Physiological and Molecular Analyses of Cold Acclimation of Plants

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To my father

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their Roman numerals:

I Pihakaski-Maunsbach K, **Puhakainen T** (1995) Effect of cold exposure on cortical microtubules of rye (*Secale cereale*) as observed by immunocytochemistry. Physiol Plant 93: 563-571

II **Puhakainen T,** Pihakaski-Maunsbach K, Widell S, Sommarin M (1999) Cold acclimation enhances the activity of plasma membrane Ca^{2+} ATPase in winter rye leaves. Plant Physiol Biochem 37: 231-239

III **Puhakainen T,** Hess MW, Mäkelä P, Svensson J, Heino P, Palva ET (2004) Overexpression of multiple dehydrin genes enhances tolerance to freezing stress in Arabidopsis. Plant Mol Biol (in press)

IV **Puhakainen T**, Li C, Boije-Malm M, Kangasjärvi J, Heino P, Palva ET (2004) Short day photoperiod potentiates low temperature-induced expression of a CBF-controlled gene during cold acclimation in silver birch (*Betula pendula* Roth.). Plant Physiol (in press)

ABBREVIATIONS

ABA	abscisic acid
ABP	actin binding protein
ABRE	ABA responsive elements
CaM	calmodulin
CBL	calcineurin-like protein
CBF	CRT/DRE binding factor
CDPK	calcium dependent protein kinase
CIPK	CBL-interacting protein kinase
COR	cold responsive
CRT	C-repeat
CRT	calreticulin
DHN	dehydrin
DNA	deoxyribonucleic acid
DRE	dehydration responsive element
DREB	DRE binding protein
ER	endoplasmic reticulum
FT	freezing tolerance
GTP	guanosine triphosphate
IEM	immunoelectron microscopy
kD	kilodalton
LD	long daylength
LEA	late embryogenesis abundant
LT	low temperature
LT_{50}	lethal temperature for 50% of the tissues
LTRE	low-temperature-responsive element
MAP	microtubule associated protein
MAPK	mitogen-activated protein kinase
PC	phosphatidylcholine
PLD	phospholipase D
PM	plasma membrane
ROS	reactive oxygen species
SAMK	stress-activated protein kinase
SD	short day length

SUMMARY

Low temperature (LT) is one of the most important factors limiting the growth, development and distribution of plants**.** Many plant species are able to increase their freezing tolerance (FT) in response to low, non-freezing temperature. This process, referred to as cold acclimation, results in various physiological and biochemical changes mainly derived from alterations in the expression of a number of cold-responsive genes. The activation of these cold-responsive genes is controlled by a set of signalling pathways triggered by exposure to the LT stimulus. The objective of this study was to gain a better understanding of the development of FT in plants.

The plant cytoskeleton has a central role in cold signalling and acclimation. Cytoskeletal reorganization serves as a link between membrane rigidification and Ca^{2+} influx in the early stages of cold acclimation, and is needed for the development of maximum FT.

Low temperature exposure also leads to a transient increase in $[Ca^{2+}]_c$, a signal that is recognized and transduced further by specific calcium binding proteins. Low-temperature-induced changes in $[Ca^{2+}]_c$ correlate with the expression of cold-responsive genes and the development of FT. Ca^{2+} homeostasis in cells is controlled by active Ca^{2+} transporters that restore $[Ca^{2+}]_c$ to resting levels after stimuli.

The effect of cold acclimation on the stability of cortical microtubules and on the activities of Ca^{2+} ATPase and H^+ ATPase in winter rye (*Secale cereale*) was studied. The results demonstrate that cold acclimation enhances the stability of cortical microtubules against freezing stress in leaves and against dehydration stress in roots of winter rye. Cold acclimation also leads to a significant increase in the activity of plasma membrane Ca^{2+} ATPase and to a slight increase in the activity of plasma membrane H^+ ATPase in winter rye leaves. Increased stability of cortical microtubules, presumably needed for growth under suboptimal temperatures, may have a role in stabilizing the plasma membrane during freezing stress. The enhanced activity of plasma membrane Ca^{2+} ATPase may reflect the increased capacity required to sustain resting levels of Ca^{2+} during cold acclimation.

Dehydrins (DHNs) are proteins which accumulate in vegetative tissues during stresses that cause cellular dehydration such as drought, salinity and cold. Accumulation of DHNs is frequently linked to the development of FT both in herbaceous and woody plants. Despite efforts to elucidate the contribution of DHNs to increased stress tolerance, their exact function remains unknown. The results demonstrate that overproduction of DHNs in Arabidopsis leads to lower ion leakage and better survival after freezing stress than in control plants. An immunoelectron microscopy study revealed partial intracellular translocation from the cytosol to the vicinity of the membranes of the acidic DHN LTI29 during cold acclimation in transgenic plants. These findings provide evidence that DHNs contribute to freezing stress tolerance in plants and suggest that this might partly be due to their protective effect on membranes.

In woody plants, the cold acclimation process is initiated by short day length (SD). Subsequent LT and freezing temperatures are needed for the development of full FT. Leaves of silver birch (*Betula pendula*) are able to recognize and respond to both SD and LT by increasing their FT. To study the molecular events during cold acclimation in birch, a *DHN* gene, *Bplti36*, encoding a 36 kD, acidic SK_2-type DHN, was cloned. This gene was responsive to LT, drought, salt and exogenous abscisic acid, and, this responsiveness was retained when *Bplti36* was introduced to Arabidopsis. Furthermore, the LT induction of *Bplti36* appears to be under the control of the CBF pathway because of its constitutive expression in a CBF-overproducing Arabidopsis line. Exposure to a SD photoperiod led to only a slight increase in *Bplti36* expression, whereas pre-exposure to SD followed by LT treatment resulted in a significant increase in *Bplti36* transcripts compared with LT-treated plants grown at long day

length. These results demonstrate that LT activation of cold-responsive genes in woody plants employs similar mechanisms as in herbaceous plants, including components of the CBF pathway. In addition, a SD stimulus appears to sensitize birch to subsequent LT exposure, as seen in both the increased FT and the markedly enhanced expression of *Bplti36* in the leaves of silver birch exposed to SD followed by LT.

1. INTRODUCTION

1.1. Cold acclimation of plants

Low temperature (LT) is one of the most important abiotic factors limiting growth, productivity and distribution of plants (Boyer, 1982; Sakai and Larcher, 1987). LT decreases biosynthetic activity of plants, inhibits the normal function of physiological processes and may cause permanent injuries, which finally lead to death. Different plant species vary widely in their ability to tolerate LT. Chilling-sensitive tropical species can be irreparably damaged even at non-freezing temperatures. Injuries are caused by overall impairment of metabolic and cellular processes and alterations in membrane properties. Chilling-tolerant but freezing-sensitive plants are able to survive temperatures slightly below zero but are severely damaged upon ice formation in the tissues. Freezing-tolerant plants are able to survive variable levels of freezing temperatures, the actual degree of tolerance being dependent on the species, developmental stage and duration of stress.

Plants from temperate and boreal regions commonly encounter freezing temperatures seasonally as well as during their active growth season. The exposure of plants to subzero temperatures leads to freezing of the tissue water. Due to the higher freezing point and presence of more active ice nucleators in the apoplastic solution compared with the cytoplasm, this freezing invariably occurs extracellularly. Ice formation outside the cells reduces the water potential of the apoplastic solution, which leads to withdrawal of water from the cells and subsequent cellular dehydration. Therefore, freezing stress on a cellular level is always accompanied by dehydration stress, and consequently, tolerance to freezing is correlated with tolerance to dehydration. Plants encountering freezing temperatures have two general strategies to survive freezing stress; either avoidance of or tolerance to freezing (Sakai and Larcher, 1987). Avoidance of freezing is mainly achieved by supercooling of tissue water. However, this mechanism has limited value since it mainly occurs in special organs such as seeds, overwintering buds or ray parenchymal cells (Sakai and Larcher, 1987). Tolerance to freezing is therefore the dominant mechanism by which plants survive freezing stress.

Several plant species have the ability to increase their degree of freezing tolerance (FT) in response to low, non-freezing temperatures, a phenomenon known as cold acclimation (Levitt, 1980; Sakai and Larcher, 1987; Thomashow, 1999). Development of FT can also be induced by osmotic stresses, including dehydration (Siminovitch and Cloutier, 1982; Lee and Chen, 1993; Mäntylä et al., 1995; Li et al., 2002) and high salinity (Ryu et al., 1995), and by treatment of cells or plants with the phytohormone abscisic acid (ABA) (Chen and Gusta, 1983; Lee and Chen, 1993; Li et al., 2003). The level of FT obtained through cold acclimation is not static but can vary seasonally and is rapidly lost upon return to a warm non-acclimating temperature. Cold acclimation is a dynamic, photosynthetic activity-demanding process (Griffith and McIntyre, 1993; Wanner and Junttila, 1999). Central to successful cold acclimation is the ability to adjust the photosynthetic apparatus to function at a low

temperature, especially in moderate to high light conditions, which otherwise expose the plant to photoinhibition and can lead to formation of reactive oxygen species (ROS) (Foyer et al.,1994; Wanner and Junttila, 1999).

The ability to cold-acclimate is a polygenic trait involving a large number of genes, whose expression is controlled mainly by low temperature. Alterations in the expression levels of these genes lead to the numerous molecular and physiological changes characteristic of the cold acclimation process (Figure 1), and the combined effect of the corresponding gene products is manifested in the level of FT obtained. The complexity of the acclimation process is reflected in the amount of genes that are affected by low temperature, which according to a recent estimate is up to 25% of the transcriptome in Arabidopsis (Krebs et al., 2002).

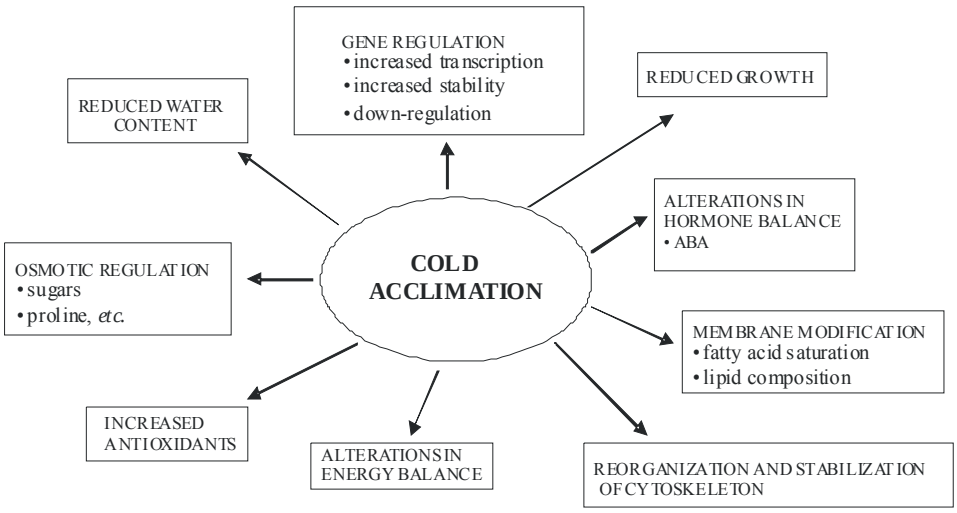

Figure 1. Cold-acclimation -induced cellular changes in plants (adapted from Xin and Browse, 2000).

In this work, three freezing-tolerant species have been employed as material; *Arabidopsis thaliana* ecotype Landbergis erecta, winter rye (*Secale cereale*) and birch (*Betula pendula*). In Arabidopsis, low temperature alone is sufficient to trigger the cold acclimation process, and light and cold requirements for enhanced FT are separable (Gilmour et al., 1988; Kurkela et al., 1988; Wanner and Junttila, 1999). The role of light in the cold acclimation process of Arabidopsis is mainly in photosynthetic carbon fixation, which is necessary for the accumulation of sucrose and other compatible solutes (Wanner and Junttila, 1999). The cold acclimation process in Arabidopsis is rapid; enhanced FT can be observed after only 12 hours of cold acclimation (Gilmour et al., 1988). A maximum FT of around –10ºC is achieved when plants are exposed for one week to 4ºC. Deacclimation is also rapid; within one day after plants are returned to normal growth temperature, they will lose the attained FT and may be killed by subsequent freezing to –7ºC (Wanner and Junttila, 1999). FT in Arabidopsis is not the same throughout the plant (Wanner and Junttila, 1999). The youngest leaves in the centre of the rosette develop FT more rapidly than older leaves, and the cotyledons are unable to increase FT at all.

In winter cereals, ultimate FT is dependent on a highly integrated system of structural, regulatory and developmental genes. The development of maximum low-temperature tolerance, up to –30ºC, is known to be associated with two developmentally controlled adaptive features; vernalization and photoperiodic requirement (Fowler et al., 1996a, 1996b,

2001; Mahfoozi et al., 2000; Danyluk et al., 2003). The short photoperiod prevents the developmental switch from vegetative to reproductive phase, thereby maintaining a higher level of expression of cold-responsive genes (Fowler et al., 1996a, 1996b, 2001; Mahfoozi et al., 2000). As a result, full expression of cold-hardiness-related genes only occurs in the vegetative phase, and plants in the reproductive phase have only a limited ability to cold-acclimate. More specifically, in winter cereals, growth at a low temperature is a prerequisite for cold-hardiness; leaves fully expanded at 20ºC will eventually die during winter, and thus, ultimate FT and survival are achieved only in leaves which have developed under low temperatures (Huner et al., 1989).

In woody plants, the cold acclimation process consists of two stages. The first stage is triggered by a short photoperiod and the second by low temperature (Weiser, 1970; Sakai and Larcher, 1987; Li et al., 2002). Photoperiod has a central role as the primary signal to induce growth cessation and dormancy development and initiate cold acclimation. Subsequent low temperature exposure is the main factor required for increased FT. FT development in deciduous trees is a rather slow process, but ultimately results in a very high tolerance (up to –196Cº in buds and stem) (Sakai and Larcher, 1987; Rinne et al., 1998). Like buds and stem, leaves are also able to respond to short days and low temperatures by increasing their FT (Li et al., 2002). The similar responses of buds and leaves to low temperatures suggest that birch leaves could provide a rapid and convenient system for studies on molecular mechanisms of cold acclimation. As in Arabidopsis and winter rye, the development of FT in birch is not uniform; buds and leaves develop FT more rapidly than the stem, and young leaves have a higher FT than old leaves (Li et al., 2002).

1.2. Freezing injuries of the plasma membrane

There is a general consensus that the plasma membrane is the primary site of freezing injury (for reviews, see Palta, 1989, 1990; Steponkus, 1984, 1990). The morphological symptom of freezing-induced injury is a water-soaked appearance of the plant tissue. The physiological consequence – loss of compartmentalization – leads to leakage of ions and organic solutes, and to the inability to regain turgor during recovery (Levitt, 1980; Palta, 1989). Membrane damages are mainly due to the dehydration that occurs during the freeze-thaw cycle. Elegant experiments have shown that freezing-induced destabilization of the plasma membrane involves different types of lesions (Steponkus, 1984; Steponkus et al., 1990; Webb et al., 1994; Uemura et al., 1995). In protoplasts from non-acclimated rye leaves, reduction in cell volume at temperatures close to –5ºC is accompanied by loss of plasma membrane surface area due to invagination of the plasma membrane, followed by budding of endocytotic vesicles. Upon rewarming, the melted water is drawn back into the cells. Consequently, rehydration results in an intolerable pressure, and the cells burst. This type of behaviour is known as expansion–induced lysis. The cryobehaviour of cold-acclimated cells is different. Cold-acclimated cells also dehydrate and shrink, but instead of budding of the endocytotic vesicles, the plasma membrane forms endocytotic extrusions which remain in association with the plasma membrane and are reincorporated during rehydration. Thus, the cells are able to swell to their original size without lysis.

At lower temperatures when dehydration becomes more severe, different cellular membranes are brought into close apposition. In non-acclimated tissues, membrane lipids undergo lateral phase separations and form lamellar-to-hexagonal-II-phase transitions in regions where the plasma membrane is brought into close apposition with subtending endomembranes, which leads to destabilization of the plasma membrane and ion leakage. In cold-acclimated cells, lamellar-to-hexagonal-II-phase transitions are prevented. Instead, in

cold-acclimated cells exposed to –20ºC, freezing injuries are associated with a phenomenon referred to as fracture jump lesions between the plasma membrane and closely appressed cytoplasmic membranes, most frequently with those of the outer membrane of chloroplasts.

Alterations in the cryobehaviour of the plasma membrane have been shown to be due to changes in the plasma membrane lipid composition during cold acclimation (Steponkus et al., 1990, 1993; Uemura et al., 1995). The accumulation of sucrose, other simple sugars and osmolytes that typically occurs with cold acclimation also seems to contribute to the stabilization of membranes (Thomashow, 1999). In addition, there is emerging evidence that certain novel hydrophilic and late embryogenesis abundant (LEA) polypeptides also participate in the stabilization of membranes against freeze-induced injury (Thomashow 1999). Solutes and polypeptides have been proposed to stabilize membranes either by direct interaction with membrane surfaces or, indirectly, by their strong interaction with water (Crowe et al., 1992; Close, 1996). Only a few experimental approaches have been applied to improve the tolerance of plasma membranes against freezing stress. The lipid composition of rye plasma membranes has been experimentally manipulated by increasing mono- and di-unsaturated species of phosphatidylcholine (PC) (Steponkus et al., 1988), and Arabidopsis has been transformed to constitutively express chloroplast protein COR15a, which is thought to interact with membranes (Artus et al., 1996). Both approaches resulted in a 1-2ºC improvement in FT.

The active transport system of the plasma membrane has been shown to be affected both in the early stages of freezing injury (review by Palta 1989, 1990) and in cold acclimation (Hellergren et al., 1983; Ishikawa and Yoshida, 1985; Iswari and Palta, 1989; Mattheis and Ketchie, 1990; Sutinen et al., 2004). The plasma membrane-associated proton-pumping ATPase (PM H^+ ATPase) is suggested to be an early site of incipient freezing injury (Palta and Li, 1980). This suggestion is supported by observations that freeze-thaw stress selectively impairs the function of PM H^+ ATPase but has no effect on marker enzymes located in other membranes (Hellergren et al., 1987; Iswari and Palta, 1989). Furthermore, recovery from freezing injury is dependent on the activity of PM H^+ATPase (Arora and Palta, 1991). The activity of PM H^+ATPase increases during cold acclimation in both herbaceous (Ishikawa and Yoshida, 1985; Iswari and Palta, 1989) and woody plants (Hellergren et al., 1983; Mattheis and Ketchie, 1990; Sutinen et al., 2004), which further indicates the involvement of PM H^+ATPase in the response to low-temperature stress. The mechanism by which cold acclimation affects PM H^+ATPase activity is unknown, but changes in the lipid content of membranes may play an important role (Carruthers and Melchior, 1986; Palmgren et al., 1988; Palta, 1989). Recently, phosphorylation-dependent binding of the 14-3-3 protein to H^+ATPase has been shown to increase its activity (Camoni et al., 2000).

1.3. Signal transduction in cold acclimation of plants

1.3.1. *Perception of low temperature signal*

The response of plants to any environmental signal is mediated by a series of reactions, collectively referred to as signal transduction (Figure 2). Low temperature signal transduction starts with the perception of the cold signal by a yet unidentified receptor located presumably at the plasma membrane. A putative sensor protein has been proposed to detect physical phase transitions in microdomains of the plasma membrane as a result of temperature shifts (Murata and Los, 1997). Ding and Pickard (1993) have shown that the tension-dependent activity of a mechanosensitive Ca^{2+} channel in onion increases when temperature is lowered. The reason for this enhancement is not known, but changes in membrane properties or properties of the

channel itself were proposed. Heino and Palva (2003) have discussed the possibility that receptor-like protein kinases act as cold sensors. These proteins could be activated by a temperature-induced conformational change in their extracellular domains, which would then induce the kinase activity on the cytoplasmic side of the receptors. Genes encoding receptor-like kinases have been demonstrated to be up-regulated in response to low temperature in Arabidopsis (Hong et al., 1997; Kreps et al., 2002), but no information about their involvement in temperature sensing has been obtained.

In prokaryotes, two component systems are central in sensing environmental signals (Mikami et al., 2002), and in blue-green algae *Synechocystis* sp.PCC6803, a histidine kinase, Hik33, has been identified as a putative low temperature sensor (Suzuki et al., 2000, see below). In higher plants, two component systems have been shown to act as ethylene and cytokinin receptors, and also the involvement of histidine kinase in osmosensing processes has been suggested (Urao et al., 2000). However, no evidence exists for direct involvement of two component regulators in temperature sensing in higher plants.

In *Synechocystis*, Hik33 autophosphorylation is induced by membrane rigidification caused by low temperature, which leads to activation of a subset of low-temperature-responsive genes, including genes for fatty acid desaturases (Suzuki et al., 2000, 2001). Increased expression of fatty acid desaturases is an adaptive response of the cyanobacterium that modulates the degree of lipid desaturation, and thus, membrane fluidity at low temperature. In *Synechocystis*, the disruption of genes encoding fatty acid desaturases rigidifies membrane lipids and enhances the expression of cold-induced genes (Inaba et al., 2003). In addition, transcription of *desA* (fatty acid desaturase gene) has been shown to be induced at 34ºC by increasing membrane rigidity through the Pd-catalysed hydrogenation of fatty acids in the *Synechocystis* plasma membrane (Vigh et al., 1993).

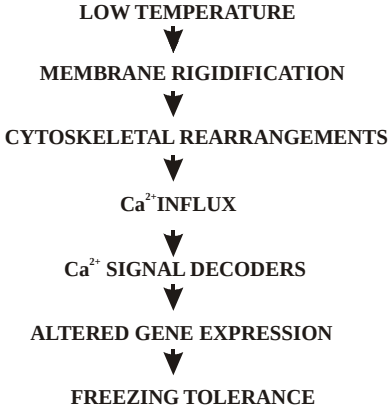

Figure 2. A model showing the initial events in cold signalling.

Membrane fluidity or viscosity is directly and reversibly affected by changes in temperature also in higher plants; an increase in temperature renders the membranes more fluid, whereas a decrease in temperature rigidifies them. A series of sophisticated studies recently demonstrated that membrane rigidification, coupled with cytoskeletal rearrangements, triggers low temperature responses in alfalfa (*Medigaco sativa*) and *Brassica napus* (Örvar et al., 2000; Sangwan et al., 2001, 2002). By using pharmacological approaches, they showed that both rigidification of the membranes and destabilization of the actin microfilaments and microtubules lead to activation of the cold acclimation response without

any low temperature treatment. Conversely, fluiditization of the membranes or stabilization of the microfilaments or microtubules prevented cold acclimation during low temperature treatment (Örvar et al., 2000, Sangwan et al., 2001, 2002). Furthermore, they showed that membrane rigidification and cytoskeletal reorganization is followed by an influx of calcium ions (Sangwan et al., 2001), which is known to be required for the acclimation process (Monroy and Dhindsa, 1995).

1.3.2. *Calcium as a secondary messenger*

Calcium is frequently involved as a secondary messenger in plant responses to external signals (Trewavas and Malhó, 1997). Various biotic and abiotic stimuli, such as cold, touch, light, pathogenic elicitors and plant hormones, cause a transient increase, known as a Ca^{2+}spike, in cytosolic calcium concentration (stimuli have been catalogued in Sanders et al., 1999; Knight and Knight, 2000; Scrase-Field and Knight, 2003). The information of different stimuli is then encoded by changing a Ca^{2+}spike´s magnitude, duration, location or frequency, and hence, each stimulus can elicit a characteristic Ca^{2+} signature that is recognized by different calcium sensors (Sanders et al., 1999, 2002). Calcium sensors then transduce calcium signatures into downstream effects, including altered protein phosphorylation, cytoskeletal rearrangements and modified gene expression patterns (Sanders et al., 1999, 2002; Rudd and Frankling-Tong, 2001).

1.3.2.1. *$[Ca^{2+}]_c$ kinetics in cold*

One of the earliest events in a plant´s response to low temperature is a transient elevation of the free cytosolic concentration of Ca^{2+}. Such elevations have been demonstrated in Arabidopsis (Knight et al., 1996; Polisensky and Braam, 1996; Lewis et al., 1997), as well as in other species, including tobacco (Knight et al., 1991). This elevation in cytosolic free Ca^{2+} levels $[Ca^{2+}]_c$ is due mainly to an influx of Ca^{2+} from external sources (Monroy and Dhindsa, 1995; Knight et al., 1996), but there is also evidence for inositol (1,4,5,)-triphosphate (IP_3-)- and cyclic ADP-ribose (cADPR-)-mediated Ca^{2+} release from the vacuole (Knight et al., 1996; De Nisi and Zocchi, 1996; Wu et al., 1997; Sangwan et al., 2001; Xiong et al., 2001). Influx of Ca^{2+} into the cytosol from outside the cell and release of Ca^{2+} from intracellular stores are energetically downhill processes that occur spontaneously when Ca^{2+} channels are open.

$[Ca^{2+}]_c$ increases as a response both to rapid cold shock (Knight et al., 1991, 1996) and to slow gradual reduction in temperature (Plieth et al., 1999; Nordin-Henriksson and Trewavas, 2003). Cold exposure results in a biphasic response of $[Ca^{2+}]_c$ (Knight et al., 1996; Kiegle et al., 2000), the first peak always being higher than the second (Plieth et al., 1999; Knight and Knight, 2000). However, the slower the cooling, the more pronounced the second peak. Vacuolar release of calcium has been shown to be responsible for the increase in the second peak of $[Ca^{2+}]_c$ during slow cooling, i.e. during cold acclimation (Knight and Knight, 2000). The magnitude of response is dependent on the cooling rate and the final temperature to which cooling occurs, the relationship between cooling rate and magnitude of calcium elevation being approximately linear (Knight, 2002). When plants are challenged successively with identical cooling regimes, the calcium response becomes attenuated; i.e. at a certain point, the plant is no longer sensitive to the cold in terms of generating a calcium signal (Plieth et al., 1999). This desensitization can be overcome simply by further reducing the temperature (Plieth et al., 1999). Therefore, although the rate of cooling is the most important parameter, the absolute temperature does have the ability to sensitize the system.

1.3.2.2. *Calcium in low temperature signal transduction*

Low-temperature-induced changes in $[Ca^{2+}]_c$ have been correlated with the expression of cold-responsive genes and the development of FT. In alfalfa, treatment of cells with Ca^{2+} chelators or Ca^{2+} channel blockers prevented calcium influx as well as expression of the low-temperature-responsive *cas15* gene and development of FT (Monroy et al., 1993; Monroy and Dhindsa, 1995; Sangvan et al., 2001). When Ca^{2+} influx was artificially increased by ionophores or Ca^{2+} channel agonists, cold-acclimation-specific genes were induced at 25ºC and FT increased in alfalfa cells and *Brassica napus* leaves (Monroy and Dhindsa, 1995; Sangvan et al., 2001). In comparable studies, Ca^{2+} channel blockers and Ca^{2+} chelators also inhibited the low temperature activation of *kin* genes in Arabidopsis (Knight et al., 1996; Tähtiharju et al., 1997; Nordin-Hendriksson and Trewavas, 2003). However, in Arabidopsis, these treatments caused only a partial inhibition of cold-induced Ca^{2+} influx and low-temperature-responsive gene expression, suggesting that an intracellular Ca^{2+} source might also be involved. In addition, *chs3*, a chilling-sensitive mutant of Arabidopsis has been reported to be impaired in cold-triggered calcium response and to show lower expression levels of cold-induced *LTI78* and *KIN1*genes (Knight, 2002). The lower levels of *LTI78* and *KIN1* were found, however, to reflect the low level of transcripts encoded by their cognate transcription factors (CBFs), the expression of which is Ca^{2+}-dependent (Knight, 2002). These findings have been verified by artificial gene construct showing that the level of expression of genes harbouring CRT element is induced by intracellular Ca^{2+} increase or is inhibited by Ca^{2+} chelators. With this construct it was possible to demonstrate that the expression of the CBF genes requires calcium. These experiments indicate that calcium regulates a whole battery of genes required for FT.

In conclusion, the cold signal appears to initially cause rigidification of the membranes, which results in reorganization of the cytoskeleton. This then leads to opening of the Ca^{2+} channels and subsequent Ca^{2+} influx. The increased $[Ca^{2+}]_c$ is then used as a signal for cold acclimation response.

1.3.2.3. *Regulation of calcium homeostasis*

The efficacy of calcium as a signalling molecule is dependent on tightly regulated transport and storage. After stimuli, the free $[Ca^{2+}]_c$ is restored rapidly to resting levels and maintained at a low level by active Ca^{2+} transporters (Sze et al., 2000). Two types of active transporters drive Ca^{2+} out of the cytosol against a steep electrochemical gradient at the plasma membrane (PM) and the endomembranes: (a) Ca^{2+} pumps/Ca^{2+}ATPases directly energized by ATP hydrolysis and (b) H^+-coupled Ca^{2+} antiporters (H^+/Ca^{2+} antiporters) driven by a proton electrochemical gradient. These transporters differ significantly in their kinetic properties. The H^+/Ca^{2+} antiporter is a low-affinity (K_{mCa}= 10-15 μM), high-capacity transporter, whereas Ca^{2+} pumps generally have a high affinity for Ca^{2+} (K_{mCa}= 0.1-2 μM) but a low capacity (Sze et al., 2000). These differences suggest that H^+/Ca^{2+} antiporters are particularly important for removing cytosolic Ca^{2+} when concentrations are high, while Ca^{2+}ATPases are responsible for fine tuning of calcium concentration.

1.3.2.3.1. *Calcium ATPases*

Ca^{2+} ATPases have been characterized from several plants, including Arabidopsis and cauliflower (Askerlund and Sommarin, 1996; Geisler et al., 2000; Sze et al., 2000). Ca^{2+} ATPases belong to a functional superfamily of P-type ATPases (see Møller et al., 1996), that form a phospho-aspartate (hence P) enzyme intermediate during the reaction cycle. Ca^{2+}

ATPases belong to two phylogenetic types: (a) type IIA Ca^{2+} ATPases (ECA for ER-type Ca^{2+} ATPase), which are similar to animal Ca^{2+} ATPases of the sarcoplasmic reticulum or the endoplasmic reticulum (ER), and (b) type IIB Ca^{2+} ATPases (ACA for autoinhibited Ca^{2+} ATPase), which are similar to animal calmodulin (CaM)-stimulated Ca^{2+} ATPases found in the PM (Askerlund and Sommarin, 1996; Geisler et al., 2000). Specific characteristics of type IIB include (1) CaM stimulation, (2) nucleotide unspecificity (in addition to ATP, ITP or GTP may function as a substrate) and (3) extreme sensitivity to fluorescein and its derivatives. Fluorescein is known to bind at or near the nucleotide-binding domain (Askerlund and Sommarin, 1996; Geisler et al., 2000; Sze et al., 2000). Type IIA characteristics are, in addition insensitivity to CaM, sensitivity to cyclopiazonic acid (CPA) and preference for ATP rather than GTP (Askerlund and Sommarin, 1996; Sze et al., 2000). In plant cells, type IIA and type IIB Ca^{2+} ATPases occur in both endomembranes and the plasma membrane and may co-exist in the same membrane system (Askerlund and Sommarin, 1996; Geisler et al., 2000; Sanders et al., 2002).

1.3.2.3.2. *Regulation of calcium ATPases*

Plant type IIB Ca^{2+} ATPases have an extended N-terminus, which contains a CaM binding domain (Malmström et al., 1997). At low cytosolic [Ca^{2+}], type IIB Ca^{2+}ATPases are kept in a state of low basal activity by an intramolecular interaction between an autoinhibitory domain and the active site of the pump. When [Ca^{2+}] levels increase, the pump is activated as a result of Ca^{2+}-induced binding of CaM to a site overlapping or immediately adjacent to the autoinhibitory sequence. All biochemical and molecular studies of plant type IIB Ca^{2+} ATPases characterized thus fur support this model (Sze et al., 2000). In addition to regulation by CaM, reversible phosphorylation seems to be a mechanism of post-translational regulation of Ca^{2+} ATPases. The activity of ACA2 (Arabidopsis Ca^{2+} ATPase, isoform 2 protein) (Hong et al., 1999) can be stimulated by Ca^{2+}/ CaM or inhibited by the phosphorylating activity of Ca^{2+}-dependent protein kinase (CDPK isoform CPK1) (Hwang et al., 2000). This complexity of the regulation of Ca^{2+} signal attenuation by feedback from two different types of Ca^{2+} sensors provides a mechanism to control Ca^{2+} efflux through opposing inhibitory and stimulatory activities (Hwang et al., 2000, Luan et al., 2002). Factors that shift this balance may alter the rate of Ca^{2+} efflux, thereby altering the magnitude or duration of a Ca^{2+} signal (Hwang et al., 2000). Furthermore, the low homology between regulatory regions of different type IIB pumps might lead to differential regulation by distinct modifications by protein kinases and phosphatases and protein-protein interactions depending on the cellular context, thus increasing the complexity of regulation (Sze et al., 2000).

The regulation of plant IIA pumps is still poorly known (Sanders et al., 2002). AtECA pumps have been suggested to be modulated by an unidentified regulatory protein(s) (Sze et al., 2000). The following two facts support this view: 1) AtECA1 is a high-affinity Ca^{2+} pump and 2) its activity is blocked by a synthetic peptide corresponding to the autoinhibitory domain of AtACA2 (type IIB pump) (Hwang et al., 2000).

1.3.2.3.3. *H^+/Ca^{2+} antiporters*

H^+/Ca^{2+} antiporter activity is found most commonly in the vacuolar membranes, although there is evidence that activity is also present in the plasma membrane (Kasai and Muto, 1990). The first plant H^+/Ca^{2+} antiporter cloned and so far best characterized, CAX1 (calcium exchanger 1), was identified by screening a cDNA library from Arabidopsis for clones able to complement a yeast mutant defective in vacuolar Ca^{2+} transport (Hirschi et al., 1996; Hirschi, 2001). CAX1 has high Ca^{2+} transport capacity and low Ca^{2+} affinity (Shigaki et al., 2001) and

seems to be located in the vacuolar membrane (Cheng et al., 2003). The activity of CAX1 is regulated at the post-translational level by an autoinhibitory N-terminal region (Pittman et al., 2002). The N-terminal part of CAX1 has also been shown to be responsible for specificity for calcium (Shigaki et al., 2001). Ectopic expression of CAX1 in tobacco leads to increased sensitivity to chilling temperatures, suggesting that CAX1 can play a role in plant acclimation to cold (Hirschi, 1999). Interestingly, the expression of CAX1 is highly induced by calcium (Hirschi, 1999) and also in response to low temperature (Catala et al., 2003). The characterization of T-DNA insertion mutants *cax1-3* and *cax1-4,* which display reduced tonoplast Ca^{2+}/H^{+} antiporter activity, demonstrated that mutants were not affected in their constitutive capacity to tolerate freezing temperature, dehydration, chilling or high levels of salt (Catala et al., 2003). However, they exhibited enhanced FT after cold acclimation, indicating that CAX1 negatively controls the cold acclimation response. Indeed, increased ability to cold-acclimate in *cax1-3* and *cax1-4* correlated with enhanced expression of genes encoding cold-responsive transcription factors CBF/DREB, as well as their downstream target genes in response to low temperature (Catala et al., 2003). These results suggest that CAX1 ensures the accurate development of the cold acclimation response in Arabidopsis by controlling the induction of *CBF/DREB* and the corresponding target genes by regulating Ca^{2+} homeostasis in response to low temperature. Studies conducted with a *cax1* mutant revealed an interplay among vacuolar transporters, a decrease in CAX1 activity leading to an increase in vacuolar Ca^{2+} ATPase activity and a reduction in V ATPase activity, suggesting that CAX1 is involved in modulating different plant responses (Cheng et al., 2003).

In Arabidopsis 11 genes are predicted to encode antiporters closely related to CAX1 (CAX2-CAX12 and MHX) (Mäser et al., 2001), but thus far only CAX2 (Hirschi et al., 1996) has been shown to have a low capacity to transport Ca^{2+}. However, indirect evidence, i.e. increased expression of CAX3 and CAX4 in *cax1*, suggests that CAX3 and CAX4 also have a role in Ca^{2+} homeostasis (Cheng et al., 2003).

1.3.3. *Calcium signal decoders in cold acclimation*

A cold-induced Ca^{2+} signal or signature is recognized by specific calcium binding proteins, which usually contain the ´EF´ hand motif(s), a helix-loop-helix structure that binds a single Ca^{2+} ion (Snedden and Fromm, 2001). To date, three major classes of EF hand Ca^{2+} sensors have been characterized in plants, based on the number and organization of EF hands and on the similarity of the amino acid sequences: calmodulins/calmodulin-like proteins, calcineurin B-like proteins (CBL) and calcium-dependent protein kinases (CDPKs) (Rudd and Franklin-Tong, 2001; Snedden and Fromm, 2001; Luan et al., 2002; Sanders et al., 2002). A fourth class of Ca^{2+} binding proteins that have been suggested to play a role in Ca^{2+} signalling because of their high Ca^{2+} binding capacity is calreticulins (CRTs) (Persson et al., 2001, 2003, and references therein).

1.3.3.1. *Calmodulin (CaM)*

Calmodulin (CaM) is one of the most conserved Ca^{2+} binding proteins in eukaryotes. CaM has no catalytic activity on its own, but upon binding Ca^{2+}, it activates numerous target proteins involved in a variety of cellular processes (Snedden and Fromm, 1998, 2001). One of the intriguing properties of CaM is its ability to activate target proteins that share very little amino acid sequence similarity in their CaM binding sites (Snedden and Fromm, 1998, 2001). In addition to the evolutionarily conserved form of CaM, plants possess an extended family of CaM isoforms and CaM-like proteins (Snedden and Fromm, 1998). In Arabidopsis and tobacco cells, environmental stimuli, including low temperature, trigger rapid transcription of

genes encoding CaM and CaM-like proteins (Braam and Davis, 1990; Braam, 1992; van der Luit et al., 1999). This low–temperature-responsive expression of CaM genes is partially regulated by Ca^{2+} (Polisensky and Braam, 1996). Studies with alfalfa cells (Monroy et al., 1993) and Arabidopsis (Tähtiharju et al., 1997) have indicated that CaM antagonist prevents cold acclimation and reduces expression of cold-regulated genes, supporting a role for CaM in low temperature signalling. On the other hand, overexpression of CaM in Arabidopsis has been shown to cause reduction in cold-responsive gene expression (Townley and Knight, 2002), implying that CaM might have a role as a negative regulator during cold acclimation. In sum, CaM appears to have both positive and negative effects on cold acclimation, probably depending on the balance of Ca^{2+}, CaM and CaM target proteins.

1.3.3.2. *Calcineurin-like proteins (CBLs) /SOS3-like calcium binding proteins (SCaBP Ca^{2+} sensors) and CBL interactin protein kinases (CIPKs)/ SOS2-like protein kinase (PKS protein kinase)*

Calcineurin-like (CBL) proteins (also called SOS3-like Ca^{2+} binding proteins, ScaBLs) are a new family of Ca^{2+} sensors, which have been identified recently from Arabidopsis (AtCBLs/SCaBPs) (Liu and Zhu, 1998; Kudla et al., 1999; Luan et al., 2002; Gong et al., 2004; Kolukisaoglu et al., 2004). These proteins are similar to the regulatory B subunit of Ca^{2+}/CaM dependent phosphatase calcineurin in animals and yeast. AtCBLs/SCaBPs are Ca^{2+} binding proteins, and like CaMs, they do not have enzymatic activity by themselves. One member of the *AtCBL* gene family, *AtCBL1*, is strongly and transiently induced by cold and drought stresses, suggesting a role of this calcium sensor in the respective signalling cascades (Kudla et al.,1999). Analyses of loss of function mutants and *AtCBL1*-overexpressing transgenic Arabidopsis lines indicate a crucial function of this calcium sensor protein in abiotic stress responses (Albrecht et al., 2003). Mutation of *AtCBL1* affects the expression of cold-regulated genes and impairs responses to drought and salt stresses but does not affect abscisic acid (ABA) responsiveness (Albrecht et al., 2003). Overexpression of *AtCBL1* reduces transpirational water loss and induces the expression of CBF/DREB transcription factors and cognate target genes in non-stressed plants, and as a consequence, enhances stress tolerance of plants (Albrecht et al., 2003).

AtCBLs interact specifically with a group of serine-threonine protein kinases designated as CBL-interacting protein kinases (CIPKs) or SOS2-like protein kinase (PKS protein kinase) (Shi et al., 1999; Kim et al., 2000; Gong et al., 2004), which are encoded by a multigene family in Arabidopsis (Shi et al., 1999; Luan et al., 2002; Kolukisaoglu et al., 2004). The interaction between CBLs and CIPKs has been demonstrated to be Ca^{2+}-dependent (Shi et al., 1999; Gong et al., 2004). One member of this family, CIPK3, has recently been characterized (Kim et al., 2003) and been shown to be induced by stresses like cold, drought and salt as well as by ABA application, implicating *CIPK3* in stress and ABA responses. The involvement of *CIPK3* is further supported by the finding that disruption of this gene alters the expression pattern of stress genes (cold, salt, ABA) (Kim et al., 2003). Interestingly, *CIPK3* does not regulate the gene expression induced by drought stress, nor does it have an effect on transcription of CBF3. Consequently, CIPK3 has been suggested to act downstream of the Ca^{2+} signal but upstream of the transcription factors regulating low-temperature and ABA-responsive promoters (Kim et al., 2003). CIPK3 appears to define a component involved in cross-talk between cold and ABA signalling during acclimation (Kim et al., 2003).

1.3.3.3. *Calcium-dependent protein kinases (CDPKs)*

Typical targets representing primary downstream transducers of calcium signals are phosphorylation cascades consisting of tightly regulated protein kinases and phosphatases. It is well established that protein phosphorylation/dephosphorylation is involved in signal transduction during cold acclimation, and the requirement of reversible phosphorylation of pre-existing proteins for cold acclimatization has been demonstrated in alfalfa, Arabidopsis and *Brassica napus* (Monroy et al., 1993, 1997, 1998; Tähtiharju et al., 1997; Sangwan et al., 2001). Furthermore, protein kinases and phosphatases have been reported to differentially regulate cold-induced gene expression (Monroy et al., 1997, 1998; Sangwan et al., 2001).

Calcium-dependent protein kinases (CDPKs) are implicated as important sensors in response to abiotic stresses, including cold (Harmon et al., 2000; Cheng et al., 2002; Ludvig et al., 2004). Monroy et al. (1993) originally demonstrated that in alfalfa cell suspension cultures changes in the phosphorylation pattern of pre-existing proteins take place during cold acclimation. They also showed that CDPK activity was needed for the full acclimation response. Transient transactivation assays of stress-responsive reporter gene constructs in maize (*Zea mays*) protoplasts transformed with genes encoding both wild-type and a mutated form of CDPKs provided the first evidence of the involvement of a particular CDPK in specific signal/response pathways (Sheen, 1996). Exposure to cold temperatures has been correlated with changes in expression of CDPK genes in various plant species. In alfalfa, expression of two CDPKs are differentially regulated by low temperature (Monroy and Dhindsa, 1995). In rice (*Oryza sativa*), the gene encoding CDPK7 is induced by cold and salt stresses (Saijo et al., 1998). The enzymatic activity of CDPKs also increases in response to cold. For example, cold treatments enhance activity of a membrane-bound rice CDPK (Martin and Busconi, 2001). Recently, overexpression of a cold and salt stress-inducible CDPK-encoding gene, OsCDPK7, has been shown to enhance low temperature tolerance of chilling-sensitive rice plants (Saijo et al., 2000). Taken together, these studies indicate that CDPKs could have a central role in mediating Ca^{2+} signals during acquisition of cold or chilling tolerance.

1.3.3.4. *Protein phosphatases*

The phosphorylation level of some proteins are affected by cold through a differential inhibition of protein kinases and phosphatases, which exhibit differential sensitivity to cold (Monroy et al., 1997). The Arabidopsis protein phosphatase 2C, *AtPP2CA*, is cold-inducible, reaching a maximum level by 12 hours and remaining high thereafter (Tähtiharju and Palva, 2001). Arabidopsis transgenic plants expressing *AtPP2CA* in antisense orientation showed that regulation of cold-responsive genes (*RAB18*, *RCI2A/LTI6*, *RD29A/LTI78*) was cold stress-dependent similar to the wild type, but they were superinduced during cold stress in *AtPP2CA* antisense plants and conferred better FT. Cold-responsive gene expression and cold acclimation were also accelerated in *AtPP2CA* antisense plants (Tähtiharju and Palva, 2001). Thus, by shifting the equilibrium between phosphorylation and dephosphorylation, low temperature may direct its signal transduction cascade through cold-specific protein phosphorylation, leading to low-temperature-responsive gene expression and development of FT (Monroy et al., 1997).

1.3.3.5. *Mitogen-activated protein kinases (MAPKs)*

MAPKs (mitogen-activated protein kinases) are serine/threonine protein kinases that play key roles in integrating multiple intracellular signals transmitted by various secondary

messengers. A MAPK cascade consists of three protein kinases. Inactive MAPKKKs are activated by a stress signal messenger; upon activation, they activate MAPKKs by phosphorylation at conserved serine/threonine. Activated MAPKKs activate MAPKs by phosphorylating MAPK at both threonine and tyrosine residues in the TXY motif. MAPKs appear to be ubiquitously involved in signal tranduction during eukaryotic responses to extracellular stimuli (Mizoguchi et al., 1997). In plants, many MAPK family members have been cloned and proposed to be involved in environmental stress responses, including cold (Mizoguchi et al., 1997). MAPK kinase activity has been shown to be enhanced by cold in alfalfa (Jonak et al., 1996; Sangwan et al., 2002) and Arabidopsis (Ichimura et al., 2000). Activation of alfalfa MAPK, SAMK (stress-activated protein kinase), by cold, by chemically modulated membrane fluidity or by cytoskeleton destabilizers is inhibited by blocking the influx of extracellular calcium and as well by an antagonist of CDPKs (Sangwan et al., 2002). Thus, it is evident that MAPKs are also decoders of the Ca^{2+} signal in cold, although the molecular targets for these kinases remain unknown.

1.3.3.6. *Calreticulins (CRTs)*

Calreticulins (CRTs) are ER-located Ca^{2+} binding proteins which have been suggested to be involved in Ca^{2+} signalling because of their high Ca^{2+} binding capacity. CRTs are very well characterized from the mammalian system and have been proposed to also be involved in chaperone activity, cell adhesion, gene expression, apoptosis and store-operated Ca^{2+} fluxes through the plasma membrane (Persson et al., 2001, and references therein). Reverse genetics approaches have been applied to clarify the function of CRTs in plants (Persson et al., 2001; Wyatt et al., 2002). Results of these studies suggest that CRT plays a key role in the regulation of Ca^{2+} status of the plant ER, that the ER, in addition to the vacuole, is an important Ca^{2+} store in plant cells (Persson et al., 2001) and that plants have access to these stores under calcium stress conditions (Wyatt et al., 2002). CRTs have been shown to possess both tissue-dependent expression patterns and stress-related regulation (Persson et al., 2003). However, the involvement of CRTs in cold responses of plants have not been elucidated to date.

1.3.4. Cytoskeleton in cold signalling and freezing tolerance

1.3.4.1. *Cytoskeletal components*

The cytoskeleton is a highly dynamic structure composed of microtubules polymerized from α- and β-tubulin subunits and microfilaments polymerized from G-actin. Tubulin and actin monomers interact with a continually changing array of monomer-binding nucleoside triphosphates or diphosphates; guanosine triphosphate (GTP)/guanosine diphosphate (GDP) for tubulin and adenosine triphosphate (ATP)/adenosine diphosphate (ADP) for actin. These cytoskeletal elements interact with Ca^{2+} (Solomon, 1977), actin binding proteins (ABP) and microtubule-associated proteins (MAPs) (reviewed by Staiger, 2000; Wasteneys and Galway, 2003). ABPs and MAPs interact with regulatory kinases, phosphatases, Ca^{2+} and other ions (Wasteney and Galway, 2003). The cytoskeleton is involved in many basic cellular processes such as cell division, cytoplasmic streaming, organelle positioning, cellular transport and signal transduction.

Based on sequence homology with well-characterized animal, fungal and protist sequences, the Arabidopsis genome contains 17 to 19 tubulin sequences, about 220 potential MAP sequences, 16 actin and actin-related sequences and approximately 150 putative ABP sequences (in addition, another 150 sequences showing weak homology to ABPs have been

detected). Altogether, about 2% of Arabidopsis genes encode cytoskeletal structural proteins or proteins linked directly to them (Meagher and Fechheimer, 2003).

For many cellular processes, the structure and function of microtubule and actin cytoskeletons are co-ordinated and their stability is interdependent (Wasteneys and Galway, 2003). The organization of microtubule arrays may involve interactions with actin (reviewed in Cyr and Palevitz, 1995), and vice versa, i.e. the organization of cortical actin arrays may be dependent on the localization and organization of the microtubules (Chu et al., 1993a). The cortical actin cytoskeleton is associated directly with both the PM and the cortical microtubules (Collings et al., 1998). A number of studies have demonstrated that cortical microtubules are linked to the PM by cross-bridges (Akashi and Shibaoka, 1991; Shibaoka, 1994; Sonobe and Takahashi, 1994). Furthermore, this linkage can extend to the cell wall (Akashi et al., 1990). The mechanism of binding of the cytoskeleton, or more specifically, of microtubules to the PM, appears to be very complex.While the proteins involved are still largely unknown in higher plants, tubulin itself may be involved (Laporte et al., 1993; Sonesson et al., 1997). The connection is possibly through a hydrophobic domain on the tubulin molecule or indirectly through interaction with an integral membrane protein (Sonesson et al., 1997). The best-characterized candidate so far is phospholipase D (PLD), which is able to bind to both microtubules and the PM (Marc et al., 1996; Gardiner et al., 2001) and is therefore suggested to act as a structural and signalling link between the plasma membrane and the cytoskeleton in tobacco and in Arabidopsis (Gardiner et al., 2003). Cytoskeletal reorganization and PLD activation are involved in many stress responses, including cold acclimation (Ruelland et al., 2002; Welti et al., 2002). The activation of PLD has been suggested to trigger cytoskeletal reorganization by releasing the cortical array of microtubules from the plasma membrane (Dhonukshe et al., 2003). The overproduction of PLDδ in Arabidopsis has been shown to lead to enhanced FT after cold acclimation (Li et al., 2004).

1.3.4.2. *Cytoskeleton in cold signalling*

The close relationship of the plant cytoskeleton with the plasma membrane, the major platform for signal perception and transduction (Gilroy and Trewavas, 2001; Wasteney and Galway, 2003), suggests that microtubules and microfilaments are downstream targets of various signalling pathways. Signalling cascades have been shown to involve changes in cytoskeletal organization. In animal cells, microtubules have been proposed to transmit signals from the receptor to the nucleus since they span the distance from the plasma membrane to the nucleus (Gundersen and Cook, 1999). Inside the cells, the cytoskeleton provides ample surface for components which transduce extracellular signals, and thus, the state of the cytoskeleton in plant cells can be critical in activating and recruiting signal molecules to a site where they interact (Eun and Lee, 1997; Gundersen and Cook, 1999).

Recent experiments indicate that membrane transport may also be regulated by the cytoskeleton in higher plants. The activities of different ion channels in plant cells have been shown to be affected by the general organization of the cytoskeleton (Thion et al., 1996, 1998; Thuleau et al., 1998; Zimmerman et al., 1999). The role of microtubules in opening the Ca^{2+} channel was suggested by Thion et al. (1996), and when cold-shocked tobacco (*Nicotiana plumbagnifolia*) was treated with oryzalin and cytochalasin, destabilizers of microtubules and microfilaments, respectively, a synergistic increase in Ca^{2+} influx was observed (Mazars et al., 1997). In contrast, compounds that interfere with the polymerization status of actin filaments had no effect on the intensity and stability of Ca^{2+} currents in carrot cell protoplasts (Thion et al., 1996). Consequently, cytoskeletal regulation of Ca^{2+} channels was concluded to be mainly due to microtubule organization and not to microfilaments.

1.3.4.3. *Cytoskeleton in freezing tolerance*

Microtubules have been of special interest in low temperature research due to their cold lability; low temperature can cause microtubules to depolymerize into their protein subunits either directly or through the complex function of $Ca^{2+}$and CaM (Cyr, 1991; Fisher and Cyr, 1993; Fisher et al., 1996). In chilling-sensitive species, depolymerization of microtubules has been associated with chilling injuries (Rikin et al., 1980, 1983). Furthermore, chilling sensitivity is closely correlated with the critical temperature that can induce microtubule disassembly (Jian et al., 1989). Microtubule cold stability has also been shown to be related to the cold-hardiness of plants (Jian et al., 1989; Abdrakhamanova et al., 2003). The role of microtubules in FT seems to be different from their role in chilling. Although it is evident that the stability of microtubules provides support for plant cells to endure freezing stress and sustain growth in low temperatures, the development of maximum cold resistance seems to require microtubule flexibility. The prevention of microtubule disassembly by taxol impairs cold acclimation in rye roots (Kerr and Carter, 1990a) and decreases FT in both non-acclimated and cold-acclimated mesophyll cells of spinach (*Spinacia oleracea* L.) (Bartolo and Carter, 1991). In wheat (*Triticum aestivum* L.) roots, the oryzalin-sensitive, i.e. flexible, microtubule cytoskeleton in differentiating vascular tissue may predict the ability to develop efficient cold resistance (Olinevich et al., 2002). Most freezing-resistant cultivars of wheat show transient and partial disassembly of microtubules during the early phase of cold acclimation, whereas the sensitive cultivar lacks this disassembly (Abdrakhamanova et al., 2003). In freezing-sensitive cultivars, artificially induced disassembly of microtubules led to enhanced FT which further supports the idea that transient disassembly of microtubules is involved in the cold acclimation process (Abdrakhamanova et al., 2003).

The molecular basis of microtubule cold resistance seems to involve the differential induction of tubulin isotypes during cold acclimation (Kerr and Carter, 1990b; Chu et al., 1993b; Abdrakhamanova et al., 2003) and phosphorylation/dephosphorylation of either the tubulin itself or MAPs (Mizuno, 1992). The interphase cortical array of BY-2 cells becomes resistant to cold-induced depolymerization following treatment with the protein kinase inhibitors 6-dimethylaminopurine (6-DMAP) and staurosporine (Mizuno, 1992).

Relatively little is known about the effects of cold on the actin cytoskeleton or the role of actin in the cold tolerance of plants. Actin itself appears to be quite resistant to cold (Quader et al., 1989; Åström et al., 1991; Chu et al., 1993a), and the effects of cold may be indirect, perhaps due to partial co-ordination and physical interaction with the microtubule cytoskeleton (Quader et al., 1989; Chu et al., 1993a; Collings et al., 1998). Recently, an actin-depolymerizing factor, ADF, has been characterized in wheat and its role in cold acclimation studied (Quellet et al., 2001). The accumulation of ADF was more pronounced in freezing-tolerant wheat cultivars than in less tolerant ones. Therefore, cytoskeletal, e.g. actin, rearrangements were proposed to occur at low temperature, and this remodelling of the actin cytoskeleton was suggested to be important for the enhancement of FT (Quellet et al., 2001). However, immunocytological studies are still needed to verify that the actin cytoskeleton undergoes major restructuring at low temperatures.

The cytoskeleton appears to play a central role in cold signalling and cold acclimation. At the early phase of cold acclimation, flexibility and re-organization of the microtubule cytoskeleton is needed, while completion of the process requires the formation of cold-stable microtubules, which ensure growth even when the temperature is not optimal.

1.4. Regulation of gene expression in response to low temperature

Cold acclimation is accompanied by altered gene expression; hundreds of genes are either up- or down-regulated (Seki et al., 2001, 2002a; Fowler and Thomashow, 2002; Xiong et al., 2002b). Many of the cold-induced genes are also up-regulated by drought, high salt concentration or ABA, suggesting that a common set of signal transduction pathways are triggered during many stress responses (Thomashow, 1999; Nuotio et al., 2001; Seki et al., 2001, 2002a, 2002b; Krebs et al., 2002; Shinozaki et al., 2003). Promoter analysis of the cold-regulated (*COR*) genes has shown that they contain sequence elements that mediate the stress induction of the genes. The best characterized of these is the dehydration-responsive element (DRE), also known as a C-repeat (CRT) or a low-temperature-responsive element (LTRE). Moreover, some of the *COR* genes contain ABA-responsive elements (ABREs), that mediate the ABA responsiveness of these genes (Seki et al., 2002b). The expression of *COR* genes is regulated by both ABA-independent and ABA-dependent pathways (Ishitani et al., 1997; Shinozaki and Yamaguchi-Shinozaki, 2000).

Analysis of the expression profiles of cold-inducible genes during low temperature treatment reveals the existence of at least two groups that have different temporal patterns of expression (Fowler and Thomashow, 2002; Seki et al., 2002a). In the first group, the expression is rapid and transient in response to low temperature, and in the second ,the expression increases gradually during cold treatment (Fowler and Thomashow, 2002; Seki et al., 2002). The analysis of the expression profiles also indicates that multiple regulatory pathways are activated during cold acclimation and that cold-induced genes can be members of more than one cold regulon (Fowler and Thomashow, 2002).

1.4.1. *CBF cold response pathway*

A family of transcription factors known as C-repeat binding factors (CBFs) (Stockinger et al., 1997; Gilmour et al., 1998) or dehydration-responsive element binding factors (DREB1s) (Liu et al., 1998; and Shinwari et al., 1998) that control ABA-independent expression of *COR* genes in response to cold stress has been identified in Arabidopsis. These transcription factors belong to the ethylene-responsive element binding protein/APETALA2 (EREBP/AP2) family (Stockinger et al., 1997; Liu et al., 1998) and bind to the cold- and dehydration-responsive DNA regulatory elements (DREs) (Yamaguchi-Shinozaki and Shinozaki, 1994), also termed C-repeats (CRTs) (Baker et al., 1994). CRT/DRE elements contain the conserved CCGAC core sequence, which is sufficient to induce transcription under cold stress (Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994). The *CBF/DREB1* genes are transiently induced by cold and their expression precedes that of the cold-inducible genes with the CRT/DRE *cis*-element (Gilmour et al., 1998; Liu et al., 1998; Medina et al., 1999). Three cold-inducible *CBF/DREB1* genes, *CBF1/DREB1B*, *CBF2/DREB1C* and *CBF3/DREB1A,* have been identified in Arabidopsis (Thomashow et al., 2001). Ectopic expression of *CBFs/DREB1s* in transgenic plants has been shown to activate downstream cold-responsive genes even at warm temperatures and to confer improved freezing, drought and salt tolerance (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Kasuga et al., 1999; Hsieh et al., 2002). Arabidopsis CBF/DREB1 orthologues have been identified in other plant species, including *Brassica napus*, wheat, rye, tomato (Jaglo et al., 2001) and birch (Ojala et al., in preparation), suggesting that the CBF transcriptional cascade is highly conserved in the plant kingdom during cold stress.

The expression of *CBF/DREB1* genes is regulated by low temperature (Shinwari et al., 1998). The cold induction of *CBF* genes is related to temperature changes such that the lower the temperature the higher the *CBF* transcription (Zarka et al., 2003). At a given low temperature, the expression of *CBF* genes becomes desensitized and resensitization requires

exposure to a higher temperature (Zarka et al., 2003). Recently, a differential temporal pattern in the expression of *CBF* genes has been uncovered in response to a low temperature, the expression of *CBF1/DREB1B* and *CBF3/DREB1A* precedes that of *CBF2/DREB1C* (Novillo et al., 2004). The expression of the *CBF* genes is apparently repressed by either their own gene products or the products of their downstream target genes, ensuring transient and tightly controlled expression of these genes (Guo et al., 2002; Chinnusamy et al., 2003). In the mutant *los1-1*, the expression of the CBF/DREB1 target genes is highly reduced, but the transcription of *CBF* genes themselves are superinduced in response to low temperature (Guo et al., 2002). *LOS1* encodes a translation elongation factor 2-like protein, and *in vivo* labelling studies indicate that protein synthesis in *los1* mutant plants is disrupted at low temperatures. As a consequence, cold-induced *CBF* transcripts are not translated to activate downstream genes, and in their absence, feedback repression can not occur, which leads to accumulation of *CBF* transcripts (Guo et al., 2002). Recently, Novillo et al. (2004) have shown that the expression of *CBF1/DREB1B* and *CBF3/DREB1A* is negatively regulated by *CBF2/DREB1C*.

1.4.1.1. *Regulation of the CBF pathway*

The promoter regions of *CBFs* have no evident DRE/CRT elements, and thus, these genes do not appear to be subject to autoregulation (Gilmour et al., 1998). Some factors and components controlling the cold-induced expression of *CBFs* have recently been characterized by mutational screens (Ishitani et al., 1997) (Figure 3.). Since *CBF* transcripts start to accumulate within 15 minutes of plants´ exposure to cold, it was proposed (Gilmour et al., 1998) that a transcription factor(s) already present in the cell at normal growth temperature recognizes the *CBF* promoters and induces *CBF* expression upon activation by cold stress. This/these hypothetical factor(s) was named the ICE (índucer of CBF expression) protein, and ICE1 has now been isolated and characterized (Chinnusamy et al., 2003). *ICE1* encodes a MYC-like basic helix-loop-helix (bHLH) transcriptional activator, which has been shown to bind to the MYC recognition sequences in the *CBF3* promoter. A dominant-negative mutation of *ICE1*, *ice1*, results in almost complete elimination of *CBF3* transcript accumulation in response to low temperatures. However, *ice1* had little effect on cold-induced accumulation of *CBF2* transcripts, indicating that differences exist in the mechanisms of activation within the *CBF/DREB1* family (Chinnusamy et al., 2003). *ICE1* is expressed constitutively, and its overexpression in wild-type plants enhances the expression of the *CBF* regulon in cold and improves FT of transgenic plants (Chinnusamy et al., 2003). *ICE1* is most probably activated by phosphorylation (Zhu J-K, unpublished result).

Another gene that has a positive role in *CBF* expression is *LOS4,* which encodes DEAD-box RNA helicase, indicating that it functions in regulation of RNA metabolism. Expression of *CBFs* and their downstream target genes as well as cold acclimation are impaired in *los4-1* mutant plants (Gong et al., 2002). The effect of the mutation can be counteracted by ectopic expression of *CBF3* (Gong et al., 2002). Interestingly, *los4-1* plants are highly sensitive to chilling when exposed to cold in darkness. This could be specifically due to impaired expression of *CBF2* in *los4-1* plants since *CBF2* alone was expressed when wild-type Arabidopsis plants were exposed to cold during darkness (Gong et al., 2002). The *CBF2* orthologue of birch shows a higher expression when exposed to cold in darkness than in light (Puhakainen, unpublished results), implicating that the expression of *CBF* genes is regulated also by light. Kim et al. (2002) have shown that light signalling mediated by phytochrome B is necessary for cold-induced gene expression through the DRE/CRT element. In addition, transient accumulation of the *CBF2* transcripts has been shown in response to far-red light, and this accumulation was found to be phytochrome A dependent (Tepperman et al., 2001).

Arabidopsis plants with *hos1* and *hos2* (*high expression of osmotically* responsive gene) mutations show enhanced expression of a set of cold-inducible genes under cold stress, indicating that *HOS1* (Lee et al., 1999, 2001) and *HOS2* (Lee et al., 1999) are negative regulators of cold signal transduction. The *hos2* and *hos1* mutations enhance the cold-inducible genes by a different mechanism (Lee et al., 1999). In *hos1* plants, the enhanced induction of genes by cold treatment correlates with increased capacity to cold-acclimate. In the *hos2* mutant plants, enhanced gene expression in the cold does not bring about an increase in FT. HOS1 is also a negative regulator of *CBF* expression since *hos1* mutation causes enhanced cold induction of both CBFs and their downstream target genes. HOS1 encodes a ring finger protein, which has been implicated as an E3 ubiquitin conjugating enzyme that targets CBF regulatory proteins, e.g. ICE1, for degradation (Lee et al., 2001). The level of *hos1* mRNA was shown to decline transiently in response to low temperature, being almost undetectable after 30 minutes of treatment and increasing to its basal level after one hour (Lee et al., 2001), suggesting that if HOS1 is a negative regulator of ICE1, then the low expression during the initial stages of cold treatment would allow ICE activation and subsequent transient induction of the CBF genes. However, the effect of *hos1* mutation is not restricted to a CBF regulon since it also has an effect on cold induction of genes that do not have a DRE/CRT element in their promoters (Ishitani et al., 1998).

Two additional negative regulators of the CBF pathway, FIERY1 (FRY1) and FIERY2 (FRY2), have been characterized (Xiong et al., 2001, 2002a). *Fry1* mutant shows enhanced levels of mRNAs corresponding to *CBF2* and stress-responsive genes in cold. However, these plants are impaired in cold acclimation. *FRY1* encodes an inositol polyphosphate 1-phosphatase which mediates the catabolism of IP_3 (inositol 1,4,5-triphosphate). Xiong et al. (2001) suggested that FRY1 has a role in the attenuation of stress responses by controlling the turnover of the second messenger IP_3. Recessive mutations in *FRY2* result also in impairment of cold acclimation despite superinduction of the *CBFs* and their target genes (Xiong et al., 2002a). This further indicates that either the downregulation of the *CBF* genes is essential for cold acclimation or that *fry1* and *fry2* mutations have pleiotropic effects on processes involved in development of FT. In addition to the signal transduction pathways regulating the CBF regulon, there are multiple parallel and converging pathways that lead to enhanced FT. To dissect the signalling pathways mediating the complex changes that result in increased FT, mutants with enhanced FT in the absence of cold acclimation (*eskimo*) (Xin and Browse, 1998) and mutants sensitive to freezing (*sfr*) (Warren et al., 1996) have been characterized. A mutant exhibiting constitutive FT, *eskimo1*, accumulated elevated levels of proline and soluble sugars but did not exhibit constitutively increased expression of CBF-regulated genes. These results suggest that the *esk1* mutant defines a cold acclimation signalling pathway that is distinct from the CBF pathway. However, plants overexpressing *CBF3* (*DREB1A*) contain increased levels of proline and soluble sugars and exhibit constitutive expression of the *COR* genes (Gilmour et al., 2000). Thus, CBF3/DREB1A may act in a pathway that leads to both the *COR* pathway and the *ESK1* pathway, as suggested by Browse and Xin (2001). *sfr* mutants are identified based on their specific failure to gain FT after cold acclimation treatment (Warren et al., 1996) but are no more sensitive to low, non-freezing temperatures than the corresponding wild type. Most of the *sfr* mutants show strong induction of cold-regulated genes, even though they are partially deficient in their ability to cold-acclimate. However, one of the *sfr* mutants, *sfr6*, shows a marked reduction in accumulation of cold-induced mRNA corresponding to genes containing the DRE/CRT element in their promoter regions and consequently a lower amount of their cognate proteins (Knight et al., 1999; Boyce et al., 2003). Notably, the cold-inducible expression of *CBFs* is unaffected, indicating that SFR6 affects either a component in the signalling pathway downstream of CBF transcription or a component in an independent

pathway that is simultaneously required for *COR* gene expression, as indicated by the *sfr6* mutation also downregulating cold-regulated genes that do not have a DRE/CRT element in their promoters (Knight et al., 1999; Boyce et al., 2003).

1.4.2. *Abscisic acid (ABA)-dependent cold signal pathway*

Several lines of evidence suggest that ABA is involved in cold acclimation of plants. ABA content has been shown to increase transiently in both herbaceous and woody plants exposed to low temperature (Chen et al., 1983; Lång et al., 1994; Li et al.,1997, 2002). Exogenous application of ABA at normal growth temperature leads to increased chilling and freezing tolerance (Chen and Gusta, 1983; Lång et al., 1989; Li et al., 1997, 2003) and *de novo* protein synthesis as well as induction of a subset of cold–responsive genes (Li et al., 1997; Seki et al., 2002b). In addition, both ABA-insensitive (*abi*) and ABA (*aba*)-deficient mutants have been shown to be impaired in cold acclimation (Heino et al., 1990; Gilmour and Thomashow, 1991; Mäntylä et al., 1995), and application of ABA can suppress the impaired cold acclimation phenotype in the *aba* mutant (Heino et al., 1990; Llorente et al., 2000). As stated above, several of the low-temperature-responsive genes are also induced by exogenous ABA, and consequently, the promoters of these genes have ABA response elements (ABREs) (Figure 3.). The ABREs, *cis* elements with a consensus sequence C/TACGTGGC, confer ABA responsiveness to many genes when more than one copy is present (Leung and Giraudalt, 1998). The class of bZIP transcription factors, ABRE binding proteins (AREBs or ABFs), can bind to ABRE and activate ABA-dependent gene expression (Choi et al., 2000; Uno et al., 2000). The genes encoding ABFs (ABF1-4) are themselves induced by ABA and show differential regulation by various environmental stresses; ABF1 is induced by cold, ABF2 and ABF3 by high salt concentration and ABF4 by cold, high salt concentration and drought (Choi et al., 2000). The differential regulation of ABFs suggests that separate ABFs are likely to function in these signal transduction cascades through common ABREs. Also cross-talk between ABA-dependent and ABA-independent regulatory systems has been suggested by genetic and molecular analyses (Ishitani et al., 1997; Krebs et al., 2002; Seki et al., 2002a, 2002b). Cross-talk occurs at the interaction between different *cis*-acting elements. Many cold-and drought-inducible genes contain both DRE/CRT and ABRE elements in their promoter. These *cis*-acting elements are thought to function independently. However, precise analysis of these elements in *RD29A* gene expression revealed that DRE/CRT functions co-operatively with ABRE as a coupling element in ABA-responsive gene expression in response to drought (Narusaka et al., 2003). In addition, *sfr6* mutant, which is deficient in the expression of cold-regulated genes that contain a DRE element, also showed lower expression levels of *KIN1* when exposed to ABA, suggesting that cross-talk occurs between the ABRE and DRE. Genetic evidence indicates that a stress signalling pathway for the activation of *LEA*-like genes that is completely independent of ABA may not exist (Xiong et al., 2002b). This suggestion is verified by a recent study demonstrating that *CBFs* are also induced by ABA (Knight et al., 2004).

Although the involvement of CBF/DREB1 elements in *COR* gene expression has been elucidated extensively (Jaglo-Ottosen et al., 1998; Liu et al., 1998), ABRE-mediated *COR* gene expression is not as well characterized. Recently, a C_2H_2-type zinc finger protein, SCOF1, that is specifically induced by cold and ABA but not by dehydration or high salt concentration has been cloned and characterized in soybean (Kim et al., 2001a, 2001b). Constitutive overexpression of *SCOF-1* induced cold-regulated gene expression harbouring either DRE/CRT or ABRE in the promoter sequence and enhanced cold tolerance of non-acclimated transgenic Arabidopsis and tobacco plants (Kim et al., 2001a). SCOF-1 was localized to the nucleus but did not bind directly to either DRE/CRT or ABRE elements (Kim

et al., 2001a). However, in *in vitro* studies, SCOF-1 greatly enhanced the DNA binding activity of SGBF-1, a soybean G-box binding bZIP transcription factor (Hong et al., 1995) to ABRE. SCOF-1 also interacted with SGBF-1 in a yeast two-hybrid system. In transactivation studies with protoplast, SGBF-1 was shown to induce ABRE-dependent gene expression, which is enhanced by SCOF-1 (Kim et al., 2001a). These results indicate that SCOF-1 is a positive regulator of *COR* gene expression mediated by ABRE via interaction with SGBF-1. Furthermore, the temporal pattern of *SCOF-1* expression, induced at three hours and reaching a maximum at 24 hours and thereafter sustained at a high level so long as plants are kept at a low temperature, and the constitutive expression of low-temperature-responsive genes in transgenic Arabidopsis overexpressing SCOF-1 suggest that SCOF-1 may act synergistically with CBF/DREB1 proteins and maintain the expression of CBF/DREB1 target genes after *CBF/DREB1* is decreased.

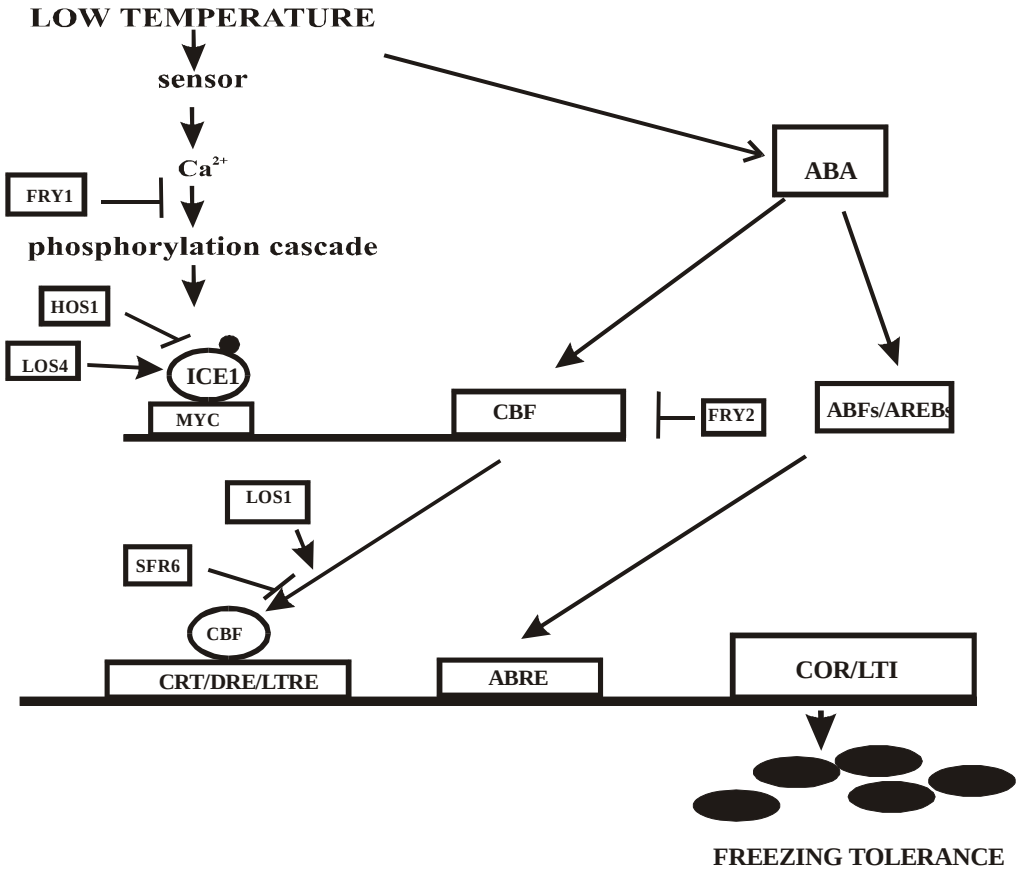

Figure 3. The low-temperature-sensing signalling pathway.

1.5. Dehydrins (DHNs)

Dehydrin proteins (late embryogenesis abundant (LEA) D11 family) are produced in plant cells in response to environmental stimuli with a dehydrative component, including low temperature, drought and salinity, and developmental stages such as seed and pollen maturation (reviewed by Campbell and Close, 1997; Close, 1996, 1997; Svensson et al., 2002). Since 1988, when dehydrin was first cloned (Mundy and Chua, 1988), over 100 *DHN* genes have been characterized from both angiosperms and gymnosperms (for review, see

Close 1996, 1997; Svensson et al., 2002). Today, it is evident that dehydrins exist in all photosynthetic organisms (Close, 1997). Characterized members of the dehydrin family have molecular weights ranging from 9 to 200 kD (Close, 1996), are thermostable, maintaining their integrity in aqueous solutions up to 100˚C, and are generally enriched with glysine and lysine residues.

DHNs are characterized by three highly conserved domains known as the K-, Y-, and S-segments. The K-segment (EKKGIMDKIKEKLPG), which is present in one or more copies, is the only conserved repeat found in all DHNs. This segment has been proposed to form an amphipathic α-helix (Dure, 1993), and 10 (IMDKIKEKLP) or 12 (GIMDKIKEKPLG) residues from the centre of the segment have been speculated to form a class A amphipathic α-helix (Close, 1996). Class A amphipathic helices have hydrophilic polar and hydrophobic non-polar faces (Segrest et al., 1990), suggesting that dehydrins might act as interfaces between hydrophobic membrane phospholipids and hydrophilic cytosol in plant cells (Campbell and Close, 1997). In the presence of SDS, α-helical structure has been induced in cowpea DHN1 and *Citrus unshui* DHN CuCor19 (Ismail et al., 1999a; Hara et al., 2001). Recently, binding of maize DHN1 to lipid vesicles was demonstrated to be associated with an increase in α-helicity of the protein (Koag et al., 2003). The Y-segment (DEYGNP) is usually found in 1-3 copies close to the N-terminal part of dehydrins. This sequence (V/T)DEYNGNP) shares significant homology with the nucleotide binding site of plant and bacterial chaperones, although binding of nucleotides to Y-segments has not been reported (Close, 1996, 1997). The S-segment consists of serine residues followed by three acidic residues, indicating phosphorylation by casein kinase II (CKII) (Close, 1997, and references therein), which has also been shown to take place (Plana et al., 1991; Godoy et al., 1994). The phosphorylation of S-segments participates in nuclear targeting of the DHN (Jensen et al., 1998).

Dehydrins are divided in to five subclasses based on the number and the order of conserved domains (Close, 1997). Y_nSK_2 dehydrins contain one, two or three Y segments, followed by one S-segment and two K-segments, and they tend to be strongly induced by dehydration. The K_n types contain no Y- or S-segment and tend to be strongly cold-induced. The K_nS types contain K-segments that begin with the consensus E(H/Q)KEG, rather than EKKG, and an S-segment near the carboxy terminus. In addition to their constitutive expression, they are also cold- and dehydration-induced, the induction of which is related to organ type and dependent on the developmental stage of tissue (Rorat et al., 2004). Two other types are SK_n and Y_2K_n. The majority of dehydrin polypeptides are composed of domains rich in glysine, and polar amino acids, called Φ-segments, that are interspersed between conserved segments. These highly polar, hydrophilic Φ-segments have been proposed to interact with hydrophobic surfaces of cytoplasmic or nuclear macromolecules to prevent their coagulation (Campbell and Close, 1997).

Dehydrins are intrinsically unstructured proteins (Ceccardi et al., 1994; Ismail et al., 1999a; Hara et al., 2001). However, *in vivo*, DHNs may fold into a more ordered structure by interacting with a number of other molecules or intracellular structures such as membranes and nucleoproteins (Close, 1997; Svensson et al., 2002).

DHNs localize in the nucleus, cytoplasm, mitochondria, vacuole and in the vicinity of the plasma membrane (Sarhan et al., 1997; Danyluk et al., 1998; Borowskii et al., 2000ab, 2002; Heyen et al., 2002). In general, localization studies have revealed tissue- and cell-type-specific accumulation of DHNs in unstressed plants (Robertson and Chandler, 1994; Rinne et al., 1999; Nylander et al., 2001). After stress, the expression patterns of some DHNs become more generalized (Egerton-Warburton et al., 1997; Nylander et al., 2001). Constitutive DHNs may carry out some basic function under unstressed conditions, and upon stress, similar functions are extended to most cells and tissues (Nylander et al., 2001). For many DHNs, the

highest accumulation is observed within vascular tissues and surroundings cells (Godoy et al., 1994; Houde et al., 1995; Danyluk et al., 1998; Bravo et al., 1999; Nylander et al., 2001), which are more vulnerable to ice nucleation and damage at low temperatures.

The exact function of dehydrins is still unclear, but based on their sequence features, they have been postulated to stabilize cell structures against dehydration (Close, 1997; Egerton-Warburton et al., 1997; Danyluk et al., 1998). In several plant species, DHNs have been shown to possess *in vitro* cryoprotective activity (Rinne et al., 1999; Wisniewski et al., 1999; Hara et al., 2001; Bravo et al., 2003; Sanchez-Ballesta et al., 2004) and *in vivo* antifreeze activity (Wisniewski et al., 1999). DHNs have also been suggested to improve enzyme activities under conditions of low water activity (Rinne et al., 1999) and to function as possible osmoregulators (Nylander et al., 2001) or as radical scavengers (Hara et al., 2003). DHNs have been purified from plants (summarized in Svensson, 2001) and by expression of recombinant DHNs in *Escherichia coli* (Svensson et al., 2000). Purification of Arabidopsis DHNs with immobilized metal affinity chromatography revealed strong binding to copper-charged columns. Since DHNs do not possess any known metal binding motif, the strong binding observed was suggested to be due to complexes formed by histidine residues within DHNs (Svensson et al., 2000). Metal binding activity has also been reported for a dehydrin from *Ricinus communis* (Krüger et al., 2002). Histidine constitutes 19% of *Ricinus* DHN (ITP, iron transport protein), and *Ricinus* DHN has been shown to transports iron (Fe^{3+}) in phloem. Purified *Ricinus* DHN also binds Cu^{2+}, Zn^{2+} and Mn^{2+} *in vitro*. Recently, DHNs/DHN-like proteins have been demonstrated to have ion (iron and calcium) binding activity (Heyen et al., 2002; Krüger et al., 2002; Alsheikh et al., 2003), suggesting that DHNs might act as ion transporters and calcium buffers or as calcium-dependent chaperones like calreticulin and calnexin. In celery (*Apium graveolens*), the phosphorylation of DHN VcaB45 results in a partial activation of its calcium-binding activity (Heyen et al., 2002). Some DHNs are modified post-translationally. DHN and DHN-like protein from blueberry and pistachio have been found to be glycosylated (Golan-Goldhirsch, 1998; Levi et al., 1999). Proper functioning of DHNs during cold/dehydration stress might involve an interplay of several mechanisms e.g. the concerted action of a group of DHNs or interaction with other protective molecules such as other LEA proteins or compatible solutes (Close, 1996; Hoekstra et al., 2001).

Six DHNs have been characterized in Arabidopsis. RAB18 accumulates in response to ABA, drought and low temperature (Lång et al., 1989; Lång and Palva, 1992; Nylander et al., 2001), LTI29/ERD10 (Kiyosue et al., 1994; Welin et al., 1994), LTI30/DHNXERO2 (Welin *et al*., 1994; Rouse *et al*., 1996) and COR47 (Welin *et al*., 1995) accumulate primarily in response to low temperature (Nylander *et al*., 2001). ERD14 is present in non-stressed plants and is upregulated by stress, particularly drought stress (Kiyosue *et al*., 1994; Nylander *et al*., 2001). *DHNXERO* is constitutively expressed (Rouse *et al*., 1992; Welin *et al*., 1995). In addition, four Arabidopsis EST clones (accession numbers *Q96261, Q9SVE4, Q9T022, Q9SLJ2*) have homology with *DHNs*. Expression of many *DHNs* has been shown to be regulated by the CBF pathway and several other pathways (Ishitani et al., 1997; Shinozaki and Yamaguchi-Shinozaki, 2000; Fowler and Thomashow, 2002).

2. AIMS OF THE STUDY

The aim of this project was to gain a better understanding of the development of FT in plants. For this purpose, different cell and molecular biological and biochemical research approaches and several plant species were used. In Studies I and II, the cold-induced changes in the structure of the microtubule cytoskeleton and in the Ca^{2+} transport activity in winter rye were investigated. Study III elucidates the effect of DHN overexpression on FT in Arabidopsis. Study IV examines the cold acclimation process at molecular level in trees by means of cloning and characterizing a *DHN* gene from birch.

3. MATERIALS AND METHODS

3.1. Plant material

Three freezing-tolerant plant species were used. Winter rye (*Secale cereale* L. cv. Voima) was used in Studies I and II, *Arabidopsis thaliana* L. Heyhn. in Studies III (ecotype Landberg *erecta*, LE) and IV (ecotype Columbia, Col-1), and silver birch (*Betula pendula* L. Roth.) in Study IV.

3.2. Methods

Growth conditions, different stress treatments and biochemical methods are described in detail in the original articles (I, II, III and IV), and no unpublished material is included.

4. RESULTS

4.1. Cold acclimation enhances stability of cortical microtubules in rye (*Secale cereale*) cells (I)

The effect of cold acclimation on cortical microtubules was examined by exposing non-acclimated (NA) and cold-acclimated (CA) rye plants as well as protoplasts isolated from leaves to sub-zero temperatures. To study the effect of dehydration response only, leaves and roots were exposed to hypertonic solutions equivalent to the dehydration response of freezing (Steponkus and Lynch ,1989). The organization and abundance of microtubules were observed by indirect immunofluorescence (IIF) microscopy. At normal growth temperatures, both NA and CA leaf cells (I, Figure 2A and 2D, respectively) had intact, random arrays of cortical microtubules and root cells (I, Figure 2G and 2J, respectively), and full arrays of microtubules in transverse orientation. After exposure to –4ºC, CA leaf cells still had complete microtubule organization (I, Figure 2E), whereas microtubules of NA leaf cells (I, Figure 2B) and of NA and CA root (I, Figure 2H and 2K, respectively) were shorter and less abundant. After –10ºC exposure, cortical microtubules were almost totally depolymerized in NA and CA root cells (I, Figure 2I and 2L, respectively) and in leaf cells of NA plants (I, Figure 2C), while CA leaf cells still had abundant microtubule arrays (I, Figure 2F). These results (I, Figure 2) were confirmed by analysing the polymerization stage of microtubules in protoplasts prepared from leaves of NA (I, Figures 4A-C and 5) and CA (I, Figures 4D-F and 5) plants.

In hypertonic sorbitol solutions corresponding to the sub-zero temperatures used in freezing treatments, microtubules of NA and CA leaf cells behaved similarily as in sub-zero treatments (data not shown). However, after hypertonic treatment of root cells of CA plants (I, Figure 3C, D), more microtubules remained intact than in NA (I, Figure A, B) root cells, suggesting that cold acclimation increases the dehydration stability of microtubules in root cells. The amount of long microtubules in isolated protoplasts (I, Figure 5) parallelled the viability of the leaf tissues both for NA and CA plants (I, Figure 1). The survival of roots was also consistent with the amount of microtubule observed (I, Figure 1). The results indicate that cortical microtubules have a role or may be used as reporters of FT in different parts of rye plants. In addition, the observation that microtubules of CA root cells could not tolerate -10ºC but still had the ability to resist equivalent dehydration stress suggests that different mechanisms may underlie protection against freezing and drought stresses.

4.2. Cold acclimation enhances the activity of plasma membrane calcium ATPase in winter rye leaves (II)

ATP-dependent Ca^{2+} transport and H^{+}ATPase activities were analysed in isolated plasma membranes of NA and CA winter rye leaves. Highly purified plasma membranes were

isolated from NA and CA rye plants using aqueous polymer two-phase partitioning. The purity of the plasma membrane fractions was estimated with glucan synthase II as a marker for plasma membrane purity and cytochrome *c* oxidase as a marker for contaminated mitochondria. A 4.6-5.5-fold enrichment of glucan synthase II was found in the upper phase containing the plasma membrane in comparison with the lower phase containing the microsomal fraction (II, Table I). Cytochrome *c* oxidase had decreased 10-fold in plasma membrane fractions from NA and CA plants (II, Table I). The activity of the marker enzyme for the ER, the NADH-dependent, antimycin A-insensitive, cytochrome *c* reductase, was low and similar in both materials.

Two weeks of cold acclimation of plants led to a two-fold increase in the plasma membrane Ca^{2+} transport activity, which was higher (P=0.021) than that in plasma membranes from NA plants (I, Figure 1; Table II). A small increase in H^{+}ATPase activity, measured as ATP hydrolysis, was observed in CA as compared with NA plasma membranes (I, Table II). The activity of the marker enzyme, glucan synthase II, was not significantly higher in CA than in NA plasma membranes (I, Table II). The effect of calmodulin on the activity of Ca^{2+}ATPase was studied with EDTA and Brij58 washed membranes. Calmodulin treatment resulted in a two-fold increase in Ca^{2+} transport in both NA and CA membranes by increasing V_{max} (I, Figure 2). The increase in the activity of ATP-dependent Ca^{2+} transport in CA rye plasma membrane probably reflects the capacity needed to sustain the resting level of cytosolic Ca^{2+} concentration during cold acclimation.

4.3. Overexpression of multiple dehydrin genes enhances tolerance to freezing stress in Arabidopsis (III)

To assess the contribution of DHNs in freezing stress tolerance in Arabidopsis, transgenic plants overexpressing multiple DHN genes were generated. Two different chimeric double constructs were generated: pTP9 for overexpression of *RAB18* and *COR47*, and pTP10 for overexpression of *LTI29* and *LTI30* (III, Figure 1). These constructs were transformed into Arabidopsis by using *in planta* transformation method. Transgenic plants obtained were screened by Western blotting to detect dehydrin overproducers with the highest production of DHNs. All transgenic lines exhibited increased accumulation of DHNs at normal growth temperature to levels similar or higher than in CA wild-type plants (III, Figure 2). Two TP9 lines (TP9-2 and TP9-3) and two TP10 lines (TP10-12 and TP10-18) were chosen for further studies. Plants overproducing dehydrins showed no phenotypic differences when compared with controls.

To reveal the effect of overproduction of DHNs on FT of Arabidopsis, both transgenic and control plants were exposed to a controlled freezing test. The determination of LT_{50} values suggested that both NA transgenic and CA (2d, 7d) *DHN*-expressing lines were more tolerant to freezing than the corresponding vector control plants (III, Figure 3). The difference in LT_{50} between control and *DHN*-expressing lines (TP9-3 and TP10 lines) was accentuated further by cold acclimation (III, Figure 3).

To study the effect of DHN overproduction on FT at the whole-plant level, frost survival experiments were performed with NA *in vitro* and soil-grown plants (III, Figure 4A, B). Two-weeks-old *in vitro* DHN-overexpressing plants showed a higher survival rate after exposure to –10ºC for eight hours than controls (III, Figure 4A). Similar results were obtained with NA three-weeks-old soil-grown plants (III, Figure 4B). In addition to a higher survival rate after freezing, transgenics overproducing DHNs showed enhanced recovery from freezing stress when compared with controls.

To get a better insight into the mechanism by which DHNs enhance FT of Arabidopsis, DHN localization in transgenic plants was analysed by immunoelectron microscopy (IEM). Due to the more pronounced effect of LTI29 and LTI30 overproduction (TP10 lines) on FT and the specificity of antibodies available, LTI29 and LTI30 were used for IEM. The results indicate that LTI29 shows partial intracellular translocation from the cytosol to the vicinity of the membranes during cold acclimation (III, Figure 5A, B). LTI30 was only detected in CA plants, where it occurred in membranes (III, Figure 5C) and occasionally in the nucleoplasm (III, Figure 5C). These results suggest that DHNs may function as membrane stabilizers in Arabidopsis during/after freezing stress.

4.4. Short-day photoperiod potentiates low-temperature-induced expression of a CBF-controlled gene during cold acclimation in silver birch (*Betula pendula* L. Roth.) (IV)

To obtain a marker gene to study the molecular mechanism of cold acclimation in woody plants, a full-length cDNA and a corresponding genomic clone of the dehydrin gene were cloned from birch. The gene, designated *Bplti36*, encodes a 36-kDa SK_2-type dehydrin (IV, Figure 1). The genomic clone of *Bplti36* included a 2kb DNA upstream of the coding sequence. Promoter analysis performed by using the PLACE program (Prestridge, 1991; Higo et al., 1999) revealed that the promoter contained several sequence motifs showing homology to *cis*-acting elements conferring stress responsiveness to previously characterized plant genes (IV, Suppl Figure). These included five CRT/DRE/LTRE elements (Nordin et al., 1993; Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994) and one ABRE element (Marcotte et al., 1989). *Bplti36* was shown to be highly responsive to LT, drought and exogenous ABA (IV, Figure 2), somewhat responsive to salt and only marginally responsive to wounding and salicylic acid (data not shown).

To elucidate the functionality of *cis*-acting elements in the *Bplti36* promoter, transgenic Arabidopsis plants carrying the *Bplti36* promoter fused to the *uidA* marker gene were generated. The transgenic plants were exposed to cold, drought and exogenous ABA. All three treatments resulted in considerable expression of *uidA* in the transgenic plants (IV, Figure 3A), suggesting that the *cis* elements in the *Bplti36* promoter are operational in Arabidopsis. To verify the functionality of CRT/DRE/LTRE elements, which act as possible binding sites for the CBF/DREB1 transcription factors in Arabidopsis, transgenics plants carrying *Bplti36* promoter-*uidA* fusion were crossed both with an Arabidopsis line overproducing CBF3 and a corresponding vector control line B6 (Gilmour et al., 2000). The F1 plants from the cross with the CBF3 overproducer exhibited constitutive expression of *uidA* at normal growth temperature and enhanced expression when cold-acclimated (IV, Figure 3B).

To elucidate the response of *Bplti36* expression to the SD signal, birch seedlings were exposed to a short two-stage acclimation process, and the accumulation of *Bplti36* mRNA was followed. Birch seedlings were exposed to either SD, LT or SD followed by LT. SD exposure led to a slight increase in the accumulation of *Bplti36* (IV, Figure 4), but not to the level observed in LT-treated plants (IV, Figure 4). SD treatment followed by LT resulted in a substantial increase in the *Bplti36* level compared with the LT treatment alone (IV, Figure 4).

5. DISCUSSION

5.1. Cold acclimation enhances stability of cortical microtubules in winter rye (*Secale cereale*) cells (I)

IIF microscopical analysis of microtubule cytoskeleton showed that cold acclimation enhances the cold stability of cortical microtubules in winter rye leaf cells as well as in protoplasts isolated from the leaves of CA plants. Moreover, the analysis suggested a relationship between the polymerization stage of cortical microtubules and FT of winter rye. Cold acclimation also increases dehydration tolerance in root cells of CA plants. These observations agree with results showing that cold acclimation enhances stability of cortical microtubules and that the stability of cortical microtubules is related to FT in plants (Jian et al., 1989; Wang and Nick, 2001; Olinevich et al., 2002; Abdrakhamanova et al., 2003). The observed increase in dehydration tolerance in root cells of rye after cold acclimation is in accord with dehydration being a component in both cold and drought stresses, and thus, cold acclimation also enhancing tolerance to dehydration stress. The most likely role for cold-stable cortical microtubules is to sustain growth under the non-optimal growing temperatures (Olinevich et al., 2002; Abdrakhamanova et al., 2003) that winter rye encounters during its life cycle. The MT cytoskeleton may support the plasma membrane during freezing and subsequent thawing. The mechanism by which cortical microtubules protect the plasma membrane during freezing might be partly due to the prevention of hexagonal II phase transition, i.e. structural hindrance of the interlamellar fusion under dehydration stress.

Recently, it has become evident that cortical microtubules and especially their dynamic reorganization during the early phases of cold acclimation are also involved in low temperature signal transduction and are thus needed for the cold acclimation process (Thion et al., 1998; Sangwan et al., 2001, 2002; Olinevich et al., 2002; Örvar et al., 2000; Abdrakhamanova et al., 2003). In Arabidopsis, the effect of microtubules on the regulation of Ca^{2+} channels was shown to be indirect and most probably due to protein-protein interactions involving microtubules (Thion et al., 1998). Cytoskeletal reorganization appears to be an integral component in low temperature signal transduction, serving as a link between membrane rigidification and calcium influx in cold acclimation (Örvar et al., 2000; Sangwan et al., 2001, 2002). Cortical microtubules thus appear to have a central role both at the beginning and at the end of the cold acclimation process in cold-tolerant herbaceous species.

5.2. Cold acclimation enhances activity of plasma membrane calcium ATPase in winter rye leaves (II)

The enhancement of plasma membrane H^+ATPase activity during cold acclimation is supported by studies done with both herbaceous and woody species (Hellergren et al., 1983; Ishikawa and Yoshida, 1985; Iswari and Palta, 1989; Mattheis and Ketchie, 1990; Sutinen et

al., 2004). The increase in H^+ ATPase activity has also been shown to be associated with increases in FT (Hellergren et al., 1983; Sutinen et al., 2004). These observations suggest the involvement of H^+ ATPase in the cold acclimation process. However, the mechanism by which these changes in H^+ ATPase activity are brought about during cold acclimation remains obscure. Membrane lipid composition is known to affect membrane protein activity (Carruthers and Melchior, 1986). Thus, changes in plasma membrane lipid composition during cold acclimation have been thought to have an impact on the activities of plasma membrane enzymes. Especially, fatty acid unsaturation, as it has a central role in the resistance to temperature stress (Nishida and Murata, 1996), has been speculated to affect the activity of H^+ ATPase. This hypothesis is supported by the finding that increasing 18:2 fatty acids in the plasma membrane results in enhanced activity of plasma membrane H^+ ATPase (Palmgren et al., 1988). However, no clear relationship was found between plasma membrane H^+ ATPase activity and bulk fatty acid unsaturation during cold acclimation and deacclimation in pine needles (Sutinen et al., 2004).

In winter rye, cold acclimation induced a small increase in H^+ ATPase activity (II). the Relative proportions of di-unsaturated fatty acid species increases during the first week of cold acclimation and virtually every lipid species changes during cold acclimation in winter rye (Lynch and Steponkus, 1987; Uemura and Steponkus, 1994). Thus, it is possible that changes in specific lipid species or an overall change in membrane properties, e.g. in fluidity, have an effect on plasma membrane H^+ ATPase activity in CA winter rye, although this relationship remains to be elucidated. Recently, plasma membrane H^+ ATPase activity has been shown to be regulated by interaction with 14-3-3 proteins (Camoni et al., 2000), and in sugar beet, regulation of plasma membrane H^+ ATPase activity by 14-3-3 proteins during cold stress has been observed (Chelysheva et al., 1999). Further support for this hypothesis comes from the observation that this interaction is abolished by protein phosphatase 2A (Camoni et al., 2000), which is known to be inactivated during cold acclimation (Monroy et al., 1998). The interaction between plasma membrane H^+ ATPase and 14-3-3 proteins was suggested to be due to Ca^{2+}-dependent phosphorylation of the C-terminal domain of H^+ ATPase (Chelysheva et al., 1999). Overall, these findings suggest that the increased activity of plasma membrane H^+ ATPase in the early phase of cold exposure/acclimation is due to Ca^{2+}-dependent phosphorylation, which is triggered by Ca^{2+} entry into the cells under cold stress.

The activity of plasma membrane Ca^{2+} transport was enhanced two-fold in CA winter rye plasma membranes. The reason for this is unknown since no clear difference in the amount of Ca^{2+} ATPase was detected. Cold-acclimation-induced changes in PM properties are a more likely explanation. Exposure of plant cells to cold or freezing temperatures leads to an increase in cytosolic Ca^{2+} and leakage of ions out of the cell, resulting in an ionic imbalance, which, if not restored, may cause cell injuries. Therefore, when plants acclimate to low temperatures, processes that control ion homeostasis are important. Low-temperature-induced Ca^{2+} influx is necessary for the expression of cold-responsive genes and the development of FT (see section 1.3.2.2., page 8). However, a prolonged high level of cytosolic Ca^{2+} is harmful, resulting in metabolic dysfunction and structural damage. Thus, an active Ca^{2+} transport system, such as the Ca^{2+} pump (Ca^{2+}ATPase) on cellular membranes, is needed to maintain Ca^{2+} homeostasis. The enhanced activity of plasma membrane Ca^{2+} ATPase activity has also been observed in winter wheat after prolonged chilling exposure (Jian et al., 1999). In chilled winter wheat seedlings, Ca^{2+} levels initially increased, but calcium homeostasis was restored in three days, when the level of Ca^{2+} was brought back to the level observed in seedlings grown in normal temperature (Jian et al., 1999). By contrast, maize, which is a chilling-sensitive species, was unable to restore Ca^{2+} homeostasis under prolonged chilling and showed cellular damages after the chilling treatment (Jian et al., 1999). Although the involvement of other Ca^{2+} transporters was not discussed by Jian et al. (1999),

changes in vacuolar Ca^{2+} were observed. Since no Ca^{2+} ATPase activity was observed in the vicinity of the tonoplast, changes in vacuolar Ca^{2+} are most probably due to the activities of vacuolar Ca^{2+} transporters, and thus indicating the involvement of vacuolar transporters, e.g. H^+/Ca^{2+} transporter, in the regulation of Ca^{2+} homeostasis during chilling. These results demonstrate that the ability of plants to restore and sustain Ca^{2+} homeostasis during cold is a key characteristic determining the chilling sensitivity or cold tolerance of plants.

5.3. Overexpression of multiple dehydrin genes enhances tolerance to freezing stress in Arabidopsis (III)

Accumulation of dehydrins (LEA D-II proteins) is frequently linked to the development of FT in plants (Close, 1996, 1997; Svensson et al., 2002). Higher expression of DHNs in more-cold tolerant lines (Ismail et a., 1999b), in cultivars (Zhu et al., 2000) or in sibling species (Artlip et al., 1997; Lim et al., 1999) suggests contribution of *DHNs* in FT. In woody plants, DHNs display seasonal expression and accumulation patterns. Up-regulation and accumulation of DHNs are closely linked to the timing and extent of cold acclimation in several woody plant species, including birch (Welling et al., 1997, 2004; Rinne et al., 1998), Scots pine (Kontunen-Soppela and Laine, 2001), peach (Arora and Wisniewski, 1994, 1996; Artlip et al., 1997), hybrid poplar and willow (Sauter et al., 1999), blueberry (Muthalif and Rowland, 1994), red-osier dogwood (*Cornus sericea*) (Sarnighausen et al., 2002) and *Rhododendron* (Lim et al., 1999).

To elucidate the contribution of DHNs to stress tolerance of plants, several overexpression studies have been conducted. Overexpression of spinach DHN had no effect on FT in tobacco (Kaye et al., 1998), nor did overexpression of resurrection plant (*Craterostigma plantagineum*) DHN on drought tolerance of tobacco (Iturriaga et al., 1992). Similarly, overexpression or antisense inhibition of *RAB18* had no impact on freezing or drought tolerance of Arabidopsis (Lång, 1993). However, recently, overproduction of a citrus dehydrin (CuCOR19) in tobacco has been shown to lead to a slight decrease in ion leakage during chilling and freezing stress (Hara et al., 2003). In a heterologous expression study with yeast, tomato DHN has been described to enhance tolerance to osmotic and freezing stress (Zhang et al., 2000). These studies indicate that one DHN does not contribute significantly to stress tolerance of plants, instead improvement in stress tolerance requires the action of several components on the cold signalling pathway, as demonstrated by the overexpression studies conducted with CBF1/DREB1B and CBF3/DREB1A (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Gilmour et al., 2000).

To elucidate the contribution of dehydrins to freezing stress tolerance in Arabidopsis, transgenic Arabidopsis plants overexpressing multiple dehydrins were generated. These findings showed that overexpression of dehydrins results in increased FT and better survival in transgenic plants than in controls when exposed to freezing stress. This difference between the LT_{50} values is further increased during cold acclimation.

DHNs have been proposed to function as membrane stabilizers during freeze-induced dehydration (Close, 1997; Danyluk et al., 1998; Thomashow, 1999). Here, overproduction of DHNs decreased ion leakage resulting from membrane injury in both NA and CA transgenic plants compared with control plants after freezing stress. IEM localization revealed DHNs in leaf cells from NA and CA transgenic plants in the vicinity of membranes and the partial translocation of the acidic dehydrin LTI29 from the cytosol to close to the membranes during cold acclimation. These results are in accordance with the results obtained by Danyluk et al. (1998), who showed that a wheat acidic dehydrin, WCOR410, accumulated in the vicinity of the plasma membrane during cold acclimation and suggested that this prevented the

destabilization of the plasma membrane in dehydrative conditions. The difference in tolerance to freezing stress between transgenic DHN-overproducing lines and corresponding controls increased during cold acclimation. The relatively small differences in DHN amounts following cold acclimation in transgenic plants can only partly explain the increased tolerance. Therefore, low-temperature-induced changes in the protective activity of DHNs must be based on either their altered localization or structure. Concerted action with other protective molecules, such as sugars, could also enhance protection of the plasma membrane in CA plants (Hoekstra et al., 2001; Hoekstra and Golovina, 2002). Accumulation of sucrose and other simple sugars typically occurs during cold acclimation in Arabidopsis (Ristic and Ashworth, 1993; Wanner and Junttila, 1999). This is likely to contribute to the stabilization of membranes, since these molecules in *in vitro* experiments can protect membranes against freeze-induced damages (Strauss and Hauser, 1986). Freezing sensitivity of the *sfr4* mutant of Arabidopsis, which is deficient in accumulation of soluble sugars during cold acclimation, is due to the susceptibility of this mutant to hexagonal II phase transition (Uemura et al., 2003). Changes in the lipid composition of the plasma membrane during cold acclimation have been shown to contribute to enhanced tolerance of the plasma membrane against freezing-induced injuries (Steponkus et al., 1993). However, changes in membrane properties are detected and tolerance to dehydration stress in membranes increases before any changes can be observed in lipid composition (Ristic and Ashworth, 1993; Kawamura and Uemura, 2002). Therefore, the enhanced tolerance of the plasma membrane during the first days of cold acclimation might mainly be due to changes in the composition of proteins associated with the plasma membrane and to accumulation of sugars (Uemura et al., 1995; Kawamura and Uemura, 2002).

Dehydrins have been proposed to contribute to increased FT by the same mechanism as chloroplastic protein COR15a (Artus et al., 1996; Steponkus et al., 1998). COR15a has similar biochemical properties as DHNs, being highly hydrophilic, soluble upon boiling and predicted to form an amphipathic α-helix. It has been proposed to inhibit the formation of hexagonal II phase transitions that occur in regions where the plasma membrane is brought into close apposition with the chloroplast envelope (Steponkus et al., 1998). Partitioning of DHNs, as endogenous amphiphilic substances from the cytoplasm into membranes during freezing/cellular dehydration may restrict the desiccation-induced oxidative damage, increases the fluidization of membranes and reduces the dehydration-induced increase in melting temperature (Hoekstra and Golovina, 2002). In transgenic tobacco, overexpressing of citrus dehydrin CuCOR19 decreased ion leakage during chilling and freezing stress (Hara et al., 2003) was probably due to the inhibitory effect of CuCOR19 against lipid peroxidation (Hara et al., 2003).

In conclusion, this study provides evidence that dehydrins contribute to freezing stress tolerance in plants. This may be partly due to their protective effect on membranes.

5.4. Short-day photoperiod potentiates low-temperature-induced expression of a CBF-controlled gene during cold acclimation in silver birch (*Betula pendula* L. Roth.) (IV)

A dehydrin gene, *Bplti36*, was cloned to obtain a marker gene for cold acclimation studies in birch. It showed high homology with acidic dehydrins (classified by Danyluk et al., 1994) found in Arabidopsis (Welin et al., 1994), wheat (Danyluk et al., 1994) and white spruce (Richard et al., 2000), and it responded to drought stress and to exogenous ABA. Furthermore, promoter analysis revealed several sequence motifs showing homology to *cis*-acting elements conferring stress responsiveness, including one ABRE and five

CRT/DRE/LTRE elements. Thus, both the expression pattern and promoter analysis indicated that this gene would be a suitable marker gene for cold acclimation studies in birch.

The functionality of CRT/DRE/LTRE elements, previously known to be binding sites of CBF/DREB transcription factors, was proven by introduction of the *Bplti36* promoter, coupled to the reporter gene *uidA*, into a transgenic Arabidopsis line overproducing CBF3. This resulted in constitutive expression of *uidA* in Arabidopsis. This finding suggests that the LT activation of cold-responsive gene expression in woody plants employs similar mechanisms as in herbaceous plants and that the CBF pathway mediating LT responses in Arabidopsis and other herbaceous plants (Stockinger et al., 1997; Liu et al., 1998; Jaglo et al., 2001) is conserved in trees. This suggestion is further supported by a birch EST database generated in Viikki Biocentre including putative *CBF* orthologues (Ojala et al., in preparation).

Development of FT in trees is a two-stage process involving sequential perception of distinct environmental signals, a SD and a LT (Weiser, 1970; Sakai and Larcher, 1987; Welling et al., 1997; Li et al., 2002). Previous studies have shown that a SD and LT lead to increased FT in leaves of silver birch (*Betula pendula* Roth.) (Li et al., 2002). The present work indicates that a synergistic effect of SD and LT is also evident in the expression of *Bplti36*. SD photoperiod at normal growth temperature did not result in significant induction of *Bplti36* in silver birch, whereas exposure to SD followed by LT treatment resulted in a marked increase in *Bplti36* expression compared with the effect of LT only. These results indicate that SD exposure sensitizes the birch to the subsequent LT, which is in agreement with the suggestion that SD induces a translocatable hardening promoting factor that could sensitize cells to perceive the LT signal (Weiser, 1970).

6. CONCLUSIONS

This study demonstrates the involvement of the cytoskeleton, especially cortical microtubules, and plasma membrane Ca^{2+}ATPase in the cold acclimation process of plants. Cold exposure is known to lead to a transient increase in $[Ca^{2+}]_c$ which is needed for cold-induced gene expression and development of FT. The cytoskeleton and its dynamic reorganization are involved both in low temperature signal tranduction and in the actual cold acclimation process. The increased stability of cortical microtubules and increased activity of plasma membrane Ca^{2+}ATPase observed in winter rye most probably ensure the proper response of the plant in the altered situation during cold acclimation and are among the key characteristics determining FT in plants.

The findings further indicate a contribution of DHNs in the FT of plants. Overproduction of DHNs resulted in increased FT and lower LT_{50} in transgenic Arabidopsis. Cellular membranes are regarded as the primary sites of cold stress damage. The localization of DHNs in the vicinity of membranes at low temperature suggests their involvement in stabilization and protection of cellular membranes. However, the ultimate mechanism by which DHNs carry out this protection remains to be elucidated.

The structural and expression analysis of a *DHN* gene, *Bplti36*, cloned from birch demonstrated similarities with DHNs previously characterized from herbaceous plants. When introduced to Arabidopsis, *Bplti36* showed similar responsiveness to different abiotic stresses and exogenous ABA as in birch. Introduction of *Bplti36* into a transgenic Arabidopsis line overexpressing the transcription factor CBF3 resulted in constitutive expression of *Bplti36* at normal growth temperature. These results demonstrate that the putative CRT/DRE elements identified in the *Bplti36* promoter are functional in Arabidopsis and indicate that cold acclimation in herbaceous and woody plants shares similar signalling pathways. The accumulation of *Bplti36* transcripts temporally correlated with the development in FT in birches exposed to SD and LT stimuli. This implicates the polypeptide encoded by *Bplti36* contributing to the freezing tolerance in birch leaves. However, cognate protein analysis is needed to verify this relationship. The involvement of the CBF pathway in development of FT in woody plants also warrants further studies.

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