Chapter 6
Genomics of Low-Temperature Tolerance for an Increased Sustainability of Wheat and Barley Production

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Abstract Stability of high yields in a changing environment becomes the main aim of the future wheat and barley breeding, oriented towards development of frost-tolerant winter and facultative cultivars together with careful selection of growth cycle adaptation and drought tolerance. Since low temperature signal influences both the cold acclimation and vernalization processes the interaction between *VRN* gene expression and freezing tolerance (FT) is discussed. Recent advances in global expression changes driven by cold are reviewed in view of the immense progress in high throughput technological platforms. Different signal transduction pathways in which several transcription factors play an important role regulating the expression of whole sets of genes are presented, including CBF-regulated and CBF-independent hubs. The knowledge acquired from genomics and transcriptome analysis has been then complemented by the description of metabolomics and proteomic approaches to help unraveling the molecular changes that occur under cold stress in the cereal plants. Finally, it is surveyed the great importance of stable and well-characterized genetic resources for future breeding for FT, that could switch from marker-assisted to genomics-assisted selection.

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6.1 Introduction

Global climate change, causing more dramatic and erratic temperature shifts and rainfall events, has increased the importance of an old problem. Due to variations in environmental stresses such as drought, flooding, heat, salinity, low temperature, etc., the increasing variation in crop yield from year to year throughout the world posses a serious problem for agricultural production and the market of agricultural commodities. Although farmers in the northern hemisphere face the possible risk of winter damage, they still grow winter cultivars. The explanation is very simple: winter cereals are sown in the fall, and provided they have adequate tolerance to survive freezing temperatures in winter, because of their longer growing period they usually have higher yield potential than spring varieties, planted later in the spring. In addition, they are usually earlier in flowering and maturity than their spring counterparts, thus they escape the summer heat and drought, the other significant abiotic stress factors which may reduce yield significantly. Maintaining yield stability while enhancing productivity in the driest and coldest regions of cereal growing areas is becoming increasingly important for securing a level of food production sufficient to meet the needs of mankind. Stability of high yields in a rapidly changing environment becomes the main aim of stress biology, from a theoretical point of view, to be applied to future crop breeding. In this context, it seems unavoidable the trend towards an increasing adoption of frost-tolerant winter and facultative cultivars in temperate cereals, coupled with careful selection of growth cycle length and drought tolerance. Thus there are reasons for further efforts to improve cereal freezing tolerance, particularly among winter materials.

The ability of plants to survive freezing is based on the effectiveness of cold acclimation process (Thomashow 1999; Skinner 2009). Cold acclimation is a relatively slow, adaptive response during fall, when the temperature, day length and light intensity decrease gradually. All these factors are important for attaining genetically determined freezing tolerance (Sandre et al. 2011; Schoot and Rinne 2011). Apart from the cold acclimation, decreasing day length and an extended cold period influence the plant development also through the vernalization process mediated by *VRN* genes (Distelfeld et al. 2009; Greenup et al. 2009). Plants are able to cold acclimate only in the vegetative developmental phase, during which in most cereal growing areas wheat and barley experience frost. From the freezing tolerance point of view, the vegetative/reproductive transition phase is the most critical period. Following the vernalization saturation, when the shoot apex reaches the double ridge phase (floral initiation), the leaf initiation is terminated. This developmental stage is irreversible and reduces the ability of the plant to maintain enhanced freezing tolerance upon cold acclimation conditions (Fowler et al. 1996; Vítámvás and Prášil 2008; Galiba et al. 2009). Although cold stress in the reproductive phase is quite infrequent, it can occur due to cold winds (Reinheimer et al. 2004). In Australia, the predominant frost damage occurs from radiation-frost events in spring during the reproductive stages of barley and wheat. Reproductive tissue is very sensitive and can be damaged by small sub-zero temperatures drops ($\leq -2\,^{\circ}\mathrm{C}$) that can lead to spikelet sterility. Genetic studies performed in barley concluded that chromosomes

2H and 5H were responsible for reproductive freezing tolerance, and showed that winter habit alleles at the *VRN-H1* vernalization gene on 5H were linked in coupling with reproductive FT (Chen et al. 2009). Since low temperature influences the cold acclimation and vernalization processes, we will discuss the interaction between *VRN* genes and freezing tolerance.

Low temperature-specific gene expression is mediated by different parallel signal transduction pathways. Two main signalling pathways have been described in *Arabidopsis*; one is dependent on the involvement of the phytohormone abscisic acid (ABA), while the other is not (Zhang et al. 2004). Both pathways trigger the expression of a range of transcription factors that bind, among others, to the C-repeats or Dehydration-Responsive Element (CRT/DRE), ABA-Responsive Element (ABRE), and MYC/MYB Recognition Sequence (MYCR/MYBR) binding sites of their target genes, thus regulating the transcription of these genes. The altered expression of these downstream genes leads to cold acclimation and to an increased level of freezing tolerance (Chinnusamy et al. 2004). During cold acclimation many molecular processes are conserved in the dicot and monocot lineages (Nakashima et al. 2009). One of the best examples of this phenomenon is the central role of the C-repeat Binding Factor or Dehydration-Responsive Element Binding Factor (CBF/DREB1) regulon in the cold acclimation process both in model plants and crops. Accordingly, this review will discuss the role of the CBF regulon in the cold acclimation process in cereals. Since *CBF* genes are important but not the only regulators involved in the cold-response regulatory pathway, our current knowledge on other transcription factors and their target genes will also be described. In addition to transcriptomic analysis, metabolomics and proteomic approaches helped to highlight the molecular changes under cold stress in plants as well (Fig. 6.1).

FT is a polygenic trait affected by several genes located on different chromosomes. Since bread wheat (*Triticum aestivum* L.) is hexaploid, its vital genes are replicated. This allowed Sears (1953) to develop a series of nullisomic lines (i.e. lines lacking one of the normal chromosomal pairs) from Chinese Spring (a freezing-susceptible spring wheat). These lines served as the basis for developing intervarietal chromosome substitution lines, thus providing one of the best means of studying the genetic control of freezing tolerance.

In the last two decades, following the omics breakthrough, from genomics (Tuberosa et al. 2002) to the introduction of high-throughput technological platforms, there has been a shift from genetic, gene-by-gene, studies to genomic ones, in which sequence as well as expression variation are studied on a global scale. Since the pioneering works to develop suitable genetic materials used for genetic studies of FT (Galiba et al. 2009), a large amount of genomic data have been generated in wheat and barley, and two international sequencing initiatives led to the recent release of the first genomic frameworks of the wheat and barley genomes. In hexaploid wheat, Brenchley et al. (2012) used a whole-genome shotgun sequencing strategy to obtain a low-pass coverage of the genome. In barley, Mayer et al. (2012) reported an integrated and ordered physical, genetic and functional sequence resource of the cultivar Morex gene-space in a structured whole-genome context.

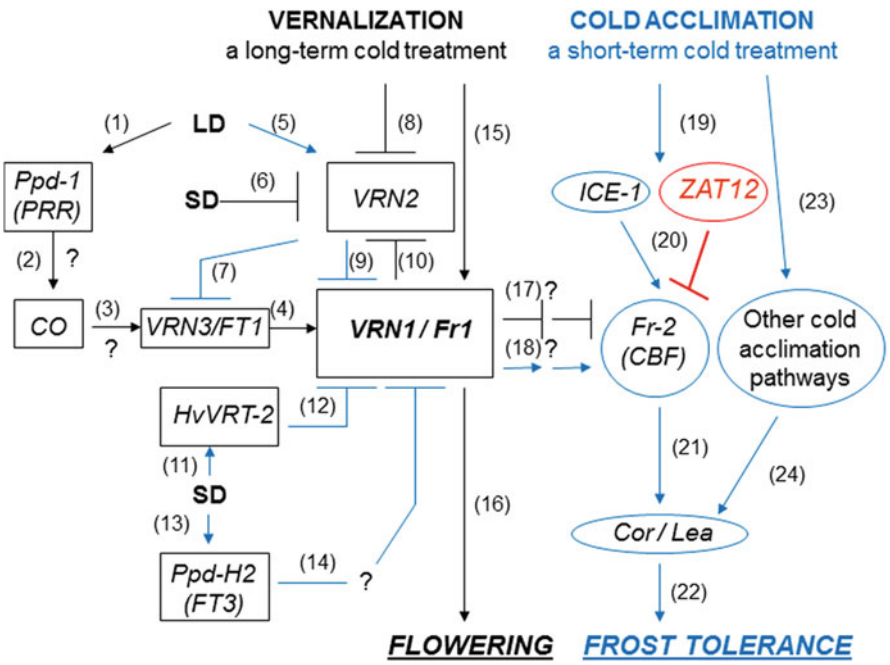

Fig. 6.1 Relationships between vernalization and FT in *Triticeae*

Descriptive scheme of the relationships between vernalization- and photoperiodically-regulated loci (their candidate genes, respectively) which control the transition to flowering and loci which regulate the development of FT upon cold acclimation (CA). (1) Laurie et al. (1994), (2) Turner et al. (2005), Beales et al. (2007), (3) Griffiths et al. (2003), Turner et al. (2005), (4) Yan et al. (2006), (5) Yan et al. (2004), Loukoianov et al. (2005), LD induces expression of *VRN2* gene, (6) Yan et al. (2004), (7) Yan et al. (2006), (8) Dubcovsky et al. (2006), Trevaskis et al. (2006), (9) when *vrn-1* is recessive and *Vrn-2* is dominant, von Zitzewitz et al. (2005), (10) possibly when *Vrn-1* is dominant or when *vrn-2* is recessive under prolonged LD treatment, Loukoianov et al. (2005), Trevaskis et al. (2006), (11) Kane et al. (2005), (12) Kane et al. (2007), (13) Laurie et al. (1995), (14) Faure et al. (2007), (15) vernalization induces expression of *VRN1* gene, Oliver et al. (2009), (16) expression of *VRN1* gene is necessary for flowering, Shitsukawa et al. (2007), (17) *VRN1* gene downregulates expression of *CBF* genes, but only under LD conditions, Danyluk et al. (2003), Kane et al. (2005), Kobayashi et al. (2005), Stockinger et al. (2007), Dhillon et al. (2010), (18) Vágújfalvi et al. (2000), Kobayashi et al. (2005), Stockinger et al. (2007), (19) cold induces expression of *ICE-1* and *ZAT12* genes, Zarka et al. (2003), Skinner et al. (2006), (20) Skinner et al. (2006), (21) Vágújfalvi et al. (2000), Choi et al. (2002), Kume et al. (2005), Skinner et al. (2005), Miller et al. (2006), (22) Houde et al. (1992), Crosatti et al. (1995), Vágújfalvi et al. (2000), Vítámvás et al. (2007), Kosová et al. (2008a),

(23) Thomashow (1999), (24) Thomashow (1999), Choi et al. (2002). Question marks indicate uncertain or unknown components of the regulatory pathways. Blue lines indicate signalling pathways active before vernalization, black lines indicate pathways active after vernalization fulfillment. Modified after Kosová et al. (2008b).

Moreover, knowledge acquired from *Triticeae* genomics is complemented by a deeper understanding of the proteome, metabolome, and more recently, epigenome. In a research environment where throughput and amount of data generated are no longer limiting, the availability of appropriate and well-characterized plant genetic resources will become a crucial issue for further knowledge advancements in stress biology. This article reviews how the best allelic variants for freezing tolerance can be combined in a single wheat or barley genotype.

6.2 "Omic" Approaches to Study Cold Acclimation

6.2.1 Transcriptomics

The effect of low temperature on the transcriptome was first investigated mainly in the model plant *Arabidopsis* (Seki et al. 2001; Fowler and Thomashow 2002; Nakashima and Yamaguchi-Shinozaki 2006) but later on, these studies were also extended to an increasing number of crop species including cereals (for review see Vij and Tyagi 2007, Sreenivasulu et al. 2007). Thus, cold-induced transcriptome changes were investigated in rice (Rabbani et al. 2003), wheat (Gulick et al. 2005; Herman et al. 2006) and barley (Svensson et al. 2006; Greenup et al. 2011). Among the cold-responsive genes, transcription factors play a pivotal role since they regulate the expression of whole sets of genes called "regulons" and therefore, they can switch on arrays of metabolic activities necessary for cold acclimation and freezing tolerance. Special attention was given to the CBF regulon controlled by the C-repeat binding factors (CBFs), also called dehydration-responsive element binding (DREB1) factors (Chinnusamy et al. 2006; Nakashima and Yamaguchi-Shinozaki 2006; Van Buskirk and Thomashow 2006). From a comparison of the expression of several *CBF* genes in *Triticeae* (rye, wheat, barley) it turned out that sample timing, induction temperature and light-related factors have significant effects on transcript levels, and must be considered in future transcriptomic studies involving functional characterization of low temperature-induced genes in cereals (Campoli et al. 2009). Although the expression of 10 *CBF* genes was compared in three rye genotypes with different freezing tolerance, it is unclear which of them have the greatest influence on FT and whether they have an additive effect. The regulation of cold acclimation by CBFs is described in more detail in Sect. 6.3.

CBF genes are arranged in clusters on the homeologous group 5 chromosomes of *Triticeae*, and coincide with *FR2* QTL for FT (Vágújfalvi et al. 2003, 2005; Miller et al. 2006; Baga et al. 2007; Francia et al. 2007; Galiba et al. 2009). Besides *Fr-A2*, *Fr-A1,* a vernalization gene, *Vrn-A1* and several additional genes involved in the response to low temperature and other stresses were localised on the long arm of

wheat chromosome 5A (Dubcovsky et al. 1995; Galiba et al. 1995; Cattivelli et al. 2002; Galiba 2002; Danyluk et al. 2003; Yan et al. 2004; Ramalingam et al. 2006), which has a major effect on freezing tolerance (Sutka 1994). The arrangement of loci and respective functions is very similar in the barley H genome (Francia et al. 2004, 2007; von Zitzewitz et al. 2005). *VRN* genes regulate the transition from the vegetative to the reproductive phase, and act as a master switch controlling the duration of the expression of low temperature-induced structural genes (Danyluk et al. 2003). Both changes in temperature and day length are involved in the control of cold-regulated (*COR*) genes by *VRN1*, since mutations in the *VRN-1* promoter, resulting in high transcript levels under both long and short days, lead to down-regulation of the *COR14b* gene under long days (LD) but not under short days (SD) (Dhillon et al. 2010). Several cold-responsive and chromosome 5A-regulated genes, including those ones of LEA proteins and antioxidants were determined by comparison of the transcript profile of a freezing-tolerant and a freezing-sensitive chromosome 5A substitution line during a 21-day-long hardening period (Kocsy et al. 2010).

Besides the *CBF* downstream cascade, other CBF-independent pathways involving ZAT12 or the homeodomain transcription factor HOS9 have been shown to control cold-regulated genes (Vogel et al. 2005; Chinnusamy et al. 2006, Nakashima and Yamaguchi-Shinozaki 2006) (Fig. 6.1). Additonal CBF-independent cold acclimation pathways were discovered by characterization of several mutants. Thus, mutations in ESKIMO1 protein having unknown function and in the transcriptional adaptor protein ADA2 resulted in constitutively increased freezing tolerance with simultaneous induction of genes distinct of CBF regulons and without influencing genes of this regulon (Vlachonasios et al. 2003; Xin et al. 2007). A detailed description of CBF-independent transcriptional regulation of cold acclimation can be also found in Sect. 6.3.

Comparison of transcript profile changes in genotypes with different freezing tolerance and vernalization requirement is a powerful tool for the identification of genes having a role both in the effective cold acclimation and in the vegetative to reproductive transition. While in most studies an abrupt decrease in temperature was used for cold acclimation (Monroy et al. 2007; Kocsy et al. 2010), in a recent experiment a gradual reduction of temperature was applied which is more similar to the natural acclimation process during autumn. In this case, the underlying rationale was that those genes having different cold induction threshold temperatures could have been discovered in this way (Winfield et al. 2009). The earlier induction of cold-responsive genes at higher temperatures in the freezing-tolerant wheat genotypes compared to the freezing-sensitive ones, which was shown in the case of *COR14b* gene (Vágújfalvi et al. 2003), ensures their more efficient cold acclimation and greater level of freezing tolerance. Both gradual (Winfield et al. 2010) and abrupt decrease in temperature (Monroy et al. 2007; Kocsy et al. 2010) induced the expression changes of more genes during long-term cold acclimation in the freezing-tolerant wheat genotypes than in the sensitive ones. During a short-term exposure to cold (2 d, 4 °C), Winfield et al. (2010) did not find a such a difference by separate investigation

of crowns and leaves, but other authors observed alterations in the level of more transcripts in the tolerant genotype whole shoots than in the sensitive one (1 d at 2 °C and 1 d at 4 °C) (Monroy et al. 2007; Kocsy et al. 2010). This contradiction may be due to the different plant organs used for the analysis in the two experimental systems.

The importance of separate investigation of leaves and crown was first supported by the results of Winfield et al. (2009), who interestingly found many genes which were affected by cold either only in the crown or only in the leaves. They investigated cold-induced changes in the transcriptome of wheat leading to the vegetative to generative transition identifying several MADS-box genes, as others related to the gibberellin pathway, which may play an important role in the onset of flowering. Another numerous set of cold-responsive genes was found common to both organs, including genes encoding DEAD-box RNA helicase, choline-phosphate cytidyltransferase and delta-1-pyrroline carboxylate synthetase (Ganeshan et al. 2011). However, the same authors, in agreement with the previous report, found more genes being affected only in one of the two organs than common ones; and emphasized how cold acclimation mechanisms were likely differently regulated in crowns and leaves.

Additional genes involved in the acclimation process were identified by transcriptome analysis in crowns of wheat subjected to subzero acclimation (−3 °C for 12–18 h) after the cultivation at non-freezing low temperature (Herman et al. 2006). These plants exhibited additional 3–5 °C increase in freezing tolerance and the expression of genes related to carbohydrate metabolism, photosynthesis and defence processes (dehydrins, ice recrystallization inhibition protein) significantly changed compared to the plants acclimated at non-freezing temperatures. The induction of dehydrins was also confirmed at proteome level (Kosová et al. 2008a, 2010). In another experimental system where the temperature was slowly decreased to −10 or −12 °C after the cold acclimation, genes involved in transcription and defence processes were affected by the subzero acclimation in wheat crowns (Skinner 2009). In summary, the results of transcriptome analysis carried out with plants acclimated at various temperatures indicate that the gradual decrease in temperature during autumn triggers continuous alterations in the transcriptome pattern in order to ensure an effective adaptation to the environment.

6.2.2 Proteomics

A wide range of proteomic studies of temperature stress in plants are currently underway, using numerous methodologies, species, and stress conditions. Despite results obtained so far, information on the systemic response to temperature stress is still quite limited because plant perception and response is often based on factors common to the response to other stresses. What is at least clear is that high and low temperature stresses cause distinct proteome responses in plant tissues (Neilson et al. 2010; Kosová et al. 2011).

Similarly to transcriptome analysis, plant cold acclimation processes at the proteome level were first studied in model plants *Arabidopsis* and rice, where large sequence data sets were available (Bae et al. 2003; Kawamura and Uemura 2003; Imin et al. 2004; Cui et al. 2005; Amme et al. 2006; Yan et al. 2006; Hashimoto and Komatsu 2007). Subsequently, proteome responses to cold were studied in a much broader range of plants including *Arabidopsis* cold- and salt-tolerant relative *Thellungiella salsuginea* (Gao et al. 2009), chicory (Degand et al. 2009), *Festuca pratensis* (Kosmala et al. 2009), soybean (Cheng et al. 2010), pea (Dumont et al. 2011), *Lolium perenne* (Bocian et al. 2011), woody plants such as peach (Renaut et al. 2008), and also wheat (Vítámvás et al. 2007; Sarhadi et al. 2010; Rinalducci et al. 2011a, b) (reviewed in Kosová et al. 2011). Unfortunately, for barley limited information is available on proteome variation following exposure to low-temperature.

Based on studies in cyanobacterium *Synechocystis* strain PCC6803 carried out several years ago, the plasma membrane has been proposed as the primary site of cold-signal sensing (Murata and Los 1997; Suzuki et al. 2000). A temperature decline leads to a decrease in plasma membrane fluidity with changes in composition of membrane phospholipids, which also affects conformation of several transmembrane protein complexes. At the protein level, changes in the composition of plasma membrane proteins in response to cold were first studied in *A. thaliana* by Kawamura and Uemura (2003). The increase in ERD10, ERD14 and COR47 dehydrins indicated protection of membrane-associated proteins against dehydration and denaturation. The increase in outer membrane, lipoprotein-like proteins belonging to the lipocalin family was associated with their important role in membrane biogenesis and repair, as in cellular adaptation to high osmotic stress (Bishop 2000). A similar behaviour was observed for two VfENOD18-like proteins, known to confer tolerance to various stresses including oxidative stress and osmotic shock. From signalling studies, it is known that cold signal is transduced from plasma membrane to nucleus via Ca^{2+} signalling pathway (reviewed in Yamaguchi-Shinozaki and Shinozaki 2006). Accordingly, changes in the abundance of several proteins involved in Ca^{2+} transduction pathway such as a homologue of tobacco DREPP-like protein, synaptotagmin-like protein and phospholipase D δ (PLD δ) have been detected.

In nucleus, cold signal induces profound changes in gene expression underlying the plant cold acclimation process. At the proteome level, Bae et al. (2003) observed in *A. thaliana* a significant increase during the first 4 h of cold followed by a decline in several transcriptional regulators involved in RNA processing and export, such as U2 snRNP-A× and DEAD box RNA helicase; the latter also involved in the regulation of *CBF* gene expression (Gong et al. 2002). A longer lasting upregulation (up to 24 h) by cold has been reported for several heat-shock proteins such as dnaK-type HSP or HSC70-1. In addition, changes in several proteins involved in cold-induced Ca^{2+} signalling, such as calmodulin isoforms, and protein metabolism, such as 20S proteasome alpha subunit G, have also been observed.

At the whole-cell level, cold induces profound changes in hydration status and increases the risk of osmotic and oxidative stress. Cold acclimation process also

displays increased demands on energy metabolism and is associated with a profound redirectioning of the whole cellular metabolism from an active growth and development to an active cold acclimation. The changes in cellular metabolism are also reflected at proteome level. Cold, similarly to other stress factors, leads to disbalances in cellular metabolism increasing the risk of oxidative stress (ROS—Reactive Oxygen Species—formation). Enhancement of antioxidative enzymes involved in ROS scavenging, namely enzymes of ascorbate-glutathione cycle, has been reported in all proteomic studies. Several classes of superoxide dismutase (SOD), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), as well as several classes of glutathione-S-transferases (GST) and thioredoxin *h* were increased by cold (e.g. Amme et al. 2006; Yan et al. 2006; Degand et al. 2009; Gao et al. 2009; Dumont et al. 2011; Vítámvás et al. 2012).

Chilling similarly to several abiotic stresses such as freezing, drought, salinity or osmotic stress, is also a dehydrative stress, i.e. it leads to a disbalance between water uptake and water release resulting in cellular dehydration. As a response, a *de novo* synthesis of several osmoprotective compounds including both low-molecular hydrophilic compounds, especially carbohydrates (mono- and oligosaccharides), and high-molecular hydrophilic proteins from COR/LEA group occur (Amme et al. 2006; Vítámvás et al. 2007; Degand et al. 2009; Gao et al. 2009; Vítámvás et al. 2012). Apart from COR/LEA proteins revealing hydrophilic and chaperone properties (reviewed in Kosová et al. 2007; Tunnacliffe and Wise 2007; Battaglia et al. 2008; Kosová et al. 2010), changes in several other proteins with chaperone functions, namely HSP proteins, have been observed. An increased abundance of HSP70 proteins and a decreased abundance of HSP90 proteins was found in cold-treated winter wheat (Vítámvás et al. 2012). HSP90 is known to conserve allele variation due to its role in protein folding. Proteins revealing differential primary structure (sequence) encoded by different alleles adopt similar tertiary structure with the aid of HSP90. A decrease in HSP90 abundance could lead to an increased variation in protein conformation which may be advantageous upon stress (Queitsch 2002). Furthermore, an increased abundance of several classes of pathogenesis-related (PR) proteins has been observed upon cold expsosure (Sarhadi et al. 2010).

An increased risk of ROS formation and an enhanced demand of osmoprotectants leads to profound changes in energy metabolism driven by cold. Degradation of polysaccharides, namely starch, followed by glycolysis and Krebs cycle respiratory pathway are upregulated (Amme et al. 2006; Gao et al. 2009). In contrast, an abundance of enzymes incorporating activated glucose into polysaccharide molecules (UDP-glucose pyrophosphorylase) is usually decreased during long-term cold treatments (Gao et al. 2009; Vítámvás et al. 2012); however, during a short-term cold response in young seedlings, it can be increased with respect to control (Cui et al. 2005).

The other major cold-affected process in plants is photosynthesis. An increased risk of ROS formation as a consequence of chilling-enhanced photoinhibition processes results in a down-regulation of electron transport in photosynthetic transport

chain. This is accompanied by a decrease in several protein components of the photosynthetic electron transport chain, as plastocyanin, cytochrome b_6-f complex, Fe-S protein (Rieske protein), and parts of OEC complex (OEE1 protein) (Amme et al. 2006; Gao et al. 2009; Kosmala et al. 2009; Dumont et al. 2011). In contrast, changes in abundance of proteins involved in CO_2 assimilation (Calvin cycle), especially Rubisco large and small subunits and Rubisco activase proteins, are more complex and less clear. Increased abundance of several Calvin cycle components is in fact often observed (Gao et al. 2009; Bocian et al. 2011; Dumont et al. 2011) since rate of enzymatically-catalyzed reactions generally decreases at low temperatures. However, cold acclimation processes reveal increased energy demands, therefore in cold conditions, the CO_2 assimilation needs to be efficiently regulated. As indicated by physiological studies comparing acquired freezing tolerance (FT) levels in long-day (LD) versus short-day (SD) grown barley plants, LD-grown plants reveal higher acquired FT levels (lower LT50 values) in comparison to SD-grown plants when all plants were in vegetative stage (Limin et al. 2007). This result is probably caused by longer photosynthetic periods and higher accumulation of soluble sugars in LD-grown plants in comparison with the SD-grown ones. Thus, a sufficient rate of CO_2 assimilation during cold conditions would be crucial for plant ability to efficiently acclimate to cold.

Another widely reported proteome change due to cold is related to metabolism of methionine and S-adenosylmethionine (SAM) (Cui et al. 2005; Amme et al. 2006; Yan et al. 2006; Vítámvás et al. 2012). SAM is well-known not only as a universal methyl donor in plant methylation reactions, but also as a precursor of ethylene and polyamine biosynthesis. Proteomic studies reporting increased abundance of methionine synthase and SAM synthase under cold thus indicate profound metabolic changes dealing with stress signalling and osmoprotection.

In *Triticeae*, proteome changes upon cold have been studied in bread wheat with respect to both cold acclimation and vernalization processes (Vítámvás et al. 2007; Sarhadi et al. 2010; Rinalducci et al. 2011a, b; Vítámvás et al. 2012). Proteome changes with respect to cold acclimation process have been studied both in spring and winter backgrounds. From physiological studies, it is well-known that upon cold spring genotypes can acquire only a limited level of FT in comparison to winter ones (Fowler et al. 1996b; Fowler 2008). The differences in acquired FT between spring and winter genotypes, but even between winter genotypes with different levels of acquired FT, are mirrored at relative abundance levels of several COR/LEA proteins, namely wheat LEA-II WCS120 proteins (Houde et al. 1992; Vítámvás et al. 2007), their barley homologue DHN5 (Kosová et al. 2008), and stromal LEA-III COR14b protein (Crosatti et al. 1995; Vágújfalvi et al. 2003).

A complex comparison of dynamics of FT acquisition in a winter and a spring wheat genotype with respect to phytohormone levels has revealed that in the winter genotype cold acclimation process is associated with a significant upregulation of several "stress-responsive" phytohormones such as ABA, jasmonic acid (JA), salicylic acid (SA), together with a significant downregulation of growth regulators such as active gibberellins (GAs), cytokinins (CKs) and auxin (IAA). On the other hand, in the spring genotype an initial upregulation of stress-responsive phytohormones,

with a downregulation of positive growth regulators is reversed in later phases of cold treatment (Kosová et al. 2012). These data thus could elucidate why spring genotypes can acquire only a limited level of FT in comparison to the winter ones. At the proteome level, a decrease of several photosynthetic proteins (OEE1, OEE2, ferredoxin NADPH oxidoreductase, fragmentation of Rubisco large subunits, several Calvin cycle enzymes) indicates an impairment of photosynthetic processes.

From studies on transgenic *Arabidopsis*, overexpression of *CBF1* is known to result in plant dwarfism and to positively regulate accumulation of DELLA proteins which act as GA inhibitors (Achard et al. 2008). Conversely, upregulation of transcripts induced by GA seems to represent a logical consequence of a downregulation of *CBFs*. At protein level, Rinalducci et al. (2011b) reported vernalization-induced upregulation of two important proteins, a glycine-rich RNA binding protein (GR-RBP), whose *A. thaliana* homologues are known to act as repressors of FLC (Quesada et al. 2005), and a lectin protein VER2, which is involved in β-N-acetylglucosamine signaling during vernalization (Xing et al. 2009). However, when accumulation of cold-inducible protective proteins (COR/LEA, chaperones) has been evaluated, no dramatic changes caused by vernalization have been reported with respect to the control (Sarhadi et al. 2010; Rinalducci et al. 2011b; Vítámvás et al. 2012). These findings are caused by the fact that the vernalization process has been studied on cold-treated plants and that both unvernalized and vernalized plants have been exposed to conditions inducing cold acclimation. Proteins, unlike transcripts, are direct effectors of plant cold response; a downregulation of cold-inducible proteins in vernalized, but still cold-treated, winter wheat plants would have fatal effects on their survival. Therefore, it can be proposed that there is a time lag between a downregulation of cold-inducible transcripts and cold-inducible proteins in vernalized winter wheat plants, especially when COR/LEA proteins are investigated (Sarhadi et al. 2010; Vítámvás et al. 2012). However, when both unvernalized and vernalized winter wheat plants were exposed to warm temperatures (cold deacclimation) and then reacclimated to cold, significant differences in WCS120 protein accumulation between unvernalized and vernalized winter wheat plants were reported (Vítámvás and Prášil 2008).

From a proteomic point of view, it can therefore be concluded that vernalization fulfillment in cold-treated winter wheat plants is associated with profound changes at the signalling level, but not with rapid changes in cold-inducible protective proteins. A rapid decline of cold-inducible proteins can be achieved only by cold deacclimation, since accumulation of cold-inducible proteins is necessary for plant survival under low temperatures.

6.2.3 Metabolomics

Low temperature-induced changes in transcriptome and proteome finally result in the reconfiguration of metabolomes. Alterations in the level of osmolytes (carbohydrates and free aminoacids), antioxidants, polyamines and other metabolites are

important in the cold acclimation process, and result in an increased freezing tolerance. A coordinated increase in the concentration of amino acids derived from pyruvate and oxalacetate, of polyamine precursors and compatible solutes was observed during cold shock experiments in *Arabidopsis* (Kaplan et al. 2004). The role of CBF regulon was confirmed also at metabolite level in the model plant, since the cold acclimation-induced extensive changes in metabolome could be mimicked by constitutive overexpression of *CBF3*. At the same time, the low temperature metabolome was depleted in metabolites controlled by the CBF regulon in a more sensitive *Arabidopsis* ecotype (Cook et al. 2004). In contrast to *Arabidopsis*, complex metabolome studies were not done with cold-treated wheat and barley. However, several metabolites being important in cold acclimation were studied in these cereals.

A central role of carbohydrate metabolism in the reprogramming of metabolome during temperature stress was suggested by Guy et al. (2008). The positive role of carbohydrates in the cold acclimation process was demonstrated several years ago in wheat, since the activity of sucrose phosphate synthase, sucrose fructosyl transferase and acid invertase increased only in the winter-tolerant genotype but not in the sensitive one, and the fructan accumulation was greater in the former one (Savitch et al. 2000). Using other wheat genotypes with different freezing tolerance, a significant correlation was found between fructose and sucrose content, and freezing tolerance. The comparison of chromosome 5A substitution lines with different freezing tolerance indicated the effect of this chromosome on cold-induced fructose accumulation (Vágújfalvi et al. 1999). The cold-induced up-regulation of sucrose synthase, with the down-regulation of beta-fructofuranosidase genes may result in sucrose accumulation (Kocsy et al. 2010).

Cold acclimation altered both the composition and amount of free amino acids in wheat (Kovács et al. 2011). The ratio of amino acids belonging to the glutamate family increased and the ratio of those ones of aspartate family decreased. Considering the individual amino acids, Asp, Glu, Gln and Pro levels were greatly induced by cold, and these changes were also observed at gene expression level in the case of Pro and Glu. Cold-induced increase in total amino acid content derived mainly from the accumulation of Pro, Glu and Gln in bluegrass (Dionne et al. 2001). The increase in Arg content would drive the greater rate of polyamine synthesis which was described in cold-hardened wheat (Kovács et al. 2010). On the other hand, the cold-induced accumulation of Glu should influence not only polyamine, but also glutathione (GSH) synthesis, the activation of which at low temperature was described in wheat (Kocsy et al. 2000). Cold treatment resulted in increased free amino acid levels in barley as in wheat, and it was suggested that Glu decarboxylation together with GABA metabolism would play a role in freezing tolerance (Mazzucotelli et al. 2006). As an osmolyte, Pro has a special role during cold acclimation, since its accumulation may prevent the water loss from the cells occurring at subzero temperatures, due to extracellular ice formation. In winter wheat, cold acclimation increased Pro content and the change was greater in the tolerant genotype than in the sensitive one (Dörffling et al. 1990; Macháčková et al. 2006).

The cold-induced increase in the amount of the amino acids, which are precursors of polyamines is thus associated with an increased polyamine synthesis, as described

in wheat (Kovács et al. 2010). In the case of putrescine (Put), the increase was not only observed at the metabolite level, but also at the transcript level of the corresponding gene. The importance of Put in the response to low temperature stress was demonstrated in tomato leaves, in which exogenous Put decreased the cold-induced electrolyte leakage, while the inhibition of Put synthesis increased membrane damage (Kim et al. 2002). The accumulation of Put was also observed in alfalfa and wheat during cold hardening, with a decrease in control plants in the activity of arginine decarboxylase, a key enzyme of Put synthesis (Nadeau et al. 1987). In addition, the Put, spermidine and cadaverine contents increased after cold hardening in a winter wheat genotype, while only the concentrations of the polyamines spermidine and spermine increased in a spring wheat variety, indicating the involvement of polyamines in the response to low temperature stress.

Combinatorial approaches that integrate metabolome and transcriptome data have recently elucidated regulatory networks acting in response to environmental stresses. Clear examples are from the model species *Arabidopsis*, in which the metabolome was analyzed using various types of mass spectrometry after cold and dehydration exposure, and metabolic profiles have been then combined into regulatory networks together with transcriptome data. Maruyama et al. (2009) coupled microarray analysis of transgenic plants overexpressing of genes encoding DREB1A/CBF3 and DREB2A transcription factors with the metabolic pathways that act in response to cold and dehydration.

6.3 Molecular Networks: Key Steps in Cold Acclimation (Genomic Role of the CBF Regulon)

The fundamental acquisition of FT in fall-sown temperate cereals is associated with several interconnected physiological and molecular changes, aimed at protecting critical cell structures and vital processes during freezing. The adaptive phenomenon of cold acclimation typically occurs when plants are exposed to low non-freezing temperatures (usually below 9 °C) for several weeks (4–6) and contributes significantly to strengthen the capacity to cope with the stress (Fowler et al. 1996a; Kosova et al. 2008a; Rizza et al. 2011). During the past two decades, molecular biology research of model and crop plants has taught us that cold acclimation is an extremely complex process, whose final outcome consists of increased levels of sugars, soluble proteins, proline and organic acids, the appearance of new enzyme isoforms, and the modifications in lipid membrane composition (Heidarvand and Maali Amiri 2010, and as already described in previous chapters). On the whole, the molecular mechanisms leading to such physiological changes can be ascribed to three main steps: (1) perception of external/physical changes related to temperature drop; (2) transduction of the signal to the nucleus, including the activation of transcription factors; and (3) activation of the low-temperature regulated gene batteries acting as the real effectors of the response (for in-depth reviews see Penfield 2008 and Nakashima et al. 2009). As one can imagine, such view of the phenomenon through

linear steps may be too reductive, especially in light of the results accumulated in the eudicot model *Arabidopsis*. This information provided plant scientists with efficient and valuable resources for co-expression and comparative studies aimed to reconstruct gene networks and pathways that are in turn used to infer functional interactions among genes, proteins and metabolites (Sasaki et al. 2010; Barrett et al. 2011; Mochida et al. 2011). Drawing on Thomashow's commentary on the recent insights gained in the molecular basis of plant cold acclimation, it seems promising that questions on: (i) how low temperature signal is sensed/processed to activate the first wave of cold-regulated genes; (ii) what regulatory logic underlies the cascading pattern of the low-temperature gene network; and (iii) what biological functions can be ascribed to the genes that constitute the various circuits of the network, should find a complete answer soon (Thomashow 2010). The aim of the present paragraph is to make a comprehensive summary of the fundamental players that act in the regulatory networks leading to cold acclimation.

6.3.1 Function of the CBF Regulatory Hub

After exposure to low temperatures, the transcriptome of the *Triticeae* undergoes an extensive progressive reorganization with thousands of genes involved being up- or down-regulated (Svensson et al. 2006; Greenup et al. 2011; Laudencia-Chingcuanco et al. 2011). As estimated in *Arabidopsis*, among these waves of cold-regulated genes, the impressive number of more than 200 transcription factors act in concert for transcriptome reconfiguration and serve as major regulatory for "hubs" acclimation (Thomashow 2010). Although a precise topology of the regulatory network activated in response to cold exposure is still far from being fully understood, the most clearly understood node from both the *cis*-regulatory and transcription factor side is represented by the CBF circuit. The CBFs are characterized by a plant specific APETALA2/ethylene-responsive element binding factor (AP2/ERF) domain flanked by a typical sequence motif, the so called "CBF signature" (Stockinger et al. 1997; Skinner et al. 2005). It is the AP2/ERF domains that interacts with the CRT elements present in the promoter region of their target genes, referred to as the CBF regulon, to increase their expression level. Starting from the early 1990s, the CRT elements, defined by the 5'-CCGAC-3' core motif, and the CBF proteins were isolated and characterized in several plant species from *Arabidopsis* to *Triticeae* crops (Baker et al. 1994; Stockinger et al. 1997; Ouellet et al. 1998; Choi et al. 1999; Xue 2003; Dubouzet et al. 2003). Much of our understanding of this circuit was built on a bottom-up approach, by linking the results of promoter binding assays and mutant analysis (for a recent review see Chinnusamy et al. 2010), whereas the top-down studies used DNA microarrays to establish components and wiring of the CBF regulon, and to add new putative components (Vogel et al. 2005; Greenup et al. 2011; Laudencia-Chingcuanco et al. 2011).

As summarized earlier, a series of stress-inducible genes that play a major role in acclimation and acquisition of FT are downstream *CBFs*. Examples of targets

within the CBF regulon are genes encoding enzymes involved in the biosynthesis of osmo- and cryo-protectant molecules (such as sucrose, raffinose and proline) but also other transcription factors, late-embryogenesis abundant (LEA) proteins, COR and cold-inducible (KIN) proteins, phospholipase C enzymes, and sugar transport proteins (Heidarvand and Maali Amiri 2010). The primary functional involvement of the target stress-inducible genes is thus the protection of membranes and proteins against the severe water deprivation stress that occurs with freezing (Penfield 2008). However, beside production/accumulation of cryoprotectants, other metabolic functions are affected by the CBF regulatory hub in contributing to the ability of plants to survive frost. A well-known phenomenon in *Arabidopsis* plants that constitutively overexpress *CBF* genes under normal growth conditions is severe growth and development retardation (Jaglo-Ottosen et al. 1998). Results accumulated in transgenic tomato plants overexpressing *AtCBF1* suggested a link between the CBFs and giberellic acid (GA) through nuclear-localized DELLA proteins (Hsieh et al. 2002). Afterwards, Achard et al. (2008) demonstrated that the CBFs enhance the expression of GA-inactivating GA2-oxidases allowing the accumulation of DELLA protein repressors, which in turn lead to dwarfism and late flowering. Although the role of DELLA-mediated change in plant architecture has still not been completely clarified, one could speculate on how this phenotype would potentially contribute to winter survival of fall sown cereals. For example, a more prostrate growth habit should result in a greater chance of the plants being covered by snow and insulated against harsh air temperatures (Roberts 1990).

It is worthy to note that the majority of the studies done on wheat and barley CBFs focused only on transcript levels, and not on CBF protein abundance, post-transcriptional and post-translational modifications. However, a further interesting perspective related to the role played by CBFs in the grasses concerns their *cis*- and *trans*-acting regulation. The rationale is that once the transcription factors involved in expression of the first wave of stress-responsive genes are identified, further studies can be followed to work back upstream into the stress-sensing system and thus understanding to which extent the activities of the transcription factors are affected. In this view, the *Arabidopsis* research community provided indications about two cold sensing pathways upstream of the CBF regulatory hub: post-translational modification and calcium signaling (Thomashow 2010).

In the first pathway, *Inducer of CBF expression1* (*ICE1*) gene is involved, and it binds to multiple MYC DNA regulatory elements in the promoters of certain *AtCBF*s to stimulate their transcription (Chinnusamy et al. 2003). Such a major positive regulator mediates cold induction via low temperature-induced activation rather than by transcription modulation. On one side, ICE1 is activated by small ubiquitin-related modifier (SUMO) so that sumoylation (i.e. SUMO conjugation) of ICE1 is directly responsible of the positive induction of the CBF regulon and is mediated by the SIZ1 protein, a SUMO E3 ligase (Miura et al. 2007). On the other side, this activation process is finely modulated by *High expression of osmotically responsive gene 1* (HOS1), a RING finger E3 ligase that mediates ubiquitination and targeted proteolysis of ICE1 (Dong et al. 2006). The SIZ1-HOS1 system thus appears to contribute to the "transient" nature of ICE1 as a master regulator controlling the

CBF regulon and many other cold-responsive genes. In wheat, the ICE1 homologs *TaICE141* and *TaICE187* are constitutively expressed and activate the wheat *CBF* genes of group IV, which are associated with freezing tolerance. Overexpression of *TaICE141* and *TaICE187*in *Arabidopsis* positively stimulated *CBF* and *COR* gene expression and enhanced freezing tolerance only after cold acclimation. This finding suggests that, similar to AtICE1, also wheat ICE1 proteins need to be activated by cold acclimation (Badawi et al. 2008).

In addition to the ICE1 mediated pathway, the CBF regulatory hub is upstream-regulated by a second cold-sensing pathway in which cytosolic signatures of the second messenger Ca^{2+} are major players. Calcium signatures are sensed by calcium sensor family proteins, namely calcium-dependent protein kinases (CDPKs), calmodulins (CaMs), and salt overly sensitive 3-like (SOS3-like) or calcineurin B-like (CBL) proteins (for a review see Reddy et al. 2011). Several studies linked the low-temperature induced intracellular Ca^{2+} spikes to *COR* gene expression in *Arabidopsis* (Knight et al. 1996; Knight 2000), and it is demonstrated that cold induction of certain CBF regulon genes is weaker either in mutants impaired in Ca^{2+} channels and Ca^{2+}/H^{+} antiporters or using chemical agents that chelate/block calcium influx into the cytoplasm (Monroy et al. 1997; Catala et al. 2003). Recently, Doherty et al. (2009) analyzed the promoter region of the three *AtCBFs* and found seven conserved DNA motifs (CM1-7) responsive to cold induction. In particular, CM2 matched the calmodulin-binding transcription activator (CAMTA) binding sequence 5'-CGCG-3' (which also overlaps the ICE1 regulatory element) and brought to cold-induced expression of *AtCBF*s. Additional information about interconnection between calcium cellular concentration and gene regulation in response to stress is beyond the scope of this paragraph and can be found reviewed in detail by Chinnusamy et al. (2010) and Reddy et al. (2011). As far as we know, no or very few studies on isolation and characterization of CAMTA genes involved in low-temperature tolerance induction in wheat and barley have been published. However, the evidence accumulated in the model plant *Arabidopsis* would suggest that the *Triticeae* research community deepen the study about the existence and diffusion of such a conserved regulatory pathway.

6.3.2 Early Events in the Cascade

An interactive reactive oxygen species (ROS)—nitric oxide (NO) concentration-dependent signaling mechanism has been recently proposed by Cantrel et al. (2011). The authors showed how, after membrane rigidification at low temperature exposure, NO act as a key player at the very beginning of the regulatory network activation in modulating the synthesis of sphingolipids signals. They also proposed a model in which the cold stress is perceived as a result of plasma membrane rigidification, and this in turn results in the production of phosphatidic acid through the activation of diacylglicerol kinase (DAGK) and/or phospholipase D (PLD). Phosphatidic acid activates a short spike of ROS and NO that is quickly modulated to a subtoxic

concentration by nonsymbiotic haemoglobins (nHb). Similarly to the first report in which either sphingolipid phosphorilation or its regulation by NO were implicated in plant cold-signal transduction, the findings by Cantrel et al. (2011) open the way for future studies aimed to further increase our understanding of these complex and inter-related processes in model and crop plants.

6.3.3 Integration of the Circadian Control

Besides the described molecular mechanisms leading to the development of freezing tolerance, it is important to briefly mention here also the influence of the circadian clock on the CBF regulatory hub. The circadian clock has been shown to control the expression of *CBF* genes in *Arabidopsis* and tomato (Fowler et al. 2005; Pennycooke et al. 2008), suggesting that this could be a highly conserved way of regulation. Expression analyses in both barley and hexaploid wheat also indicate that certain *CBF* genes are circadian-regulated, while others are not (Badawi et al. 2007; Stockinger et al. 2007), with the maximal cold-induced increase in CBF transcription that occur when cold stress is imposed 4 h after dawn. Determining how the low temperature regulatory network and the circadian clock are integrated will not be trivial. However, some insights were recently obtained in *Arabidopsis* where a complex molecular circuit seems to directly affect the CBF regulatory hub both in a negative and in a positive way of action. On one hand, it was demonstrated as the *phytochrome-interacting factor 7* (PIF7), a bHLH transcriptional repressor, interact with *timing of cab 1* (TOC1), a component of the central circadian oscillator, and a red light photoreceptor PHYB (Kidokoro et al. 2009). On the other hand, a direct positive action of two transcription factors that are core components of the clock, i.e. *Circadian Clock-Associated 1* (CCA1) and *Late Elongated Hypocotyl* (LHY) has been found (Dong et al. 2011). In this latter report, the authors proposed a first comprehensive model for circadian regulation and gated cold induction of *AtCBF -1*, *-2*, and *-3* in response to day/night oscillation coupled to warm/cold temperature exposure, and also include the mechanism of action of the above mentioned factors ICE1 and CAMTA (Dong et al. 2011). Due to the expected conservation of the circadian clock regulation system between *Arabidopsis* and the *Triticeae*, one could predict that in the near future the orthologous components of the regulatory network should be identified in wheat and barley as well.

6.3.4 CBF-Independent Hubs

Genes induced by abiotic stresses (mainly dehydration and cold) can be classified according to two main signal transduction pathways that able to convert the external stimulus into cellular responses: the ABA-independent and the ABA-dependent. The CBF regulatory hub discussed so far functions in the ABA-independent pathway. However, transcriptomic analyses involving *Arabidopsis* mutants identified through

genetic screening for freezing tolerance, revealed that several classes of transcription factors are affected by ABA and are related to multiple stresses (for recent reviews, see Nakashima et al. 2009; Chinnusamy et al. 2010). For example, after a reporter-gene genetic screening (based on P_{RD29A}*:LUC* construct) two constitutively expressed transcription factors, HOS9 (a homeodomain protein) and HOS10 (a R2R3-type MYB), which are necessary for developing low-temperature tolerance were identified (Zhu et al. 2004, 2005). Through transcriptome analysis it was possible to identify the HOS9 regulon as distinct from the CBF regulon (Zhu et al. 2004) and, since HOS10 positively regulates NCED3 (9-cisepoxycarotenoid dioxygenase), this transcription factor has been assigned to ABA-dependent cold acclimation pathways (Zhu et al. 2005). Among the CBF-independent regulons those under the control of ZAT12 and HOS9 show remarkable characteristics (Vogel et al. 2005; Chinnusamy et al. 2010) (Fig. 6.1). The ZAT12 regulon contains 9 cold-induced and 15 cold-repressed genes and the overexpression of *ZAT12* gene resulted in increased freezing-tolerance. In *hos9-1* mutant seedlings, the expression of 140 genes was higher and that of 35 genes was lower than in wild type, and from the highly expressed genes 41 appeared to be cold-inducible (Zhu et al. 2004). Both *ZAT12* and *HOS9* genes are negative regulators of some genes of the CBF regulon (Zhu et al. 2004; Vogel et al. 2005), which indicates a possible interaction between the various cold-responsive regulons. *Eskimo1* (*esk1*) is a constitutively freezing-tolerant *Arabidopsis* mutant characterised by a 30-fold higher level of proline than wild-type plants, supporting an important role of the osmolytes in stress resistance (Xin et al. 2007). ESK1 is constitutively expressed and encodes the protein domain of unknown function which mechanism of action has yet to be revealed. However, transcriptome comparison of CBF2-overexpressing plants and *esk1* mutants showed that different sets of genes are regulated by CBF2 and ESK1 (Fowler et al. 2005).

Arabidopsis showed its potential as a well-established model system also through the transgenic analysis of cold-inducible transcription factors identified in crop species and helped in validation of functions of some transcription factors in cold tolerance. As intriguing examples, overexpression in *Arabidopsis* of the cold-regulated rice transcription factors OsMYB4 (an R2R3-type MYB) and OsMYB3R-2 (an R1R2R3 MYB) enhanced freezing tolerance (Vannini et al. 2004; Dai et al. 2007). Similarly, transgenic *Arabidopsis* overexpressing *TaERF1*, a wheat gene induced by cold, drought salinity, ABA, ethylene, salicylic acid, and infection by *Blumeria graminis* f. sp. *tritici*, exhibited enhanced tolerance to cold, salt, and drought stresses, as well as pathogens (Xu et al. 2007). Finally, although still not deeply characterized, the two transcription factors *wheat low-temperature-induced protein 19* (WLIP19) and *T. aestivum ocs-element bindingfactor 1* (TaOBF1), two bZIP-type proteins, were found to form heterodimers that were able to activate the expression of *COR/LEA* genes in the development of abiotic stress tolerance. In conclusion, these results suggest that a number of transcriptional networks operate during cold acclimation and cold stress tolerance in addition to the major role played by the CBF hub. Comparative analysis of gene expression patterns between *Arabidopsis* and the Triticeae are important future tasks, and further research efforts are expected to better clarify the interconnection of the whole gene regulatory network involved in cold acclimation.

6.4 Exploiting Genetic Resources and Genomic Selection for FT

6.4.1 Genetic Resources

The progenitors of current wheat and barley varieties come from the Fertile Crescent and most wild *Triticeae* species possess a winter growth habit (Kosová et al. 2008b) as they are adapted to climatic conditions where the annual rainfall is concentrated in the autumn and spring, followed by hot and dry summers. Plants develop vegetative organs during rainy autumns, and use vernalization to delay flowering until winter is over, while the photoperiod sensitivity allows them to flower and complete grain filling before the hot summer begins. The first domesticated cereals shared the growth habit of their wild relatives. Farmers selected for spring forms which could be sown and harvested in a shorter season and allow growing two crops in succession each year (Cockram et al. 2007b). Mutations in spring barley resulted in reduced photoperiod responsiveness alleles that removed the promotion of flowering in response to long days (Turner et al. 2005). Those factors are likely to have favoured expansion of barley production to higher latitudes allowing it to avoid injury during cold winters (Cockram et al. 2009), while taking advantage of the long cool and wet summers of northern Europe (Pourkheirandish and Komatsuda 2007; Comadran et al. 2012). Distinct clustering of types classified as winter or spring has been maintained and is reported in all surveys of cultivated crops diversity (Cockram et al. 2007b; Comadran et al. 2012). Some, rather infrequent, materials are classified as "alternative" or "facultative". No unambiguous description for this type exists; von Zitzewitz et al. (2005) classified them as cold-tolerant and vernalization unresponsive, while according to the International Union for the Protection of New Varieties of Plants (UPOV) they display an intermediate flowering time (respect to that of winter and spring types) when grown under inductive photoperiods without vernalization (Cockram et al. 2009). Many winter, most facultative, and few spring barleys are sensitive to short days, whereas winter and facultative barleys are more cold-tolerant than spring types. Since those traits are controlled by different genes, cold-tolerant, facultative varieties could be the best choice (www.barleyworld.org). Wheat is considered to have the broadest adaptation of all cereal crops and is cultivated in a wide range of environments due, largely, to its tolerance to cold. Winter and facultative wheats are grown on one third of the 220 million ha devoted to wheat worldwide. The most winter-hardy wheat cultivars are required for areas in the northern Great Plains of North America, the Russian Federation and Ukraine and, to less extent, ineastern, central and northern Europe, eastern Turkey, northwest Iran and China (Braun and Sãulescu 2002).

Breeding for uniformity for the development of high-yielding cultivars as requested by modern agriculture, rapid population growth and economical changes led to a drastic intraspecific narrowing of the genetic base of barley and wheat, leaving behind many potential useful genes, and enhancing the risk of losing adaptation to abiotic stress such as frost. The conservation and availability of genetic diversity of crops and their wild relatives provide farmers and breeders with materials for

improving and adapting the crops to face future environmental, climatic and economic changes in a sustainable way (FAO 2010). Harlan and de Wet (1971) were the first authors to classify plants using the "three gene pool" concept. The primary gene pool consists of species which can be easily intercrossed; the secondary gene pool includes related species; their crossing with the target produces at least some fertile hybrids. Species in the tertiary gene pool can be crossed only by applying techniques such as embryo rescue, bridge crossing or protoplasm fusion (Acquaah 2006). In the case of barley, wild barley (*H. vulgare* spp *spontaneum)* belongs to the primary genepool; *H. bulbosum*is the only member of the secondary genepool, while the tertiary genepool of barley comprises about 30 *Hordeum* species (Pickering and Johnston 2005). The primary pool of wheat comprises all *Triticum* species, the secondary pool—all *Aegilops* species, the tertiary wild relatives pool includes some remote members of the tribe *Triticeae*.

In order to preserve the existent crop diversity, major cereal collections have significantly increased in recent years. In total 466,531 barley accessions are held worldwide, and major holders are Plant Gene Resources of Canada (PGRC) with 9 % of them, and National Small Grains Collection (NSGC) USA with 6 % (FAO 2010). If we consider the division of resources according to the growth habit, the European Barley Database (EBDB) of the Institute of Plant Genetics and Crop Plant Research (IPK) reports for advanced/improved cultivars 1,431 winter; 2,875 spring and 10 facultative accessions. For wild barley there are 135 winter, 320 spring and 2 facultative, while among landraces there are 1,485 winter, 14,180 spring and 90 facultative accessions (http://barley.ipk-gatersleben.de/ebdb.php3).

According to a FAO's recent report there are 856,168 total world accessions of wheat, with major holders being CIMMYT (13%) and NSGC (7 %) (FAO 2010). If we consider the division according to the growth habit, there are 19,179 accessions of winter, 11,419 of spring and 119 intermediate advanced/improved cultivars in the European Wheat database (EWDB) (http://genbank.vurv.cz/ewdb/) that contains data of wheat collections stored in European countries. Among landraces we find 8,058 winter, 9,921 spring and 180 intermediate accessions. As far as wild *Triticum aestivum* is considered, there are 126 winter accessions and 142 characterized as spring. For durum wheat, there are 614 winter, 3,145 spring and 11 intermediate improved/advanced accessions, 644 winter, 4,044 spring and 109 intermediate traditional varieties/landraces.

Variation in physiological traits associated with salt, cold and drought tolerance and N-starvation has been reported in the highly variable wild progenitor *H. spontaneum* (Hussain 2006). Perennial *H. bulbosum* that includes both diploid and tetraploid forms is an obligate outbreeder (self-incompatible) and the only member of the secondary genepool of *Hordeum* that represents a valuable source of genetic diversity for barley crop improvement. A set of diploid introgression lines (ILs) containing chromatin introgressed from *H. bulbosum* into cultivated barley (*H. vulgare*) were generated that represent a significant germplasm resource likely to contain genetic diversity that can be mined for improvement of traits of interest for barley breeders and researchers (Johnston et al. 2009).

A large number of accessions of *A. tauschii* have been screened for FT and found to contain cold hardy accessions, although none of these were as cold hardy as winter wheat cultivars (Limin and Fowler 1991). Moreover, the cold hardiness levels of synthetic hexaploid wheat, produced by combining tetraploid wheat with *A. tauschii*, to introduce new cold hardiness genes into the common hexaploid wheat gene pool, did not identify transgressive segregates for improved cold hardiness, even if the cold hardiness levels of hybrids ranged from similar to equal to the hardy parent. These observations led Limin and Fowler (1993) to conclude that the close wheat relatives, sharing common genomes with *T. aestivum*, cannot be considered promising sources of new genes for cold hardiness improvement. On the other hand, recently wild emmer *T. dicoccoides* has been reported to be a rich, mostly untapped genetic resource for improvement of cultivated wheat and genetic traits of economic significant such as earliness, yield and cold tolerance (Nevo 2011).

Related cultivated species could provide another potential source of genetic variability. The much greater winter hardiness of rye than that known in wheat and barley proves that its potential to improve wheat for the trait has been largely unused. Hexaploid or octaploid *Triticales* are synthesized by crossing rye either with tetraploid or hexaploid wheats, respectively. Secondary hexaploid triticales, that nowadays are the most common commercial *Triticales* worldwide, derive from intercrosses between different primary hybrids, after backcrosses with wheat. At present, winter *Triticale* varieties are as winter hardy as the best winter wheat varieties, but less than winter rye (Alberta Agriculture, Food and Development 2001). Rizza et al. (1997), comparing the behaviour of the most frost resistant *Triticale* (cv. Aubrac) with a winter wheat, demonstrated that this genotype could perform much better than the winter wheat and, under specific test conditions, even as good as a typical frost-resistant rye.

6.4.2 Genomics-Assisted Breeding

Until present, prerequisite for any application of DNA-based technologies to plant breeding is sufficient knowledge of the genetic bases for agronomically relevant traits: inheritance, number of loci and weight on the traits of single loci. Secondly, sufficient knowledge of markers associated (in linkage disequilibrium) with loci supporting the trait: nature and information content, position in the genome, distance from the gene responsible for the trait. Gene and QTL cloning allow in this case moving from associated, linked, markers, to "perfect" candidate gene-derived markers.

As underlined in the previous paragraphs, genomic research has allowed in the last decade to make significant advances in knowledge of the genetic bases for freezing tolerance in plants, as well as in *Triticeae*. Although considered a polygenic trait, since the early 90's QTL mapping has led to the identification in *Triticeae* of a relatively small number of quantitative trait loci having major effects on the ability of the plant to survive freezing (Galiba et al. 2009; Pecchioni et al. 2012). After the first reports that identified a highly significant genomic region of chromosome

group 5, associated with *VRN-1* vernalization requirement locus (Galiba et al. 1995), Francia et al. (2004) demonstrated that FT was mainly controlled by two linked QTLs, *FR-H1* and *FR-H2*, thanks to the Nure x Tremois (winter x spring), unique mapping population where both FR- QTLs were segregating in the *Triticeae*; in wheat *FR-A2* (Vágújfalvi et al. 2003) and *FR-A1* were mapped in different genetic systems. A cluster of at least 12 C-repeat binding factor (*CBF*) genes are the best candidate genes underlying *FR-2*, whereas *FR-1* was later identified with the *VRN-1* vernalization response locus on chromosome 5A, and then cloned by Yan et al. (2004). The two QTLs together explain a large part of variation for the trait (e.g. 65.9 % of tolerance in a controlled freeze test in barley, Francia et al. 2004), and this fact limited the success of identification of other, minor effect, QTLs for freezing tolerance through such linkage mapping approach. A probably hortologous locus, *FR-B1*, with a smaller effect than *FR-1*, was mapped on the homoeologous chromosome 5B (Tóth et al. 2003). In barley, Reinheimer et al. (2004) indicated two chromosomes, 2H and 5H, as implicated in the genetic control of reproductive FT. A pioneering work by Tuberosa et al. (1997) found a slightly more complex situation in a winter x winter type barley cross Arda x Opale. Nine freezing tolerance QTLs were mapped on chromosomes 2H, 3H, 6H and 5H, after a screening conducted in controlled environment, with the one of 5H roughly coinciding with *FR-1* (Tuberosa et al. 1997). For other identified loci, due to the nature of markers used, it was not possible to identify precisely colinearity regions with more dense genetic maps (Cattivelli et al. 2002). Recently and still in barley, 285 spring type cultivars were evaluated for FT and genotyped with 1,536 gene-based SNPs for an incremental association mapping approach and significant new marker/trait associations have been detected on chromosome 4H and 5H, that do not co-localize with *FR-H1* and *FR-H2* loci (Tondelli et al. 2009).

Varshney et al. (2005) discussed how for some traits it could be necessary to use crop wild relatives to introgress some of the diversity that was lost during domestication. That might be the case of freezing tolerance too. In the cross Arta x *Hordeum spontaneum 41-1,* it was shown that whereas cultivated barley contributed higher scoring alleles for the major effect QTLs for cold tolerance, for some QTLs with minor effects (on 2H, 4H and 6H), the allele from the *H. spontaneum* conferred tolerance (Baum et al. 2003), suggesting a deeper investigation of the wild resources. The use of association mapping to position loci controlling economically important traits in a wild crop progenitor was tested on a collection of 318 accessions from the Fertile Crescent, Central Asia, and North Africa assembled to form the Wild Barley Diversity Collection (WBDC) genotyped with 50 SSR and 1090 DArT markers, with 3,000 additional SNP markers to follow and evaluated for resistance to six barley diseases and 25 agronomic and morphological traits (Steffenson et al. 2007).

Although it is possible that in the future new introgressions, new crossing schemes, especially among either winter or spring types, or very large populations like NAM (Nested Association Mapping; Buckler et al. 2008), will allow to identify other QTLs, it would be less likely that they have a large influence on tolerance to frost. For this reason, the routine application of DNA-based technologies to breeding for this trait could be focused more on marker-assisted selection (MAS) than on genomic selection (GS) approaches. Owing to Kumar et al. (2012), the first approach could be

named LD-MAS, i.e. MAS using markers in LD with a QTL. Moreover, this breeding strategy could be based on candidate genes (CGs) and "functional" markers rather than on anonymous markers.

6.4.2.1 LD-MAS for Freezing Tolerance

Availability of functionally characterized genes, ESTs collections and ongoing genome sequencing projects have facilitated in important crops like wheat and barley the development of molecular markers directly from the transcribed regions that are commonly referred to as "genic" or "functional" markers (FMs) as their putative functions can be derived from homology searches. Such markers can target directly the functional polymorphism within the gene that is causing variation in the influenced trait, enabling selection in different genetic backgrounds without the need to validate the marker. That is why they are also commonly named "perfect markers" (Varshney et al. 2005).

The development of a reliable molecular test for GH in wheat and barley could find numerous applications. Crosses within GH classes, predominant in modern breeding contribute to corresponding genotypic division of spring and winter types, diagnostic markers for *VRN* genes could be thus deployed in breeding programs in order to increase genetic diversity by utilizing winter × spring crosses (Cockram et al. 2008, 2009). For example, the identification of *VRN-H1* and *VRN-H2* genes in barley allows screening of germplasm collections and classification of the alleles and allele combinations present in modern cultivars, as well as discovery and characterization of novel alleles and allele combinations controlling vernalization requirement (Cockram et al. 2007a). Since a highly conserved VRN-1 peptide is likely to be essential for the plant (for both GH), the functional marker is based in barley on an In/Del polymorphism in putative *cis*-regulatory regions of VRN-H1 intron 1 (von Zitzewitz et al. 2005). Similar intron 1 deletions of the orthologous genes of the hexaploid and tetraploid wheats are associated with spring alleles suggesting that those deletions are responsible for the differences in GH (Fu et al. 2005). Because coincident with *FR-1*, the same GH molecular marker for *VRN-H1* (*HvBM5*) was also reported as the best predictor for marker-assisted selection within highly frost-tolerant accessions from Turkey and other winter, facultative and spring barley germplasm. The CG marker can thus be used not only for selection of GH types in winter x spring crosses, but also for fast routine selection of frost tolerant genotypes (Akar et al. 2009). Similar indications came from the work of Rapacz et al. (2010) that found variation in the promoter region of *Vrn-H1* (Hv*BM5*) directly connected with freezing tolerance of plants partially de-acclimated in the field.

In a study by Akar et al. (2009), only one out of the three markers designed on *CBF* genes was moderately associated with FT. Polymorphisms used and derived from linkage mapping were in fact not associated with any functional diversity in the transcripts. In barley, it is still not clarified if a distinct CBF element could be causative of the *FR-2* effect on the trait, as tentatively hypothesized by Fricano et al. (2009), or if, more likely, the composition of the gene cluster in terms of copy

number variation (CNV) of at least two elements could constitute the functional difference between freezing-tolerant and susceptible genotypes (Knox et al. 2010). Yet, further studies would be necessary to validate the feasibility of a MAS approach based on single *CBF* elements rather than on the introgression of the whole *CBF* cluster region; especially in winter x winter genotype crosses, where recombinants between *CBF* sub-clusters could be not easy to obtain. The simplest approach to select with the LD-MAS is the introduction of (SNP) functional marker panels into normal pedigree breeding schemes. The CG markers can be introduced as support or substitution of phenotypic selection also in case of normal backcrosses, or doubled-haploid (DH) line evaluation. An interesting application of LD-MAS is the parent building by means of candidate gene pyramiding (Sabatini et al. 2013). Mainly used to cumulate pathogen resistances into a single genotype (Mago et al. 2011), it could also be used to cumulate CGs for freezing and other abiotic stress tolerances.

6.4.2.2 Genomic Selection (GS)

Genomic selection (Meuwissen et al. 2001) could be defined as a method of prediction of the breeding value of lines by analyzing phenotype together with high-density marker scores. Basically, GS simultaneously estimates all locus, haplotype, or marker effects across the entire genome to calculate genomic estimated breeding values (GEBVs). It incorporates all marker information in a prediction model, thereby avoiding biased marker effect estimates and capturing more of the variation due to small-effect QTL. Genomic selection (GS) has been proposed to overcome the limits of application of LD-based MAS to polygenic traits selection. While GS should substantially accelerate the breeding cycle, it would also dramatically change the role of phenotyping, that could be used more to update the prediction models driving GS than to select lines (Heffner et al. 2009).

By using high-density SNP panels, the genotype that would better fit with the genomic prediction model should be selected in order to combine and cumulate the positive effects from all beneficial alleles at contributing genes and minor QTLs. Heffner et al. (2010) demonstrated in a population of 374 winter wheat characterised for 13 agronomic traits that the average prediction accuracies for GS would be 28 % higher than MAS, while 955 as accurate as phenotypic selection. Since a high-density SNP panel could be excessively costly per single analysis, Habier et al. (2009) proposed to use a panel of low-density evenly spaced SNPs. Diffusion of GS in practical breeding thus strongly depends on the increasing availability of cheap high throughput markers systems.

Moreover, GS can be better proposed for species where genomic constitution is known in terms of sequences and their physical position, and for which cultivar resequencing projects are in progress, as in apple (Kumar et al. 2012). In this view, the success in sequencing all gene-containing regions of barley and wheat is a necessary requirement to allow GS-based schemes. Recently, Paux et al. (2012) report that GS methods are under evaluation for crops such as maize and wheat and in some cases are being applied in wheat commercial breeding programs, although details have yet to be published.

Genomic selection could be particularly useful to cumulate durable resistance QTLs in wheat as in other plants. Rutkoski et al. (2011) propose a GS-based wheat breeding scheme for quantitative (durable) resistance to stem rust, where the multigenic nature of adult plant resistance hampers the efficiency of MAS-based pyramiding. Due to the lack of mapped minor QTLs in wheat as in barley, all affecting the final level of freezing tolerance, GS for FT could be an option, together with other agronomically relevant traits. Once high density SNP panels can be made available and at reasonable assay costs, there should not in fact be the need to know all the (minor) QTL positions to select associated markers. Rather, and as proposed by Meuwissen et al. (2001), GS analyzes jointly all markers on a population attempting to explain the total genetic variance with dense, genomewide marker coverage through summing the marker effects to predict the final breeding value of individuals.

6.5 Conclusions and Perspectives

Global climate change poses increasing constraints on the ability of crops to acclimate to abiotic stresses. Cereal breeding for an enhanced FT and winter hardiness will remain an important part of winter cereal breeding programmes in temperate climate zone. New approaches of structural as well as functional genomics will facilitate the identification of candidate genes underlying FT and winter-hardiness QTLs as well as the functional characterization of their protein products. Recently, fine mapping of the cluster of *CBF* transcriptional activators at *Fr-2* locus in Triticeae and their further characterization has revealed genetic differences in *CBF* gene sequence and gene-copy number between frost-tolerant winter and frost-sensitive spring genotypes of *Triticeae* (Knox et al. 2008, 2010). Differences in *TmCBF12* sequence at AP2 CRT/DRE promoter-binding domain between winter line G3116 and spring line DV92 of einkorn wheat and differences in the number of *HvCBF2* and *HvCBF4* paralogues (*HvCBF* gene copy number) between winter barley Nure and spring barley Tremois can be utilised as genetic markers to distinguish frost-tolerant winter and frost-sensitive spring genotypes of *Triticeae* (reviewed in Tondelli et al. 2011). Combination of "omics" approaches with transgenic techniques will help us to identify key factors at all levels (genes, transcripts, proteins and metabolites) involved in the processes of FT acquisition and vernalization. Transgenic techniques will help us to functionally characterize key proteins involved in cold acclimation and vernalization. An example is provided by the progress in understanding the role of the major cereal vernalization gene *VRN1* using *mvp* (*maintained vegetative phase*) mutants of einkorn wheat coding for a non-functional *VRN1* gene (Dhillon et al. 2010). A better understanding of the physiological mechanisms involved in cold acclimation and FT acquisition will lead to breeding progress using modern genetic approaches (e.g. MAS, gene pyramiding, etc.). Modern high-throughput screening methods will enable us to carry out genome-based selection of genotypes with desired sets of alleles of multiple genes regulating stress tolerance as well as grain quality and other desired agronomic traits. The new approaches described herein

will eventually lead to the development of new cultivars with tailored characteristics and with an improved versatility underlying the ability to cope with environmental challenges.

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