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Cycle XXXVI

Dissecting Cold Acclimation and Frost Resistance Using Reciprocal Near Isogenic Lines in Barley

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Abstract

Abiotic stresses impact barley and cereals growth and production, among them freezing temperature is one of the major limitations. especially in the climate change era

Barley increases its freezing tolerance in response to low, non-harmful temperatures; a phenomenon known as cold acclimation. Two major quantitative trait loci (QTL), *Frost Resistance 1* (*FR-H1*) and *Frost Resistance 2* (*FR-H2*), located on the long arm of chromosome 5, play a prominent role in cold acclimation and freezing tolerance. *FR-H2* encompasses a cluster of 13 different C-repeat Binding Factor (*CBF*) genes, which encode transcription factors that activate effector genes.

The aim of the present thesis was to investigate the role of *FR-H1/VRN-H1* and *FR-H2/CBF* genes transcription factors in the regulation of cold acclimation thus frost tolerance. To test the effects of *FR-H1* and *FR-H2* on cold acclimation and freezing tolerance, four reciprocals Near Isogenic Lines (NILs) were developed carrying the winter allele at each locus introgressed into the spring background, and vice versa. To the authors knowledge, this thesis was the first work using QTL-NILs genotypes to dissect the effect and the interaction of *FR-H1* and *FR-H2* loci to study acclimation and freezing resistance in barley.

One of the aspects that this thesis investigated was phenotyping, and two freezing protocols, both in short-day conditions were applied in a controlled condition in a growth chamber showing that the NILs carrying Nure allele of *FR-H2* introgressed in the spring/susceptible background increased the freezing survival. In addition, open field trials were assessed to evaluate the agronomical parameters and winter survival rate of QTL-NILs.

The second aspect investigated, was gene expression modulation of four *CBFs* (*HvCBF2*, *HvCBF4*, *HvCBF9* and *HvCBF14*) *VRN-H1* and two effector genes (*HvCOR14b* and *HvDHN5*) during the control (20/15 °C), pre-hardening (10/8 °C) and hardening phases (3/1 °C). The experiments were design to analyze the cold induction. Quantitative real time (qRT-PCR) results showed that use of these plant materials made it possible to quantify the effect of the allelic form of the two loci in the resistant and susceptible backgrounds, highlighting interesting differences observed between resistant and susceptible genotypes already under control condition.

These results represent a significant step toward the understanding of the genetic basis of the cold acclimation and frost resistance mechanism in barley crop.

Chapter 1

The *Triticeae CBF* Gene Cluster - to Frost Resistance and Beyond

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1.1. *Triticeae* Crops and Abiotic Stress

1.1.1. *Triticeae* as Staple Food and Adaptable Crops

The Green Revolution had been able to meet the demand for food, reducing world hunger of the growing population (from 2.519 billion in the 1950 to 4.435 billion in the 1980) thanks to a unprecedented increase in crop yield and agricultural production (Pingali, 2012; *World Population Prospects 2022, Population Growth Rate File, Estimates Tab. United Nations Department of Economic and Social Affairs. 2022. Date of Access: 29-11-2022.*, n.d.). New irrigation techniques, massive use of fertilizers and plant protection products, mechanization, crop breeding and adoption of improved varieties were the determining factors in the observed increase in productivity (Bailey-Serres et al., 2019; Evenson and Gollin, 2003; Hedden, 2003). Cereal crops, in particular, saw significant improvement with yields tripling despite a small increase of arable land (Foley et al., 2011; Wik et al., 2008). However, beside the positive effects, the excessive agricultural intensification created the conditions for the rise of environmental problems, such as pollution, soil degradation and loss of genetic diversity (Kopittke et al., 2019; Pingali, 2019). For example, in many breeding programs, genotypes were selected for the high-input systems driving to gene pools erosion, especially for those alleles responsible for the adaptation to the environment (Kole et al., 2015; Schröter et al., 2005; Zhu et al., 2010). However, new issues emerged: yield seems to have reached plateau and a contraction of genetic diversity is observed (Grassini et al., 2013; Khoury et al., 2022) and as a result, the adaptation to biotic and abiotic stresses of cereals crops has been reduced (Corrado and Rao, 2018; Doebley et al., 2006; Haussmann et al., 2004; Zenda et al., 2021; Zhao et al., 2015). In a scenario where the population is still growing (based to UN estimation, planet Earth will be populated by 8.5, 9.7, 10.9 billion of people by 2030, 2050 and 2100, respectively (*World Population Prospects 2022, Population Growth Rate File, Estimates Tab. United Nations Department of Economic and Social Affairs. 2022. Date of Access: 29-11-2022.*, n.d.), one of the goals of the global food production system is to provide higher yield and food quality, reducing however, the environmental pollution (Wik et al., 2008).Furthermore, extreme weather conditions, reduction of arable lands, increasing demand of fertilizers and irrigation water are putting under stress conditions the crops cultivation in open fields, affecting significantly agricultural production in all continents (Intergovernmental Panel on Climate Change, 2022). A novel approach is required to cope with the climate issue. Crop breeding programs need to develop new genotypes with a higher adaptation to the weather fluctuations (Challinor et al., 2014; Kovak et al., 2022) and to contribute to global food security (Reynolds et al., 2016).

Triticeae tribe, a grass tribe of the *Poaceae* family that includes cultivated wheats (durum wheat *Triticum turgidum* L. ssp. *durum* Desf., bread wheat *Triticum aestivum* L.), barley (*Hordeum vulgare* L.), and rye (*Secale cereale* L.) is by far the most important source of energy and nutrients worldwide (Muehlbauer and Feuillet, 2009; Wang et al., 2018). For example, wheat and barley together were the most cultivated herbaceous crops in the world in 2021, with a harvested area of 220 and 48 million hectares and a total grain production of 770 and 145 million tons, respectively (*FAO.STAT. License: CC BY-NC-SA 3.0 IGO. Extracted from: https://www.fao.org/faostat/en/#home. Data of Access: 07-10-2022.*, n.d.). Rye is an important crop for the Northern and Eastern European countries, with a harvested area of 3.5 million hectares and a total production of 11 million tons (*FAO.STAT. License: CC BY-NC-SA 3.0 IGO. Extracted from: https://www.fao.org/faostat/en/#home. Data of Access: 07-10-2022.*, n.d.). The *Triticeae* tribe comprises about 350 species, including also the so-called minor cereal, such as triticale, spelt emmer, and einkorn wheats, poulard, polish and khorasan wheats (Barkworth and Von Bothmer, 2009).

Temperate grasses species are characterized by winter growth habit (WH) in their natural environments (Hyles et al., 2020; Schreiber et al., 2019). The two key traits of WH genotypes are the vernalization requirement and the cold acclimation. Vernalization is defined as the induction of flowering after a prolonged exposure to cold. Moreover, *Triticeae* are usually classified as long day (LD) plants because most varieties flower earlier when exposed to longer days. This mechanism synchronize plants to flower after cold harmful temperatures in wintertime (Bond et al., 2011; Trevaskis et al., 2006, p.). The cold acclimation is the ability of the crop to adapt to cold temperature and then to survive to frost events (Thomashow, 2010). The winter habit (WH) genotypes are usually sown in winter due to their higher productivity. In Mediterranean climates, sowing is performed in autumn to take advantage of the rainiest seasons, and the plants are harvested during the drier summer. Winter habit is a limiting factor in the widespread of cultivation of *Triticeae* in environments where winter is too cold to survive or too warm to satisfy vernalization requirement (von Bothmer et al., 2003). To overcome this limit, spring habit (SH) and facultative habit (FH) genotypes were selected for their lack of vernalization requirement (Stockinger, 2009; von Zitzewitz et al., 2011). SH genotypes are sown in spring, whereas FH genotypes can be alternatively sown either in autumn or spring. Most SH cultivars are frost susceptible and, due to a shorter crop cycle, may be exposed to drought. For these reasons, in the last years, FH genotypes are gaining more and more interest since they show high level of frost tolerance (FT) and do not require vernalization (Muñoz-Amatriaín et al., 2020; Rosicka - Kaczmarek et al., 2016). The *Triticeae* crops are thus adaptable to several environments, ranging from sub-arctic to tropical climates, allowing their

cultivation across a wide geographical area (Deng et al., 2016; Feldman and Levy, 2015), even if the highest yields are achieved in temperate regions (Kumlehn et al., 2010).

1.1.2. Cold and Drought Issues for *Triticeae* in the Climate Change Era

Abiotic stresses are described as environmental conditions that can impact on plant's growth and production (Zhang et al., 2022). The abiotic stress response is a mechanism regulated by interactions and crosstalk of many molecular pathways resulting in metabolic changes (Figure 1). Metabolic changes include the repair of stress-induced damage, the rebalancing of cellular homeostasis and growth modification to levels suitable for the stress conditions (Zhang et al., 2022). These stresses can be perceived in various cellular compartments and the signal is transduced by protein kinases or Ca^{2+} ions and transmitted to the cytoplasm. Inside the cytoplasm, the signal, whether hormonal or non-hormonal, undergoes through signal transduction pathways. These pathways pass through the nucleus, where transcription factors (TFs) are up-regulated. TFs are "*the orchestra conductor"* of the abiotic stress mechanism. TFs are specific proteins that regulate stress responses by modifying gene expression interacting with specific effector genes in their promoter regions. TFs bind the cis-elements and form a complex of transcription initiation on the TATA box (core promoter) upstream of the transcription start sites (Ciarmiello et al., 2014). The result of this interaction is the up-regulation of the effector genes. After the effector genes activation, stress-related proteins are activated and trigger physiological and biological changes that lead to an adaptive response within the plant, enabling it to cope with and respond effectively to the stress (Cramer et al., 2011; Mickelbart et al., 2015; Udawat and Deveshwar, 2018).

Several families of TFs are involved in abiotic stress response, the main are C-repeat binding factors or Dehydration responsive element CBF/DREB1 and DREB2, NAC (NAM, ATAF, and CUC), zinc-finger homeodomain (ZF-HD), ABA-responsive element binding protein/ABA binding factor (AREB/ ABF), and myelocytomatosis oncogene/myeloblastosis oncogene (MYC/MYB) (Ciarmiello et al., 2014). We know that C-repeat binding factors or Dehydration responsive element (*CBF/DREB1*) transcriptional factors are involved in cold acclimation and frost tolerance. Recent research suggests that they are also involved in the drought response (Dong et al., 2018; Guo et al., 2019; Yang et al., 2020; Yin et al., 2018).

Temperate cereals usually experience frost during the vegetative/tillering phase, whereas drought usually negatively influence flowering, heading and ripening phases. Extreme temperatures and drought are associated with plant cell dehydration, and represent some of the major issue for agronomical and global food security in *Triticeae* (Kole et al., 2015; Maccaferri et al., 2009). Frost damage is caused by the ice

formation in intercellular spaces resulting in a drop in water potential (Thomashow, 1999), influencing root water uptake and photosynthesis (Hassan et al., 2021). Drought stress affects plant growth differently depending on the phenological phase (Praba et al., 2009); early events reduce stomatal conductance, transpiration and CO_2 assimilation, affecting tiller formation, while water deprivation during the reproductive phase, reduces grain number and size (Farooq et al., 2009). In some environments, a combination of frost and drought (the so-called harsh winter) may happen, causing a severe reduction of enzymatic activities and membrane disintegration, leading to stunted growth and compromising yield (Ejaz et al., 2023; Preston and Fjellheim, 2020).

The autumn, winter and spring seasons are becoming nowadays more and more variable, with a higher alternation between warm-dry and cold-rainy periods with extreme frost episodes (Cohen et al., 2021; Huang et al., 2021; Ozturk et al., 2015). This alternation may putatively cause an overlapping of low temperature and water deprivation, in regions – such as the Mediterranean basin – where prolonged droughts followed by/overlapped with cold and rain events become more frequent with consequences still unclear on crops (Hussain et al., 2018; Rizza et al., 2016). Moreover, based on the United States Drought Monitor and JRC European Drought Observatory, data collected since 2011 reveal that drought in fall-winter and during early spring has been increasing in temperate areas, where *Triticeae* crops are manly cultivated ("Current Map | U.S. Drought Monitor," n.d.; "Drought Reports - European Drought Observatory - JRC European Commission," n.d.). Furthermore, the warmer temperatures in the artic pole might affect mostly the northern hemisphere increasing the severity of winter and frost events in early spring (Cohen et al., 2021; Francis et al., 2017; "Graph | U.S. Climate Extremes Index (CEI) | National Centers for Environmental Information (NCEI)," n.d.). The climate fluctuations in temperate regions are impacting on the crop cycle and the phenological responses of barley and wheat crops (Tao et al., 2014; Wang et al., 2018). The alternation of warm and cold periods might induce de-acclimatation process in the WH genotypes, reducing their frost tolerance and producing serious yield losses (Chen et al., 2020; Willick et al., 2021). Additionally, drought stress, which occur due to a reduced amount of rain and a lower level of soil moisture during the sowing period (autumn/early winter) and in initial phenological stages, can decrease the rate of germination and early seedling development (Barlow et al., 2015). Furthermore, the vernalization requirement may not be totally satisfied resulting in delayed flowering and exposing the plants to a different incidence of biotic and abiotic stresses (Penfield et al., 2021). Even spring genotypes are not immune to this problem as the alternation of drought stress and late frost events can compromise germination and development (Frederiks et al., 2015; Trnka et al., 2014); moreover, SH

genotypes may face an increased risk of heat waves exposure during the grain filling phase (Faranda et al., 2023; Perkins-Kirkpatrick and Lewis, 2020).

Due to the multitude of factors involved in the climate change, a prediction of yield loss is complex to assess. Several authors have reported that a reduction in the yield of barley and wheat is linked to the geographical area and the consequent impact that climate change has on that specific region (Asseng et al., 2015; Neupane et al., 2022; Wang et al., 2018). For example, a study on the impact of the climate change in a period between 1981-2009 on wheat production in China, stated that due to 1 °C increase, the yield was reduced by 1 to 10% (Tao et al., 2014). As far as growth habit is regarded, Gammans and colleagues analyzed the effect of a wide range of climate models and emissions scenarios on winter/spring barley and wheat yield reduction in France for the period 2037-2065 (Gammans et al., 2017). Similarly, Cammarano et al. stated that climate change could impact the yield up to 9% for barley genotypes with different vernalization requirement cultivated in the Mediterranean basin (Cammarano et al., 2019).

Numerous research has been undertaken in recent years to elucidate the molecular basis of adaptation process in *Triticeae* cereals for the development of new improved varieties (in Figure 1 we present a simplified model of the cellular adaptative response to osmotic and temperature variations). The crucial role of genetic resources on vernalization response (*VRN-1*, *VRN-2* and *VRN-3*) and frost tolerance (*FR-1* and *FR-2*) has been defined already by multiple Authors (Dhillon et al., 2010; Francia et al., 2004; Kobayashi, 2005; Szùcs et al., 2007; Vágújfalvi et al., 2005). *FR-2* is known to encompass a cluster of C-repeat binding factors (*CBF*) genes that are involved in cold acclimation and frost tolerance. In addition, recent reports suggest that they are also involved in drought response (Dong et al., 2018; Guo et al., 2019; Yang et al., 2020).

Maintaining yield performance under various unfavorable conditions is the main concern for breeders regarding genetic improvement of resistance/tolerance to abiotic stresses. Starting from the comprehensive description of the *CBF* gene cluster locus structure and function, this review aims at better understanding the complex signal transduction pathway(s) resulting in abiotic stress tolerance of *Triticeae*. The availability of different linkage maps and genomic resources, such as transcriptomes and whole genome sequences, can boost the efficacy of breeding programs for wider climatic adaptability and stress tolerance. Thus, our aim is to describe the pivotal role of *Triticeae* CBF transcriptional activators in frost tolerance and to show their putative role in drought tolerance. Integration of omics

with substantial trait variation existing in genetic resources, would pave way for cereal crops improvement against abiotic stresses.

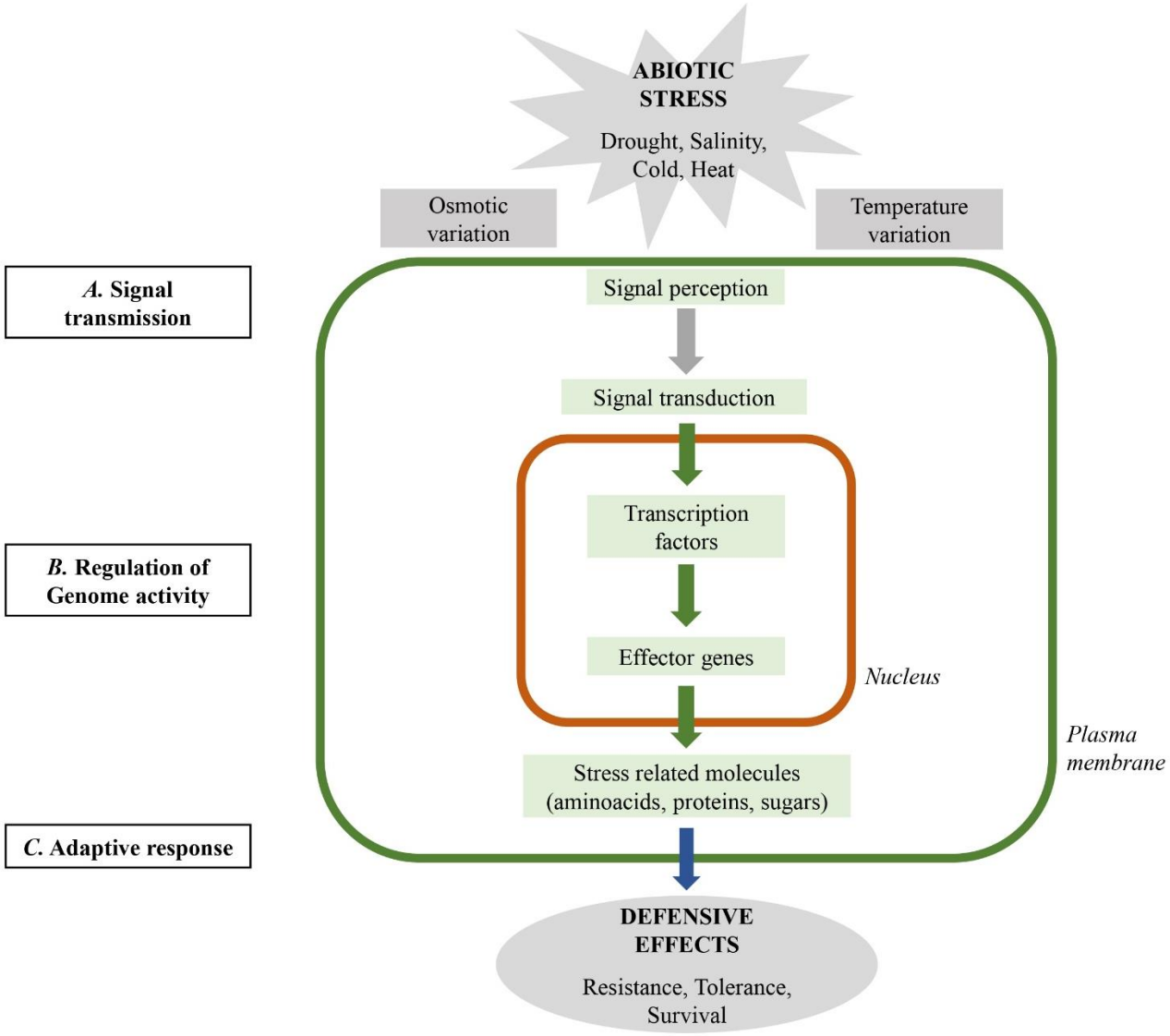

Figure 1. Simplified model of cellular response to temperature and osmotic variations. Starting from perception and transduction of the signal (A), a complex regulation of genome activity is initiated in the nucleus (B). Key transcriptional regulators target regulons of effector genes that lead to the production of stress-related molecules in the adaptive response (C). The final result is an increased ability of the cell, tissue and plant to cope with and respond effectively to the stresses (modified from Mastrangelo et al., 2005; Zhang et al., 2022).

1.2. *CBF* Gene Cluster and its Central Role in Response to Frost and Drought

1.2.1. C-Repeat Binding Factors and Cluster Organization in *Triticeae*

C-repeat binding factors or dehydration responsive element (*CBF/DREB1*) are a larger subfamily of transcription factors that belong to the APETALA2/ethylene-responsive element binding factor (AP2/ERF) protein family and are induced/activated in response to osmotic stresses as cold or drought. The AP2/ERF domain binds to the C-repeat/dehydration responsive elements (CRT/DRE) in the promoter region of a variety of genes involved in the abiotic stress response, also known as "CBF's regulon," such as, cold-responsive genes (COR) encoding the late embryogenesis abundant protein (LEA), dehydrin family gene (DHN) that protects against the adverse effect of losing water caused by frost and drought, cold-inducible (*KIN*), responsive to desiccation (RD) family and genes involved in biosynthesis of osmoprotectant protein, carbohydrate metabolism related, and sugar transport. (Akhtar et al., 2012; Choi et al., 1999; Heidarvand and Maali Amiri, 2010, p. 3; Park et al., 2015). The distinctive element of *CBFs* within the AP2/ERF family is the specific "CBF signature" flanking the AP2 domain (Akhtar et al., 2012; Skinner et al., 2005).

CBF1 was the first *CBF* gene isolated and characterized by Stockinger and colleagues in *Arabidopsis thaliana* (Stockinger et al., 1997). Subsequently, other important works discovered the *CBF* family and its role in the model plant (Jaglo-Ottosen et al., 1998; Medina et al., 1999) and then, in other 54 genera: 31 dicotyledons, 23 monocotyledons including 13 woody species (Cheng et al., 2015; Hu et al., 2020; Li et al., 2020; Tondelli et al., 2011; Welling and Palva, 2006). In *Poaceae*, multiple elements of the family were isolated and characterized, either in chilling sensitive (e.g.: rice and maize) or in frost tolerant species (e.g.: wheat, barley, and rye) (Dubouzet et al., 2003; Nakano et al., 2006; Qin et al., 2021; Tondelli et al., 2011). The *CBF* genes are characterized by short, mono-exon coding sequences (average length 700 bp) with no introns. Interestingly, Shi et al. (Shi et al., 2018) performed a phylogenetic analysis and found that the *CBF* gene structure is remarkably conserved across various species (monocots/dicots), independently of their degree of frost tolerance. As reported by Campoli et al. and Badawi et al. (Badawi et al., 2007; Campoli et al., 2009), *CBF* genes are classified in four phylogenetic groups, each with two or more sub-groups. Some elements of the CBF family are scattered along the genome, while the other, more frequently, are organized in cluster of tandemly duplicated genes on the long arm of homoeologous chromosome group 5 of *Triticeae* (Francia et al., 2007, 2004; Hayes et al.,

1993; Vágújfalvi et al., 2003). The genomic portion harboring the *CBFs* coincides with a QTL for the frost tolerance, namely *Frost Resistance 2* (*FR-2*) in barley (*FR-H2*), diploid (*FR-A^{m}2*) and polyploid wheats (*FR-A2* and *FR-B2*), and rye (*FR-R2*) (Båga et al., 2022; Francia et al., 2004; Vágújfalvi et al., 2003). In *Triticeae* crops, beside *FR-2*, part of the phenotypic variation for frost tolerance is attributed to another QTL located about 25-30 cM apart from *FR-2* on the long arm of homoeologous chromosome group 5: *Frost Resistance 1* (*FR-1)*. This locus was identify by Hayes et al. in 1993 and Galiba et al. in 1995 (Galiba et al., 1995; Hayes et al., 1993) in barley and wheat, respectively, and reported to co-segregate with *VRN-1*, the vernalization requirement gene (Francia et al., 2004), whose expression leads the plant to become competent for flowering (Amasino, 2004).

The number of *CBF* genes identified in *Triticeae* species has been increasing during the last two decades with novel studies being performed and new genomic data being obtained. The first comprehensive studies reported at least 15 *CBF* genes present in each of the A, B, and D genomes in wheat (Badawi et al., 2007) and 20 in barley (Skinner et al., 2005). In rye, only 12 *CBF* genes have initially identified (Campoli et al., 2009; Rabanus-Wallace et al., 2021). Up to date, the estimate number of *CBF* orthologs harbored only at *FR-2* is 13, 54, and 21, for barley, wheat and rye, respectively (The International Wheat Genome Sequencing Consortium (IWGSC) et al., 2018). *Triticeae CBFs* can be classified into groups sharing similar structural characteristics and a common phylogenetic origin. Six of these groups, i.e IIIc, IIId, IVa, IVb, IVc and IVd had been identified only in subfamily *Pooideae* suggesting the recent adaptation of *CBFs* to the temperate habitats (Badawi et al., 2007). Figure 2 shows a simplified model of the *FR-2* cluster organization in *Triticeae.*

Barley *CBF*s were classified by Skinner et al. (Skinner et al., 2005) in three phylogenetic clades: HvCBFI, HvCBFIII and HvCBFIV. Seven *CBF* genes of the HvCBFI clade are widespread across the genome, while *FR-H2* encompass 13 *CBF*s genes of HvCBFIII and HvCBFIV clades organized in a single cluster (Pasquariello et al., 2014). The cluster is divided into three different portions: proximal (*HvCBF 2*, *4* and *9*), central (*HvCBF 3*, *12*, *13*, *14*, *15*, *16*) and distal part (*HvCBF 6* and *10*). *HvCBF* genes in *FR-H2* cluster are surrounded by non-coding sequences enriched in multiple repetitive elements (Mareri et al., 2020).

Thirteen *TmCBF* were described in *Triticum monococcum* L., eleven of them were mapped on *FR-A^{m}2*, while *TmCBF15* and *TmCBF18* were mapped on chromosomes 7A^m and 6A^m, respectively (Miller et al., 2006). Vágújfalvi et al. (Vágújfalvi et al., 2003) attributed the locus for FT to chromosome 5A and

subsequently Knox and colleagues (Knox et al., 2008), divided the *FR-A2* locus into: proximal (*CBF 2*, *4*, *9* and *17*), central (*CBF 12*, *14*, and *15*) and distal (*CBF 3*, *10*, *13* and *16*).

The genome of hexaploid wheat encode 65 *TaCBF*s (Mohseni et al., 2012), 27 of which are paralogs with 1-3 homoeologous A, B, D copies (Mohseni et al., 2012). As reported by The International Wheat Genome Sequencing Consortium (IWGSC) (The International Wheat Genome Sequencing Consortium (IWGSC) et al., 2018), 54 *TaCBF*s are located on chromosomes Group 5; 17 genes on 5A, 19 on 5B, and 18 on 5D chromosomes. Other *TaCBF*s are located on homoloegous chromosomes 6; A, B, D.

Rye encodes 21 *ScCBF*s, most of which reside at *FR-R2* and were mapped to the chromosome 5RL (Badawi et al., 2007; Båga et al., 2022; Campoli et al., 2009).

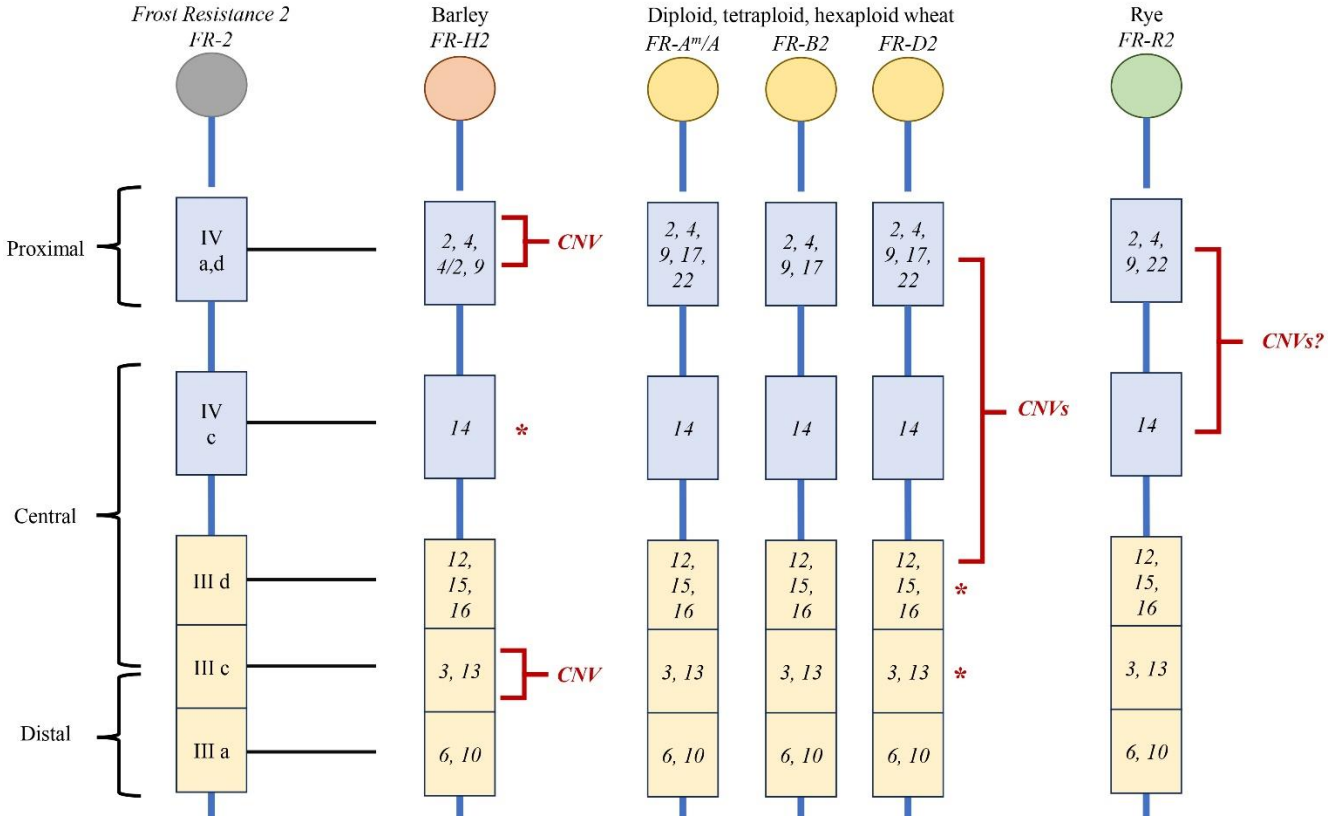

Figure 2. Model of the structural organization of the *CBF* cluster at *Frost Resistance-2* in *Triticeae* genomes. The locus is depicted in proximal, central, and distant part highlighting different phylogenetic classification in *CBF* subgroups. Pale blue boxes - *CBFs* of groups IV; yellow boxes - *CBFs* of III groups; CNV - copy number variation; question mark - CNV still not confirmed; red asterisks - SNV (single nucleotide variation).

1.2.2. Role of the ICE-CBF-COR Pathway in Cold Acclimation

In winter cereals, cold acclimation, also known as “hardening”, has the vital function to protect crown and young leaves from ice damages (Hüner et al., 2016); even after a severe stress episode, if the crown and young leaves survive, the plant maintains the potential to restore from tillering nodes (Kosová et al., 2014). This peculiarity is linked to the ability of the meristematic tissue to survive thanks to the physiological phenomenon of cold acclimation (Hüner et al., 1993). Phenolic compounds, sugars, soluble proteins and new enzyme isoforms, proline and organic acids, modification of the fatty acids composition in the phospholipid membrane and higher levels of antioxidants are all proactive compound connected to the reduction of frost damage (Fiust and Rapacz, 2020; Heidarvand and Maali Amiri, 2010; Hüner et al., 2016, 2013).

In winter barley, wheat and rye, cold acclimation occurs only in the vegetative phase, and it has two different signaling pathways: abscisic acid or ABA-dependent (ABA pathway) and ABA-independent (also known as the ICE-CBF-COR pathway) (Shi et al., 2015). Although the ABA and CBF signal transmissions were considered distinct from each other, recent studies suggested a cross talk between these two pathways (Kashyap and Deswal, 2019).

In short-day condition, the ICE-CBF-COR pathway is promptly activated after a brief exposure to low, non-harmful temperature (Galiba and Tóth, 2017; Maibam et al., 2013) and the *CBF* gene have a pivotal role in the coordination of the acclimation processes (Kurepin et al., 2013). In Arabidopsis a marked increase in *CBF* transcript levels was observed 15 minutes after cold exposure, followed by up-regulation of the effector genes about 2 hours later (Thomashow, 2010; Vaultier et al., 2006). On the other hand, in wheat and barley an increase of *CBF* transcript levels was observed 4-12 hours later after the cold exposure (Jin et al., 2018; Vágújfalvi et al., 2005; Xue, 2003). The gene induction rely on a temperature threshold dependent on the species and occurs in a 10 °C to 12 °C range in winter barley, wheat and rye (Fowler, 2008; Rizza et al., 2011). The result of the ICE-CBF-COR pathway cascade is the activation of the effector genes that modify the plant metabolism conferring frost tolerance (Crosatti et al., 2003). The temperature must be below 10 °C for 4-6 weeks in short-day conditions to complete the adaptive response in *Triticeae* (Cha et al., 2022; Xu and Chong, 2018); once the process is completed, crops can withstand freezing at -7/12 °C for barley, -9/18 °C for wheat and -18/-30 °C for rye (Galiba and Tóth, 2017; Pecchioni et al., 2014).

Interestingly, no receptors receiving the low temperature signal were identified so far (Fiust and Rapacz, 2020). The ICE-CBF-COR pathway is activated by an increase in intracellular Ca^{2+} concentration by either rigidification of the plasma membrane or ligand activated channels. After calcium influx into cytosol and its binding by Ca-sensors (such as calmodulins), a signal cascade based on calcium binding proteins (CBPs) is started to target the *ICE* (Inducers of CBF-gene expression) transcription factors that upregulate the *CBF* genes (Miura and Furumoto, 2013; Wang et al., 2016). ICE transcription factors belong to the MYC family and MYC subfamily of bHLH (Basic Helix–Loop–Helix) (Guo et al., 2019) and are known as positive *CBF* expression regulators, considered to act upstream of the low-temperature signaling pathway (Badawi et al., 2008; Chinnusamy et al., 2003; Guo et al., 2019).

In addition, as shown in Figure 5, temperature variation is not the only environmental stimulus influencing the expression of the *CBFs*, as also circadian rhythms and light characteristics (i.e., quality and quantity) have been reported to be involved in cold acclimation (Maibam et al., 2013). For example, recent studies showed that the expression of some barley *HvCBF* genes (*HvCBF2A*, *HvCBF4B*, *HvCBF6* and *HvCBF14*) is regulated by the circadian rhythm and day-length (Dhillon et al., 2017; Gierczik et al., 2017; Maibam et al., 2013). In warm condition *CBF* genes show high expression late in the afternoon and continue to decrease early in the night (Gierczik et al., 2017). The peak of expression is 8-12 hours after the dawn either in short- or long-day conditions. However, the amplitude of the peaks is wider in short-day compared to long-day conditions (Lee and Thomashow, 2012). This peak does not coincide with the coolest period of the day but, it may putatively be functional for the preparation of the cell to the subsequent cold of the night (Badawi et al., 2007). The circadian clock regulates the expression of several genes. The G-Box-Like motifs are necessary for transcriptional regulation by the circadian pseudo-response regulators binding basic helix-loop-helix transcription factor (Liu et al., 2016). Other environmental stimuli are the light spectra and intensity; several works elucidated that the variation of light spectra and light intensity might modulate the expression of *CBF* genes and also increase the frost tolerance (Ahres et al., 2020, 2021, 2022; Kovács et al., 2020; Monostori et al., 2018; Novák et al., 2017).

The vernalization process is controlled by three major genes: *VRN-1*, *VRN-2* e *VRN-3* (Cao et al., 2021; Szùcs et al., 2007). *VRN-1* is a flowering promoter that was shown to be an AP1-*like* MADS-box transcription factor, whose expression leads the plant to the transition from the vegetative to the reproductive phase (Distelfeld et al., 2009; Trevaskis et al., 2007). Moreover, it was also proved to be involved in cold acclimation and the frost tolerance (Dhillon et al., 2010). *VRN-2* is a dominant flowering repressor down-regulated by vernalization treatment and includes two tandem zinc finger-CCT domain

genes (*ZCCT1* and *ZCCT2*) (Karsai et al., 2005; Trevaskis et al., 2006). *VRN-3*, the main integrator of the photoperiod and vernalization signals that lead to the transition of the apical meristem (Fernández-Calleja et al., 2021), is homologous to the flowering integrator *FLOWERING LOCUS T* gene in Arabidopsis (Faure et al., 2007; Yan et al., 2006). Due to its diploid nature, WH barleys can be considered as a model for the vernalization in *Triticeae* crops (Muehlbauer and Feuillet, 2009). *VRN-H2* is expressed in long and neutral day condition (Monteagudo et al., 2019), in autumn, when plants are still in the seedling stages, *VRN-H2* is highly expressed and repress the *VRN-H3* which is the flowering induction gene (Fernández-Calleja et al., 2021; Hemming et al., 2009)). The repression of *VRN-H3* also limits the expression of *VRN-H1* (Distelfeld et al., 2009; Trevaskis et al., 2007). Exposure to cold temperatures activates *VRN-H1* and results in the down-regulation of *VRN-H2* and consequently in the release of *VRN-H3* from repression (Szùcs et al., 2007; Trevaskis et al., 2006). After a prolonged cold exposure the expression level of *VRN-H1* reaches a threshold necessary to induce the transition phase, upregulating *VRN-H3*, to initiate the flowering process (Hemming et al., 2009); exposure to long day conditions mediated by the photoperiod genes *PPD-H1* and *PPD-H2* is also necessary (Rizza et al., 2016).

The expression of *VRN-H1* change in function of the plant growth habit; as mentioned above, in winter genotypes the expression of the recessive *vrn-h1* allele is induced by prolonged periods of cold (Cuesta-Marcos et al., 2015, 2010). The quantity of time under cold and short-day conditions necessary to satisfy the vernalization requirements vary with the geographical origin of the genotype and the environmental condition, changing from 6 to 10 weeks of temperatures in a range between 6 °C to 2 °C under short day conditions (Cha et al., 2022; Maeda and Nakamichi, 2022; Trevaskis et al., 2006; Xu and Chong, 2018). In spring genotype the dominant *Vrn-h1* allele has a constitutive high expression that rapidly induces the transition (Shcherban et al., 2015). The vernalization in wheat is more complex compared to barley due to the presence of three homoeologous *VRN-A1, VRN-B1* and *VRN-D1* loci were mapped on the long arm of the chromosome group 5 (Tóth et al., 2003), with the major effect of *VRN-A1* in determining of the growth habit (Todorovska et al., 2014).

The interaction between *VRN-1/FR-1* and *FR-2* (*CBFs*) has also been demonstrated (Stockinger et al., 2007); *VRN-H1* can bind promoter regions of the *CBF* genes inducing a reduction of their transcription levels, nevertheless the mechanism is still not fully understood (Deng et al., 2015; Mareri et al., 2020) (Figure 3).

However, a question remains: how does the ICE-CBF-COR pathway confer frost tolerance?

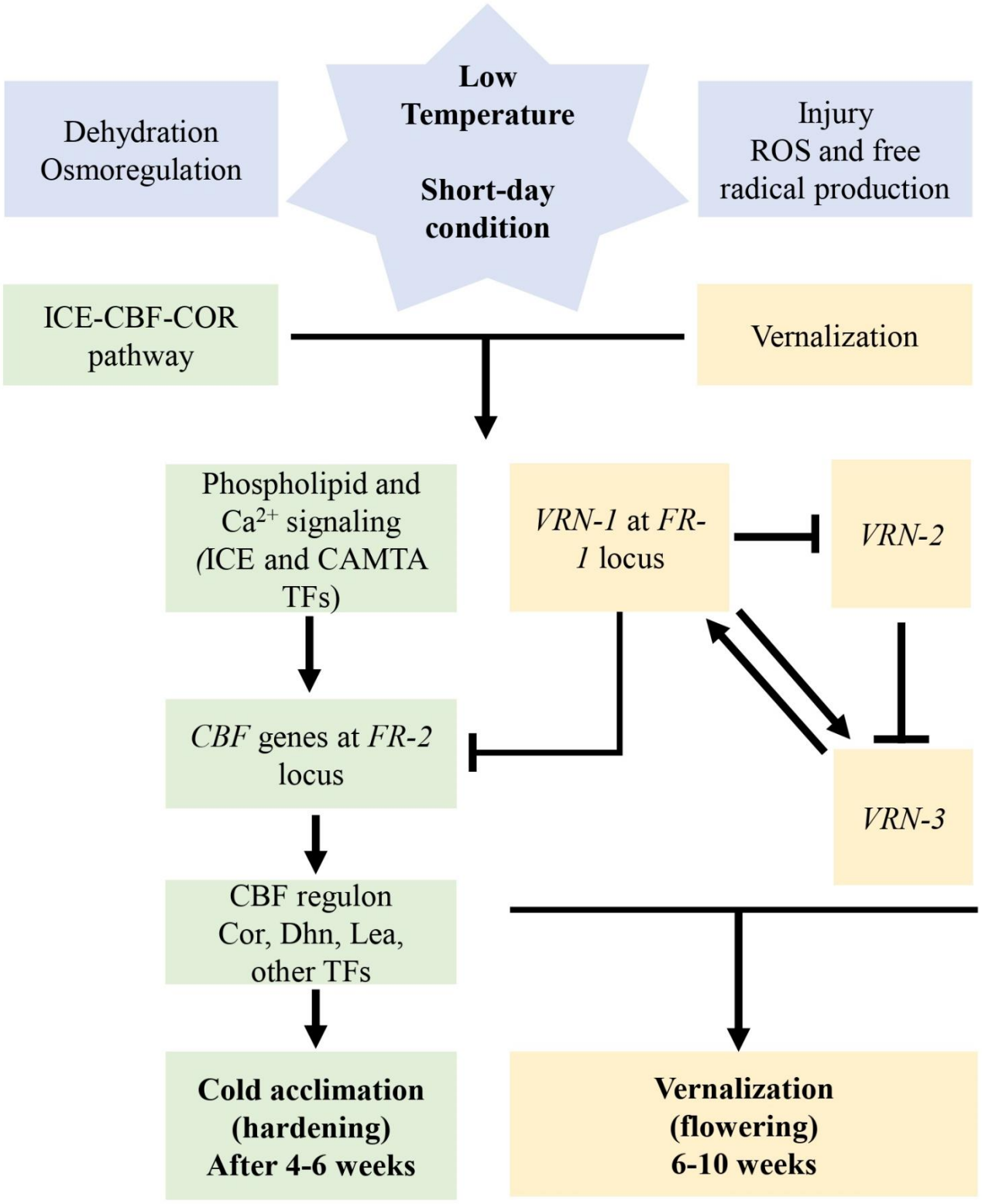

Figure 3. Schematic diagram connecting cold acclimation and vernalization as they are thought to occur in WH genotypes (taken as reference). Black arrows - gene induction; black lines with blunt ends - gene repression; green boxes - the ICE-CBF-COR pathway; yellow boxes - the vernalization requirement genes.

1.2.3. *FR-2* in Barley - A Synergistic Action of CNV and *HvCBF14*?

The efforts to identify the molecular mechanisms underlying *FR-2* in *Triticeae* crops were based on integration studies on structural and functional aspects of the locus (Figure 2). Several barley genotypes have been sequenced, and a pan-genome has been assembled (Jayakodi et al., 2020). Thanks to this data, *FR-H2* was studied in different frost susceptible and tolerant genotypes to evaluate the *CBFs* position in the cluster (Figure 4), the variability in the structure, in the *CBF* coding sequences and in the promoter regions (Galiba et al., 2013; Muehlbauer and Feuillet, 2009; Pasquariello et al., 2014; Stein and Muehlbauer, 2018; Visioni et al., 2013).

Initially, four *HvCBF* genes (*HvCBF3, HvCBF6, HvCBF9* and *HvCBF14*) have been selected as candidate genes due to the presence of homologs in other *Triticeae* already reported to be involved in cold resistance (Badawi et al., 2007; Fricano et al., 2009). Then, *HvCBF14* has emerged as the major candidate for the frost tolerance in barley in several works (Ahres et al., 2020; Fricano et al., 2009; Guerra et al., 2022; Mareri et al., 2020; Novák et al., 2016, 2017; Tondelli et al., 2011). Two SNP linked to *HvCBF14,* associated to frost tolerance were identified by Fricano and colleagues (Fricano et al., 2009) in an association analysis in a panel of European cultivars, landraces and *H. spontaneum* accessions. Later on, a correlation between frost tolerance and the same *HvCBF14* gene in spring haplotypes was demonstrated by Guerra et al. (Guerra et al., 2022) who investigated a panel 403 accessions with exome sequencing-based allele mining.

Structural variation is recognized as a common feature and evolutionary force of genomes, where copy number variations (CNV) and resulting gene dosage effects determined a number of trait/phenotypes in plants (Cook et al., 2012; Dolatabadian et al., 2017; Francia et al., 2015; Maron et al., 2013; Yin et al., 2021). One of the first clear association of CNV and phenotype was reported for the boron-toxicity tolerance in barley (Sutton et al., 2007). The first indication on the involvement of CNV at the *FR-H2* locus and frost tolerance in *Triticeae* was reported by Knox et al. (Knox et al., 2010). Two *HvCBF2* paralogs (*HvCBF2A* and *HvCBF2B*) and multiple copies of the *HvCBF2A-HvCBF4B* genomic segment were identified in the frost tolerant genotypes ‘Dicktoo’ and ‘Nure’. On the other hand, genomic clones of ‘Morex’ and ‘Tremois’ showed only single paralogs of *HvCBF4* and *HvCBF2.* Results on CNV were confirmed by sequencing the same physical region in the tolerant ‘Nure’ (Mareri et al., 2020) and susceptible ‘Morex’ (Pasquariello et al., 2014) genotypes, in successive, independent experiments. Francia et al. (Francia et al., 2016) and Rizza et al. (Rizza et al., 2016) confirmed that frost resistant

varieties of barley were characterized by a high number of copies for *HvCBF2* and *HvCBF4* genes and maintained two distinct *HvCBF2* paralogs (*HvCBF2A* and *HvCBF2B*). In summary, the influence of structural variation on determining the *FR-2* effect remains a long-standing conundrum and leave an open question: is the phenotype influenced by the expression of *HvCBF14* gene alone, or multiple copies of other *CBFs* are involved? Is the number of copies at *HvCBF2A–HvCBF4B* segment relevant for modulation of the *HvCBF14* expression level and the resulting phenotype?

In the previous cited work, Francia et al. (Francia et al., 2016) evaluated a panel of 41 genotypes using two phenotyping method (F_v/F_m and field survival) combined with RT-qPCR. The results showed a correlation between the number of copies of the *HvCBF2A–HvCBF4B* segment and frost tolerance. Winter and facultative genotypes showed higher number of copies and greater frost tolerance compared to spring ones.

The influence of the gene dosage (i.e., the pool of transcripts) of a specific *CBF* on the expression of other elements of ICE-CBF-COR pathway was tested/evaluated in two elegant experiments. The overexpression of *HvCBF2* in the spring susceptible cultivar 'Golden Promise' resulted in higher transcript levels of COR genes; *HvCOR14B* and *HvDHN5* already at warm temperature, raising then strongly at cold temperatures. Moreover higher transcription levels of *HvCBF12*, *HvCBF15*, and *HvCBF16* and greater frost tolerance were observed in overexpressed lines (Jeknić et al., 2014). According to Authors, *HvCBF2* may activate target genes at warm temperatures and transcript accumulation for some of them is greatly enhanced by cold temperatures.

The influence of CNV at *HvCBF2A-HvCBF4B* on the expression levels of *HvCBF12*, *HvCBF14*, and *HvCBF16* was investigated using the high frost tolerant variety 'Admire' and different descendent genotypes (namely, Missouri barley - MO B lines) by Dhillon and colleagues (Dhillon et al., 2017). MO B lines harboring a higher number of copies of *HvCBF2A-HvCBF4B* had higher expression levels of all three genes under normal growth conditions.

In addition, Mareri et al. (Mareri et al., 2020) investigated the expression levels and relationship between *HvCBF14* and CNV at *HvCBF2A*, *HvCBF4B* in barley winter, spring, and facultative cultivars with varying degrees of frost tolerance. Authors found higher expression levels for *HvCBF2A* and *HvCBF4B* in winter lines (with higher copy number) under warm-light conditions. Moreover, putative motifs recognized by other AP2-CBF were identified in promoters of *HvCBF2C*, *HvCBF12*, *HvCBF12C*, *HvCBF14*, *HvCBF15* and *HvCBF16*, suggesting an extensive interplay of *CBF* gene family in response

to external stimuli. This observation might indicate that CNV present in frost tolerant genotypes might play a role in accumulation of higher levels of transcripts under warm daylight conditions; a kind of "steady-state defense system" ready to react when winter cold arrives. In contrast, *HvCBF14* was inducted by cold in dark (6 °C) suggesting its role in the activation of the response to cold stimulus.

These observations, therefore, seem to outline a complex scenario in which the *CBFs* in the proximal-central portion of *FR-2* (phylogenetically classified in subgroup IV) are involved for the response to low temperatures through two; putatively complementary and interacting, mechanisms; "pre-cold priming" and "cold induction." The former represents a kind of constitutive CBF expression level that fluctuates however, driven by circadian cycle and light quality and/or intensity. In case of frost resistant genotypes this may lead to accumulation of higher levels of transcripts, due to their higher CNV of *HvCBF2A-HvCBF4B* (Dhillon et al., 2017; Knox et al., 2010; Mareri et al., 2020) and as a result this may be associated with higher expression levels after the cold stimulus, of other *CBFs* and then, effector genes (Jeknić et al., 2014). Otherwise, some post-translational mechanisms could be involved that activate the proteins accumulated at "pre-cold priming" only after the cold stimulus (Kopeć et al., 2022).

The presence of a basal, light-independent, cold-responsive activation of the HvCBF–COR14B pathway was proposed by Vashegyi et al. 2013 (Vashegyi et al., 2013). The expression of *HvCBF14* was shown to be induced by temperature shift and blue light (Novák et al., 2016), while a significant response to cold, light intensity and far-red supplementation for *HvCBF14* and consequently *HvCOR14B* gene was reported(Ahres et al., 2020). The second mechanism "cold induction" is thus based on the major contribution of *HvCBF14* (exhibiting single nucleotide variations) that is up-regulated with the cold stimulus and may regulate effector genes or other *CBF* genes, leading to cold acclimation via activation of ICE-CBF-COR pathway (Novák et al., 2017, 2016). Whether this mechanisms can be effectively exploited for the breeding of superior genotypes remains to be proved and represents an interesting starting point for applied research. (Novák et al., 2017, 2016).

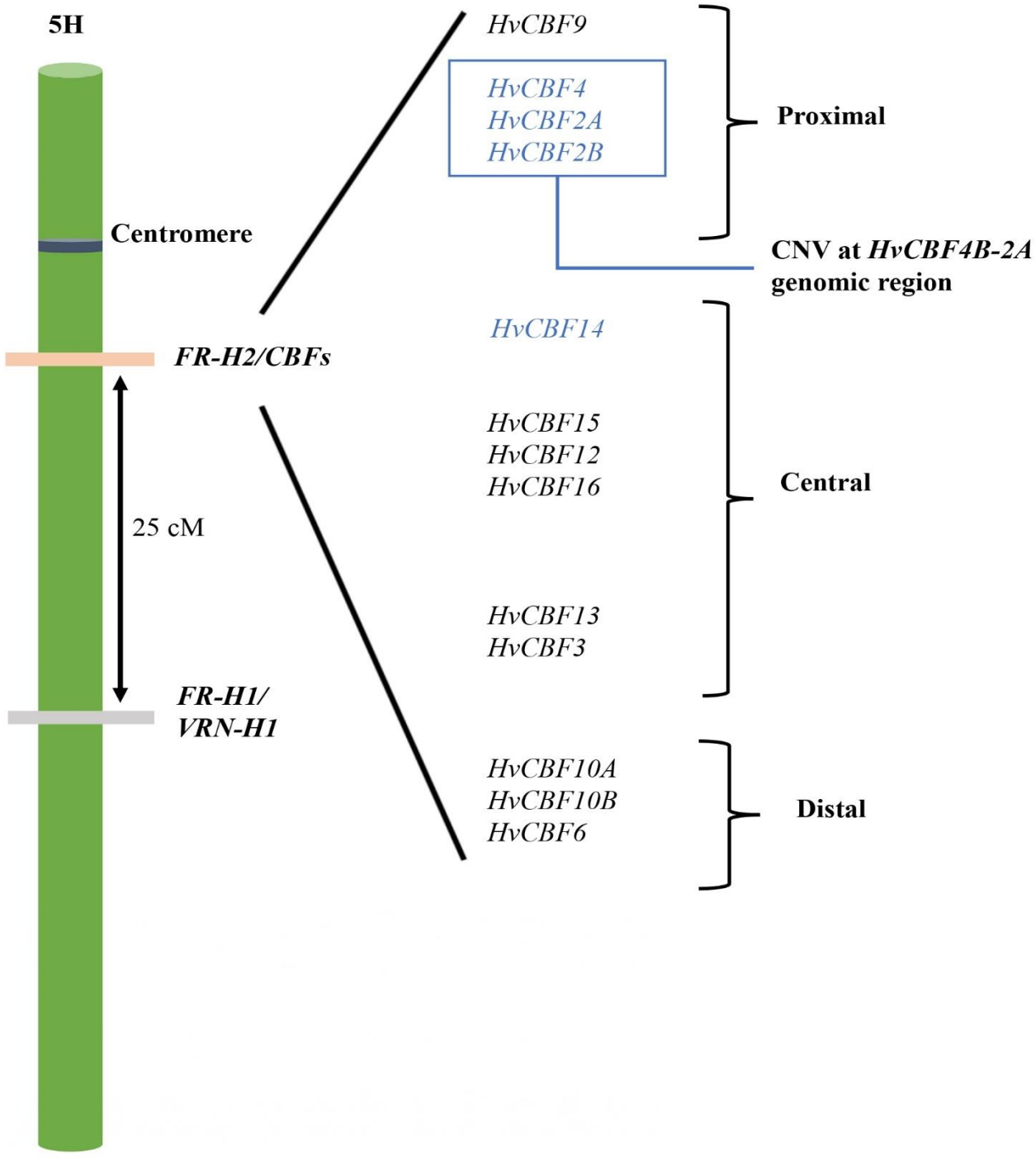

Figure 4. Model for *CBF* gene cluster in barley. *Fr-H1* was mapped about 25-30 cM apart from *Fr-H2. Fr-H2* locus is divided into three different portions: proximal (*HvCBF 2*, *4* and *9*), central (*HvCBF 3*, *12*, *13*, *14*, *15*, *16*) and distal part (*HvCBF 6* and *10*). In blue, candidate *CBF* genes involvement in response to cold stress; *HvCBF14* and the *HvCBF2A–CBF4B* segment with different the copy number variations (modified from Francia et al., 2007).

1.2.4. *FR-2* in Wheats - *CBF* Cluster Ploidy

While barley has a diploid genome (2n=2x=14, HH) of 5 giga base pairs (Gbp) (Ogihara et al., 2015), tetraploid durum wheat (2n=4x=28, AABB) has 12 Gbp (Maccaferri et al., 2019) and hexaploid wheat (2n=6x=42, AABBDD) approximately 17 Gbp (Smith and Flavell, 1975). Thereby, *FR-2* organization in wheat is more complex compared to barley due to the contribution of one/multiple homoeologous chromosome regions redundancy caused by the ploidy level (Bolouri et al., 2023; Li et al., 2023; Pearce et al., 2013). Wheat exhibits a high variability in frost tolerance trait, given that hexaploid wheat genotypes (AABBDD) exhibits greater frost tolerance than diploid (AA) and tetraploid genotypes (AABB) (Fowler et al., 1977; Limin and Fowler, 1981).

First works on *CBF/FR-2* in wheat were carried out in mapping populations of einkorn diploid wheat (*Triticum monococcum L*) which is the ancestor of A genome in hexaploid wheat and is considered a practical model for the functional genetics of wheat (Båga et al., 2006; Knox et al., 2008; Miller et al., 2006; Saripalli et al., 2023; Tóth et al., 2003; Vágújfalvi et al., 2005, 2003). First expression studies showed the association of *CBF* genes at the *FR-A^{m}2* with expression of COR genes and frost tolerance (Vágújfalvi et al., 2005, 2003).

TmCBF12, *TmCBF14* and *TmCBF15* and *TmCBF16* (central cluster) expression levels were significantly associated with frost tolerance measured as regrowth capacity after stress. Moreover, a high-density mapping study confirmed that the *TmCBF12*, *TmCBF14* and *TmCBF15* were the candidate for the observed differences (Knox et al., 2008).

Thanks to the works carried out on *T. monococcum*, number and position of *CBF* genes in bread wheat were identified in different works. While in barley a CNV has never been associated with central cluster at *FR-H2* (see above), in diploid and polyploid wheat, a lower copy number of *CBF14* in the B genome compared to the A and D genomes was reported (Dhillon and Stockinger, 2013). Consistent with this finding, Zhu et al. (Zhu et al., 2014) reported that differences in *TaCBF-A14* copy numbers, separated susceptible and resistant haplotypes into two distinct panels of winter and spring. Moreover, CNV at *CBF14* in the winter panel was associated with higher expression levels in tolerant haplotypes. *TaCBF-A12* and *TaCBF-A15* were characterized by two insertion/deletions and ten single nucleotide polymorphisms, and in addition CNV was reported. The importance of CNV at *FR-A2* was confirmed by Sieber and colleagues (Sieber et al., 2016) in durum wheat where approximately 90% of the genotypic variance at *FR-A2* was explained by CNV at *CBF-A14*. Three large deletions which eliminated 6, 9 and

11 *TaCBF* genes, respectively, were identified in *FR-B2* locus in tetraploid and hexaploid wheat (Pearce et al., 2013). These deletions were mainly located in the central part of the cluster which encompass *TaCBF-B12 TaCBF-B14* and *TaCBF-B15* and were observed in cultivated spring wheat, either tetra or hexaploid, associated with lower levels of frost tolerance compared to the wild-type *FR-B2* locus without deletion (Pearce et al., 2013). Würschum and colleagues (Würschum et al., 2017) evaluated different levels of phenotypic variance in a panel of f 407 diverse European winter wheats (Würschum et al., 2017) confirming the importance of CNV of *TaCBF-A14*. New highly conserved amino acid substitutions in TaCBF-A3, TaCBF-A15, VRN3 and PPD1 genes were found associated with frost tolerance in wheat (*Triticum aestivum* L.) revealed (Babben et al., 2018).

TaCBF14 and *TaCBF15* were associated with increased frost tolerance in doubled haploid (DH) mapping populations of 'Norstar' x 'Winter Manitou' and 'Norstar' x 'Cappelle-Desprez' (all WH genotypes) (Båga et al., 2006). Higher levels of *TaCBF14* induced by temperature shift and blue light were reported in winter wheat 'Cheyenne' (Novák et al., 2016).

Recent studies expanded the investigation of ICE-CBF-COR interconnection with other environmental stimuli with high throughput functional analysis (Guo et al., 2019; Li et al., 2023; Pan et al., 2022; Singh et al., 2022; Wang et al., 2023; Zheng et al., 2020). Guo et al. (Guo et al., 2019) carried out RNAseq and qPCR analysis in wheat tissues under different stress conditions observing the expression of 53 genes belonging to the ICE-CBF-COR signaling cascade that revealed tissue-specific expression patterns of the *ICE*, *CBF* and *COR* genes under different stress conditions. Six genes related to ICE-CBF-COR pathway (T*aCBF11a*, *TaCBF16b*, *TaICE1a*, *TaICE1d*, *TaCOR5a* and *TaCOR6d.1*) were induced by all treatments (drought, heat, drought, cold). Three genes, two *CBFs* and one *COR* (*TaCBF1b*, *TaCBF4a*, *TaCOR3b)*, were induced specifically by cold (Guo et al., 2019).

Zheng et al. (Zheng et al., 2020) carried an isoform sequencing experiment at four leaf stage, under frost stress (at -6 °C) and expression levels of *TaCBF8a* and *TaCBF14a* resulted decreased, while *TaCBF6a*, *TaCBF9a*, *TaCBF10a*, *TaCBF13a*, and *TaCBF15a* expression levels increased. Recently, Wang et al. (Wang et al., 2023) performed a transcriptome analysis during vernalization (4 °C) time-course with sampling from one to six weeks. Six *CBF* genes of the III subgroup, were highly expressed exclusively before vernalization ("steady state" at 22 °C), while 10 *CBFs*; mainly from the IV subgroup were not expressed before, and were highly induced by vernalization, reaching the highest level of expression after three weeks, and decreasing after five/six weeks of treatment. Two different homologs of the MYC-like

bHLH transcription factor *ICE* were identified in wheat as *TaICE41* and *TaICE87* (Badawi et al., 2008), and their overexpression in Arabidopsis enhanced frost tolerance after hardening. The recent availability of the wheat genome allowed to locate three *TaICE1* on the long arm of homoeologous chromosome group 3; these genes were shown to be induced by drought and cold treatment (Guo et al., 2019). In addition, Wang et al. (Wang et al., 2023) reported that *TaICE41* was expressed at extremely high levels after five weeks of vernalization.

1.2.5. *FR-2* in Rye - Evidence of *ICE1* Involvement in the Tolerance

Compared to other *Triticeae* crops, rye is uniquely tolerant to biotic and abiotic stresses, showing high yield potential under marginal condition (Alptekin et al., 2017; Jung and Seo, 2019; Mago et al., 2005) however, received little attention in terms of breeding efforts and genomic research due to its limited distribution worldwide. Likewise barley, rye has a diploid genome (2n=2x=14, RR), however, it has not become a reference crop for genomic analysis in *Triticeae* tribe due to its elevated level of allogamy and the fact that the first chromosome-scale assembly of its large 7.9 Gbp genome was released only recently, in 2021 (Rabanus-Wallace et al., 2021) showing 92% of repetitive elements (Bartoš et al., 2008; Flavell et al., 1974; Jung and Seo, 2019; G. Li et al., 2021; Martis et al., 2013).

Investigation of rye genome evolution and chromosome synteny (G. Li et al., 2021) revealed, as expected, that the chromosome 5R harboring *FR-2* and *FR-1* loci is entirely collinear with wheat homoeologous chromosome group 5. Initially, eleven *ScCBF* genes were isolated in a winter rye genome and nine of them were mapped on the chromosome 5R with a cluster organization *(FR-R2)* (Campoli et al., 2009). Subsequently, Jung and Seo (Jung and Seo, 2019) identified 12 new *CBF* genes and five new *CBF* gene alleles. The genome assembly (Rabanus-Wallace et al., 2021) reported CNV for 4 member of CBF Group IV between tolerant and resistant varieties.

Concerning the structure of the locus, *FR-R2* haplotyped variation has been associated with different frost tolerance levels in different rye genotypes (Li et al., 2011; Rabanus-Wallace et al., 2021). In a recent work aimed at evaluation of different haplotypes of *FR-R2*, 259 marker-trait-associations (MTAs; $p < 0.01$) were found in 96 genotypes (Båga et al., 2022). The ten most significant markers associated with winter frost survival (WFS) corresponded to nine strong candidates, including *ICE1*. Moreover, three MTA identified at lower significance level, matched *CBF* genes at *FR-2R*, namely *CBFIIId-19, CBFIVa-2.2*, and *CBFIIIa-6.2*. What is interesting, *ICE1*, showed 97% sequence identity to orthologous *TaICE41* of hexaploidy wheat, and *CBFIIId-19, CBFIVa-2.2* are putatively orthologous to hexaploid wheat genes

reported to be induced by vernalization (Pan et al., 2022; Saripalli et al., 2023) (see above). For ICE1, amino acid variation within the DNA binding bHLH region and/or start of zipper region resulted associated with traits, such as WFS and low temperature tolerance. Authors hypothesized that *ICE1* gene identified, could be allelic with *ICE2* gene in rye, whose allelic variation had been already reported to be associated with variation for winter hardiness and frost tolerance, and that different *ICE* alleles could be important for frost tolerance in rye (Li et al., 2011). Thus, specific *ICE* alleles, for example coding for ICE1 proteins with reduced affinity for the MYC binding sites in the promoters of *CBF* and *COR* genes could be important for winter hardiness (Båga et al., 2022).

1.2.6. New Frontiers for *CBF* Genes? *CBF* Genes in the Drought Stress Adaptative Response

CBF genes are members of a large protein family of the C-repeat binding factor/Dehydration responsive element-binding 1 (*CBF/DREB1*), known to be involved in the growth and development processes and responses to different environmental stress (cold, heat, drought, salt, etc.) (Wu et al., 2022). *CBF* genes could thus have in *Triticeae* a role in a cross-talk between the cold and drought response pathways as already reported for Arabidopsis (Haake et al., 2002; Li et al., 2020).

The *CBF/DREB1* regulon modify the plant metabolism in conditions of water deficiency and their activation might be triggered also by drought conditions in seedling phase (Guo et al., 2019; Hrmova and Hussain, 2021). In several drought responsive genes, such as *AtRD29A* (responsive to desiccation), *HvDHN1* (dehydrin) or *AtCOR6.6*, a DRE/CRT motif is present in the promoter regions. When drought conditions occur, the plant reduces its water uptake by closing the stomata, which also reduces their CO_2 uptake that results in the reduction of the photosynthesis and physiological activities. To cope with drought stress, plant activates several morphological and physiological modifications to conserve water and reduce its loss. The molecular response to drought follows a similar pathway to cold acclimation due to the same trigger of water scarcity, which activates both the responses. As already summarized (see, Figure 1), water deficit activates, like low temperature stress, two different signaling pathways; ABA-dependent and ABA-independent (Kashyap and Deswal, 2019). The interaction between *CBF* genes in hormone-mediated acid abscisic (ABA) pathways has been reported (Muhammad Aslam et al., 2022; Tuteja, 2007). In *A. thaliana* the ABA-independent pathway is regulated by *AtCBF4* that increases the production of a class of small, highly-expressed, and stress-inducible proteins called late embryogenesis abundant (LEA) protecting the cellular membranes and the cytoskeleton from desiccation (Haake et al.,

2002; Kaur and Asthir, 2017). Interestingly, it has been showed in *A. thaliana,* that *ABA-responsive* genes contain in the promoter regions both the ABA response cis-element ABRE/ABF and the CRT/DRE motif (Uno et al., 2000). Overall, drought stress in barley and wheat can have a significant negative impact on plant growth, yield, and grain quality, however, plants have evolved mechanisms to cope with water scarcity and to survive in dry environments (Salehi-Lisar and Bakhshayeshan-Agdam, 2016). Nevertheless, which *CBF* genes and which pathways are activated have not been determined yet (Choi et al., 1999; Gierczik et al., 2017; Wu et al., 2022). In a study of *A. thaliana* transgenic lines, the overexpression of *AtCBF1* and *AtCBF3* genes resulted in an increasing of drought tolerance (Xu et al., 2014). A review on a conservative role of *CBF* genes throughout *Poaceae* family reported rice *OsDREB1A* localized in the cluster *OsDREB1H*, syntenic with the *FR-2* locus on the chromosome 5 of *Triticeae* involved in the chilling tolerance (Tondelli et al., 2011). However, few examples of studies of the role of *CBF* genes in drought tolerance are available for barley and wheat.

A common phenotypic response observed in transgenic lines overexpressing *CBF* genes in different crops can be identified as an increased tolerance to frost and/or drought and modified growth and development as originally reported for Arabidopsis (Wu et al., 2022; Xu et al., 2014). Overexpression of two *CBF/DREBs* (*TaDREB3* and *TaCBF5L*) in wheat and barley was reported to lead to an increase in drought and frost tolerance of transgenic barley. Moreover, in transgenic wheat, *TaCBF5L* gene significantly increased the grain yield under severe drought during flowering (Yang et al., 2020). Javadi and colleagues mined available GeneChip microarray data (Javadi et al., 2021) in order to detect key genes involved in drought tolerance in barley, and identified hub genes, from AP2 and NAC families, that might be among key TFs that regulate drought-stress response in barley. What is interesting *HvCBF6* (distal cluster of *FR-H2*) was included among the hub genes. Also in rye, the PEG treatment (drought condition simulation) revealed that there is a specific type of response to stress among *ScCBF* genes; most of them were highly responsive to cold stress, whereas *ScCBF2* and *ScCBF7b* were induced by water deprivation and were almost insensitive to low temperature (Jung and Seo, 2019). Guo and colleagues (Guo et al., 2019) characterized the expression profile of the *ICE-CBF-COR* pathway in different wheat tissues under different stress conditions. Authors showed that *TaCBF11a, TaCBF16b, TaICE1a, TaICE1d, TaCOR5a* and *TaCOR6d.1* were induced by drought and the induction level was higher in tolerant genotypes (Singh et al., 2022).

The overlapping of cold/frost and drought conditions is a relatively an unexpected new form of combination of abiotic stress and it usually happens during the late autumn, after sowing, when winter

genotypes are in seedling phase. The drought stress in seedling phase induce root architecture modification that might act as a constitutive resistance mechanisms, useful when the stress re-occur in other phenological phases (Canè et al., 2014; Manschadi et al., 2008; Sallam et al., 2019). As observed in numerous studies in the past years, *CBFs* overexpression in model plant Arabidopsis, enhances abiotic stress tolerance but, on the other hand, reduces growth. *CBF* genes are known to interact with plant hormones (Kashyap and Deswal, 2019) and the current model of CBF-GA (gibberellic acid) interplay proposes that overexpression of *CBFs*, either via cold induction or by transgenic means, stimulates the accumulation of DELLAs. Those growth-repressing proteins act downstream in the GA signaling pathway leading to stunted growth. As far as the underlying molecular mechanism is regarded, in warm temperature, DELLAs interact with JAZs to prevent JAZs binding to *ICE1,* leading to its inactivation. In cold temperature, *ICE1* is modified to gain the function for activation of *CBF* transcription (Zhou et al., 2011). Understanding the relationship between *CBF* genes, GA and DELLA proteins might help to get an overall picture of the role of *CBFs* in plant physiology. One of the new frontiers that regard *CBF* genes is to evaluate their contribution in the tillering phase, crucial in growth and development of wheat and barley, as it directly influences the potential yield and overall productivity of cereal crops (Ye et al., 2019). Moreover, in this phase, winter cereals reach the maximum of their stress tolerance (Galiba and Tóth, 2017; Hyles et al., 2020; Pecchioni et al., 2014). The main actors of tillering formation are gibberellic and abscisic acids, moreover the role of *VRN-1* and *VRN-2* and the photoperiod response gene *PPD-1* has been described (Hussien et al., 2014; Riaz et al., 2022; Shang et al., 2021). All these components interact with *CBF* genes; however, mechanisms of interaction are still not clear (Figure 5).

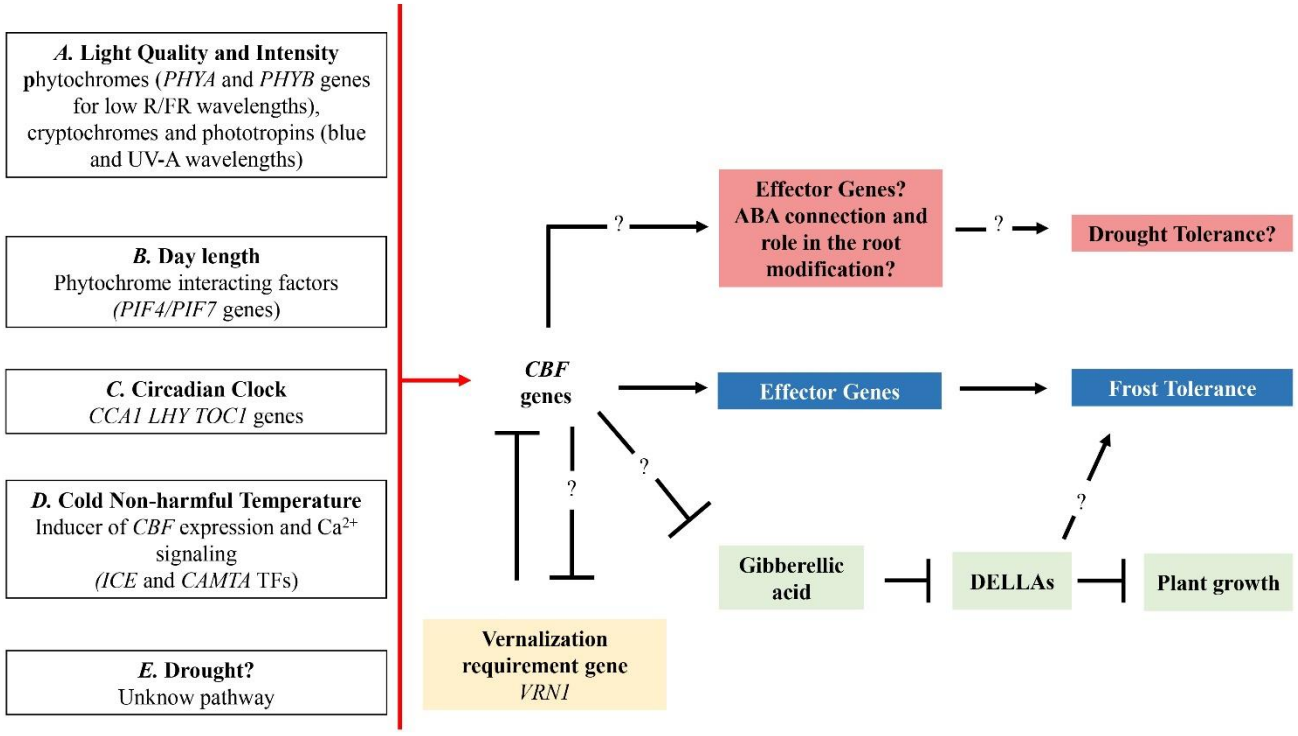

Figure 5. Outline of the integration of environmental stimuli that putatively influence the action of CBF genes. White boxes - environmental stimuli that influence (induce or repress) *CBF* genes; colored boxes - the cascade or putative cascade pathways activated by *CBF* genes; black arrows - induction or up-regulation of gene expression; black lines with blunt ends - repression of gene expression; ? - hypotheses still not proven in *Triticeae* crops.

1.3. Prospects for *Triticeae* Improvement Against Abiotic Stresses

Crop improvement is pursued through different approaches that range from traditional breeding to marker-assisted and genomic selection. Molecular markers facilitated identification of superior plant materials; however, they still present limitations for complex quantitative traits and in case of reduced genetic variability. Genomic selection (GS) uses high-throughput genotyping and phenotyping, and can be further integrated with the so-called speed-breeding methods and modeling (Cha et al., 2022), leading to an approach called integrated genomic selection (IGS) (Kaur et al., 2021; Sinha et al., 2023). Doubled haploid (DH) breeding and single seed descent (SSD) method can be implemented to reduce the length of breeding cycles and fasten genetic advances (Forster et al., 2000; Watson et al., 2018). Speed breeding has also been proposed in combination with marker-assisted selection (MAS) for efficient introgression/combination of desired traits in superior genotypes (for a recent review, see e.g. (Leng et al., 2017)). The efficacy of breeding programs can take advantage of multi-dimensional omics; existing germplasm collections can be investigated with the information derived from genome sequences and

connected to high-throughput phenotyping data. Incorporating, through climate change models, environmental information sourced from past weather databases or forecasted into the analysis of crop models, can yield valuable metadata regarding phenology (Sinha et al., 2023).

Climate change effects that we have been facing in the last decades are increasing the demand for resilient crops, that would harbor traits required to tolerate multiple stresses in combination with optimum yield and efficient biomass partitioning (Bhatta et al., 2021; Sinha et al., 2023). The availability of different -omics tools (e.g. whole genome sequences, transcriptomes, molecular markers, and linkage maps) could be used to increase and incorporate into new resistant varieties the necessary phenotypic variability (Kumar et al., 2023).

Extensive literature sources regarding the complicated biological pathways involved in abiotic stress tolerance of *Triticeae* exist. Breeding of wheat and barley is based on their strong autogamous mating system (outcrossing rate are less than 2% in most situations). For this reason, most new varieties are still obtained via conventional strategies (e.g. inter-varietal crosses, backcrosses, and multi-parent schemes); genotypes harboring the desired traits are artificially crossed and selection of desirable traits combinations is applied to their progeny. In the case of MAS, functional/diagnostic markers are ideal, in particular when derived from major genes at *VRN-1/FR-1* and *FR-2*. Haplotypes based on multiple markers (mainly SNPs) with strong linkage disequilibrium are more informative and thus were proposed to be applied to target the traits of interest (Bevan et al., 2017), and examples of genome-wide association-based haplotypes were reported in barley (Lorenz et al., 2010) and wheat (Voss-Fels et al., 2018). The contribution of GS (based on prediction of genomic estimated breeding values of individuals modeled from a full-characterized training population) has been also demonstrated in major crops (Crossa et al., 2017). As the inclusion of functional markers linked to determinant genes can greatly improve prediction accuracy (Lozada et al., 2018; Sun et al., 2023), molecular markers associated to variation of *CBF* genes could become a useful tool to be integrated in the barley and wheat programs (Hassan et al., 2021; Stockinger, 2021; Zhou et al., 2011). Genotypes harboring higher copy numbers at relevant genomic regions within *FR-2* (i.e. *HvCBF2A-HvCBF4B* or *TaCBF14* and *TaCBF15*), in combination with resistant alleles of other *CBF*s (e.g. *HvCBF14* in barley), should be used to create new resistant *Triticeae* cultivars (Båga et al., 2006; Soleimani et al., 2005). For example, for the development of new, winter malting barley lines, Stockinger (Stockinger, 2021) suggested to advance a “breeder-friendly” molecular marker approach capable to speed up the quantification of the CNV in order to integrate it in modern breeding programs (Dhillon et al., 2017). Nowadays most genotypes used for

malting are spring varieties, however due to the climate change, in the future, the actual areas of their cultivation, such as US, Canada, UK, Denmark, Germany, Poland, and Hungary might see the introduction of new winter varieties due to the increasing temperature in winter. In wheats the contribution of homoeologous *FR-2* loci could also be taken into account; a KASP (Kompetitive allele-specific PCR) assay was developed for an SNP at *FR-B2* which would be included in a set of markers used for breeding and research (Eagles et al., 2018).

Due to minor worldwide distribution of rye and issues for genetic analyses (given by its cross-pollinator nature with a genetic self-incompatibility mechanism), genomic tools in this species have been developed only recently (Miedaner et al., 2019). Rye genetic resources provide a valuable source of new alleles for the improvement of the species. Although marker-assisted selection was useful for monogenic traits, such as fertility restoration or some disease resistance, only the recent increase of genomic resources has boosted hybrid rye breeding (Miedaner et al., 2019; Wilde and Miedaner, 2021) giving the possibility to operate on larger scale than MAS. Rye has strong potential for adaptation to a changing climate and thus, as far as breeding for frost resistance is regarded, genetic resources should harbor promising alleles for the improvement of this trait in winter elite lines. Whole-genome prediction models assigning a high weight to the *FR–R2* locus allow for increasing the selection intensity by genome-based pre-selection of promising candidates (Erath et al., 2017).

The above-described approaches proved their efficiency and contributed to developing abiotic stress tolerance in *Triticeae*, however the process may take years, not in line with demands of evolving requirements of farmers, growers, industry and commerce (Kumar et al., 2023). Therefore, efficient technologies with fast impacts are required to face those issues and challenges (Driedonks et al., 2016). Genome editing enables precise changes in an organism's DNA by introducing targeted mutation, insertion/deletion, and specific sequence alterations. CRISPR/Cas9 (Jiang et al., 2013) is considered to be the most successful genome editing system for a wide range of organisms (Chen et al., 2019). The genes involved in regulatory networks, signal transduction and metabolite production may be targeted via CRISPR/Cas9 technologies to develop stress-tolerant crops (Jain, 2015). However, there have been only a few examples of CRISPR-based genome editing approaches in plants for improvement of abiotic stress tolerance, especially those regarding cold/drought resistance and involving genes from the ICE-CBF-COR pathway. Mutants were generated by CRISPR/Cas9 to characterize the *UGT79B2* and *UGT79B3* genes of the UDP-glucosyltransferase (UGTs) of Arabidopsis showing that *CBF1* regulates *UGT79B2/B3* and improves stress resistance (Li et al., 2017). *CBF1* was shown moreover (using

CRISPR/Cas9-based *cbf1* mutants), to protect the tomato plant from cold/chilling damage and decrease electrolyte leakage (Wang et al., 2017). To augment the plant's resistance to cold, genome editing was employed to target a few of the transcription factors in rice, e.g. *OsMYB30* that regulates the amylase gene and negatively affects cold tolerance (Lv et al., 2020). The first CRISPR/ Cas9-based gene editing attempt was conducted in wheat protoplasts targeting *TaDREB2* and *TaERF3* and the results support the positive regulation of both genes under drought stress and the potentiality of the method (Kim et al., 2018). However, it should be taken into consideration that editing single/multiple *CBF*s could not result in a desired phenotype. In fact elegant CRISPR/ Cas9 experiment conducted in Arabidopsis clarified the involvement of the *CBF* cluster in the response to multiple abiotic stresses and in plant development (Zhao et al., 2016). Single, double, and triple mutants in either *AtCBF1*, *AtCBF2* and *AtCBF3* were phenotyped for tolerance to chilling/freezing and to other abiotic stresses, and in development. Transcriptome analysis in wild type and mutants after cold induction (4 °C) revealed complexity of the regulon with the underlying redundancy and interplay of the genes connected to abiotic tolerance and growth.

1.4. Conclusions

Aiming to improve crops by combining agronomic practices and phenotyping with genetics and biotechnological tools (i.e., molecular markers, QTLs, and haplotype mapping), we reported the latest updates regarding the mechanism involved in abiotic stress response that has the *CBF* genes as the main hub. In this review, the potential role of the ICE-CBF-COR pathway in frost tolerance and the putative involvement in drought tolerance of *Triticeae* were discussed aiming at increased knowledge on crop growth, stress responses, and tolerance mechanisms. Understanding the structural organization and the expression regulation of the *CBF* cluster harbored by the homoeologous chromosome group 5 entails significant potential for genetic improvement of cereals within the *Triticeae* tribe. Retrieving, evaluation, and synthesis of pertinent literature outlined a complex scenario in which the *CBFs* in the proximal-central portion of *FR-2* (phylogenetically classified in subgroup IV) are mainly involved in tolerance to low temperatures. In barley, taken as a diploid model, a mechanism that integrates the "steady state" level of some element(s) under CNV (e.g. *HvCBF2A* and *HvCBF4B*) with a strong induction of other gene(s), among which *HvCBF14* exhibiting single nucleotide variations, seems to have an important role. However, the response/phenotype appears to be governed by two complementary and interacting modes of action, that we called; "pre-cold priming" and "cold induced". The former represents a kind of constitutive CBF expression level that, however, fluctuates driven by circadian cycle and light

quality/intensity. In case of frost resistant genotypes, harboring higher copies of the *HvCBF2A-HvCBF4B* segment may lead to accumulation of higher levels of transcripts at "steady state" ready to be translated into proteins. Otherwise, some post-translational mechanisms could be involved; CBF proteins accumulated at "pre-cold priming" are activated only after the cold stimulus. The second mode of action is mainly based on the contribution of *HvCBF14* that is strongly up regulated by cold, and the final scope of the whole mechanism is a fine-tuning of the CBF regulon that leads to cold acclimation. Whether this molecular regulation can be effectively exploited for breeding superior genotypes, remains to be proved and represents an interesting starting point for applied research. Concerning wheat, the organization of the *FR-2* seems more complex than in barley, due to polyploidy, which appears to confer species-specific levels of resistance. Moreover, specific *CBF*s such as *TaCBF14* and *TaCBF15,* carrying variations in gene copy numbers, have been associated with frost tolerance. Recent studies focused on ICE-CBF-COR pathway elucidating an important involvement of the ICE transcription factor that was linked to winter hardiness and frost tolerance in rye. As far as the Authors know, no such association has been never reported neither for wheat nor barley and could therefore explain the greater tolerance of (diploid) rye, even if *FR-R2* hosts a similar number of *CBFs* (per haploid genome). The demonstrated overlapping nature of the adaptive responses to cold and drought, however, appears to be based on the involvement of different CBF/DREB1 factors. While response to cold seems to be driven by subgroup IV, we report works suggesting the potential role of *CBF* members of subgroup III (mapping in the distal region of the *FR-2* cluster) in drought resistance. In conclusion, given the redundant involvement of *CBFs*, an integrated approach based on (i) low-cost markers capable of detecting CNV of key genomic segments, (ii) introduction of new allelic variants (generated by CRISPR/Cas), (iii) haplotype selection, and (iv) accurate phenotype selection techniques are needed.

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Chapter 2

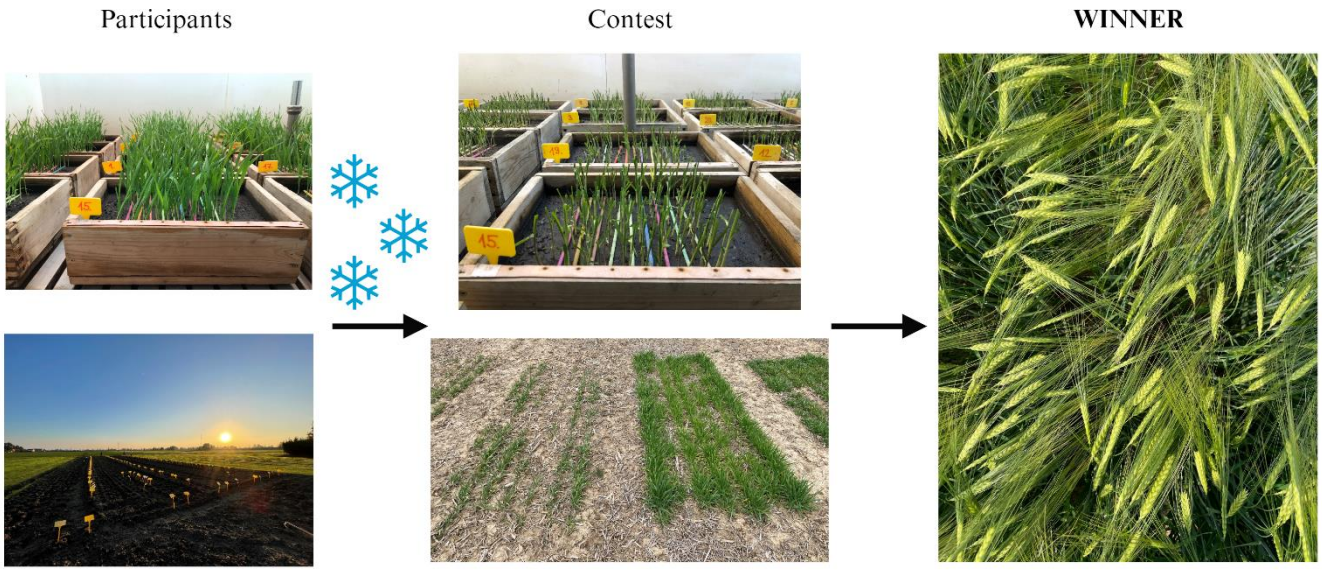
Aim of the Thesis

Since the discovery of the two QTLs (*FR-H1* and *FR-H2*) and their structural variations involved in the acquisition of frost tolerance in barley (*Hordeum vulgare* L.), important efforts were made to uncover the mechanisms underlying their functions. The present thesis aims to further investigate the role of *FR-H1* and *FR-H2* in the cold acclimation and frost stress by applying a multidisciplinary approach ranging from phenotyping in different environment conditions to single gene expression profile using novel genotypes such as QTL-Near Isogenic Lines (NILs) carrying different allele combination at *FR-H1* and *FR-H2* in different background.

- In **Chapter 3** entitled "**Phenotyping a set of QTL-NILs Carrying Alternative Alleles at *FR-H1* and *FR-H2*: a Step Towards Mendelizing the Effect of Frost Tolerance Genes in Barley**", a phenotyping approach to evaluate the frost tolerance was described for Nure (winter, frost resistant), Tremois (spring, frost susceptible) and four QTL-NILs selected by the cross between parental lines (Nure and Tremois) and double haploid (DH derived by Nure x Tremois cross NT) lines. The combined application of growing chamber freezing tests and winter survival rate in open field trials allowed generating information regarding the role of the *FR-H1* and *FR-H2* loci in determining the phenotype. Two different growing conditions were applied to analyze the cold acclimation, and two locations were selected representing different winter conditions. Such comparison highlighted a role of *FR-H2* to increase the frost resistance in susceptible background.
- In **Chapter 4** entitled "**Gene Expression Analysis of Barley FT Candidates, Dissecting the Effect of Low Temperature and Light Stimuli During Acclimation**", a quantitative real time (qRT-PCR) approach was used to analyze the single gene expression profile of four *HvCBF* (*2*, *4*, *9* and *14*), *VRN-H1* and two *COR* genes (*HvCOR14B* and *HvDHN5*) to dissect the cold stimuli. Results herein obtained globally confirmed the pivotal role of *CBFs* in the cold acclimation and highlighted putative effect of the winter background in determining the expression of *HvCBF* and *COR* genes. An influence by the light was highlighted influencing the gene expression.
- Finally, obtained results are summarized and discussed in **Chapter 5**.

Chapter 3

Phenotyping a Set of QTL-NILs Carrying Alternative Alleles at *FR-H1* and *FR-H2*: a Step Towards Mendelizing the Effect of Frost Tolerance Genes in Barley



3.1. Introduction

Phenotyping small-grained cereals for frost tolerance at vegetative stage aims to evaluate plant ability to acclimate and cope with the stress after exposure to natural or artificial freezing events. In controlled conditions such as phytotron, growth chambers or greenhouse, F_v/F_m parameter (a measure of a plant's capacity to convert light energy into chemical energy), re-growth test, the electrolyte leakage (related to plasma membrane cation conductance) and high throughput image analysis are the most common methods (Qiu et al., 2022). On the other hand, to evaluate the FT in open field trials involving numerous genotypes/replicates, the winter survival rate and visual assessment are the most used methods. Freezing injury by LT_{50} is a method used either in control or open field condition (Prášil et al., 2007; Rizza et al., 2011; Skinner and Garland-Campbell, 2008). Depending on the nature of the study, these methods can be combined to obtain the best possible phenotypic evaluation.

Chlorophyll fluorescence measurement is a non-destructive method which evaluates the change in photosystem II (PSII) photochemistry inducted by several stresses (Maxwell and Johnson, 2021). To evaluate frost damage in barley, chlorophyll fluorescence is a rapid and convenient method (Baker and Rosenqvist, 2004) and it is widely used because it can handle a large numbers of genotypes (Rizza et al., 2011). Measurements of F_v/F_m furnish an indirect assessment of the frost damage as an indicator of the breakdown of cellular compartmentalization due to membrane damage through the subsequent decline in maximum quantum yield of photosystem II photochemistry (Steponkus and Webb, 1992). Several works reported the use of F_v/F_m value to evaluate the frost response in populations with a large number of individuals (Mareri et al., 2020; Rizza et al., 2011, 2016; Tondelli et al., 2014). For example, an accurate phenotyping in 41 barley genotypes (winter, spring and facultative growth habit) combined F_v/F_m and field survival score with *FR-H2* locus variants (Francia et al., 2016). Electrolyte leakage tests and/or survival screening after exposure to a range of freezing temperatures are also performed (Ahres et al., 2020). However, they are less accurate and requires more labor, especially when studying the dynamic effects of frost through space and time (Costa et al., 2019). Therefore, more precise and non-invasive methods like image phenotyping is raising its application thanks to the huge range of available experimental settings that permit a real time evaluation of plant during growth (Cabrera-Bosquet et al., 2012; Kim et al., 2021).

The FT of a specific barley genotype can vary in different locations and phenological phase (Rapacz et al., 2008). The maximum of the FT is during the vegetative phase, and it begins to decrease constantly

from the stem elongation (reproductive stage). Consequently, determining FT through field observations can be challenging, however it is necessary to confirm the results observed in control conditions and/or raise new questions concerning plant stress resistance in nature (Kopecká et al., 2023). Favorable conditions for FT assessments may occur infrequently, especially in regions prone to unpredictable severe frost events. For this purpose, controlled and repeatable experiments in growth chambers and freezing tests are essential for precise FT quantification at specific hardening levels at vegetative stage; these approaches help researchers to comprehensively study FT, providing valuable insights into their response to cold and freezing temperature (Badeck and Rizza, 2015).

Quantitative genetic studies have shown that a large part of the observed phenotypic variation in cold acclimation and resistance to frost is driven by the combined effect of the two major genes/QTLs *FR-H1* and *FR-H2*, located approximately 25 cM apart on the long arm of chromosome 5H (Francia et al., 2004; Tondelli et al., 2014). *FR-H1* co-segregate with the *VRN-H1* vernalization response locus; *FR-H2* encompass a cluster of at least 13 *CBFs* and co-localizes with QTL influencing COR protein accumulation. The two QTLs have a crucial role in controlling multiple traits related to abiotic stress response (for review regarding QTLs in barley, see e.g. Ahmad et al., 2018; Alqudah et al., 2020). Although several genetic resources were employed/developed so far to identify the molecular basis of *FR-H* loci, a major issue remains on how their interaction can be disentangled from the background influence of the rest of the genome. In this context, the production of QTL-Near Isogenic Lines (NILs) can be preparatory to mendelize the QT loci effects and for studying specific genome characteristic (Liu et al., 2023). In fact, when a QTL is introgressed into a NIL, the effect of the gene can be studied in an uniform background removing the confounding effect of other factors involved in the same trait (Alonso-Blanco and Koornneef, 2000). During the introgression, the size and number of segments depend on the target trait, usually transferring a small segment from a donor parent into the genetic background of another parent. In a typical backcross program, a donor parent from segregating populations (e.g. BC, DH, or RI lines) is chosen to carry a genomic segment precisely mapping at the QTL location, preferably with a low portion of non-targeted genome. Through repeated backcrossing (usually 4-6 cycles) with the recurrent parent, and using specific molecular markers to select/counter-select the foreground/background genome, undesired introgressions are removed until only the desired chromosome segment from the donor remains (Rafalski et al., 1996). After stabilizing this introgression via selfing or sibling mating, a homozygous Near Isogenic Line (NIL) is obtained. NILs, with a uniform chromosome background except for the target QTL, enable efficient analysis of the desired trait (Kooke

et al., 2012). This approach allows for precise confirmation of the presence of the QTL in the introgressed region, facilitating in-depth analysis and comparison with the recurrent parent's phenotype (Upadhyaya, 2007). Several studies have demonstrated the efficacy of such approach in defining the contribution of the QTL to a specific trait in barley . (Bernardo et al., 2012; Gao et al., 2023). Regarding abiotic stresses, NILs were used to identify 146 genes involved in salinity tolerance pathway at QTL *QSl.TxNn* on chromosome 2H (Zhu et al., 2020). In another elegant work, Shrestha et al. developed NILs with *P5CS1* introgressed from wild barley to dissect the drought resistance physiological mechanism. To the best of the author's knowledge, no data has been reported regarding the development of QTL-NILs differing at *Frost Resistance*-locus system *FR-H1/VRN-H1* and *FR-H2/CBFs*. Different allelic combinations in resistance and susceptible background would provide valuable material for a more comprehensive study into the genetic basis of vernalization and FT, as the effect of each QTL could be tested separately.

In order to dissect the cold acclimation and the frost tolerance, combined F_v/F_m and field trials in two contrasting locations, was considered as the best approach to measure the phenotyping differences of Nure (frost resistance), Tremois (frost susceptible) and the derived QTL-NILs with different allelic combinations at *FR-H1/FR-H2*.

3.2. Materials and Methods

3.2.1. Plant Materials and QTL-NILs Development and Genotyping

Four QTL near isogenic lines (QTL-NILs) and two parental commercial variates were selected to dissect the cold acclimation and the FT in the present thesis.

As far as varieties are regarded, Nure and Tremois were chosen due to numerous already available studies conducted. Nure is an Italian two-rowed winter genotype with high degree of FT. Nure was derived applying the Pedigree method from the crossing FO1236 = [(Fior 40 x Alpha^2) x Baraka] at the Genomic Research Centre (CREA-GB) in Fiorenzula d'Arda, Italy. Tremois [(Dram x Aramir) x Berac] is a French tow-rowed spring variety with high malting quality and low level of frost tolerance.

The QTL-NILs development started in 2004 at CREA-GB (D. Pagani and E. Francia) according to the selection scheme reported in Figure 1. Four lineages were started from the cross between the two parents (Nure or Tremois), used as recurrent, and four double-haploid (DH) lines selected according to their allelic status at *FR-H1, FR-H2, VRN-H2, VRN-H3, HvCEN* and *HvPPD* genes (Francia et al., 2004). The four DHs, used as donor and selected to possess alternative alleles at the two QTLs (i.e., either *FR-H2*–

fr-h1 or *fr-h2–FR-H1* haplotype) were: NT-42 (39.7 % of Nure, 57.9 % of Tremois), NT-64 (47.9 % Nure, 47.2 % Tremois), NT-92 (49.1 % of Nure, 47.7 % of Tremois) or NT-26 (35.4 % Nure, 60.7 % Tremois). From the F_1 generations onward, the recurrent cultivars were used in the backcrossing program to increase either the Nure or the Tremois genomic background. During each selection cycle, the obtained BCxF1 seeds were field-sown to obtain at least 12 spaced plants; these were labelled, and their DNA extracted to test the allelic state at *FR-H1* and *FR-H2*. In the applied marker assisted selection (MAS) scheme, two PCR-based codominant markers – *HvCBF3* and *HvBM5* (Francia et al., 2007, 2004) – targeting respectively *FR-H2/CBF* and *Fr-H1/VRN-H1* were used to monitor the allelic status of the introgressed segments and background. While no selection for *VRN-H2, VRN-H3, PPD-H1* and *PPD-H2* was initially performed, the genotypic status of the background was evaluated at the BC_3F_1 generation using 19 SSR markers scattered in the barley genome. Moreover, lines *CBF-Nu*/Tremois (Cod42 [BC_5S_4]) and *CBF-Tr*/Nure (Cod44 [BC_5S_3]) were also genotyped (unpublished data) with the barley 50 k Illumina Infinium iSelect (TraitGenetics GmbH, Gatersleben, Germany).

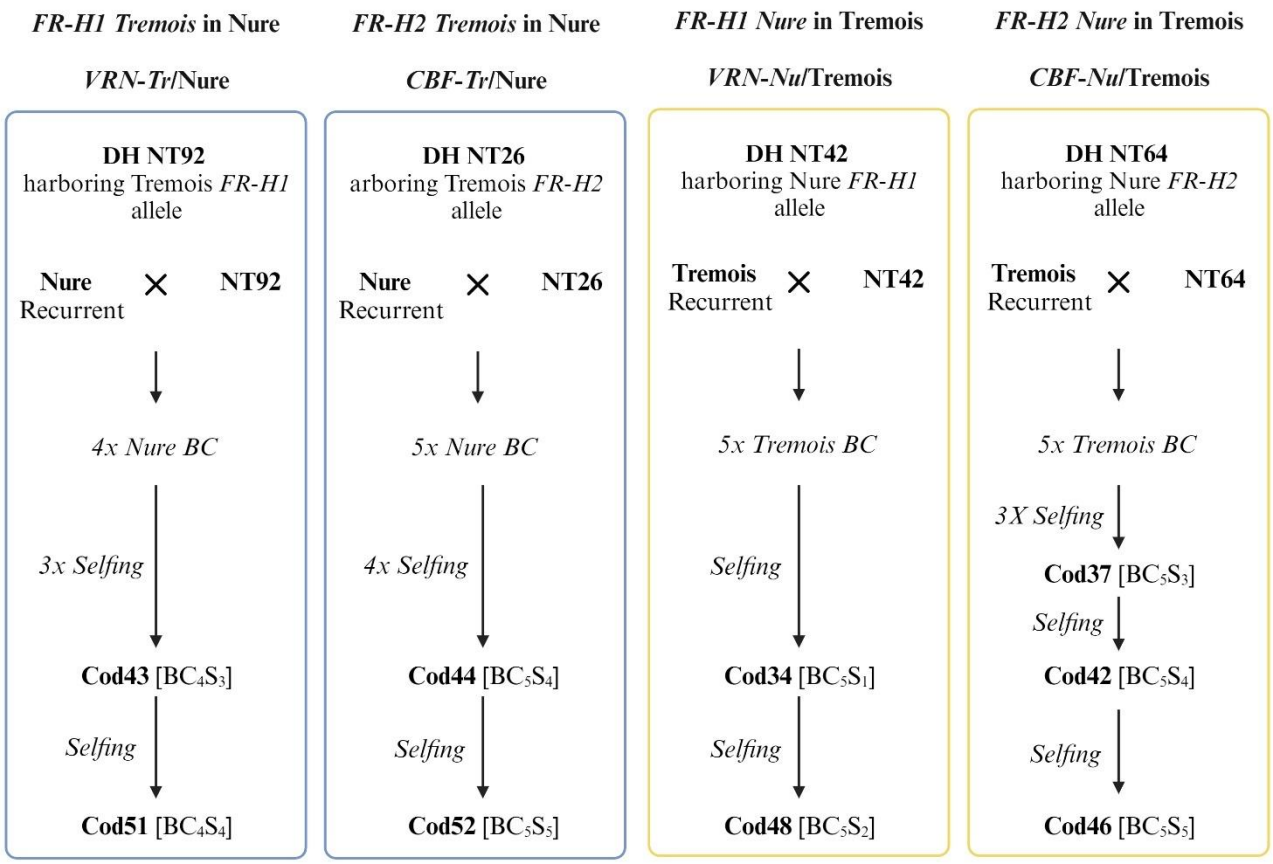


Figure 1. Development of QTL-NILs lines.

During this project, four QTL-NILs were selected based on the haplotype, backcross generation and selfing level (Table 1). All lines were in BC_4 or BC_5 generation and the percentage of recurrent genome is expected to be 96,875 % and 98,44 %, respectively. After field multiplication in 2021/2022 at experimental field of "Zanelli" Agricultural Highschool, Reggio Emilia, Italy, a sufficient quantity of seed for the subsequent controlled condition and open field trials was obtained:

- Cod34 [BC_5S_1] → Cod48 [BC_5S_2] *Frost Resistance-H1-Nure* in Tremois (*VRN-Nu*/Tremois).
- Cod42 [BC_5S_4] → Cod46 [BC_5S_5] *Frost Resistance-H2-Nure* in Tremois (*CBF-Nu*/Tremois).
- Cod43 [BC_4S_3] → Cod51 [BC_4S_4] *Frost Resistance-H1-Tremois* in Nure (*VRN-Tr*/Nure).
- Cod44 [BC_5S_3] → Cod52 [BC_5S_4] *Frost Resistance-H2-Tremois* in Nure (*CBF-Tr*/Nure).

Hereinafter, for better readability, the 4 QTL-NILs will be indicated as follows: *VRN-Nu*/Tremois, *VRN-Tr*/Nure, *CBF-Nu*/Tremois and *CBF-Tr*/Nure; where *VRN* and *CBF* are used for *FR-H1* and *FR-H2* respectively, combined with the acronym of donor genotype Nure (*Nu*) and Tremois (*Tr*), while the background genome is reported after the slash.

Table 1. Haplotype for Nure (blue), Tremois (yellow) and the four QTL-NILs (color based on the allelic status of the parental). Nure and Tremois haplotypes were derived by the literature (Francia et al., 2004, 2016; Rizza et al., 2016; Tondelli et al., 2014). QTL-NILs haplotype were derived from the genotyped with the barley 50 k Illumina Infinium iSelect (TraitGenetics GmbH, Gatersleben, Germany) (unpublished data); [A] The frost resistance gene *FR-H3* was recently identified on the short arm of chromosome 1H as one of the candidate Frost Resistance locus (Fisk et al., 2013); [B] *VRN-H2* and *PPD-H2* are different alleles between Nure and Tremois according to Rizza et al. 2016; [c] *VRN-H3* and *HvCEN* (no color) are the same allele for Nure and Tremois according to Francia et al., 2004; [D] *PPD-H1* is the same allele for Nure and Tremois according to Rizza et al. 2016; [E] *ICE* was found on chromosome 3H and it is a candidate for the ICE-CBF-COR because it refers to the orthologous in wheat *ICE41*. *ICE41* showed an influence on the cold acclimation according to Wang et al. 2023; [F] *ICE2* was found on chromosome 7H and it is one of the candidate *ICE* transcription factor for the ICE-CBF-COR (Francia et al., 2004); * Growth habit refers to the classification proposed by Rizza et al., 2016 based on the allele combinations at *VRN-H1/VRN-H2* locus.

Haplotype	***VRN-Tr*/Nure**	***CBF-Tr*/Nure**	**Nure**	**Tremois**	***VRN-Nu*/Tremois**	***CBF-Nu*/Tremois**
FR-H2/CBF	***Nu – Cbf***	***Tr – cbf***	***Nu – Cbf***	***Tr – cbf***	***Tr – cbf***	***Nu – Cbf***
FR-H1/VRN-H1	***Tr - Vrn-H1***	***Nu - vrn-H1***	***Nu - vrn-H1***	***Tr - Vrn-H1***	***Nu - vrn-H1***	***Tr - Vrn-H1***
[A] *FR-H3*	*Fr-H3*	*Fr-H3*	*Fr-H3*	*fr-H3*	*fr-H3*	*fr-H3*
[B] *VRN-H2*	*Nure*	*Nure*	*Nure*	*Tremois*	*Tremois*	*Tremois*
[C] *VRN-H3*	*Vrn-H3*	*Vrn-H3*	*Vrn-H3*	*Vrn-H3*	*Vrn-H3*	*Vrn-H3*
[D] *PPD-H1*	*ppd-H1*	*ppd-H1*	*ppd-H1*	*ppd-H1*	*ppd-H1*	*ppd-H1*
[B] *PPD-H2*	*Nure*	*Nure*	*Nure*	*Tremois*	*Tremois*	*Tremois*
[E] *ICE*	*Nure*	*Nure*	*Nure*	*Tremois*	*Tremois*	*Tremois*
[F] *ICE2*	*Nure*	*Nure*	*Nure*	*Tremois*	*Tremois*	*Tremois*
[C] *HvCEN*	*Nure*	*Nure*	*Nure*	*Tremois*	*Tremois*	*Tremois*
* Growth Habit	Facultative	Winter	Winter	Spring	Facultative	Spring

3.2.2. Measuring FT in Controlled Conditions

3.2.2.1. FT test -11 °C at CREA-GB

A first -11 °C freezing test was settled in collaboration with CREA-GB (Fiorenzuola d'Arda, Italy) with the following protocol. 10 seed for each genotype were planted into Styrofoam plateaus (522 mm x 330

mm x 50 mm) filled with sterilized neutral commercial peat (23% organic carbon, 0.5% organic nitrogen and dry apparent density 214 kg m–3, Dueemme S.r.l., Reggio Emilia, Italy) with randomized block design. The plantlets were grown for 11 days at 20/15 °C (day/night) and 11/13 h day/night photoperiod, light intensity of 200 μmol m^{-2} s^{-1}. After 11 days at warm conditions, barleys were put at the beginning of day 12 in the acclimation chamber (Sanyo Gallenkamp Model SGC970, Loughborough, UK) for 3 days at 12/7 °C with the same photoperiod and light intensity. After three days, 30 minutes before the dawn, temperature started to decrease to reach 3 °C when the light switched on. Once the temperature of 3 °C was reached, barleys started the hardening phase that lasted for 21 days at 3/1 °C d/n with the 11/13 h day/night photoperiod, light intensity of 200 μmol m^{-2} s^{-1}. After three weeks of cold acclimation, the freezing test was carried out in the dark in a temperature test cabinet (Vötsch VT 3050V, Weiss Technik, Magenta [MI], Italy) as described in (Rizza et al., 2001). The temperature was gradually decreased (2 °C/h steps) for seven hours to reach the freezing temperatures of -11 °C, then applying frost treatment for 12 h in the dark. After frost treatment, temperature was increased by 2 °C/h steps to + 1 °C for 7 h and then 24 hours at 20/15 °C as a recovery. Freezing tolerance was quantified by measuring chlorophyll fluorescence using the last fully expanded leaf by using a PAM-2000 fluorometer (Walz, Effeltrich, Upper Franconia, Germany). The functionality of the PSII reaction centers was measured by the ratio of variable (F_v) to maximal (F_m) fluorescence in a dark-adapted state, and expressed as F_v/F_m (Rizza et al., 2011). Measurements were performed according to Rizza et al. (2001) before and immediately after the freezing treatment, and after 24 h of recovery under control conditions (20/15 °C, 200 μmol m−2 s−1).

3.2.2.2. FT test -11 °C at ATK-MTA

A second -11 °C freezing experiment was carried out at ATK-MTA, Agricultural Institute, Centre for Agricultural Research, Martonvásár, Hungary. After 3 days of germination in Petri dishes (first day at room conditions, three days at 4 °C in dark conditions and one day at room conditions). 10 seedlings were planted into wooden boxes (30 cm × 25 cm × 10 cm) with randomized block design. The growing medium was a 2:1:1 (v/v/v) mixture of soil, sand, and humus. The plantlets were grown in plant growth chambers (Conviron PGV36; Controlled Environments Ltd.; Winnipeg, MB, Canada) in control conditions for 14 days at 20/15 °C (day/night) with short-day photoperiod 8/16 h, 70–75% RH, and 180-220 μE of light intensity. At the end of the fourteenth day, the shift of temperature was applied during the night, and it was gradually decreased for two days to reach hardening conditions (3/1 °C, same photoperiod, and light intensity), and seedlings exposed to acclimation for four weeks. Soil moisture was monitored daily in each box using an Electro-Conductivity meter (Type OK-102/1, Radelkis) and EC

adjusted to 120–170 µS. Before the freezing test, three plants for each genotype were collected to evaluate the apex development according to the protocol developed in a previous project (ADAPTAWHEAT, 2015). Most of our knowledge about the genetic control of the transition phase in barley comes from the dicotyledonous model plant *Arabidopsis thaliana*. For this purpose, the aim of the apex development assessment was to make a preliminary assessment of floral development in barley with different allele combinations of *VRN-H1* in winter and spring background. After four weeks of cold acclimation, the temperature was gradually decreased (2 °C/h steps) to reach the freezing temperatures of -11 °C, then applying frost treatment for 24 h in the dark. After the frost treatment increase the temperature by 2 °C/h steps to + 1 °C for 24 h. FT was quantified with a PAM-2000 fluorometer (Walz, Effeltrich, Upper Franconia, Germany) following the same procedures reported in the previous paragraph, before and immediately after the freezing treatment and after 24 h of recovery. A regrowth test was then applied (Hincha and Zuther, 2020) cutting all barleys 3 cm above the first internode and maintaining the plants in a recovery chamber (17/16 °C 14/10 d/n) for three weeks. The regrowth score was assessed with the visual assessment at the end of the recovery phase.

3.2.2.3. FT test -13 °C at ATK-MTA

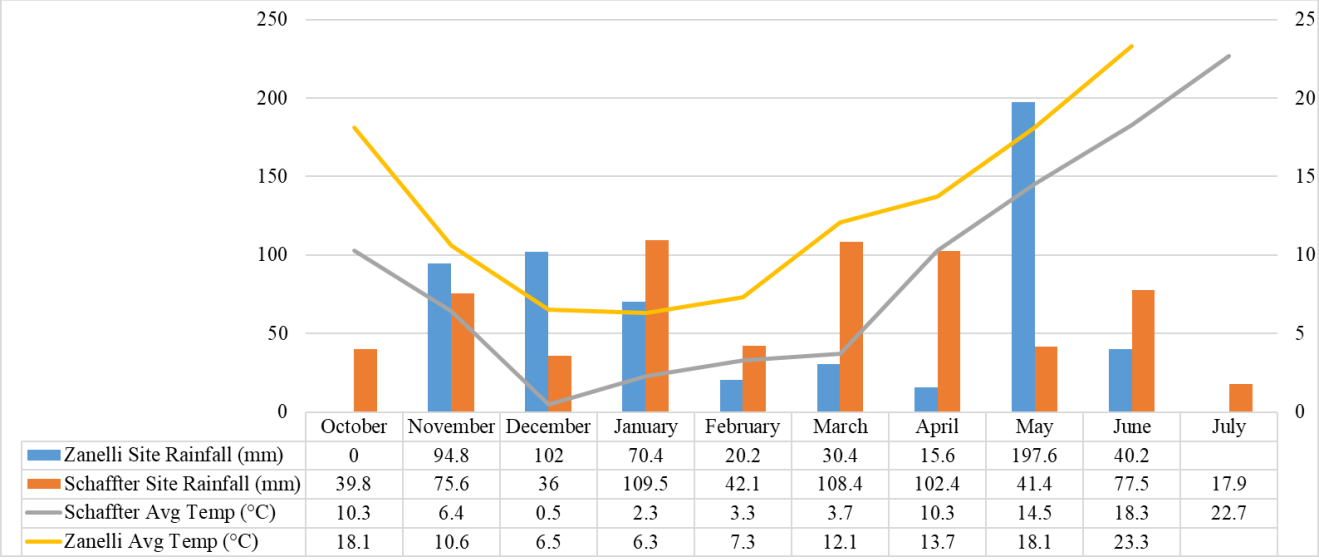
A third freezing experiments at -13 °C was finally carried out at ATK-MTA in which, apart from the freezing temperature, the entire protocol was followed as described in the previous section.

3.2.3. Open Field Trials

Nure, Tremois and the four QTL-NILs were grown in two contrasting locations during the season 2022/2023: experimental field of "Zanelli" Agricultural Highschool, Reggio Emilia, Italy (44°41'27.1"N 10°36'22.2"E) and "Schaffter Farm" at Ohio State University (OSU), Wooster, Ohio, USA (40°45'22.1"N 81°53'55.4"W). Based on USDA soil texture classification, "Zanelli" site had silty clay loamy, while "Schaffter" site silt had loam soil type. The experimental design at "Zanelli" farm was a randomized block with three replications (1 x 1 m), while at "Schaffter" farm was a single plot (1 x 3 m) due to the lack of a sufficient quantity of seed.

As expected, the climate/environmental conditions in the two locations were very different. "Zanelli" site had a typical temperate subcontinental climate, characterized by hot and humid summers followed by mild/cold and rainy winters. "Schaffter" site had the typical humid continental climate characterized by cold winters and hot, humid summers. In Italy, planting and harvest dates were October 25, 2022, and

June 22, 2023, respectively (for a crop cycle of 242 d), while in OSU planting and harvest dates were October 7, 2022, and July 5, 2023, respectively (for a crop cycle of 272 d). The weather conditions of the growing seasons in the two locations are reported in Figure 2 (rainfall is reported in the left-side axis, while temperature in the right-side axis) and Table 2.



	October	November	December	January	February	March	April	May	June	July
Zanelli Site Rainfall (mm)	0	94.8	102	70.4	20.2	30.4	15.6	197.6	40.2	
Schaffter Site Rainfall (mm)	39.8	75.6	36	109.5	42.1	108.4	102.4	41.4	77.5	17.9
Schaffter Avg Temp (°C)	10.3	6.4	0.5	2.3	3.3	3.7	10.3	14.5	18.3	22.7
Zanelli Avg Temp (°C)	18.1	10.6	6.5	6.3	7.3	12.1	13.7	18.1	23.3	

Figure 2. Weather of "Zanelli" and "Schaffter" sites during the growing season 2022/2023.

Table 2. Minimum and maximum average temperature for Zanelli and Schaffter sites.

Month	Zanelli Max Avg Temp (°C)	Zanelli Min Avg Temp (°C)	Schaffter Max Avg Temp (°C)	Schaffter Min Avg Temp (°C)
October	23.8	14.2	17.3	4.0
November	13.9	7.8	11.9	1.5
December	8.3	5.0	4.7	-3.1
January	9.0	4.1	5.4	-0.9
February	12.5	3.2	10.4	-2.5
March	17.8	7.0	9.0	-1.1
April	19.5	8.4	17.1	3.4
May	22.8	14.0	21.5	6.9
June	28.6	18.2	24.5	11.8
July			28.5	18.3

At both sites, winter survival rate was measured at end of tillering - beginning of stem elongation phase (Zadoks growth stage Z29-Z30); in addition, grain yield was calculated to evaluate the impact of the stress on the crop.

3.2.4. Statistical Analysis

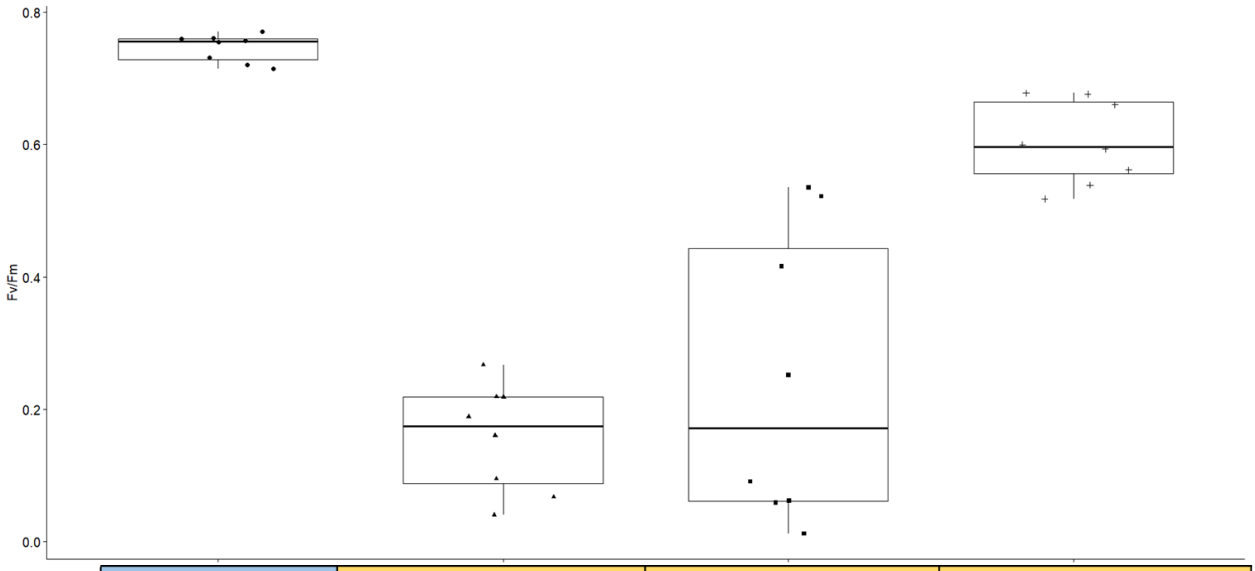
The F_v/F_m and the winter survival rate as indicator of frost damage and survival data have a non-normally distribution. The data, bounded within 0 – 1 value or 0 - 100% intervals with skewed distributions. In addition, when damaged and nondamaged plants constitute the statistical ensemble bimodal distributions often result; therefore, all analyses were performed with nonparametric tests. The Kruskal–Wallis test (Kruskal and Wallis, 1952) was applied to evaluate genotype differences, with subsequent nonparametric multiple comparison using RStudio version 4.1.3 (R Core Team, 2022) the dplyr package (Wickham et al., 2023). On the rank-transformed data Post Hoc Analysis with Benjamini-Hochberg was used to test genotype × treatment interactions using RStudio version 4.1.3 (R Core Team, 2018) agricolae package (Mendiburu, 2023). For the boxplot graphs RStudio version 4.1.3 the package ggpubr were used (Kassambara, 2023).

The agronomical and morphological data were analyzed using analysis of variance (ANOVA) followed by Duncan post hoc test (p value < 0.05) in GENSTAT 17th software (VSN International, Hemel Hempstead, UK).

3.3. Results

3.3.1. Freezing Test

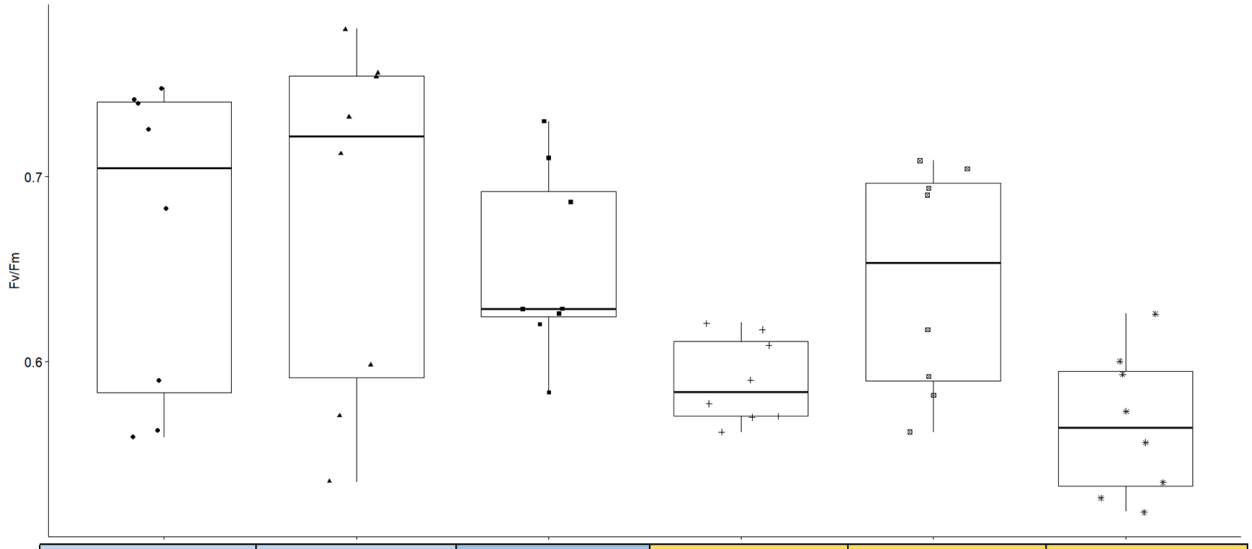
The aim of the freezing test performed at CREA-GB at -11 °C was to establish, in a preliminary way, the effect of frost tolerant alleles in a susceptible background. F_v/F_m analysis was performed on plants at a seedling stage (Fig. 3) that experienced 3 days of pre-acclimation (12/7 °C) and 21 days of hardening (3/1 °C, 11/13 d/n photoperiod). The data showed high variability of FT among Nure, Tremois, and NILs with Tremois background. Genotypic differences were significant when measured directly after stress (pvalue Chisq <0.001) as well as during recovery (pvalue Chisq <0.001). The data measured at 48 h of recovery are reported in Figure 3. The treatment at -11 °C showed the highest F_v/F_m value for Nure (nonparametric multiple comparison), and interestingly, *CBF-Nu*/Tremois showed statistically higher value compared to *VRN-Nu*/Tremois and Tremois.



	Nure	Tremois	*VRN-Nure*/Tremois	*CBF- Nu*/Tremois
F_v/F_m– Rank	0.746 - a	0.157 - c	0.244 – c	0.603 – b
FR-H2	Nure	Tremois	Tremois	Nure
FR-H1	Nure	Tremois	Nure	Tremois

Figure 3. -11 °C Freezing test at CREA GB. Result of the Kruskal-Wallis test performed on the Fv/Fm parameter. a–c means followed by different letters are statistically significant at pvalue Chisq <0.001.

At ATK-MTA a preliminary analysis of apex development was performed, just before freezing test, after four weeks of hardening temperature at 3/1 °C and 8/16 h day/night coinciding with the third leaf stage showed that Nure plants were the only not yet in transition phase, *CBF-Tr*/Nure was at the beginning of the transition phase, while Tremois and the remaining NILs were at the beginning of double-ridge phase (i.e., the phenological signal of the attained flowering competence of the apical meristem in winter cereals). As far as the two FT tests at ATK-MTA (-11 °C and -13 °C) are regarded, F_v/F_m was measured on plants at seedling stage after 21 days of acclimatization (3/1 °C, 8/16 d/n photoperiod). All four QTL-NILs were used in the experiments. PSII functionality data did not reported significant result for the -11 °C test (results not shown), while showed slightly differences between genotypes for the -13 °C test (Figure 4). The only difference was between the genotypes with Nure background and the *VRN-Nu*/Tremois line. Probably due to non-optimal growth conditions, no regrowth (0-score for all genotypes) was observed after two weeks of recovery.



	VRN-Tr/Nure	*CBF-Tr*/Nure	Nure	Tremois	*VRN-Nu*/Tremois	*CBF-Nu*/Tremois
F_v/F_m– Rank	0.669 - a	0.680 - a	0.652 - a	0.59 - ab	0.566 - b	0.644 - ab
FR-H2	Nure	Tremois	Nure	Tremois	Tremois	Nure
FR-H1	Tremois	Nure	Nure	Tremois	Nure	Tremois

Figure 4. -13 °C Freezing test. Result of the Kruskal-Wallis test performed on the Fv/Fm parameter. a and b means followed by different letters are statistically significant at pvalue Chisq 0.02.

3.3.2. Field Trials

The situation at the "Zanelli" site was abnormal during the early stages of plant development. In fact, a severe drought affected Northern Italy and the Po valley between September and October 2021 (see Fig. 2 and Table 2, section *2.4.*). These conditions were found to be associated with positive thermal abnormalities and negative rainfall anomalies that rekindled the conditions of extreme drought (SNPA - Sistema nazionale protezione ambiente, 2023). The amount of rainfall between September and October was 52.6 mm, of which only 2.4 mm in October (a month normally rainy). The season average is 57 and 84 mm of rain in September and October, respectively. The first rainy days were November 3 and November 4 with 3.8 mm of rain each. However, the first important event occurred November 22 with an amount of 60.4 mm (Arpae, n.d.), 3 weeks after the plant emerged. The seedling emergence and development were influenced by these abnormal conditions and, owing to Barlow et al., (2015) may have influenced the entire growth cycle. Regarding winterhardiness, no prolonged frost conditions occurred during 2021/2022 cold season, and no differences were observed at the end of tillering/beginning of stem

elongation phase. Probably due to harsh weather conditions, grain yield values showed high variability within blocks, and no significant difference among genotypes was observed (Table 3). The yield values of the trial ranged between 3 and 4 t/ha, except for the NIL carrying the Tremois's *CBF* allele in Nure (*CBF-Tr*/Nure), which yielded less than 1.97 t/ha. These values confirm the negative impact of the climate in the emerging and developing phases, as the average of barley production per hectare in the Nord of Italy is up to 5 t/ha from 7 t/ha. In addition, barleys at Zanelli's site have been analyzed for morphological and agronomical data (Table 3). Thousand kernel weight showed statistical difference between genotypes, as Nure and NILs with Nure background had a higher seed weight compared to Tremois and its NILs. For the hectoliter weight the only difference is the *CBF-Nu*/Tremois that showed a lower value compared to other genotypes. For the morphological data, spike height showed significant differences between genotypes. Nure had a longer spike compared to Tremois and there are not significant differences between NILs and their parentals.

Table 3. Yield and Zanelli's morphological data. Ns - not significant. Result of the ANOVA test performed on the yield, agronomical and morphological parameters. A–c means followed by different letters are statistically significant at $p < 0.05$; ns, not significant.

Yield Data

	Zanelli(t/ha)		Schaffter (t/ha)	
***CBF-Nu*/Tremois**	3.53	ns	5.72	ns
***VRN-Nu*/Tremois**	3.97	ns	2.10	ns
***VRN-Tr*/Nure**	3.41	ns	6.30	ns
***CBF-Tr*/Nure**	1.97	ns	7.31	ns
Nure	3.00	ns	6.38	ns
Tremois	3.08	ns	2.77	ns
Pvalue	0.282			

Zanelli Agronomical Data

	1000 seed (g)		Hectoliter weight (kg/hL)	
***CBF-Nu*/Tremois**	42.43	b	57.27	b
***VRN-Nu*/Tremois**	42.83	b	62.49	a
***VRN-Tr*/Nure**	50.91	a	61.05	a
***CBF-Tr*/Nure**	51.42	a	60.79	a
Nure	51.11	a	62.23	a
Tremois	42.36	b	62.11	a
Pvalue	<.001		0.01	

Zanelli Morphological Data

	Height (cm)		Spike height (cm)		Spike number		Caryopses/spike	
***CBF-Nu*/Tremois**	70.27	ns	7.233	c	26.4	ns	24.87	ns
***VRN-Nu*/Tremois**	68.80	ns	8.32	ab	28.67	ns	27.4	ns
***VRN-Tr*/Nure**	69.27	ns	8.913	ab	29.4	ns	27.93	ns
***CBF-Tr*/Nure**	68.53	ns	8.84	ab	28.8	ns	27.67	ns
Nure	70.00	ns	9.08	a	28.8	ns	27	ns
Tremois	69.07	ns	7.853	bc	26.87	ns	25.93	ns
Pvalue	0.989		0.002		0.158		0.247	

The result of yield and winter survival rate (WSR) at Schaffter farm should be considered only as a preliminary test of the QTL-NILs in a severe environment. Unfortunately, due to an insufficient quantity of seed, we were only able to sow only one plot per genotype; therefore, no statistical analysis could be performed. However, a sufficient quantity of seed for the following 2023/2024 growing season was

obtain and it will be used to perform a proper experimental design to validate the interesting data obtained in this work. The season at Schaffter's site was slightly warmer, considering the area, but still cold enough to exert environmental pressure on barleys. During the crop cycle, the plants experienced 40 days with a daily average temperature below 0 °C, including 8 days with minimum temperatures below -10 °C, among which 4 consecutive days had temperatures of -21.2 °C, -19 °C, -14.1 °C, and -13.2 °C. Based on these data, the conditions were severe enough to differentiate the genotypes for winter survival rate with the visual score ranged from 1 (minimum) to 9 (maximum). As shown in Figure 5, Nure had a WSR of 7 and Tremois of 2, data consistent with actual literature. As regards NILs carrying different Nure alleles in Tremois background (Fig. 6), it is interesting to note that *CBF-Nu*/Tremois line scored the maximum WSR of 9; conversely, the lowest score (WSR of 1) was obtained by the *VRN-Nu*/Tremois line. NILs carrying different Tremois alleles in Nure background are shown in Figure 7. The *CBF-Tr*/Nure line scored WSR of 8, while the NIL carrying the VRN-H1 spring allele scored WSR of 7. Overall, these observations might suggest a putative role of the background in controlling cold acclimation and winter survival rate.

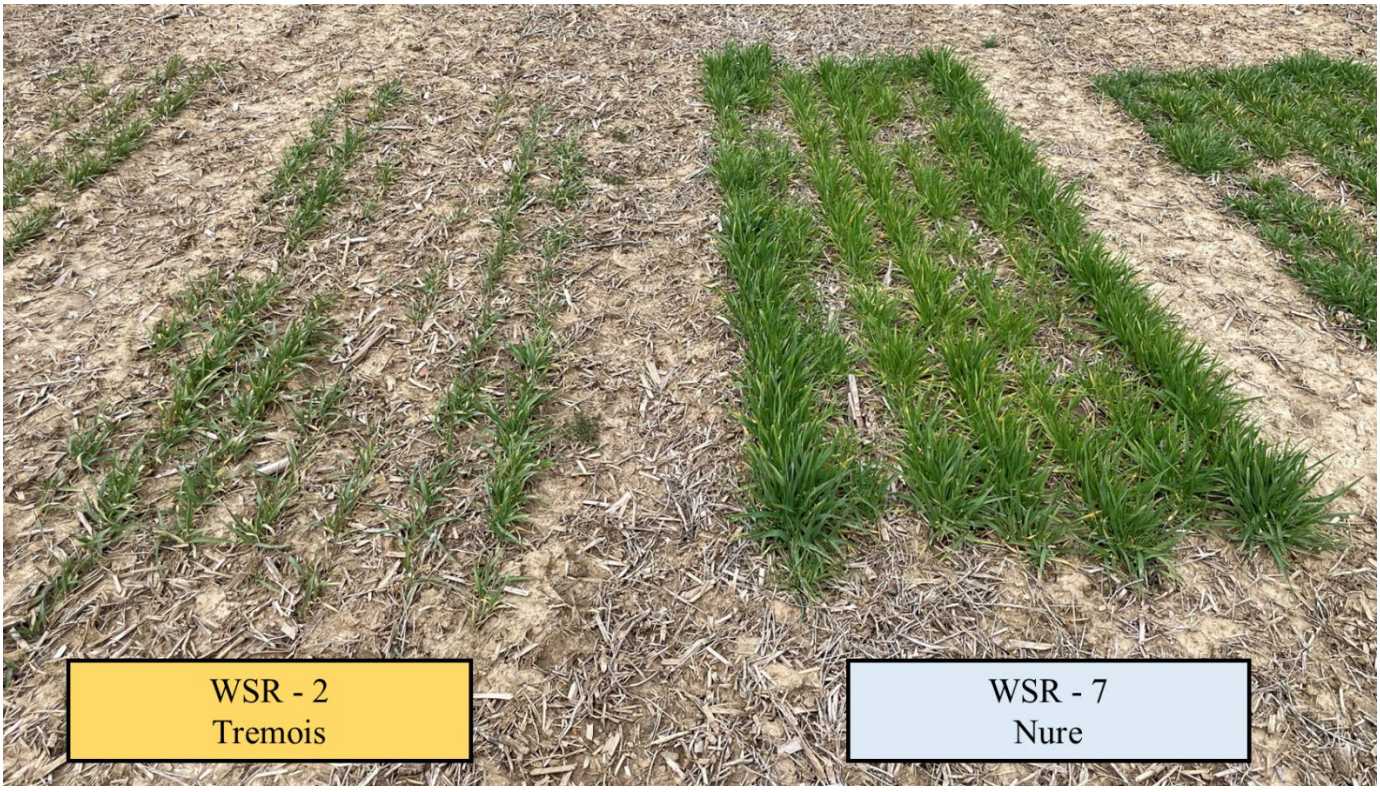


Figure 5. WRS of parental lines Nure and Tremois.

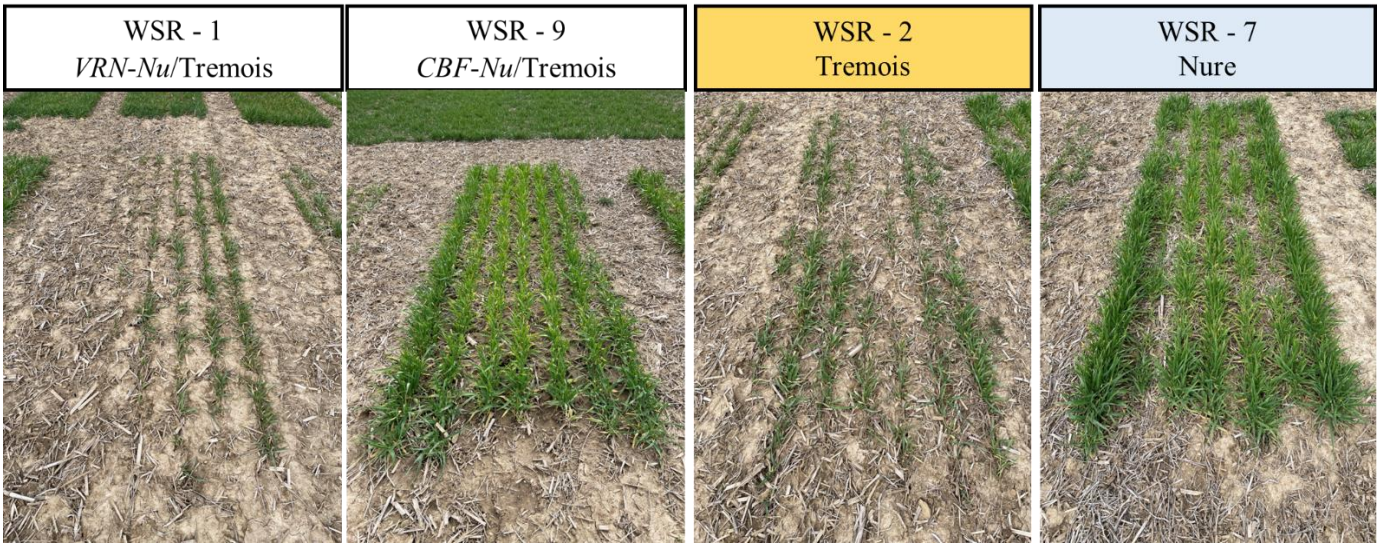


Figure 6. WSR of NILs with Nure alleles in Tremois background.

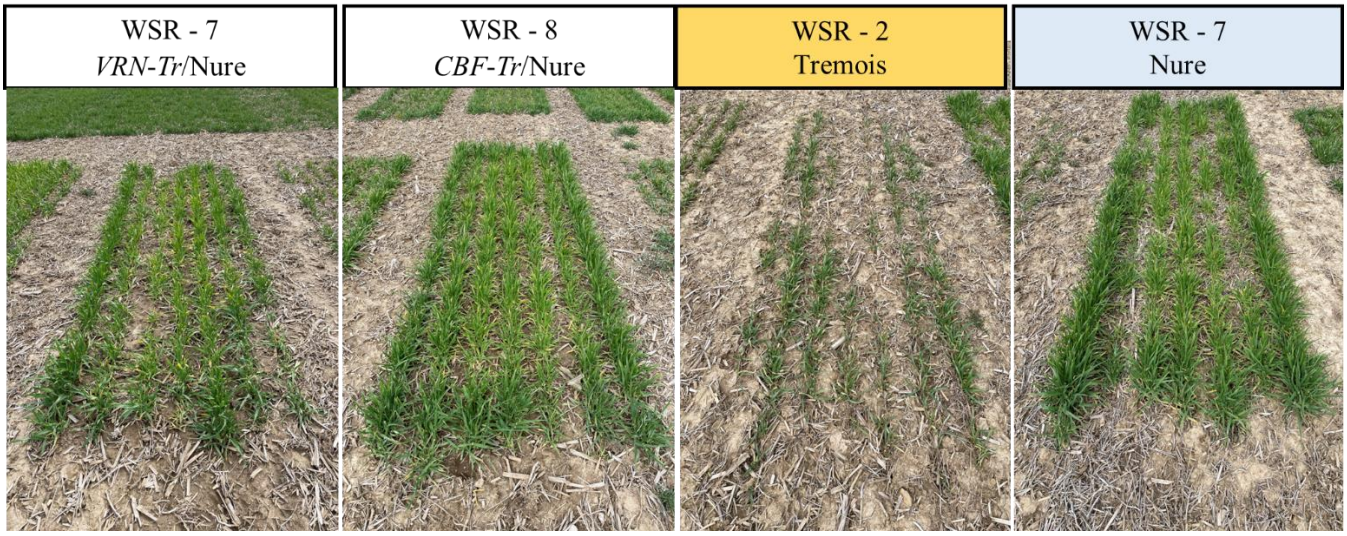


Figure 7. WSR of NILs with Tremois alleles in Nure background.

Analyzing the putative effects of the *VRN-H1* and *CBF* alleles on WSR, it has been observed that *VRN-H1* allele from Nure did not improve the WSR in Tremois background. Whereas *VRN-H1* allele from Tremois did not reduce the WSR in Nure background. Regarding the *CBFs*, Nure's allele significantly improved the frost resistance in the NIL with Tremois background. The *CBF* allele of Tremois did not reduce resistance in the Nure background.

As reported for the winter survival rate, the yield data regarding the Schaffter farm are just an observation, with *CBF-Tr*/Nure showing the highest yield followed by Nure and *VRN-Tr*/Nure (Table

3). *VRN-Nu*/Tremois and Tremois showed the lowest yield, on the other hand *CBF-Nu*/Tremois had higher yield.

3.4. Discussion

The present study was, to the authors' knowledge, the first reports of using QTL-NILs with different allele combinations at *Fr-H1/VRN-H1* and *Fr-H2/CBF* developed to mendelize the genetic basis of frost tolerance (FT), winter hardiness and vernalization. Regarding the ATK-MTA experiment, no significant differences in the F_v/F_m and the 0 score in the regrowth in -11 °C and -13 °C freezing test were obtained. Data of the freezing test at CREA-GB and the phenotypic observation at Ohio open field trials highlighted a putative major effect on phenotype given by Nure's *CBF* (*FR-H2*) compared to Nure's *VRN-H1* (*FR-H1*) while expressed in the spring background. On the contrary, in NILs with winter background, no significant influence of spring alleles was observed on reducing the frost resistant. This result also confirmed phenotypic data obtained previously at CREA-GB, under the same conditions, for other materials from the same lineage during the development of the QTL-NILs (such as Cod37 [BC_5S_3] *CBF-Nu*/Tremois, Fig. 1) (Mareri, 2018).

The selection of the proper site for open field trials was based on the germplasm origin of the parental lines of QTL-NILs. Nure and Tremois are genotypes that come from European gene pool, Nure is a fall-sown variety adapted for Mediterranean weather conditions, while Tremois is a spring-sown variety that was developed for the Central Europe. For this reason, two different environments were selected: Zanelli was chosen for its humid subcontinent climate with mild winters and Ohio for its weather comparable to the conditions of Central Europe with prolonged and cold winters. Ohio was selected with the aim of having a strong phenotyping on the FT trait. FT depends on barley phenology and during the vegetative phase it reaches its maximum (Ibrahim et al., 2016). However, FT depends also on the environmental conditions to which barleys are exposed (Badeck and Rizza, 2015). FT increases through cold acclimation once exposure to low non-harmful temperatures, but the exposure to mild temperature can de-acclimate barleys exposing them to severe frost damages (Wójcik-Jagła and Rapacz, 2023). In this term, Ohio weather represented the perfect conditions for FT evaluations on open field trial (as shown in Figures 5-7). During the crop cycle the temperature decreased to reach its minimum average values during the wintertime after several weeks of acclimation and without any mild period, thus, no de-acclimation process (for weather data see Figure 2 and Table 2). When barleys were exposed to freezing temperatures, they fulfilled the hardening phase and the frost permitted to discriminate plants by their

degree of frost resistance. The results obtained under controlled conditions at CREA-GB confirm the observations in Ohio showing the importance of having a proper growing chamber protocol to combine with open field trials (Badeck and Rizza, 2015).

CREA-GB growing protocol had been used for similar experiment in barleys for several publications (Francia et al., 2016; Guerra et al., 2022; Rizza et al., 2011) with the aim to reproduce conditions that mimic as much as possible the open field conditions. In fall, after sowing, the temperature is generally mild and only gradually reduce, first, to non-harmful cold temperature, and then to freezing temperature. Thus, the combination of short-day conditions (11/13 h d/n) and a short phase at mild acclimation temperature (12/7 °C) before hardening temperature (3/1 °C) in the growing chamber should have activated the acclimation process like in the open field experiment. The protocol selected for ATK-MTA experiment differed from the one used at CREA-GB for three aspects: (i) the circadian rhythm (8/16 h d/n), the (ii) temperature drop, and the (iii) chamber used for the freezing test. (i) The two circadian rhythms (11/13 h and 8/16 h d/n) were selected based on the literature (Dhillon et al., 2017; Mareri, 2018; Rizza et al., 2011); however, no differences should be occurred because both protocols are in short day conditions (Dhillon et al., 2017; Novák et al., 2017). Thus, the circadian rhythm may have not influenced the phenotype. (ii) The temperature drop at the ATK-MTA was more rapid and in a shorter period of time (2 days) and it was selected based on work that discriminated the frost resistance of 121 barleys genotypes including Nure, Tremois and double haploid NT lines (Rizza et al., 2016). So, (iii) the "chamber effect" may have been putatively caused by the freezing chambers: at ATK-MTA cold hardened barleys were subjected to -11 °C and -13 °C temperatures in walk-in freezing chamber, while at CREA-GB the freezing at -11 °C for 12 hours was in a cabinet test. The structure of the walk-in chamber was different from the cabinet test; the position of plants on the different shelves may have changed the perceived temperature, thus increasing the data variability. Whereas this aspect was strongly reduced in the tests carried out in the cabinet because all the plants were on the same shelf during the stress. Freezing chambers have been widely used in studies of the frost tolerance; however it has been shown that although they are highly controlled, they are not uniform, which can lead to considerable degrees of variability in plant response data (Lee and Rawlings, 1982). Variation in the response of barleys to frost is normally due to natural genotypic and phenotypic variation; however, the so-called "chamber effect," might cause variability in the data with the same genotypes due to growing plants in different chambers (Porter et al., 2015).

As could be expected introgression of resistant allele at *FR-H2* into susceptible genotype (*CBF-Nu*/Tremois) led to improved frost tolerance. However, this improvement was not observed for the line with resistant *FR-H1* allele expressed in susceptible background. This would mean that *FR-H2* winter allele of *CBF* genes is sufficient to induce tolerance. What's interesting, on the other hand, introgression of spring alleles at either of the two loci into winter background didn't let to reduced resistance. Thus, even if winter *CBFs* are sufficient to induce frost tolerance in spring background, they seem not necessary in the winter background, showing the complexity of the trait. *CBF* genes at *FR-H2* have a pivotal role in the coordination of the acclimation processes which is driven by the ICE-CBF-COR (Barrero-Gil and Salinas, 2018; Francia et al., 2007). After cold exposure, *ICE1* transcription factor induce the *CBFs* expression that upregulate Cold-Related (*COR*) genes activating the cold acclimation pathway (Barrero-Gil and Salinas, 2018; Stockinger, 2009; Tondelli et al., 2011). After several days of cold exposure, barley acquires the tolerance to frost temperatures (Galiba et al., 2009; Tolera Angessa and Li, 2016). This process has been described either in winter or in spring genotypes, however with different degrees of resistance (Guerra et al., 2022; Rizza et al., 2011, 2016). The key aspect of this work is that the winter allele, with its structural variations (i. e., CNV at *HvCBF2-4* segment), was able to increase the degree of frost resistance in spring genotype. Interestingly, on the other hand *FR-H2* spring allele did not reduce the frost resistance in the winter NIL and that. might be explained by other actors of the ICE-CBF-COR pathway, controlled independently of the *CBF* allele (Wang et al., 2016). In barley, the role of *ICE1* was suggested by a higher transcripts accumulation of transcription factors that control its expression in transcriptome profile analysis of Nure and Tremois. Moreover, the role of *ICE* transcription factor in the cold response reporting that different degree of frost tolerance linked to its different alleles were reported in rye (Båga et al., 2022; Li et al., 2011) and wheat (Guo et al., 2019; Pan et al., 2022; Saripalli et al., 2023). Thus, the highest level of frost tolerance might be putatively explained by the combination of winter allele of *ICE* and *CBFs*; however, only one of them, either a winter allele of *ICE* or *CBFs* might be sufficient to confer a high degree of frost tolerance. As it was observed in the present study in the case of the NIL carrying the spring *CBF* allele in winter background (winter *ICE* allele), or in the case of the NIL carrying the winter *CBF* allele in spring background (spring *ICE* allele).

The quite novelty of this work is that Winter allele of *vrn-H1* did not increase the resistance in the spring growth and spring allele *Vrn-H1* did not reduce the resistance in the winter growth habit. Although in Arabidopsis vernalization was reported to have an independent pathway compared to cold acclimation (Bond et al., 2011; F. Li et al., 2021), our results seem to point out for the first time that this phenomenon

is also present in cereals. *VRN-H1* is known to be the key gene in the vernalization requirement which is included in the more complex trait of flowering induction (Distelfeld et al., 2009). *VRN-H1* is a flowering promoter whose expression leads the plant to the transition from the vegetative to the reproductive phase (Trevaskis, 2010). Moreover, *VRN-H1* co segregate with *FR-H1* involved in the frost resistance (Dhillon et al., 2010). However, vernalization, that might be considered as a kind of "side effect" of the frost resistance is activated after a long period of exposure to cold temperature, and thus coincide con Cold acclimation, but it seems not to contribute directly to frost resistance. The reason might reside in the fact that the response to low temperatures (cold acclimation) and vernalization seem to be controlled by two distinct signaling pathways (Bond et al., 2011). In terms of evolution, winter habitus genotypes are adapted not to flower until environmental conditions, such as temperature, circadian rhythm and light are optimal (Thomashow, 2010). This transition to reproductive phases depends on a complex mechanism that involves other vernalization genes such as *VRN-H2* and *VRN-H3*, the earliness *per se* gene *HvCEN*, and photoperiod *PPD* genes (see recent review Muñoz-Amatriaín et al., 2020). However, vernalization may be potentially unlinked from the cold acclimation and frost resistance for three putative major differences. First, cold acclimation occurs in a wide variety of plant tissues, including mature leaves (Kim and Sung, 2014). On the other hand, vernalization is effective either at the shoot apical meristem (SAM) or young leaves, indicating that rapidly dividing cells are responsive to vernalizing cold (Kim and Sung, 2014). Second, cold acclimation pathway starts after a short exposure to cold non-harmful temperatures and requires 4 to 6 weeks to reach the peak of the frost tolerance (Galiba et al., 2009). Whereas, vernalization is a quantitative response to low temperatures that requires from 6 to 10 weeks (Trevaskis et al., 2006); short periods of cold exposure are less effective at promoting flowering time than longer periods (Sheldon et al., 2006). The third point that may be hypothesized by the mechanism described in Arabidopsis, where none of the signaling component of the ICE-CBF-COR pathway are involved in the induction of *VERNALIZATION INSENSITIVE 3* (*AtVIN3*), which is the first gene in the vernalization pathway. In fact, barleys with facultative growth habitus do not require vernalization to flower, at the same time are highly resistant to frost temperatures. The lack of vernalization combined together with high degree of frost tolerance suggest that vernalization is not involved in the frost tolerance. On the other hand, apex development, including flowering, is intimately linked to stress responses, and both traits are reported to be influenced by the expression of genes such as *FR-H1/VRN-H1*, *FR-H2/CBF*, and *COR* genes (Crosatti et al., 2003; Hazrati et al., 2021; Kosová et al., 2010). The apex evaluation, performed in the present study was intended to identify the plant morphogenesis during the cold acclimation and the vernalization in genotypes with different FT/growth habit. This evaluation

aimed to recognize the transition phase and the role of *FR-H1* and *FR-H2*. Data showed that Tremois, *VRN-Tr*/Nure and the NILs with the background of Tremois were all in the transition phase, on the other hand Nure, and the *CBF-Tr*/Nure still not. Based on the *VRN-H1*/*VRN-H2* haplotype classification suggested by Rizza et al., 2016, the latter two genotypes are classified as winter habitus. The spike development is known to be controlled by cold acclimation, tillering and vernalization pathway genes (Shaaf et al., 2019), and among them *VRN-H1* (Cuesta-Marcos et al., 2015). The apex development and the transition from vegetative to reproductive phase depend on the *VRN-H1/VRN-H2* allele combination and growth habit (Digel et al., 2015; Gol et al., 2017). In winter habitus (winter recessive allele of *vrn-H1* and dominant of *Vrn-H2*), the apex development is governed by cold acclimatization, vernalization and photoperiod and it may last for several weeks until the spikelet initiation (Dennis and Peacock, 2009; Greenup et al., 2009). In this work, the preliminary date regarding the development of the apex may suggest that the transitions phase is regulated by factors present in the background of spring variety (notwithstanding *VRN-H1* and *CBF* alleles) or by the sole presence of *Vrn-H1* allele /notwithstanding the background. This hypothesis is consistent with the result obtained by Cuesta-Marcos et al. Authors used barley's NILs targeting the *VRN-H1/H2/H3* alleles. Result showed that the substitution of *VRN-H1* allele is sufficient to eliminate vernalization requirement. It can be assumed that the major influence of the apex development comes from *Vrn-h1* spring allele and/or spring background which encodes the spring allele of *VRN-H2* and *VRN-H3* (Fowler et al., 2001; Prasil, 2004). Finally, while Nure and Tremois carry different alleles at *VRN-H2* (von Zitzewizt et al., 2005), the coding region of *HvFT1* (determinant of *VRN-H3* on barley chromosome 7H; (Yan et al., 2006) has no sequence polymorphism and both varieties carry the recessive *vrn-H3* allele (Tondelli et al., 2014).

The present study enabled moreover to highlight the issues given by the ongoing climate changes. Barleys faced an unprecedented combination of drought and high temperatures during the seedling development in the open field study at Zanelli site. Unfortunately, this combination influenced the all the barley crop cycle. In addition, the winter was milder compared to the average of the area and thus no prolonged frost events during the cycle could be measured. The environmental conditions impacted also the agronomical and morphological data reducing the yield based on the average production of the area. Such a type of weather modification is becoming more and more frequent opening up new scenarios to the cultivation of fall-sown barleys (Rizza et al., 2016). The alternation of warm and cold periods might cause a de-acclimatation process in fall-sown barleys, reducing their frost tolerance and producing serious yield losses (Willick et al., 2021). Furthermore, the vernalization requirement may not be totally

satisfied, resulting in delayed flowering, and exposing the plants to a different incidence of biotic and abiotic stresses. Additionally, drought stress in early phenological phases might decrease the rate of germination and early seedling development (Barlow et al., 2015).

The study of the role of the *CBF* genes in the drought stress in early stages of the development could give a putative solution to such issues. *CBF* are members of a large protein family that is involved in the growth and development processes and responses to different environmental stress factors (cold, heat, drought, salt, etc.; Wu et al., 2022). *CBF* genes could thus have a role in barley in a cross-talk between the cold and drought response pathways, as already reported for Arabidopsis (Haake et al., 2002). Barley is one of the most important cereals cultivated and frost stress limits the amplification of its areal of cultivation. Dissect the molecular mechanisms behind the frost response linked to the drought stress could unlock the development of improved barley varieties for frost thus widespread the areal of cultivation (Hlaváčková et al., 2013).

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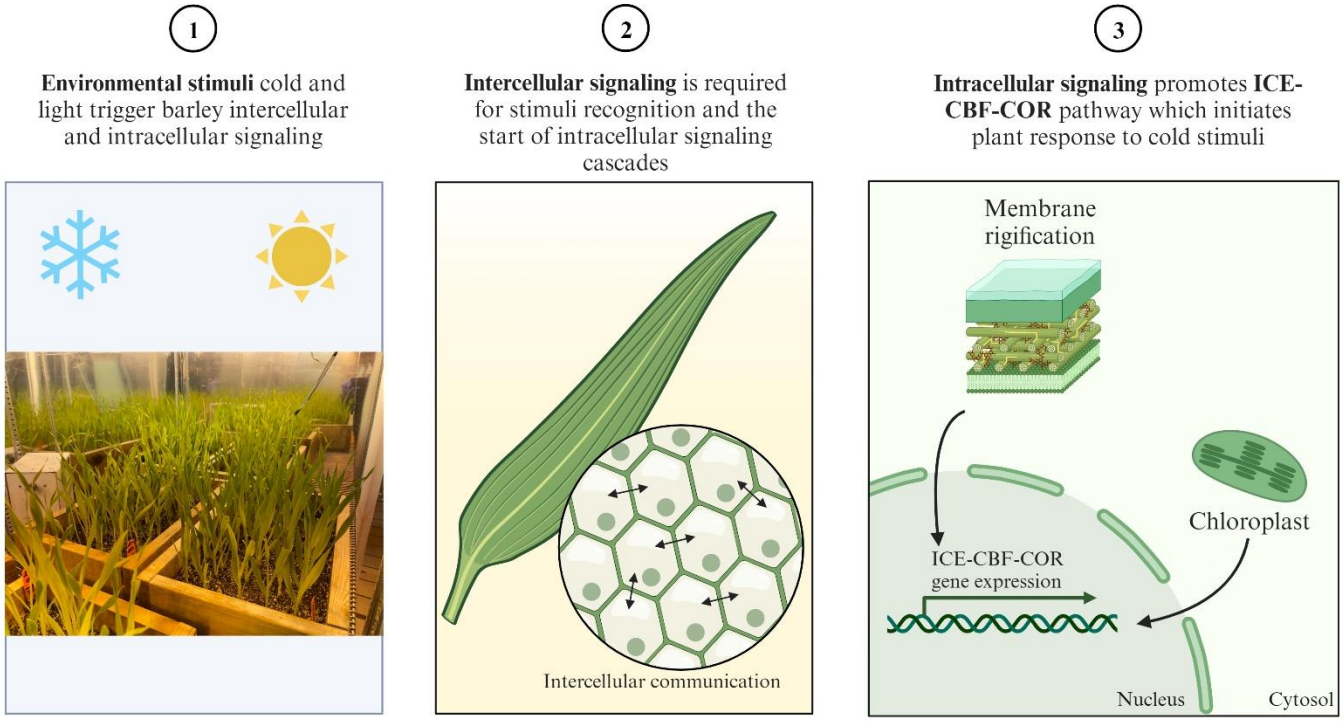
Chapter 4

Gene Expression Analysis of Barley FT Candidates, Dissecting the Effect of Low Temperature and Light Stimuli During Acclimation



4.1. Introduction

Plants have developed adaptive mechanisms to integrate different environmental signals such as temperature, circadian cycle, light and osmotic variations (Ciarmiello et al., 2014). The three most important external factors, temperature, light and circadian cycle, are crucial for proper acclimation and crop development (Franklin, 2009). The key factors in plant acclimation to low temperature are exposure to cold non-harmful conditions and circadian cycle in short day photoperiod (Colinet et al., 2012). This adaptive response requires the orchestration of transcriptional, biochemical, and physiological changes involving different genomic region (Kurepin et al., 2013; Peppino Margutti et al., 2023; Vaitkevičiūtė et al., 2022).

The *CBF* genes at *FR-H2* locus are well-known for their central role in the cold acclimation process (Francia et al., 2004), and through the CBF-COR pathway barley acquires the ability of surviving frost events. This process occurs when barleys sense a shift in the daily temperature below 12/10 °C, a shortening of day length, and an alteration in the light spectrum (Sandve et al., 2011; Thomashow, 2010). Of the total of 13 *CBF* orthologs harbored at *FR-H2*, the main gene elements involved in cold acclimation belong to the HvCBFIV-phylogenetic subgroup, which includes *HvCBF2*, *4*, *9* and *14* (for a review see (Caccialupi et al., 2023). The portion of the genome including *HvCBF2* and *HvCBF4* has been identified as a 22 kb segment present in multiple copies, and several authors reported that a higher copy number of the *HvCBF2A-HvCBF4B* segment correlate with higher degree of frost tolerance (FT) (Dhillon et al., 2017; Francia et al., 2016; Knox et al., 2010; Mareri et al., 2020). Studies on *HvCBF9* and *HvCBF14* reported these two genes as the major candidates for FT in barley (Campoli et al., 2009; Fricano et al., 2009; Novák et al., 2016; Todorovska et al., 2014); however, no structural variations have been reported. Several works analyzed the expression of the *HvCBF2*, *4*, *9* and *14*, highlighting that their induction and transcript accumulation after the cold stimulus leads to the induction of *COR* genes such as *HvCOR14B* and *HvDHN5* (Ahres et al., 2020; Novák et al., 2017; Vashegyi et al., 2013) that act as effectors in the cold acclimation process. The overexpression of *HvCBF2* was reported to increase *HvCOR14B* and *HvDHN5* transcript accumulation in spring genotypes (Jeknić et al., 2014) that reached a FT level similar to the winter hardy cultivar 'Dicktoo'.

A third QTL for frost resistance, named *FR-H3*, was discovered on the short arm of chromosome 1H that accounted for up to 48 % of the phenotypic variation in field survival in two bi-parental populations derived from either facultative x facultative or facultative x winter barley lines (Fisk et al., 2013). Three

candidate genes for *FR-H3* were then identified that could be acting in concert as a complex locus. A protein belonging to UDP-glycosyltransferase superfamily (putatively involved in FT via flavonoid glycosylation; (Schulz et al., 2016; Zhao et al., 2019), a low temperature-induced protein lt101.2 (Brown et al., 2001) that may slow plant growth under low temperatures and increase tolerance to water stress (Choi and Hwang, 2015), and a tetraspanin family protein putatively involved in plant development and response to stresses, including low temperatures (reviewed by Reimann et al., 2017).

Beside the cold stimulus, circadian regulators like *CCA1/LHY* and light spectra have been shown to contribute to cold acclimation through the control of the CBF pathway in Arabidopsis (Dong et al., 2011; Franklin and Whitelam, 2007). Similar regulatory mechanisms were also observed in barley; circadian cycle and different light spectra were reported to regulate the expression of *HvCBF* genes, especially HvCBFIV-phylogenetic subgroup differently (Ahres et al., 2021, 2020; Maibam et al., 2013; Novák et al., 2017). Circadian and light putative regulatory elements in the promoter regions of the *CBF* genes at *FR-H2* cluster were also identified in a silico analysis (Mareri et al., 2020). The light impact on the expression of *HvCBFs* affects also the *COR* transcripts accumulation and the increased frost tolerance (Gierczik et al., 2017). The main light spectra influencing the CBF-COR pathway seems to be far-red (FR) wavelengths and white light illumination (Ahres et al., 2022, 2020). However, temperature and light signals relayed to *CBF* expression follow separate signaling routes and the cascade mechanism is still not clearly understood (Deng et al., 2015; Novák et al., 2017).

Induction of the *CBF* transcription factors was reported to precede an increase in expression of *VRN-H1* transcripts as well, and the overall similarities between the expression patterns of *HvDHN5*, *HvCOR14B*, and *VRN-H1* during short-term cold treatment suggest that common signaling pathways regulate cold acclimation and the initial stages of the vernalization response (Oliver et al., 2013). Vernalization is the process by which prolonged exposure to cold induces flowering and thus constitutes the most critical factor determining grain yield in temperate cereals (Trevaskis et al., 2007). The action of *VRN-H1*, that coincides with *FR-H1*, occurs after low-temperature exposure and mediates the cold acclimation and vernalization response (Dhillon et al., 2010; Distelfeld et al., 2009; Hemming et al., 2009). The higher expression of *VRN-H1*, after vernalization was accompanied by lower *CBF* expression levels suggesting a correlation and/or dampening effect in interaction of those loci (Greenup et al., 2011; Stockinger et al., 2007). The effect of vernalization is cumulative, so cold exposure extents until a point when the vernalization response is saturated; once reached the point of saturation the frost tolerance begins to decrease (Trevaskis, 2010).

The study of gene expression in response to environmental stimuli is critical for understanding complex mechanisms, particularly in crops with a large genome such as barley (*Hordeum vulgare* L.). This chapter delves into the intricate mechanisms of gene expression analysis during the cold acclimation, focusing on four major *HvCBF* candidates (*2, 4, 9* and *14*), *VRN-H1*, and two well-known *COR* genes (*HvCOR14B* and *HvDHN5*). The aim is to dissect the effects of low temperature and light stimuli, two pivotal environmental factors influencing plant growth and survival, during cold acclimation. The plant growing/stress conditions applied in the present gene-expression experiment followed the schemes reported in the previous chapter, finetuned in function of the results obtained in the growth chamber/field experiments and using for the very first time *FR-H1/Fr-H2* QTL-NILs.

4.2. Materials and Methods

4.2.1. Plant Materials

Nure, Tremois and four QTL-near isogenic lines (QTL-NILs) were evaluated in this work. Nure is an Italian winter genotype, derived from crossing FO1236 = (Fior 40 x Alpha^2) x Baraka at Consiglio per la Ricerca e la Sperimentazione in Agricoltura, Genomics Research Centre, 29017, Fiorenzuola d'Arda, PC, Italy. Nure is a two-rowed winter-feeding barley with high FT, while Tremois [(Dram x Aramir) x Berac] is a French two-rowed spring malting barley with very low FT. The four QTL-NILs developed to mendelize the effect of the two loci were: *VRN-Nu*/Tremois (*vrn-h1*), *CBF-Nu*/Tremois (*Cbf*), *VRN-Tr*/Nure (*Vrn-h1*) and *CBF-Tr*/Nure (*cbf*) (for details see section *2.1*.).

4.2.2. Growing Conditions at The Centre for Agricultural Research-Hungary (ATK-MTA Experiment) – Rapid Temperature Decrease, no Pre-hardening Step, Led White Light

For the ATK-MTA experiment (Figure 1), plantlets were grown in growth chambers (Conviron PGV36; Controlled Environments Ltd.; Winnipeg, MB, Canada) under control (warm) conditions for 14 days with short-day photoperiods 8/16 h at 20 °C/15 °C (day/night), with 70–75% RH, at 180-220 µE light intensity under white light with a narrow peak at 450 nm LED (Philips Lumileds, LXZ1-PA01). After 14 days of control conditions, barley seedlings were exposed to 3 days of hardening (3/1 °C, 8/16 h day/night). The strong temperature shift (2 hours -red lines-) was applied during the dawn between the last day of control conditions and the beginning of hardening. Leaf samples at third leaf stage for RT-qPCR analysis were collected in three separate times during the day (morning, afternoon, and night) for

three consecutive days (black arrows): last day of warm condition (20/15 °C, 8/16 day/night) and the first and the second day at hardening conditions (3/1 °C, 8/16 day/night).

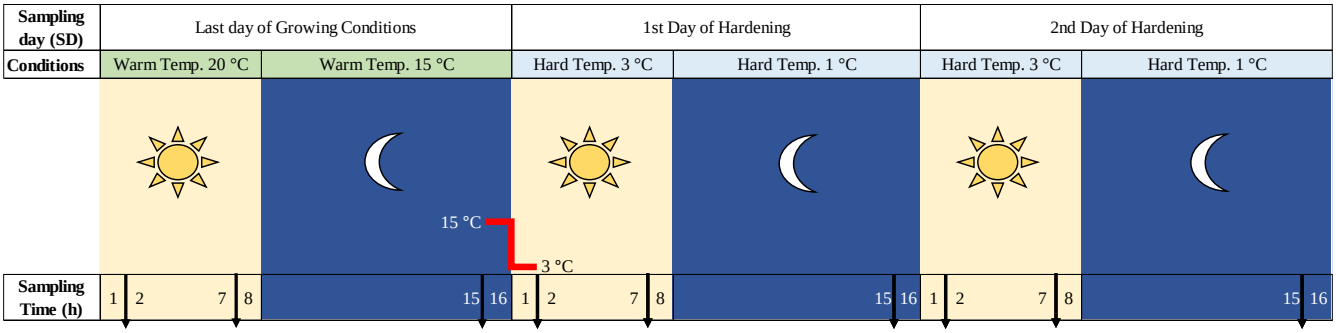


Figure 1. Growing conditions during the sampling timings for ATK-MTA experiment. Black narrow – sampling time. Red lines -. Cold temperature application.

4.2.3. Growing Conditions at Ohio State University-USA (OSU Experiment) – Gradual Temperature Decrease, Pre-hardening Step, MH/HSP Light

For the experiment held at Ohio State University- (Figure 2), expression analyses were carried out using plant material grown in Conviron BDR16 growth chambers (http://www.conviron.com/). Warm growth conditions (10 days once reached the third leaf phase) were as follows: 20/15 °C, 8/16 circadian d/n using a light intensity of 210 μM m^{-2} s^{-1} with 70–75% RH, metal halide (MH) and high-pressure sodium (HPS) lamps peaking at 450 and 600 nm for. During the last night in control conditions, the temperature was decreased (red triangle) from 20 °C to reach the pre-hardening temperature of 10 °C one hour before the lights switched on. A pre-hardening phase 10/8 °C, 8/16 circadian d/n using a light intensity of 210 μM m^{-2} s^{-1} from metal halide (MH) and high-pressure sodium (HPS) lamps was then applied for 6 days. Again, during the last night of in pre-hardening, the temperature was decreased (red triangle) from 10 °C to 6 °C one hour before the lights switched on. Hardening temperature treatment was set at 6/4 °C, 8/16 circadian d/n, and a low light intensity of 210 μM m^{-2} s^{-1} (MH lamps) was used. Relative humidity was set constant at 60%. Leaf samples at third leaf stage for RT-qPCR analysis were collected in two separate times during the day (morning and afternoon) in the last day of warm condition, in the first day of pre-hardening and in the first, fifth and twentieth day of hardening conditions. During the dark period (night) samples were collected just after the temperature shift (decrease) from warm do pre-hardening, and from pre-hardening to hardening, respectively (light blue arrows).

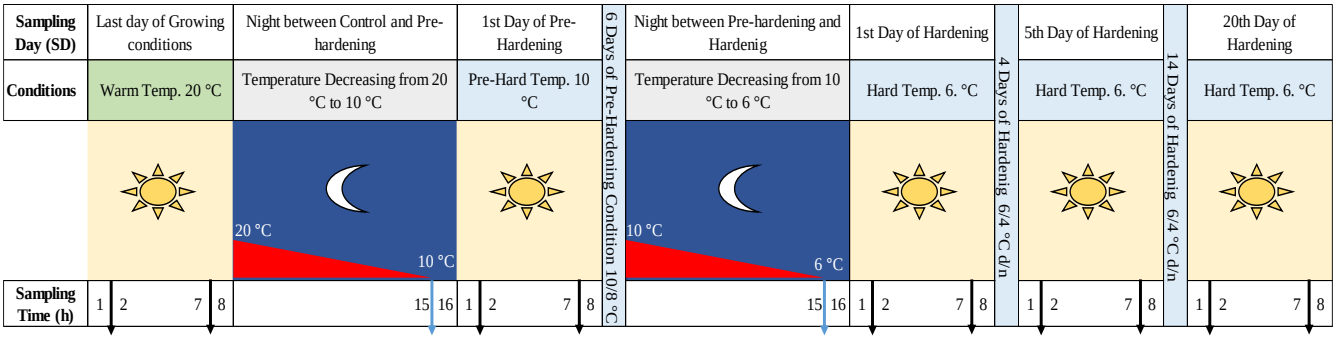


Figure 2. Growing conditions during the sampling time for the OSU experiment. Black narrow – sampling time. Light blue narrow – sampling time at the end of the shifting temperature. Red triangle – shifting temperature.

4.2.4. RNA Isolation, Quality Control, and cDNA Synthesis

4.2.4.1. ATK-MTA Experiment

For the ATK-MTA experiment, total RNA was extracted from 50 mg of leaf samples stored at −80 °C using the Direct-zol™ RNA MiniPrep kit (Zymo Research, Irvine, CA, USA) and quantified by Nanodrop 1000 (Thermo Scientific, Wilmington, DE, USA).

For each genotype, 3 biological replicates from 3 different plants were selected. The second leaf was cut 1 cm above the node, to obtain between 0.05-0.1 g of sample. Samples were put in eppendorf tubes containing small glass balls used for grinding and placed in nitrogen liquid. The eppendorf tubes containing the leaves were placed in the TissueLyser II (Qiagen, Hilden, Germany) tube container, left overnight in the -80 °C fridge. The next day samples were grinded by TissueLyser II (Qiagen, Hilden, Germany) for 30 seconds and once the process was completed, tubes were placed back in the -80 °C fridge. During the sample shredding process, the cold chain was maintained at all times.

RNA Purification was performed using Direct-zol™ RNA MiniPrep kit (Zymo Research, Irvine, CA, USA) supplied protocol.

Synthesis of cDNA was done from 1 µg of total RNA using M-MLV Reverse Transcriptase (Affymetrix, Santa Clara, CA, USA) according to supplier's protocol.

4.2.4.2.Ohio State Experiment

For the Ohio State experiment, total RNA was extracted from 50 mg of leaf samples stored at −80 °C using the RNeasy® PowerPlant® Kit (Qiagen, Hilden, Germany) and quantified by Nanodrop 1000

(Thermo Scientific, Wilmington, DE, USA). Synthesis of cDNA was done from 1 µg of total RNA using QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany) according to supplier's protocol.

4.2.5. RT-qPCR of Candidate Genes and Statistical Analysis

Primers were selected from literature as reported in Table 1. Candidate genes evaluated were *VRN-H1*, four *CBF* genes *HvCBF2, 4, 9* and *14* and two effector genes *HvCor14b* and *HvDHN5*. *HvCyclophilin* was selected as house-keeping gene (Burton et al., 2004).

Table 1. Primes selected for the candidate genes analyzed in this work. A - Burton et al., 2004; B - Morran et al., 2011; C - Ahres et al., 2020; D - Schmitz et al., 2000; E - Wójcik-Jagła et al., 2020; F - Francia et al., 2016; G - Mareri et al., 2020; H - Gierczik et al., 2017.

Target gene	Type		Primer sequences (5' → 3')	Gene ID NCBI	Amplicon length	Melting Temperature °C	GC %	Primer length	Literature
HvCyclophilin	Housekeeping	Forward	CCTGTCGTGTCGTCGGTCTAAA	AK253120.1	122	62.26	54.55	22	A
		Reverse	ACGCAGATCCAGCAGCCTAAAG			62.94	54.55	22	
HvCor14b	Effector	Forward	TTGAGGATGTGAGCAAATGAG	XM045114576.1	103	56.24	42.86	21	B C
		Reverse	TACATCGTCAATGACGAGACC			57.58	47.62	21	
HvDHN5	Effector	Forward	TGGCGAAGTTCCACCGTATGC	XM045098426.1	106	63.28	57.14	22	B C
		Reverse	ACGAAAACTGTTGCCACACTG			59.87	47.62	21	
HvBMA5 - VRN1	MADS	Forward	CTCCCCCTGTCGCAGATACCAG	XM_045128907.1	277	64.13	63.64	22	D
		Reverse	CATTCCTGCAGCTTGCTGCTTTAC			63.19	50	24	
HvCBF2A	DREB/AP2	Forward	ATGATGCGTGCCTCAACTTC	XM_045091538.1	104	58.91	50	20	E
		Reverse	GACGGCGTCCTTGATCTCTT			59.83	55	20	
HvCBF4	DREB/AP2	Forward	AGCGCCGCTCTGTTTTACA	XM_045091535.1	208	60.3	52.63	19	F G
		Reverse	AGCAGTCGAACAAATAGCTCCA			60.03	45.45	22	
HvCBF9	DREB/AP2	Forward	AGCACTACTGTCAACATGTAG	NC_058522.1	162	55.37	42.86	21	B H
		Reverse	CCTTGATTTCGATTCATGGAG			54.73	42.86	21	
HvCBF14	DREB/AP2	Forward	AGCCGTTGACGAGAAGGAAGTC	XM045092011.1	112	62.54	54.55	22	F G
		Reverse	GTAGCATGATCCGGCATCCAT			60.34	52.38	21	

Quantitative real-time PCR was performed using the QuantStudio™ 3 System (Applied Biosystems, Foster City, CA, USA) in 20 µl reactions containing 10 µl of SYBR Green PCR Master Mix (Applied Biosystems), 2.5 µl of each primer (0.1-0.8 µM), 5 µl of cDNA (2 ng/µl). PCR conditions were holding

stage for 95 °C for 10 min, PCR stage 40 cycles of 95 °C for 15 s and 60 °C for 1 min and melt curve stage 95 °C for 15 s 60 °C for 1 min and 95 °C for 1 s. In this study, each single biological replicate corresponds to leaves collected from three independent plants, which were homogenized for RNA isolation, then used for cDNA syntheses. Then three biological replicates for each sample were pooled together and three technical replicates for each pool per plate were performed. The relative gene expressions were calculated using the ΔΔCt method (Livak and Schmittgen, 2001) with Ct values normalized by the Ct values of house-keeping genes *HvCyclophilin* (Burton et al., 2004).

4.3. Result

4.3.1. RNA Isolation and Quality Control

After extraction, RNA yield per sample ranged from 200 to 600 ng/uL, while Nanodrop absorbance at 260 nm and 280 nm were used to assess the purity: only samples with ratios greater or equal of 2.0 were generally accepted as "pure RNA" for further analysis.

In addition, regarding the RNA samples extracted at OSU, the RNA quality was assessed by Agilent spectrophotometric analysis, which showed that the extracted RNA was intact and of high purity. Good RNA samples are characterized by the absence of smaller well-defined peaks between the two main ribosomal peaks. RNA quality is determined by quantification of the 25S to 18S ribosomal RNA ratio using the RNA integrity number (RIN) an algorithm method (Schroeder et al., 2006). Samples were selected for the cDNA synthesis only with a RIN ratio higher than four.

4.3.2. RT-qPCR of Candidate Genes and Statistical Analysis

4.3.2.1. *VRN-H1*

A quantitative real time was performed to determine the relative gene expression levels of *VRN-H1,* selected *HvCBF* candidate genes, effector genes and consider the circadian.

In these experiments, barleys grew up in short day conditions (8/16 hours day/night) to mimic the natural circadian cycle during the cold periods of temperate regions (Fig. 1 and Fig. 2). In both experiments (Figure 3), the accumulation of *VRN-H1*'s transcripts at steady state (20/15 °C d/n) and after the cold/hardening induction was significantly different between Nure and Tremois in case of either short (first and second day at 3/1 °C d/n), medium (fifth days at 6/4 °C d/n) or long (twentieth days at 6/4 °C d/n) exposure to low temperature (Fig. 3). A higher transcripts accumulation was observed in Tremois

compared to Nure at all the timings especially after 5 and 20 days when an induction was observed for all genotypes. *VRN-H1* expression levels in NILs with Tremois backgrounds were analogous to Tremois highlighting that other than only *FR-H1* and/or *FR-H2* factors in the spring background affect the expression levels bringing expression of winter allele to levels similar to those of spring allele. On the other hand: the NIL carrying *Vrn-H1* spring allele from Tremois in Nure background (*VRN-Tr*/Nure) showed expression levels similar to Tremois, and thus was not influenced negatively by the winter background factors, while the NIL with *cbfs* from Tremois (*CBF-Tr*/Nure) had identical expression levels and profiles of Nure as expected.

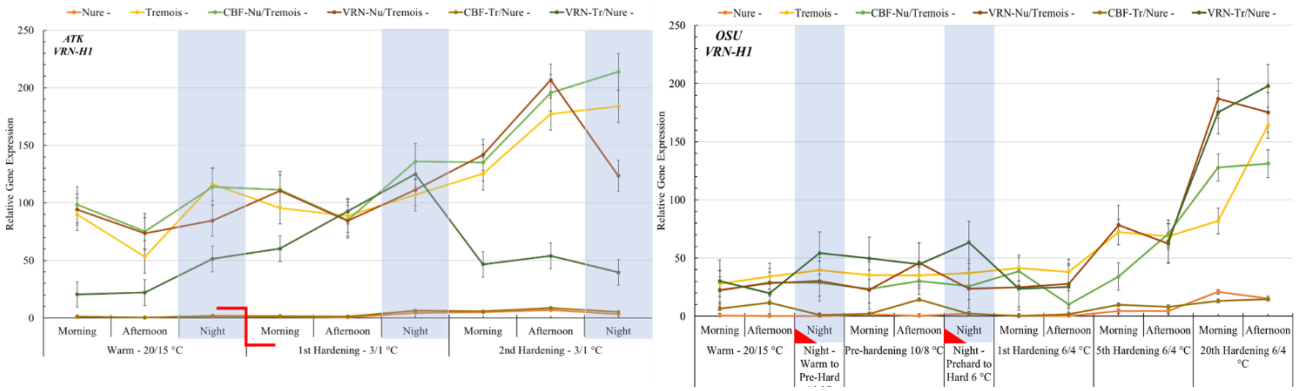


Figure 3. *VRN-H1* expression levels for ATK (left) and OSU (right).

4.3.2.2. *HvCBFs*

The expression levels of *CBF* genes were compared among genotypes and experiments. Expression profile obtained in the experiments conducted at obtained in experiment conducted at ATK-MTA (rapid temperature decrease to hardening condition, white led light) are shown in Figure 4, and those at Ohio (pre-hardening step, gradual temperature decrease, metal halide (MH) and high-pressure sodium (HPS) lights) are presented in Figure 5.

A clear induction of expression by cold exposure was observed for all *CBFs* but *HvCBF2*. As it can be observed, as a general rule, the levels of expression (included control, warm condition) were higher in the former experiment, the only exception was *HvCBF4* for which similar expression levels were obtained between the two experiments. The pattern of expression of this gene observed for all genotypes was similar both in control and after the first temperature shift in both experiments, suggesting that the temperature variation already to pre-hardening condition was sufficient to trigger similar changes in expression to what is observed when the hardening temperature is applied immediately (ATK-MTA). No

further induction was then observed when hardening temperature was applied in the OSU experiment (Fig. 5). As far as *HvCBF4* gene is regarded in ATK-MTA experiment (Fig. 4 and Fig. 6), its expression was four times higher in *CBF-Nure*/Tremois NIL respect all other genotypes, both under the control conditions, and after the cold was applied and thus this difference between this genotype and all the others seems not temperature-dependent. What's interesting in OSU experiment *HvCBF4* pattern (two distinct peaks; one during control condition and one after cold application) was observed for all genotypes (Fig. 7), however the level of expression *CBF-Nu*/Tremois NIL remained four times higher than other genotypes, apart from *VRN-Tr*/Nure that reached similar levels, but only after cold application. In the experiment held at ATK-MTA, the peak expression of *HvCBF4* alle in Nure and NILs with Nure background was "delayed" (night sampling 24 hours after cold induction) respect to what was observed for Tremois, and for all genotypes in the other experiment. Given that the differences between the two experiments were observed already under control conditions, they might putatively be attributed to the different light applied. It is worth noting, however, that no differences were observed for *CBF-Nu*/Tremois NIL.

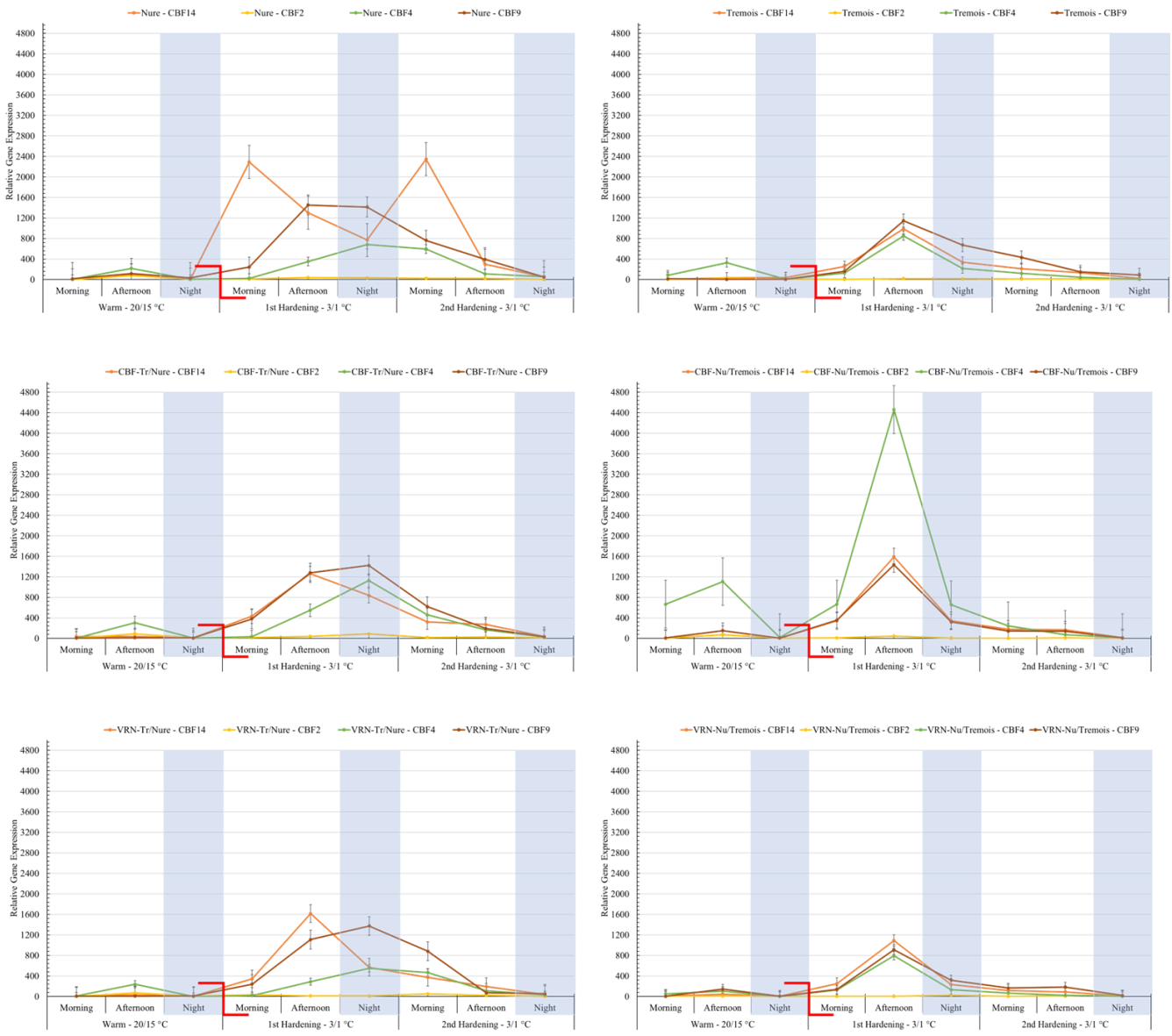


Figure 4. Comparison of all the *CBFs* expression levels for all the genotypes in ATK.

Figure 5. Comparison of all the *CBFs* expression levels for all the genotypes in OSU.

Moreover, the expression levels of winter *HvCBF4* allele in OSU experiment (Fig. 7; MH/HSP light) were the same regardless the winter or spring background, while the expression of the *HvCBF4* spring allele was slightly higher under influence of winter background than in the parental spring genotype Tremois. On the other hand, while the genotypes were grown under white light (ATK experiment, Fig. 6), winter *CBF* allele followed the same pattern (the peak "delayed" as described before but only with the winter background, in the presence of spring background (NIL *CBF-Nu*/Tremois) its pattern follow Tremois (spring parental genotype) showing peak expression during the afternoon. The spring *HvCBF4* allele was expressed in the same way in the spring background, while in the winter pone follows the

expression pattern of the winter allele. This suggest and influence of background factors on *CBF* alleles expression, while at OSU experiment (MH/HSP light) when the maximum peak of expression has the same timing in all genotypes is less visible.

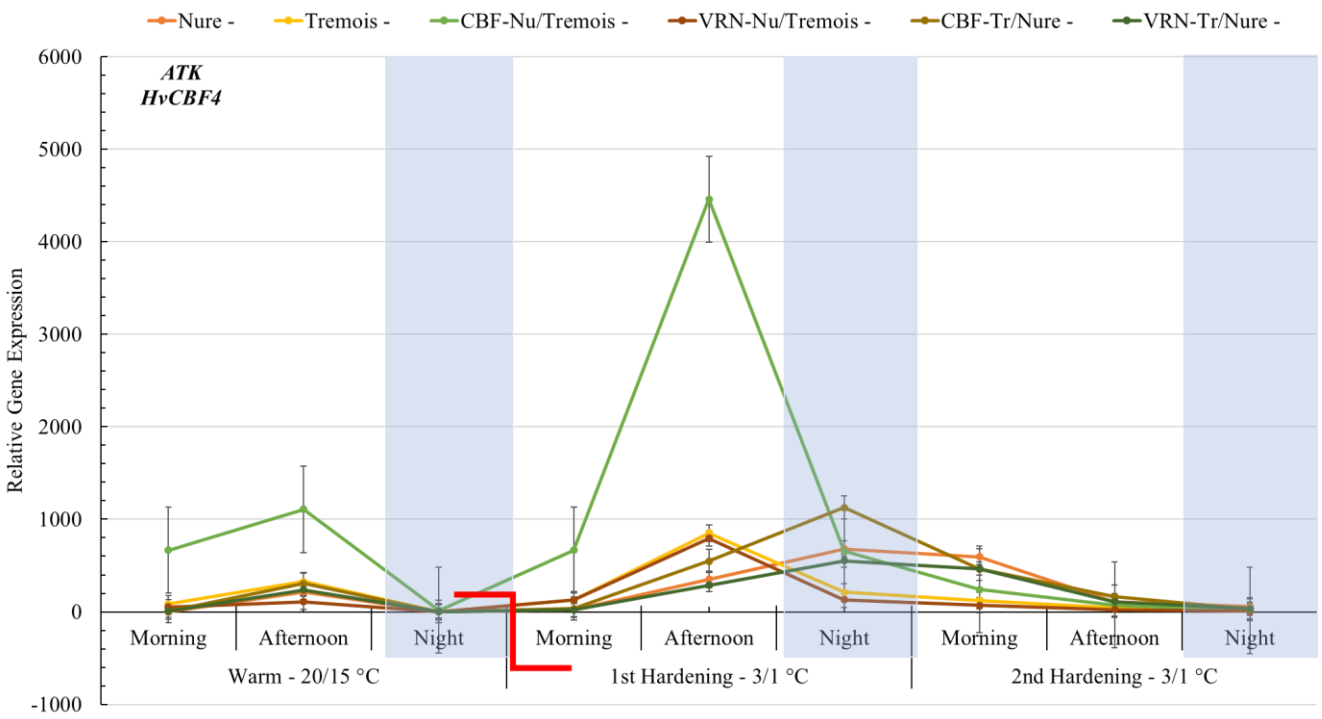


Figure 6. *HvCBF4* expression levels in ATK.

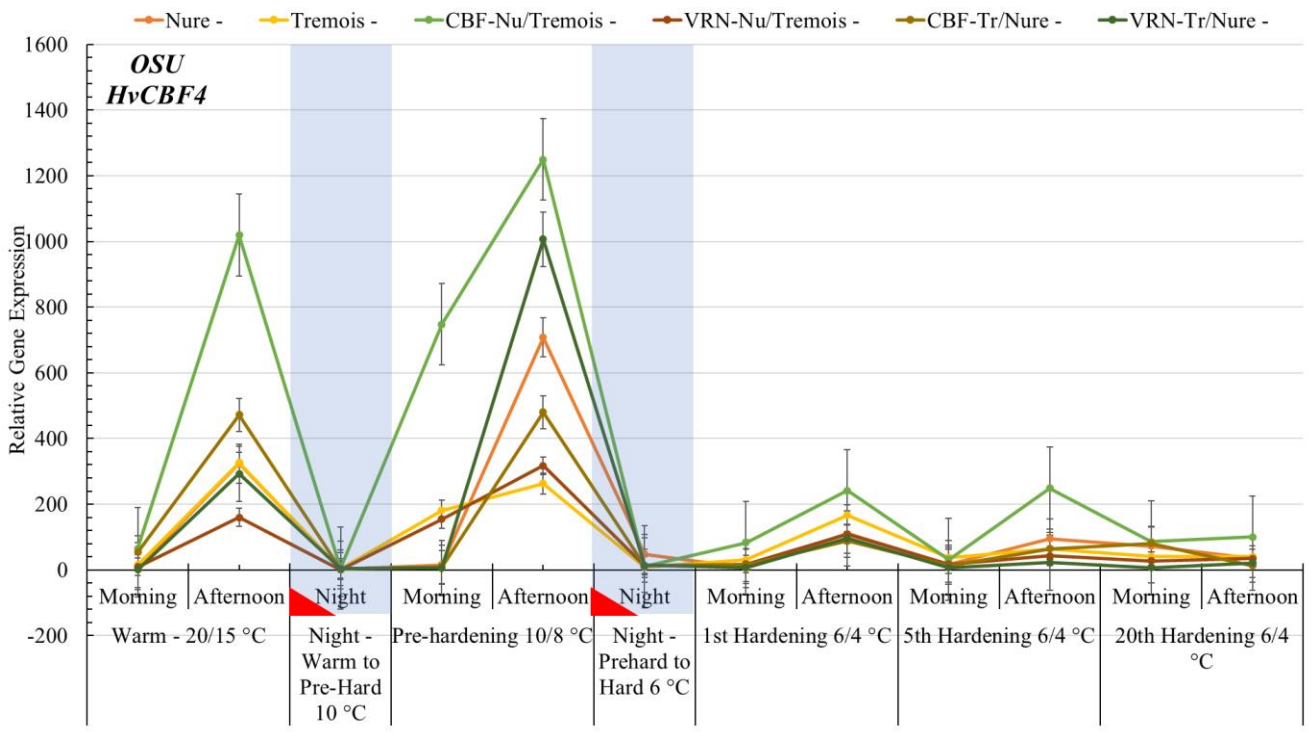


Figure 7. *HvCBF4* expression levels in OSU.

As far as expression profiles of *HvCBF2* are regarded it was expressed at very low levels at all timings in the ATK-MTA experiment (Fig. 4 and Fig. 8) when plants were grown under white light, while its levels resulted higher, similar to that of other *CBFs* in the OSU experiments (Fig. 5 and Fig. 9) were MH/HSP light was applied. What's interesting in that experiment *HvCBF2* expression levels were particularly high in two NILs with Nure background, especially under the control conditions, in which no circadian effect was observed for his gene, contrary to what was observed for other genotypes and to what has been already reported in literature. Such situation was not observed when white light was applied (ATK). Circadian regulation seems thus to depend not only on the combination of alleles at *HvCBF2* and *VRN-H1*, but also light spectrum that regulate some factors in the "background."

The use of reciprocal NIL lines enabled to highlight the interesting observation that *HvCBF4* winter allele is overexpressed in the spring background, while for *HvCBF2*, expression of spring allele is highly induced when expressed in winter background (ATK, Fig. 8; OSU, Fig. 9).

All genotypes expression seems reduced after temperature shift to hardening condition in both experiments (Fig. 4 and Fig. 5).

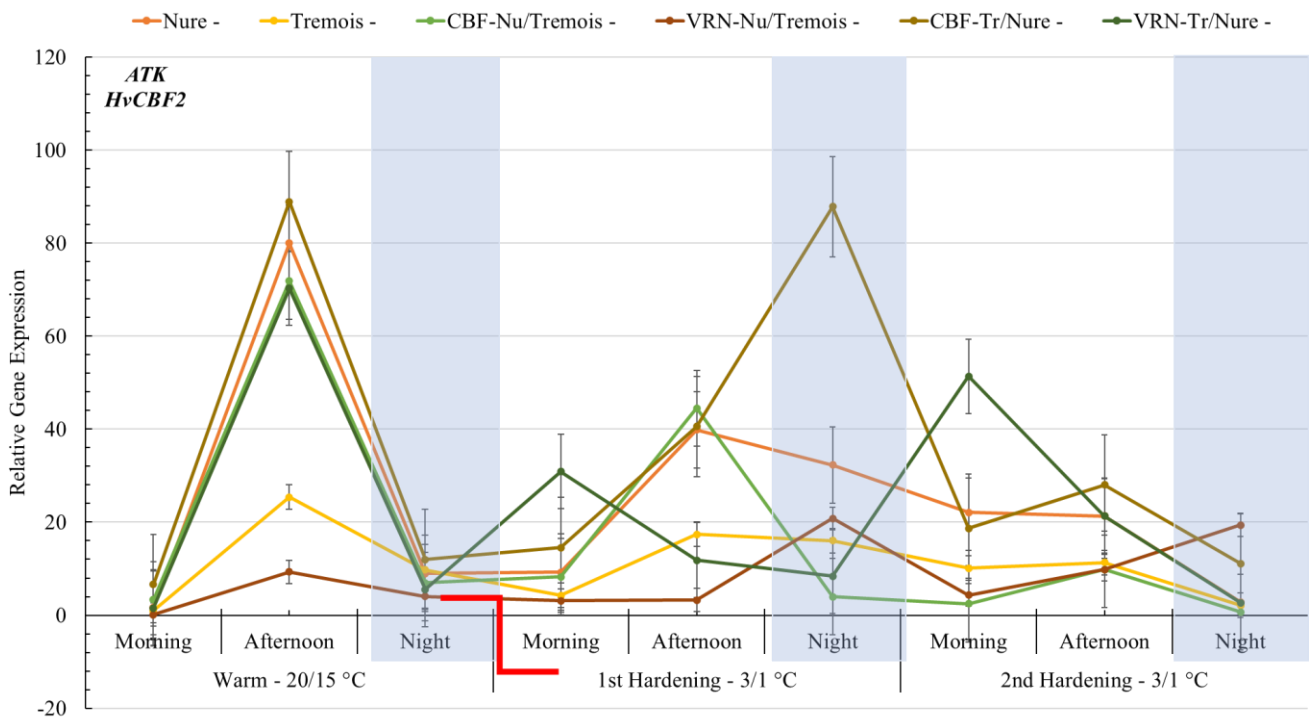


Figure 8. *HvCBF2* expression levels for all the genotypes in ATK experiment.

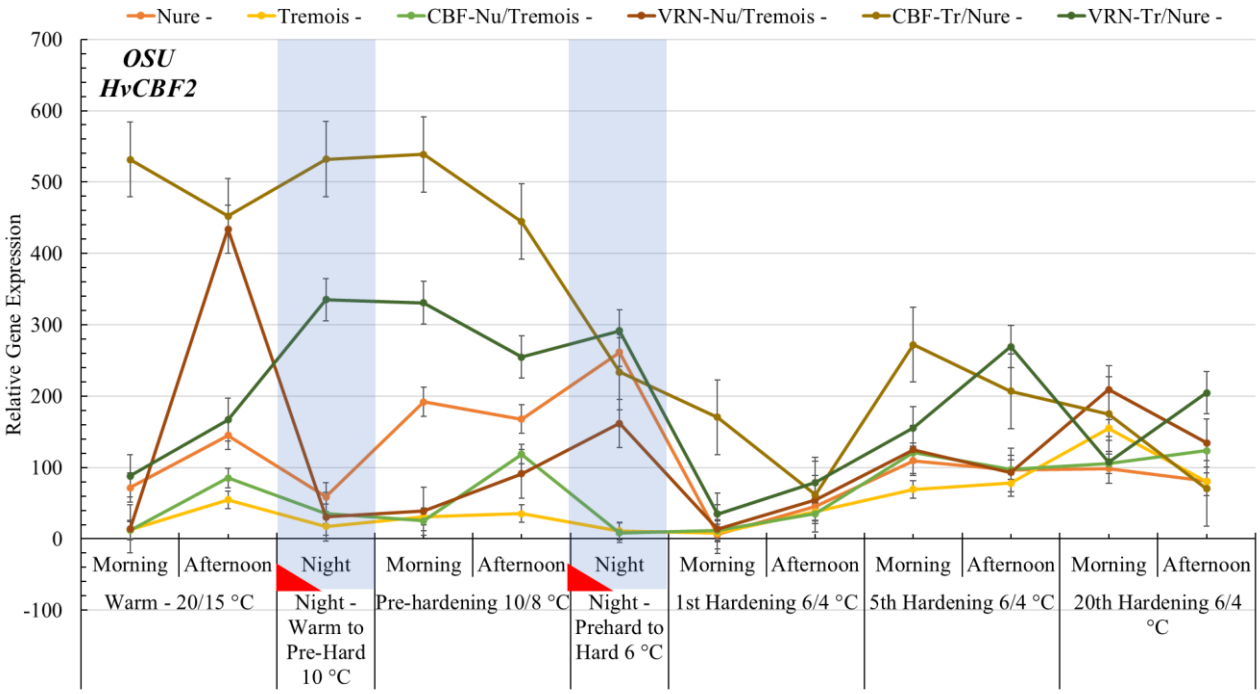


Figure 9. *HvCBF2* expression levels for all the genotypes in OSU experiment.

For *HvCBF9* interesting differences were observed between the two experiments (Fig. 10 and Fig. 11). First of all, lower levels of *HvCBF9* expression were observed for all genotypes in the ATK-MTA experiment already in the warm condition and that suggest a strong influence that light change (ATK-MTA vs OSU) has on the *HvCBF9* expression levels.

Moreover, higher induction (10-fold) was observed in ATK-MTA experiment, when the hardening temperature was applied and then the levels drop down being however constantly much higher than those obtained in OSU experiment in which only a slight induction was observed for both pre-hardening and hardening temperature. This observation suggests a strong influence of both light quality and temperature applied. Moreover, for ATK-MTA experiment a different pattern was observed for Nure and NILs with Nure background vs Tremois and NILs with Tremois background. Both reached the maximum expression levels after cold induction in the afternoon, however in the former group the high level was then maintained, while dropped immediately in the latter. Such difference was not observed in OSU experiment, suggesting, that the light spectrum influence on the gene expression, also in this case, as observed for *HvCBF4* and *HvCBF2*.

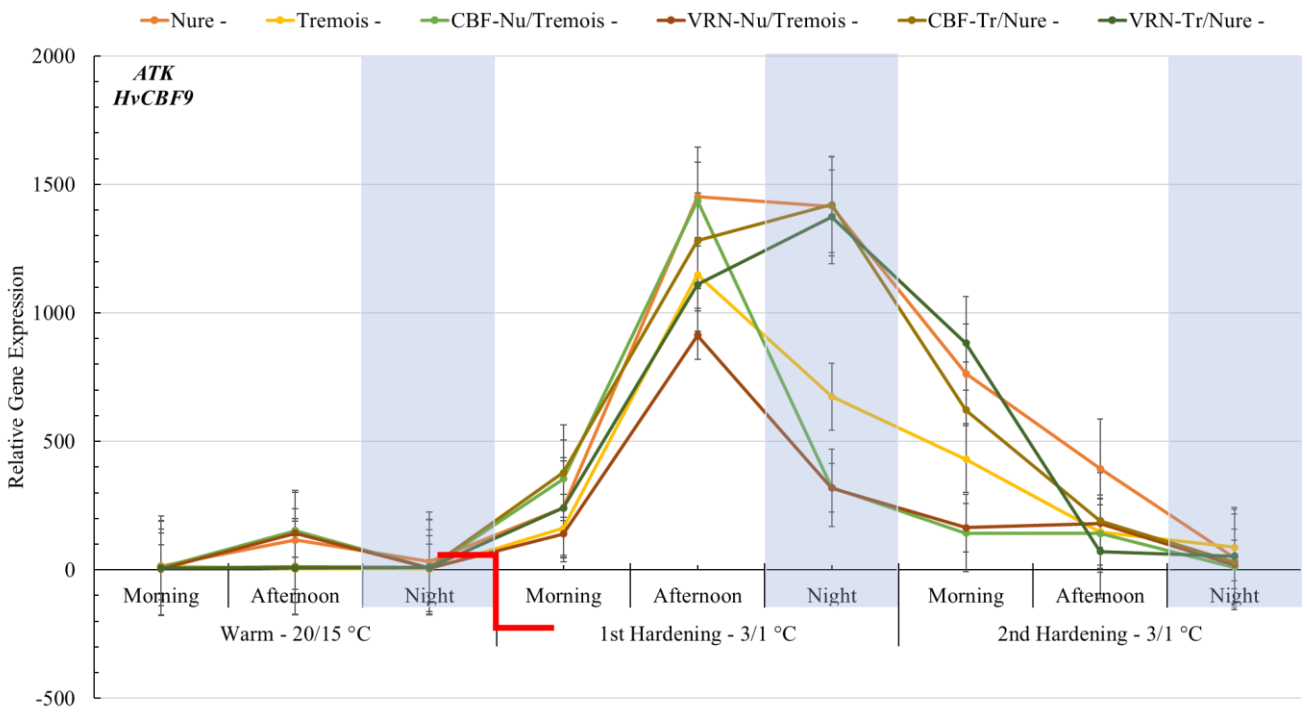


Figure 10. *HvCBF9* expression levels for all the genotypes in ATK experiment.

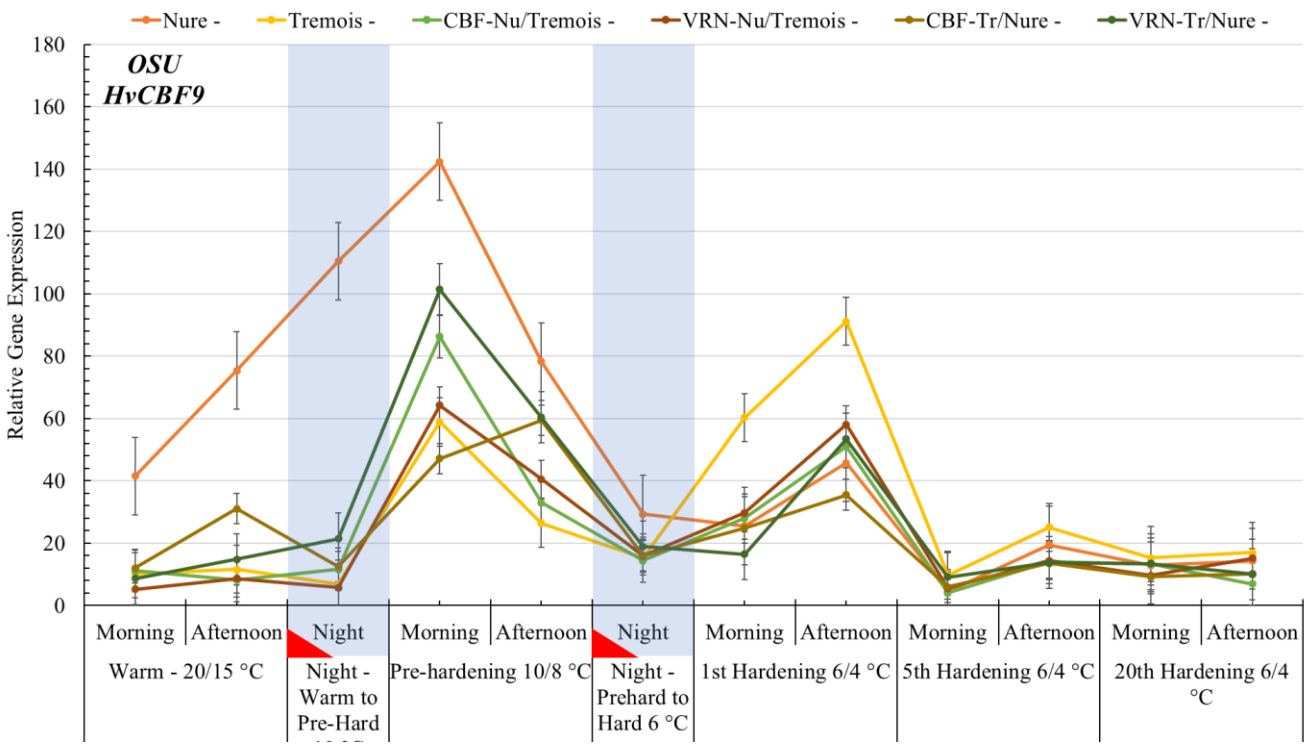


Figure 11. *HvCBF9* expression levels for all the genotypes in OSU experiment.

For *HvCBF14*, In ATK-MTA experiment (Fig. 12) expression levels and pattern are different in Nure than in all other genotypes – the expression reaches its maximum level at the first timing following the cold induction and then were induced for the second time the day after. For all other genotypes only one peak of maximum expression was observed at the second timing (afternoon) after the cold induction. Moreover, the expression levels reached by *HvCBF14* in Nure were higher than in all other genotypes. In OSU experiment (Fig. 13) this characteristic Nure pattern after cold induction stimulus was not observed, and as the expression levels in control conditions were the same in both experiments it seems that the determining factor was the rapid and strong temperature decrease.

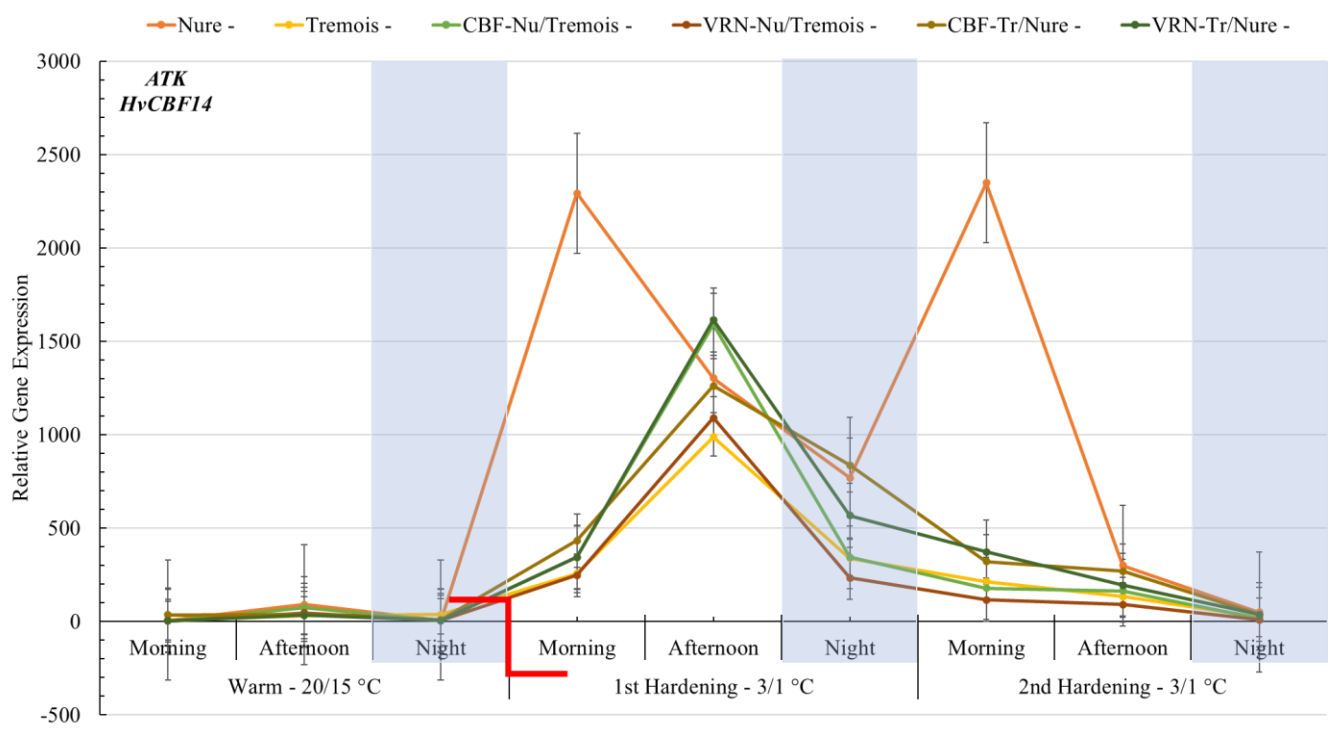


Figure 12. *HvCBF14* expression levels for all the genotypes in ATK experiment.

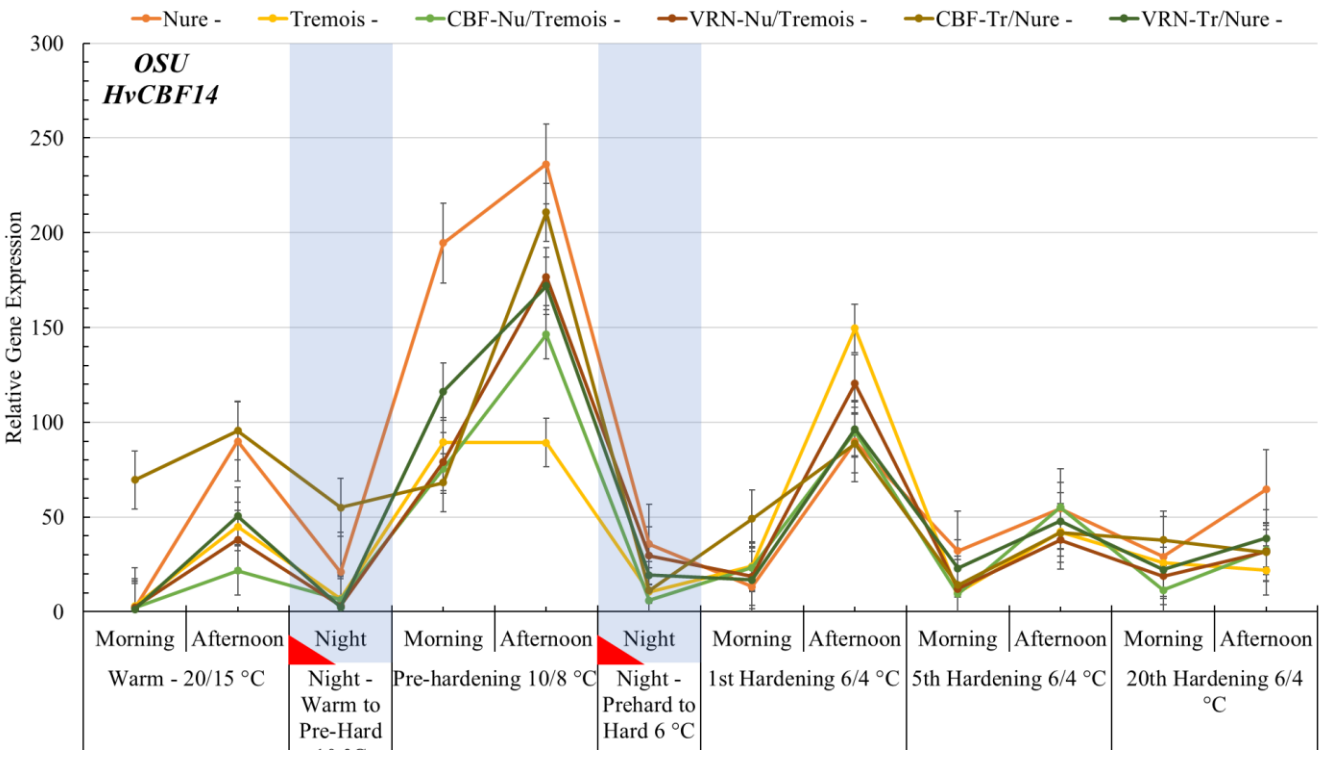


Figure 13. *HvCBF14* expression levels for all the genotypes in OSU experiment.

4.3.2.3. *HvCOR14B* and *HvDHN5*

All the genotypes showed similar expression pattern for the *HvCOR14B* (Fig. 14; left figure - ATK; right figure - OSU); very low accumulation during the warm phase and induction that didn't follow immediately temperature decrease in neither of the studies but was delayed for few hours and was strongly upregulated only after 24 hours. This "shift" response to low temperature might suggest that its expression depends on factors that in turn are regulated by decrease in temperature. However, differences in the expression levels between Nure and Tremois were observed only after prolonged exposure to low temperature: for the ATK-MTA experiment (Fig. 14, left) differences occurred only during the second day, while huge differences were observed in OSU (Fig. 14, right) experiment when the temperature shifted to hardening temperatures. Higher expression levels were observed for Nure and both NILs with Nure background respect Tremois and NILs with Tremois background.

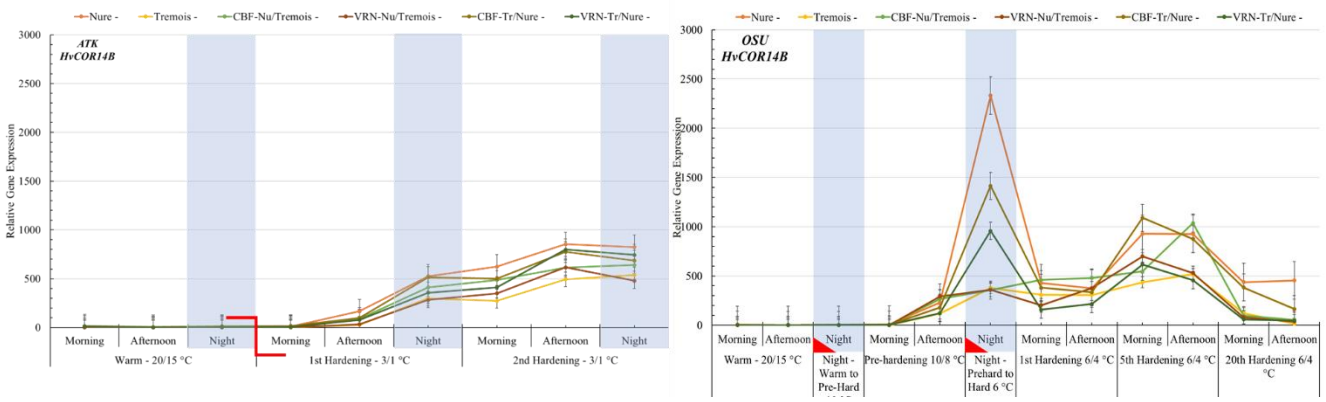


Figure 14. Expression levels for *HvCOR14B* in ATK (left) and OSU (right).

The expression profiles obtained for *HvDHN5* in the ATK-MTA experiment (Fig. 15; left figure - ATK; right figure - OSU) corresponded to those observed for *HvCOR14B*; and consisted in induction delayed after temperature shift and higher induction only after 24 hours. For the OSU experiment (Fig. 15, right), the shift from warm to pre-hard was sufficient to upregulate *HvDHN5* expression to its maximum level already few hours after exposure however, what must be stressed, both the peak expression and the overall expression level were much lower than those observed in ATK-MTA experiment. Interestingly, after five days the transcripts accumulation were higher in Nure and NILs harboring reciprocal *CBF* alleles, (*CBF-Tr*/Tremois and *CBF-Nu*/Nure; Fig. 15, right).

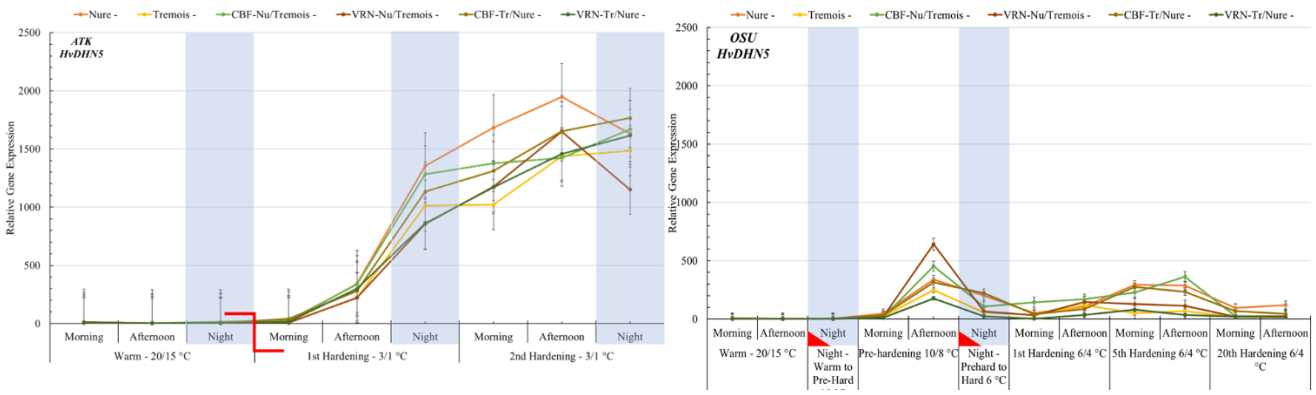


Figure 15. Expression levels for *HvDHN5* in ATK (left) and OSU (right).

4.4. Discussion

Since the discovery of the two QTLs (*FR-H1* and *FR-H2*) and their structural variations involved in the acquisition of frost tolerance in barley (*Hordeum vulgare* L.), important efforts were made to uncover the molecular mechanisms underlying their function and interaction. The present thesis aims to further investigate the role of *FR-H1* and *FR-H2* in the cold acclimation pathway, which results in the acquisition of frost tolerance. Accordingly, a multidisciplinary approach ranging from phenotyping in diverse conditions to single gene expression profile was applied to a set of Near Isogenic Lines (NILs) that carry different allele combination at *FR-H1* and *FR-H2* in reciprocal backgrounds.

The combined application of growing chamber freezing tests and winter survival rate in open field trials allowed generating information regarding the role of *FR-H1* and *FR-H2* in determining the phenotype. To the author knowledge, this is the first time that mendelizing *Frost Resistance* loci in *ad hoc* Near Isogenic Lines (NILs) developed has been used to the test their effects on frost tolerance. Hence, three different experiments were conducted in controlled conditions as an explorative trial, and the four QTL-NILs had not yet been tested together for resistance. As the conditions of experiment needed fine-tuning, the freezing study was based on literature review concerning FT testing (Francia et al., 2020; Rizza et al., 2016; Tondelli et al., 2014). Trials performed at ATK-MTA and CREA-GB differed only for the step when the temperature was shifted from warm control condition (20/15 °C) to hardening (3/1 °C), and in particular, it was more gradual for CREA-GB experiment where an additional step at 12/7 °C was included. While no statistically significant differences were observed for genotypes in freezing experiment at ATK-MTA; a positive effect on frost tolerance was observed for a line harboring *FR-H2* winter allele in spring susceptible background at CREA-GB experiment. Moreover, two open field trails

were performed in two climatically distinct habitats, Northern Italy, a typical temperate subcontinental climate, characterized by mild/cold and rainy winters followed by hot/humid summers, and Ohio - USA a typical humid continental climate characterized by severe cold winters and hot/humid summers. Interesting results were observed in the Ohio field trial for the NILs; while substituting the Tremois *FR-H2* with winter allele led, as expected, to frost resistance comparable to that of Nure, unexpectedly the substitution of one of the Nure *FR-H1* or *FR-H2* loci with spring allele did not affect field FT in both NILs with Nure background.

Profound differences were observed between the two trials, Ohio conditions turned out to be very discriminative for studying differences in barley FT; however, due to the seed shortage, the experiment could only give a preliminary indication and has to be repeated in coming years. All those trials highlighted the fact how much FT is dependent on the conditions in which the experiment is conducted, and it would be thus preferable to carry them out in different location characterized by different climatic conditions (within the geographical area of cultivation of a given species). The same rationale must thus be applied also to the growing conditions/ protocols used in the gene expression experiments.

In the present thesis, two kinds of experiments were thus conducted to study the gene expression during the hardening stage; one where the hardening temperature was obtained by a rapid shift (ATK-MTA) and another where the gradual decrease of temperature also included 6 day of pre-hardening condition (OSU). The latter was set to mimic the conditions that actually can be observed in field, when the shift of temperature from 20/15 °C (warm control conditions applied in the growing chamber) to 3/1 °C (hardening temperature) is quite improbable, or at least not the most frequently observed. Moreover, trying to dissect the effects of light and cold stimuli on *CBF* gene expression two different light spectra were used that led to interesting differences observed for the gene expression induction profile and intensity.

Nowadays, always more often, lighting based on light-emitting diodes (LEDs) is being used in plant indoor cultivation, and different combination of wavelength, included white light with extended spectrum range, are being applied optimized to booster plant photosynthesis and productivity (Sena et al., 2024). Other type of lamps that are commonly used in greenhouses and plant growth chambers are high-intensity discharge (HID) lamps, such as high-pressure sodium lamps (HPS), and or/metal halide (MH) (Paradiso and Proietti, 2022). The latter, moreover, are used to supplement sunlight during low solar radiation periods (Paradiso and Proietti, 2022). After the discovery of a notable influence of light quality on the expression of *CBFs* genes, in recent years both kind of illuminations were used in several studies

conducted on gene expression regulation by cold and/or light (Dhillon et al., 2017, 2017; Gierczik et al., 2019).

Under the same temperature and photoperiod (i.e., in control conditions), we observed striking differences in expression profiles of analyzed genes between the two experiments that were most probably due to the spectral compositions of the light sources.

FR-H2 encodes a cluster of 13 *CBF* genes which interact with a complex network of genes and it is influenced by cold, circadian and light stimuli following distinct pathways (Gierczik et al., 2017; Hwarari et al., 2022; Novák et al., 2017). *HvCBF2*, *HvCBF4 HvCBF9* and *CBF14* were selected for this thesis as reported previously to be involved in cold acclimation and frost resistance (Badawi et al., 2007; Campoli et al., 2009; Francia et al., 2020; Fricano et al., 2009) or as their expression resulted light-regulated either at warm or during the cold acclimation (Ahres et al., 2020c; Gierczik et al., 2017). In addition, *HvCBF4* and *HvCBF2* were selected for the presence of CNV, whereas for *HvCBF9* and *HvCBF14* no CNV was reported so far. Several authors reported that higher copies of the *HvCBF2A-HvCBF4B* segment correlate with higher degree of FT (Dhillon et al., 2017; Francia et al., 2016; Knox et al., 2010; Mareri et al., 2020). As far as *HvCBF9* and *HvCBF14* are regarded, they have emerged as the major candidates for the frost tolerance in barley in several works (Campoli et al., 2009; Fricano et al., 2009; Novák et al., 2016; Todorovska et al., 2014).

Under white LED light, nearly the same profiles were obtained for Nure and NILs with Nure background and Tremois and NILs with Tremois background, respectively; the only two exceptions were *HvCBF14* in Nure (an additional peak) and *HvCBF4* in *CBF-Nu*/Tremois NIL (higher peak expression amplitude). What is outstanding is that expression profiles of all *HvCBF4*, *HvCBF9* and *HvCBF14* seemed nearly synchronized, showing exactly the same pattern and similar level in Tremois and NILs with Tremois background – such a behavior was not observed neither for Nure at ATK-MTA nor for both genotypes in the OSU experiment.

Differences were observed for *HvCBF4* expression patterns between the two experiments that could be attributed to both cold and light stimuli. Under control condition, lower levels were observed in OSU experiment for all lines but one, suggesting clear light influence. However, it is interesting that on the other hand, the white light had the same effect than MH/HSP on *CBF-Nure*/Tremois line characterized by extremely high expression, higher that all other genotypes. Under MH/HSP light the expression levels of winter allele were the same regardless the background (winter or spring), while for spring allele an

increase of expression was observed in winter background (observed under white light as well). What is interesting in ATK-MTA experiment is that the peak of expression was "delayed" and lower in Nure and NILs with Nure background than in genotypes with Tremois background and respect to what was observed for all plants grown under MH/HSP. Exactly the same effect was observed for *HvCBF9* expression. Similar results were obtained by Gierczik et al. (2017), who reported peak "shifting" as a function of light for *HvCBF4* and *HvCBF9* in Nure, and that might suggest that differences observed in the present study are due to light spectra changes.

A similar influence of light on expression profile was observed for *HvCBF2*; that was expressed at particularly low levels at all timings in the ATK-MTA experiment when plants were grown under white light. The expression levels in that experiment were much lower than those recorded for all other *CBFs*, while its levels resulted higher, similar to that of other *CBFs* in the OSU experiments were MH/HSP light was applied. Noteworthy, in that experiment *HvCBF2* expression levels were particularly high in two NILs with Nure background, especially under control conditions, in which no circadian profile was observed and contrary to what observed for other genotypes and reported in literature (Gierczik et al., 2017; Hwarari et al., 2022; Novák et al., 2017). Such situation was not observed when white light was applied (ATK-MTA). Circadian regulation seems thus to be regulated by light spectrum that control some factors in the "background", and by the combination of alleles at *HvCBF2* and *VRN-H1*. On the other hand, the cold induction acted rapidly after the stimulus on the expression levels in the same way for both the experiments, regardless the hardening or pre-hardening temperatures were applied.

Metal halide (MH) and high-pressure sodium (HPS) had two different wavelength peaks (450 nm and 600 nm, respectively). The perception of changes in the spectral composition was already reported to lead to modification of the *CBF* expression through the phytochrome-CBF regulon pathway (Gierczik et al., 2017). Phytochromes play an important role in light-mediated signaling to activate cold-induced gene expression through the CBF pathway (Crosatti et al., 1999; Kim et al., 2002). Different grades of light spectra responses were associated with transcriptional reprogramming, plastid signals, and photosynthesis. regulated by transcription factors from MADS-box, WRKY, and NAC families, and in specific photoreceptors such as phytochromes (PHYA, PHYB, PHYC) and cryptochromes (CRY) during vernalization (Rodríguez Del Río et al., 2023; Szűcs et al., 2006). In barley, several work highlighted the role of the light spectra in the frost tolerance that modifying the *CBF* transcripts accumulation (Ahres et al., 2022, 2020c); however, this is not supported by the presence of any G-Box-Like and CAMTA3 binding site in the promoter of *HvCBF2* (Mareri et al., 2020). G-Box-Like motifs are necessary for

transcriptional regulation by circadian pseudo-response regulators binding basic helix-loop-helix transcription factor, Phytochrome-interacting factor 4 (Liu et al., 2016). *CAMTA3* transcription factors are not directly influenced by the lights, however this same stimulus influences their expression through PI4K-PLC-Ca^{2+} pathway (Gierczik et al., 2017; Liu et al., 1998; Ranty et al., 2016; Xiong et al., 2002). The effect of the light spectra in the cold acclimation has been proven, however its related pathways connected to *CBF* genes in barley is still under investigation (Rodríguez Del Río et al., 2023). The lack of binding sites in the promoter, as reported by Mareri and colleagues, suggests that different intermediate light-regulator related to the photoreceptors in winter background might influence the expression of the *HvCBF2*. Thus, the phytochrome-CBF regulon might be the putative reason of the background effect in modifying the expression of *HvCBF2* under different light spectra. The marked difference in the light spectrum at the same temperature might have activated two different types of receptors, which turned in different signal cascade that had *HvCBF2* as its final terminal. Future investigations require to highlight which phytochrome receptors influence most the *HvCBF2* and evaluate any allelic variations between winter and spring growth habit. The possible difference between background may lie in the allelic state of the phytochrome or cryptochrome and signal cascade they activate. The use of reciprocal NIL lines enabled to highlight the interesting observation that *HvCBF4* winter allele is overexpressed in the spring background, while for *HvCBF2*, expression of spring allele is highly induced when expressed in winter background (ATK, Fig. 8; OSU, Fig. 9). In accordance with the present study, the overexpression of *HvCBF4* in a susceptible plant has been observed in transgenic rice, which is chilling sensitive crop (Oh et al., 2007). The over-expression of *HvCBF4* in rice increased in tolerance to drought, high-salinity and low-temperature stresses without stunting growth suggesting the putative role of this *CBF* in response to low temperature (Oh et al., 2007).

For *HvCBF9* interesting differences were observed between the two experiments that could be attributed to both temperature and light spectrum (ATK, Fig. 10; OSU, Fig. 11). Higher induction (10 times) was observed in ATK-MTA experiment, when the hardening temperature was applied, and then the expression levels dropped down being however constantly much higher than those obtained in OSU experiment, in which only a slight induction was observed for both pre-hardening and hardening temperature. Moreover, for ATK-MTA experiment a different pattern was observed for Nure and NILs with Nure background vs Tremois and NILs with Tremois background, both reached the maximum expression levels after cold induction in the afternoon, however in the former group the high level was

then maintained, while dropped immediately in the latter. As for *HvCBF4* the peak of expression was "shifted" for Nure in ATK-MTA experiment.

In ATK-MTA experiment (Fig. 12), *HvCBF14* expression levels and pattern were different in Nure than in all other genotypes – the expression reached its maximum level at the first timing following cold induction and then got induced for the second time the day after. For all other genotypes similar levels and only one peak of maximum expression was observed at the second timing (afternoon) after the cold induction. Moreover, the expression levels reached by *HvCBF14* in Nure were higher than in all other genotypes. In OSU experiment (Fig. 13) this characteristic Nure pattern after cold induction stimulus was not observed. On the other hand, the expression levels of *HvCBF14* in all genotypes in control conditions were the same in both experiments but much stronger induction (10 times) was observed when the hardening temperature was applied directly, suggesting that the rapid and strong temperature decrease was determining for such a characteristic increase of expression.

Contrary to what might have been expected, in the present study no clear differences in expression levels were observed for winter and spring *CBFs* alleles under cold temperatures. In a previous analysis of putative promoter regions of Nure and spring cultivar Morex (Mareri et al., 2020) higher numbers of binding sites for *ICE* were reported for *HvCBF4* and *HvCBF14* winter alleles, however in the present study no clear differences due to *CBF* allele can be seen neither for induction timing, nor the expression level. However, such differences seem to be observed between groups of genotypes sharing the same background (ATK, Fig. 4; OSU, Fig. 5). On the other hand, the two parental genotypes have different CNV at *HvCBF2-HvCBF4* segment, and a difference in expression levels could have been expected. This difference could be seen at the control and pre-hardening temperatures in the OSU experiment for both genes, while no differences were observed when the hardening temperature was applied in that experiment. Moreover, in the ATK-MTA experiment such differences were observed for *HvCBF2* but not for *HvCBF4* under control condition. Taken together the observed results seem to be given more the light spectrum differences that by the CNV at *FR-H2*.

VRN-H1 is a MADS-box transcription factor, and its expression is a key regulator of vernalization (Distelfeld et al., 2009), controlling the plants response to winter cold and influencing its reproductive competence and flowering time (Dhillon et al., 2010). While in winter growth habit genotypes, *vrn-H1* expression increases after a long exposure to cold temperature (Cuesta-Marcos et al., 2010), in spring growth habit ones, *Vrn-H1* has a high constitutive expression (Shcherban et al., 2015).

The expression of *VRN-H1* may not depend only on *VRN-H1/VRN-H2* allelic combination and might be putatively linked to cold exposure, plant development and their combined action on the promoter region (Sasani et al., 2009; Trevaskis et al., 2006). The accepted model for vernalization (Kippes et al., 2018) in winter growth habit cereals establishes that, during the fall, *VRN-2* represses the expression of *VRN-3* (Yan et al., 2006). *VRN-3* encodes a mobile protein that moves from the leaves to the apex (Corbesier et al., 2007) where it creates a complex with FD-like and 14-3-3 proteins. This complex binds the *VRN-1* promoter and induces flowering (Li et al., 2015; Taoka et al., 2011). During the winter, a slight accumulation of *VRN-1* is sufficient to repress *VRN-2*, which favors *VRN-3* up-regulation when days get longer during spring (Chen and Dubcovsky, 2012). The interaction between *VRN-1/2/3* result in a positive feedback-loop that irreversibly promotes flowering in the spring (Kippes et al., 2018). Although the floral repressor *VRN-H2* is required to delay flowering prior to vernalization (Yan et al., 2004), cold induction of *VRN-H1* takes place in conditions where *VRN-H2* is not actively expressed and can occur in the absence of the *VRN-H2* gene (Hemming et al., 2008; Sasani et al., 2009; Trevaskis et al., 2006).

An epigenetic regulation was reported to maintains chromatin at the *VRN-H1* locus in an inactive state by histone modifications deposited by a plant Polycomb Repressor Complex 2 (PRC2), resulting in low transcript accumulation. Afterwards, when plants are exposed to prolonged cold, the promoter of *VRN-H1* becomes more active, leading to increased transcription and higher accumulation. This triggers change in the state of chromatin at *VRN-H1*, with a shift towards an active state of the locus (Alonso-Peral et al., 2011).

NILs can help scientists to separate the effects of different alleles at *VRN-H1* in different backgrounds. However, further analysis is needed evaluating other genes responsible for vernalization such as *VRN-H2* and *VRN-H3*. In this present work, expression patterns obtained for *VRN-H1* gene in Nure and Tremois were in accordance with what expected for varieties with winter and spring growth habits (Casao et al., 2011; Hemming et al., 2009; Stockinger et al., 2007). However, interesting new data was observed for the NILs. The *CBF-Tr*/Nure line showed expression levels similar to Nure, whereas the expression in all other NILs, including the one harboring *VRN-H1* winter allele in the Tremois background, had a pattern similar to the spring parent, showing that the expression was influenced also by regulation factors present in the background and not only by the winter *VRN-H1* allele.

After prolonged exposure to cold temperature in OSU experiments, the levels of *VRN-H1* transcripts strongly increased in all lines but Nure and *CBF-Tr*/Nure, where only slight induction could be observed.

Nure carries the combination of *vrn-H1 (5200)* allele, that has the full length first intron, and *Vrn-H2*, which is the functional dominant allele; such a combination determining a high vernalization requirement (Fernández-Calleja et al., 2021; Rizza et al., 2016). Tremois –spring growth habit that does not require vernalization– carries *Vrn-H1-7*, a dominant allele with a larger intron 1 introgression (Hemming et al., 2009; Rizza et al., 2016), and *vrn-H2* allele, which encodes the null recessive allele given by the absence of the whole ZCCT-H gene cluster on the very distal part of chromosome 4H (Karsai et al., 2005). Several potential regulatory motifs at the promoter of the *VRN-H1* locus has been detected such as: ERE - ethylene response element (GCCGCC), CRT/DRE C-repeat transcription factor core binding site (CCGAC), MYC - MYC transcription factor binding site (CANNTG), B-ZIP transcription factor binding site (ACGT) and VRN Box putative vernalization regulatory motif (Alonso-Peral et al., 2011; Pidal et al., 2009).

The winter *vrn-H1* allele in a spring background showed, in the present study, a high expression level due to the lack of functional repressors present in the winter background. On the other hand, spring allele in winter background was not downregulated because it might hypothetically lack of the binding site for the repressor. Moreover, NIL *CBF-Tr*/Nure showed two different *vrn-H1* expression patterns at the steady state in two experiments. For the ATK-MTA experiment, the expression was identical to that observed for Nure, while in OSU experiment, the expression level at the steady state was intermediate between Nure and Tremois and after the cold induction decreased to levels observed in Nure. Those differences observed at the steady state might putatively be due to different light influence as observed and already discussed for *HvCBF2*.

In this study, winter *vrn-H1* higher accumulation in Nure was observed for the timing 20 days of hardening, in accordance with what observed in a similar work which reported transcript growth after 21 and 35 (stronger) days under of vernalization conditions (temperature 7 °C, circadian 8/16 d/n; Casao et al., 2011). A correlation, or a "dampening" effect, of *VRN-H1* high expressions levels on *CBF* expression were reported (Stockinger et al., 2007); however, such an effect could be observed only under long day condition, and thus was not observed in the present study where plants were grown under short day. In rye, a study of eleven candidate genes involved in frost response (including several *ScCBFs*, *ScICE2*, and *ScVRN1*) (Li et al., 2011) found no association of frost tolerance with *ScVRN1*, although the authors did detect significant epistatic effects involving it (Li et al., 2011; Visioni et al., 2013). That meaning it is not/or not only the level of expression on *VRN-H1* that might influence *CBF* expression, but rather a right photoperiod input is necessary.

Cold-inducible genes designated as Cold-responsive or Cold Regulated Genes (*HvCOR14B* gene) and dehydrin (*HvDHN5*) genes carry the CRT/DRE cis-acting element (CCGAC) target by CBF protein through the *CBF*-dependent pathway (for a recent review, see Hwarari et al., 2022). *HvCOR14B* is the best characterized among the molecular genetic factors of frost tolerance in cereals (Crosatti et al., 2003). The dehydrin 5 (*HvDHN5*) gene is the most cold-inducible dehydrin in barley and is an orthologue to the *WCS120* gene in wheat (Kosová et al., 2008). In wheat, the *WCS120* gene can be used as a marker gene for frost tolerance (Kosová et al., 2008). Whereas in barley, the *HvDHN5* gene is also considered as a marker gene for FT, however, in contrast to the *WCS120* gene, the expression level of the *HvDHN5* gene is only a part of the whole system (Kosová et al., 2013), the so-called COR regulon.

In the present study all genotypes showed similar expression pattern for the *HvCOR14B* (Fig. 14); very low accumulation under control condition and induction that didn't follow immediately temperature decrease in neither of the experiments but was delayed for few hours and was strongly upregulated only after 24 hours. This "shift" of response to low temperature might suggest that its expression depends on factors that in turn are regulated by decrease in temperature. The expression profiles obtained for *HvDHN5* in the ATK-MTA experiment (Fig. 15, left) corresponded to those observed for *HvCOR14B*; and consisted in delayed induction rising strongly only after 24 hours. For the OSU experiment (Fig. 15, right), the shift from warm to pre-hardening temperature was already sufficient to upregulate *HvDHN5* expression to its maximum level just few hours after exposure, however, what must be stressed, both the peak of expression and the overall expression level were much lower than those observed in ATK-MTA experiment. Differences in expression level of both genes were observed between Nure and Tremois in OSU experiment when the temperature shifted to hardening for *HvCOR14B* and after five days of hardening for *HvDHN5*. Moreover, while for the former gene higher expression levels were observed in winter Nure genotype and NILs with Nure background, for the latter the highest expression was reported for Nure and in NILs with winter *CBF* allele in Tremois background, and vice versa. Thus, while for *HvCOR14B* winter background seems necessary, with or without Nure *CBF* allele for induction, for *HvDHN5* gene either only background Nure or winter *CBF* allele were sufficient to induce higher expression.

Interesting studies showed that higher expression levels of *CBFs* such as *HvCBF2*, *HvCBF4*, *HvCBF9* and *HvCBF14* resulted in higher expression levels of *HvCOR14B* and *HvDHN5* (Ahres et al., 2020c; Hazrati et al., 2021; Jeknić et al., 2014). In the present study some interesting observation can be made when we consider three lines with the highest expression level for each gene. Nure and lines with

reciprocal substitution of *CBF* allele (*CBF-Tr*/Nure and *CBF-Nu*/Tremois) were characterized by the highest expression levels for both *COR* genes at 5 days after hardening, when moreover two very distinct group of three varieties different for expression levels, could be observed (ATK, Fig. 14; OSU, Fig. 15). That suggests that either only winter background or winter *CBF* allele are sufficient to induce higher expression of those genes during hardening. On the other hand, the highest expression levels for *HvCBF2* and *HvCBF9* genes were observed at the pre-hardening temperature, for Nure and lines with Nure background (*CBF-Tr*/Nure and *VRN-Tr*/Nure) suggesting that that winter background is necessary to induce stronger *HvCBF2* and *HvCBF9* expression during the pre-hardening.

While a distinct group of lines with lowest expression levels could be clearly identified for those genes, the levels of expression obtained for the lines where the highest induction was observed were different, however clearly separated from the former group (ATK, Fig. 4; OSU, Fig. 5). No clear division due to expression levels could be observed between genotypes for *HvCBF4* and *HvCBF14* expression. It is very worth noting the lines with highest expression level for *COR* genes, and *HvCBF2* and *HvCBF9*, respectively, resulted all frost tolerant in the phenotypic trials.

Recent studies on cold acclimation and frost tolerance in cereals (Wang et al., 2023; Guo et al., 2019; Hwarari et al., 2022; Panahi and Shahi, 2024; Wang et al., 2016) focused on the entire cold pathway also known as ICE-CBF-COR pathway, and *ICE1* might be the putative "upstream factor" that influenced the *CBF* and *COR* expression in this work. The differences observed linked to the winter background might be a combination of differences allele at *ICE1* and/or by its posttranslational modification by the ABA signaling gene *OST1* (Ding et al 2015*).* Inducer of CBF expression 1 (*ICE1*) is a transcription factor that belongs to MYC-type basic helix-loop-helix family, and several works reported their involvement in upregulating the expression of *CBF* genes under low temperature conditions in a wide range of species (Båga et al., 2022; Chinnusamy et al., 2003; Guo et al., 2019). In Arabidobsis, wheat and rye different degrees of cold tolerance are due to different alleles of *ICE1* (Båga et al., 2022; Chinnusamy et al., 2003; Guo et al., 2019; Wang et al., 2023). In wheat, two different homologs of the *ICE* were identified as *TaICE41* and *TaICE87* (Badawi et al., 2008), and their overexpression in Arabidopsis enhanced FT after hardening. In rye, an ScICE2 protein has been evaluated showing amino acid variation within the DNA-binding bHLH region and/or start of the zipper region resulted in traits such as winter survival rate and low temperature tolerance (Li et al., 2011). Authors hypothesized that the *ICE1* gene identified could be allelic with the *ICE2* gene in rye, whose allelic variation had been reported to be associated with variation for winter hardiness and frost tolerance, and that different *ICE* alleles could be important for frost

tolerance in rye. Similar pathways might be expected to exist in barley, given the conservation of cold response mechanisms across plant species of the *Triticeae* tribe. A greater transcripts abundance in Nure than in Tremois was reported for OST1 homologs and PYL5 homologs (ABA signaling) suggesting posttranslational variation of ICE1 protein (Wang et al., 2016). This hypothesis may also be partly supported by the previous reports between the interaction of *CBF* genes in hormone-mediated acid abscisic (ABA) pathways (Muhammad Aslam et al., 2022; Tuteja, 2007). Therefore, the *ICE* activation with specific *ICE* alleles (for example, coding for ICE1 proteins with reduced affinity for the MYC binding sites in the promoters of *CBF* and *COR* genes) and/or posttranslational differences might be the focal point in the observed variation between backgrounds acting on the CBF-COR pathway, and thus should be included in future gene expression studies.

Apart the ICE-CBF-COR pathway, some other genes might have contributed to the winter background effect. Among these genes, *FR-H3* could have a crucial role to confer high levels of resistance in NILs with winter background. *FR-H3* QTL was mapped on the short arm of chromosome H1 and it was identified in barley populations with superior low temperature tolerance (winter x facultative and facultative x facultative; Fisk et al., 2013). At *FR-H3* locus, three candidate genes encoding proteins involved in the cellular membrane modifications by the cold temperature (Li et al., 2017; Schulze et al., 2012) were identified (Muñoz-Amatriaín et al., 2020). In the present study, two markers in the were identified (barley 50 k Illumina Infinium iSelect, unpublished data) in the putative region of *FR-H3*, reporting allelic differences between Nure and Tremois in their NILs. Therefore, *FR-H3* might have contribute to confer frost tolerance in NILs with winter background carrying susceptible alleles at *FR-H1* and *FR-H2*.

Overall, the OSU experiment where the pre-hardening step was included and where MH/HSP illumination was applied to mimic the natural field conditions observed during vernalization period, produced more clear results than the ATK-MTA experiment. In OSU experiment, lower expression levels were observed; however, in the warm pre-hardening phases *CBF* and *COR* genes showed the diversification linked to the winter allele of *CBF* or winter background. On the other hand, during the ATK-MTA experiment, with the “hard” temperature shift higher expression levels were observed for *HvCBF9* and *HvCBF14*, albeit the differences between alleles were smaller.

This study elucidates the complex interplay of the ICE-CBF-COR pathway and its connections with the light signaling system. Future experiments will require more in-depth analysis such RNAseq or

proteomic evaluation to dissect the cold acclimation mechanisms choosing the more representative timings in order to discover all the actors involved in the cold acclimation pathway and the differences between the frost resistant e susceptible barleys.

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Chapter 5

General Conclusion

5.1. General Conclusion

In the research carried out during this PhD project, the attention was focused on barley (*Hordeum vulgare* L.). In 2022, barley ranked fourth in terms of crop production after wheat, maize, and rice, with a worldwide harvested area of 47.2 million hectares (FAO, 2023). Its primary role for cultivation is as a source of animal feed (about 75% of the global production), with subsidiary uses in alcoholic and non-alcoholic beverages (20%), and in human nutrition (5%) (FAO, 2023). During the 20th century, barley proved to be an excellent model organism for both basic and applied research thanks to a diploid (2n) genome, a haploid complement of only seven chromosomes, and its self-pollinating reproduction system. Climate change significantly impacts agricultural productivity, with increasing incidents of extreme weather conditions posing a substantial threat to barley yield (Zhang et al., 2022). Particularly in temperate regions, unpredictable temperature fluctuations and extreme cold events have emerged as critical challenges to maintaining stable cereals outputs (Wang et al., 2018). In the context of this PhD thesis, two main experiments were set-up using specifically developed QTL Near Isogenic Lines. Such novel plant material was intended to mendelize the QTL effect of *FR-H1* and *FR-H2* in order to elucidate their role in the cold acclimation and frost tolerance. In Chapter 3, we reported extensive phenotypic characterization of the NILs by means of freezing tests and open field trials under different conditions. This allowed us to better understand the role of the two QT loci on the phenotype. In Chapter 4, we summarize results from complex single gene expression profile experiments used to understand the regulation of candidate genes at *FR-H1* and *FR-H2* locus during the cold acclimation. In addition, the effect of different types of light spectra on gene expression was analyzed in order to try to separate the effect of the light and the cold stimuli on the gene expression.

The findings of the present work have highlighted new insights as a basis for future research on cold acclimation and frost tolerance in fall-sown cereals. Since the genetic material we used derives from the Nure (winter) x Tremois (spring) cross, in which the resistant parent (Nure) harbors both alleles with positive effect on resistance, the NILs enabled isolation of the effect of the single loci in alternative backgrounds. Summarizing the main results, three QTL-NILs exhibited degree of frost resistance similar to Nure, while only one line showed a degree of the susceptibility similar to Tremois. Noteworthy, the resistant NILs were those with the Nure background and the NIL with Nure's *CBFs* in the Tremois background. A pivotal aspect of the findings is given by the fact that *FR-H1*, generally considered a candidate QTL for frost resistance, seemed not to have any improving (NIL with winter allele of *FR-H1* in Tremois) or worsening effect (NIL with spring allele of *FR-H1* in Nure) on the phenotype. This

outcome suggests that the cold pathway may not be directly linked with the flowering pathway. Conversely, winter *FR-H2* in the spring background improved the frost resistance, increasing the genotype *CBF-Nu*/Tremois to a degree of frost resistance comparable to winter genotypes. An observation worthy of note from the expression analysis study concerned the accumulation of transcripts of *HvCBF4* after cold induction in the *CBF-Nu*/Tremois. *HvCBF4* is one of the *CBF* candidates in *FR-H2* and it showed significantly higher expression levels in the NIL compared to other *CBFs*, thus suggesting its putative role in phenotype improvement. Additionally, the role of other factors apart from the *FR-H1/FR-H2* system in conferring cold resistance was demonstrated. The main candidates we assume operate from the (winter) background could be represented by *ICE* genes activators of the ICE-CBF-COR pathway and residing on the long arm of chromosome 3H, or the *FR-H3* locus on the short arm of chromosome 1H.

5.2. Future Prospectives

Winterhardiness in cereals like wheat and barley refers to their ability to withstand and survive harsh winter conditions. This trait is vital for their growth and development in regions with cold climates, and it encompasses several physiological and genetic adaptations, including the ability to tolerate frost and to become reproductively competent. Research conducted over the last 25 years has highlighted that barley's response to cold stress and vernalization is a multifaceted process involving a complex network of genetic and molecular interactions. The AP2 *CBF* gene family emerged as a central player in the process, its expression being finely tuned by external cues such as temperature fluctuations, circadian rhythm, and light quality.

Laboratory experiments typically use well-controlled and homogeneous light and temperature growing conditions that differ significantly from the natural variation observed in the field. As a result, the complexity of the effects of all of the environmental factors that act on plants in open field is not well-understood (Kopecká et al., 2023). Herein, the need to develop extensive field trials to validate laboratory findings and to understand the cold acclimation and the vernalization under open field conditions. Phenotypic experiments alone are not enough to elucidate the cold mechanism, they must necessarily be supplemented with new omics technologies. Future scenarios require to investigate cold acclimatization, vernalization and winter hardiness in combined growth chamber/open field experiments, with integrated omics approaches that combine transcriptomics, proteomics, and metabolomics.

In the attempt to link gene expression analyses with phenotypic data, researchers should try to elucidate the molecular mechanisms governing frost tolerance and developmental processes in barley. This integrated approach enhances the robustness of findings, helping breeders to pinpoint key genetic elements for targeted improvement of novel varieties with enhanced frost tolerance and optimized growth patterns, crucial for agricultural sustainability in diverse climates. The introgression of winter allele of *CBF* genes into spring malting barley could be an interesting new starting point for malting breeding. Incorporating *CBF* genes into spring malting barley, a traditionally less cold and frost tolerant crop, breeders can significantly improve the ability to withstand frost conditions. The capacity to overwinter provides an advantage by allowing fall sowing (especially at high latitudes), which benefits from fall and winter rainfall and extends the crop cycle that means higher yield (Caccialupi et al., 2023; Stockinger, 2021). In addition, the fall-sown barleys mature earlier compared to spring barleys avoiding problems caused by the increased risk of heat wave exposure and drought during the grain filling phase in summertime (Faranda et al., 2023). The extension of the growing season in colder regions also ensures the stability of yield and quality (Abi Saab et al., 2019), crucial for the malting and brewing industries. The introgression of *CBF* genes represents a key advancement in agricultural biotechnology, offering a sustainable solution to the challenges posed by changing climate conditions on crop production.

In conclusion, knowledge obtained in this PhD thesis might be helpful for developing barley varieties better equipped to withstand the adverse future effects of climate, thereby ensuring sustained agricultural productivity and food security in the face of environmental uncertainties (Challinor et al., 2014; Kovak et al., 2022). The collective insights from this manuscript pave the way for innovative strategies to enhance barley's resilience to cold stress, which is crucial for ensuring stable crop yields.

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