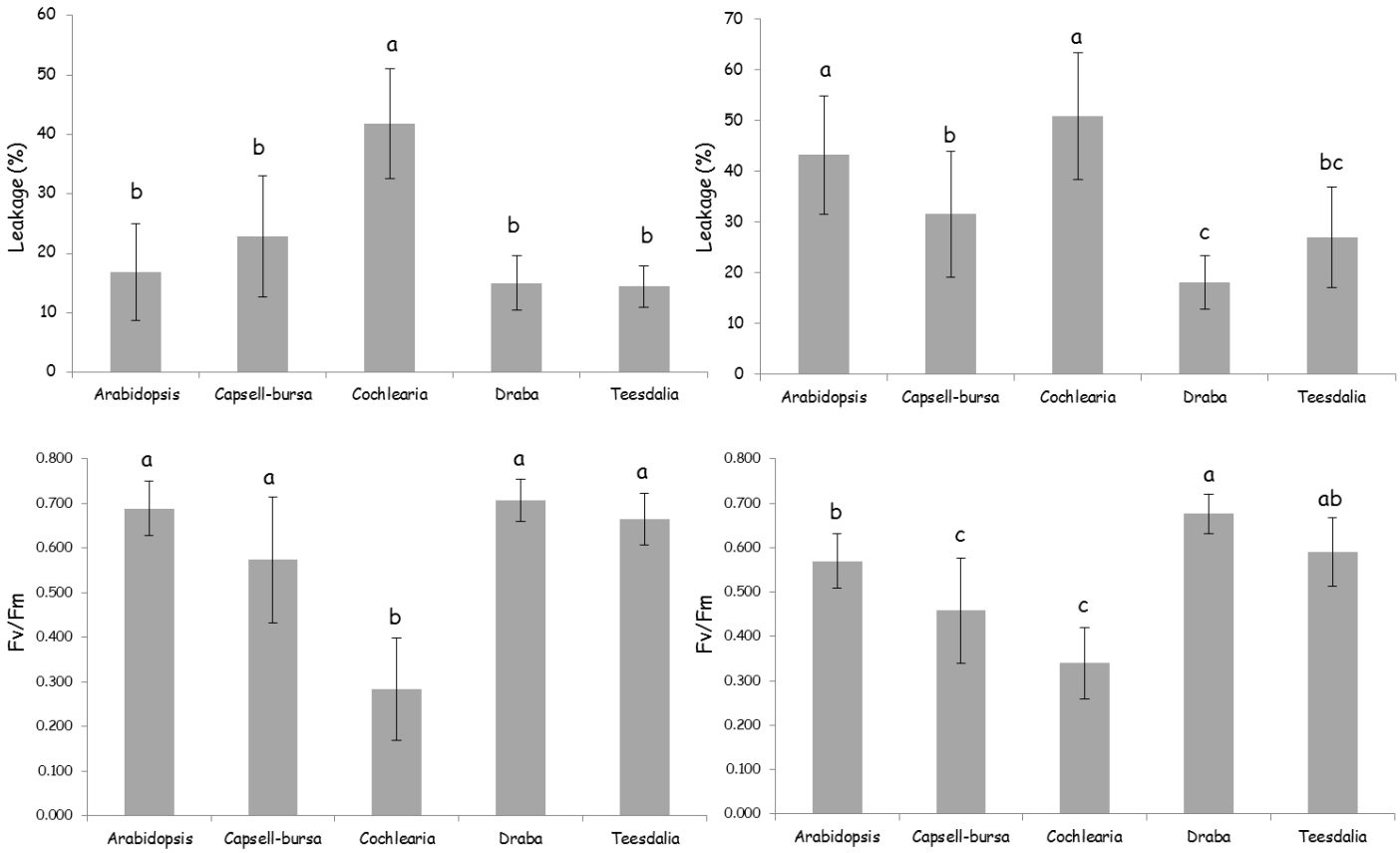

Figure 3. Electrolyte leakage (%) and maximum quantum yield PSII (Fv/Fm) measured during the first freezing event on 1 December, 2010. ANOVA post-hoc (Turkey's b test) are indicated on the graphs, different letters means a significant difference at $P<0.05$. Left and right panel for each assay indicate the result obtained from mid-day and mid-night measurement, respectively. Bars indicate standard errors (n=8).

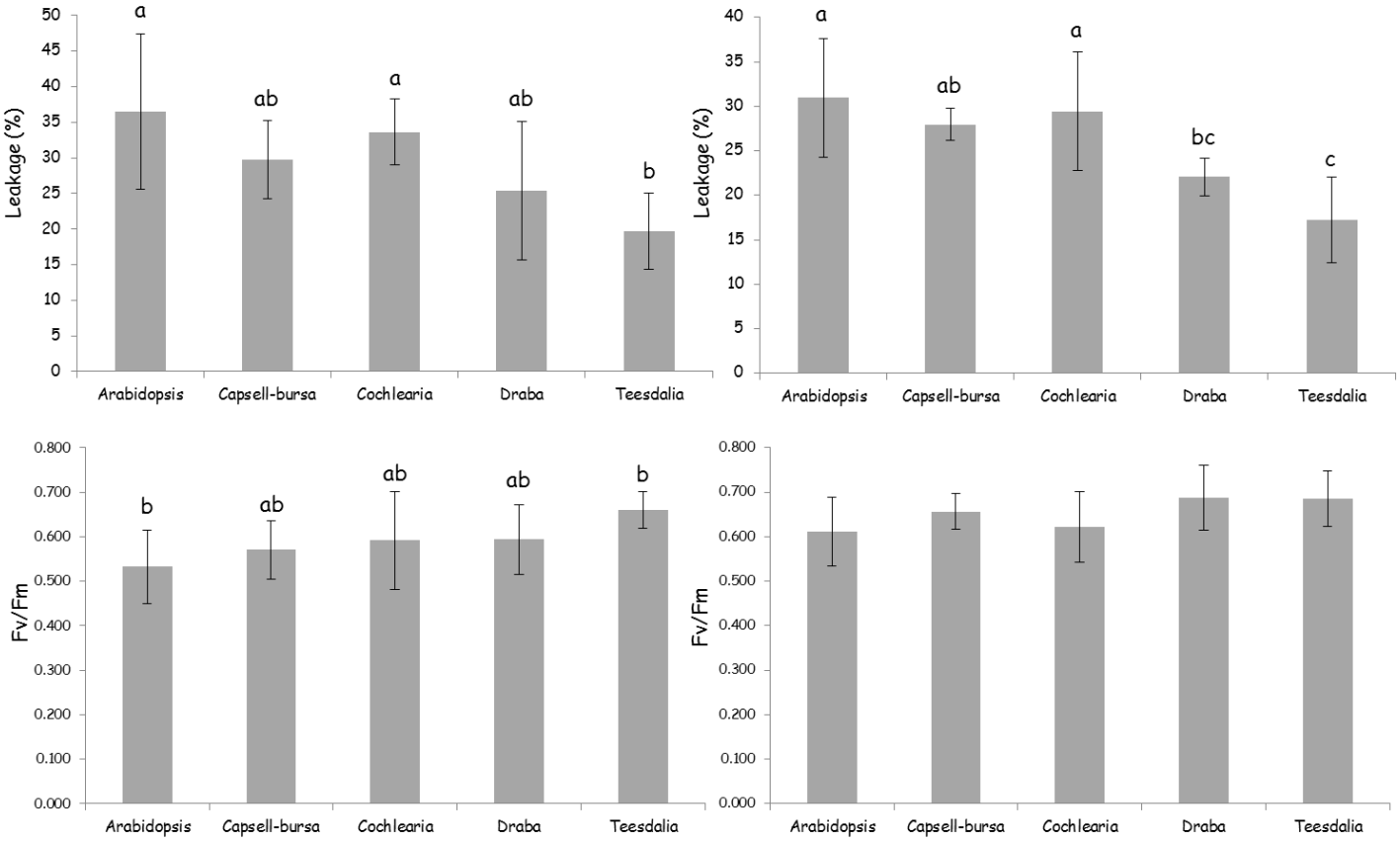

Figure 4. Electrolyte leakage (%) and maximum quantum yield PSII (Fv/Fm) measured during the freezing event in late winter (23 February, 2011). ANOVA post-hoc (Turkey's b test) are indicated on the graphs, different letters means a significant difference at $P<0.05$. Left and right panel for each assay indicate the result obtained from mid-day and mid-night measurement, respectively. Bars indicate standard errors (n=8).

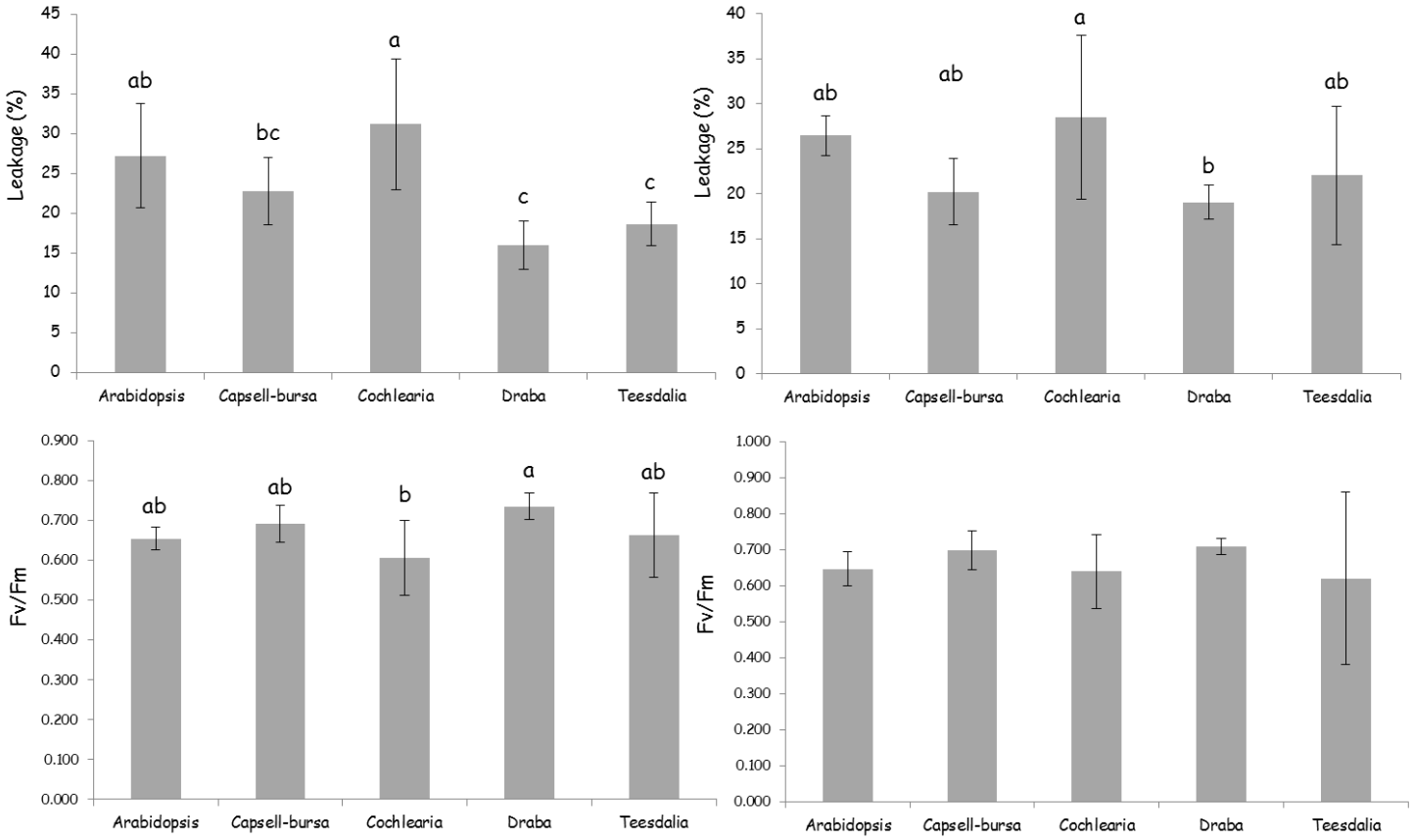

Figure 5. Electrolyte leakage (%) and maximum quantum yield PSII (Fv/Fm) measured at the end of March. ANOVA post-hoc (Turkey's b test) are indicated on the graphs, different letters means a significant difference at $P<0.05$. Left and right panel for each assay indicate the result obtained from mid-day (12:00) and mid-night measurement (24:00), respectively. Bars indicate standard errors (n=8).

flowering, which differed from the situation in the preceding months. Significant damage to cellular membranes even under the mild freezing was observed on 23-03-2011, unlike similar conditions in winter. During the last freezing event, *A. thaliana*, i.e. the species the earliest developmental shift toward reproduction in the experiment, was least tolerant to freezing and showed more damage than, for instance, early flowering *D. verna*, which was most tolerant to freezing in winter and even *C. danica*, which was the most sensitive species earlier, and *T. nudicaulis* which had the latest flowering time in nature (Figure 4). After freezing in late winter, the leakage in *A. thaliana* maintained a higher value, which could mean that recovery from frost-damage did not progress although temperature has risen (Figure 2). In contrast, cellular membrane of other species seemed to be recovered from frost damage but their status was not fully recovered to the initial level. Among all species, *T. nudicaulis* was a tolerant species and seemed to have the benefit from starting regrowth relatively late. In addition, *D. verna* had a high ability to recovery from frost damage and loss of freezing tolerance was slow in spring compared to other species, even though it already started flowered (Figure 5).

MAXIMUM QUANTUM YIELD OF PHOTO-SYSTEM II(Fv/Fm) OF PLANTS GROWING OUTDOOR ENVIRONMENT

During initial harvesting times, Fv/Fm in species on midday has shown ranging from approximately 0.782 to 0.726 (Figure 2). Fv/Fm in some species on midnight was slightly reduced, ranging from 0.779 to 0.704. Fv/Fm was stable during winter and Fv/Fm did not frequently occur, even under subzero condition. Only when minimum temperature dropped down to -2°C in early or late winter, slight reductions in Fv/Fm were observed, and Fv/Fm in all species declined further when the temperature reached to -8°C. The decline was obviously more pronounced in species that exhibited higher increase in leakages (Figure 3). These results suggested that components of the photosynthetic apparatus are likely damaged in freezing sensitive species, while freezing tolerant species were less affected. Similar to the electrolyte leakage, the average of Fv/Fm in five species was fairly constant from January to middle of February, but decreased in spring, as was the case for electrolyte leakage (Figure 4 and 5). Although low R^2 Linear level has been investigated in some species, a statistically significant correlation (significant level at $P<0.01$) between electrolyte leakage and Fv/Fm were investigated in all species (Figure 6).

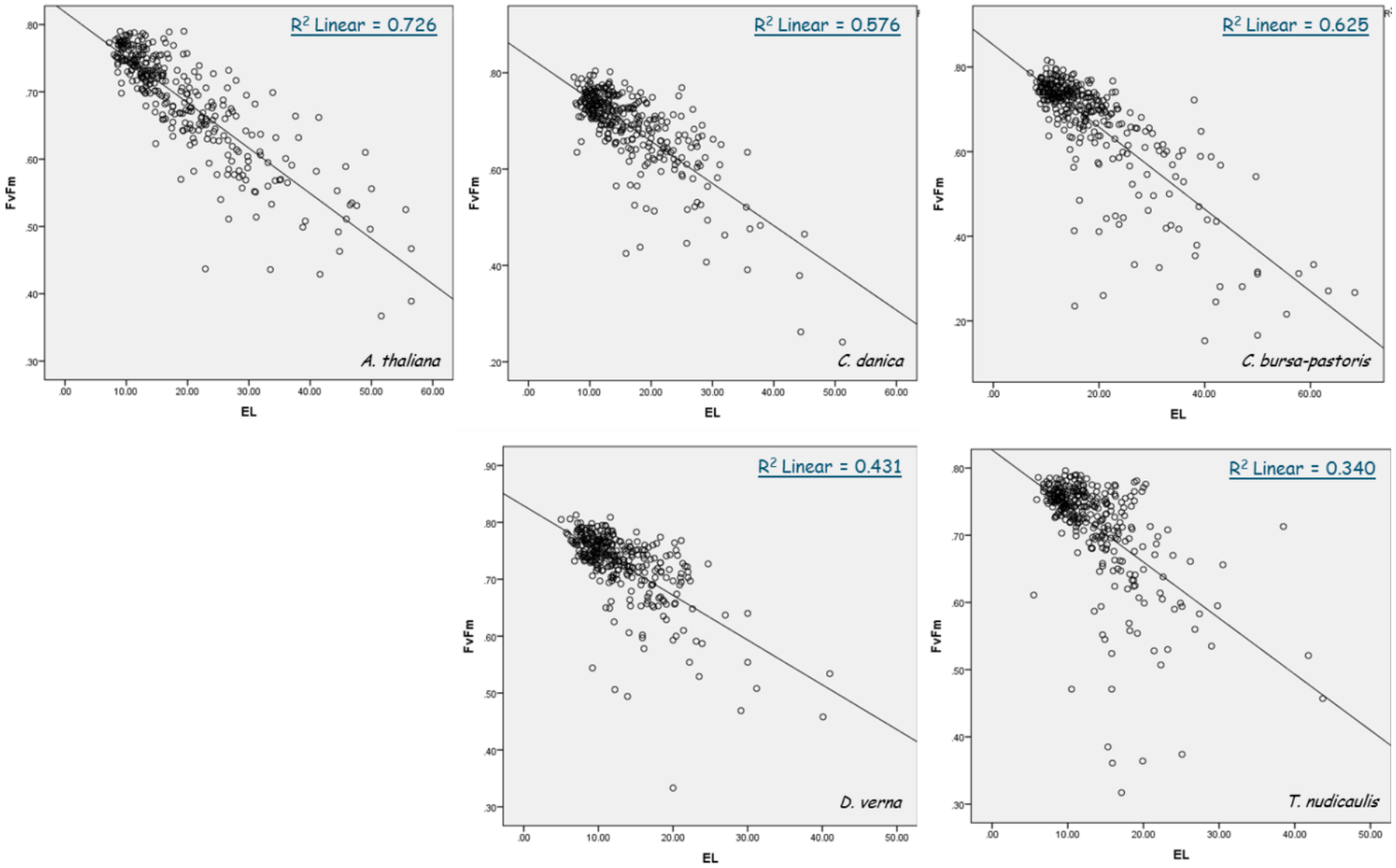

Figure 6. Significant correlation between electrolyte leakage (%) and maximum quantum yield PSII (Fv/Fm).

FREEZING TOLERANCE OF FIVE SPECIES GROWING IN A CONTROLLED ENVIRONMENT

The calculated relative freezing tolerance based on scoring of the seedling as either dead or alive after cold acclimation are shown in Figure 7. The freezing tolerance of the different species was quantified as the lethal temperature for 50% survival (LT_{50}), and estimated from fitted response curves. When plants were grown under standard conditions (20/16°C day/night, 14h photoperiod), levels of freezing tolerance did not differ among selected species (data not shown). In all species, LT50, values decreased during cold acclimation for 2 days. LT50 values for *A. thaliana*, *C. bursa-pastoris* and *C. danica* slightly decrease a lot for 2 days. In contrast, freezing tolerance in *D. verna* and *T. nudicaulis* rapidly increased. Their relative order for LT50 values in the cold acclimation were *T. nudicaulis*, *D. verna*, *A. thaliana*, *C. danica* and *C. bursa-pastoris* (Figure 7).

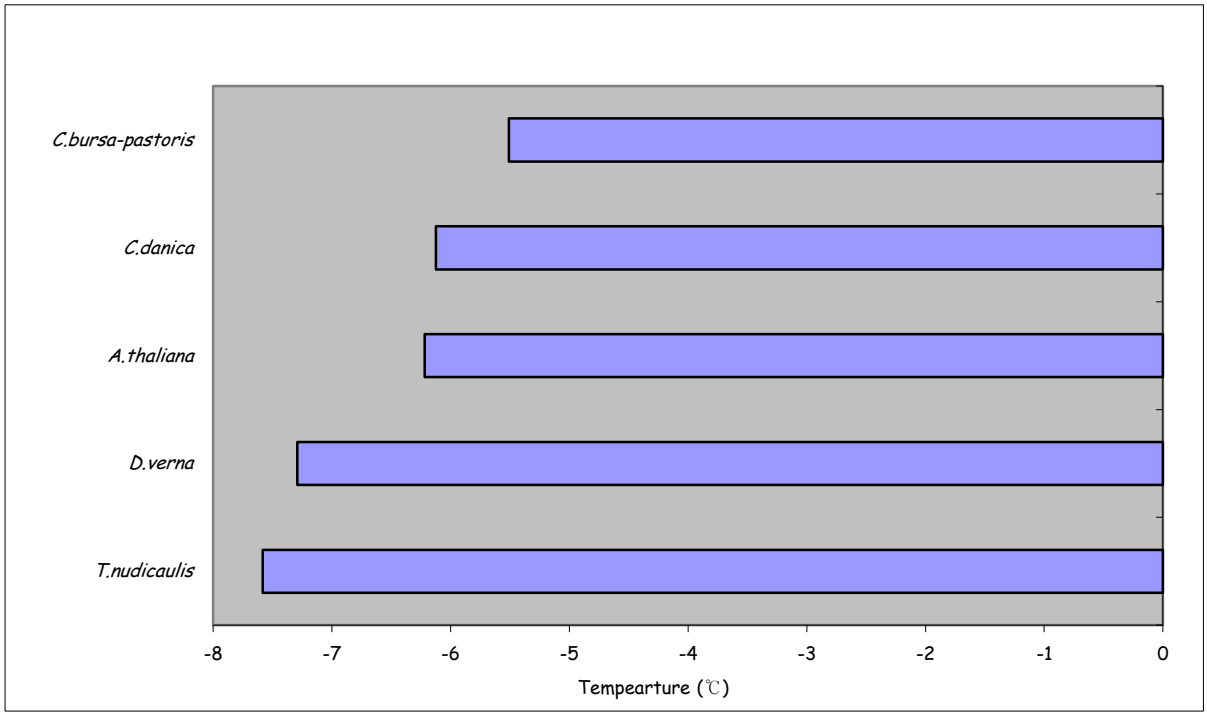

Figure 7. Relative freezing tolerance in seedlings of five winter annual species after cold acclimation under controlled condition.

IV. DISCUSSION

FREEZING TOLERANCE IN PLANTS CAN BE ASSOCIATED WITH THEIR EVOLUTIONARY HISTORY OR SPECIFIC GENETIC COMPONENT

The samples used for our study were harvested from late November onwards, with low initial leakages for all species. In our outdoor experiment, the most discriminating results were obtained from freezing events on December 1 and 8, 2010. Based on these results, we could arrange the five species from freezing tolerant (*D. verna*), semi-tolerant (*T. nudicaulis*), intermediate (*A. thaliana* and *C. bursa-pastoris*) to sensitive (*C. danica*). The rank order obtained from the outdoor experiment nearly matched with the result for LT50 from seedling survival test. Hence, it seemed that plants with greater ability for cold acclimation may be the ones that are less susceptible to winter freezing in nature. It has been demonstrated that the freezing tolerance of natural accessions in *A. thaliana* was correlated with the habitat winter temperature (Hannah et al., 2006), so it should be noted that all comparison are made for natural accessions of the Netherlands. Our result showed that *D. verna* is the most tolerant species to freezing. *D. verna* is the earliest flowering species that already starts flowering in late winter, which means that it has to cope with low temperatures during growth and reproduction, and have to be adapted accordingly.

A further interesting result was observed from the controlled environment experiment. Cold acclimation process at 4°C for 2 days resulted in increase of freezing tolerance in all species. *T. nudicaulis* and *D. verna* showed a somewhat better cold acclimation than *A. thaliana*, *C. bursa-pastoris* and *C. danica*. This result indicated that *D. verna* acclimated to cold rapidly, suggesting that gene induction during cold acclimation can differ among species. Late-flowering *T. nudicaulis* was, perhaps surprisingly, tolerant to freezing events and recovered the stability of its cell membranes fast after frost damage, and was also very responsive to acclimation. Two possible explanations for its high freezing tolerance are as follows. Plants have adapted to various stress conditions and evolved specific tolerance mechanisms for successful reproduction and growth. In nature, stresses act in concert (Mahajan and Tuteja, 2005), and drought, freezing and salinity may all damage the membrane systems of cells. Various genes are up-regulated in response to these stresses and lead to adjustment of the cellular membrane and tolerance to stresses (Sanghera et al., 2011). *T. nudicaulis* is mainly found in sandy dunes and is known as a drought tolerant species with a deep root of arid soils (Newman, 1964). It, therefore,

may have developed the mechanism to protect cellular membranes to various stresses. Another hypothesis could be found from the specific genetic characteristics and distribution of *T. nudicaulis*. The geographic distribution of *T. nudicaulis* was compared to that of other winter annual *Teesdalia* R. Br. species in Europe (Hoffmann, 2000). The author found they generally show a wide distribution in Europe, whereas *T. nudicaulis* is nearly absent from the Mediterranean region. Other species, for instance *A. thaliana* and *C. bursa-pastoris*, are widely distributed, in the Mediterranean region as well as other regions in Europe. Hence, *T. nudicaulis* might have an evolutionary history of coping with low temperatures and thus could have a high freezing tolerance.

In any case, our results suggest that even in same local community considerable natural variation of freezing tolerance exists among species, and their specific genetic component or evolutionary history can be of significant importance to determine freezing tolerance of plants.

RESISTANCE TO COLD DE-ACCLIMATION IN SPRING IS LOOSELY CONNECTED WITH FREEZING TOLERANCE IN WINTER AND THEIR FLOWERING TIMES

From late winter, the electrolyte leakages in all species tended to be gradually increased with re-growth or flowering, indicating that cold de-acclimation was progressed, regardless of environmental conditions. Dhillon et al. (2010) demonstrated that a regulatory component of freezing tolerance is linked to a developmental shift between the vegetative and reproductive stage. Rapacz (2002) also reported that degree and level of cold de-acclimation and re-acclimation differ among developmental stages of plants. It, therefore, should be noted that our experiment was not perfectly suited to compare the level of de-acclimation in spring because of the differences in flowering times and developmental stages among the species of this study. Nevertheless, some of our results provide the chance to understand genetic variation of de-acclimation. Our results showed that plant responses of species with seemingly similar life histories can differ considerably. *A. thaliana* and *D. verna* both being earlier flowering showed contrasting responses from late February onward. *D. verna* kept higher freezing tolerance even after it started to flower, while *A. thaliana* being only slightly later in nature dropped its cold tolerance in spring. Another noteworthy species difference comes from comparing *T. nudicaulis* and *D. verna*. *T. nudicaulis* had the advantage to keep their freezing tolerance in spring from late initiation of growth, but seemed to lose their freezing tolerance fast with regrowth and reproduction compared to *D. verna*, *T. nudicaulis*. Our results suggest

that there is abundant variation in resistance to cold de-acclimation in species studied, and flowering time is loosely connected to it, if at all, whereas high freezing tolerance in winter may not always be associated with resistance to cold de-acclimation in spring. Global warming may lead to warmer winter and faster growth in native species as well as important cultivars, ultimately making them more susceptible to cold stress in late winter and early spring, especially if the ability to maintain freezing tolerance diminishes after transition from the vegetative to the reproductive stage. From this point of view, the characteristic of *D. verna* investigated in spring was interesting. In outdoor experiment, freezing tolerance of *D. verna* with earlier flowering time decreased like other species but the timing and degree seemed to be different. These results suggest that different mechanisms regulate cold de-acclimation. Therefore, further indoor and outdoor experiments on the interactions between temperature, acquisition and loss of freezing tolerance, and the molecular and physiological changes using a comparative approach would help us to understand how cold de-acclimation evolved.

PHOTOSYNTHETIC PERFORMANCE IS IMPORTANT FOR MAINTAINING FREEZING TOLERANCE

It has been demonstrated that decrease in Fv/Fm reflects a progressive inactivation of Photosystem II(PSII)-mediated electron transport, which may be the result of Calvin cycle disturbances that delay redoxidation of QA- and induce photosystem II down-regulation or damage thylakoid membrane electron transport (Galle et al., 2002). Hence, the low value in Fv/Fm may indicate portion of PSII reaction center is possibly damaged by stressful conditions (Baker and Rosenqvist, 2004; Souza et al., 2004). Although we did not investigate detailed quenching changes of PSII during cold acclimation period, we could observe they had different PSII status by measuring Fv/Fm ratio in late November indirectly. In this study, we observed that Fv/Fm in *C. danica*, the least tolerant species to freezing of our study, decreased to 30 % in late November, much more than the other species, and it thus could be assumed that it was not (yet) properly acclimated to low temperature. Winter frost event on December 1, 2010 allowed us to evaluate the relationship between freezing tolerance and photosynthetic performance. On middays, we observed that freezing stress resulted in significant reduction in Fv/Fm for all species. At midnight with prolonged freezing stress, more dramatic changes for some species were observed. Some species during outdoor freezing stress showed a marked decrease in Fv/Fm. The freezing sensitive *C. danica* had the lowest Fv/Fm and *A. thaliana* and *C.*

bursa-pastoris also had lower Fv/Fm than two freezing-tolerant group species. These results suggest that freezing causes a greater inhibition of the status of photosystem in species with larger damage of cellular membrane. Our results also showed that two freezing tolerant species had higher capacity for cold acclimation under a controlled condition and more stable status of PSII under outdoor condition during the winter than those of other less freezing tolerant species. These results strongly indicate that freezing tolerance links to photosynthetic performance, and higher level of freezing tolerance could benefit from abilities to acclimate cold and photosynthetic apparatus to environmental conditions in autumn.

In spring, the status of PSII was degenerated, and was more affected by environmental conditions compared to winter. Different growth phages and generating new leaves among species, however, made it difficult to compare the degree of de-acclimation with the degradation of photosynthetic performance among all species. Hence, more controlled and outdoor experiments should be needed to explain the relationship between development, tolerance and photosynthesis.

Chapter 6. General discussion

Low temperature stress impairs several cellular processes, leading to various types of injury that affect plant growth, development and reproduction (Ruelland et al., 2009). Plants have evolved unique and diverse mechanisms to withstand harmful growth conditions (Uemura and Hausman, 2013). To understand cold-responsive mechanisms of plants several approaches have been applied, and progress has been made (Thomashow 1999; Zhu et al., 2007). However, many studies have just focused on the use of some model species. We anticipated that some cold response pathways of *Boechera stricta* would differ from those of *Arabidopsis thaliana*, and performed a series of genetic and molecular studies to identify genetic regulators controlling freezing tolerance in *Boechera stricta*. We demonstrated that major genetic components responsible for freezing tolerance in *B. stricta* could be same (Chapter 2 and 3), but also different (Chapter 4). In chapter 5 of this thesis, we carried out a study of natural variation of cold acclimation and deacclimation using five *Brassicaceae* winter annual species with similar life histories that all occur in the Netherlands. Our results clearly showed that the ability to maintain freezing tolerancein spring differs among species, as is already known for cold acclimation. Some interesting findings and possible interpretations of this thesis and suggestions for future work will be discussed below.

Experiments across environments and analysis of QTL by environment interactions identified promising genetic regulators for freezing tolerance.

In chapter 2, freezing tolerant-related traits such as electrolyte leakage and LT50 were evaluated in two environments. A trait related to photosynthesis was also evaluated by measuring Fv/Fm ratio of RILs grown in three different environments. As all environmental conditions gave differential stress responses to RILs, the level of freezing tolerance and photosynthetic performance were varied. Numerous reports have already indicated that the mechanism for freezing tolerance is very complex (Thomashow, 1999; Yamaguchi-Shinozaki and Shinozaki, 2006; Uemura and Hausman, 2013). The pattern of observed differential QTLs (both in detection and effect size of major QTLs across environments) in this study corroborates this. In particular, our results showed that genotype x environment interactions (G x EIs) result in variable significance levels of QTL effects across environments and experimental settings. Indeed the lack of stable QTLs for freezing tolerance can be a main constraint to identify promising genetic

regulators for marker assisted breeding in crop species. Our research, however, showed that G x EIs can be broken down into its constituent QTL x Environment interactions (QTL x EIs), which can help to reveal relatively stable QTLs. By QTL x EIs analysis, QTLs identified in this study could be categorized into three groups; (1) a QTL (on LG 3) is expressed in one indoor environment but not in another indoor and outdoor environments; (2) a QTL (on LG 4) is expressed strongly in one indoor environment, weakly in another indoor environment, but not detected under outdoor conditions; and (3) a third QTL (on LG 7) is expressed strongly in outdoor environments but only weakly in indoor environments, as indicated by the variation in its effects across environments. Our results show that analysis of QTL x EIs based on diverse environmental experiments can provide a good prediction of the position, effects and stability of QTLs controlling freezing tolerance, and combined can be a starting point to improve the freezing tolerance of the plants.

Our QTL analysis may contribute to enhance understanding mechanism of freezing tolerance in plants.

Interestingly, the QTL explaining high percentage of freezing tolerance phenotype variation was also detected on LG 7, which was CBF loci containing tandemly arranged CBF genes. CBF transcript activators are master regulars for freezing tolerance in a number of cold adaptable plants (Liu et al., 1998; Skinner et al., 2005; Welling and Palva, 2008). In chapter 3 of this thesis, we tried to characterize the potential function of tandemly clustered *CBF* genes in *B. stricta,* labelled *Bs*CBF1, *Bs*CBF2, and *Bs*CBF3. We demonstrated that *BsCBF* genes have similar gene structures as found in *Arabidopsis CBF* genes and share essential domains and motifs for the functionality of the gene. We also showed that the *Bs*CBF genes exhibit similar expression patterns of *Arabidopsis CBF1, CBF2*, and *CBF3* under cold treatments, followed by the induction of CBF targeted-genes. These results suggested that *Bs*CBF genes may have similar functions as in *Arabidopsis*. Most importantly is that a genotypic difference between LTM and SAD12 is obvious in the levels of CBF genes and cold stress-responsive genes, suggesting that CBF pathway can also be involved in freezing tolerance in *B. stricta*.

In this study other QTLs were detected on LG 3 and 4. But those QTLs were not detected in outdoor conditions. These QTLs could be minor QTLs with amplified effects due to environment. Therefore consecutive growth and evaluation of traits may be essential to detect minor QTLs and to reduce errors from environmental effect. As a trait used for QTL analysis is

a quantitative trait, it was also affected by developmental stages and a differential QTL was detected in seedlings. In seedling freezing tolerance study of chapter 4, QTL was only detected on LG 5. Until now the genes located on this QTL controlling freezing tolerance were not identified. Using orthologous gene informations and comparative mapping between *Arabidopsis* and *Boechera*, some candidate genes could be identified. Because damage of membrane systems caused by freezing leads to direct harmful effects on plants, plants increase the cryostability of membranes during cold acclimation to minimize the frost damage later on. Hence, a lipid biosynthesis gene leading to stabilization of membranes in response to freezing stress can be highly relevant. Diacylglycerol Transferase-1 (DGAT1), one of important lipid biosynthesis genes, is located on this loucs and is a key mediator for Triacylglycerols (TAGs) that are suggested to have a potential role to protect critical cell structures during freezing stress (Moellering et al., 2010). We tested its potential role for freezing tolerance and showed it could be associated with freezing tolerance. It, however, should be noted that identification of genes for quantitative traits such as freezing tolerance is difficult by using any single approach due to complex inheritance of the traits and limited resolving power of individual techniques (Pandit et al., 2010), and only a few instances has been possible to positively identify the functional alleles of genes underlying the QTLs despite the developed technologies. More efforts as combining fine mapping and trascriptome profiling, therefore, should be required to identify novel genes controlling freezing tolerance within this locus in the near future.

Status of photosystem II is genetically associated with freezing tolerance and chlorophyll fluorescence can be used as a high-throughput phenotypic proxy for freezing tolerance. Inhibition of photosynthesis (photoinhibition) is a common occurrence for plants in response to cold stress (Huner et al., 1993). Because severe photoinhibition causes the death of plants by hindering the transfer of energy necessary for growth and establishment of plants, an increased resistance to cold stress-induced photoinhibition during cold acclimation is regarded as one of the most important adaptive strategies (Huner et al., 1998). It has been demonstrated that the status of PSII is positively associated with freezing tolerance (Rizza et al., 2001; Rapacz and Wolniczka, 2009), but genetic basis of relationship study between two factors had not yet been investigated using molecular markers and QTL mapping population. In the chapter 2, freezing tolerant and photoinhibition-related traits were evaluated, and QTL regions controlling these traits were compared. Like in previous studies, we found significant correlations between

freezing tolerance and photoinhibition-related traits during freezing or post-freezing. Freezing treatments caused photoinhibition in the RILs, and the decrease in Fv/Fm was much higher in freezing sensitive RILs than in freezing tolerant RILs. These results indicated that components of PSII can be significantly damaged in freezing sensitive RILs. Results of our QTL analysis presented in Chapter 2 of this thesis supported that these tarits are genetically associated, suggesting that key genes involved in the freezing tolerance also control photo-inhibition related traits. This result implies that a study of genes regulating reduced inhibition of photosynthesis in freezing tolerant genotypes will provide new insights on the complexity of freezing tolerance in plants.

From a methodological point of view, we found that chlorophyll fluorescence can be used as a reliable high-throughput phenotyping proxy to measure freezing tolerance in plants. Chlorophyll fluorescence has been already suggested as one of promising methods to quantify freezing damage (Ehlert and Hincha, 2008). A few studies, however, have been done to support it. In this thesis, Chlorophyll fluorescence and electrolyte leakage assays were applied for the natural variation study across diverse environments (Chapter 2 and 5). We demontrated there are significant correlation between routine freezing tolerance assays and chlorophyll fluorescence. Routine frost damage assay such as electrolyte leakage is accurate, but so laborious, and thus has a disadvantage that is not easily applicable for a large-scale screening purposeThe result of our extensive comparative studies strongly supports that chlorophyll fluorescence that is more simpler freezing damage assay than electrolyte leakage assays can be used for a powerful tool to study extentisive freezing tolerance in plants.

Natural variation in de-acclimation

Global warming can increase average temperatures and extreme temperature fluctuation events, which can have negative impacts on the regulation of freezing tolerance of plants. One of the major potential negative impacts may be an increase of the risk of frost damage by untimely or premature de-acclimation in late winter or early spring. De-acclimation can be initiated by diverse exogenous factors, including warm temperatures, day length, phenological changes and restart of growth (Kalberer et al., 2006). It has been shown that in woody plants de-acclimation is a faster process compared to cold acclimation (Pagter et al., 2011). In herbaceous species, it has been reported that even one day cold acclimation could lead to acquisition of freezing tolerance. These results indicate that a de-acclimation process in herbaceous species can be

activated within even a few hours depending on environmental stimuli. Very little is still known of the natural variation in deacclimation in herbaceous species under natural conditions.

Hence, the experiment of the chapter 5 was performed to study de-acclimation under natural conditions. Variable weather conditions during winter provided us the opportunity to detect genetic variation of cold acclimation and deacclimation. Freezing tolerance in all species was consistently maintained during winter even though it was a relatively warm winter. Loss of freezing tolerance progressed in spring after the restart of growth. These results suggested that the risk of frost damage by untimely deacclimation in winter could be relatively small, but by premature deacclimation in spring could have significant effects. Furthermore, in the outdoor experiment species had different flowering times, which made it difficult to compare the degree of deacclimation among all species, because being in a different developmental stage affect freezing tolerance. Nevertheless, two species, *D. verna* and *A. thaliana*, with almost the same flowering time exhibited contrasting freezing tolerance in spring. *D. verna* tended to maintain freezing tolerance even after flowering, whereas *Arabidopsis* lost its freezing tolerance after initiation of flowering. Because *D. verna* differs in resistance to deacclimation from *A. thaliana*, comparative molecular and physiological studies between these species could provide novel information about deacclimation and the genes and pathways involved. In addition to this study, variation underlying deacclimation can be efficiently exploited by *A. thaliana* with similar flowering times and determination of their freezing tolerance before and after their flowering time. *A. thaliana*, with its range of molecular and genetic resources, probably provides the best chance to study deacclimation than any other plant, both in the laboratory and field.

Summary

Understanding genetic variation for freezing tolerance is important for unraveling an adaptative strategy of species and for finding out an effective way to improve crop productivity to unfavorable winter environments. The aim of this thesis was to examine natural variation for components of freezing tolerance beyond what has been done using the model organism *Arabidopsis thaliana*. We, therefore, used a number of related Brassicaceae species. Our goal was to identify potentially novel and beneficial traits and loci not identified in *Arabidopsis* (due to experimental approaches, lack of genetic diversity and/or traits not present in this model species).

In chapter 2, we identified genomic regions that explain the variation in freezing tolerance between two genotypes of *Boechera stricta* collected from different environments in the US. We identified three genomic regions with major QTL effects across experimental environments, and were able to select the most promising QTL for further studies by analysis of QTL x Environment interactions. A significant QTL involved in freezing tolerance across all stress conditions was mapped on the middle of the linkage group VII. The QTL region corresponds to a syntenic region in *Arabidopsis thaliana* containing three tandemly arranged CBF genes. CBF genes have been shown to have a key role for resistance to freezing in many cold adaptable species. Accordingly, in the chapter 3, we isolated three tandemly arranged CBF genes from *B. stricta,* and studied their potential role in development of freezing tolerance in *B. stricta*. Our results showed that they can be highly associated with freezing tolerance in *B. stricta*. In the chapter 4, we performed a seedling freezing survival assay and QTL analysis. As a result, we identified a novel QTL that has not been reported in any other species and found evidence that a lipid biosynthesis gene can be involved in enhancing freezing tolerance. In the final research chapter, we investigated natural variation of cold acclimation and deacclimation in five *Brassicaceae* wild species, and revealed that species have contrasting responses in cold acclimation and deacclimation. Our results strongly indicate differential regulatory mechanisms are involed in cold acclimation as well as cold deacclimation. Although we are still far from understanding those mechanisms, we have shown that exploiting natural variation using wild species provides new perspectives on ecologically important adaptation to cold, and may contribute to improve tolerance in crucifer species.

Samenvatting

Om een effectieve manier te vinden om gewas productiviteit te beïnvloeden in een winterse omgeving en om de adaptatie van soorten aan vorst te begrijpen moeten we meer weten van de genetische variatie van vorsttolerantie. Het doel van deze thesis was om te onderzoeken wat de natuurlijke variatie voor vorsttolerantie is buiten het modelorganisme *Arabidopsis thaliana*. Hiervoor hebben we een aantal *Brassicaceae* soorten onderzocht. Ons doel was om mogelijk nieuwe en voordeel opleverende kenmerken en genetische loci te identificeren die (nog) niet in *Arabidopsis thaliana* zijn ontdekt (dit laatste komt door experimenten met alleen deze modelplant waardoor er een gebrek aan genetische diversiteit en/of een gebrek is aan kenmerken).

In hoofdstuk 2 hebben we genomische locaties geïdentificeerd die de variatie in vorsttolerantie verklaren tussen twee genotypen van *Boechra stricta*, die verzameld zijn van verschillende locaties in de VS. We hebben drie genomische loci met major QTL effecten over verschillende experimentele omgevingen geïdentificeerd. Bovendien konden de meest belovende QTL voor verder studies selecteren door een interactie analyse van QTL x Omgeving. Een significante QTL betrokken bij vorst tolerantie voor alle stress condities zat in het midden van linkage group VII. Deze QTL regio correspondeert met een regio die syntenisch is in *Arabidopsis thaliana*, deze laatste heeft drie CBF genen achterelkaar. Het is al aangetoond dat CBF genen een sleutelrol spelen voor weerstand tegen vorst in veel soorten die aangepast zijn tegen kou. Vervolgens hebben we in hoofdstuk 3 de drie achtereen liggende CBF genen geïsoleerd uit *B. stricta* en hun mogelijke rol in de ontwikkeling van vorsttolerantie in *B. stricta* onderzocht. Onze resultaten lieten zien dat ze erg geassocieerd zijn met vorsttolerantie in *B. stricta.* In hoofdstuk 4 hebben we zowel een zaailing overleef assay gedaan als een QTL analyse. Hiermee hebben we een nieuwe QTL geïdentificeerd, die niet in een andere andere soort is gevonden, en vonden we bewijs voor een lipide biosynthese gen die betrokken is bij het vermeerderen van tolerantie tegen vorst. In het laatste onderzoekhoofdstuk hebben we de natuurlijk variatie van kou acclimatie en de-acclimatie onderzocht in vijf wilde *Brassicaceae* soorten. Hiermee hebben we laten zien dat soorten contrasterende responses in kou acclimatie en de-acclimatie hebben. Onze resultaten wijzen sterk op verschillende regulatoire mechanismen in kou acclimatie en de-acclimatie. Alhoewel we nog niet al deze mechanismen begrijpen, hebben we laten zien dat het onderzoeken van wilde soorten nieuwe perspectieven kan bieden aan ecologisch belangrijke adaptatie aan kou, dit kan een toevoeging zijn om tolerantie in cruciferen te verbeteren.

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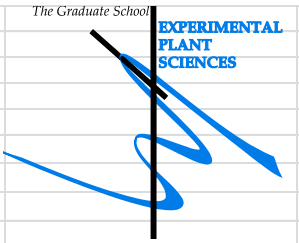
마지막은 한국에 계신 가족들을 위해서 한글로 마무리 하고자 합니다. 지금까지 부족한 자식을 믿어주시고 희생하신 부모님께 깊은 감사의 말씀을 드립니다. 앞으로도 더욱 성장하는 모습을 보여 드릴 수 있도록 항상 건강하셨으면 합니다. 아울러 언제나 제 편이 되어 힘을 주시고 기도를 아끼지 않으신 장인어른, 장모님께도 감사의 말씀을 전합니다. 주위에서 격려를 아끼지 않으시고 오늘이 있기까지 듬직한 힘이 되어 주신 우리 형제, 처가 식구분들께도 감사의 말씀을 전합니다. 오늘의 작은 결실에 자만하지 않고 꾸준히 노력하는 좋은 모습을 보이도록 하겠습니다. 마지막으로 학위과정 막바지에 여러 가지 이유로 인해서 어려움을 겪고 오던 저의 모든 근심 걱정을 잊게 해준 나의 사랑하는 아들 연우에게도 가득한 감사와 사랑을 드리며 이 결실의 기쁨을 함께 하고자 합니다.

About author

Jae-Yun Heo was born on the 15th day of January 1982 in Masan, Korea. After finishing high school in 2000, he studied at Kangwon National University and received a BSc degree in Horticultural Science in 2005. He then continued to study at graduate school of the same University from 2005 to 2008, and successfully obtained his MSc degree in Horticultural science in 2008. While he conducted his project for MSc degree, he got a temporary researcher position at National Institute of Horticultural & Herbal Science in Korea and performed cooperation research for 8 months. After graduation, he won a competitive PhD scholarship through a NIIED-funded program to pursue further graduate studies in the Netherlands. On 1st day of April 2010, he started his PhD program in Experimental Plant Systematics group at University of Amsterdam, and later transferred to Biosystematics group at Wageningen University and Research Centre. The thesis is the results of four years of research carried out in both groups at University of Amsterdam and Wageningen University. Since 17th day of March 2014, he started to work at Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries as a research.

Education Statement of the Graduate School

Experimental Plant Sciences



Issued to: Jaeyun Heo
Date: 16 June 2014
Group: Biosystematics, Wageningen University & Research Centre

1) Start-up phase		*date*
▶	**First presentation of your project**	
	Variation in cold hardening and dehardening in annual winter species	Apr 05, 2010
▶	**Writing or rewriting a project proposal**	
	Variation in cold hardening and dehardening in annual winter species	Apr 2010
▶	**Writing a review or book chapter**	
▶	**MSc courses**	
▶	**Laboratory use of isotopes**	
	Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure		*date*
▶	**EPS PhD student days**	
	EPS PhD student day, Univeristy of Amsterdam, NL	Nov 30, 2012
▶	**EPS theme symposia**	
	EPS theme 4 symposia 'Genome Biology', Radboud University, Nijmegen, NL	Dec 07, 2012
	EPS theme 4 symposia 'Genome Biology', Wageningen University. NL	Dec 13, 2013
▶	**NWO Lunteren days and other National Platforms**	
	NWO-ALW meeting 'Experimental Plant Sciences', Lunteren, The Netherlands	Apr 22-23, 2013
▶	**Seminars (series), workshops and symposia**	
	IBED seminar by Keith Brander (Climate impacts on marine ecosystems and fisheries)	May 27, 2010
	IBED seminar by Dr. Geoff Abbott (Exploring phenols in a northern peatland ecosystem)	Sep 17, 2010
	IBED seminar by May Berenbaum (Coevolutionary interactions on three continents; worldwide	Sep 23, 2010
	IBED seminar by Pierre Taberlet (Next generation sequencers and biodiversity)	Sep 23, 2010
	IBED seminar by Tanja Schwander (Mechanisms favoring and constraining the transition to asexuality)	Oct 28, 2010
	IBED seminar by David A. Vasseur (A tale of two synchronies: intra and interspecific dynamics in space and time)	Nov 25, 2010
	IBED seminar by Santiago Madriñan (Patterns and processes of Páramo plant diversification)	Dec 16, 2010
	IBED seminar by Gregor Fussmann (The multifaceted nature of plankton dynamics: from cell cycles to meta-communities)	Jan 27, 2011
	IBED seminar by A.P. van Wezel (Nanonext; program Environmental risk assessment of manufactured nanomaterials)	Feb 24, 2011
	IBED seminar by Dr. David Burney (TIME'S MOST IMPORTANT MOMENT: Tracing the Global Footprint of Human Arrival through Paleoecology)	Mar 24, 2011
	IBED seminar by Axel Son (Impact of land use changes on soil carbon - quantification and process understanding)	Sep 22, 2011
	IBED seminar by Jonathan F. Wendel (Genes, jeans, and genomes: exploring the mysteries of polyploidy in cotton)	Oct 13, 2011
	Invited seminar by Peter Fields (Neutral and selective determinants of FST in a metapopulation of Silene latifolia)	Oct 28, 2011
	SILS seminar by Alain Tissier (Glandular trichomes and terpenoid biosynthesis in the Solanaceae)	Nov 24, 2011
	IBED seminar by M.O. Gessner (Drivers of Litter Decomposition in a Changing World: A Fresh Water Perspective)	Nov 24, 2011
	IBED seminar by M. Knaden (Smells like home - how desert ants use olfaction for navigation)	Dec 15, 2011
	IBED seminar by Pascal Boeckx (How isotopes help to understand ecosystem dynamics and	Jan 21, 2012
	IBED seminar by Martin Kaltenpoth (The Brotherhood of the Wolf: Symbionts Provide Antimicrobial Combination Prophylaxis to Beewolf Wasps)	Feb 23, 2012
	IBED seminar by Christian Körner (The significance of plant diversity in a global change context)	Mar 22, 2012
	IBED seminar by Stefano Allesina (Type of Interaction and the Stability of Large Ecological Networks)	Apr 26, 2012
	IBED seminar by Jeremy Jackson (Ocean Apocalypse: What will the oceans be like by 2035?)	May 24, 2012
	Seminar by Yukihiro Sugimoto ('Strigolactones, new plant hormones. Importance of their stereochemistry for bioactivity as germination stimulant')	Oct 16, 2012
	Plant Sciences Seminar: Ecological principles of plant-plant interactions: linking crops and natural systems	Dec 11 , 2012
	Seminar by Tim Sharbel (The dynamics of asexual genome evolution and candidate apomixis factors in the genus Boechera (Brassicaceae))	Feb 20, 2013
	Plant Sciences Seminar on Bioinformatics (Is your Research becoming Digital? Time to call the BioinforMagician!)	Mar 12, 2013

	EPS Flying Seminar by Graham Farquhar ('Integrating photosynthetic carbon assimilation from the leaf to the canopy')	Mar 13, 2013
	Seminar by Arjen Biere ('Iridoid glycosides: biochemistry and role in biotic interactions in ribwort	Apr 10, 2013
	Seminar by Alain Tissier ('Glandular trichomes of tomato: from terpene biosynthesis to trichome differentiation')	May 03, 2013
	EPS-Flying Seminars by Howard S. Judelson and Rays H.Y. Jiang	May 07 , 2013
	Plant Sciences Seminar The role of Plant Breeding in improving quality of crop plants	Jun 11, 2013
	Seminar by Hanhui Kuang (Using the Nicotiana-TMV system to study resistance gene evolution and plant genome stability)	Sep 11, 2013
	Seminar by Stephane Blanc (New insights into the relationship between plant viruses and insect vectors)	Sep 18, 2013
	Biosystematics Group seminar by Ronald Pierik (Multiple ways to deal with shade in dense vegetations)	Oct 01, 2013
	Plant Sciences Seminars on Plant Metabolomics	Oct 08, 2013
	Seminar by Ying Zeng (Elucidating the Biosynthetic Pathway for Vibralactone in the Basidiomycete Fungus Boreostereum vibrans)	Oct 24, 2013
	Plant Sciences Seminars: Statistics, genetics and omics in research	Dec 10, 2013
	Seminar by Niklas Wahlberg (The 215 million years of Lepidoptera diversification: lessons from an ever changing world)	Dec 13, 2013
	Seminar by Jos Raaijmakers (exploring and exploiting the plant microbiome)	Jan 07, 2014
	Seminar by Dani Zamir (Geno-Pheno in Plant breeding)	Febr 10, 2014
►	**Seminar plus**	
►	**International symposia and congresses**	
	9th International Plant Cold Hardiness Seminar	Jul 17-22, 2011
	International Workshop Photosynthesis: from Science to Industry	Oct 08-12, 2012
►	**Presentations**	
	Natural variation of cold hardening and dehardening in Brassicaceae (poster presentation)	Jul 17, 2011
	Oral presentation to co-workers/collaborators in the US at Poineer Hybrid	Feb 16, 2012
	Oral presentation to co-workers/collaborators in the US at Poineer Hybrid	Jun 21, 2012
	Quantitative trait loci associated to freezing tolerance in B.stricta (poster presentation)	Oct 08, 2012
	Oral presentation to co-workers/collaborators in the US at Poineer Hybrid	Nov 15, 2012
	Oral presentation to co-workers/collaborators in the US at Poineer Hybrid	Mar 21, 2013
	Oral presentation to co-workers/collaborators in the US at Poineer Hybrid	Jul 18, 2013
	Oral presentation to co-workers/collaborators in the US at Poineer Hybrid	Dec 05, 2013
►	**IAB interview**	
►	**Excursions**	
	Subtotal Scientific Exposure	*16.8 credits**

3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	Postgraduate course 'The Power of RNA-seq', Wageningen, NL	Jun 05-07, 2013
	Postgraduate course 'Phylogenetics', Wageningen, NL	Oct 14-19, 2013
►	**Journal club**	
	Participant in literature discussion group at Biosystematics	2010-2013
►	**Individual research training**	
	worked/visited other laboratories (plant physiology group in University of Amsterdam, 3 weeks)	2013
	Subtotal In-Depth Studies	*7.4 credits**

4) Personal development		*date*
►	**Skill training courses**	
	English conversation training course	2010
	IBED Lustrum Celebration Day (networking)	Sep 14, 2010
	IBED day (career orientation)	Sep 15, 2011
►	**Organisation of PhD students day, course or conference**	
►	**Membership of Board, Committee or PhD council**	
	Subtotal Personal Development	*2.1 credits**

TOTAL NUMBER OF CREDIT POINTS*	**33.8**
Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS	
** A credit represents a normative study load of 28 hours of study.*	