The Role of Calcium and Protein Phosphatases in Cold Signal Transduction in *Arabidopsis thaliana*

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Academic dissertation
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"When You are
A Bear of Very Little Brain,
and you Think of Things,
you find sometimes that a Thing
which seemed very Thingish inside of you
is quite different when it gets
out into the open and
has other people looking at it."

Winnie-the-Pooh
"The House At Pooh Corner"
A.A. Milne

To Juha

Cover figure "*Arabidopsis thaliana* in the snow" by MSc Roosa Laitinen

TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS

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

I **Tähtiharju, S.,** Sangwan, V., Monroy, A.F., Dhindsa, R.S., and Borg, M. (1997) The induction of *kin* genes in cold-acclimating *Arabidopsis thaliana*. Evidence of a role for calcium. Planta, 203:442-447.

II **Tähtiharju, S.,** and Palva, T. (2001) Antisense inhibition of protein phosphatase 2C accelerates cold acclimation in *Arabidopsis thaliana*. Plant J. 26, 461-470.

III Vranová, E., **Tähtiharju, S.**, Sriprang, R., Willekens, H., Heino, P., Palva, E.T., Inzé, D., and Van Camp, W. (2001) The AKT3 potassium channel protein interacts with the AtPP2CA protein phosphatase 2C. J. Exp. Bot. 52, 181-182.

IV **Tähtiharju, S.,** Heino, P., and Palva, E.T. (2001) Protein phosphatase 1 is a positive regulator of cold and oxidative stress signalling in *Arabidopsis thaliana.* (Submitted to Plant J.)

ABBREVIATIONS

ABA	abscisic acid
ABRE	abscisic acid responsive element
AKT	*Arabidopsis* K+ transporter
AREB	abscisic acid-responsive element binding protein
AtPP2CA	*Arabidopsis thaliana* protein phosphatase 2C
bp	base pair
bZIP	basic leucine zipper
Ca^{2+}	calcium
Ca^{2+}_i	intracellular calcium
cADPR	cyclic adenosine diphosphate ribose
CaM	calmodulin
CBF	C-repeat binding factor
cDNA	complementary DNA
CDPK	calcium-dependent protein kinase
CRT	C-repeat
DNA	deoxyribonucleic acid
DRE	dehydration-responsive element
DREB	DRE-binding protein
EGTA	ethylene glycol-bis (β-aminoethyl ether) N, N, N′, N′,-tetra-acetic acid
Gd^{3+}	gadolinium
H_2O_2	hydrogen peroxide
H7	1-(5-isoquinoline-sulfonyl)-2-methylpiperazine dihydrochloride
IP_3	inositol-1,4,5-triphosphate
La^{3+}	lanthanum
LTRE	low temperature responsive element
NAD(P)H	nicotinamide adenine dinucleotide phosphate (reduced form)
mRNA	messenger RNA
PCR	polymerase chain reaction
PIP_2	phosphatidylinositol-4,5-bisphosphate
PI-PLC	phosphoinositide-specific phospholipase C
PK	protein kinase
PP	protein phosphatase
RNA	ribonucleic acid
ROS	reactive oxygen species
RyR	ryanodine receptor
TOPP	type one protein phosphatase
W7	N-(6-amonihexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride

ABSTRACT

Plants are continuously exposed to a wide range of environmental stresses, such as low temperature, which is one of the most important factors limiting the growth and distribution of plants in the world. Many plant species are able to increase their freezing tolerance in response to low, non-freezing temperature. This photosynthesis requiring response, referred as cold acclimation, results in various cellular changes including altered expression of cold-responsive genes. Despite extensive research into the mechanisms that regulate the expression of these genes, little is known about low temperature perception or about transducers of the cold signal.

Calcium (Ca^{2+}) and reactive oxygen species (ROS) are central signalling components in various stress responses. In addition, stress hormones such as abscisic acid (ABA) act as mediators of stress signalling. The transduction of stress signals via these mediators is in many cases controlled by protein phosphorylation and dephosphorylation, catalysed by protein kinases and phosphatases, respectively. Although implicated in cold acclimation, the precise role of various factors in cold signal transduction is unclear. In this study the functional role of Ca^{2+}, and protein phosphatases of type 1 and 2C (TOPP1 and AtPP2CA, respectively) in cold acclimation of *Arabidopsis thaliana* was studied.

The results demonstrate that Ca^{2+}, as a second messenger, is required for cold-induced gene expression and development of freezing tolerance in *Arabidopsis*. Furthermore, this study shows that Ca^{2+} mediated cold signal transduction is a complex process, which involves elevation of cytosolic Ca^{2+} levels through the action of both plasmalemma and tonoplast located Ca^{2+} channels in response to decline in temperature as well as transduction of this signal via various cold-upregulated Ca^{2+} binding proteins to the level of gene expression.

A reverse genetic approach was used to study the role of protein phosphatases 1 and 2C, putative regulators of ABA signalling in cold acclimation. For this purpose, transgenic plants were generated. First, studies with transgenic *AtPP2CA* antisense plants showed clearly accelerated development of freezing tolerance in response to both low temperature and exogenous ABA. Furthermore, the expression of cold- and ABA-induced genes was enhanced in these plants. Using the yeast two hybrid screen the potassium channel protein AKT3 was identified as a specific interacting partner of AtPP2CA. Firstly, taken together, the data indicates that AtPP2CA is a negative regulator of ABA responses during cold acclimation. Secondly, studies with transgenic *TOPP1* sense plants indicated that plants overexpressing *TOPP1* were more tolerant to oxidative stress than wild-type and *TOPP1* antisense plants. Thirdly, *TOPP1* antisense plants were impaired in their ability to cold acclimate when exposed to low temperature under high light conditions further suggested that TOPP1 is a positive regulator in cold stress responses. Enhanced expression of ABA-dependent genes in *TOPP1* sense plants after cold acclimation under high light conditions suggested a role for TOPP1 as a positive regulator of ABA signalling in cold stress response. In conclusion, TOPP1 positively regulates oxidative stress and ABA-mediated cold stress signal transduction.

This study confirms the important role for Ca^{2+}, ABA and ROS in cold signal transduction. Furthermore, evidence has been found that cold signal transduction is a complex process in which parallel and branched signalling pathways converge and cross-talk leading to the development of freezing tolerance. Also, this study has shown that by modification of signalling components, such as protein phosphatases, it is possible to enhance tolerance against oxidative stress and freezing. Thus, it is evident that understanding the cross-talk between different signalling pathways will become increasingly important in the future. The understanding of complex signalling networks will open new possibilities to design crops with increased abilities to adapt to a variety of stresses.

INTRODUCTION

Overview of signal transduction in plants

Plants and plant cells continuously respond to various stimuli, which alter their physiology, morphology and development. External cues such as the water status of soil, light and temperature are the most important signals affecting plant growth (for a review see e.g. Boyer, 1982; Trewavas and Malhó, 1997). In addition to external signals, a variety of internal signals such as hormones and solutes modify plant cell metabolism, growth and development. Perception of and response to these various stimuli triggers a cascade of complex events involving several interacting components, some of which are required for initial recognition of the signal and others in subsequent transduction of the signal to the physiological response. This cascade of events is called signal transduction. Signal transduction normally acts through second messengers, which are either formed or released from intra- or extracellular stores, and which modulate the activity of different effector proteins, like enzymes, whose activity then trigger the molecular events leading to the physiological response, usually by modification of gene expression (for a recent review see Trewavas and Malhó, 1997).

Signal transduction uses a network of interactions within cells, among cells and throughout the plant. Moreover, different signals affect the transduction network in different ways and at different places (for a recent review see Trewavas and Malhó, 1997). Various stimuli may evoke the same end response via different signalling pathways or they may activate signalling pathways leading to different end responses. These signal pathways can operate totally independently of each other or they may regulate the flux of information of the other pathways either positively or negatively. Different signalling pathways may also share components and second messengers to mediate their end responses. This convergency of signalling pathways is defined as cross-talk. As a result, many signals interact co-operatively with each other to produce a final response (Knight and Knight, 2001).

Although plant cells shares some common signalling elements with animals, as photosynthetic organisms, plants, also have some unique signalling components, which are not found in animal cells. There are also some differences in signal transduction between plants and animals due to anatomical and physiological differences. For instance, virtually all plant cells can sense and respond to environmental stimuli (Trewavas and Malhó, 1997), whereas animals have cells that are specialized for signalling (Alberts *et al*., 1989). In addition, animal cells are able to transduce signals to other cells in direct physical contact (Alberts *et al*., 1989), whereas cell walls separate individual plant cells preventing direct membrane contact. However, plant cells are connected to their living neighbours by fine cytoplasmic channels called plasmodesmata, which pass through the intervening cell walls. Plasmodesmata allow signalling molecules to pass directly from cell to cell enabling intercellular communication (reviewed by Zambryski and Crawford, 2000). Some animal cells are also able to form gap junctions that directly join the cytoplasms of the integrating cells, thereby allowing exchange of ions and small molecules (Alberts *et al*., 1989). In contrast to gap junctions, plasmodesmata also transport macromolecules including endogenous proteins like transcription factors (reviewed by Zambryski and Crawford, 2000).

Our knowledge about the signalling pathways in plants leading from stimulus to end response has increased in recent years. A number of signalling molecules have been isolated and characterised. The completion of the sequence of the *Arabidopsis* genome revealed that as many as 10% of *Arabidopsis* genes encode predicted signalling components (The Arabidopsis Genome Initiative, 2000). Still, very little is known about their biological function and role in plants.

Cold acclimation and tolerance to freezing stress

Plants encounter a wide range of environmental stresses during their lives. Consequently, plants have evolved a variety of mechanisms that enable them to tolerate stress and survive in adverse conditions.

Low temperature is one of the most important factors limiting the growth and distribution of plants worldwide (for a general review see Sakai and Larcher, 1987).

Freezing stress in plants

Exposure of plants to subzero temperatures may lead to freezing stress due to intracellular or extracellular ice formation. Intracellular freezing is always lethal due to membrane damage caused by ice crystals growing inside the cells. However, intracellular freezing is rare and thus the stress experienced by plants is mainly caused by extracellular freezing (originally reviewed by Levitt, 1980). The formation of extracellular ice moves water from the cell to the growing ice crystals on its surface (originally reviewed by Sakai and Larcher, 1987) causing cellular dehydration, which resembles that caused by water deficit (originally reviewed by Levitt, 1980). Depending on the extent of the freezing stress and plant species in question, some injury may occur (originally reviewed by Sakai and Larcher, 1987). Freezing-induced cellular dehydration causes various perturbations to membrane structure and function indicating that membranes are the primary targets of freezing injury (originally reviewed by Steponkus, 1984). In addition to dehydration, other factors such as protein denaturation, mechanical stress caused by intercellular ice, and production of reactive oxygen species may also contribute to freezing-induced cellular damage (reviewed by McKersie and Bowley, 1998; Thomashow, 1999). Also upon thawing, when the extracellular ice melts and water re-enters the cell, injury may occur. Uneven expansion of the cell wall and cytoplasm due to rapid thawing rates may cause plasmolysis followed by cell death (Palta and Weiss, 1993). Thus, if a cell is to survive the freeze thaw cycle, its plasma membrane must be able to withstand the major efflux and influx of water. After thawing, if the injury is not too severe, injured cells are able to repair the damage and they will recover.

Plants have two mechanisms to survive freezing stress; either by avoidance of or tolerance to freezing. Ice formation can be avoided mainly through the absence of freezable water or by supercooling. However, avoidance of freezing is of limited value since it mainly occurs in special organs such as seeds and overwintering buds. Therefore, tolerance to freezing is the dominant mechanism used by plants to adapt to freezing stress. Freezing tolerance includes either tolerance of freeze-induced dehydration or avoidance of freeze-induced dehydration mainly by lowering of the freezing point of the cellular sap by accumulating solutes (originally reviewed by Levitt, 1980).

Reactive oxygen species

In plants several reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radicals, are generated by the reduction of molecular oxygen (for a recent review see Dat *et al.*, 2000). Although ROS are formed in normal cell matabolism mainly during photosynthesis and their regulation is a common cellular event, oxidative damage is often associated with plant stress. Under stress conditions ROS can either act as signal molecules that activate multiple defense responses or exacerbate damage (Dat *et al.*, 2000). Indeed, a common denominator in various stresses, such as dehydration and freezing is oxidative stress, which is due to excess production of ROS (McKersie and Bowley, 1998). Studies with winter wheat and alfalfa present several types of evidence that link oxidative stress with freezing (reviewed by McKersie and Bowley, 1998). Furthermore, low temperatures, particularly in combination with high light intensities, cause excess production of ROS (Inzé and Van Montagu, 1995). ROS can be extremely reactive and oxidize biological molecules, such as DNA, proteins and lipids resulting in their damage. The reaction of ROS with these molecules depend on concentration and on the scavenging capacity of the cell (Dat *et al.*, 2000). Plants have several antioxidant enzymes and metabolites, which are involved in scavenging of ROS. The enzymes involved in detoxification of ROS, such as superoxide dismutases (SODs), catalases, glutathione reductases and ascorbate peroxidases have been found to be upregulated upon exposure to low temperature (Inzé and Van Montagu, 1995). Transgenic alfalfa plants overexpressing *SOD*s exhibited higher survival rates than

wild-type plants after two subsequent winters in field trials. This supports the hypothesis that enhancement of tolerance to oxidative stress might improve a plant´s ability to survive various stresses associated with winter (McKersie and Bowley, 1998).

Cold acclimation

Many plant species growing in temperate climates are able to increase their freezing tolerance in response to low, non-freezing temperatures (Levitt, 1980; Sakai and Larcher, 1987). This photosynthesis requiring response, referred to as cold acclimation, can also be triggered by other stresses such as moderate desiccation (Levitt, 1980; Palva and Heino, 1998) or high salinity (Ryu *et al*., 1995). Controlled photosynthesis at low temperature is important for cold acclimation since the energy provided by photosynthesis is required for successful acclimation (Wanner and Junttila, 1999). Furthermore, if a plant is unable to control phosynthesis reactions at low temperature, excitation energy may accumulate leading to photoinhibition and the formation of destructive ROS (Foyer et al., 1994).

In addition to being an inducible process, cold acclimation is also transient. Upon returning to normal growth temperatures the freezing tolerance is lost and active growth is restored. This process is called deacclimation (Levitt, 1980; Sakai and Larcher, 1987). There are differences in kinetics of acclimation and deacclimation processes between plant species. Overwintering perennials and annuals exhibit seasonal acclimation, which is a slow process involving several stages and leads to freezing tolerance down to –30 - -50ºC. In contrast, the acclimation for daily fluctuations during the growth season happens rapidly and results in freezing tolerance of about -15ºC (originally reviewed by Sakai and Larcher, 1987).

Cellular changes during cold acclimation

Cold acclimation involves numerous physiological, biochemical and molecular changes. These changes have been the subject of a wide range of extensive studies during the past two decades and have been reviewed in many excellent articles (Graham and Pattersson, 1982; Guy, 1990; Levitt, 1980; Steponkus, 1984; Thomashow, 1999; Xin and Browse, 2000). The cold-induced alterations in plant cell structure and metabolism presented in Figure 1 also occur during cold acclimation of the model plant *Arabidopsis thaliana*. Exposing *Arabidopsis* to low temperature leads to reduction in water content and transient increase in endogenous abscisic acid (ABA) levels (Lång et a., 1994). Cellular hydrogen peroxide (H_2O_2) concentration together with the activity of antioxidant enzymes also increase upon exposure to low temperature (O´Kane *et al*., 1996). The most common changes also include the accumulation of osmolytes, such as proline and soluble sugars (Wanner and Junttila, 1999), and changes in both structure and lipid composition of membranes (Ristic and Ashworth, 1993; Uemura *et al*., 1995). Recently, these complicated responses have also been studied by using different freezing sensitive and freezing tolerant mutants of *Arabidopsis*. However, the results obtained from these genetic studies are somewhat contradictory (Xin and Browse, 2000). Thus, the precise role that each of these changes has in the cold acclimation process is still uncertain

Low temperature-induced genes of Arabidopsis

In a wide range of plants, including *Arabidopsis*, most cellular changes during cold acclimation are associated with alterations in gene expression (for a review see Thomashow, 1999). These changes include increased or decreased levels of existing transcripts and proteins as well as the appearance of novel ones (reviewed by Guy, 1990). Indeed, numerous cold-inducible genes have been isolated and characterised (for a recent review see Thomashow, 1999). Some of these genes encode various regulatory proteins and proteins involved in signal transduction or proteins with known enzymatic functions. In addition, some cold-inducible genes encode proteins, which are similar to antifreeze proteins, such as KIN1 and KIN2 (Kurkela and Borg-Franck, 1992; Kurkela and Franck, 1990), or to proteins involved in dehydration responses, such as RAB18 (Lång and Palva, 1992). However, a large set of cold-induced

genes including *LTI78* (Nordin *et al*., 1991) and *RCI2A/LTI6* (Capel *et al*., 1997; Nylander *et al*., 2001) encodes proteins with unknown function (for a review see Thomashow, 1999). Several studies have tried to elucidate the role of these individual genes in a cold acclimation process and development of freezing tolerance. However, overexpression of a single cold-inducible target gene in transgenic plants has not resulted in a significant increase in freezing tolerance (Artus *et al*., 1996), Neither has inhibition of target gene expression in mutants resulted in any defectiveness in the ability to cold acclimate (Leyva *et al*., 1995). Thus, it is becoming more evident that the ability to cold acclimate is a complex, quantitative trait which involves several genes as reviewed earlier by Guy (1990) and Thomashow (1990).

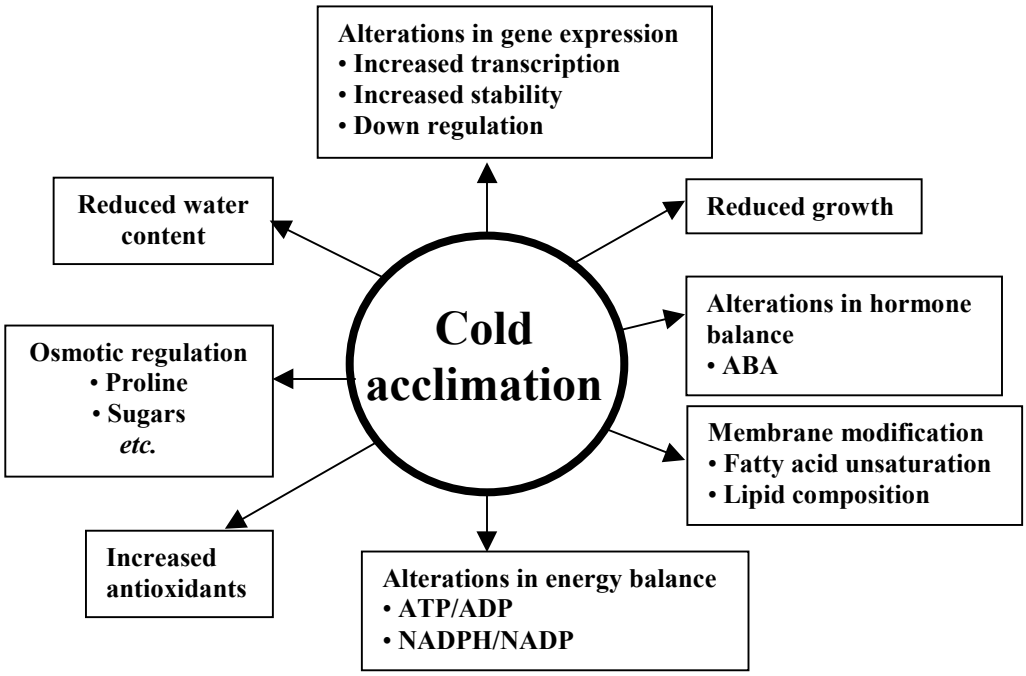

Figure 1. Cold acclimation induces many cellular changes in plants. The figure shows some of the changes commonly observed in *Arabidopsis* when it is exposed to low, non-freezing temperature. (Adapted from Xin and Browse, 2000).

Regulation of low temperature-responsive genes in Arabidopsis

In addition to low temperature, drought, salt, and application of ABA also trigger the expression of most of the cold-induced genes (Table I) (Palva and Heino, 1998; Thomashow, 1999). Analysis of the promoter regions of the cold-inducible genes has revealed several DNA sequences, which are involved in the regulation of the expression of these genes. ABA-independent gene expression under cold as well as dehydration and salt stress have been shown to be dependent on a *cis*-acting element named the dehydration-responsive element, DRE (Yamaguchi-Shinozaki and Shinozaki, 1994). The DRE-motif, also referred to as the C-repeat (CRT) (Baker *et al*., 1994) or low temperature responsive element (LTRE) (Nordin *et al*., 1993), occurs in the promoters of several cold- and drought-inducible genes (Table I). Many of them also contain ABA-responsive elements (ABRE) (Marcotte *et al*., 1989; Mundy *et al*., 1990). Furthermore, several low temperature responsive gene promoters contain the G-box element, which is the core motif of ABRE and functions in the regulation of plant genes in a variety of environmental conditions (Giuliano *et al*., 1988; Williams *et al*., 1992). In addition, recognition sites for transcription factors MYB and MYC have been found in promoters of cold-responsive genes. However, insufficient data exist to show the importance of ABREs, G-boxes as well as MYB- and MYC-motifs in the regulation of low temperature-induced gene expression.

Recently, five genes encoding transcription factors that bind to the DRE-motif have been cloned. These transcription factors belong to an unique family of AP/EREBP proteins (Okamuro *et al*., 1997). Three of the corresponding genes; *CBF1*, *CBF2*, and *CBF3* (C-repeat Binding Factor)/*DREB1B*, *DREB1C*, *DREB1A* (DRE-Binding protein), seem to be specifically cold inducible (Gilmour *et al*., 1998; Liu *et al*., 1998; Stockinger *et al*., 1997), whereas the other two, *DREB2A* and *DREB2B*, seem to be specifically dehydration- and salt stress-inducible (Liu *et al*., 1998). Transgenic plants overexpressing the cold-regulated transcription factors, CBF1/DREB1B or CBF3/DREB1A, exhibited increased freezing tolerance as well as expression of cold-inducible genes, even under non-inducing conditions (Jaglo-Ottosen *et al*., 1998; Liu *et al*., 1998). Moreover, under these conditions overexpressors also exhibited increased tolerance to drought (Liu *et al*., 1998) and salt stress (Kasuga *et al*., 1999). Exposure of CBF3/DREB1A as well as DREB2A overexpressors to stresses, such as cold, or exogenous ABA also resulted in the enhanced expression of cold-inducible genes (Liu *et al*., 1998). Taken together, these studies demonstrate an important role for the DRE in mediating the induction of cold-inducible genes and suggest a fundamental role of these genes in protection of plant cells against cellular dehydration as proposed by Jaglo-Ottosen *et al*. (1998).

Several stress-inducible transcription factors that can specifically bind to ABREs have been reported (Choi *et al*., 2000; Uno *et al*., 2000). They contain the basic leucine zipper (bZIP) motif and thus belong to the large protein family of bZIP transcription factors (Singh, 1998). The genes encoding these bZIP proteins, AREBs (ABA-Responsive Element Binding protein) or ABFs (ABRE Binding Factors) are ABA responsive. In addition, they respond differentially to various environmental stresses. However, only one of the corresponding genes is specifically cold-inducible suggesting that they have divergent functions during different stresses (Choi *et al*., 2000; Uno *et al*., 2000). To date, two AREBs have been shown to activate dehydration and ABA-induced expression of *LTI65* gene (Table I). However, their role in the regulation of low temperature-induced expression of this gene has not been demonstrated.

Recently, a soybean zinc finger protein, SCOF-1, was shown to enhance the DNA binding activity of SGBF-1, a soybean G-box binding bZIP transcription factor, to ABRE. Constitutive overexpression of the cold- and ABA- inducible *SCOF-1* gene induced cold-regulated gene expression and resulted in enhanced freezing tolerance of *Arabidopsis* (Kim *et al*., 1997). Kim *et al*. (2001) suggested that SCOF-1 might function as a positive regulator of cold-responsive gene expression mediated by ABRE via protein-protein interaction, which in turn enhances cold tolerance of plants. Several zinc finger family SCOF-1 homologs that are induced by cold stress have also been reported in *Arabidopsis*

(Sakamoto et el, 2000). In addition to bZIP, MYC and MYB type helix-loop-helix transcription factors also mediate the transcriptional activation of genes in response to ABA and dehydration (Abe *et al*., 1997). However, at the moment the involvement of these types of transcription factors in regulation of low temperature induced gene expression remains to be elucidated.

Table I. Summary of the current knowledge about induction and regulation of some of the most extensively studied cold-inducible genes of *Arabidopsis thaliana.*

Gene	**Motif**[a]	**Induction**[b]	**Transcriptional activator**[c]	**Reference**
LTI78/ RD29A/ COR78	DRE, ABRE	LT, ABA, D, S,	CBF1, DREB1A/CBF3, DREB2A	Horvath et al., 1993; Jaglo-Ottosen et al., 1998; Liu et al., 1998; Nordin et al., 1991; 1993; Yamaguchi-Shinozaki & Shinozaki,1993; 1994
LTI65/RD29B	DRE, ABRE, MYB	LT, ABA, D, S	AREB	Nordin et al., 1993; Uno et al., 2000; Yamaguchi-Shinozaki & Shinozaki,1993; 1994
COR15A	DRE, G-box	LT, ABA, D	CBF1, DREB1A/CBF3	Baker et al., 1994; Hajela et al., 1990; Jaglo-Ottosen et al., 1998; Liu et al., 1998
KIN1	DRE, G-box	LT, ABA	DREB1A/CBF3	Kurkela & Franck, 1990; Kurkela & Borg-Franck, 1992; Liu et al., 1998; Nordin et al., 1993; Wang et al., 1995
KIN2/COR6.6	DRE, G-box	LT, ABA, D, S	CBF1, DREB1A/CBF3	Gilmour et al., 1992; Jaglo-Ottosen et al., 1998; Kurkela & Borg-Franck, 1992; Liu et al., 1998; Nordin et al., 1993; Wang et al., 1995
RAB18	ABRE, DRE	LT, ABA, D	unknown	Lång & Palva, 1992; Nordin et al., 1993
RCI2A/LTI6	DRE, G-box, MYB, MYC	LT, ABA, D, S	unknown	Capel et al., 1997, Medina et al., 2001, Nylander et al., 2001

a *cis*-acting element present in the promoter of the corresponding gene
b LT= low temperature, ABA= abscisic acid, D= drought, S= salt
c transcription factor involved in the regulation of gene expression

The mode of action of CBF

Recently, Stockinger *et al*. (2001) showed that the ability of CBF1 to activate transcription is dependent upon the activities of a histone acetyltransferase protein (HAT) GCN5 and the transcriptional adaptor proteins ADA2 and ADA3 in yeast. These proteins, together with other proteins, form an adaptor complex, which makes a promoter more accessible to RNA polymerase II and other components of the transcriptional apparatus. The existence of *Arabidopsis* genes encoding GCN5, ADA2 and ADA3 proteins, and interaction of these proteins with CBF1 suggested the occurrence of HAT-containing adaptor complexes also in *Arabidopsis*. Thus, Stockinger *et al*. (2001) proposed that CBF1 might function through the action of one or more of these complexes by recruiting them to promoters.

CBF activity has been proposed to be transcriptionally regulated (Gilmour *et al*., 1998; Liu *et al*., 1998). Gilmour *et al*., (1998) have suggested that there is a specific transcription factor, ´ICE` (Inducer of CBF Expression), present at warm temperature that recognises a putative cold regulatory element, ´ICE box´, in the CBF promoters. At warm temperatures ICE is in an inactive state or in a form that does not bind to DNA or does not activate transcription effectively. However, a decrease in temperature triggers a signalling pathway that activates ICE, which in turn induces CBF expression resulting in low temperature-induced gene expression.

On the other hand, CBF1 has been suggested to act as a repressor, which transforms to an activator along with reduction in temperature (Kanaya *et al*., 1999). It has been proposed that local low temperature-induced cold denaturation of CBF1 initiates the transcriptional activation of cold-responsive genes, because extension of the molecule resulting from cold denaturation facilitates interaction with the factor(s) that activate or initiate transcription of the genes. On the other hand, Kanaya *et al*. (1999) also proposed that other cofactor(s) might associate with CBF1 for the repression of transcription at normal growth temperatures. At low temperature these factors then would be released from the locally denatured CBF1 protein resulting in transcriptional activation. They further proposed that CBF1 overexpression induced cold-regulated gene expression without a low temperature stimulus as reported by Jaglo-Ottosen *et al*., (1998) was probably due to shortage of some repressor in plants. Even though this result seems to support the theory by Kanaya *et al*., the exact function and activation of CBF1 as well as other transcription factors of plants still remain to be elucidated.

Cold signal transduction

Cold signal transduction can be determined as a process, which leads from perception of low-temperature stimulus to expression of genes encoding proteins, which are involved in cold acclimation and freezing tolerance. Despite extensive research on the mechanisms that regulate the expression of various low temperature-induced genes, little is known about the transducers of the cold signal or about the primary sensor that detects a change in temperature.

Perception of a low temperature signal

Biological membranes have been proposed to play a major role in the perception of low temperature signals. At normal growth temperatures fatty acids of membrane lipids in higher plants are highly unsaturated enhancing membrane fluidity. However, a reduction in temperature leads to a decrease in membrane fluidity, which in turn might trigger a signalling cascade (Murata and Los, 1997). Recently, it was shown that membrane rigidification at normal growth temperature activates cold signalling whereas membrane fluidization at low temperature inhibits it (Örvar *et al*., 2000). Thus, the primary signal upon a temperature shift might be a change in the fluidity of plasma membrane as suggested by Murata and Los (1997). A putative temperature sensor in the plasma membrane might detect such a dramatic conformational change in microdomains of plasma membrane. As a result, the hypothetical sensor protein might undergo a conformational change or a cycle of phosphorylation and dephosphorylation as the primary event in transduction of the temperature signal (Murata and Los, 1997).

Firstly, a sensor for perception of a downward shift in temperature might be accomplished by a two-component system, which is composed of a sensory histidine kinase and a response regulator. Two-component systems have been identified to play a role in osmosensing processes as well as to act as ethylene and cytokinin receptor in plants (Urao *et al*., 2000). Interestingly, two histidine kinases and a response regulator have been identified as components of the pathway for perception and transduction of low-temperature signals in a photosynthetic bacterium, *Synechocystis* (Suzuki *et al*., 2000). Inactivation of these components depressed the transcription of several low temperature-responsive genes (Suzuki *et al*., 2000, Suzuki *et al*., 2001). However, a set of cold-inducible genes in cyanobacterium is not regulated by the histidine kinase identified suggesting that the expression of these genes is modulated by multiple cold sensors (Suzuki *et al*., 2001). A cold-inducible histidine kinase gene, referred to as *AtHK1*, has been isolated from *Arabidopsis* (Urao *et al*., 1999). However, Urao *et al*. (1999) suggested that AtHK1 only senses changes in osmolarity in cells caused by dehydration, salinity, and cold stresses and not changes in membrane fluidity.

Secondly, the putative cold sensor in higher plants might be a calcium (Ca^{2+}) channel. According to this hypothesis, the Ca^{2+} channel opens at low temperatures when there is a decrease in membrane fluidity, and the Ca^{2+} ions that enter activate a signal transduction pathway for upregulation of the expression of low temperature-inducible genes (Monroy and Dhindsa, 1995). Indeed, membrane fluidity regulates the influx or mobilisation of Ca^{2+} ions in animal cells (Murata and Los, 1997) and a decrease in temperature enhances tension-dependent activity of the plasmalemma mechanosensitive Ca^{2+}-selective cation channels in plants (Ding and Pickard, 1993a). Thus, mechanosensory Ca^{2+} channels probably serve to detect not only mechanical stress but also thermal stimuli (Ding and Pickard, 1993b). The temperature dependence of the mechanosensitive channels suggests that they might well be temperature sensors for a variety of responses to temperature (Ding and Pickard, 1993b). Pickard and Ding (1993) presented the plasmalemmal control centre (PCC) model, which suggests that mechanosensitive Ca^{2+} channels are clustered and physically connected to clusters of regulatory plasmalemmal proteins and cytoskeletal elements grouped around a set of wall-to-membrane and transmembrane linkers. These centers of activity, the PCCs, might in turn permit not only mechanical but also biochemical interactions based on mechanical connections and on second messenger Ca^{2+} (Pickard and Ding, 1993). However, no Ca^{2+} channels have yet been identified at the molecular level in plants (Sanders *et al*., 1999) nor is their role in cold signal perception clear.

Thirdly, it was suggested that the cytoskeleton serves as a primary sensor for cold stimulus (Örvar *et al*., 2000). According to this proposal cold-induced membrane rigidification results in re-organization of cytoskeletal components, microfilaments and microtubules, which are attached to the plasma membrane. Cytoskeletal re-organization then in turn triggers cold signal transduction by opening Ca^{2+} channels. Indeed, cold depolymerizes microtubules (Bokros *et al*., 1993) and distruption of microtubules and actin microfilaments stimulates cold-induced Ca^{2+} influx in tobacco protoplasts (Mazars *et al*., 1997). Furthermore, it has been shown that disruption of both microtubules and actin microfilaments is required for cold signalling in plant cells as well as in intact plants (Sangwan *et al*., 2001; Örvar *et al*., 2000). However, there appears to be also actin cytoskeleton independent mechanisms that modulate cold-induced processes (Sangwan *et al*., 2001; Örvar *et al*., 2000) suggesting that the cytoskeleton alone does not act as the primary cold sensor. Furthermore, Ding and Pickard (1993a) showed that prevention of cytoskeleton organization sensitises tension-dependent Ca^{2+} channel opening rather than activating the channel independently. In addition, it has been shown that the changes in intracellular Ca^{2+} concentration affect the assembly and disassembly of microfilaments and microtubules either directly or indirectly (Rudd and Franklin-Tong, 1999). Ca^{2+} enhances cold-induced depolymerization of microtubules (Bokros *et al*., 1993). Furthermore, low temperature alters the expression of tubulin genes resulting in changes in levels of tubulin isoforms in *Arabidopsis* (Chu *et al*., 1993). One effect of

these changes would be to replace cold-labile microtubules with cold-stable ones (Bokros *et al*., 1993). Therefore, the temporal order of events is not so certain. However, there is increasing evidence that the cytoskeleton, as well as having a structural role, also has a signalling role (Rudd and Franklin-Tong, 1999).

In sum up, the existence of histidine kinase- and cytoskeleton-independent mechanisms in modulation of cold signal transduction as well as increasing evidence about the importance of Ca^{2+} influx favours the role for Ca^{2+} channels as a primary cold sensor. However, these same results also suggest that there might be more than just one receptor for a cold signal. It is evident that the relationship between membrane fluidity and plant temperature responses is very complex and further studies are needed to identify the cold sensor as well as to determine the temporal order of events after the perception of a cold stimulus. The identification of the Ca^{2+} channel gene and the corresponding knockout mutant would give an answer to many of these questions.

Calcium as a second messenger

Ca^{2+} has been implicated as a modulator of metabolism and development and to serve as a second messenger in the transduction of environmental stimuli in a variety of organisms, including plants (Sanders *et al*., 1999). In tobacco as well as in *Arabidopsis* rapid and transient exposure to cold (cold shock) evokes transient increases in cytosolic Ca^{2+} levels (Ca^{2+}_i) (Knight *et al*., 1991; 1996; Lewis *et al*., 1997; Polisensky and Braam, 1996). Monroy and Dhindsa (1995) proposed that this cold-induced Ca^{2+} influx plays an essential signalling role in cold acclimation. Indeed, cold acclimation of alfalfa cells and intact *Brassica napus* (canola) leaves in the presence of calcium chelators or of calcium channel blockers inhibited the influx of extracellular Ca^{2+} as well as expression of cold-acclimation specific genes and development of freezing tolerance (Monroy and Dhindsa 1993; 1995; Sangwan *et al*., 2001). The addition of a Ca^{2+} ionophore or a Ca^{2+} channel agonist to nonacclimated alfalfa cells or to intact *Brassica napus* leaves caused an influx of extracellular Ca^{2+}, induced the expression of cold acclimation-specific genes and increased the freezing tolerance at 25ºC (Monroy and Dhindsa, 1995; Sangwan *et al*., 2001). Elevated cytosolic Ca^{2+} levels in response to low temperature are mainly due to Ca^{2+} influx from extracellular stores (Monroy and Dhindsa, 1995). Indeed, increases in $[Ca^{2+}_i]$ and cold-dependent gene expression in cold-shocked *Arabidopsis* seedlings can also be blocked by plasma membrane Ca^{2+} channel blockers as well as by an extracellular Ca^{2+} chelator (Knight *et al*., 1996; Polisensky and Braam, 1996). However, these treatments caused only a partial inhibition of both cold shock Ca^{2+} elevation and cold-dependent gene expression in *Arabidopsis*, suggesting that also an intracellular Ca^{2+} source might be involved. Studies with the inhibitors of PI-PLC activity and IP_3 release from intracellular stores have suggested also a role for inositol-1, 4,5-triphosphate (IP_3)-mediated Ca^{2+} release from a vacuole in cold responses (Knight *et al*., 1996).

Phosphoinositides play an important role in ABA signalling and osmotic stress responses (Chapman, 1998). Their role in mediating signalling during cold acclimation is also emerging. In maize, cold shock and elevated intracellular Ca^{2+} result in hydrolysis of membrane phospholipids, in particular phosphatidylinositol-4, 5-bisphosphate (PIP_2), leading to the production of IP_3, which acts as a second messenger (De Nisi and Zocchi, 1996; Trewavas and Malhó, 1997). Hydrolyzation of PIP_2 is mediated by an activated phosphoinositide-specific phospholipase C (PI-PLC) (Trewavas and Malhó, 1997). Interestingly, low temperature and ABA-inducible gene encoding PI-PLC, whose activity is dependent on Ca^{2+}, has been isolated from *Arabidopsis* (Hirayama *et al*., 1995). Thus regulation of both expression and activity of PI-PLC during stress might control IP_3-mediated signalling. Furthermore, PI-PLC may be one of the primary sensors of Ca^{2+} signals through which Ca^{2+} regulates its own signal (Sanders *et al*., 1999).

Regulation of calcium homeostasis

Ca^{2+} channels in the plasma membrane and in intracellular membranes do not control the cytosolic Ca^{2+} concentration alone. Active Ca^{2+} transport out of the cytosol, mediated by primary and secondary transporters in the

plasma membrane and in intracellular membranes, ensure that the cytosolic Ca^{2+} level is brought back to resting level. Ca^{2+}/H^{+} antiporters and Ca^{2+} ATPases, regulators of Ca^{2+} efflux from cytosol, modulate Ca^{2+} concentrations in the cytosol, load Ca^{2+} into intracellular compartments and supply Ca^{2+} to organelles to support biochemical functions. Several genes encoding Ca^{2+} ATPases have been cloned from *Arabidopsis* (Sanders *et al*., 1999) but their role in cold stress is unclear. However, Ca^{2+} ATPase activity increases in winter rye leaves in response to low temperature (Puhakainen *et al*., 1999). A Ca^{2+}/H^{+} antiporter gene has also been isolated from *Arabidopsis*. Transgenic tobacco plants overexpressing *Arabidopsis* Ca^{2+}/H^{+} antiporter gene displayed sensitivity to cold shock suggesting that antiporter activity is essential for adaptation to cold stress (Hirschi, 1999). A major function of Ca^{2+}/H^{+} antiporters and Ca^{2+} ATPases may be to terminate Ca^{2+} signals by restoring cytosolic Ca^{2+} levels to prestimulus values (Sanders *et al*., 1999). Both Ca^{2+} ATPase and Ca^{2+}/H^{+} antiporter seem to be subject to regulation. However, the signals that control these efflux systems are unknown. An increase in Ca^{2+}_{i} in response to low temperature results in transient closure of plasmodesmata, thus controlling cell-to-cell movement of ions and small molecules (Holdaway-Clarke *et al*., 2000). Taken together, control of both influx and efflux systems of Ca^{2+} in cold signalling are of equal importance allowing sensitising and desensitising the cell for cold-induced Ca^{2+} signal, which acts as a second messenger.

Calcium binding proteins

One way in which cytosolic Ca^{2+} may pass on information within the cell is through Ca^{2+}-binding proteins, such as calmodulin (CaM) (Rudd and Franklin-Tong, 1999). CaM is a highly conserved multifunctional protein, which has been considered as the primary sensor for changes in cellular free Ca^{2+} levels. Genes encoding CaM or CaM-like proteins have been isolated and characterised from a number of plant species, including *Arabidopsis* (Snedden and Fromm, 1998). In *Arabidopsis* and tobacco cells, stimuli such as touch, wind, or temperature shocks induce the rapid accumulation of CaM and CaM-related protein mRNAs (Braam, 1992; Braam and Davis, 1990; van der Luit *et al*., 1999). The cold-induced expression of the *CaM* genes is partly regulated by intracellular Ca^{2+} levels (Braam, 1992; Polisensky and Braam, 1996). Elevated intracellular Ca^{2+} levels modulate, in addition to *CaM* gene expression, also the activity of CaM itself. Ca^{2+} binding activates CaM, which in turn either directly or indirectly regulates the activity of numerous effector proteins involved in a variety of cellular processes (Rudd and Franklin-Tong, 1999; Snedden and Fromm, 1998). Cold acclimation of alfalfa cells in the presence of an antagonist of CaM resulted in inhibition of development of freezing tolerance and reduced accumulation of transcripts of cold-acclimation specific genes suggesting a role for CaM as a mediator of cold-induced Ca^{2+} signalling (Monroy and Dhindsa 1993).

Ca^{2+} signalling may also be mediated by Ca^{2+} binding protein kinases may also mediate and phosphatases, which phosphorylate and dephosphorylate target proteins, respectively (Rudd and Franklin-Tong, 1999). Numerous Ca^{2+} dependent protein kinases (CDPKs) have been identified in *Arabidopsis* (Harmon *et al*., 2000). Transient expression studies with various CDPK isoforms have proposed that there are specific CDPK isoforms for different stress signalling pathways (Sheen, 1996). Yet, none of these have been shown to be involved in cold signal transduction in *Arabidopsis*. However, based on studies with a putative CDPK inhibitor it has been proposed that CDPK mediates cold-induced changes in protein phosphorylation, accumulation of transcripts of cold-acclimation specific genes and development of freezing tolerance in alfalfa (Monroy and Dhindsa 1993). Two alfalfa sequences corresponding to CDPKs, which were markedly cold inducible, have been isolated (Monroy and Dhindsa, 1995). A cold and salt stress inducible *CDPK*, *OsCDPK7*, has also been isolated from rice. Notably, overexpression of this gene enhanced the chilling tolerance of rice plants (Saijo *et al*., 2000) suggesting that a coupling of Ca^{2+} and protein phosphorylation might play an important role during the acquisition of cold tolerance.

A role for protein phosphatases as primary sensors of Ca^{2+} signals is also emerging (Sanders *et al*., 1999). The expression of an

Arabidopsis gene encoding an important effector of Ca^{2+} signalling, Ca^{2+} binding calcineurin-B-like protein, AtCBL1, is highly up-regulated by cold in addition to drought and salt (Kudla *et al*., 1999). Calcineurin B-like proteins (also known as protein phosphatase 2B, PP2B) have been demonstrated to play a role in salt stress signalling (Hasegawa *et al*., 2000). Whether Ca^{2+} regulated protein phosphatases also play roles in cold signal transduction remains to be elucidated.

Genetic dissection of cold signal transduction pathways

Recently, mutational screens, which have been employed to dissect the mechanisms of freezing tolerance, have revealed a large number of cold signalling mutants in *Arabidopsis*. One approach involved the screening for mutants with altered expression of cold- and osmotic stress-responsive target genes. Consequently, the isolated mutants were named as *cos* (for constitutive expression of osmotically responsive genes), *los* (for low expression of osmotically responsive genes), and *hos* (for high expression of osmotically responsive genes) (Ishitani *et al*., 1997). Plants with *hos1* and *hos2* mutations cold acclimated slower than wild-type plants and showed altered expression of a set of cold-inducible genes under cold stress indicating that HOS1 and HOS2 are important negative regulators of cold signal transduction in plant cells (Ishitani *et al*., 1998; Lee *et al*., 1999a). However, the *hos2* and *hos1* mutations enhance gene induction in the cold through very different mechanisms (Lee *et al*., 1999a). HOS1 is also a negative regulator of *CBF* expression since *hos1* mutation also causes enhanced cold-induction of the genes encoding CBF2 and CBF3 transcription factors (Lee *et al*., 2001). *HOS1* encodes a RING-finger protein that resides in the cytoplasm at normal growth temperatures but appears in the nucleus when plants are subjected to low temperature (Lee *et al*., 2001). Since some RING-finger proteins can help to degrade specific proteins, Lee *et al*. (2001) proposed that HOS1 might target the transcriptional activators, CBFs, for degradation. The role of HOS1 in low temperature signalling is not restricted to gene regulation and cold stress tolerance. The *hos1* mutation also impair the vernalization response (Ishitani *et al*., 1998; Lee *et al*., 1999a). Lee *et al*. (2001) suggested that HOS1 might interact with a negative regulator of vernalization thus modulating flowering during cold acclimation.

A second approach involved the isolation of mutants that fail to develop full freezing tolerance after cold acclimation. These mutants were named *sfr* for sensitive to freezing (Warren *et al*., 1996). Most of the *sfr* mutants showed strong induction of cold-regulated genes even though they are partially deficient in the ability to cold acclimate. However, one of these *sfr* mutants, *sfr6*, was shown to be deficient in cold-inducible expression of CBF1-regulated genes, confirming the importance of the CBF1 pathway in cold acclimation. The mechanism of SFR6 action is not known neither has the gene encoding SFR6 or any other SFR been identified yet. Notably, low temperature-induced accumulation of CBF transcripts is normal in *sfr6* mutant indicating that SFR6 may act somewhere between CBF transcription and induction of the CBF regulon. Another possibility is that SFR6 acts as a component in an independent pathway that is simultaneously required for cold-regulated gene expression (Knight *et al*., 1999).

A third approach involved the isolation of *constitutively freezing-tolerant* (*cft*) mutants, i.e. mutants that are more freezing tolerant than wild-type plants in the absence of cold acclimation. One of the mutants, *eskimo1* (*esk1*), results in markedly increased freezing tolerance in both non-acclimated and cold-acclimated plants. The *esk1* mutants contain high levels of proline and soluble sugars but do not express the cold-regulated genes in the absence of cold acclimation. Furthermore, the *esk1* mutation does not affect cold-induced expression of several CBF1-regulated genes. However, during cold acclimation *esk1* has higher levels of transcripts for *RAB18*, a cold-responsive gene encoding a dehydrin protein, which is not part of the CBF regulon. These results suggest that *esk1* defines a cold acclimation signalling pathway that is distinct from the CBF cold acclimation pathway. Since *esk1* mutant was more freezing tolerant without expressing CBF1-regulated genes, Xin and Browse (1998) proposed that distinct signalling

pathways activate different aspects of cold acclimation and that activation of one pathway can result in considerable freezing tolerance without activation of other pathways. The gene encoding ESK1 has not been identified yet and the function of ESK1 still remains to be elucidated. However, it seems that, in addition to HOS1 and HOS2, ESK1 also acts as a negative regulator of cold acclimation (Xin and Browse, 1998).

Genetic dissection of ABA signalling pathways during cold acclimation

The role of phytohormone abscisic acid (ABA) in cold acclimation has been the cause of much debate (Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000). However, several lines of evidence obtained from studies, which have been conducted mainly by using mutants affected in ABA biosynthesis or ABA responsiveness (Koorneef *et al*., 1998; Leung and Giraudat, 1998; McCourt, 1999; Rock, 2000) suggest that the ABA may have an important role in the cold acclimation process. First, the ABA-deficient mutants of *Arabidopsis*, *aba1* and *aba4*, are severely impaired in their ability to cold-acclimate (Heino *et al*., 1990; Gilmour and Thomashow, 1991). However, application of ABA could suppress the impaired cold-acclimation phenotype (Heino *et al*., 1990). In addition, application of ABA at normal growth temperatures can induce an increase in freezing tolerance in a wide range of plants, including *Arabidopsis* (Guy, 1990; Lång *et al*., 1989). Furthermore, ABA levels increase transiently in response to low temperature (Lång *et al*., 1994).

Recent studies with various mutants with altered regulation of cold- and osmotic stress-responsive gene expression (Ishitani *et al*., 1997) and reduced freezing tolerance (Llorente *et al*., 2000) have further confirmed the involvement of ABA signalling during cold acclimation. The *Arabidopsis* mutant *los5* shows a dramatic reduction in the expression of stress-responsive genes under both cold and osmotic stress conditions. Furthermore, *los5* plants are more susceptible to damage by freezing and osmotic stresses, suggesting that LOS5 is critical for plant stress tolerance. The *los5* mutation, which is allelic to *aba3*, also results in ABA deficiency (Xiong *et al*., 2001a). Llorente *et al*. (2000) obtained similar results with the freezing sensitive mutant *frs1*. ABA3/FRS1/LOS5 encodes a molybdenum cofactor (MoCo) sulphurase, which catalyses the generation of the sulphurated form of MoCo, a cofactor required by aldehyde oxidase that functions in the last step of ABA biosynthesis in plants. Although ABA3 is a key regulator of ABA biosynthesis the regulation of cold responsiveness by ABA3 is not dependent on ABA. It was shown that treatment of *los5* mutant plants together with ABA and cold did not restore expression of stress-responsive genes in *los5*, suggesting that the reduced gene induction by cold in *los5* mutants is not a result of ABA deficiency as in *aba1*. Thus it was proposed that in addition to its role in ABA biosynthesis, ABA3/FRS1/LOS5 might have additional roles in cold regulation. At present, it is unclear how ABA3/FRS1/LOS5 is involved in the cold or ABA regulation of gene expression (Xiong *et al*., 2001a).

In addition to mutants impaired in ABA biosynthesis also *Arabidopsis* mutants defective in ABA responsiveness show alterations in their cold acclimation response. The ABA-insensitive mutant *abi1* is impaired in its development of freezing tolerance (Mäntylä *et al*., 1995). Furthermore, the cold-induced expression of several cold- and ABA-responsive genes has been reported to be reduced in *abi1* mutant plants (Lång and Palva, 1992; Nordin *et al*., 1993). ABI1 encodes protein phosphatase 2C (Leung *et al*., 1994; Meyer *et al*., 1994), which has been shown to play a role in drought tolerance (Gosti *et al*., 1999). However, the function of ABI1 in cold responses remains to be elucidated.

Analysis of cold-induced gene expression in ABA-signalling mutants in response to cold acclimation promoting treatments has revealed that the stress-induced expression of these genes is controlled by a multitude of signalling pathways (Nordin *et al*., 1991; Nordin *et al*., 1993; Shinozaki and Yamaguchi-Shinozaki, 1996). In addition, the *hos*, *los* and *cos* mutants discussed earlier had distinct responses to cold, drought and salt stress and ABA signals or a combination of them suggesting that both ABA-dependent and independent signal pathways are essential in cold signal transduction (Ishitani *et al*., 1997). For instance, *Arabidopsis* mutant *hos5* exhibits

enhanced expression of osmotic and cold stress-responsive genes in response to osmotic stress but not in response to cold. This osmotic stress hypersensitivity was shown to be ABA-independent. Thus, various authors have suggested that HOS5 is a negative regulator of cold and osmotic stress-responsive gene expression shared by ABA-dependent and ABA-independent osmotic stress signalling pathways (Xiong *et al*., 1999).

Furthermore, there is increasing evidence of cross-talk between ABA and Ca^{2+} signalling during cold acclimation. A role for IP_3-mediated Ca^{2+} release from the vacuole in the regulation of cold-responses was suggested by Knight *et al*. (1996). This proposal is supported by the recent studies with the *Arabidopsis* mutant *fiery1*. FIERY encodes an inositol polyphosphate 1-phosphatase, which mediates the catabolism of IP_3. Upon exposure to ABA, *fiery* mutants accumulate more IP_3 than wild-type plants and exhibit super-induction of ABA, cold and osmotic stress-responsive genes. However, *fiery* mutant plants are defective in cold acclimation suggesting that phosphoinositols mediate ABA and stress signal transduction in plants and their turnover is critical for attenuating ABA and stress signalling (Xiong *et al*., 2001b).

Taken together, the above discussion clearly demonstrates that ABA is an essential component of the protective mechanisms against cold stress. It is also evident that ABA-independent and ABA-dependent stress signalling pathways cross-talk to activate stress gene expression in the manner proposed by Ishitani *et al*. (1997).

Protein phosphorylation and dephosphorylation

A common way cells relay molecular messages is reversible protein phosphorylation and dephosphorylation catalysed by protein kinases and protein phosphatases, respectively. Recently the involvement of these proteins has been established also in cold signal transduction. It has been shown that cold acclimation of alfalfa and canola is mediated by protein phosphorylation (Monroy *et al*., 1993; 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). Low temperature alters the phosphorylation level of some proteins through a differential inhibition of protein kinases and phosphatases, which exhibit differential sensitivity to cold (Monroy *et al*., 1997). It has been proposed that cold sensitivity of kinases and phosphatases might alter the equilibrium of their actions on substrate proteins and thereby change the phosphorylation level of the product favouring hyperphosphorylation when a phosphatase is much more inhibited than a kinase. 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 freezing tolerance (Monroy *et al*., 1997).

Protein kinases

Protein kinases have been divided into numerous subgroups based on their substrate specificity and sequence relationships. Most of the subgroups belong to a large family of serine/threonine protein kinases like the mitogen activated protein kinases (MAPKs) (Stone and Walker, 1995). MAPK pathways are intracellular signal modules that are involved in the transduction of extracellular signals to intracellular targets. Distinct MAPK pathways are regulated by different extracellular stimuli, such as abiotic stresses, and are implicated in a wide variety of biological processes. The MAPK cascades consist of a specific set of three functionally interlinked MAP kinases (MAPKKK-MAPKK-MAPK) that are activated sequentially by an upstream kinase (Jonak *et al*., 1999). The first *Arabidopsis* MAPK cascade, consisting of AtMEKK1, AtMEK1/AtMKK2, and AtMPK4, was identified on the basis of extensive yeast two-hybrid analysis and mutant complementation in yeast (Ichimura *et al*., 1998). In *Arabidopsis*, gene expression and protein activity studies have shown that AtMEKK1 and AtMPK4 are induced in response to low temperature as well as touch and dehydration stress (Ichimura *et al*., 2000; Mizoguchi *et al*., 1996). Low temperature also induces rapid and transient activation of *Arabidopsis* MAP kinases

ATMPK3, and AtMPK6, which is also activated by ROS (Ichimura *et al*., 2000; Mizoguchi *et al*., 1996; Yuasa *et al*., 2001). Recently, Kovtun *et al*. (2000) demonstrated that these MAPKs are activated by ANP1 and NPK1, orthologous MAPKKKs from *Arabidopsis* and tobacco, respectively (Kovtun *et al*., 2000). Tobacco plants constitutively expressing NPK1 display enhanced tolerance to multiple environmental stress conditions including cold stress without activating previously described drought, cold, and ABA signalling pathways (Kovtun *et al*., 2000). The roles of various stress-inducible MAPKs are considered to be different, because they are structurally dissimilar, have specific interactions with each other, and their kinetics upon activities are not identical (Ichimura *et al*., 2000). Furthermore, the duration of the activated state of MAPK is considered to determine stimulus-dependent responses (Ichimura *et al*., 2000). Despite these differences a role for various MAPKs in cold signal transduction remains to be characterized.

A role for the histidine kinase AtHK1 in perception of cold signal has been proposed based on its upregulation by cold stress (Urao *et al*., 1999) (see Cold signal perception). In addition, there is evidence that the transcript levels for an *Arabidopsis* receptor-like protein kinase (RLK) accumulate in response to low temperature (Hong *et al*., 1997). The RLKs, the dominant type of cell surface receptors in plant cell, are transmembrane protein kinases that transduce extracellular signals across the plasma membrane and into the cell. Genetic and expression studies have revealed RLK function in developmental processes and plant defence responses (Lease *et al*., 1998). However, their role in perception and transduction of cold signal remains to be elucidated.

A role for other protein kinases in cold signalling is also emerging. By complementation of yeast with *Arabidopsis* cDNA library, *DBF2* cDNA was isolated. In yeast *DBF2* encodes a serine threonine protein kinase, which is a component of a multisubunit general transcriptional complex. In yeast this kinase is a cell cycle-regulated phosphoprotein, which is active only in a dephophorylated form. Overexpression of a functional *Arabidopsis* homologue of *DBF2*, *AtDBF2*, enhanced salt, drought, heat and cold stress tolerance both in yeast and in transgenic plants. Furthermore, overexpression also enhanced the expression of stress-inducible genes suggesting a role for AtDBF2 in the regulation of gene expression. Yet little is known about how it functions at the cellular and molecular levels (Lee *et al*., 1999b). Based on inhibitor studies a role for various other protein kinases, such as a phosphoinositide kinase, a tyrosine kinase and a protein kinase C in cold-induced gene expression have also been reported (Sangwan *et al*., 2001). Taken together, the data suggests that cold signalling is a complex multistep process that involves several types of protein kinases (Sangwan *et al*., 2001).

Protein phosphatases

Based on their substrate specificity and structural differences protein phosphatases are classified into two major groups; serine/threonine and tyrosine phosphatases (Luan, 1998). The protein tyrosine phosphatases (PTPases) are further divided into three subgroups: receptor-like PTPases; intracellular PTPases; and dual-specificity PTPases (Luan, 1998). Recently, the first plant cDNA encoding putative PTPase, AtPTP1, was isolated from *Arabidopsis* (Xu et a., 1998). Surprisingly, the expression of the *AtPTP1* gene is transiently down-regulated by low temperature. Such down-regulation by stress factors has not been reported for PTPases in any other organism before, implicating AtPTP1 in a unique mechanism for plant response to environmental factors (Xu *et al*., 1998). However, the function of PTPases in signalling pathways remains to be identified in plants.

Based on their biochemical and pharmacological properties, the serine/threonine protein phosphatases (PPases) are also categorized into four types; types 1, 2A, 2B, or 2C (also known as PP1, PP2A, PP2B and PP2C) (Luan, 1998). Numerous genes encoding these various PPases have been isolated from different plant species, including *Arabidopsis* (Kudla *et al*., 1999; Smith and Walker, 1996). Although little is known about the roles of these PPs in plants, recent studies indicate

that PPases are involved in developmental processes and in various signalling cascades, including those for ABA, pathogen and stress responses (Luan, 1998). However, only little is known about their role in cold signal transduction. Expression of cold-responsive genes is enhanced at normal growth temperature by the protein phosphatase 1 and 2A inhibitor (Monroy *et al*., 1998; Sangwan *et al*., 2001). These inhibitors also caused an increase in freezing tolerance at normal growth temperature (Sangwan *et al*., 2001). Moreover, low temperature caused a rapid and dramatic decrease in protein phosphatase 2A (PP2A) activity, which is dependent on Ca^{2+} influx. Therefore, it was suggested that PP2A might be an early target for cold-inactivation in low temperature signal transduction (Monroy *et al*., 1998). Studies with unspecific inhibitor of PP1 and PP2A also suggest a role for PP1 and PP2A in regulation of ABA signalling and expression of ABA and cold-responsive genes in *Arabidopsis* (Wu *et al*., 1997). An interacting partner for an *Arabidopsis* PP2A has been identified (Harris *et al*., 1999). This protein is a homolog of yeast protein involved in the target-of-rapamycin (TOR) signalling pathway, which may regulate protein synthesis. Interestingly the gene encoding this PP2A interacting protein is cold-inducible (Harris *et al*., 1999). However, the function of the target PP2A as well as the presence and function of the TOR pathway in plants and in plant stress response remains to be elucidated.

The *Arabidopsis* genome has approximately seventy genes encoding putative PP2Cs (The Arabidopsis Genome Initiative, 2000). However, to date only one of them has been suggested to play a role in cold signal transduction. The *abi1* mutation has been shown to affect both cold-inducible gene expression as well as development of freezing tolerance (Lång *et al*., 1992; Nordin *et al*., 1991; 1993; Mäntylä *et al*., 1995). Both *ABI1* and the homologous *ABI2* encode PP2Cs, which act as negative regulators of ABA signalling (Gosti *et al*., 1999; Leung *et al*., 1994; Leung *et al*., 1997; Merlot *et al*., 2001; Meyer *et al*., 1994; Sheen, 1998). The expression of *ABI1* and *ABI2* is upregulated by ABA and osmotic stress (Leung *et al*., 1997). Transcript levels of common ice plant PP2Cs are in addition to these treatments also regulated by cold (Miyazaki *et al*., 1999). However, nothing is known about their role in signalling. Instead, ABI1 acts as a negative regulator of ABA-mediated responses to drought (Gosti *et al*., 1999). Another PP2C, AtPP2CA, which is also a negative regulator of ABA signalling, inhibits expression of barley *HVA* gene (Sheen, 1996), which is both ABA and cold inducible (Straub *et al*., 1994). However, the role for these various regulators of ABA signalling during cold acclimation waits to be revealed.

Summary

A schematic presentation summarising the current knowledge about cold signal transduction in *Arabidopsis* is presented in Figure 2. The mechanism of low temperature perception is still unknown. However, endogenous levels of ABA and intracellular Ca^{2+} have been shown to increase in response to cold, suggesting that they play a role as second messengers. Blocking of ABA biosynthesis in *aba1* and *aba3/los5/frs1* mutants inhibits development of freezing tolerance. In addition, *aba1* mutation leads to decreased cold-induced expression of *RAB18* and *RCI2A/LTI6*. These ABA-dependent genes are regulated by different ABA-signalling pathways since cold-induced expression of *RAB18* is decreased in *abi1* mutant whereas the expression of *RCI2A/LTI6* is not. The role of ABA in regulation of *LTI78* and *KIN* genes is unclear. Instead, ABA-independent pathway mediated by Ca^{2+} signalling regulates cold-induced expression of *LTI78*, *KIN1* and *KIN2* genes. The transcriptional activator CBF modulates the expression of these genes. The CBF-regulated expression is diminished in the *sfr6* mutant, which is deficient in the ability to cold acclimate. Furthermore, *hos1* and *hos2* mutations have reduced development of freezing tolerance and which affects the expression of CBF-regulated genes, suggesting that HOS1 and HOS2 are negative regulators of cold signal transduction. In addition, ESK1 also acts as a negative regulator of cold acclimation and expression of *RAB18*. Even though numerous genes encoding putative signalling proteins, such as Ca^{2+}-binding proteins, protein kinases and protein phosphatases have been isolated from *Arabidopsis* their role in cold signalling and in the development of freezing tolerance remains to be elucidated.

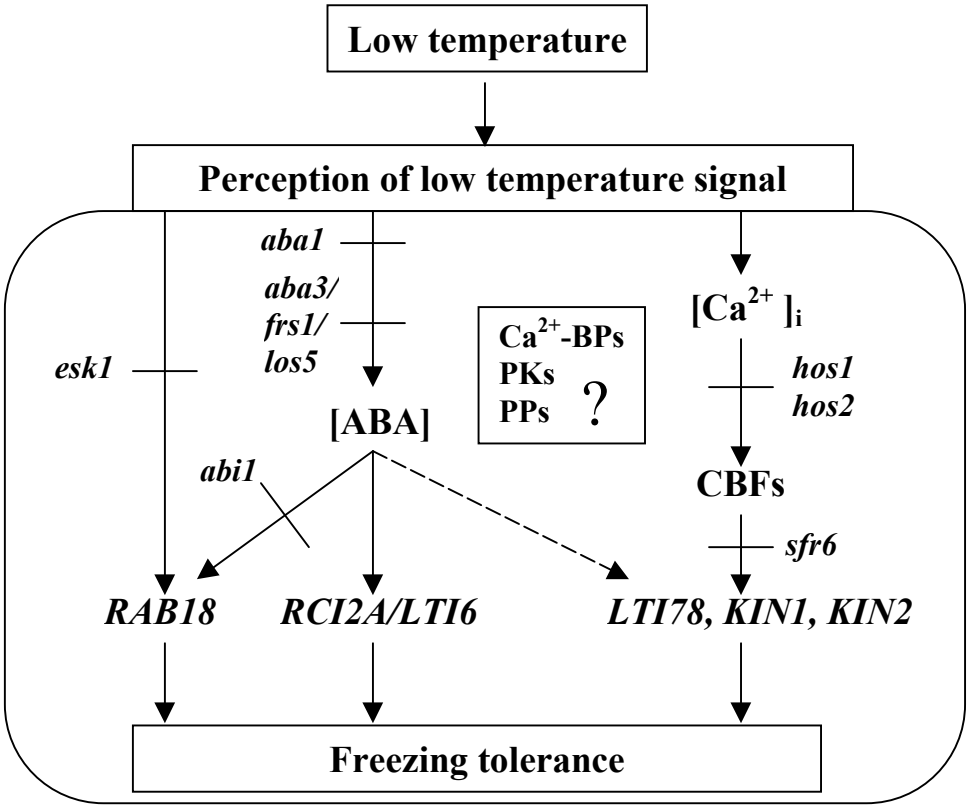

Figure 2. Summary of signalling pathways regulating the expression of several cold-inducible genes during cold acclimation in *Arabidopsis thaliana.* The signalling pathways presented in this picture has been shown to be involved in regulation of gene expression and development of freezing tolerance as discussed in the text.

AIMS OF THE PRESENT STUDY

The general objective of this study was to understand how plants sense cold temperature and how this signal is transduced to the level of gene expression by identifying and characterising key elements regulating the plant responses to low temperature. The specific objectives were:

1. To examine the involvement of calcium signalling during cold acclimation of *Arabidopsis thaliana* and in the cold induction of gene expression.

2. To elucidate the functional role of PP2C, AtPP2CA, in cold acclimation of *Arabidopsis thaliana* and to identify the protein, which interacts with AtPP2CA.

3. To characterise the involvement of PP1, TOPP1, in abiotic stress responses *in Arabidopsis thaliana*.

MATERIALS AND METHODS

Plant material

The object of this study was *Arabidopsis thaliana* L. Heynh. (2n= 10), which is a small weed in the *Brassicaceae* (mustard) family (Rédei, 1992). *Arabidopsis* has many advantages which make it an excellent model plant in plant physiology and molecular biology, such as a short generation time, a large number of offspring and a small size of a plant. Furthermore, *Arabidopsis* is relatively easy to cross (Rédei and Koncz, 1992) and it can be easily transformed by using the *Agrobacterium* mediated transformation method (Bechtold *et al*., 1993). Recently, the small nuclear genome of *Arabidopsis thaliana* was completely sequenced (The Arabidopsis Genome Initiative, 2000). Moreover, there are numerous *Arabidopsis* mutants available (Rédei and Koncz, 1992). As a chilling tolerant plant, *Arabidopsis* is also an excellent model plant for studying the cold acclimation process because it is capable of increasing its freezing tolerance in response to low temperature (Gilmour et. al., 1988; Kurkela *et al*., 1988).

In this study *Arabidopsis thaliana* L. Heynh. ecotypes Landsberg *erecta* (L*er*) and Columbia (Col-1) (Rédei, 1992) were used. Plants were grown and exposed to various stress and chemical treatments as described in more detail in the articles (I, II, IV).

Methods

Technique:	used and described in paper:
Isolation of plant DNA	IV
Isolation of plant RNA	I, II, IV
PCR cloning	II, III, IV
Vector construction	II, III, IV
DNA gel blot analysis	IV
RNA gel blot analysis	I, II, IV
Plant transformation	II, IV
Yeast two-hybrid analysis	III
Ion leakage measurements	I, II, IV
RC4D based cDNA amplification	I
Measurement of leaf chlorophyll content	IV

RESULTS

Calcium signalling is required for cold-induced gene expression and development of freezing tolerance in *Arabidopsis* (I)

The involvement of calcium signalling in the development of freezing tolerance in *Arabidopsis thaliana* was examined by using a pharmacological approach. Treatments of *Arabidopsis* plants with chemicals which either chelate extracellular calcium (EGTA) or block the plasma-membrane Ca^{2+}-channels (La^{3+} and Gd^{3+}) inhibited cold acclimation as well as cold-induced *KIN* gene expression (Figures 1 and 2) suggesting that an influx of extracellular calcium is required for these responses. An inhibitor of calcium release from intracellular stores (ruthenium red) also inhibited *KIN* gene expression and development of freezing tolerance but only partially (Figures 1 and 2) suggesting that also a release of intracellular calcium is required for full development of freezing tolerance and expression of *KIN* genes. The Ca^{2+}-independent protein-kinase inhibitor (H7) reduced the expression of *KIN* genes in response to low temperature but not the freezing tolerance of *Arabidopsis* (Figures 1 and 2) suggesting that Ca^{2+}-independent protein kinases regulate *KIN* gene expression but not the cold acclimation process. In contrast, an inhibitor of CDPKs and CaM (W7) also prevented cold acclimation as well as reduced the cold-induction of *KIN* genes (Figures 1 and 2) indicating the involvement of these proteins in both responses. Using the RC4D protocol, five CDPK clones were identified which exhibited homology to known plant *CDPK* genes (Figure 4). These *CDPK*s were upregulated by cold stress in *Arabidopsis* (Figure 3) suggesting that they play a role in calcium signalling during cold acclimation. This study thus proposes an important role for Ca^{2+} influx and for Ca^{2+} binding proteins during the cold acclimation of *Arabidopsis*.

AtPP2CA negatively regulates ABA responses during cold acclimation and interacts with a potassium channel protein (II & III)

To elucidate the role of PP2Cs in the development of freezing tolerance in *Arabidopsis* the expression of *AtPP2CA* and the related *PP2C* gene, *ABI1* in response to abiotic stresses were studied using the Northern blot technique. Cold, dehydration and salt stresses as well as exogenous ABA induced the expression of both *PP2C* genes (Figure 2). Utilizing various ABA signalling mutants the cold and drought-induced expression of these genes was shown to be ABA-dependent, but divergent in different mutants (Figure 2). In addition, the two *PP2C* genes, *AtPP2CA* and *ABI1,* exhibited differences in their temporal expression patterns in response to low temperature (Figure 1b). To further elucidate the function of AtPP2CA in cold acclimation and freezing tolerance, the corresponding gene was silenced by antisense inhibition (Figure 3). Both exposure to low temperature and exogenous ABA resulted in clearly accelerated development of freezing tolerance in transgenic *AtPP2CA* antisense plants compared to wild-type plants (Figures 4a and b) suggesting a negative role for AtPP2CA in controlling cold and ABA responses. Enhanced ABA sensitivity of transgenic *AtPP2CA* antisense plants during cold acclimation (Figure 4b) suggested that AtPP2CA might negatively regulate ABA signalling. The cold and ABA-induced expression of stress and ABA-responsive genes, *LTI78*, *RAB18* and *LTI6,* but not the cold specific *CBF1* gene, were enhanced in transgenic *AtPP2CA* antisense plants compared to wild-type plants (Figure 5) indicating that enhanced expression of stress-inducible genes was not due to enhanced expression of *CBF1*. The potassium channel protein, AKT3 was identified as an interacting partner of AtPP2CA by a yeast two-hybrid screen. Deletion analysis showed that the catalytic domain of AtPP2CA was essential for the interaction with AKT3. The interaction between AKT3 and ATPP2CA was shown to be specific since the related PP2C, ABI1, did not interact with AKT3 in yeast (Figure 1). Taken together, the results suggest that AtPP2CA, which interacts with AKT3, is a negative regulator of ABA responses during cold acclimation.

TOPP1 positively regulates oxidative stress and ABA-mediated cold stress signal transduction (IV)

Induction of *TOPP1* expression in response to abiotic stresses, low temperature, drought

and salt, as well as to exogenous ABA (Figure 1) suggested that TOPP1 might have a role in abiotic stress responses. To elucidate the function of TOPP1 in this stress response, transgenic plants with altered levels of *TOPP1* expression were generated (Figure 2). Since accumulation of ROS is a common denominator in various stresses the oxidative stress tolerance of transgenic *TOPP1* sense and antisense plants together with wild-type plants was examined by exposing them to methyl viologen. The results indicated that plants overexpressing *TOPP1* were more oxidative stress tolerant than wild-type or transgenic *TOPP1* antisense plants, which were even more sensitive than wild-type plants (Figure 3). Cold acclimation under low light conditions showed that there was no difference in freezing tolerance between wild-type and transgenic *TOPP1* sense and antisense plants (Figure 4b). In contrast, exposure to low temperature under high light conditions demonstrated that *TOPP1* antisense plants were impaired in their ability to cold acclimate (Figure 4a) suggesting a role for TOPP1 in cold stress responses. Enhanced cold-induced expression of ABA-dependent genes in TOPP1 sense plants but abolished in antisense plants under high light conditions (Figure 5) indicated a role for TOPP1 as a positive regulator of ABA signalling.

DISCUSSION

All four studies forming this thesis are discussed in detail in the relevant scientific context in the corresponding articles. Therefore only selected conclusions are presented in this chapter.

IP_3 and cADPR-regulated Ca^{2+} release from the vacuole is required for cold responses (I)

This study shows that influx of extracellular Ca^{2+} is required for development of freezing tolerance in *Arabidopsis*. Together with previous studies with alfalfa (Monroy and Dhindsa, 1993) this study indicates that Ca^{2+} is a common denominator involved in the triggering of the development of freezing tolerance in plants. Recent studies with canola support this suggestion (Sangwan *et al*., 2001). Further support comes from the studies which show that intracellular Ca^{2+} levels increase transiently in response to cold shock in *Arabidopsis* (Knight *et al*., 1996; Lewis *et al*., 1997; Polisensky and Braam, 1996).

This study also demonstrates that, in addition to extracellular Ca^{2+}, also Ca^{2+} release from intracellular stores is involved in the cold acclimation process. IP_3-mediated Ca^{2+} release from the vacuole has been implicated in the cold shock-elevated increase in cytosolic Ca^{2+} levels (Knight *et al*., 1996). IP_3 has also been reported to induce expression of cold- and ABA-responsive genes *LTI78* and *KIN2* (Wu *et al*., 1997). Heparin, a competitive antagonist of the IP_3 receptor blocked this IP_3-induced gene expression (Wu *et al*., 1997) confirming a role for IP_3-mediated Ca^{2+} signalling in the regulation of the gene expression. In addition to IP_3, cyclic ADP-ribose (cADPR), a mediator of ABA signalling in plants, induces the release of Ca^{2+} from the vacuole (Wu *et al*., 1997). A putative receptor for cADPR is the ryanodine receptor (RyR) (Wu *et al*., 1997). Notably, Wu *et al*. (1997) did not study the effects of agonists and antagonists of ryanodine receptors on the expression of *LTI78* and *KIN2* genes. Instead, this study shows that such an inhibitor, ruthenium red, partially inhibits *KIN1* and *KIN2* transcript accumulation and freezing tolerance in cold-stressed *Arabidopsis* suggesting a role for cADPR in these responses. Recent studies with canola supports this proposal. Sangwan *et al*., (2001) demonstrated that application of cADPR at normal growth temperature increased cold-responsive gene expression and freezing tolerance. Therefore, both IP_3 and cADPR regulated Ca^{2+} release from the vacuole is involved in regulation of cold responses.

Furthermore, this study implicates CDPKs and CaM as mediators of cold-induced calcium signal transduction in *Arabidopsis*. The isolation of cold-inducible *CDPK* genes from *Arabidopsis* suggested an important role for these proteins in transduction of cold-induced calcium signal. Indeed, inhibition of the Ca^{2+} binding proteins, CaMs and CDPKs, decreased the cold-induced *KIN* gene expression and prevented cold-acclimation. Antagonist of Ca^{2+} - independent protein kinases also inhibited expression of *KIN* genes but not the development of freezing tolerance. Thus, it was concluded that partially different signalling pathways lead to cold acclimation and *KIN* gene expression. Wu *et al*. (1997) showed that the cADPR mediated ABA signalling pathway is regulated by protein phosphorylation and that inhibition of protein kinases also blocks ABA-induced expression of *LTI78* and *KIN2*. Therefore, it is possible that protein kinases regulate Ca^{2+} release from intracellular stores, which seems to be involved in both development of freezing tolerance and the transcriptional activation of these genes in response to low temperature.

AtPP2CA may regulate cADPR-mediated ABA signalling (II)

This study shows that AtPP2CA as a negative regulator of ABA signalling also modulates expression of cold and ABA-responsive genes. In addition to ABA-dependent genes, such as *RAB18* and *RCA2A*/*LTI6* (Capel *et al*., 1997: Lång and Palva, 1992; Nylander *et al*., 2001), also the cold-induced expression of *LTI78* (Nordin *et al*., 1991) was enhanced in transgenic *AtPP2CA* antisense plants. In contrast to previous studies (Nordin *et al*., 1993), the results suggest that the ABA signalling pathway is also involved in regulation of *LTI78* gene expression during cold

acclimation. Thus, both ABA-independent and ABA-dependent signalling pathways regulate cold-induced expression of *LTI78*. Since *KIN1* and *KIN2* belong to the same class of genes in CBF regulon as *LTI78* (Thomashow, 1999) it is tempting to suggest that Ca^{2+} influx through plasmalemma channels is also required for the expression of *LTI78* as for the expression of *KIN* genes (I). Furthermore, since ABA-induced expression of *LTI78* and *KIN2* is regulated by cADPR mediated Ca^{2+} release from the vacuole (Wu *et al*., 1997, I) it seems possible that cold-induced ABA-dependent expression of *LTI78* is mediated through this signalling pathway. Taken together, this study shows that AtPP2CA regulates ABA-dependent cold signalling possibly through cADPR.

AKT3 may be involved in pH signalling (III)

This is the first study identifying a substrate of PP2C implicated in ABA signalling. The results show that AtPP2CA specifically interacts with potassium channel AKT3, also known as AKT2 (Lacombe *et al*., 2000). The interaction between AtPP2CA and AKT3 indicates that this K^+ channel may be directly dephosphorylated by PP2C. Still, it is unclear how this affects AKT3 activity. However, phosphorylation of another K^+ channel, KAT1, by CDPK results in inactivation suggesting that AKT3 might be also regulated by phosphorylation. Indeed the AKT3 channel is blocked by Ca^{2+} ions (Marten *et al*., 1999) suggesting that CDPK may negatively regulate AKT3 channel activity whereas dephosphorylation of AKT3 would activate the channel. However, further studies are needed to prove this hypothesis.

Based on RNA blots and promoter activity studies, AKT3 was shown to be predominately expressed in leaves (Dennison *et al*., 2001; Lacombe *et al*., 2000; Marten *et al*., 1999). Since AKT3 was shown to be expressed mainly in the phloem and it was capable of mediating K^+ fluxes, AKT3 was proposed to be involved in K^+ transport in phloem tissues and thus play a role in sugar translocation (Lacombe *et al*., 2000; Marten *et al*., 1999). An impairment of AKT3 activity in sugar translocation would be expected to affect growth. Yet, studies with plants containing T-DNA insertion mutation in AKT3 showed that AKT3 does not contribute to seedling growth rate (Dennison *et al*., 2001). Thus, the function of AKT3 is still unknown.

Based on the amino acid sequence homology to previously identified plant genes encoding K^+ channels, AKT3 was classified as an inward rectifying K^+ (K^+_{in}) channel (Cao *et al*., 1995; Ketchum and Slayman, 1996). However, recent studies have revealed that AKT3 has unique functional features that are not displayed by any other K^+ channel studied thus far. In addition to inward K^+ currents AKT3 also mediates K^+ efflux. Most strikingly, both extracellular and intracellular acidification decreases the activity of AKT3 (Lacombe *et al*., 2000; Marten *et al*., 1999) whereas the other K^+_{in} channels are activated by a decrease in pH (Zimmermann *et al*., 1999). Thus, it is evident that AKT3 is a unique type of K^+ channel.

Since cytosolic pH is strictly regulated, it has been proposed that it serves a second messenger (Zimmermann *et al*., 1999). However it is not know how this signal is sensed. It has been put forward that either pH sensitivity of the channels themselves or a membrane-delimited signalling pathway mediates pH sensing (Zimmermann *et al*., 1999). Interestingly, ABA is known to evoke a considerable alkalisation of the cytoplasm (Zimmermann *et al*., 1999). The unique response of AKT3 to pH (Lacombe *et al*., 2000; Marten *et al*., 1999) as well as ABA-induced upregulation of AKT3 gene expression (Lacombe *et al*., 2000) suggests a role for AKT3 in pH-mediated ABA signalling.

TOPP1 may positively regulate ROS-mediated Ca^{2+} influx (IV)

TOPP1 was shown to positively regulate both oxidative stress and ABA-mediated cold stress signalling. Recently, both *abi1-1* and *abi2-1* mutations were reported to disrupt the ROS-mediated ABA signal transduction pathway, which regulates Ca^{2+} influx (Murata *et al*., 2001). Notably, these mutations also abolish the ABA-dependent cold-induction of *RAB18* (Lång and Palva, 1992; Tähtiharju and Palva, unpublished results) suggesting that ROS-mediated ABA

signalling is involved in cold-induced expression of *RAB18*, which is positively regulated by TOPP1. Since this pathway leads to Ca^{2+} influx, TOPP1 may also regulate Ca^{2+} signalling. Indeed, Ca^{2+} influx through specific plasmalemma Ca^{2+} channels is a major requirement in the ABA signalling chain leading to *RAB18* expression (Ghelis *et al*., 2000). The ROS-mediated ABA activation of plasma membrane Ca^{2+} channels was shown to require cytosolic NAD(P)H suggesting that NAD(P)H oxidases contributes to ABA signal transduction (Murata *et al*., 2001). In addition to cell wall NAD(P)H oxidase regulated ROS production, there are also several other known sources of ROS in plants (Dat *et al*., 2000). Indeed, TOPP1 was shown to mediate tolerance against oxidative stress, which was generated via ABA-independent production of ROS, indicating that TOPP1 acts downstream of ROS. Notably, ABA-independent ROS signalling pathways may also regulate Ca^{2+} influx as shown by Price *et al*. (1994), who demonstrated that treatment of tobacco seedlings with H_2O_2 resulted in transient increase in cytosolic Ca^{2+} concentration. Thus, it is likely that TOPP1 positively regulates ROS-mediated Ca^{2+} influx.

A hypothetical model for calcium and ABA-mediated cold signal transduction (I-IV)

The role of calcium and protein phosphatases 1 and 2C in cold acclimation is summarized in Figure 3. The Roman numerals I-IV refer to the original publications, in which the results were reported. For simplicity only signalling pathways discussed in this study are presented.

At the onset of cold acclimation low temperature triggers Ca^{2+} influx through plasmalemma calcium channels. Increase in intracellular Ca^{2+} concentration is further mediated to the level of gene expression via CaMs and CDPKs allowing a rapid response to the decline in temperature. Low temperature also evokes production or release of ABA, which activates several signal transduction cascades such as the one involving cADPR and the other involving ROS mediated signalling. Low temperature also leads to ABA independent production of ROS. The ABA-dependent ROS pathway is abolished in *abi1* and *abi2* mutants and positively regulated by TOPP1. ROS production through this pathway promotes Ca^{2+} influx leading to expression of a set of ABA-dependent genes. The cADPR mediated ABA signal transduction pathway regulates Ca^{2+} release from intracellular Ca^{2+} stores. Ca^{2+} regulated activity of PI-PLC regulates production of IP_3 which also regulates Ca^{2+} release from intracellular stores. AtPP2CA negatively regulates both of the ABA signalling pathways probably through AKT3.

Cold acclimation involves cross-talk between various signalling pathways (I-IV)

Taken together this study shows that it is not appropriate to consider the events that result in cold acclimation as a simple linear pathway. Indeed, studies with various cold signalling mutants have shown that in most cases activation or blocking of one pathway leads to only partial enhancement or inhibition in capacity to cold acclimate (Ishitani *et al*., 1998; Knight *et al*., 1996; Lee *et al*,. 1999; Mäntylä *et al*., 1995; Xin and Browse, 1998) also suggesting a role for other pathways in this response. At present, it is unclear which genes and signalling pathways are essential for the development of freezing tolerance and which are activated as a general response to low temperature, but are not directly involved in the development of freezing tolerance. The sequence of events in cold signalling is also unclear. Given that injury during freezing is caused mainly by cellular dehydration (Steponkus, 1984), but also by ROS (Thomashow, 1999), it is likely that various signalling pathways leading to tolerance against these stresses are all involved in the full development of freezing tolerance. Furthermore, it seems that cooperation between these pathways reinforce the cold signal. Indeed, low temperature and ABA are additive in induction of gene expression (Xiong *et al*., 1999). The important role of Ca^{2+} as a second messenger in abiotic stress signalling has suggested a role for Ca^{2+} as an important nodal point at which cross-talk can occur (Knight and Knight, 2001). Although it is not yet clear, the amplitude, duration and frequency of stimulus-induced cytosolic Ca^{2+} elevations may encode information

about the particular stimulus providing specificity in signalling (Rudd and Franklin-Tong, 1999). IP_3-mediated Ca^{2+}-induced Ca^{2+} release from intracellular stores as well as cADPR-mediated Ca^{2+} signalling may be involved in this decoding of signal messages (Rudd and Franklin-Tong, 1999). In conclusion, it is becoming increasingly evident that cold signal transduction is a complex process in which parallel and branched signalling pathways converge and cross-talk leading to development of freezing tolerance.

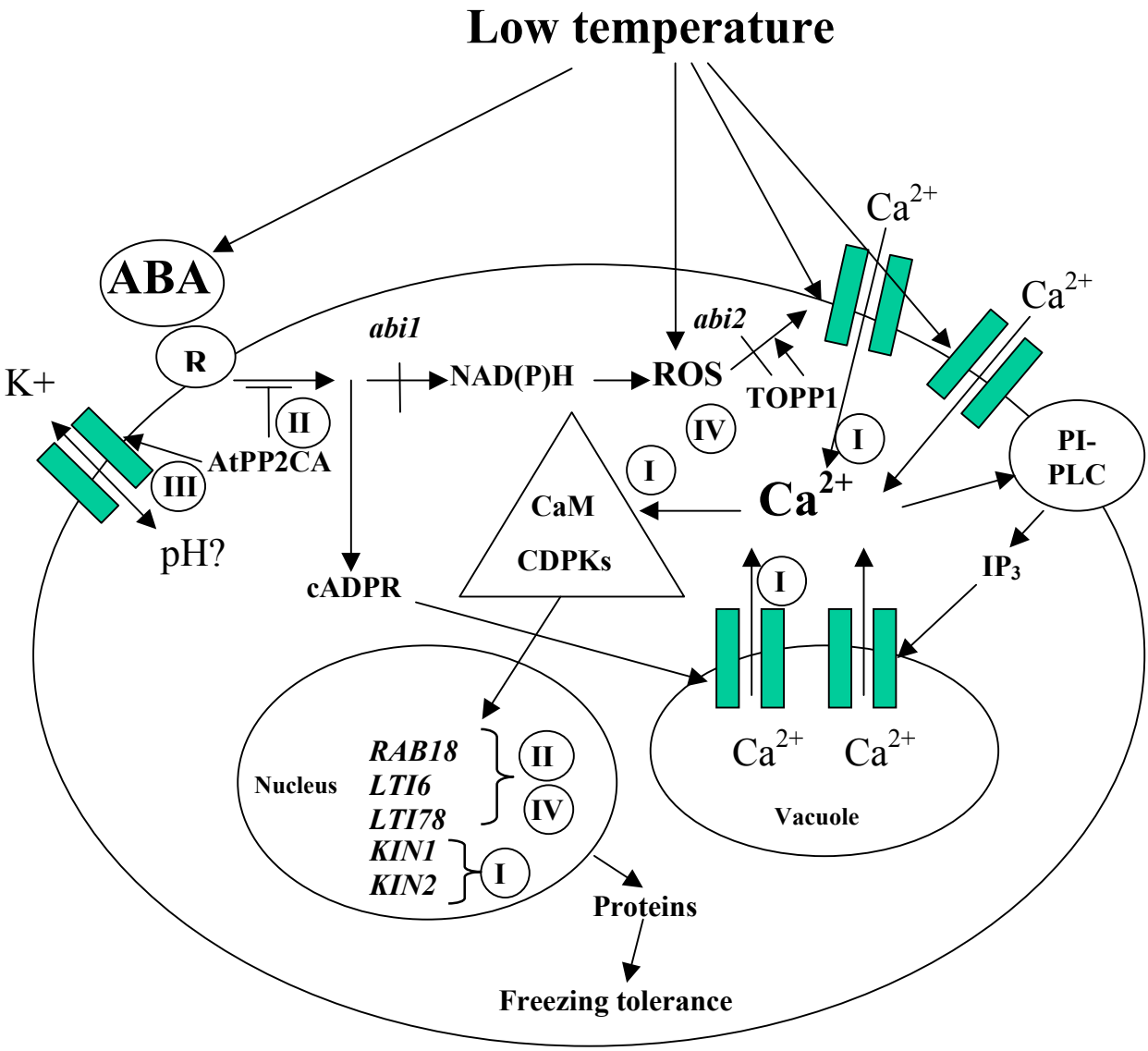

Figure 3. The hypothetical model for Ca^{2+}- and ABA-mediated cold signal transduction in *Arabidopsis thaliana*. Cold signal transduction is a complex process in which several parallel and branched signalling pathways converge and cross-talk leading to development of freezing tolerance as discussed in the text. The Roman numerals I-IV refer to the original papers where the results are reported. R symbolises the yet unidentified ABA receptor.

CONCLUDING REMARKS

This study demonstrates the importance of Ca^{2+} as a second messenger in cold signal transduction in *Arabidopsis*. Furthermore, it shows that Ca^{2+} mediated cold signal transduction is a complex process, which involves an increase in cytosolic Ca^{2+} levels through the action of both plasmalemma and tonoplast Ca^{2+} channels and transduction of this signal via various cold-upregulated Ca^{2+} binding proteins to the level of gene expression. It was also demonstrated that in addition to Ca^{2+}, also ABA and ROS play an important role in cold signal transduction. Regulation of cold-induced ABA signalling positively by protein phosphatase 1 (TOPP1) and negatively by protein phosphatase 2C (AtPP2CA) indicates that this hormonal signalling is tightly regulated. Involvement of TOPP1 also in oxidative stress tolerance suggested that the signalling pathways regulated by these protein phosphatases might cross-talk. Together with the studies the results further propose a role for Ca^{2+} as an important node at which cross-talk occurs.

Taken together, it is increasingly evident that common cellular signal transduction pathways mediate overlapping responses to different environmental stresses. Thus, understanding the cross-talk between different signal transduction pathways will become increasingly important for our understanding of complex signalling networks. The use of micro array technology will help to reveal which signalling pathways are active in different cell types during various stresses. Furthermore, the role of the putative signalling components in specific stress and cross-talk can be elucidated by phenotypic analysis of corresponding knockout mutants. In the long term, targeting the genes encoding components of stress-related signal transduction pathways may be more profitable than manipulation of individual genes at the termini of these cascades. This may open new ways for the engineering of crops with an increased ability to adapt to several stresses experienced concurrently in the field.

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