Unravelling the complexity of cold acclimation in plants

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To my daughter Tanya

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Abstract

Many plants respond to low non-freezing temperatures by increasing their freezing tolerance in a process known as cold acclimation. Microarray studies have shown that hundreds of genes are differentially expressed during the cold acclimation process in *Arabidopsis*. To predict the gene regulatory interactions amongst these differentially expressed genes a rule based bioinformatics model was developed. The inferred regulatory network correctly identified several previously characterized interactions and predicted several new interactions under combinatorial control of many TF families (Chapter 3.1, Paper I). As a continuation of this work, detailed combinatorial studies on promoters were done to understand weather the key regulon DREB1/CBFs in turn is regulated by several other TFs in *Arabidopsis* and rice (*Oryza Sativa L.*). The results showed that bioinformatics can correctly predict combinatorial regulation and can be used to identify previously known promoters motifs and predict new ones involved in co-regulated genes (Chapter 3.1, Paper II).

In Sweden, cultivated oat (*Avena sativa L.*) is grown only as a spring crop as no suitable winter oat exists. To develop such a cultivar, a model system to detect differences between spring and winter oats on the molecular level is required. To this end 294 winter oat lines from throughout the world were collected, tested in the field in Sweden and rated based on their survival and vigor. The best performing lines were further characterized in the laboratory by physiological, biochemical and molecular analysis. The tests showed that while the German cultivar LPWH992209 performed best in the field, the American cultivar Win/Nor-1 outperformed the others in the controlled tests. Six cultivars including two spring, two intermediate and two winter cultivars were finally selected to make up the winter oat model system. Metabolic analysis revealed several metabolites such as sugars, amino acids as well as unknown metabolites that were differentially expressed in the winter oat model lines (Chapter 3.2, Paper III).

Finally, an EMS mutagenized oat TILLING (Target Induced Local Lesions In Genomes) population consisting of 2,500 different mutated lines was generated. The genetic variation of the library was verified by various molecular analysis and proven by the identification of mutations in the *AsPAL1* and *AsCslF6* genes. Several mutants producing low levels of lignin in their husk were identified by biochemical analysis. This TILLING population will now be used to identify mutants with increased freezing tolerance (Chapter 3.3).

Keywords: bioinformatics, genetic networks, cold acclimation, freezing, oat, Avena sativa, Arabidopsis, microarray, electrolyte leakage, abiotic stress, metabolomics, TILLING
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Papers discussed

The thesis is based on the following papers.

Paper I
Chawade A., Bräutigam M., Lindlöf A., Olsson O., Olsson B. (2007) Putative cold acclimation pathways in *Arabidopsis thaliana* identified by a combined analysis of mRNA co-expression patterns, promoter motifs and transcription factors.
BMC Genomics, 8:304.

Paper II
Lindlöf A., Bräutigam M., **Chawade A**., Olsson O., Olsson B. (2009) *In silico* analysis of promoter regions from cold-induced genes in rice (*Oryza sativa* L.) and Arabidopsis thaliana reveals the importance of combinatorial control.
Bioinformatics, 25 (11):1345-1348.

Paper III
Chawade A., Lindén P., Bräutigam M., Jonsson R., Jonsson A., Moritz T., Olsson O. (2011) Identification of differentially expressed metabolites during cold acclimation in a winter oat model system.
(Manuscript)

Paper IV
Chawade A., Sikora P., Bräutigam M., Larsson M., Vivekanand V., Nakash M.A., Chen T., Olsson O. (2010) Development and characterization of an oat TILLING-population and identification of mutations in lignin and β-glucan biosynthesis genes.
BMC Plant Biology, 10:86.

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1. Introduction

During the process of cold acclimation, several physiological and developmental changes take place in the plant to avoid the detrimental effects of freezing temperatures [1-3]. Freezing tolerance varies greatly amongst plant species and between ecotypes/cultivars within the species because of the differences in their ability to cold acclimate. The key questions that arise while studying cold acclimation are: How does the low temperature response signal the activation of the cold acclimation process? Which genes are involved in the process? How are they regulated and what are their functions? What physiological changes are brought about in the plants to increase freezing tolerance? Answering these key questions could not only lead to greater understanding of how plants interact and respond to the changing environment but also to the development of more freezing tolerant plants.

According to Abraham Blum [4] winter survival is "the final integrated plant response to a multitude of stresses involved during and after freezing stress, including both external, physical and biotic stresses". The main cause for the winter kill on the fields is the low temperature that is below the crop's critical survival temperature. However, several additional factors lead to extended damage to the crop in winter, such as, improper hardening because of delayed sowing or germination in autumn, cold-induced desiccation [5], freeze thaw cycles [6] extended periods of extremely low freezing temperatures and ice encasement [7]. The data from the last 100 years of winter survival of the wheat crop on the fields in Ukraine showed in those cases when significant winter damage occurred low temperature was the main cause in 35% of the years followed by freeze thaw cycle in 26% and ice encasement in 22% [8].

In-spite of these problems, winter crops are preferred as they normally give huge benefits. Winter crops are sown in autumn well before the onset of winter, which in Sweden usually starts in December. Once the winter is over, the surviving plants recover and begin the seasonal growth several weeks earlier than corresponding newly planted spring crops, thus producing higher biomass and increased yield (Figure 1). Moreover, less pesticides and herbicides can normally be used, as the crop is better established. Pest damage during the winter is not a problem. Furthermore, since the ground is covered, less soil erosion and leakage of nourishments occur from the winter crop field. Among common commercial crops, potato (*Solanum tuberosum*), rice (*Oryza sativa* L.) and corn (*Zea mays*) originate from tropical and sub-tropical regions and are chilling sensitive, while others like oat (*Avena sativa* L.) are chilling tolerant but freezing sensitive. Other common crops, including barley (*Hordeum vulgare* L.), common wheat (*Triticum aestivum* L.) and rye (*Secale cereale* L.) are more cold hardy and therefore are well adapted to survive winters with freezing temperatures [9].

Figure 1. Winter wheat planted in autumn (left) and spring wheat planted in the following spring (right). The difference in developmental stage is clearly seen, revealing the distinct advantages of growing winter crops. Reproduced with permission of Prof. Olof Olsson.

Developing cultivars with increased freezing tolerance has been a long term aim for breeders in temperate regions. However, several difficulties must be overcome when breeding for winter crops, the first and foremost the reduced yields seen in less adapted crops. Moreover, increased freezing tolerance is usually associated with delayed flowering to avoid low temperatures in the reproductive phase. However, in locations where heavy rains, drought or high temperatures are common immediately after the winter, delayed flowering characteristics are not desirable. Winter oat breeding and commercial cultivation is extensively done in the USA, UK and Ireland and to some extent in northern Europe. Cold hardiness and freezing tolerance is a critical requirement in such crops but unpredictable combinations of factors such as snow and ice cover, winter duration, light, drought, freeze-thaw cycles, diseases, insects etc make selection of hardy lines in the field difficult. As a solution to this problem, Fowler et al. developed a scoring system termed ‘Field survival index’ (FSI) which can be used to measure the relative hardiness of cultivars in relation to each other [10]. Using FSI, they later studied the magnitude of the variability in winter stress levels experienced under field conditions in Canada [11].

Amongst cereal crops, rye is the most freezing tolerant, followed by wheat, barley and oat [9]. In places with harsh winters, rye, barley and wheat are therefore the winter crops of choice. Breeding for winter oats, on the other hand, has been of limited success. Winter oat Cv. Wintok released in USA in 1940 and Cv. Norline released by Purdue University in USA in 1960 are among the most cold hardy winter oat cultivars [12, 13]. In UK, winter oat Cv. Gerald released in 1993 by

the Institute of Biological, Environmental and Rural sciences (IBERS) is well adapted to the English winters and widely used as a commercial winter oat cultivar. However the English lines are not hardy enough for Swedish conditions. To select better lines, several different physiological tests can be done following cold acclimation such as whole plant freezing, crown freezing, electrolyte leakage, plant weight and growth measurements, chlorophyll fluorescence measurements etc. Fowler et al. analyzed spring and winter cultivars of rye, wheat, barley and oats collected from plants cold acclimated under field conditions in the fall [9]. They measured crown freezing, crown fresh weight, dry weight and water content. They identified several developmental and physiological differences between cultivars and concluded that acclimation to cold is more complex than simply the regulation of tissue water content [9]. The crown is the tissue that connects roots with shoots and has the ability to regenerate new roots and shoots under optimal growth conditions. Marshall et al. developed an improved crown freezing technique for winter oats and proved the usefulness of the technique in progeny testing and selection for winter hardiness [14]. The crown plays an important role in winter survival as the plant is completely dependent on the ability of the crowns to survive winter and to restart growth in spring. Upon freezing most of the leaf tissue of the plant is killed whereas the crown and the roots of the plants that are underground and often also covered by snow and ice often survives. Thus the key to survival of the plant is the survival of the crown. Livingston et al. studied freeze induced damage to specific regions of oat crowns and confirmed that the apical meristem was the tissue in the crown most tolerant of freezing [15]. Livingston et al. also studied the relationship between carbohydrate accumulation in various sections of crowns and plant survival. They found that after 3 weeks of cold acclimation at 3^{o}C, carbohydrates accounted for approximately 40% of the dry weight of oats and 60% of the dry weight of rye. Moreover, various carbohydrates were allocated at different levels between different genotypes, suggesting the importances of carbohydrate levels and quality and their allocations in crowns for survival [16].

In Sweden, winters are too harsh for oat cultivation. Figure 2 shows testing of a spring oat line from Sweden in the field in southern Sweden in the year of 2006-2007. As can be seen from the figure, the spring oat is completely killed, where as the more hardy Germany LPWH 002205 line and the winter hardy control triticale survived. However, a selection in the field alone has proven not to be sufficient to develop more freezing tolerant cultivars. Instead, an in-depth understanding of the damages caused by freezing temperatures and the genetic processes involved in the cold acclimation could would lead to a more effective development of better winter cultivars.

Figure 2. Winter Oat trials in Skåne, Sweden 2006-2007. Left: winter hardy control (Triticale); Middle: complete winter kill of spring oat as indicated by the arrow; Right: Survival of the oat line LPWH992205. Reproduced with permission of Prof. Olof Olsson.

1.1 Responses to low temperatures

Responses to low temperatures vary depending on the duration of exposure, the temperature difference before and after the shift and also the absolute temperature. Different plant species also respond differently. Although low temperature responses include chilling stress (mild low temperatures), cold and freezing stress, this work was mostly focused on cold stress i.e., the cold acclimation process leading to increased freezing tolerance. Low temperatures affect membrane structure and composition, metabolic rates, induce transcriptomic changes, cellular dehydration, inhibits photosynthesis, disturb functioning of ion channels, cellular signaling etc. [2, 17-24]. Furthermore, freezing temperatures lead to extracellular ice formation which in turn leads to changes in the chemical potential in the cellular environment and migration of cellular water to extracellular spaces causing dehydration and shrinkage [17]. Eventually, extracellular ice penetrates the symplast causing further damage of intracellular structures [25]. Some of these changes can later be manifested as reduced growth rate and loss of turgor, eventually leading to plant death (Figure 3) [26].

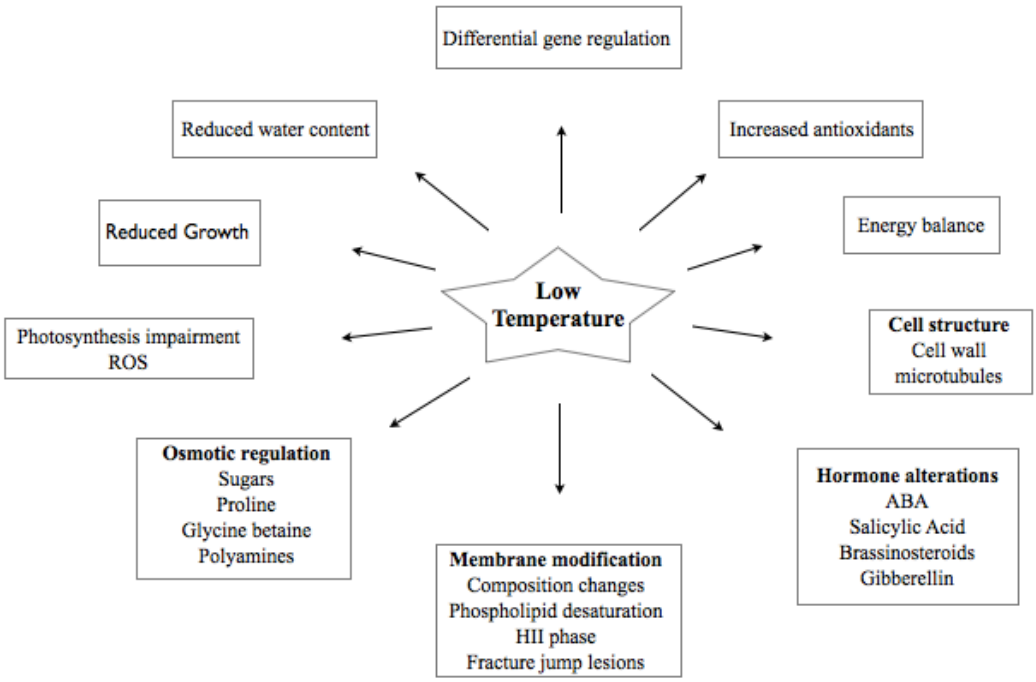

Figure 3. Responses and effects of low temperatures (Adapted from Xin et al. [26]).

Cell architecture

a. Cell wall

The plant cell wall has an important role in maintaing the cell structure upon extracellular ice formation and freeze induced dehydration as they prevent collapse and/or cell deformation. The characteristics of cell wall pores are also important in protection against freezing. Fewer or smaller pores can prevent the extracellular ice from penetrating the cells. In suspension cultures of apple and grapes, it has been shown that upon cold acclimation, the cell wall rigidity increased while the pore size decreased. In addition, there was a correlation between decrease in pore size and decrease in intracellular ice formation [27]. In pea (*Pisum sativum*), upon cold acclimation, the freezing resistance of plants increases along with an increase in cell wall weight and composition. Arabinosyl content increased by 100%, hydroxyproline content increased by 80% and other cell wall glycosyl residues and cellulose increased by 80% [28]. In winter rye, cell wall thickness was shown to be increased upon cold hardening [29, 30]. In rice, protein levels of phenylalanine ammonia-lyase (PAL) were shown to be induced upon cold [31]. PAL is a regulatory enzyme in the phenyl-propanoid biosynthesis pathway involved in production of lignins and other secondary metabolites. Although the role of lignins in cold tolerance is not known, increased lignin content was found in *ex vitro* poplar seedlings grown at 10^{o}C but the same was not true for *in vitro* seedlings [32].

b. Microtubules

Microtubules are structural components within the cell and are involved in various cellular processes. Microtubules are destabilized upon cold stress and are replaced by more cold stable ones. In the root-tip cells of cucumber (*Cucumis sativus* L.), it was shown that after 5hrs of cold treatment

at 4°C, chilling stable microtubules were found near plasma membrane and in the cytoplasm [33]. Tobacco mutants screened for a microtubule assembly blocker aryl carbamate or exposed to chilling treatment showed that those mutants that were resistant to aryl carbamate were also chilling resistant. This suggests that increased microtubule resistance to cold increases the cold tolerance of cells [34].

c. Plasma Membrane

The plasma membrane plays an important role in maintaining the integrity and structure of the cell and is the primary site of freezing injury [17, 24, 35]. Membrane damage leads to cellular dehydration during the freeze thaw cycles [2]. In low freezing temperatures, ice nucleation occurs in the apoplasts leading to imbalance in the osmotic potential. This imbalance causes movement of solutes to apoplasts leading to dehydration. Low freezing temperatures causes multiple forms of membrane damage such as formation of endocytotic vesicles leading to expansion induced lysis upon thawing, fracture jump lesions and lamellar-to-hexagonal II phase transitions [36]. In cold acclimated cells, exocytotic extrusions occur instead of formation of endocytotic vesicles, therefore thawing will not lead to expansion induced lysis and the cells retain their structure [36, 37]. Low temperatures also cause membrane rigidification leading to activation of mechano-sensitive or ligand-activated Ca^{2+} channels, leading to increasing levels of cytosolic Ca^{2+}. The *Arabidopsis fad2* (fatty acid desaturase-2) mutant defective in oleate desaturase exhibits membrane rigidification at 18°C, compared with 14°C in the wild type. These plants contain reduced levels of polyunsaturated fatty acids [38]. In *Arabidopsis*, *sfr2* (sensitive to freezing-2) mutants showed extensive membrane rupture in leaves during freezing recovery [39]. It was recently shown that the SFR2 protein stabilizes the plasma membrane and avoids the formation of hexagonal II phase transitions [40].

Photosynthesis

During cold acclimation, light is required for the accumulation of sugars and other compatible solutes through carbon fixation [41]. During photosynthesis photosystem I (PSI) and photosystem II (PSII) converts light energy into ATP and NADP which are used in the Calvin cycle to produce triose-phosphate using CO_2 in stroma. Triose-phosphate is later used for sucrose synthesis in cytosol. Low temperatures reduce the rate of sucrose metabolism [42] which leads to accumulation of phosphorylated intermediates, depletes inorganic phosphates and inhibits ATP synthesis necessary for continued CO_2 fixation [18, 23, 43]. This creates an imbalance between the amount of light energy that is trapped and the amount that is utilized [44, 45] leading to formation of Reactive Oxygen Species (ROS) such as hydrogen peroxide (H_2O_2), superoxide (O_2^-) and hydroxyl radical (·OH). It has been shown that PSI photo-inhibition in low light at chilling temperatures occurs both in the cold sensitive plants cucumber and potato [46] and in the cold resistant plants barley [47], winter rye [48] and *Arabidopsis* [49]. The differences in PSI activity in different plants lie in the amount of sensitivity to the ROS scavenging enzymes and in the ability to cope with the excessive light [50].

Signal transduction

To initiate the cold acclimation process, upon exposure to low temperature, plants need to identify the signal induced by the temperature change and transduce it to the nucleus. Here the signal has to be converted to a genetic response in order to initiate differences in gene expression leading to physiological and metabolic changes in the cell to protect it against the stress. The temperature sensors could either be transmembrane receptor proteins or ion channels or both. Mechano-sensitive Ca^{2+} channels with activity modulated by low temperature have been studied [51]. One of the earliest events of low temperature exposure is the elevation of free cytosolic Ca^{2+} levels. The Ca^{2+} flow to cytoplasm is both from extracellular sources and from internal organelles. This elevation correlates with a differential expression of several genes. When Ca^{2+} influx was artificially increased by ionophores or Ca^{2+} channel agonists, cold-acclimation-specific genes were induced at 25^{o}C and freezing tolerance increased in alfalfa cells [52].

Ca^{2+} signals are transmitted primarily through Ca^{2+} regulated proteins called calcium sensors, which change their phosphorylation status when they sense the elevation of Ca^{2+} ions [53]. Some of the major sensors are calmodulin (CaM), CaM domain containing protein kinases (CDPKs), calcineurin B-like proteins (CBLs) and CBL-interacting protein kinases (CIPKs).

Mitogen activated protein kinase (MAPK) cascades are also involved in cold stress signaling. A MAPK cascade consists of three protein kinases. Inactive MAPKKKs are activated by a stress signal messenger; upon activation, they activate MAPKKs which in turn activate MAPKs. In addition to low temperature, MAP kinase activities also increase during drought stress in both alfalfa and *Arabidopsis* [54, 55].

Transcriptional regulation

Several studies have shown that major changes in gene expression profiles are noticed upon exposure to low temperatures. Many genes are either up- or down-regulated [56, 57]. Seki et al., monitored the expression patterns of 1200 *Arabidopsis* genes under drought and cold stress using cDNA microarrays. They identified 19 stress inducible genes out of which 11 contained the dehydration-responsive/C-repeat element (CRT/DRE motif) in their promoter regions [57]. The two motifs are defined as 5' TGGCCGAC 3' and 5' TACCGACAT 3' respectively with the shared motif 5' CCGAC 3'. The transcription factors (TF) that interact with the CRT/DRE element are the C-repeat Binding Factor/DRE Binding factor 1 (CBF/DREB1), first found in a yeast one-hybrid screen [58]. All three CBF encoding genes in *Arabidopsis CBF1*, *CBF2* and *CBF3* are tandemly located on chromosome 4 and contain an AP2/ERF (APETALA2/Ethylene Responsive element binding factor) domain. AP2/ERF is a DNA-binding domain that recognizes the CRT/DRE element which is found in the promoters of many *COR* (Cold Responsive) genes.

Global expression studies of transgenic *Arabidopsis* plants with ectopic expression of *CBF1*, *CBF2* or *CBF3* revealed that there is a large overlap in their gene regulons. Using cDNA microarrays, expression profiles of 8000 *Arabidopsis* genes were analyzed in over-expression lines and 30 genes were found to be differentially expressed by more than 3 folds in all the lines [59].

Vogel et al. showed that up to 16.5% of the cold inducible genes are also expressed in over-expression lines of *CBF2* [60]. Molecular analysis of the *cbf2* mutant in *Arabidopsis* revealed that CBF2 is a negative regulator of CBF1 and CBF3 [61]. Furthermore, CBFs are also negatively regulated by MYB15 TF (a member of the R2R3 MYB family) in *Arabidopsis* [62]. MYB15 recognizes and binds to MYB recognition sites in the promoters of *CBFs*. ICE1 (Inducer of CBF Expression-1) is the most upstream TF in cold stress. It was first found in a screen of the CBF3 promoter fused with luciferase [63]. ICE1 is constitutively expressed and by screening for mutants impaired in luciferase activity the *ice1* mutant was identified. In *ice1* mutant plants, the expressions of *CBF1*, *CBF2* and *CBF3* are altered. Zarka et al. showed that ICEr1 and ICEr2 motifs located in the *CBF2* promoter affect the induction of *CBF2* in a promoter deletion assay [64].

HOS1 is a RING E3 ligase that negatively regulates cold signal transduction. It physically interacts with ICE1 and mediates the ubiquitination of ICE1 both *in vitro* and *in vivo* [65, 66]. Vogel et al. showed that over-expression of *ZAT12* in *Arabidopsis* caused a small but significant increase in freezing tolerance of plants. It was shown that ZAT12 down-regulated the expression of *CBF* genes, indicating a role for ZAT12 in the negative regulatory circuit that reduces the expression of the CBF cold response pathway [60]. Several other negative regulators of *CBFs* are also identified including FVE [67], STRS (Stress Response Suppressor) [68] and FIERY2 [69]. Repressors independent of CBF pathway have also been identified. HOS9 [70], HOS10 [71] and HOS15 [72] were found to repress *CORs* while the *CBFs* were not affected indicating CBF independent regulation of *CORs.* For more in-depth reviews on genetic regulation under cold acclimation refer to [37, 73-75] (Figure 4). CORs are hydrophilic proteins predicted to form an amphipathic α-helix. Their mechanism of protection is poorly understood. However, in one study, transgenic *Arabidopsis* lines containing double constructs of the dehydrins LT129 and LT130 or RAB18 and COR47 exhibited lower LT_{50} during the freezing stress signifying a role of dehydrins in freezing tolerance. It was also shown that in transgenic plants LT129 localized to cytoplasm and intracellular membranes [76]. Danyluk et al. showed that in wheat the WCOR410 protein accumulate in the vicinity of the plasma membrane and suggested that this protein is involved in the cryoprotection of the plasma membrane against freezing or dehydration stress [77].

Abscisic acid (ABA) has also been shown to have a minor role in increased freezing tolerance. Studies showed that both ABA-deficient (*aba*) and ABA-insensitive (*abi*) mutants are impaired in cold acclimation [78-80]. Analysis of promoter regions of ABA responsive genes revealed a conserved motif designated as ABA-responsive element (ABRE; PyACGTGGC) [81]. The ABRE TFs that bind to these elements were identified by yeast one-hybrid screening [82].

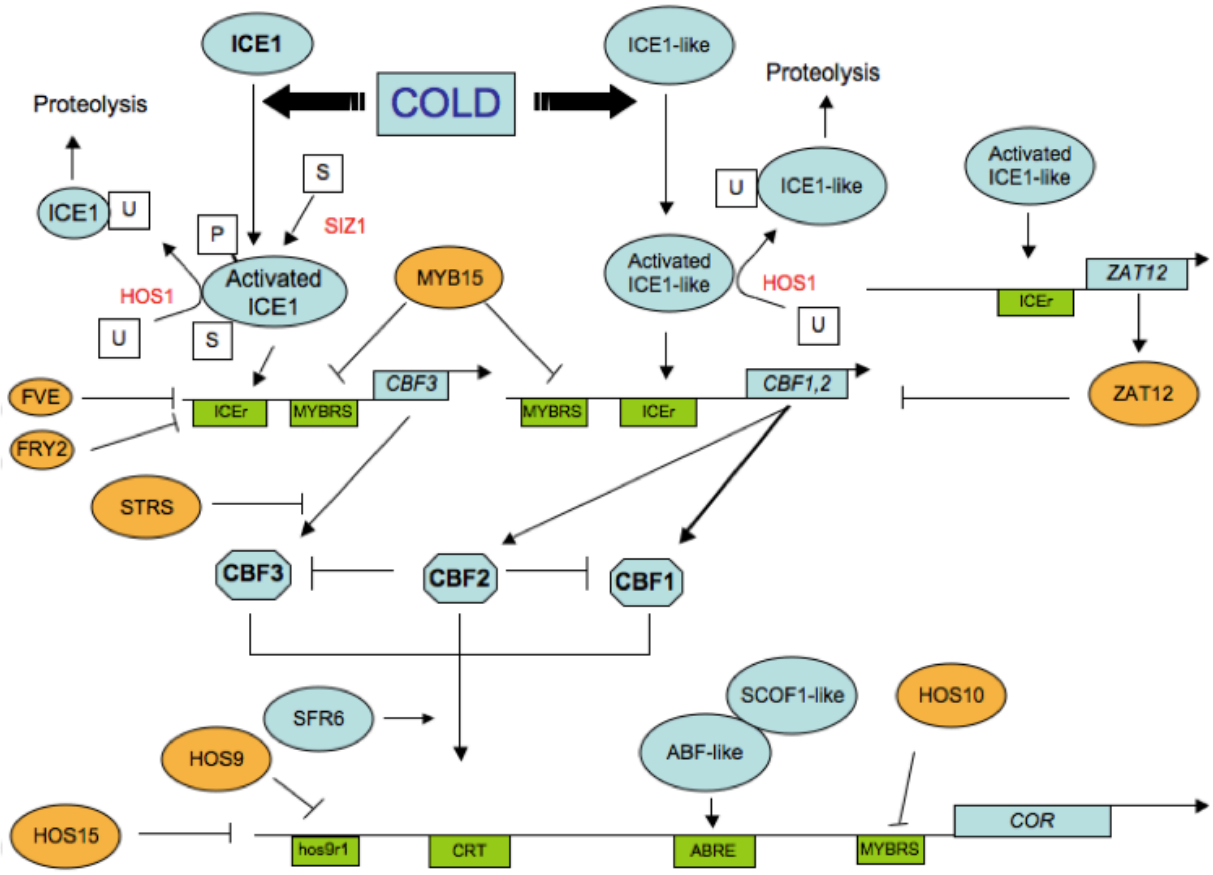

Figure 4. Regulators of CBF dependent and independent pathways. Redrawn with permission from Ruelland et al. [73]

Vernalization

Wheat, barley and rye are grown in temperate regions and flowering in these cereals is accelerated upon exposure to low temperatures for several days, a process known as vernalization. The vernalization process is a requirement for winter hardy cereals and occurs in the temperature range of 0º to 12ºC [83]. In barley three genes, *VRN1*, *VRN2* and *VRN3* previously shown to have a role in vernalization, were cloned [84-86]. Vernalization induces *VRN1* that promotes the shift from the vegetative to reproductive stage [87]. It has been shown that *VRN1* is constitutively expressed and its expression is not affected by low temperatures in spring cultivars. On the contrary, in winter cultivars, *VRN1* is induced only after several days/weeks of exposure of plants to low temperatures. The time it takes to induce the expression of *VRN1* and the extent to which it is induced indicates the vernalization requirement of the cultivar. It has also been shown that up-regulation of *VRN1* in vegetative meristems of oat was significantly later than in leaves [87]. This suggests distinct and conserved roles for *VRN1*, first, in inducing flowering competency and second in meristems to activate genes involved in floral transition [87]. *VRN2* is a zinc finger protein which mediates DNA binding and is controlled by the circadian clock during long days [85]. It has been suggested that VRN2 blocks flowering during long days by repressing *VRN3*. VRN3 promotes flowering in long days [86].

Sugars & other metabolites

Various carbohydrates are accumulated at different levels upon cold acclimation. Most commonly accumulating carbohydrates are glucose, sucrose, fructose, raffinose and fructans. Many different mechanisms have been proposed for the role of sugars in protection against damage from low temperatures. Under sub-zero temperatures, ice is formed in the apoplasts. This causes a difference in water potential inside and around the cells leading to cellular dehydration. Carbohydrates and other osmolytes changes the osmotic potential of the cells and reduces the rate of water flow from the cells. Carbohydrates also reduce the rate of ice nucleation and stabilize the plasma membranes under low temperatures by replacing the water molecules lost from the membrane. Another protection mechanism against freezing temperatures that can also occur is a process known as vitrification, i.e. the formation a super cooled liquid through a glass transition avoiding the formation of ice crystals.

Livingston et al. analyzed the expression levels of simple sugars and fructans in apical and basal regions of spring and winter oat crown tissues. They concluded that levels of the simple sugars (glucose, sucrose and fructose) were not correlated with increased freezing hardiness upon cold acclimation, whereas the levels of high DP (degree of polymerization) fructans correlated well with cold acclimation [16]. To understand the role of raffinose in cold acclimation, Zuther et al. studied *Arabidopsis* lines that overexpressed the galactinol synthase gene from cucumber [88]. These lines contained up to 20 times more raffinose than the wild type under non acclimated conditions - up to 2.3 times more after 14 days of cold acclimation at 4^{o}C. Additionally, they also studied an *Arabidopsis* raffinose synthase knockout line in which raffinose was completely absent. The study revealed that the freezing tolerance of the over-expressors and the knock-out mutants were similar to wild type plants both under non acclimated and cold acclimated conditions. Thus they concluded that raffinose is not essential for basic freezing tolerance or for cold acclimation of *Arabidopsis*. In alfalfa (*Medicago sativa* L.), raffinose, stachyose and sucrose levels were found to be increased much earlier in field grown winter hardy cultivar compared to spring cultivar planted at the same time in autumn indicating the importance of these sugars in cold hardiness [89]. Thus, although carbohydrate levels increase many folds in the plants during cold acclimation, their role in mediating increased freezing tolerance has been arguable.

Kaplan et al. performed metabolic profiling to determine temporal dynamics in various metabolites associated with the induction of acquired thermo-tolerance in response to heat shock and freezing tolerance in response to cold shock [90]. They monitored 81 known metabolites and 416 un-identified mass spectral tags and identified several metabolites responsive to cold shock including glucose, proline, GABA (γ-aminobutyric acid), arginine, fructose-6-phosphate, maltose, galactinol and raffinose. Korn et al. conducted a metabolomics study for freezing tolerance in *Arabidopsis* to identify metabolites that are most representative of freezing tolerance and heterosis in tolerance [91]. To accomplish this they profiled five parental accessions (C24, Col-0, Co-2, L*er*,

Te) and eight F1 populations generated by manually crossing both C24 and Col-0 with the respective four accessions. This analysis revealed 20 metabolites that could predict freezing tolerance in C24-crosses while 14 metabolites were sufficient to predict freezing tolerance in Columbia crosses. The results showed that the most diagnostic metabolites were fumaric acid, succinic acid, various simple sugars, glycine, proline etc. Thus metabolomic analysis can be used to screen for breeding lines with greater freezing tolerance ability. An overview of the sequence of events during the response to low temperature stress is shown in figure 5.

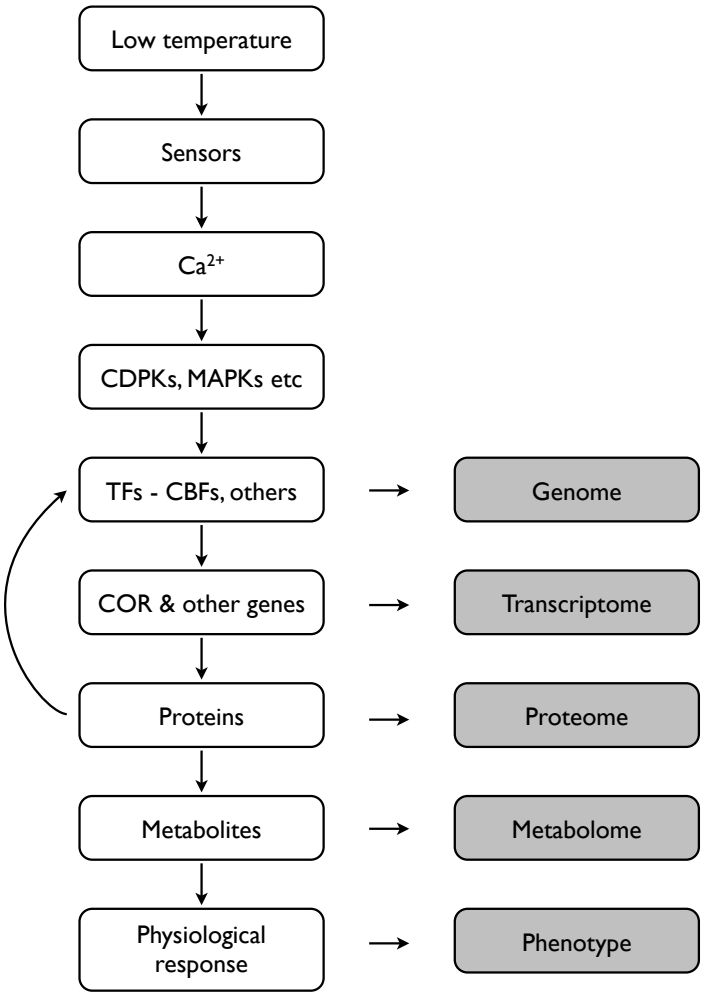

Figure 5. Schematic representation of responses to low temperature stress and how they can be studied

1.2 Constructing genetic networks

Approaches

The development of whole-genome microarrays and the resulting public availability of gene expression data have made it feasible to develop and test computational approaches to predict genetic networks. Understanding how genes are regulated can help us identify the most important upstream transcription factors (TFs) regulating a given response. TFs are proteins with DNA binding domains that bind to a specific sequence (motif/cis regulatory element) in the 5' upstream region of the target gene. Although TFs that are significantly induced in a given condition can be

identified from the microarray analysis, a further identification of the motif to which it binds could be a difficult task. It is known that TFs from the same family generally bind to motifs with similar sequences, which share a common subsequence (core motif). Public databases such as PlantCARE [92] can therefore be used to retrieve information on TFs and their binding motifs. Upstream promoter sequences can then be screened for the known motif sequences. This approach will identify down stream targets of TFs. For example in microarray data analysis from *Arabidopsis* plants over-expressing the *CBF2* gene, out of 85 up-regulated genes, 68 (80%) contained the DRE element in the 1kb upstream promoter region indicating that these genes are part of the CBF2 regulon [60].

Microarray data analysis

Microarrays are useful tools to identify the genes that are differentially expressed. Several parameters should be considered while analyzing microarray data, such as background noise, ways of normalization, fold change threshold values and false discovery rate. Several different algorithms exist for background correction such as Affymetrix Microarray Suite 5 (mas5) [93], Robust Multi-array Analysis with (gcrma [94]) and without (rma [95]) correction for the GC content of the oligo. These algorithms are most commonly used and selecting one for the analysis is in most cases a matter of preference and availability. Differentially expressed genes can then be identified using statistical tests for variance such as t-test, Significance analysis of Microarrays (SAM) or Analysis of Variance (ANOVA) [96]. Optionally fold change criteria for gene expression can also be used for further filtering. Fold change thresholds are set anywhere from 2 to 5 folds and the genes that pass this criterion in at least one of the time-points are considered as differentially expressed genes. Once the highly expressed genes are identified they can then be clustered based on their expression pattern using different clustering algorithms.

Clustering is often done after filtering genes on any of the above mentioned criteria. The goal of clustering is to group genes with similar expression dynamics together while genes with dissimilar dynamics fall into different clusters. This helps with better visualization of expression profiles. It is known that co-expressed genes could be co-regulated, thus genes from the same cluster could also be co-regulated. Two of the most commonly used clustering measures are Euclidean distance and Pearson correlation coefficient. The most common clustering methods are hierarchical clustering, *k*-means clustering and self organizing maps (SOM).

Motif over-representation

Motifs/*cis*-acting elements act as activators or repressors in gene transcription by allowing the recognition and binding of TFs. Several motifs are identified by different molecular experiments and are documented in online databases such as plantCARE. These known motifs can be quickly searched in a given promoter sequence by a DNA pattern matching algorithm. Once a match to the known motif is found, the question arises if the found motif is actually regulating the target gene *in vivo*. This can be tested by promoter deletion assays. On the other hand, since these motifs are usually very short in length, with bioinformatics analysis it becomes necessary to calculate over-

representation of motifs assuming that the over-represented motif will have a higher probability of being involved in the regulation of the gene. Over-represented motifs can be identified in the promoter regions of all the genes in a cluster. It is also possible to calculate the over-representation of different motif combinations thus indicating the role of combinatorial control in gene regulation.

Over-represented gene ontology terms

Upon clustering, the genes in the cluster can be analyzed for their gene ontology (GO) annotation terms concerning cellular location, biological process and molecular function. Over-represented GO categories for a given cluster indicate that the co-expressed genes also have similar putative functions. Over-representation in this case is calculated using hypergeometric distribution.

Identifying co-regulated genes usually involves microarray data analysis, identifying over-represented motifs and some times also over-represented gene ontology terms.

Putative networks

Upon exposure of plants to low temperatures, hundreds of genes are differentially expressed, indicating the complexity of the cold regulation reaction. However, most of these genes have been identified by microarray studies, whereas very limited in-depth genetic studies have been performed. The scale of the genetic response makes the task of experimentally proving all possible regulations very laborious. For this reason, computational analysis is being increasingly used to identify the most probable genes and cis regulatory elements with important roles in stress response. In one approach, a putative network around *ICE1* was identified using a combination of different publicly available microarray data together with *in silico* mutagenesis [97]. *In silico* mutagenesis is a computational technique wherein the behavior of a motif is studied by analyzing the expression profiles of genes containing the given motif with mismatches in one or more positions. In a different study, Cooper et al. built a genetic network in rice for genes associated with developmental and stress responses by identifying interaction domains for 200 proteins from stressed and developing tissues using a yeast two hybrid assay. This was achieved by measuring gene expressions in different conditions and by localizing the genes to regions of stress-tolerance trait loci. Their work suggested that similarly expressed genes respond to environmental cues and stresses in a similar way. They showed that the data can also be used to predict gene function in both monocots and dicots and identified five genes that contribute to disease resistance in *Arabidopsis* [98].

A network closest to a genome-scale network reconstruction comes from an analysis of transcriptome data based on a regularized graphical Gaussian model (GGM) [99]. Through GGM a network of 2,917 interactions among 1,986 genes were inferred and defined as a “universal abiotic stress response” network which contains genes known to respond to stress, participate in carbohydrate, lipid, amino acid, secondary metabolism and transport. In a different approach, a gene regulatory network of 1,609 genes involved in production of brassinosteroids was developed that included data from chromatin immuno-precipitation, Arabidiopsis tiling arrays (ChiP-chip) and gene expression studies [100]. Online tools and databases are available to cluster genes based on

expression profiles, gene annotations, protein-protein interaction data etc. MultiGo is used to cluster genes using hierarchical clustering from expression data and identifies statistically significant gene sets using gene annotations [101], whereas GeneMania [102] and STARNET-2 [103] use previously known interactions from the literature and gene expression profiles to elucidate the possible interacting partners of various genes and proteins. These different approaches indicate that there is a requirement for computational methods to identify putative interacting genes and proteins in order to scale down the number of possible candidates for experimental testing of the cold regulon.

1.3 TILLING

Applications of molecular markers in oat breeding will facilitate more efficient development of cultivars with desired phenotypes. A good way to study functional roles of genes in plants by generating mutated lines by transposons, T-DNA or RNAi techniques. Unfortunately, implementing these techniques on oats is difficult due to lack of an efficient transformation system. Oats also have a very large genome size with an estimated 1C genome weight of 13.23 pg or 13,000 Mbp [104]. A more practical way of inducing mutations in the oat genome is to use chemical mutagenesis. EMS (ethyl methanesulfonate) is one such mutagen that preferentially alkylates guanine bases leading to the DNA-polymerase favoring the placement of a thymine residue instead of a cytosine residue opposite to the O-6-ethyl guanine in the subsequent DNA-replication step. This results in a random point mutation wherein GC base pairs are switched to AT pairs [105]. The resulting mutations can be silent, missense or nonsense when in the coding region or cause gene induction or repression when present in promoter regions. TILLING (Targeting Induced Local Lesions IN Genomes) is a technique wherein a mutant library of very densely induced point mutations is generated and thereafter screened with high precision molecular techniques to identify those mutations [106]. Successful applications of TILLING have been demonstrated in several plant species. Several alternate techniques for screening for mutations in the TILLING-population can be used such as direct DNA sequencing of the gene of interest, Li-COR and MALDI-TOF based techniques, non-denaturing polyacrylamide gel based techniques, high resolution melt and next-generation sequencing of the entire genome. Once the mutation is identified and confirmed, it can be scored based on its characteristics and the location in the coding region. The plants carrying the identified mutations can then be phenotypically characterized. Figure 6 gives an overview of different analysis methods and techniques used for marker identification. Experimental lines could be TILLING lines or *Arabidopsis* mutants that could be screened using various techniques to identify new markers such as genes, SNPs or quantitative trait loci (QTLs). The identified markers can then be used in selection during plant breeding.

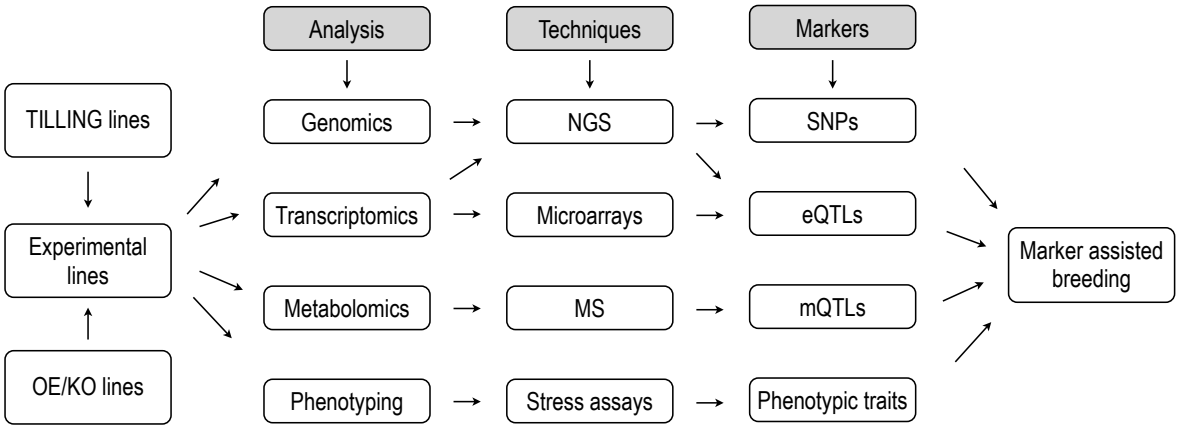

Figure 6. Flow chart of analysis methods and techniques to identify novel Quantitative Trait Loci (QTLs) for marker assisted breeding. OE/KO: Over expression or knock-out NGS: Next Generation Sequencing; MS: Mass spectrometry; SNPs: Single Nucleotide Polymorphisms; eQTLs: expression QTLs; mQTLs: metabolome QTLs

2. Scientific aims

Overall aim

The overall aim of this thesis was to better understand the genetic mechanisms underlying the cold acclimation process in plants and to implement this knowledge towards development of cold hardy Scandinavian winter oat.

Specific aims

- Develop a computational method to predict genetic networks involved in the cold acclimation process in plants and to identify cellular processes regulated by various cold inducible transcription factor families (Paper I).

- Analyze if the promoters of cold inducible genes are under combinatorial control by identifying statistically significant combinations of known motifs in upstream promoter sequences (Paper II).

- Develop a winter oat model system though a combination of field trials and controlled laboratory tests. Using the winter oat model system identify metabolites that are differentially expressed in spring and winter oat genotypes during cold acclimation (Paper III).

- Develop an oat TILLING population for the identification of oat lines carrying mutations in specific cold regulatory genes (Paper IV).

3. Present study

3.1 Genetic regulation

(Paper I)

Putative cold acclimation pathways in Arabidopsis thaliana identified by a combined analysis of mRNA co-expression patterns, promoter motifs and transcription factors

In *Arabidopsis*, under the cold acclimation process several hundred genes are differentially expressed and are either up- or down-regulated. Understanding how these genes are regulated will increase our understanding of the genetic processes responsible for the physiological changes that occur to protect plants against low temperatures. Thus, in this work, an effort was made to build a rule based computational model to predict the putative genetic regulatory network active during the cold acclimation process in *Arabidopsis* by combining Affymetrix microarray data and known transcription factor (TF) binding sites in promoter regions. The microarray data used contained the expression profiles of approximately 24,000 genes monitored during the cold acclimation process in *Arabidopsis* and was generated by AtGenExpress project. Promoter regions, defined as sequences 1kb in length and located immediately upstream of the translational start site of predicted genes, were downloaded from the TAIR database. A list of previously known TF-binding sites was collected from the literature.

A combined rule based and statistical approach was used to build genetic networks in which genes were grouped into disjoint clusters if they fulfilled the following criteria 1) all genes in a given cluster must contain in their upstream regions the same known motif or combination of motifs, for which there is a known binding TF; 2) the earliest recorded time-point of significant expression of each gene in the cluster must occur at the same time as the first recorded time-point of significant expression of the gene encoding the regulating TF, or at the immediately following time-point; 3) the expression profiles of the genes in the cluster must show a higher correlation with each other than the expression profiles of randomly selected genes.

Analysis of the data revealed that 3,495 genes were differentially expressed as a result of cold stress by more than 2.5 folds. Out of these 1,665 genes were up-regulated and 1,830 genes were down-regulated. These 3,495 genes were selected to be analyzed further. In the putative genetic network created in this work, 670 up- and 1,358 down-regulated genes passed all the required criteria and were therefore included for generation of networks for up- and down regulated genes (Figure 2 in Paper I). The results showed that several previously known interactions were correctly predicted and new putative interactions were identified and suggested to be under combinatorial control (Figure 1 in Paper I). It was found that specific TF families preferentially regulate specific cellular processes. In up-regulated genes, cell rescue, defense and virulence is preferentially regulated by the AP2 family (CBFs, $p \leq 10^{-4}$), the transcription process by WRKY ($p \leq 10^{-4}$), DOF ($p \leq 10^{-4}$) and NAC families ($p \leq 10^{-3}$); metabolic processes by BHLH ($p \leq 10^{-2}$) and energy processes by the BZIP family ($p \leq 10^{-3}$) (Table 4 in Paper I). It was also predicted that most of

the TF families are involved in both up- and down-regulation of their target genes. The DOF TF family was predicted to regulate more than 50% of the differentially expressed genes, while TCP2, MADS and AtERF families regulate smaller numbers of genes. The BHLH family seem to regulate significantly more down regulated genes than up-regulated ones (Figure 3 in Paper I).

Discussion

An attempt was made to to design a new computational approach for predicting putative genetic regulations. Several new previously unknown pathways were discovered that are putatively regulated by BHLH, BZIP, HSF, AP2, NAC, WRKY, DOF, MADS, MYB and TCP2 families of TFs. The approach identified several previously known interactions and suggested new putative genetic interactions. From the obtained results it could be suggested that TF families regulate gene targets independently as well as in combination with other TF families. Moreover, significant functional categories under regulation by various TF families were identified. Although this approach was applied to the cold stress of plants, due to its generic data requirements, it can very well be used on the data from any organism as long as the data requirement conditions are met. Since the genomes of several organisms already have been sequenced and new genomes are continuously being added, and a massive amount of array data is around, the presented method may serve as an additional tool to validate important regulatory networks in various biological systems.

(Paper II)

In silico analysis of promoter regions from cold-induced genes in rice (Oryza sativa L.) and Arabidopsis thaliana reveals the importance of combinatorial control

In this work it was investigated if the expression of DREB1/CBFs and co-expressed genes rely on combinatorial control by several TFs. For this purpose, publicly available *Arabidopsis* microarray data and rice microarray data developed in-house was used. Genes were clustered using Pearson correlation (PC≤0.95) and clusters that included CBFs were considered for further analysis. A list of known motifs that were previously identified and experimentally confirmed was downloaded from PLACE and plantCARE. Motif over-representation was estimated using Fisher's exact one-sided test (P≤0.01) and significant motif combinations were identified using a Benjamini-Hochberg false discovery rate (BH FDR) adjusted P-value threshold of ≤ 0.01.

This led to identification of several single motifs and motif combinations that could play important roles in the regulation of cold acclimation in *Arabidopsis* and rice. For example, ABRE-related MACGYGB was found in all but one of the CBF clusters. This motif was previously shown to be present in genes responsive to Ca^{2+} [107]. Furthermore, the GT-1 binding site is represented in two of the *Arabidopsis* clusters and one of the rice clusters. This motif is found in many light regulated genes. Several other motifs including W-box, TATA boxes, POLASIG3 and the well

known CCGAC motif also passed the criteria set up and could therefore be important players well worth studying further.

Discussion

This study further supports the notion that cold acclimation is a complex trait with activation and de-activation of several genes by combinatorial control of TFs. In *Arabidopsis*, the results indicate that a combination of ABRE-related, GT-1, WRKY and AT-rich motif is of particular importance in the gene regulatory response that follows from cold induction. Interestingly some of the well known motifs involved in cold regulation such as ICEr3 and MYB15 were selected by the approach in *Arabidopsis* but not in rice. Instead, the derived combinations in rice indicate a coupling to ABA, drought and light stress signaling pathways. The expression of the rice *OsDREB1* gene has previously shown to increase cold, drought and high salt [108] correlating well with motif combination results.

3.2 Metabolic profiling

(Paper III)

Identification of differentially expressed metabolites during cold acclimation in a winter oat model system.

Since little is know about cold acclimation in oats and as no winter oat exists, development of Swedish winter oat requires a model system in which to study the different causative mechanisms underlying cold tolerance in oats. By comparing winter oat with spring oat, differential gene expression and regulatory elements can be identified. To this end, 294 oat cultivars from various winter oat collections around the world were collected and tested in the field in Skåne. After testing for four consecutive winters, 14 cultivars showing especially good winter survival and vigor were selected for a further more detailed analysis in controlled growth conditions. Several assays were conducted including electrolyte leakage, crown freezing, expression analysis of known cold induced genes, sugar analysis and flowering time determination. The various tests indicated that different but overlapping causative mechanisms are potentially involved in the field survival and the controlled freezing tests. The cultivar of German origin LPWH992209 performed the best in the field whereas the American cultivar Win/Nor-1 was the best performer in laboratory tests addressing cold hardiness. Metabolic profiling by GC-MS identified expression levels of 245 metabolites. Two-way ANOVA test identified 107 metabolites that showed significant interactions with the parameter cultivar (73) or time-point (73) or both (4). The quality of the data was confirmed with PCA and OPLS-DA. Levels of specific sugars, including maltose and raffinose, were highly increased in all cultivars. The 20 most prominent metabolites were ranked and galactinol received the highest S-score for correlation with freezing tolerance characteristics.

Discussion

This work is a step towards development of a Swedish winter oat. No such variety currently exists. A winter oat would be important for agriculture especially in Sweden and Finland, since it

would yield more and also contribute to a better environment. However, cold tolerance is a complex trait with several different factors contributing to the character. In our studies a systematic comparison between characteristics important for winter survival in the field and characteristics contributing to frost hardiness in a simulated environment are shown to be slightly different. This work identified a German and an American variety as especially useful for analysis of cold tolerance. Further in-depth understanding of molecular processes involved in cold tolerance in these varieties is essential for the development of winter oat. From the results of the field studies and the controlled freezing experiments it is clear that a number of different genetic markers are required for further development of winter oats. Metabolic profiling showed differences in the expression levels in various metabolites. Expression analysis of various metabolites indicated that several spring and winter oat cultivars differ in their response to low temperatures. In a previous study in wheat, the expression levels of 947 genes in winter and spring wheat were monitored and 65 genes, differentially expressed in the two cultivars, were identified [26]. Since DNA sequencing costs have been reduced multifold in the past decade, massive parallel sequencing is now an alternative to identify differentially expressed ESTs between a good commercial spring variety and the most cold hardy lines defined here. Later we will also take advantage of a recently published oat TILLING-population to screen for mutations in important cold acclimation regulating genes.

3.3 TILLING

(Paper IV)

Development and characterization of an oat TILLING-population and identification of mutations in lignin and β-glucan biosynthesis genes

In this work, an EMS mutagenized TILLING (Targeting Induced Local Lesions IN Genomes) population of a commercial hexaploid oat cultivar Belinda was developed. To determine the optimal survival rate and mutation frequency, an EMS titration curve for oat seeds was established at different EMS concentrations. In total eight different EMS concentrations were used and ~ 200 seeds were treated at each concentration. A concentration of 0.9% (v/v) EMS was finally chosen and ca 9000 seeds mutagenized were sown in a green house. Seeds of the 2880 surviving M1 plants were harvested and one seed from each re-sown under the same conditions. 2600 of these M2 seeds germinated and 2550 produced M3 seeds, which were pooled from each plant to make up the TILLING population. When bringing up the M2 population, several different phenotypic changes were observed including chlorosis (2.3%), semi-dwarfness (1.3%), giants (0.6%), dwarf and high tiller (0.6%). In total, 5% of the mutagenized Belinda population had visually detectable phenotypic alterations. Developing leaves were collected from 2600 M2 population and the genomic DNA extracted by the CTAB method. Average mutation frequency in the population was estimated by RAPD-PCR, DNA sequencing and MALDI-TOF analysis. RAPID-PCR indicated a mutation

frequency of one mutation per 20 kb. DNA sequencing of *AsPAL* and *AsCslF6* genes indicated a mutation frequency of one mutation in 33.3 kb and one mutation in 38 kb by MALDI-TOF.

To assay differences on the post-DNA level, M3 seeds were stained for lignin by phloroglucinol-HCL assay. The assay was performed on 1824 seeds to identify mutants with lower or higher lignin quantities in the seed husk. From this screen 17 mutant lines were identified that showed lower stain intensity in the husk compared to Belinda. Mutant lines identified in the staining assay were then confirmed by the quantitative acetyl bromide method. The sequences of *AsPAL* and *AsCslF6* were submitted to Genbank (accessions GQ373155 and GQ379900) and the mutant library will be submitted to Nordgen when enough seeds from each line have been propagated.

Discussion

The aim of this project was to develop a mutant population where mutations in all genes can be found with a high redundancy without having to produce a very large population. In combination with a high precision and efficient screening method, it is realistic to assume that productive mutations in all alleles in any gene of interest will eventually be identified. Since the genetic basis of a particular mutation then will be known, by repeated back crosses productive mutations can be introduced to any chosen oat variety by marker assisted selection while the majority of the other mutations will be eliminated in the same process. Several visible phenotypes such as chlorosis, dwarfness, etc. were found in the population, furthermore low lignin mutant lines were also identified. This indicates that the probability of finding the mutants in important cold regulating genes in the population should be high. Thus efforts now could be made to screen the population for mutant lines with higher freezing tolerance.

4. Conclusions

In this work we analyzed the microarray data and the promoter sequences from *Arabidopsis* to understand gene regulation and combinatorial control under cold acclimation process in plants. In oats, we collected the best lines from winter oat breeding programs from all over the world and performed field trials, physiological, molecular and biochemical tests followed by metabolic profiling. Finally an EMS mutagenized oat TILLING library was created.

Major results from this work

- Identification of genetic regulators of the cold acclimation process in *Arabidopsis*. New insights into complexities of genetic regulation under cold acclimation was gained. Several previously known and unknown genetic interactions were identified.
- The importance of combinatorial control in gene regulation was demonstrated. New significantly overrepresented motif combinations were identified that could play a role in the regulation of the CBF TFs.
- A winter oat model system was developed from field studies and controlled experiments. Such a model system will facilitate the identification of differences in the cold acclimation process in winter and spring oats and in identifying factors necessary for cold acclimation in plants. Using the model system, differentially expressed metabolites were identified during cold acclimation in spring and winter oats.
- An EMS mutagenized TILLING population was generated. This can be used to screen for mutations in key genes identified from above mentioned approaches.

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