Cold Acclimation in Oats and Other Plants

Dissecting Low Temperature Responses Using a Comparative Genomic Approach

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To my wife Anna

"The path to our destination is not always a straight one. We go down the wrong road, we get lost, we turn back. Maybe it doesn't matter which road we embark on. Maybe what matters is that we embark"

- Barbra Hall

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Abstract

Cold acclimation protects plants from temperate regions of the world from the deleterious effects of low and freezing temperatures. This is through a series of transcriptional, regulatory and metabolic changes that enable continued growth and survival. The focus in this thesis is to increase our understanding of the cold acclimation process and there by open the door to development of cold hardy oat (*Avena sativa*) varieties for the Nordic climate conditions. We started by sequencing 9,792 oat ESTs from a cDNA library prepared from pooled total RNA extracted from cold induced oat plants. These sequences were assembled into a UniGene ser of 2,800 sequences, 398 displayed homology to genes previously reported to be involved in cold acclimation. The CBF factor family have a key regulatory role during cold acclimation and in our UniGene set we found four oat CBF sequences.

To infer regulatory networks we developed a rule-based method, which combined data from microarrays with promoter sequences and known *cis*-elements. The method was tested on the cold acclimation process in *Arabidopsis* and could indentify both known and novel network connections. We also performed a comparative transcriptome study between rice and *Arabidopsis* during low temperature stress to explore the molecular differences between chilling sensitive and freezing tolerant plants. Interesting observations were that the dynamics of the response of key genes appears to be higher in *Arabidopsis* than in rice. Several important downstream genes encoding proteins with freezing protective activities in *Arabidopsis* are not present in rice or important cis-elements. Also stress mediated hormone signalling seem to be absent in rice. Together these observations partly explain why rice is unable to cold acclimate to the same extent as *Arabidopsi*s.

Finally we developed a TILLING (Targeting Induced Local Lesions IN Genomes) population in the oat consisting of 2,600 independent events. By random sequencing of two genes involved in the lignin (*AsPAL1*) and ß-glucan (*AsClsF6*) synthesis we estimated the mutation frequency in the population to be approximately 1 per 26,000 bp. This means that each gene is mutated ca 250 times looking at the entire population and assuming an average gene size of 2 kb. This TILLING population will now be an important tool for both breeding and genetic studies in oats.

Keywords: cold acclimation, oat, EST, microarray, transcriptome, chilling, freezing, rice, *Arabidopsis*, TILLING, mutation

Papers discussed

This thesis is based on the following papers, which will be referred to by their Roman numerals.

Paper I

Bräutigam, M., Lindlöf, A., Zakhrabetkova, S., Gharti-Chhetri, G., Olsson, B. and Olsson, O. (2005) Generation and analysis of 9792 EST sequences from cold acclimated oat, *Avena saitiva*.

BMC Plant Biology 5:18

Paper II

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

BMC Genomics.

Paper III

Bräutigam, M., Lindlöf, A., Chawade, A., Gharti-Chhetri, G., Olsson, B. and Olsson O. (2008) Transcription profiling of cold stress response in rice and comparative analysis to *Arabidopsis thaliana*

(manuscript)

Paper IV

Chawade, A., Sikora, P., **Bräutigam, M.**, Larsson, M., All Nakash, M., Chen, T. and Olsson, O. (2008) Development and characterisation of an Oat TILLING population and identification of mutations in lignin and ß-glucan biosynthesis genes.

(manuscript)

List of Contents

Introduction

Low temperatures limit the geographical distribution and yield of many commercial crops species. Many plant species from temperate regions has developed sensory mechanisms to sense changes in the environment that signal the coming winter, which opens the door for the plants to adopt to the change, which lead to an increased freezing tolerance (FT). The primary environmental factor responsible for increased FT is low none freezing temperatures, a phenomenon known as cold-acclimation (Thomashow, 1999). Both farmers and modern plant breeders have exploited plants ability to survive freezing temperatures with the aim to develop cultivars with increased FT. However, in the past few decades, there has been little improvement of the FT of important crop species. FT of wheat (*Triticum aestivum*) varieties today, for instance, is only marginally better than those developed in the early part of last century (Fowler and Gusta, 1979). Revealing the nature of the genetic mechanism behind cold acclimation and FT will provide potential for new development strategies for FT crops. The main objective with the works included in this doctoral thesis was to increase the knowledge underlying genetic mechanisms of freezing tolerance in plants, with the long-term goal to use these results in the development of a Scandinavian winter oat.

Oats

Oat is a cereal crop of the family Gramineae (Poaceae) with uncertain centre of origin but likely it originates from the Mediterranean or Middle East (Rines et al., 2006). It occurs at three ploidy levels, diploid, tetraploid, and hexaploid, with a base chromosome number of seven. The most cultivated oat in the world today is *Avena sativa L.* is a hexaploid, originating from a combination of three diploid genomes (AA, CC and DD) (*ref*). There are several diploid oat species with AA and CC genome. The origin of the D genome is unknown but it's quite similar to the A genome and therefore it's probably only a recent variant of it. The genome size of hexaploid oat has been approximated to 13Gb (giga base pairs) (Bennett and Smith, 1976), which is approximately four times larger than the human genome.

Cultivated hexaploid oat is considered as a secondary crop, which means that it evolved from weeds infesting cultivated fields (Ladizinsky, 1998). Wheat and barley (*Hordeum vulgare*) was first domesticated about 10,000 years ago in the Middle East region (Desai, 2004), and they could initially compete well with the wild oat species. Later these crops where introduced to central- and northern Europe, with harsh climate condition, then the oat weeds became more successful and could therefore dominate the field. Eventually farmers started to domesticate the wild oat species by the selection of non-shattering oat weed seeds, which were present as contaminants in the wheat and barley fields. Some of the earliest records of oat grown as grain crop date bake to the period of the Roman occupation of Europe. Initially oat was primarily used as feed for horses and livestock but in writings from the 18th century it's also stated that oats constituted an important part of the human diet in Scotland, Wales and Ireland (ref). European colonists also brought the spring habit of *A. sativa* oats to North America, Australia and New Zealand. The winter-habit red oats, *A. byzantine*, extant in Spain and Portugal were imported from America, where they were grown as forage crop as well as grain for horses. In the temperate region

of Asia the naked type of *A. sativa* (hulless) evolved and today this is the dominant oat cultivar in China (Wang, 2004).

Currently, oat remains an important grain and forage crop in many parts of the world grown on 13.2 million hectares with a total grain production of 25,6 million metric tons in 2008 (http://www.fas.usda.gov/wap/current/toc.asp). Among the cereals cultivated in Sweden oats is in third place with an average production of 855,000 ton/year compared to wheat, which is the largest crop in Sweden, with an average production of 2,000,000 ton/year (Jordbruksverket, 2008). Compared to other cereal crops, oat is well suited for production under marginal environments, including cool-wet climates and soils with low fertility (Buerstmayr et al., 2007). Because oat also flowers and matures quickly in short seasons with long day length regimes, oat has until today been an important spring crop in the Nordic countries. The majority of the oat production today are grown between latitudes of 40° and 50°N in North America, Europe and Asia, and only a small portion of total oat production is produced in the southern hemisphere, i.e. South America, Australia and New Zealand (USDA, 2008). The main usage of oats is as on-farm feed, which accounts for at least 60 % of the total world production, and only 13 % is used for food production. One drawback with oats, from the farmer's perspective, is that the average yield is less than for wheat, maize and soybean. In Sweden, for example, the average yield for oats the last five years has been approximately 3,9 ton/ha, whereas the average yield for winter wheat has been 6,1 ton/ha (Jordbruksverket, 2008). In addition, since oat kernels has a hull that makes up almost 25% of the total grain weight, the energy yield per hectare is even less than that of wheat. Therefore there has been a continuous decline in the world oat production the past decades and this can be attributed to the decreased demand for oat as on-farm feed. Farmers have switched to crops with higher yield like wheat, barley, maize and soybean (Rines et al., 2006). Thus, to keep oat as a crop with in the modern agricultural practice there is need of development of a higher yielding oats, which is easer to digest when used as feed.

Low temperature stress

Low temperature (LT) stress includes chilling (<20°C) and/or freezing (0<°C) temperatures, adversely affects both productivity and geographical distribution of important agricultural crops. LT restrains the expression of the full genetic potential of plants owing to its direct inhibition of metabolic reactions and, indirectly, through cold-induced osmotic (chilling-induced inhibition of water uptake and freezing-induced cellular dehydration), oxidative and other stresses(Chinnusamy et al., 2007).

Physiology of chilling

Many plant species originating from tropical and sub-tropical areas suffer from injuries when exposed to temperatures above the freezing point but below 15° C; this is called chilling injury to distinguish it from freezing injury. Chilling injury is a serious problem during germination and early seedling growth in many plant species like maize and rice (Bedi and Basra, 1993). Visual symptoms of chilling injury can take a variety of forms, depending on the species, age of the plant, and the duration of low-temperature exposure. Young seedlings typically show signs of reduced leaf expansion, wilting, and chlorosis. Extreme cases will result in accelerated senescence, a reduced growth and eventually death. In some plants, the reproductive development is especially sensitive to chilling temperatures. Exposure of rice plants, to chilling temperatures at the time of anthesis (floral opening) results in sterile flowers.

In chilling sensitive plants, major effect is the physical transition of cell membrane from a flexible liquid-crystalline to a solid gel phase. This change in physical state of the membrane affects the cellular function in a number of ways. The most immediate effect is increased permeability leading to cellular leakiness and ion imbalance. As a consequence of abnormal metabolism, injured cells accumulate toxic metabolites and active oxygen species.

Physiology of freezing

Many plant species found in the temperate regions of the world differ from their tropical counterparts in their ability to survive temperatures below 0°C. Freezing temperatures pose a significant threat to plant survival and growth for multiple reasons.

Several studies has shown that the membrane systems of the cell are the primary site of freezing injury in plants (Webb et al., 1994; Steponkus et al., 1998) and it's well established that freezing-induced membrane damage results primarily from the severe dehydration associated with freezing (Pearce, 2001). As temperature drops below 0°C ice nucleation generally begins in the intracellular spaces, partly because of a difference in the solute concentrations leading to a higher freezing point for the intracellular fluid (Thomashow, 1999). The chemical potential of ice is less than of liquid water and therefore the formation of ice results in a decrease in the water potential outside the cell. As a consequence the unfrozen water moves from the higher potential in the cell to the lower potential in the intracellular space. This water movement cause the severe cellular dehydration during freezing.

Acclimation

Most temperate plants can acquire freezing tolerance upon prior exposure to sub lethal LT stress at temperatures above 0°C, a process called cold-acclimation. Chilling-sensitive plants from the tropical and sub-tropical regions are incapable of cold-acclimation and they cannot tolerate ice formation in their tissues. Nevertheless, the temperature threshold for chilling damage is lowered even in some chilling-sensitive crop species by prior exposures to suboptimal low temperatures (Anderson et al., 1994; Sthapit and Witcombe, 1998), this process is called chilling-acclimation. The molecular basis of chilling-acclimation is still poorly understood but recent studies shows that it in part is related to the process of cold-acclimation (Rabbani et al., 2003; Usadel et al., 2008).

A key function of cold-acclimation is to stabilize membranes against freezing injury (Webb et al., 1994; Uemura and Steponkus, 1997) and multiple mechanisms appear to be involved in this stabilization. The best documented are the changes in lipid composition (Uemura et al., 1995; Uemura and Steponkus, 1997) but also the accumulation of simple sugars seem to also contribute to the stabilization (Strauss and Hauser, 1986). Numerous plants have also been found to possess antifreeze proteins that are involved in the membrane stabilization during freezing (Yeh et al., 2000). The underlying molecular mechanisms to these physiological changes there are extensive in alterations in the expression by a number of cold responsive genes (*CORs*) (Guy et al., 1985; Thomashow, 1999; Chinnusamy et al., 2007).

Much of the injury caused to plants during LT stress can be associated with reactive oxygen species (ROS), especially in chilling sensitive plants (O'Kane et al., 1996; Guo et al., 2006). Plants have developed effective oxygen-scavenging systems consisting of several antioxidant enzymes, such as superoxide dismutase, ascorbateperoxidase, glutathione reductase (GR) and catalase and none-enzymatic antioxidants, such as ascorbic acid and reduced glutathione. These antioxidants protect membranes from the deleterious effect of ROS. It has been showed that chilling tolerant cultivars have higher activates of antioxidant enzymes than susceptible cultivars in several crops, such as rice and maize (Anderson et al., 1994; Guo et al., 2005). During both cold- and chilling-acclimation, a plant activates scavenging enzymes, which helps to detoxify the cell (Apel and Hirt, 2004; Gadjev et al., 2006), which then result in an increased tolerance to LT stress.

Temperature perception

Plants exhibit a range of responses to the temperature of their environment. Some of these responses are fast, while others require involve accumulation temperature data for several days or weeks, such as the floral promotion pathway of vernalization. Even though many of the molecular, biochemical and physiological changes associated with low temperature change have been well documented, still little is known about the mechanisms through which plants sense and transmit low temperature signals.

From studies in cyanobacteria and yeast it has been hypothesized that the temperature mediated alteration of membrane fluidity may itself be the primary temperature senor, and there has been much speculation if this is also primary sensor in higher plants (Vigh et al., 1993; Murata and Los, 1997). In a pharmacological approach, using dimethylsulfoxide that rigidified the cell membranes, it has been shown that it's possible to induce *COR* genes

and Ca^{2+} influx at room temperature in alfalfa and *Brasica napus* (Orvar et al., 2000; Sangwan et al., 2001). Orvar *et al.* also showed that *COR* gene expression and calcium (Ca^{2+}) influx could be prevented by an actin microfilament stabiliser at 4°C but induced at 25°C by an actin microfilament destabiliser. This suggests that cytoskeletal reorganization is part of the sensor mechanism. The differential activation thresholds for cold induced signalling diaglycerol kinase (DAGK) in *Arabidopsis* suspension cultures possessing altered "resting" membrane fluidity due to saturase mutations also support the concept of that plants sense the temperature through a membrane rigidification process *(Vaultier et al., 2006)*.

Cold signal transducers

The elevation of cytosolic Ca^{2+} is an early event in the response LT stress. Our knowledge of these cytosolic Ca^{2+} oscillations come from transgenic studies in *Arabidopsis* and *Tobacco* (Knight et al., 1991). Cytosolic Ca^{2+} oscillations can be detected within seconds or minutes after the transfer of the plants to LT and they are associated with membrane depolarization. Furthermore, they have characteristic waveforms that are dependent on both magnitude and absolute temperature of the temperature shift (Knight et al., 1996; Plieth, 1999). The magnitude of the Ca^{2+} oscillation is also dependent of the plant's previous experience of temperature stress, repeated low temperature treatment results in a damped Ca^{2+} oscillation, which implies that plants appear to have a Ca^{2+} signature memory of earlier temperature experiences. It has been shown that there is a positive correlation between cold-induced cytosolic Ca^{2+} influx and accumulation of *COR* genes in both alfalfa (Monroy and Dhindsa, 1995) and *Arabidopsis* (Henriksson and Trewavas, 2003). Different chelators and channel blockers have been used to show that the cytosolic influx of Ca^{2+}, and its role as second messenger in cold-responsive signal transduction (Monroy et al., 1993; Knight et al., 1996). It is also observed that effective Ca^{2+} signatures are produced only in particular tissue or organs. During LT stress the cytosolic Ca^{2+} influx occurs in the whole plant, in contrast to drought, where it is present only in roots (Knight and Knight, 2000).

If the plant fails to control the Ca^{2+} oscillation it will lead to prolonged elevated levels of cytosolic Ca^{2+}, which can be toxic for the cells. Experiments have shown that chilling sensitive maize is unable to restore the lower resting levels of intracellular Ca^{2+} after low temperature influx, while freezing tolerant wheat quickly do so (Jian et al., 1999). This observation has been suggested to partly explain the incapacity among chilling sensitive plants to cope with longer periods of cold stress, since prolonged periods with elevated levels of cytosolic Ca^{2+} is suggested to cause ROS accumulation, metabolic dysfunction, and structural damage to the cell.

Cytosolic Ca^{2+} signal is transmitted primarily through Ca^{2+} regulated proteins called calcium sensors, which change their phosphorylation status when they sense the elevation in Ca^{2+} (Monroy et al., 1993). The major calcium sensors in plants are calmodulin (CaM), CaM domain-containing protein kinases (CDPKs), calcineurin B-like proteins (CBLs) and CBL-interacting protein kinases (CIPKs). Many CDPKs are up regulated by cold stress in different plant species. Monroy and co-workers used antagonists and inhibitors of CDPK and CaM and showed that alfalfa cells in suspension culture was inhibited in their ability to cold acclimate (Monroy et al., 1993). Similar results have also been showed in *Arabidopsis*

(Tahtiharju et al., 1997). Kim and co-workers identified a Ca^{2+} regulated protein kinase in *Arabidopsis*, CIPK3, and they used a mutant, *cipk3*, to study this protein during abiotic stress (Kim et al., 2003). In this study Kim and his co-workers showed that CIPK3 mediates the Ca^{2+} signal and positively regulates the ABA- and cold-induced expression of stress related genes. They also suggest that CIPK3 is working as a cross-talk node between the ABA- and cold signal transduction, because disruption of CIPK3 function simultaneously alter the gene expression induction pattern of RD29A by ABA-, salt-, and cold-treatments. This is an interesting result since the cold-induced expression of RD29A previously has been shown to be independent of ABA (Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000). Finally Kim *et al* also proposed that CIPK3 is located upstream of the transcription factors and downstream of the initial Ca^{2+} signal. The over-expression of CIPK genes in rice have also confirmed improved tolerance to cold, drought and salt stress (Xiang et al., 2007). Ray and co-workers found four up regulated CDPK genes during a global expression study of cold stressed (+4°C) *Indica* rice. They also showed that overexpression of one of this CDPK genes (OsCDPK7) resulted in an improved tolerance to cold-, salt-, and drought stress.

Mitogen activated protein kinase (MAP kinase) cascades are also involved in cold stress signalling. The activities of MAP kinases have been shown to increase at low temperature and this activity increase could also bee observed during drought stress in both alfalfa and *Arabidopsis* (Jonak et al., 1996; Mizoguchi et al., 1996). Similarly, Teige and co-workers identified the map kinase MKK2 in *Arabidopsis* as an important mediator of the cold- and salt stress signals – defective mkk2 plants were hypersensitive to freezing and germination on salted media, while MKK2 overexpressers showed enhanced freezing and salt tolerance (Teige et al., 2004).

As a result of cold stress there is also an oxidative burst due to the generation of ROS like superoxide, hydrogen peroxide (H_2O_2) and hydroxyl radicals. These oxidative bursts induce synthesis ROS scavenger enzymes and other protective mechanism (Apel and Hirt, 2004; Mittler et al., 2004). The mechanism by which plants are able to sense the oxidative burst in response to cold is still unknown. It has been shown that ROS can activate MAP kinase cascades in *Arabidopsis*. The MAPKKK ANP1 mediates H_2O_2-induced activation of MPK3 and MPK6, and stable overexpression of ANP1 is resulting in plants more tolerant to heat, freezing and salt stress (Kovtun et al., 2000). The homologue to ANP1 in tobacco, NPK1, is active in cold signal transduction cascades and influence auxin signalling (Kovtun et al., 1998). Overexpression of NPK1 in maize also enhances the low freezing- and drought tolerance (Shou et al., 2004). This shows that ROS could be a missing link between MAP kinases and stress signalling.

Another important signalling molecule during LT stress appears to be inositol 1,4,5-trisphospahte (IP_3). *Arabidopsis* plants with mutations in the FRY1 gene have defective inositol polyphospahte 1-phosphatase enzyme, which functions in the degradation of IP_3. This defect leads to super induction of ABA and cold responsive genes during stress and reduced freezing tolerance while the plants maintain unusually high levels of IP_3 both before and after the exposure to stress (Xiong et al., 2001). It seems that the initial perception of abiotic stress (or exogenously applied ABA) by plants result in a transient increase in IP_3. This data is not in conflict with the hypothesis that Ca^{2+} function as second

messenger, since IP_3 have been shown to mediate transient increases in the cytosolic Ca^{2+} in plant cells (Allen et al., 1995). The identification of another allelic mutation in the FRY1 gene, the *hos2* mutation (resulting in an almost identical phenotype but lacking the superinduction of ABA responsive genes) enhanced our understanding of the role of FRY1/HOS2. Xiong and co-workers showed that FRY1/HOS2 might work as a negative regulator CBF2 and CBF3 expression, since the transcripts level of these two transcription factors were significantly higher in the *hos2* mutant (Xiong et al., 2004). The *hos2* allele of FRY1 was specifically temperature sensitive, with the enzymatic activity only affected in the cold, which could explain why it lacked the superinduction of ABA responsive genes versus the *fry1* counterpart.

Key regulators of cold acclimation

Transcription factors and the *cis*-acting elements in the promoters of the stress responsive genes are important components of the signal transduction network. The importance of *cis*-elements for COR gene expression during cold stress in crops was first shown in preliminary promoter studies in wheat and barley. In these studies they showed that phosphorylation-dependent events are involved in the LT stress signalling and the phosphorylation status controls the binding of nuclear factors to the promoters of the LT responsive WSC120 and BLT101.1 in wheat and barley respectively (Vazquez-Tello et al., 1998; Brown et al., 2001). Vazquez-Tello and co-workers also for the first time identified the LTRE element in wheat, which contains the 5-bp core motif common to the CBF/DREB1 transcription factor (see below).

The first evidence of an ABA-independent pathway during cold stress came from studies of the RD29A/COR78/LTI78 gene in *Arabidopsis*. RD29A/COR78/LTI78 is induced by cold, drought and ABA (Yamaguchi-Shinozaki and Shinozaki, 1993). However, this gene is also induced in ABA-deficient and ABA-insensitive mutants by both cold and drought stresses, which indicates that RD29A/COR78/LTI78 is under control of both ABA-dependent and ABA independent pathways (Gilmour and Thomashow, 1991). Analyses of the RD29A/COR78/LTI78 promoter revealed a 9-bp conserved sequence, TACCGACAT, named the dehydration response element (DRE), were essential for induction of dehydration or ABA response (Yamaguchi-Shinozaki and Shinozaki, 1994). A similar *cis*-acting element, named C-repeat (CRT) or LT responsive element (LTRE), containing A/GCCGAC motif that forms the core of the DRE sequence, have been shown to regulate LT inducible promoters in *Arabidopsis* (Baker et al., 1994; Stockinger et al., 1997), *Barascica* (Jiang et al., 1996), rice (Rabbani et al., 2003) and wheat (Takumi et al., 2003). The transcription factors that interact with the CRT/DRE element are the C-repeat Binding Factor/DRE Binding protein 1 (CBF/DREB1), which first was found in a yeast one-hybrid screen by Stockinger *et al* (1997).

Arabidopsis contains three CBF encoding genes, namely, *CBF1*, *CBF2*, and *CBF3*, all present in tandem on chromes 4, and they are rapidly induced by LT (Stockinger et al., 1997; Gilmour et al., 1998; Liu et al., 1998; Shinwari et al., 1998). These transcription factors all contain an AP2/ERF (APETALA2/Etylen Responsive element binding Factor) DNA-binding domain that recognise CRT/DRE elements, which are present in many COR genes, and they belong to the AP2/ERF superfamily of transcription factors. Analyses in transgenic plants have shown that ectopic expression of CBF/DREB1 genes is sufficient to

activate expression of many *COR* genes and thereby induce cold acclimation even at warm temperature (Gilmour et al., 2000; Maruyama et al., 2004; Vogel et al., 2005). Thus, the CBF/DREB1 transcription factors belong to a master switch that controls the expression of many important COR genes.

Global expression studies of transgenic *Arabidopsis* plants with ectopic expression CBF1, CBF2 or CBF3 revealed that there is a large, if not complete, overlap in the regulons of genes controlled by the various CBFs/DREB1 (Fowler and Thomashow, 2002). Vogel and co-workers also did extensive expression studies in *Arabidopsis* were they identified 514 cold responsive genes, referred to as COS ('cold standard') and by using ectopic overexpressers of CBF2 they concluded that 16.5 % of the cold inducible COS genes fall under the control of the CBFs/DREB1 regulon. CBF/DREB1 genes whose transcripts accumulate rapidly in response to low temperatures have been isolated in rapeseed, tomato, rye, wheat, barley, rice, oat, and many other plants species(Jaglo et al., 2001; Dubouzet et al., 2003; Zhang et al., 2004; Brautigam et al., 2005; Skinner et al., 2005; Ito et al., 2006). It therefore appears that the CBF/DREB1 pathway is partly conserved in flowering plants.

Interestingly, the promoters of CBF/DREB1 genes themselves do not contain any CRT/DRE elements. This led Gilmour *et al* (1998) to speculate about the existence of unidentified factors; called ICE (for Inducer of CBF Expression) which presumably recognised novel cold regulatory elements, or "ICE boxes," present in the CBF/DREB1 promoters. According to their model, ICE was present but inactive in plant cells at warm temperatures and was activated upon transfer to low temperature, in turn, rapidly activating CBF transcription. After years of research Chinnusamy *et al* (2003) presented the first evidence of the existence ICE, namely ICE1 . ICE1 were a MYC-type basic-helix-loop-helix transcription factor that was constantly expressed and it could bind to a MYC target in the CBF promoter. They also showed that a mutated *ice1* failed to induce *CBF3* under cold acclimation and it was hypersensitive to freezing. On the other hand, overexpression of *ICE1* enhanced expression of *CBF3, CBF2* and down stream *COR* genes, but only during LT stress. This suggested that LT stress posttranslational modification is necessary to activate ICE1. Recently there was also a report about a ICE2 in *Arabidopsis* (Fursova et al., 2009) and a homolog to ICE1 in barley (Skinner et al., 2006).

In *Arabidopsis* many transcription factor genes are transiently induced during LT stress (Fowler and Thomashow, 2002), which suggests a feedback regulation mechanism. Molecular analysis of a *cbf2* mutant in *Arabidopsis* suggests that CBF2 is a negative regulator of *CBF1* and *CBF3* (Novillo et al., 2004). Further more, *CBF* genes are negatively regulated by an upstream transcription factor, MYB15 (a member of R2R3-MYB family) in *Arabidopsis* (Agarwal et al., 2006). MYB15 is also expressed in the absence of LT stress and MYB 15 binds to MYB recognition sites in the promoters of *CBFs.*

Also a LT induced C2H2 zinc finger transcription factor ZAT12, seems to function as a negative regulator of *CBF* (Vogel et al., 2005). *ZAT12* is under control of the circadian clock and is out of phase with the rhythm of *CBF2* (Fowler et al., 2005). Transgenic overexpression of *ZAT12* decrease expression of *CBF* under cold stress (Vogel et al., 2005). I simplified overview of the CBF pathway is given in Figure 1 below.

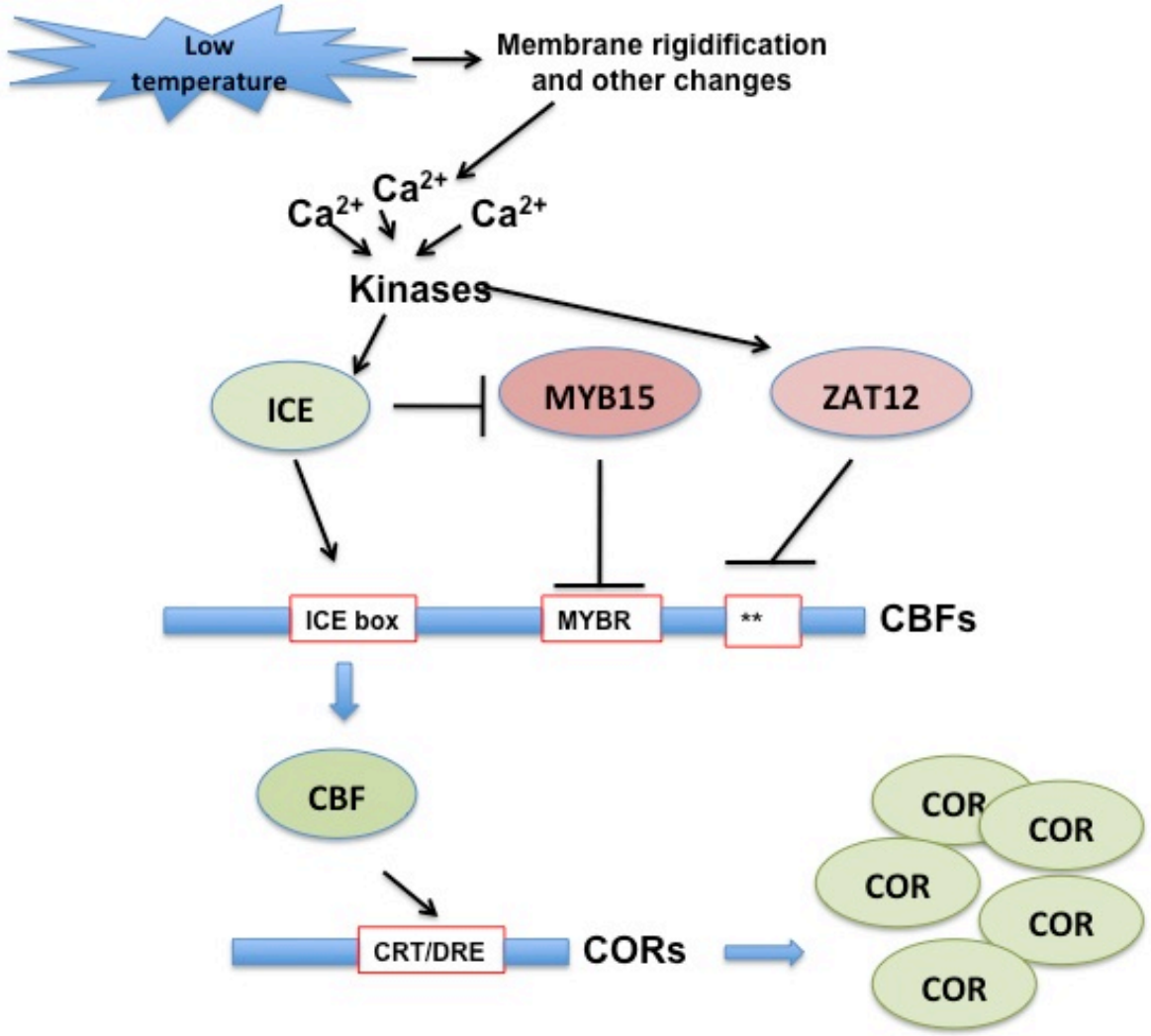

Figure 1. Overview of the proposed CBF regulatory pathway in *Arabidopsis*. Plants sense low temperatures through membrane rigidification and/or other cellular changes, which release cytosolic calcium and thereby activate protein kinases necessary for cold acclimation. Constitutively expressed ICE is activated by phosphorylation, and ICE there by activates *CBF* expression and repress *MYB15*. CBF is activating *COR* genes. Finally, ICE is deactivated and *CBF* is repressed by *ZAT12* and *MYB15* in a negative feed back control.

Although the CBF/DREBl regulon is a key component for a successful cold acclimation there have been reports about other important players. One such example is the ESKl mutant in Arabidopsis, which is constitutively freezing tolerant but it did not effect the expression of genes in the CBF/ DREB1 regulon (Xin and Browse, 2000).

Vernalization

Wheat and barely are grown in temperate regions throughout the world. Flowering in these cereals can be accelerated by prolonged exposure to LT, a process known as vernalization. Vernalization occurs during winter when the temperatures are between 0° to 12° C

(Trevaskis et al., 2007). A few weeks of cold are often sufficient to promote flowering, and varieties that require vernalization are sown in the early autumn. There are also varieties in both wheat and barely that do not require vernalization to flower, these are spring sawn.

Genetic analysis in barley and wheat have shown that there are three genes controlling the vernalization requirement; *VRN1*, *VRN2* and *VRN3* (Yan et al., 2003; Yan et al., 2004; Yan et al., 2006). *VRN3* has been mapped to known ortholog in *Arabidopsis*, the *Flower Locus T (FT)* gene (Yan et al., 2006).

Vernalization induces the *VRN1* gene that promotes the switch from vegetative to reproductive development and thereby promotes flowering. *VRN1* encodes an APETALA1-like MADS box transcription factor. In varieties that require vernalization to flower, *VRN1* is initially expressed at low levels and it is significantly induced by vernalization (Yan et al., 2003). The extent to which *VRN1* is induced depends on the length of vernalization treatment. In varieties that flower without vernalization, expression of *VRN1* increases during the initiation inflorescence and remain high during subsequent steps in the development (Trevaskis et al., 2007).

VRN2 encodes a protein with a zinc finger motif, which mediates DNA binding, and a CTT domain (Yan et al., 2004). *VRN2* is controlled by the circadian clock during long days but are not expressed during short days. On the basis of its expression pattern, and the nature of the protein, it has been suggested that VRN2 blocks flowering during long days by repressing *VRN3*. VRN3 is promoting flowering in long days (Yan et al., 2006).

Scientific aims

Overall aim

The overall aim with the works included in this doctoral thesis is to increase our knowledge about the underlying genetic mechanisms of freezing tolerance in plants, with the long-term goal to use knowledge in the development of a Scandinavian winter oat.

Specific aims

The specific aims were to:

- Indentify genes related to cold stress in oat, by analyzing EST sequences collected from a cDNA library that was based on RNA from LT treated oat plants (*Paper I*).
- Systematic investigate possible LT stress regulatory circuits in Arabidopsis. Our strategy was to take advantage of the vast amount of information and microarray data that was present in Arabidopsis to identify new pathways that could be functionally studied in Arabidopsis and later picked up in oat (*Paper II*).
- Investigate potential differences in the transcriptional dynamics between chilling sensitive and freezing tolerant plants during LT stress, with the aim to increase our understanding of the underlying mechanism leading to freezing and chilling tolerance. In this case we used the rice (*Oryza sativa*) (chilling sensitive), and made a comparison to *Arabidopsis* (freezing tolerant) (*Paper III*).
- Develop a TILLING population in oats.

Methodology

EST generation

Messenger RNA (mRNA) represents copies from expressed genes in the cell. As RNA cannot be cloned directly, they are reverse transcribed to double-stranded cDNA using a specialized enzyme, reverse transcriptase. The recovery of single-pass sequences (known as expressed sequence tags, or ESTs) from random cDNA clones has been pursued as relatively inexpensive and rapid means to access many of the expressed genes of an organism (Newman et al., 1994; Hillier et al., 1996). These ESTs usually range in size from 100–800 bp. In paper I, we isolated and pooled total RNA from oat plants (*cv* Gerald) that had been cold-acclimated at +4°C for 4, 16, 32 and 64 hours. This RNA pool was used for cDNA library construction, which were used for EST sequencing.

EST clustering and assembly

The purpose of EST clustering is to collect overlapping ESTs from the transcript of a single gene into a unique cluster to reduce redundancy. A simple way to cluster ESTs is by measuring the pair-wise sequence similarity in an all-versus-all comparison. Then, these distances are converted into binary values, depending on whether there is a significant match or not, such that the sequence pairs can be accepted or rejected from the cluster being assembled. EST clustering can be either 'stringent' or 'loose' (Ptitsyn and Hide, 2005). The stringent clustering method is conservative, uses single-pass grouping of ESTs resulting in relatively accurate clusters, but generates shorter-sequence and more singletons (sequences that are not in clusters). In contrast, loose clustering is 'liberal' and repeats low quality EST sequence alignments many times to generate less accurate but longer-sequence consensuses. In Paper I, the oat EST sequences were assembled with the Paracel Transcript Assembler (PTA) software (Paracel, 2002). PTA uses a HASTE (Hash Accelerated Search Tool) algorithm for the pairwise comparison during clustering, which is an adaption of the Smith Waterman algorithm (Smith and Waterman, 1981) to save computer time.

Annotation and functional classification of ESTs

The assembled ESTs represent putative genes and through an annotation and functional classification procedure we can put these genes into their biological context. Annotation of putative genes is accomplished via similarity searches versus public databases. A universal tool for database similarity searches is the BLAST program (Altschul et al., 1997) suite from NCBI (www.ncbi.nlm.gov), where BLASTN is used to search with ESTs against nucleotide sequence databases and BLASTX to search against protein databases. BLASTX translates a nucleotide sequence (query) into protein in six reading frames followed by comparisons with protein databases. EST data can also be correlated directly with protein-centric annotations, such as Pfam (Finn et al., 2008), INTERPRO (Mulder et al., 2007) and Conserved Domain Database (CDD) (Marchler-Bauer et al., 2009), by polypeptide translations, since proteins are better templates for indentifying domains and motifs. In any large-scale sequencing project it is extremely important to be able to store, organize and annotate sequences. Therefore we developed our own in house semi automated analysis pipeline. Appropriate PERL scripts were developed to pipeline the process of running tools in sequence searches, parsing result files and loading the data into our local database. We have also developed a public interface to the annotated oat sequence data, which is

available through www.agod.org (presently under reconstruction). These tools have been extensively used in all papers presented in this thesis.

Northern blotting

With Northern blotting the researcher can detect induced gene expression. The method is based on radioactive labelling of a gene specific cDNA probe, which is hybridized to a denatured, size-separated, and membrane-immobilized population of total RNA isolated from a particular tissue of interest, followed by subsequent washing steps to remove non-specifically bound probes from the membrane, researchers were able to track changes in the amounts of mRNA for genes expressed at reasonable levels. The advantage with Northern blots is that they are relatively sensitive and simple in terms of the sample preparation and equipment needed to perform the detection. However, for genes with many paralogs of high sequence similarity, genes expressed at very low levels, or for the analysis of large number of genes within the same sample, Northerns possess problems that are circumvented by more recently developed methods of gene quantification. In Paper I, we successfully used a Northen blott to detect the induction pattern of the oat *COR410* gene.

RT-PCR

The reverse-transcriptase polymerase chain reaction (RT-PCR) is sensitive, specific and scalable, which makes it the superior choice for detection of transcripts with low copy numbers. In the two-step RT-PCR approach used to quantify the gene expression in Papers I, isolated RNA was first reverse-transcribed into cDNA. This cDNA could then be used as template in subsequent gene-specific PCR amplifications. By designing primers to anneal at positions which differed between paralogs and/or which produced products whose sizes differed significantly, specificity could be ensured. Although RT-PCR is scalable, and it circumvents the problems with Northern described above, this technique is not convenient for genome wide transcriptome studies.

Global gene expression analysis with microarray

One way of examining gene expression at the genomic scale is through the use of cDNA or oligonucleotide microarrays, originally developed by Schena *et al* (1995) and Fodor *et al* (1993) respectively. These techniques measure differences in transcript abundance between two samples on "omic" scales. The fractional detection limit (the relative number of a specific transcript mRNA compared to total number available mRNAs in the sample) of this method is thought at least 1/300,000. Comparison of the strength of the fluorescent signal at each spot location gives an estimation of the relative abundance of the transcript in question in each sample. In actuality, the process of image/signal analysis is somewhat more complicated, as the data collected needs to be filtered (to remove "false positives" created by dust specks on the slide, saturated and missing spots), normalized (to account for differences in dye incorporation rates, background signal intensity, etc) and statistically treated to identify the populations of genes that are consistently differentially expressed between the two samples across technical and biological replicates. At each step of this process (sample preparation, hybridization, image capture, normalization, statistical analysis), the choices made concerning methods, design, and replication can have significant effect on the reliability and sensitivity of the microarray results.

In Paper III we performed a microarray analysis, using the Affymtrix Rice Genome Arrays. Total RNA was isolated from three weeks old rice seedlings that had been stressed at +4° for 0, 0.5, 2, 4, 8, and 24 hours. We used non-amplified target cRNAs for hybridization from 4 µg total RNA extracted from leaf tissue. Two biological replicates were analysed per time point. The gene expression data form was analyzed using methods implemented in the GeneSpring® software, version 7.3 (Agilent Technologies). Per-gene normalization was applied and the arrays were adjusted for background noise using the GC-RMA algorithm. With in GeneSpring a one-sample t-test were calculated for replicated data and we only used probe-sets that had a p-value $\leq$ 0.05 in 3 out of six conditions. To identify probe-sets that were differentially expressed we used the fold change. A problem with the fold-change masseurs is to find an appropriate level for cut, since to low cut-off levels tends to give to many false positives. In a previous microarray study, using the Affymetrix platform, Fowler and Thomashow (2002) showed that a 3-fold cut off efficiently excluded false positives. Based on this information we decided to consider only probes sets that were at lest 3-fold induced/repressed in at least one time point as being responsive to cold stress.

Present study

Cold acclimation in oats

Since, from the molecular point of view, oat were a relatively unstudied crop with few public available sequences, the work in Paper I was initiated by EST sequencing of a large number of cDNA clones from a LT induced library. This library was constructed with total RNA extracted from cold acclimated leaves of oat variety Gerald (as described in Methodology). To confirm that cold induced genes were present in RNA collection before we constructed the cDNA library, we performed a Northern analysis on an oat gene corresponding to a previously described cold inducible wheat gene, COR410 (Thomashow, 1999). We then sequenced 9,792 ESTs which resulted in 8508 high-quality ESTs of 100 bp or longer, with an average sequence length of 710 bp. These ESTs were assembled to an oat UniGene (AsCI UniGene) set of 2,800 putative genes. Of these, 1,726 could be functional classified into classes defined by MIPS (Munich Information Centre for Protein Sequences), which left a relatively large proportion of the genes (approx. 40%) unclassified.

Perception of the stress stimuli, transduction of the signal and a molecular response are necessary activities if the plant is to react to abiotic stress. In oat, however, very little was known about cold stress response at the molecular level, although we suspected that the general mechanisms were conserved among other temperate plants. In order to identify the oat genes involved in the LT response in Paper I we created a database denoted CSDB (cold stress data base), in which the cold stress related proteins available in the public domain were collected. This database was then used to compare all AsCI UniGene sequences with the sequences in the CSDB, using a unidirectional BLASTX search. With this methodology we could identify 398 oat sequences with significant homology (BLASTX e-value $\leq$ 1.0 e^{-10} cut off) to at least one sequence in the CSDB, indicating that at least 14% of AsCI UniGenes could be associated to cold stress related processes. Among these, sequences encoding activities related to perception, signal transduction and transcriptional regulation were overrepresented (Table X). To judge whether this high portion of LT stress related oat genes in relation to the total number of genes in the oat UniGene set were realistic we did similar comparisons with other UniGene datasets from cold acclimated barley (HvCI) and wheat (TaCI). We then found that the around of 10 % of the genes in both the TaCI and HvCI UniGene could be related to cold stress (Table X). We also created a second oat UniGene set from untreated oat leafs (AsNI). When the CSDB was searched with AsNI UniGene set only 5.1% of the genes were found to be similar (Table X2), a dramatic difference to the AsCI UniGene set. Generalising, it seems like at least 10% of all expressed genes in cold acclimating plants are involved in various cellular responses needed to prepare the plant to freezing temperatures.

The functional analysis of the AsCIUniGene set in Paper I showed that transcription factor genes were represented by 107 sequences, belonging to at least 14 different families. Of these, 51 were homologous to cold-induced genes from other systems. Of special importance for cold acclimation is the CBF/DREB transcription factor family, since these transcription factors have been shown to regulate several downstream genes important for freezing tolerance (Stockinger et al., 1997; Fowler et al., 2005; Vogel et al., 2005).

However, the regulation is known to be complex and several different CBF/DREB factors are involved. From the AsCIUniGene set we identified four putative oat *CBF/DREB* genes, denoted *AsCBF1*, *AsCBF2, AsCBF3* and *AsCBF4*. In Paper I we investigated their expression profiles during cold acclimation using semi quantitative RT-PCR. Their expression ranged from early induction already after 15 min (*AsCBF3*) to induction after 1h (*AsCBF4*) and from peaking at 4 h (*AsCBF1* and *AsCBF3*) to peaking at 8h (*AsCBF4*). Despite several attempts using different primer pairs we could not obtain a reproducible expression pattern of the *AsCBF2* gene. The reason for this is still not known. The complex regulation of the *AsCBF* genes is different from what was previously described in *Arabidopsis* (Gilmour et al., 1998) where the *AtCBF1*, *AtCBF2,* and *AtCBF3* genes follow more or less the same expression pattern with a rapid induction after 15 min and a peak after 2h. This could indicate that CBF/DREB factors have intricate and different individual roles in inducing and maintaining cold acclimation in oat. This is supported by findings in barley, which has at least 20 different genes encoding CBF/DREB factors, which are all differentially regulated (Skinner et al., 2005). Thus, a more detailed analysis of the promoter structure and regulation of *CBF* genes in cereals may reveal why some cereals like rye and wheat are more cold hardy (freezing tolerant) than oat and barley.

There were a number of genes encoding proteins with unknown functions in the AsCIUniGene set. These were divided into two groups, one in which homologous to proteins with unknown function, and the other group contained genes where no significant similarities could be found to any other sequence, i.e. genes that could be oat specific. In order to rule out that small "non-real" peptides contributed to this group, only sequences with open reading frames of 100 aa or more were included. This resulted in a potential oat specific group with 427 genes. Assuming that approx. 10% of these is cold related, more than 40 completely new oat genes involved in cold acclimation could be present in this collection. Such genes are potentially very interesting and could encode hitherto uncharacterised proteins or regulatory factors involved in adaptation to LT and freezing protection.

In parallel to the EST project we also started a field study were we during 4 consecutive years screened more than 300 oat varieties for winter survival. After the first two years, the 25 lines with the highest winter survival were selected for further evaluation during the winters of 2005/2006 and 2006/2007. All lines were evaluated according to percentage of survival and according to a nine-degree vigour scale, where the highest score means excellent agricultural performance (time of flowering, height of plants at bolting, straw strength, yield, etc.). From this screen we selected six lines that will be used for molecular characterisation of freezing tolerance in oat (Table 1).

Finally, after we had published the oat EST paper we identified a vernalization gene among the sequences in the AsCIUniGene set, *AsVRN1*. This gene was found to be homolog to the wheat VRN1 protein. VRN1 promotes transition from vegetative to floral growth and *VRN1* expression is induced by vernalization in winter wheat. On the other hand the expression of *VRN1* in spring wheat increases during the initiation inflorescence and remain high during subsequent steps in the development.

	Winter survival (%)				Agronomic value (1-9)			
	2004	**2005**	**2006**	**2007**	**2004**	**2005**	**2006**	**2007**
PI 555736	30	50	30	90	3	7	4	6
CIav 9349	50	70	40	90	7	7	2	3
CIav 8213	30	70	20	90	5	3	4	3
Wich CIav 9340	30	60	40	90	7	5	5	5
CIav 9346	40	50			7	5		
Win/Nor-1	50	40			3	3		

Table 1. Winter survival among selected oat lines in field trials. Lines are evaluated according to percentage of survival and according to a nine-degree vigour scale, where the highest score means that the agricultural performance (time of flowering, height of plants at bolting, straw strength, yield, etc.) is according to commercial standards.

Since *VRN1* has this characteristic expression patterns in wheat, it should be possible to use *VRN1* as a marker for freezing tolerance. Thus we tested *AsVRN1* as a marker for winter survival among the selected lines in Table 1. We also include the English winter oat variety Gerald and as control we used a two Swedish spring oat varieties Belinda and Stork (Figure 2.) In this study total RNA were extracted from plants that had been cold treated at +4° C for 0, 7, 15 and 42 days.

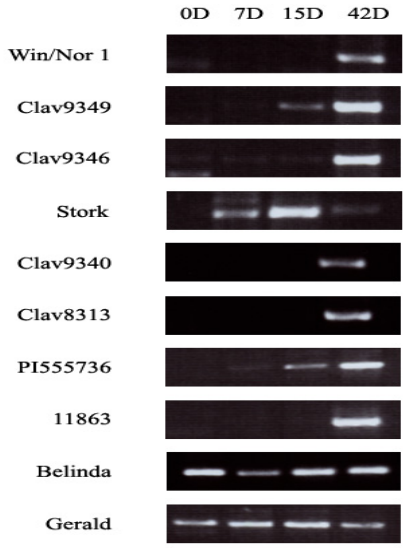

Figure 2. RT-PCR experiment on the expression of the *AsVRN1* gene on selected oat lines. Name of the lines are indicated at left. Exposure time (days) at +4° indicated at top of the figure.

This study clearly shows that *AsVRN1* can be used as a marker of freezing tolerance. Another interesting observation is that the English winter oat variety Gerald has the same expression pattern for *AsVRN1* as the Swedish spring oat Belinda. This indicates that Gerald is just a more hardy spring oat.

Regulatory networks in *Arabidopsis* during cold stress

One key factor in understanding cold acclimation in plants is to elucidate gene regulatory networks in the cold acclimation process. During the recent years Arabidopsis microarray data from cold acclimating plants have become available in the public domain. Such data has been used to deduce regulatory relations between different genes and to define transcription factors at different levels in the gene hierarchy from the initial signal to the final cellular response, but still there were many missing links in the cold regulatory models. Therefore we in Paper II initiated a more systematic investigation of possible LT stress regulatory circuits in Arabidopsis. Our strategy was to take advantage of the vast amount of information and data that was present in Arabidopsis to identify new pathways that could be functionally studied in Arabidopsis and later picked up in oat. Thus, we developed a combined rule-based and statistical approach to infer genetic regulatory networks from microarray data by integrating the information from: 1) known promoter-binding-site (motifs) and corresponding transcription factors (TFs); 2) the time-order relationships between TFs and their target genes in terms of expression response; and 3) motif synergies identified by gene expression profile similarities. In our approach, genes containing known over-represented motifs are grouped into disjoint clusters fulfilling three constraints. First, all genes in each cluster had to contain the same known motif or combination of motifs in their promoters, for which there is a known binding TF. Second, the earliest recorded time-point of significant induction/repression of each gene in the cluster must occur at the same time as the first recorded time-point of significant induction/repression of the gene encoding the regulating TF, or at the immediately following time-point. Third, the expression profiles of the genes in the cluster must show a higher correlation than the expression profiles of randomly selected genes. Applying these three constraints leads to formation of a grouping of the genes, based on which a regulatory network is derived by linking each known TF to the group(s) of co-expressed genes that it regulates.

Motifs or *cis*-elements are the regulatory regions found within the promoter of any gene, to which transcription factors specifically binds and thereby control the gene expression. Thus, in order to identify the motifs to be used for the first constraint, we searched the upstream sequences (positions -1000 to -1 bp) for the presence of known motifs using the PatMatch tool (Yan et al., 2005). Usually the mere presence of a motif in the upstream region of a gene is not sufficient proof of its role in regulation, thus our method only consider a motif if its frequency of occurrence in the promoter is greater than the frequency by which it is expected to occur by chance. The second constraint was implemented by identifying whether the first time-point of significant induction/repression of each target gene occurred at or after the first time-point of significant induction of the TF-encoding gene. Any genes not fulfilling this constraint were excluded from the further analysis. For the third constraint, we used a method where the average similarity of the expression profiles of a group of potentially co-regulated genes is compared with that of an equal number of randomly selected genes (Pilpel et al., 2001). Genes were only considered to be co-regulated, if and only if, their degree of similarity was significantly higher than that of the randomly selected genes.

Thus, by combining available microarray data on cold induced plants in Arabidopsis with our method we could develop high-resolution interaction map. More specifically, by

integrating information from motif synergies, gene expression profile similarities, known binding-site motifs for transcription factors (TF), the time-order relationships between TFs and their target genes in terms of expression initiation combined with a functional annotation, networks could be established. This confirmed many of the earlier known cold-acclimation pathways and also predicted novel putative regulatory interactions (Fig X).

Low temperature stress in rice

In Paper III we used two fully sequenced model plants, rice and *Arabidopsis,* and with the aim to expose molecular differences between chilling sensitive and freezing tolerant plants during low temperate stress at +4°C. Thus, we performed a comparative microarray analysis. From our data we identified 1,438 LT responsive rice genes and using the same selection criteria we identified 1,967 LT responsive *Arabidopsis* genes from public microarray data. All genes were functionally classified using the MapMan ontology scheme and divided into two groups, 'rapid' and 'slow' response genes. A gene was defined as a rapid response gene if it was induced/repressed during the initial 2 hours of LT treatment, whereas genes that were induced/repressed later than 2 hours of LT treatment were defined as slow responsive. When comparing the two data sets several interesting observations could be made.

In LT stressed *Arabidopsis* a larger number of genes were induced/repressed and the response was more dynamic than in rice, i.e. a larger portion of the *Arabidopsis* genes responded transiently (22.9% in *Arabidopsis* versus 7.9% in rice). There were a significantly larger number of rapid response genes in rice than in *Arabidopsis* (181 versus 285). Since rice is chilling sensitive and +4°C is an extreme temperature for this plant, one interpretation is therefore that rice is incapable of handling the "excess stress" and responds by "hitting the panic button" i.e. not only LT genes were induced/repressed, but also a large number of genes not directly involved in temperature stress.

In both data sets, more the 20% of the LT responsive genes were related to gene regulation and signalling and many different TF families were identified. However, again there were clear differences in the expression dynamics of the TFs. The famous CBF/DREB family can exemplify this. We identified six LT responsive *OsDREB1* genes and when compared to *AtCBF1-3* it was clear that the *Arabidopsis* gene was strongly induced and transiently regulated, whereas the expression of all the *OsDREB1* genes was much less pronounced and there was no clear transient trend. Since the transient expression pattern of the *Arabidopsis* TFs was true for a majority of the LT responsive genes, while most of the rice TFs were either constantly induced or repressed, it indicates that the regulatory system for handling LT stress is not working in an optimal way. The repressors that are responsible for the negative feedback are either not activated or not present at all.

Both type-A and B ARR genes are induced by cytokinin (To et al., 2004; Muller and Sheen, 2007), they work as antagonists, since type-A genes act as a negative feedback regulator and repress the cytokinin signal, whereas type-B genes work as a positive regulators. Almost all *Arabidopsis ARR* genes found in this study were transiently induced, but the rice *ARR* genes were permanently down-regulated. In addition, *CRF* and *cytokinin oxidase* genes were induced in *Arabidopsis* but not in rice. CRF are TFs belonging to the AP2/EREBP family, which are known to be positive regulators responding to cytokinin signalling. This data indicates that in *Arabidopsis* the cytokinin pathway is repressed,

which will delay growth and development and thereby protect the plant. In rice a similar repression is not in place. An effective delay of growth through the repression of the cytokinin signal is one important mechanism for LT tolerance.

Finally, *Arabidopsis* induced a significantly larger number of genes encoding proteins responsible for the actual long-term defence against LT than rice. Among these we observed *AtCOR15A-B* and *AtRD29A-B,* which are known targets of CBF and are strongly induced (more than 100-folds) in *Arabidopsis*. Surprisingly, although these proteins are crucial for LT tolerance in *Arabidopsis*, there are no homologous sequences to *AtCOR15A-B* and *AtRD29A-B* in any of the freezing tolerant crops like wheat, barley or rye. This implies that these proteins are not essential for LT tolerance in monocotyledons. On the other hand, another strongly induced CBF target gene, *At4g30650* encoding a protein of unknown function, homologous sequences were identified in both rice and wheat. However, the promoter of the homologous rice gene did not posses any CRT/DRE element, which indicates that this pathway is not active in rice.

Development of a TILLING population in oat

Although oat is the sixth most important cereal in the world, relatively few genetic resources are presently available for this crop. Cultivated oats are hexaploid with a very large genome (approximately 13,000 Mbp), and an efficient transformation system is lacking. Therefore systematic generation of lines mutated by transposons, T-DNA or RNA interference techniques is technically difficult and has so far nor been attempted. On the other hand, the use of chemical mutagens such as EMS (Ethyl-Methan-Sulfonate) and MNU (Methyl-NitrosoUrea) to induce single nucleotide changes by alkylation of specific nucleotides should be feasible. TILLING (Targeting Local Lesions IN Genomes) results in a population with point mutations more or less randomly distributed in the genome. If the mutation frequency is high enough and the population size is big enough all genes will be mutated in such a population.

In Paper IV, we used EMS to develop a TILLING population in hexaploid oat (*c v* Belinda) consisting of 2,600 individual mutant lines. We also cloned genes involved in the lignin (*AsPAL1*) and ß-glucan (*AsClsF6*) biosynthesis pathways from non-mutated Belinda. By random sequencing of these two genes we estimated the mutation frequency in the population to be approximately 1 per 26,000 bp. This means that each gene is mutated ca 250 times looking at the entire population and assuming an average gene size of 2 kb.

We observed higher mutation frequency in our oat TILLING population than what was previously found in hexaploid wheat (Slade et al., 2005) with about one mutation per 24,000 bp. It is much higher than barley (Caldwell et al., 2004), maize (Till et al., 2004), rice (Till et al., 2007) and dicots (Perry et al., 2003; Till et al., 2003; Triques et al., 2007). The molecular mechanisms for these differences are not yet known but presumably bigger polyploid genomes can absorb more mutations. One example of this is the mutation of the *GPC* (Grain Protein Content) gene in rice and wheat. A null mutation of *GCP-B1* in the B genome of hexaploid wheat caused a few days' difference in maturity, whereas in diploid rice RNAi (RNA interference) of the rice *GPC* gene results in seed sterility (Dubcovsky and Dvorak, 2007).

It is known from hexaploid wheat that the three genomes contribute differently to the expression of certain alleles and can even vary in different tissues of the plant (Nomura et al., 2005). Such a redundant genomic organization is a potential complication when identifying specific phenotypes from the TILLING-population. The presence of a specific allele on all three genomes would mean that even if one is mutated, other alleles could compensate for the lost function. In such a case similar alleles on all three genomes would have to be knocked-out to archive null expression of the product. On the other hand, already in the M2 segregating TILLING-population we could see several phenotypes obviously emanating from mutations in single alleles (Figure 2 Paper IV). This means that for some alleles the hexaploid may not lead to practical complications, but for others it could.

With the help of a MALDI-TOF based technique, we proved the feasibility of identifying mutations in individual genes by screening for mutations in the *AsPAL1* and the *AsClsF6* genes. We identified 4 mutations in *AsPAL1* and *9* mutations in *AsClsF6,* several of which mediate amino acid changes**.**

Finally, oats have many unique agronomical characteristics that cannot be studied in *Arabidopsis*. Wheat and soybean TILLING population exists but it is not always possible to implement observations from those plant species to oat. Thus our oat TILLING population will be a very useful breeding tool in the future.

Concluding remarks

Today the low temperatures negatively affect crop yields in many areas. A better understanding of the molecular differences between low temperature susceptible and tolerant crops will give the breeders new tools that opens the door for the development of higher yielding crops. It could also increase the geographical distribution of staple crops like rice and soybean.

During the course of this work we have analyzed oat EST data and microarray data from both rice and *Arabidopsis* using a comparative genomic approach, with the aim to increase our knowledge about the underlying mechanisms of cold acclimation. We have also opened for new possibilities with functional genomics in oats through the development of an oat TILLING population.

More specifically, based on this work, the following conclusions can be made:

- Oat is low temperature responsive and it contains genes that are homolog to well known genes related to the process of cold acclimation process in other plants.
- The oat genome contains a number CBF/DREB factors that are cold responsive and differently regulated during low temperature stress.
- In silico analysis of regulatory networks in *Arabidopsis* during low temperature stress suggests that specific combinations of transcription factors may be important for the low temperature response.
- The chilling sensitive Nepalese variety Jumaa Marchii is cold responsive, but essential genes for the cold acclimation process is either missing or not responding to low temperature stress.
- The low temperature response in freezing tolerant *Arabidopsis* is stronger and seems to be more dynamic than in rice. Many of the key regulatory transcription factors also seem to be transiently expressed.
- We have successfully developed a TILLLING population in oats and thereby contributed with a new tool for breeds and scientists studying oats.

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