Overwintering in Woody Plants: Involvement of ABA and Dehydrins

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which will be referred to in the text with their Roman numerals.

I **Welling A, Kaikuranta P and Rinne P** (1997) Photoperiodic induction of dormancy and freezing tolerance in *Betula pubescens*. Involvement of ABA and dehydrins. Physiol Plant **100**: 119-125

II **Rinne P, Welling A and Kaikuranta P** (1998) Onset of freezing tolerance in birch (*Betula pubescens* Ehrh.) involves LEA proteins and osmoregulation and is impaired in an ABA-deficient genotype. Plant Cell Environ **21**: 601-611

III **Welling A, Moritz T, Palva ET, Junttila O** (2002) Independent activation of cold acclimation by low temperature and short photoperiod in hybrid aspen. Plant Physiol **129**:1633-1641

IV **Welling A, Rinne P, Viherä-Aarnio A, Kontunen-Soppela S, Heino P, Palva ET** (2003) Photoperiod and temperature differentially regulate the expression of two dehydrin genes during overwintering of birch (*Betula pubescens* Ehrh.). (Submitted manuscript)

ABBREVIATIONS

ABA	abscisic acid	kPa	kilo Pascal
ABI	ABA insensitive	LD	long daylength
ABRE	ABA responsive elements	LEA	late embryogenesis abundant
AFP	antifreeze proteins	LFR	low-fluence response
AM	apical meristem	LT	low temperature
ANOVA	analysis of variance	LT_{50}	lethal temperature for 50% of the tissues
ATP	adenosine triphosphate	LTE	low temperature exotherm
B	blue light	LTRE	low temperature responsive element
BSP	bark storage proteins	M	mitosis in cell cycle
CBF	CRT/DRE binding factor	mRNA	messenger RNA
COR	cold responsive	phot	phototropins
CRT	C-repeat	phy	phytochrome
cry	cryptochromes	Pfr	far-red-light absorbing conformation of phytochrome
DHN	dehydrin	Pr	red-light absorbing conformation of phytochrome
DNA	deoxyribonucleic acid	R	red light
DRE	dehydration responsive element	RH	relative humidity
DREB	DRE binding protein	RNA	ribonucleic acid
DTA	differential thermal analysis	ROS	reactive oxygen species
EOD	end-of-day response	S	DNA synthesis in cell cycle
FR	far-red light	SD	short daylength
G_1	first gap in cell cycle	HTE	high temperature exotherm
G_2	second gap in cell cycle	T_m	membrane phase transition temperature
GA	gibberellic acid	UV	ultraviolet
HIR	high-irradiance response	VLFR	very-low-fluence response
HTE	high temperature exotherm	WS	water stress
kD	kilo Dalton		

SUMMARY

Boreal and temperate zone trees have been able to colonize the northern areas of the world by an overwintering process, during which they are in a dormant stage and have high tolerance against freezing. Freezing tolerance increases in a cold acclimation process that is initiated by short daylength (SD). Subsequent low and freezing temperatures are needed for full acclimation. One of the most important factors in cold acclimation is the increased tolerance against cellular dehydration caused by extracellular freezing. Abscisic acid (ABA) is a plant stress hormone involved in plant responses to abiotic stresses with a dehydration component, such as low temperature and drought. ABA is involved in the regulation of a number of genes that respond to dehydration and cold stress. Dehydrins are proteins that have been shown to accumulate in vegetative tissues during stresses that cause cellular dehydration, such as drought, salinity and cold and in seeds during embryogenesis. Some of the dehydrins are also responsive to ABA. The objective of the present study was to gain information about the involvement of ABA and dehydrin proteins in the overwintering process of trees. In addition to different latitudinal ecotypes of pubescent birch (*Betula pubescens* Ehrh.), ABA deficient birch (*Betula pubescens*. f. *hibernifolia* Ulvinen) and transgenic hybrid aspen (*Populus tremula* L. x *P. tremuloides* Michx.) overexpressing oat *PHYA* gene were used as model systems.

The results demonstrate that both abscisic acid (ABA) and dehydrins are involved in overwintering process in woody plants. Photoreceptor phyA plays a role in perceiving the short daylength signal that initiates growth cessation and dormancy development in woody plants. ABA might participate in signalling cascade leading to growth cessation and dormancy, but indirectly, through changes in sensitivity to ABA under SD conditions. Growth cessation under SD conditions enables resource allocation to storages that are needed during cold acclimation at freezing temperatures. Similarly to herbaceous species, woody plants have both ABA dependent and ABA independent pathway for cold acclimation, but these functions both during SD induced first stage of acclimation and during subsequent stages of acclimation, induced by low and freezing temperatures, respectively. ABA dependent and independent pathways may also converge, this was especially demonstrated under SD conditions, resulting more quick freezing tolerance in the field or higher freezing tolerance under SD conditions. Two types of dehydrins were characterized in birch. The others were mainly under developmental regulation and played a role in anticipation of stress, especially during overwintering, while the others were induced rapidly during the actual experience of stress. These distinct types of *DHN*s were expressed in sequential order during overwintering, in response to SD and freezing temperatures, suggesting that in trees DHNs participate in protection of cellular dehydration during SD induced programmed dehydration and during freezing induced cellular dehydration.

1. INTRODUCTION

Trees are among the largest and longest-living organisms on the earth. Because of their size and long lifespan, they have to be able to cope with extremes of environmental conditions during their lifetime. This study is focusing on freezing tolerance of trees growing in the boreal and temperate zones. These trees have been able to colonize the northern areas of the world despite the conditions during winter when temperature may drop down to –50ºC or even lower and night can last for two months. This has been enabled by an overwintering period, during which trees are in a dormant stage and have high tolerance against freezing. One of the critical components of the overwintering process is cold acclimation that leads to increase in freezing tolerance. Cold acclimation has been studied mostly in herbaceous plants, in particular in the model plant *Arabidopsis thaliana*. Freezing tolerance of *Arabidopsis*, exposed to low, non-freezing temperature (LT) increases very rapidly, within a day, and provides protection over a transient cold period during the growing season. Although this type of short-term acclimation shares common components with freezing tolerance development in trees, overwintering process of trees is complicated with simultaneous dormancy development. In addition, photoperiod, that is the main factor determining the initiation of freezing tolerance development of trees, is of less importance in cold acclimation in herbaceous species.

One of the most important factors in cold acclimation is increased tolerance against cellular dehydration caused by extracellular freezing. Accumulation of compatible solutes, sugars and special proteins including dehydrins, which all protect cellular components under drought stress, have been shown to be important factors also in cold acclimation. Abscisic acid (ABA) is a plant stress hormone involved in plant responses to abiotic stresses with a dehydration component, such as low temperatures and drought. ABA is involved in the regulation of a number of genes that respond to dehydration and low temperature stress. Although a wealth of information regarding cellular signalling and regulation of gene expression in response to LT in herbaceous species has accumulated during the last years, the overwintering process of trees is still largely uncharacterized. The aim of this study was to elucidate the involvement of ABA and dehydrins in the overwintering process of trees, with special emphasis on development of freezing tolerance and dormancy.

1.1. Dormancy

1.1.1. Phenology of dormancy

Dormancy on its broadest definition could be defined as lack of visible growth. It is a survival strategy that enables plants or plant parts to survive through periods unfavourable for

growth. Although roots and vascular cambium may also become dormant, bud dormancy is by far the most important for trees, since it determines the synchronisation between seasonal growth and rest, and is also controlling the growth habit and tree form (Rohde et al. 2000b). Many annual plants overwinter as seeds and some features of seed dormancy resemble bud dormancy of trees. These include for example chilling requirement for dormancy release and involvement of growth regulators for both dormancy maintenance and release (Dennis 1996). The mechanism of dormancy is complex and regulated by various different factors. Consequently, it has been difficult to create a simple nomenclature that would describe unambiguously the factors that keep the organ in a dormant state. Lang et al. (1987) have introduced terms endo-, eco- and paradormancy. Briefly, endodormancy refers to dormancy that is caused by internal factors within the organ, ecodormancy is regulated by environmental factors and paradormancy is regulated by physical factors outside the organ, like in the case of apical dominance. However, dormancy is a result of complex interactions between environmental, physiological and anatomical factors, and both the meristematic tissue itself and the surrounding tissues are involved in the regulation of dormancy development and maintenance. Thus, this nomenclature as such is too simple and in order to be precise, it is still necessary to describe the environmental factors and physiological mechanisms that control the dormancy in question (Junttila 1988).

Apical bud, bearing the apical meristem (AM), is formed during embryogenesis, whereas axillary or lateral buds are formed in axis of leaf petiole as a function of AM. During growth, cell proliferation and organogenesis occur at AM, whereas elongation occurs in the subapical meristem immediately beneath the AM. Bud is a short axis with leaf primordia covered by bud scales. After formation, axillary buds are in paradormancy, *i.e.* the presence of the apical bud prevents their growth. If the apical bud of the stem is removed, all buds along the stem show increased activity, but soon one of the uppermost buds is transformed to an apical bud and apical dominance is restored (Rohde et al. 1997). In many boreal and temperate zone trees the growth of the apical bud stops in response to the shortening daylength in late summer. Young trees are more sensitive to photoperiod than adult trees, which usually stop growth in mid-summer, independently of photoperiod (Junttila 1976). A few weeks after growth cessation both apical and axillary buds enter into endodormancy, in which they are incapable of growth even under favourable growth conditions and removal of the apical bud does not induce growth of the lateral buds. Endodormancy is broken by chilling treatment, which is defined usually as hours at temperatures between 0 to +7°C (Hänninen 1990), although also freezing temperatures have been shown to release buds from endodormancy (Rinne et al. 1997, Cox and Stushnoff 2001), as well as sublethal heat stress (Shirazi and Fuchigami 1995, Wisniewski et al. 1997). After a certain amount of chill units endodormancy of the buds is broken, and buds are in ecodormancy. The optimum chilling temperature varies between species. In addition, other species show early release from endodormancy and a long chilling requirement, whereas others are released slowly from endodormancy and are almost immediately ready to grow (Heide 1993a). Some species, such as *Fagus sylvatica*, require also long daylength for bud burst (Heide 1993b). At this stage, buds measure accumulation of two contrasting matters, chilling and the heat sum, the latter being hours at temperature above +5°C. The more they get chilling units, the less they need heat sum before they burst. The time buds need before they burst under favourable conditions has been defined as “thermal time” (Murray et al. 1989). This mechanism prevents too early bud burst during transient warm weather late in autumn or early in spring.

1.2. Ultrastructural changes during dormancy

1.2.1. Changes in the apical meristem

Although dormancy development affects the physiology of the whole tree, a small group of cells is responsible for initiation and release of dormancy. Above mentioned shoot apical meristem (AM) contains a group of cells that generate the shoot of the plant by initiating new organs and new tissues, communicating with the rest of the plant and maintaining itself as a formative region (Medford 1992). All the cells in AM are connected via plasmodesmata, allowing symplastic communication between cells, thereby facilitating direct intercellular diffusion, current flow and trafficking of proteins and mRNA (Jian et al. 1997, van der Schoot and Rinne 1999, Rinne et al. 2001). Short daylength (SD) leads to closure of plasmodesmata (Jian et al. 1997, Rinne and van der Schoot 1998) and it has been suggested that growth cessation and dormancy development is caused by this altered cell-to-cell communication (Jian et al. 1997, Rinne and van der Schoot 1998). In birch plasmodesmata are closed under SD conditions by callose that is synthesized by 1,3-β-glucan synthase (Rinne and van der Schoot 1998). Breakage of bud dormancy by chilling involves restoration of the symplasmic organization of the meristem. Restoration is likely to be mediated by 1,3-β-D-glucanase. This enzyme is located in small spherosome-like vacuoles that arise *de novo* in the cytoplasm during dormancy induction. When buds are released from dormancy during chilling, these vacuoles become aligned with the plasma membrane and are often associated with plasmodesmata, enabling 1,3-β-D-glucanase to degrade the callose and open the plasmodesmata (Rinne et al. 2001).

1.2.2. Cytoplasmic alterations

SD induced growth cessation leads to changes in source-sink relationships, allowing accumulation of photosynthesis assimilates and proteins in overwintering organs. Although these changes might not be directly connected to dormancy, growth cessation is a prerequisite for this storage accumulation. During growing season plant cells are characterized with a large central vacuole, which is surrounded by thin peripheral layer of cytoplasm (Niki and Sakai 1981, Wisniewski and Ashworth 1985, Sauter et al. 1996). During autumn cells become temporarily rich in the organelles that are involved in protein synthesis, such as vesicular endoplasmic reticulum, polysomes, dictyosomes and vesicles. Plastids containing starch granules, protein-lipid bodies and mitochondria are also abundant (Kuroda and Sagisaka 1993). During autumn central vacuole is displaced with numerous small vacuolar compartments such as protein storage vacuoles or protein bodies (Sauter et al. 1988, van Cleve et al. 1988) and the number of oleosomes, storing fat, is increased (Sauter et al. 1996). Plastids are initially filled with large starch grains, which disappear when plants are exposed to cold temperature (Rinne et al. 1994b, Sauter et al. 1996).

1.3. Cold acclimation of woody plants

1.3.1. Sequential cold acclimation during overwintering

Simultaneously as trees enter dormancy, their freezing tolerance starts to increase. Cold acclimation of deciduous trees during overwintering has been shown to be a sequential process, which proceeds most effectively when each inductive phase is completed before proceeding to the next (Howell and Weiser 1970, Fuchigami et al. 1971). The first stage is

initiated by short photoperiod and favours relatively high temperature since it involves several metabolic processes that are hampered by low temperature (LT) (Fuchigami et al. 1971, Christersson 1978). During SD leaves play an essential role in receiving the SD signal (Fuchigami et al. 1971) and probably providing energy and metabolites that are used during subsequent second stage of acclimation induced by LT (see Kacperska 1989). Leaf removal before growth cessation leads to an impaired cold acclimation of the stem, after growth cessation it has less effect (Fuchigami et al. 1971). SD induced cold acclimation of stem is relatively slow and freezing tolerance increases after the SD has lead to cessation of growth (Fuchigami et al. 1971, Junttila and Kaurin 1990). Although prolonged SD treatment can increase freezing tolerance significantly, up to LT_{50} of −40°C, subsequent low and freezing temperatures enhance freezing tolerance rapidly (Junttila and Kaurin 1990) and are needed for the development of maximum hardiness (Howell and Weiser 1970, Christersson 1978, Harrison et al. 1978, Greer and Warrington 1982). When temperate zone woody plants are fully acclimated, they can survive even the temperature of liquid nitrogen (-196ºC) (Stushnoff and Junttila 1986, Junttila and Kaurin 1990, Cox and Stushnoff 2001). Photoperiod or leaf removal has no influence at this stage (Howell and Weiser 1970, Bigras and D'Aoust 1993).

1.3.2. Other freezing tolerance inducing factors in woody plants

In general, cold acclimation is initiated by environmental conditions that precede the freezing stress. In addition to acclimating against freezing stress during overwintering, trees are also able to acclimate in response to LT stimulus under LD conditions, reflecting their ability to protect themselves against episodic, unexpected frost encountered during the growing season analogously with herbaceous plants (Junttila and Kaurin 1989, Li et al. 2002). Low non-freezing temperature functions as a trigger for cold acclimation in *Arabidopsis* and many other herbaceous species. In addition, factors that increase tolerance against drought stress usually increase also freezing tolerance. Thus, mild water stress or application of the plant hormone abscisic acid (ABA) has been shown to lead to increased freezing tolerance both in herbaceous and woody species (Irving 1969, Chen et al. 1983, Heino et al. 1990, Tanino et al. 1990, 1991, Lee and Chen 1993, Anderson et al. 1994, Lång et al. 1994, Mäntylä et al. 1995, Li et al. 2002, 2003a). Some of these environmental factors have an additive effect on freezing tolerance if plants are exposed to them at the same time. Combination of two or more of the acclimation inducing factors such as water stress with SD, LT, or ABA results in combined degrees of freezing tolerances (Chen and Li 1978, Li et al. 2002, 2003a). However, the suggestion by Chen and Li (1978), that water stress, LT and SD all induce cold acclimation independently and have an additive effect is perhaps too simple. For example, sequential exposure to SD and LT increases freezing tolerance of woody plants more efficiently than if they are given at the same time (Junttila and Kaurin 1990). It is obvious that cold acclimation causes a biological load on plants and unnecessary high level of freezing tolerance would lead to misuse of resources. On the other hand, too low a level would be mortal. Plants responses to various combinations of these factors can provide a high degree of physiological flexibility that enables plants to acclimate and resist low temperature stress (Howell and Weiser 1970). By observing how serious the conditions are, plants can acclimate adequately.

1.4. Perception of photoperiod and temperature signals

1.4.1. Photoreceptors of plants

Plants are able to detect and respond to the presence, absence, wavelength, intensity, directionality and diurnal duration of impinging light signals (Quail 2002). Light is perceived by several photoreceptors, which detect different facets of the solar spectrum. The cryptochromes (cry) and phototropins (phot) monitor the blue/ultraviolet region of the spectrum, whereas phytochromes (phy) are responsible for the detection of far-red light (FR) and red light (R), but sense also blue and UV light. The cryptochromes and phytochromes control growth and developmental responses according to variation in the wavelength, intensity and diurnal duration of irradiation (Smith 2000), whereas the phototropins function primarily in controlling directional (phototropic) growth in response to directional light and/or intracellular chloroplast movement in response to light intensity (Quail 2002). For example *Arabidopsis* has two chryptochromes (cry1 and cry2), two phototropins and (phot1 and phot2) and five phytochromes (phyA-E). It is not completely understood how photoreceptors mediate the signaling cascades upon light perception and what are the photoreceptors involved in each response. However, it is becoming evident that although early steps of signal perception are specific for a given photoreceptor, eventually signals have interaction and integration (Fankhauser and Staiger 2002).

1.4.1.1. Phytochrome photoreceptors

Phytochromes are the best characterized photoreceptor family. Phytochromes are dimeric chromoproteins with monomers of 120-130 kD. They exist in two spectrally interchangeable forms. For example, after absorbing far-red light (FR), they transform to an inactive red-light (R) absorbing conformation (Pr) and after absorbing R they transform to an active FR absorbing form (Pfr). In addition, the Pfr forms of all types of phytochromes are converted back to Pr not only by treatment with FR but also in darkness – the so called dark-reversion (Figure 1). Based on this photoreversibility, one can infer that responses that exhibit

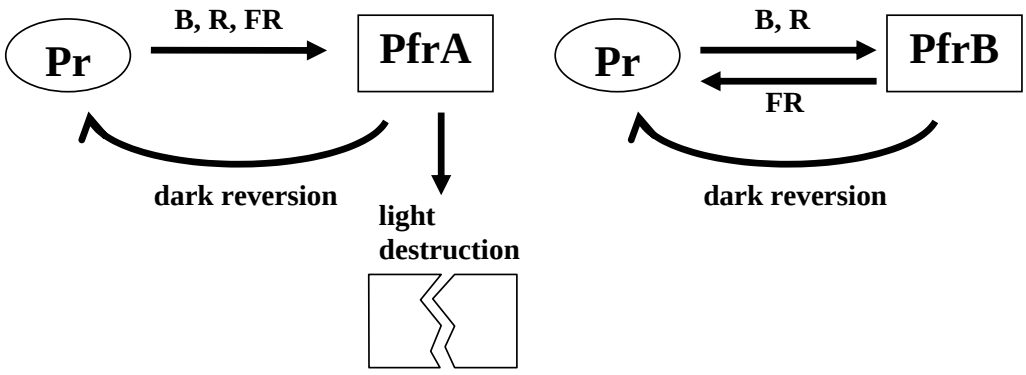

Figure 1. Schematic presentation of conformational changes of phyA and phyB in response to different wavelengths and darkness. Abbreviations for wavelengths are B, blue light; R, red light and FR, far-red light. (Adapted from Chory 1997).

R/FR reversibility are controlled by phytochrome. This R/FR reversibility functions in light levels of 1 to 1000 μmol/m^2 and is called low-fluence-response (LFR). On the other hand, the two other modes of phytochrome action, very-low-fluence-response (VLFR) (0.1 to 1 μmol/m^2) and high-irradiance response (HIR, > 1000μmol/m^2) are not R/FR reversible (Neff et al. 2000). Phytochromes are encoded by a small gene family. There are five distinct phytochromes in *Arabidopsis*, termed from phyA to phyE. PhyA is type I phytochrome. It is much more abundant in dark-grown seedlings than type II, which consists of phytochromes from phyB to phyE. During dark periods PHYA protein is accumulating in Pr form. In light PHYA is rapidly degraded after photoconversion to its Pfr form (Figure 1). Other phytochrome proteins are more light stable, only their conformation is changed in response to R and FR (Sharrock and Clack 2002). The amount of different phytochromes varies in different light conditions and they might respond antagonistically to the R:FR ratio of light, the amount of light energy and duration of light and dark periods. For example phyB is necessary for continuous R perception, whereas phyA is necessary for continuous FR perception (FR-HIR), while other phytochromes are unable to detect continuous R or FR light (Quail et al. 1995). PhyB to phyE predominantly regulate light responses under continuous red and white light in classical photoreversible R-FR responses. PhyA is responsible for VLFR and HIR responses. Members of the family have been shown to detect identical environmental signals but employ those signals in different functions. The mechanism of action might be either selective expression of target genes or modulation of cellular ion balances (Smith 2000). The overall picture is that different phytochromes may act additively, synergistically, and sometimes even antagonistically (Casal 2000). In addition, different phytochromes modulate plant growth differently in distinct developmental stages, *i.e.* seeds respond differently than green plants. PhyB probably has a role at all stages of the life cycle, whereas phyA, phyD and phyE exert their principal functions at selected stages (Smith 2000).

1.4.1.2. Phytochrome responses

Photoperiodic control of bud set and cold acclimation is analogous to phytochrome control of SD-induced flowering in annual plants. The determining factor in short day is not the length of the day but the night, *i.e.* duration of the dark period. If long dark period is interrupted by giving 15 min of R in the middle of the night, plants sense it as a short night. The effect of R night break treatment is reversed with FR, demonstrating the involvement of phytochrome for SD detection (Williams et al. 1972, Howe et al. 1996). Daylight contains roughly equal proportions of red and far-red light (red:far-red ≈ 1.2). The ratio of R:FR is decreased during dusk and plant use this ratio to measure the length of the day. This is so called end-of day (EOD) FR response. By giving plants FR at the end of the day, the amount of Pfr present is decreased and the length of the critical night period is reduced. McKenzie et al. (1974) showed that FR EOD treatment for LD grown plants induced terminal bud formation and cold acclimation in red-osier dogwood. In hybrid aspen, EOD FR was able to induce cold acclimation, but this was not connected to terminal bud formation (Olsen et al. 1997).

1.4.1.3. Endogenous clock

Plants have an internal timekeeper, the endogenous clock that allows the anticipation of regular fluctuations in the availability of the most important resource to plants, sunlight. The clock imposes a 24-hour rhythm on certain physiological processes so that they always occur at the optimal phase of the light-dark cycle. Plants have a self-sustaining oscillator, consisted of clock proteins that oscillate with approximately 24-hour rhythm. They are rhythmically

transcribed and after a certain delay feed back to inhibit transcriptional activity of their own genes (Hayama and Coupland 2003). In addition to this, plants adjust their endogenous clock on a daily basis in response to light. Photoreceptors play a role in this clock resetting, although none of them have been shown to be an essential element. PhyA, phyB, phyD and phyE mediate red-light effects on the pace of the clock, and cry1 and cry2 mediate blue light input (Somers et al. 1998). The endogenous clock provides also an internal estimate of the season. Resetting the clock by light enables a plant to detect the gradual shift in the time of sunrise during the progress of the season.

1.4.1.4. Photoperiodic ecotypes

Photoperiod provides a reliable and repetitive signal for the imminent unfavourable season. Studies where freezing tolerance is measured from the same trees during consecutive years show that cold acclimation initiates always at the same time despite of the annual differences in temperature (Howell and Weiser 1970, Greer et al. 1989). The adaptive value of initiation of hardiness is so crucial that in many temperate zone woody species latitudinal ecotypes have developed, which differ in their timing of growth cessation. Ecotypes are genetically different populations of the same species that respond differentially to certain environmental factors (Begon et al. 1990). Northern ecotypes have longer critical daylength than southern ecotypes, resulting in earlier growth cessation of northern populations during growing season (Håbjørg 1978, Junttila 1980). This leads to earlier cold acclimation of the northern populations (Junttila and Kaurin 1990). The response to photoperiod shows a clinal pattern of genetic variation that is associated with both latitudinal and elevation gradients (Håbjørg 1972a, b, 1978). Ecotypic differences are inherited and offspring of northern and southern ecotype shows intermediate phenotype (Junttila and Kaurin 1990, Hurme et al. 2000). The genetic basis for different latitudinal ecotypes is currently not known. Factors that keep the ecotypes separated are probably different for northern and southern ecotypes. If southern ecotypes are transplanted to the north, they may start to acclimate too late and can be killed by early frosts. Northern ecotypes growing in the south have too short growing season to compete with the ecotypes adapted to local conditions.

1.4.2. Low temperature perception

The identity of ‘a plant thermometer’ has not been established. Plant receptors for low temperature have not been found yet, but they could resemble cold sensors of animals. Recently, a membrane-associated histidine kinase (Hik33) in *Synechocystis* was suggested to represent one of the cold sensors or cold signalling pathways, as it was found to transduce the cold signal to a subset of cold-regulated genes. The function of the other cold sensor in animals, TRP (transient receptor potential) is based on changes of membrane currents after opening of cation channels for calcium ions. The latter type of cold sensor could function also in plants as they show rapid cold induced cytosolic Ca^{2+} influx (Sung et al. 2003). It has been shown that changes in membrane fluidity in response to a decrease in temperature function as a trigger for calcium influx from vacuoles or extracellular storage and initiates a signalling cascade leading to changes in the expression of genes that are responsible for increased freezing tolerance (Örvar et al. 2000).

1.5. Mechanism of freezing tolerance in plants

1.5.1. Acclimation capacity of plants

Plants differ in their capacity to cope with sub-optimal temperatures. Chilling sensitive plants, often growing in tropical areas, are injured at temperatures just below +10°C. Chilling tolerant plants, such as many species in the *Solanaceae* family, can tolerate low, non-freezing temperatures, but are killed in temperatures a few degrees below zero. Plants that can tolerate freezing temperatures employ two major strategies. They either avoid freezing or tolerate extracellular freezing (Sakai and Larcher 1987). The characteristics of water limits the distribution of plants with freeze-avoidance strategy. Water molecules come together to form a stable ice nucleus, either spontaneously (homogeneous nucleation) or catalysed by another substance (heterogeneous nucleation). Homogenous nucleation temperature of pure water is –38°C, at which it freezes spontaneously. Distribution of plants with freeze-avoidance strategy is thus limited to areas where temperatures below –40°C are not encountered (Sakai and Larcher 1987). Perennial plants able to tolerate dehydration caused by extracellular ice formation are the most cold hardy ones and they can grow in the coldest areas of the earth. When fully acclimated, these plants can tolerate temperatures of liquid nitrogen (-196ºC) (*e.g.* Stushnoff and Junttila 1986)

1.5.2. Control of the freezing process

Freezing of the tissues is a controlled event in freezing tolerant species. Plants themselves can secrete heterogeneous nucleators to initiate ice formation in xylem vessels and in discrete regions where extracellular ice may cause minimal physical damage. These nucleators may contain proteinaceous or carbohydrate components as well as phospholipids or polysaccharides (Griffith and Antikainen 1996). In addition, organic and inorganic debris, ice-nucleation-active bacteria (INA), other biological molecules and structures, and snow and sleet can act as heterogeneous nucleators (Pearce 2001). Ice-nucleation sites in freezing-tolerant plants have defined compositions, their amount may fluctuate seasonally and they are active in specific tissues, thus determining the temperature and the location at which the extracellular ice forms in plants (Griffith and Antikainen 1996). Antifreeze proteins (AFP) are other factors controlling freezing in plants. The role of these proteins is to bind to and modify the growth of ice crystals, whether they are present in a fish that avoids freezing or in a plant that tolerates freezing. Some plant AFPs have been shown to be similar to pathogenesis-related proteins, induced in plants in response to a pathogen attack (Griffith and Antikainen 1996).

Ashworth and Pearce (2002) demonstrated that initial freezing is always extracellular, both in cold-acclimated and non-acclimated plants, as well as in plants that have no capacity to acclimate. Freezing of the dilute apoplastic water around –5ºC occurs almost in all hardwood species, resulting in high temperature exotherm (HTE) in differential thermal analysis (DTA). If cells undergo deep supercooling, and temperature decreases below the supercooling capacity, they may freeze rapidly, producing a low temperature exotherm (LTE) in DTA (Pearce 2001). Depending on woody plants species, they might show different strategies in different organs. In the cortex ice forms extracellularily, but xylem parenchyma exhibits either deep supercooling or extracellular freezing, depending on species (Pearce 2001).

1.5.3. Injuries caused by sub-optimal temperatures

Injury caused in plants by low temperature may arise from various factors. In chilling sensitive plants injury is considered to be the consequence of physiological or metabolic dysfunction caused by the influence of low non-freezing temperatures on physiological processes. Plants that tolerate freezing temperatures by deep supercooling will die if the temperature decreases so low that their capacity for supercooling is exceeded and they freeze rapidly. Tolerant plants that freeze extracellularily may be injured by cellular dehydration: The water potential of ice is lower than that of liquid water. Consequently, extracellular ice crystals grow by drawing water from the cells until the water potential of ice and cytosolic water are equal, thus dehydrating the cell contents. The water potential of ice is lower the lower the temperature is, hence cellular dehydration becomes progressively more severe when temperature falls, down to a limit set by vitrification. Membrane structures are damaged when the freeze-induced dehydration exceeds the dehydration tolerance of the cell (Steponkus 1984). Injuries may also be caused indirectly. In trees cavitation of vessels in response to drought during winter may impair water movements later in the spring (Utsumi et al. 2003). In addition, large ice-masses in the apoplast can affect tissue or organ structure and frost-burn caused by exposure to wind and sun, diseases entering through lesions and ice-encasement causing hypoxic stress may damage plants further during winter.

1.5.4. Protection against freezing

1.5.4.1. Protection of membranes against dehydration

Water is the driving force for the assembly of phospholipids into biological membranes in cells and, in part, for the conformation of proteins. Dehydration damage can be lethal when cells are not able to maintain their cellular organization, a situation leading to structural changes in membranes and protein denaturation (Hoekstra et al. 2001). In fully hydrated cells, membranes are in a liquid-crystalline phase (Figure 2A), where lipids of the membrane have both lateral and kinetic motion. During dehydration water molecules are no longer helping to maintain the spacing between the phospholipid headgroups, leading to a closer packing of the lipid molecules and an increase in the membrane phase transition temperature (T_m). This can result in phase transition of the membrane into the gel phase (Figure 2B) (Steponkus 1984). With further dehydration, membranes undergo transition to hexagonal II phase in which membranes no longer form bilayers but three dimensional structures with long tubes of lipid surrounding water (Figure 2C). During rehydration membranes undergo new phase transitions, resulting in transient leakage of soluble cell contents trough membranes (Oliver et al. 2002). During mild drought tolerant plants accumulate compatible solutes and sugars. These are preferentially excluded from the surface of the proteins and membranes, thus forming a cohesive water layer around macromolecules and membranes, providing them a hydrated surrounding (Hoekstra et al. 2001). Under more severe cellular dehydration, when water is almost absent, sugars are suggested to replace water in the hydration shell of the membranes, maintaining the spacing between phospholipid molecules and reducing the T_m. Sugars can form a carbohydrate glass with a high melting temperature (Oliver et al. 2002).

1.5.4.2. Sugars in cold acclimation

Wanner and Junttila (1999) demonstrated that a combination of low temperature and light is required for the enhancement of freezing tolerance in *Arabidopsis*. Plants exposed to

LT in darkness failed to cold acclimate, as they were unable to accumulate sugars without light. During cold acclimation *Arabidopsis* accumulates sucrose, glucose and fructose, and the increase of sucrose has been shown to correlate well with the increase in freezing tolerance (Wanner and Junttila 1999). Moreover, *Arabidopsis* mutants incapable of sucrose accumulation are impaired in the development of cold-induced freezing tolerance (Uemura et al. 2003). Correlation with sucrose accumulation and increase in freezing tolerance has been shown also in woody plants during overwintering. Short daylength in controlled conditions or during autumn in the field triggers accumulation of sugars, which, after growth cessation, are stored as starch in the stem and buds (Nelson and Dickson 1981, Fege and Brown 1984, Kuroda and Sagisaka 1993, Rinne et al. 1994b, Imanishi et al. 1998). Starch is converted to maltose and then to sucrose and its galactosides in response to low and freezing temperatures (Sauter and van Cleve 1991). As deciduous trees have shed their leaves usually by the time of freezing temperatures, and are thus unable to provide sucrose, accumulation of starch during SD provides a source for sucrose needed for the increase in freezing tolerance at low and freezing temperatures (Sauter and Wellenkamp 1998).

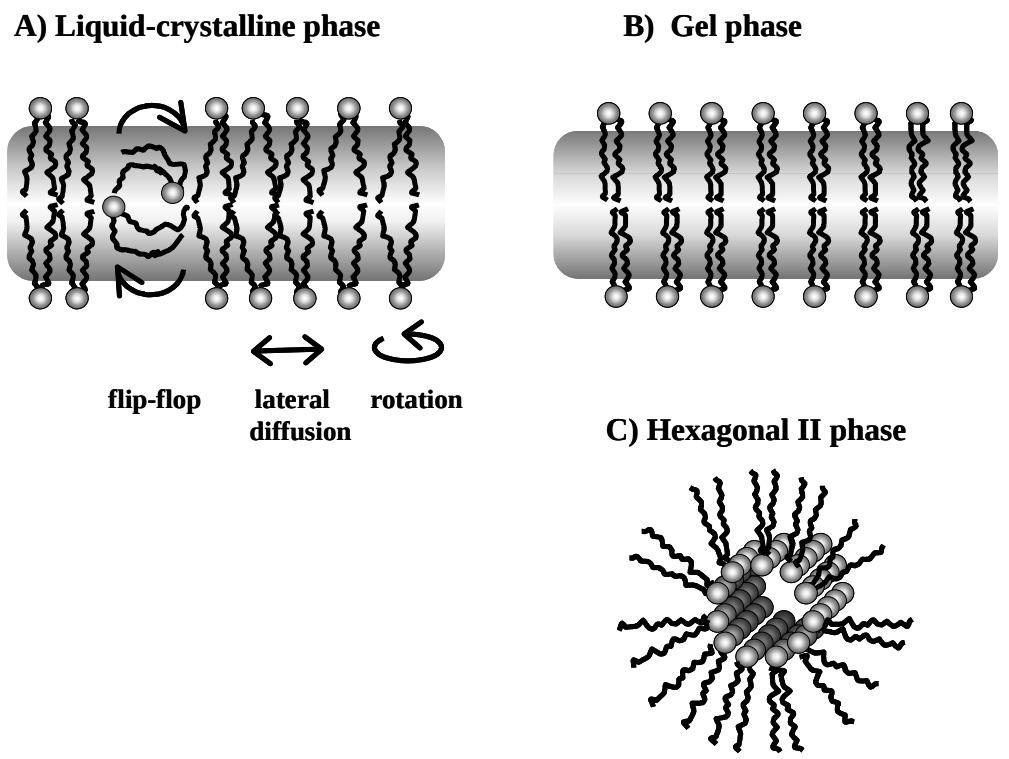

Figure 2. Illustration of phospholipid layers in liquid-crystalline phase (A), in gel phase (B) and in hexagonal II phase (C). In liguid-crystalline phase phospholipids have both lateral and kinetic motion whereas in gel phase lipids have less kinetic energy. In hexagonal II phase lipids are not in a bilayer but they form long tubes surrounding water.

1.5.4.3. Protection against oxidative stress

Oxidative damage caused by reactive oxygen species (ROS), such as superoxide, hydrogen peroxide and hydroxyl radicals, is often associated with plant stress. Low temperature and drought have been shown to cause excess production of ROS. ROS, especially hydroxyl radicals, react rapidly with proteins, lipids and DNA, causing rapid cell damage (Inze and van Montagu 1995). Especially evergreen plants are easily exposed to an additional stress caused by a combination of light and low temperature. Leaves continue to absorb light under conditions in which this absorbed energy cannot be used productively because of the low-temperature inhibition of photosynthetic CO_2 assimilation. Prevention or total inhibition of photosynthesis at low, non-freezing and freezing temperatures leads to an overexitation of the photosynthetic apparatus, which, in turn, increases the potential for photooxidative damage (Öquist and Huner 2003). Plants can decrease the energy flow through the photosynthetic apparatus by reducing the light harvesting antenna size, by a partial loss of photosystem II and by increasing energy loss by heat (Huner et al. 1998). In addition, plants have evolved several enzymes and metabolites that are able to scavenge ROS. These include superoxide dismutases (SOD), catalases, glutathione reductases and ascorbate peroxidases. The level of these enzymes is increased in response to cold, thereby preventing cellular damages caused by ROS (Inze and van Montagu 1995).

1.6. Hormonal control of cold acclimation and dormancy

Unlike animal hormones that are synthesized in one organ and transported to another organ to control a specific physiological event, plant hormones usually do not have a clear locus of synthesis but they are synthesized in various tissues. There is no evidence that transport of plant hormones is essential part of their action nor are there specific target tissues where a certain plant hormone would affect (Weyers and Paterson 2001). Trewavas (1982, 1991) raised discussion about sensitivity concept to plant hormone research. He suggested that controversy and versatility of results in plant hormone research results in the fact that usually changes in concentration are not necessarily the determining factor in plant hormone action, but sensitivity of the tissue for a given hormone may change depending on the developmental stage or environmental factors may affect the sensitivity. It is also possible that changes both in concentration and sensitivity control the action of a hormone (Weyers and Paterson 2001).

Physiological studies have demonstrated that plant growth and development require the coordinated action of multiple hormones (McCourt 1999). It is very likely that the all five “classical” plant hormones, auxin, gibberellic acid (GA), ethylene and cytokinins together with abscisic acid (ABA) participate together for most of the growth processes in plants. For example seed dormancy and germination are probably net result of a balance between many promoting and inhibiting factors. Auxin and cytokinins are involved in paradormancy *e.g.* to control distinct growth capacity of the apical versus the axillary buds (Rohde et al. 2000b). Recent results suggests that ethylene is involved in regulating antifreeze activity in winter rye in response to cold and drought, as ethylene level increases during cold acclimation and endogenous ethylene is able to induce number of AFPs (Yu et al. 2001).

In addition to ABA, GA is the most studied plant hormone in respect of growth, dormancy and cold acclimation. GA has been suggested to function as an ABA antagonist in growth processes, accelerating growth. Although more than 120 different gibberellins have been isolated from plants, only few acts as endogenous plant growth regulators, most are precursors or inactive forms. GA deficient mutants are dwarfs with short internodes, reduced leaf size, delayed flowering time, and male sterility (Yamaguchi and Kamiya 2000). All these

defects can be reversed by application of bioactive GA species. GA_1 is likely to be the determining factor for elongation growth and growth cessation in woody species (Junttila et al. 1991, Olsen et al. 1995). It has been suggested that photoperiodic regulation of elongation growth is mediated by regulation of GA biosynthesis, the levels being higher under LD than under SD (Jackson and Thomas 1997). SD appears to block certain steps in the biosynthesis of GA_1 and this effect can be mediated through phyA (Olsen et al. 1997). GA_1 affects cell divisions in the subapical meristem in response to SD and LD, effecting growth cessation and growth initiation in these conditions, respectively (Hansen et al. 1999).

1.6.1. Abscisic acid (ABA)

Although abscisic acid (ABA) was originally described in the 1960s as a dormancy-inducing and abscission-accelerating substance, it was soon realized to have many other physiological effects in plants. When synthetic ABA became available, it was noted that it was a potent inhibitor in several bioassays and counteracted the effects of growth-promoting hormones. The level of ABA has been shown to increase tremendously under water deficit and applied ABA can cause stomatal closure. Soon ABA was realized to be a general stress hormone involved in plant responses to abiotic stresses, such as low temperature, drought and salinity as well as in the regulation of plant growth and development, including embryogenesis, seed dormancy, shoot and root growth, and leaf transpiration (Koorneef et al. 1998, Leung and Giraudat 1998, McCourt 1999, Rock 2000). ABA can induce rapid closure of stomata by ion effluxes from guard cells, thereby limiting water loss through transpiration (Assmann and Wang 2001) or it can trigger slower changes in gene expression, which are thought to reprogram the cell to withstand dehydration stress (Chandler and Robertson 1994, Ingram and Bartels 1996). Studies with mutants that are either deficient in ABA biosynthesis or insensitive to ABA have revealed that both types of responses require the action of common signalling elements, together with second messengers or components of phosphorylation cascades (Finkelstein et al. 2002). There is evidence for multiple, redundant ABA perception and signalling mechanisms, and interaction between signalling by ABA and ethylene, brassinosteroids, light and sugars, demonstrating that ABA signalling is not simply linear but composed of a complex signalling network.

1.6.1.1. ABA regulated genes

Physiological responses to ABA are brought about by changes in gene expression. In vegetative tissues ABA regulated genes are involved in response to abiotic stresses that result in cellular dehydration (Ingram and Bartels 1996, Shinozaki and Yamaguchi-Shinozaki 2000, Xiong et al. 2002). In maturing seeds ABA regulated genes are involved in the synthesis of storage reserves and acquisition of desiccation tolerance (Rock 2000). In addition to these high-abundance transcripts, ABA regulates also low-abundance transcripts that encode signalling components (Rock 2000, Finkelstein et al. 2002). Hundreds of genes in various species have been shown to be responsive to ABA (Finkelstein et al. 2002) and recent genome-wide expression profiles have revealed that in *Arabidopsis* over a thousand genes are either up- or down regulated by ABA (Hoth et al. 2002, Seki et al. 2002a). Many of these genes encode proteins associated with stress, such as water channels, dehydrins, chaperonins, enzymes for osmolyte and cell wall biosynthesis, proteinases and detoxifying enzymes (Rock 2000). Many ABA-inducible genes have been shown to contain a conserved ABA responsive element (ABRE) in their promoter regions (Ingram and Bartels 1996, Bonetta and McCourt 1998, Leung and Giraudat 1998, Finkelstein et al. 2002). A single copy of ABREs is not enough for ABA responsiveness; either repeated copies of ABREs or ABREs together with

other *cis*-acting elements are required (Leung and Giraudat 1998). Another element, so called dehydration responsive element (DRE), also named as C-repeat (CRT) or low temperature responsive element (LTRE), can function as a coupling element for ABRE, suggesting interaction between osmotic stress and ABA signalling (Narusaka et al. 2003). Four main groups of *cis*-acting sequences are known to be involved in ABA inducibility: the G-box elements inside ABA responsive elements (ABRE), the functionally equivalent coupling element 3 (CE3)-like sequences, the RY/Sph elements and MYB and MYC *cis*-acting elements (Finkelstein et al. 2002). ABREs are known to bind basic leucine zipper proteins (bZIP), and RYs are bound by B3 domain proteins. In *Arabidopsis*, AtMYC2 and AtMYB2 has been shown to function as transcription factors for MYC and MYB sites, in ABA responsive genes (Abe et al. 2003).

1.6.1.2. ABA signalling

Similarly to other plant hormones, ABA is thought to act through signal transduction pathways in which binding of the hormone to a receptor elicits a transduction cascade, leading to expression of gene(s) that are responsible for the physiological effect (Bonetta and McCourt, 1998). Mutants that have normal ABA biosynthesis but show increased or decreased sensitivity to ABA under certain conditions, have been used to identify components in ABA signalling pathways. In *Arabidopsis* ABA insensitive mutants *abi1* to *abi5* have been shown to be impaired in the expression of certain signalling components. *ABI1* and *ABI2* encode proteins belonging to protein phosphatase 2C family, *ABI3* encodes a B3-domain transcription factor, *ABI4* encodes an AP2 (APETALA2) domain transcription factor and *ABI5* encodes a bZIP domain transcription factor (Finkelstein et al. 2002). Three other genes important in ABA signalling in seeds are *LEC2* (*LEAFY COTYLEDON2*) and *FUS3* (*FUSCA3*) that encode members of the B3 domain family (Stone et al. 2001) and *LEC1* that encodes a transcription factor belonging to HAP3 subunit of CCAAT binding factors (Lotan et al. 1998). The three transcription factors, ABI3, ABI4 and ABI5 participate in combinatorial control of gene expression, possibly by forming a regulatory complex mediating seed specific and/or ABA inducible expression. LEC1, LEC2 and FUS3 primarily regulate the transition from embryogenesis to germinating growth (Holdsworth et al. 1999). Although ABA signalling mutants are identified and studied mostly in seeds, they also appear to function in other processes during vegetative growth.

1.6.1.3. ABA in bud dormancy

One of the first reports of ABA described it as a bud dormancy initiating substance in birch (Eagles and Wareing 1964). Endogenous concentrations of ABA can rise and fall dramatically in response to either environmental or developmental cues (Zeevaart and Creelman 1988). Therefore, approaches where endogenous concentrations of ABA have been correlated with physiological responses or effect of applied ABA have been widely used to study the role of ABA in dormancy. Seasonal variation of ABA level in leaves, buds and xylem sap has been studied in various woody species. ABA content has been shown to fluctuate similarly in leaves, buds and xylem of various tree species, the level being highest during mid-summer or autumn and declining during winter. In xylem sap a transient increase in ABA content can be seen also in mid-winter. Some of these studies show correlation between the level of ABA and dormancy cycling (Davison and Young 1974, Harrison and Saunders 1975, Alvim et al. 1976, Webber et al. 1979), while in other studies the correlation is not so clear or the function of ABA is thought to be indirect (Mielke and Dennis 1978, Seeley and Powell 1981, Barros and Neill 1986, 1987, 1989, Rinne et al. 1994a). Although

SD and chilling, the main factors inducing and releasing bud dormancy, respectively, lead to changes in bud ABA content, no unambiguous connection between ABA level and bud dormancy has been shown (Lenton et al. 1972, Emmerson and Powell 1978, Mielke and Dennis 1978, Odén and Dunberg 1984, Qamaruddin et al. 1993, Rinne et al. 1994a, Li et al. 2002). El-Anatby et al. (1967) demonstrated the ability of applied ABA to induce growth cessation and bud dormancy under non-inductive LD conditions. Later studies have confirmed the ability of ABA to function as growth retardant, but also shown that ABA alone is not sufficient to induce dormancy (Junttila 1976, Johansen et al. 1986, Barros and Neill 1987). However, applied ABA can prevent bud bursting of ecodormant buds (Altman and Goren 1974, Rinne et al. 1994a) and continuous *in-situ* ABA biosynthesis is required for endodormancy maintenance (Le Bris et al. 1999). Barros and Neill (1986) demonstrated increased sensitivity of willow buds to ABA under SD conditions, simultaneously as buds entered dormancy. Chilling treatment was shown to release buds from dormancy and at the same time sensitivity to applied ABA was decreased (Barros and Neill 1986). In conclusion, it seems that the level of ABA does not play a role in bud dormancy, but ABA may well be involved in dormancy through sensitivity changes brought by changes in daylength and temperature.

1.6.1.3.1. Similarities in bud and seed dormancy

Seed and bud dormancy share some common features, such as chilling requirement for a given genotype, antagonism of ABA and GA in growth and dormancy, acquisition of desiccation tolerance and dehydration of the cells during dormancy induction, and accumulation of reserve proteins and lipids (Powell 1987, Dennis 1996). Ease of dormancy research in seeds and availability of various mutants have expanded the knowledge of hormonal control of seed dormancy enormously during the last years. As some of these controlling mechanisms function in vegetative tissues under stress conditions, they might be functional also in bud dormancy of trees. In seeds ABA have a clear role in embryo morphogenesis, storage protein synthesis, desiccation tolerance and the onset and maintenance of dormancy (Rock 2000, Finkelstein et al. 2002). The above mentioned transcription factors ABI3, ABI4 and ABI5 together with LEC1, LEC2 and FUS3 have been shown to be crucial for seed dormancy and have also a central position in ABA signal transduction (Giraudat et al. 1992, Parcy et al. 1994, Merlot and Giraudat 1997). ABI3 seems to have also broader function as a global regulator of cell fate that allows cellular maturation (Bonetta and McCourt 1998, Rohde et al. 2000c). ABI3 has been shown to affect flowering time, inflorescence morphology and plastid differentiation in seedlings of *Arabidopsis* (Robinson and Hill 1999, Rohde et al. 2000a). ABI3 also participates in vegetative quiescence of the shoot apex in *Arabidopsis* (Rohde et al. 1999). In poplar *ABI3* (*PtABI3*) is expressed transiently in organs and cells that grow actively but will undergo growth arrest during SD induced dormancy development, as in the young embryonic leaves, the subapical meristem, and the procambial strands (Rohde et al. 2002). As ABA level of apical bud peaked at the same time as *PtABI3* expression, it was suggested that ABA might be the factor that causes growth cessation under SD conditions and ABI3 slows down the growth cessation and allows appropriate differentiation of tissues under conditions that promote growth cessation. Thus, PtABI3 is an essential factor in bud development, which in turn has an impact on successful overwintering.

1.6.1.4. ABA in cold acclimation

Several lines of evidence suggest that ABA is involved in the cold acclimation of plants. ABA level has been shown to increase under conditions that lead to increased freezing tolerance both in woody plants (Li et al. 2002) and in herbaceous species (Chen et al. 1983, Lång et al. 1994). In addition, this increase in the ABA level is restricted to species that are able to cold acclimate (Chen et al. 1983). As mentioned, applied ABA can increase freezing tolerance in non-inductive conditions (Irving 1969, Chen et al. 1983, Heino et al. 1990, Tanino et al. 1990, 1991, Lee and Chen 1993, Anderson et al. 1994, Lång et al. 1994, Mäntylä et al. 1995, Li et al. 2002, 2003a). In addition, ABA deficient and insensitive mutants of *Arabidopsis (aba1* and *abi1*, respectively) have impaired cold acclimation (Heino et al. 1990, Gilmour and Thomashow 1991, Mäntylä et al. 1995), but the cold acclimation capacity of *aba1* mutant can be restored by exogenous ABA (Heino et al. 1990). Gilmour and Thomashow (1991) questioned the central role of ABA in cold acclimation by pointing that ABA has an important function in plant growth and development. Thus, plants unable to synthesize or respond to ABA are weak in general and therefore impaired in cold acclimation. It has been known for a long time that gene expression is required for the increase in freezing tolerance (Guy 1990). Overexpression of DRE/CRT /LTRE regulon (Stockinger et al. 1997, Liu et al. 1998) of *COR* (cold responsive) genes (*COR78*, *COR47*, *COR15a* and *COR6.6*) of *Arabidopsis* results in an increase in freezing tolerance at normal growth temperatures, pointing to their integral role in cold acclimation (Jaglo-Ottosen et al. 1998, Kasuga et al. 1999). Exogenous application of ABA leads to high expression of these genes simultaneously with an increase in freezing tolerance (Thomashow 1999). However, expression of these genes in *aba* mutants is essentially normal at low temperature (Gilmour and Thomashow 1991, Nordin et al. 1991) and although *abi* mutation prevents *COR78*, *COR47*, and *COR6.6* gene expression in response to ABA, it has no effect on the expression of these genes at low temperature (Gilmour and Thomashow 1991, Nordin et al. 1991). These results have led to conclusion that cold-regulated expression of these genes occurs through ABA-independent and ABA-dependent pathways (Gilmour and Thomashow 1991, Nordin et al. 1991). However, Ishitani et al. (1997) have proposed that these two pathways are not totally independent, but instead have points at which they cross-talk. Moreover, Shinozaki and Yamaguchi-Shinozaki, (2000) proposed that low temperature initiates a rapid increase in freezing tolerance independently of ABA, and *de-novo* ABA biosynthesis followed by ABA responsive genes is required for the slow and adaptive stress response process.

1.6.1.5. ABA biosynthesis

Mutants that have low endogenous level of ABA, and do not have increased levels in response to *e.g.* water stress have been found in several plant species (Schwartz et al. 2003). They have been shown to be defective in some step of ABA biosynthesis, resulting in impaired ABA accumulation. Characterization of these mutants has enabled the unravelling of the ABA biosynthesis route and provided information about physiological processes that require increased ABA levels. The general pathway leading to ABA biosynthesis has now been well established (Taylor et al. 2000, Bray 2002, Seo and Koshiba 2002). ABA biosynthesis branches from the carotenoid biosynthetic pathway. The first gene known to be involved in ABA biosynthesis was isolated from tobacco, where the ABA-deficient mutant *aba2* was shown to be impaired in the first step of ABA biosynthesis pathway, the zeaxanthin epoxidation reaction. The isolated *ABA2* gene encoded an enzyme called zeaxanthin epoxidase (ZEP), which epoxidates zeaxanthin to form all-*trans*-violaxanthin in a two-step process (Marin et al. 1996). *Arabidopsis* mutant *aba1* is impaired in this step of ABA

biosynthesis (Marin et al. 1996). The next step in ABA biosynthesis involves the conversion of all-*trans*-violaxanthin to 9-*cis*-violaxanthin or 9'-*cis*-neoxanthin (Schwartz et al. 1997b). Mutants affected this step have not been identified (Tan et al. 1997). The oxidative cleavage of 9-*cis*-violaxanthin and/or 9'-*cis*-neoxanthin to produce xanthoxin is catalysed by 9-*cis*-epoxycarotenoid dioxygenase (NCED), which appears to be the key regulatory step in ABA biosynthesis (Schwartz et al. 1997b, Qin and Zeevaart 1999, Chernys and Zeevaart 2000, Iuchi et al. 2000, Thompson et al. 2000a). Overproduction of *NCED* has been shown to lead in increased ABA levels (Thompson et al. 2000b, Iuchi et al. 2001, Qin and Zeevaart 2002). The maize *viviparous 14* and the tomato *notabilis* mutants both are impaired in the reaction catalysed by NCED (Tan et al. 1997, Burbidge et al. 1999). After the cleavage of 9-*cis* epoxycarotenoids, xanthoxin is converted to ABA in the cytosol. Xanthoxin is first converted to ABA-aldehyde by short-chain dehydrogenase/reductase (SDR) (González-Guzmán et al. 2002). *Arabidopsis* mutant defective in this step is named *aba2* (Schwartz et al. 1997a). Oxidation of ABA-aldehyde to ABA involves *ABA3* and abscisic aldehyde oxidase (*AAO*) genes (Seo et al. 2000a, b). The tomato *sitiens* mutant is impaired in this step of ABA biosynthesis (Sagi et al. 2002). Alternatively, oxidation of xanthoxin to xanthoxic acid, and further oxidation and rearrangement to ABA, has been proposed (Milborrow 2001). ABA-alcohol pathway appears to be a minor pathway in wild-type plants but might play a significant role in mutants, such as *flacca* and *sitiens* in tomato, impaired in their capacity to oxidize ABA-aldehyde to ABA directly (Seo and Koshiba 2002).

1.7. Gene expression involved in freezing tolerance and dormancy

Most cellular changes during cold acclimation are associated with alterations in gene expression (Thomashow 1999). Microarray technology has enabled the study of the expression of thousands of genes simultaneously, demonstrating numerous changes in gene expression during cold acclimation (Fowler and Thomashow 2002, Seki et al. 2002b). In general, stress-inducible genes can be divided into two groups: genes whose products directly participate in the protection of the cells under stress conditions and genes encoding components of the signal transduction pathways that regulate gene expression in response to stress (Shinozaki and Yamaguchi-Shinozaki 1997, Thomashow 1999). Logically, genes encoding transcriptional activators are among the first ones that are up-regulated in response to cold, followed by their respective target genes (Fowler and Thomashow 2002, Seki et al. 2002b). However, the same pattern can be seen several times during exposure to cold, showing that different transcription factors are activating distinct regulons during cold acclimation. Genes responsible for sugar metabolism, or whose products are protecting against excessive light, or dehydration, are long-term up-regulated during exposure to cold. Rapid decrease in temperature causes accumulation of ROS, such as hydrogen peroxide (Huner et al. 1998). Genes participating in the detoxification of ROS are induced transiently during cold acclimation. The genes that participate in the regulation of other stress responsive genes are in general also transiently expressed. In addition, a number of genes are down-regulated, either transiently or for longer periods during cold acclimation. These encode proteins participating in energy production, transcription, cellular signalling, cell wall biogenesis and defence against pathogens (Fowler and Thomashow 2002, Seki et al. 2002b).

1.7.1. Dehydrins

1.7.1.1. Dehydrin structure

Membranes are the primary sites of freeze-induced injury resulting largely from the severe dehydration associated with freezing (Steponkus 1984). Thus, genes whose products protect cells against dehydration are pivotal in freezing tolerance. One group of such genes encodes dehydrins (DHN), known also as group 2 late embryogenesis abundant (LEA) proteins. First *DHN* genes were isolated in late 1980's from cotton and rice (Baker et al. 1988, Mundy and Chua 1988). Nowadays, genes encoding DHNs have been cloned from numerous plant species belonging to such diverse groups as angiosperms, gymnosperms, mosses and lycopods (Svensson et al. 2002). In addition, there is immunological evidence of DHNs from ferns and liverworts (Close 1997). DHNs are characterized by highly conserved sequence motifs. By definition, DHNs contain a lysine rich domain called the K-segment, (EKKGIME/DKIKELPG), which is repeated up to eleven times and is often located in the C-terminal part of the protein (Close 1996). The other conserved domains are the S-segment [$(LHRSGS_{4\text{-}10}(E/D)_3$] that usually precedes the K-segments, and the consensus Y-segment (T/VDEYGNP), when present, is located in the N-terminus (Close 1996, Campbell and Close 1997). By using the numbers of Y, S and K-segments in DHNs, it is possible to classify them in sub-classes and five distinct types of DHNs have been found in higher plants: Y_nSK_n, SK_n, K_n, Y_nK_n, and K_nS (Campbell and Close 1997). It has been suggested that if the different YSK structural types have a distinct function, all species could in principle have at least one of the each type of dehydrins (Svensson et al. 2002).

Dehydrins contain a large amount of glycine and charged and polar residues, making them highly hydrophilic. This may partly explain their characteristic boiling stability. DHNs are not very likely to form oligomers (Svensson et al. 2000) and they are intrinsically unstructured proteins (Ceccardi et al. 1994, Ismail et al. 1999b, Hara et al. 2001). However, K-segments may form amphipathic α-helices (Dure et al. 1989, Close 1996). It has been shown that in the presence of SDS, DHNs can form α-helical structures, suggesting that DHNs may *in vivo* fold into a more ordered structure by interacting with other molecules or membranes (Ismail et al. 1999b, Hara et al. 2001). Recently, Koag et al. (2003) demonstrated that binding of maize DHN1 to lipid vesicles was associated with an increase in α-helicity of the protein. Ser-clusters of DHNs can be phosphorylated (Plana et al. 1991), which has been shown to participate in nuclear targeting of the DHNs in maize (Jensen et al. 1998). On the other hand, phosphorylation of DHNs has been shown to be related to their ability to bind calcium (Heyen et al. 2002). DHNs can also be glycolysated (Golan-Goldhirsh 1998, Levi et al. 1999). The Y-segment is similar to a portion of the nucleotide binding site motif in chaperonins of plants and bacteria (Martin et al. 1993) but nucleotide binding by dehydrins has not been reported (Close 1996, Campbell and Close 1997).

1.7.1.2. Involvement of dehydrins to freezing tolerance

Dehydrins are induced by stresses that cause cellular dehydration, such as low non-freezing and freezing temperature, drought and high salinity (Close 1996, Svensson et al. 2002). Karlson et al. (2003) suggested that accumulation of DHNs is triggered by a decrease in water content also in response to conditions such as SD. DHNs are also part of the maturation process of seeds, accumulating during late stages of embryogenesis, prior to seed drying (Ingram and Bartels 1996). In addition, some of the DHNs accumulate in response to the plant hormone abscisic acid (ABA), whose level increases in response to osmotic stresses (Zeevaart and Creelman 1988, Skriver and Mundy 1990, Chandler and Robertson 1994).

Some of the *DHN*s are also constitutively expressed, although the expression level is increasing in response to stress treatment (Choi et al. 1999, Zhu et al. 2000, Nylander et al. 2001). The contribution of DHNs in freezing tolerance of plants has been investigated by exploring the natural genetic variation of *DHN* genes and by using transgenic approaches. Although not all the DHNs are induced by cold, usually expression of at least one member of the *DHN* gene family of a given species is induced by cold (Zhu et al. 2000, Nylander et al. 2001). Higher *DHN* expression in more cold tolerant lines (Ismail et al. 1999a), varieties (Zhu et al. 2000) or sibling species (Artlip et al. 1997, Lim et al. 1999) suggests contribution of *DHN* gene expression in freezing tolerance of plants. Moreover, DHNs in woody plants display seasonal expression and accumulation patterns, the level being lowest during the active growth period and highest during winter (Arora and Wisniewski 1996, Arora et al. 1996, Wisniewski et al. 1996, Artlip et al. 1997, Sauter et al. 1999, Kontunen-Soppela and Laine 2001, Sarnighausen et al. 2002). Overproduction of single DHN has not been shown to improve the freezing tolerance on the whole plant level (Kaye et al. 1998). However, it has been suggested that during cold acclimation DHNs act in concert together with other cold-regulated proteins. Many *DHN* genes, similarly to other cold induced genes, contain the cold and drought responsive *cis*-acting element DRE/CRT/LTRE in their promoter regions (Baker et al. 1994, Yamaguchi-Shinozaki and Shinozaki 1994). The transcription factor family CBF/DREB (CRT/DRE binding factor/DRE binding protein), bind to these *cis*-acting elements and function as transcriptional activators for genes with DRE/CRT/LTRE element (Stockinger et al. 1997, Liu et al. 1998). By overexpressing these transcription factors it is possible to induce constitutive expression of a group of cold-regulated genes, including several DHNs, resulting not only in increased freezing tolerance of the plants, but also in increased drought and salt tolerance (Jaglo-Ottosen et al. 1998, Kasuga et al. 1999, Gilmour et al. 2000).

1.7.1.3. Function of dehydrins

The universal occurrence of DHNs in any studied plant species and their accumulation in response to various stresses support their participation in abiotic stress adaptation in plants. The defined mechanism of action of DHNs is still unresolved. Based on their hydrophilic structure DHNs can act as compatible solutes under mild water stress, and under more severe cellular dehydration, when water is almost absent, DHNs can interact with other proteins with their hydroxylated residues stabilizing structures of macromolecules and membranes. Thus, DHNs could be solubilizing agents with detergent and chaperone activities (Close 1996). The biased amino acid composition of DHNs towards extreme hydrophilicity and their abundance argue in favour of a mass action rather than a direct enzymatic role (Close 1997, Svensson et al. 2002). DHNs have also been shown to have in vitro cryoprotective (Close 1996, Rinne et al. 1999, Wisniewski et al. 1999, Hara et al. 2001) and antifreeze (Wisniewski et al. 1999) activity in several plant species, confirming participation of DHNs in cold acclimation of plants. The proper function of DHNs during cellular dehydration might involve complex processes involving the concerted action of group of DHNs or interaction with other protective molecules such as other LEA proteins or compatible solutes (Hoekstra et al. 2001). For example, it has been shown that sugars together with LEA proteins have higher glass transition temperature and average strength of hydrogen bonding than dehydrated sucrose alone, suggesting LEA proteins to function together with sugars in dehydration tolerance (Wolkers et al. 2001).

1.7.2. Genes involved in changes in plasma membranes

The plasma membrane and associated proteins undergo biochemical changes during cold acclimation. As mentioned earlier, lipid bilayers exist mainly in two states, liquid crystalline and gel phase. The phase transition temperature (T_m) between these two depends on the fatty acid length, the number and position of double bonds, the lipid head group and the surrounding medium. For example saturated phospholipids are usually in gel phase at physiological temperatures; insertion of one or two double bonds per pair of acyl chains typically reduces the T_m to below 0°C. As membrane processes are optimal only within a limited viscosity range, plants adjust the fluidity of the membranes to accommodate variation in temperature. Lipid unsaturation thus is an important adjustment for decreasing temperature to maintain membrane fluidity and proper viscosity. Expression of fatty acid desaturases (*FAD*) is induced in response to low temperature (Berberich et al. 1998) and shows annual variation in trees (Martz et al. 2003). Plasma membrane proteins, especially glycoproteins, show seasonal fluctuations (Yoshida 1984, 1986). Plasma membrane ATPase activity increases during the fall, because cold acclimation results in more efficient ATP catalysis and increased substance binding capacity (Mattheis and Ketchie 1990). On the other hand, expression of the genes encoding H^+ATPase in endodormant buds is low, but increases after chilling, when buds are in ecodormant state (Gévaudant et al. 2001).

1.7.3. Cell cycle genes

So far, only few genes have been shown to have a role exclusively in bud dormancy. These include genes that participate in the regulation of the cell cycle, which is arrested during endodormancy. Cell cycle consists of four phases: mitosis (M) is followed by gap1 phase (G_1), after which DNA synthesis (S) occurs, followed by a second gap (G_2) and again mitosis. Most of the cells in apical meristem are in the G_1-phase, although a reasonable number of cells in G_2 and S-phase have also been observed (Rohde et al. 2000b). The progression through the cell cycle is regulated by cyclin-dependent protein kinases (CDKs) in association with regulatory subunits called cyclins. Among others, *Arabidopsis* has two cell cycle genes: *cdc2a*, encoding a CDK which is synthesized during all four phases of the cell cycle, and *cyc1At*, which encodes the G_2-to-M-phase specific cyclin. Rohde et al. (1997) studied the role of these genes in buds of *Populus*. They showed that the expression of *Pcyc1At* (*Populus cyc1At*) was associated with tissues with active cell divisions, such as roots and apical meristems, while *Pcdc2a* was expressed in tissues with a high competence for cell divisions, such as buds, the vascular cambium, root tips, pericycle and periderm. Expression of both genes was downregulated by SD, which is known to induce dormancy in *Populus*.

1.7.4. Genes related to metabolism

Changes in gene expression in response to dormancy breaking by cold, heat or chemical treatments have been reviewed by Rowland and Arora (1997). When buds break respiratory metabolism changes from the pentose phosphate pathway providing reducing power to glycolysis that provides energy for growth (Rowland and Arora 1997). Activity of enzymes in the pentose phosphate pathway, such as glucose-6-phosphate dehydrogenase and 6-phosphate gluconate dehydrogenase decrease, whereas enzyme activities of the glycolytic and citric acid pathways, such as glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase and isocitrate dehydrogenase increase (Wang et al. 1991, Faust and Wang 1993). Bud break is further characterized with free radical removal through activated peroxide-scavenging systems, such as catalase, superoxide dismutase, ascorbate peroxidase, dehydroascorbate reductase and

glutathione reductase. However, it is not clear whether this free radical removal is a prerequisite for resumption of growth or, more likely, response to oxidative stress caused by bud bursting (Rowland and Arora 1997).

1.7.5. Bark storage proteins (BSP)

In trees numerous genes exhibit seasonal variation in their expression, the level being high in winter and low during active growth. The two most abundant of the genes whose expression increases in winter encode above discussed dehydrins and bark storage proteins (BSP) (Rowland and Arora 1997). BSP participate in nitrogen recycling from senescing leaves to bark and back to growing leaves in the spring (Wetzel et al. 1989, Clausen and Apel 1991, Gomez and Faurobert 2002). Major protein storage sites include shoot and root bark and xylem ray cells (Wetzel et al. 1989, Sauter and van Cleve 1990), where BSPs are stored in special vacuoles called protein bodies (Herman et al. 1988, Sauter et al. 1989). *Populus*, *Salix* and *Acer* have been shown to accumulate BSPs during fall (Wetzel et al. 1989, Clausen and Apel 1991), to levels which account from 30% to 50% of soluble bark proteins during winter (Wetzel et al. 1989, Coleman et al. 1991). During spring, BSPs undergo rapid degradation (Coleman et al. 1993) and are thought to provide N and C for new spring growth (Wetzel et al. 1989). Shoot growth is a prerequisite for BSP degradation regardless of plant dormancy status (Coleman et al. 1993). Poplar BSP is encoded by a small multigene family (Coleman et al. 1992). One of these genes, (*bspA*), has been cloned (Coleman and Chen 1993), and shown to be related to a gene family of wound-inducible genes (*WIN4*) that are not responsive to photoperiod (Davis et al. 1993). Photoperiod (Coleman et al. 1991, 1992, Langheinrich and Tischner 1991), nitrogen availability (van Cleve and Apel 1993, Coleman et al. 1994), temperature (van Cleve and Apel 1993), and wounding (Davis et al. 1993) all influence *bsp* expression. In northern ecotypes of poplar the level of *bsp* expression increases earlier in fall compared to southern ecotypes (Black et al. 2001). Although *bsp* expression is regulated by phytochrome, regulation is indirect, as it is coupled to changes in growth and probably to changes in sink-source relationship during growth cessation (Zhu and Coleman 2001). Arora et al. (1992) have shown that in peach BSP accumulation correlated with dormancy induction in a deciduous genotype, while an evergreen genotype did not enter dormancy and the level of BSP remained low. This suggests that BSP accumulation is connected to dormancy.

2. AIMS OF THE STUDY

The main objective of the present study was to gain information about the involvement of plant hormone abscisic acid (ABA) and dehydrin proteins in the overwintering process in trees. In addition to different latitudinal ecotypes of pubescent birch (*Betula pubescens* Ehrh.), ABA deficient birch (*Betula pubescens*. f. *hibernifolia* Ulvinen) and transgenic hybrid aspen (*Populus tremula* L. x *P. tremuloides* Michx.) overexpressing oat *PHYA* gene were used as model systems. More specifically, the following objectives were formulated:

1) To examine if ABA is involved in dormancy or freezing tolerance or in both during overwintering of birch (I, II).
2) To study the involvement of dehydrins in dormancy and freezing tolerance (II, III, IV).
3) To dissect the different factors involved in the regulation of dehydrins in woody plants (II, III, IV).

3. MATERIALS AND METHODS

3.1. Plant material

In addition to wild type birch (*Betula pubescens* Ehrh.), a spontaneous mutant of pubescent birch (*Betula pubescens* f. *hibernifolia* Ulvinen) that does not shed leaves during autumn and is impaired in accumulating ABA under stress conditions (Rinne et al. 1992) was used in the experiments. Seeds (I, II) and tissue culture material of birch (II, IV) came from trees originated from Oulu, central Finland (65°05'N, 26°02'E). In experiment IV trees of three different latitudinal ecotypes of birch were also used. These trees originated from seed material that was collected from Kittilä, northern Finland (67°40'N), Pyhäjärvi, central Finland (63°40'N) or Kangasala, southern Finland (61°20'N). In the hybrid aspen experiment, transgenic lines 22 and 8 overexpressing oat *PHYA* gene and wild type T89 (*Populus tremula* L. x *Populus tremuloides* Michx) (Olsen et al. 1997) were used (III).

3.2. Growth conditions

Birch seedlings and cloned birch and hybrid aspen ramets were grown either in controlled climate chambers (I, III) or in the greenhouse (II, IV) under LD control conditions described in Table 1 before use in the experiment. Various growth conditions used during experiments are described in Table 1. In addition, field-grown trees of 7 to 8 years (II) or 12 to 16 years (IV) of age were used in the experiments. Seedlings and ramets (both will be referred to as seedlings) were exposed to growth conditions described in Table 1 in order to study the effect of daylength and temperature to freezing tolerance and dormancy.

3.3. External application of ABA and ABA biosynthesis inhibitor

Abscisic acid (ABA) (Fluka) was applied by spraying LD grown (Table 1) birch leaves with 25 ml 50 µM ABA once a day (I) or with 5 ml of 50 µM ABA twice a week (IV). ABA biosynthesis was prevented by fluridone, an ABA biosynthesis inhibitor. Fluridone treatment was done by adding 25 ml of 0.1 gL^{-1} of fluridone (1-methyl-3-phenyl-5-(3[trifluoromethyl]phenyl-4-(1H)-pyridinone) (Riedel-de Haën) to roots of SD grown (Table 1) birch seedling once a day to each pot.

Table 1. Growth conditions used in the original papers I-IV.

Treatment	Daylength	Temperature	Light intensity	Humidity
LD (control)	24h (I) 22h (IV) 16h (II, III)	18ºC (I, III, IV) 21ºC (II)	85 µ mol $m^{-2}s^{-1}$ (I) 90-100 µ mol $m^{-2}s^{-1}$ (II, IV) 150-200 µ mol $m^{-2}s^{-1}$ (III)	0.5 kPa (I, III) 85 % RH (II) 75% RH (IV)
SD	12h (I, IV) 10h (III) 8h (II)	18ºC (I, III, IV) 21ºC (II)	85 µ mol $m^{-2}s^{-1}$ (I) 90-100 µ mol $m^{-2}s^{-1}$ (II, IV) 150-200 µ mol $m^{-2}s^{-1}$ (III)	0.5 kPa (I, III) 85 % RH (II) 75% RH (IV)
LT	16h (III) 22h (IV)	0.5ºC (III) 4ºC (III, IV)	150-200 µ mol $m^{-2}s^{-1}$ (III) 90-100 µ mol $m^{-2}s^{-1}$ (IV)	0.5 kPa (III)
SD+LT	10h (III) 12h (IV)	0.5ºC (III) 4ºC (IV)	150-200 µ mol $m^{-2}s^{-1}$ (III) 90-100 µ mol $m^{-2}s^{-1}$ (IV)	0.5 kPa (III)
Chilling (after SD)	0h (II)	5ºC (II)	Darkness (II)	75% RH (II)

3.4. Water stress treatment

Water stress treatments were done in LD conditions (Table 1) in following ways: 50 ml of water was added to each pot every 4th day for 3 weeks (I), water content of the soil was reduced by 30% (w/w) relative to the well watered plants (II), or water was withheld until seedlings wilted after one week (IV).

3.5. Freezing tests

Freezing tolerance was measured by exposing plants to controlled freezing after which injuries were estimated either by visual estimation (I, II, III, IV) or by ion leakage method (III). Freezing tolerance was defined as percentage of undamaged buds (I) or as a temperature where 50% of the tissues were injured (II, III, IV) or caused 50% of ion leakage from tissues (III). LT_{50} refers to lethal temperature for 50% of the tissue.

3.6. Dormancy evaluation

Dormancy was evaluated indirectly by measuring bud bursting under forcing conditions (24 h light, 18ºC). Both percentage of burst buds and speed of bud burst were measured. Bud bursting was evaluated from whole seedlings (I) or single node cuttings (II, IV). Bud bursting was expressed as a Σ_{11} value (I), which incorporates both the speed of bursting and the number of burst buds. Bud bursting percentage (II, IV) shows if buds are released from endodormancy and thus in ecodormant stage. Speed of bud bursting in days (II, IV) gives information about the decrease of thermal time (see introduction). In this thesis the term

endodormancy is used when we were able to show that buds are unable to burst under favourable conditions and are not under the control of the apical meristem. The term ecodormancy is used when buds under these conditions finally burst, although this may take several weeks.

3.7. Water status measurements

Water content was calculated using the formula [(fresh weight - dry weight)/fresh weight] x 100% (I, II, III, IV). Osmotic potential, water potential and turgor were measured with Scholander pressure bomb following the pressure-volume curve technique (II) (Beadle et al. 1993).

3.8. Protein analysis

Bud or bark proteins were extracted and their accumulation was studied either by studying the protein pattern in Coomassie-stained one-dimensional SDS-PAGE (I) or after they were subjected to western-analysis. As a probe, dehydrin antibodies raised against dehydrin consensus K-segment (I), or drought-specific polypeptides DSP16 (II, III) and DSP15 (II) from *Craterostigma plantagineum* were used.

3.9. Northern analysis

Total RNA was extracted and subjected to northern analysis. In experiment III dehydrin clone pcC6-19 corresponding to gene *DSP16* [X74067] of *C. plantagineum* was used as a probe. In experiment IV dehydrin clones *P42* [AJ555331] and *P32* [AJ555332] from birch (*Betula pubescens* Ehrh.) corresponding birch genes *BpuDHN1* and *BpuDHN2*, respectively, were used as probes.

3.10. Isolation of dehydrins from *B. pubescens*

For obtaining probes for detection of birch *DHN*s in northern blotting, PCR with degenerative primers obtained from conservative regions of *DHN*s were used (IV).

3.11. ABA measurements

ABA content of the leaves (I) and buds (I, II, III) was measured using GC-MS SIM and [$^{2}H_{4}$]ABA as an internal standard.

3.12. ABA sensitivity

Sensitivity of the buds to applied ABA was studied by monitoring ability of applied 50 µM ABA to prevent bud bursting of single-node cuttings in water cultivation under forcing conditions (24h day, 18ºC) (II).

3.13. Statistics

The effect of various treatments to ABA and water content were tested against control treatments at the same daylength using Scheirer-Ray-Hare extension of Kruskal-Wallis test (I). The genotypic differences in ABA levels and bud water content were tested with Mann-Whitney U-test. The correlation between ABA and water status was tested by Spearman rank correlation (II). Two-way analysis of variance (ANOVA) was used to test the influence of daylength, line, and temperature on ABA content in hybrid aspen (III). One-way ANOVA was used to test the significant difference in water content between the different treatments within the lines in hybrid aspen (III). One-way ANOVA was used to test whether differences in speed of bud bursting between the ecotypes in each month were statistically significant and Tukey's honestly significant test was used for *post hoc* comparisons (IV). Kruskal Wallis test was used to analyze whether differences in water content between different ecotypes in each month were statistically significant (IV).

4. RESULTS

4.1. The level of ABA is more directly connected to freezing tolerance than to dormancy in birch (I)

The first experiment was designed to investigate if development of dormancy and freezing tolerance under long day (LD) and short day (SD) conditions could be affected by manipulation of the endogenous ABA content in birch. SD led to cessation of growth, decrease in water content of the buds and induction of dormancy. At the same time freezing tolerance increased. The level of ABA increased transiently in buds under SD conditions. Plants grown under 95% RH had no transient increase in bud ABA content, but otherwise they behaved similarly to SD control plants. If plants grown under SD were treated with fluridone, an ABA biosynthesis inhibitor, the level of ABA decreased gradually. Fluridone treatment did not have an effect on growth cessation or on dormancy development, but freezing tolerance development was impaired. Application of ABA or water stress (WS) treatment under LD conditions led to a significant increase in ABA content, and decreased growth, but was not able to induce dormancy development. However, freezing tolerance increased in both of these treatments. Results in article I show also that birch encodes a number of DHN proteins, some of which are responsive to SD. There is an error in the original article (I: Fig. 5) in molecular mass markers of the immunoblot. The 17.7 kD protein marker should be transferred to the place of 7.1 kD protein marker. The immunoblot showed that number of dehydrin proteins (DHN) was accumulating constitutively in birch buds under all the treatments. In addition, SD induced the accumulation of 34 kD and 36 kD DHNs, the levels of which were little lower in fluridone treated and 95% RH grown birch seedlings. Taken together, these results show that physiological changes induced by SD and leading to dormancy and freezing tolerance development are complex and could not be controlled only by manipulation of the ABA level. The level of ABA had no effect on dormancy development, but may be involved in the regulation of freezing tolerance. Since the main environmental factor inducing DHN protein accumulation was SD, it may be connected to SD induced programmed dehydration of the buds.

4.2. Birch cold acclimates through ABA-dependent and -independent mechanisms (II)

The role of ABA and DHNs in dormancy and freezing tolerance of birch was studied further by using an ABA-deficient mutant of birch, which has impaired capacity to increase ABA level under stress conditions. Development and alleviation of dormancy were the same in both birch genotypes and there was no difference in the sensitivity to ABA between these two birches. ABA-deficient birch had a higher basal level of ABA, but it did not increase in

autumn under field conditions, or in response to SD or WS treatments as in the WT. ABA deficient birch had also impaired osmoregulation as seen in an inability to decrease water content or water and osmotic potential under SD or WS. As ABA deficient birch had delayed freezing tolerance in the field and impaired freezing tolerance under SD and WS conditions, it was concluded that enhanced ABA level is an important factor in freezing tolerance development. Since ABA deficient birch could develop some freezing tolerance under SD conditions without any increase in ABA level and it overwintered successfully, there must be an operational pathway for cold acclimation that is independent of ABA. ABA may thus have a role in osmotic adjustment and accurate timing of cold acclimation.

The pattern of two LEA proteins was studied with immunoblotting. LEA 14, belonging to group 3 of LEA proteins detected a 19 kD protein and showed a similar pattern of accumulation in both birch genotypes, being high during winter and low during the active growth period. In addition, it was accumulating similarly in both birch genotypes in response to WS. The other antibody, directed against RAB 16 like DHN, detected three proteins in the WT birch, size of 24, 30 and 33 kDs. ABA deficient birch accumulated only the 33 kD protein. The level of these proteins was high during winter and low during active growth, but the three proteins showed also temporal separation in their accumulation. Also, in midwinter the level of 33 kD protein was higher in the ABA deficient birch than in the WT. These results show that birch may have a way to compensate the low accumulation of certain dehydrins by overaccumulating other members of the DHN family. The external ABA was able to rescue the WT phenotype under SD conditions resulting in normal leaf shedding and accumulation of 24 kD protein after SD and chilling treatments. Since ABA could not induce accumulation of the 24 kD protein under LD conditions, it may be that ABA is needed as a signal transduction component in SD and LT induced gene expression.

4.3. Short photoperiod and low temperature induce cold acclimation independently in hybrid aspen (III)

Woody plants can acclimate in response to both SD and LT separately. We used a transgenic approach to dissect these two environmental factors in cold acclimation. Transgenic hybrid aspen line 22, overexpressing oat *PHYA* was not able to respond to SD by growth cessation, resulting in impaired cold acclimation under SD. However, under LD conditions the line 22 was able to cold acclimate in response to LT, suggesting that photoperiodic and temperature can induce cold acclimation independently. Cold acclimation was accompanied with growth cessation both in SD and LT. In contrast to WT, dehydration of the line 22 buds and bark was prevented under SD conditions. Transfer to LT prevented further dehydration of the buds in the WT, suggesting that this decrease in water content is an active process requiring a relatively high temperature. In the WT, ABA levels of the lateral buds were significantly lower under short than under long photoperiod. However, in the line 22 no significant differences could be observed between the photoperiods. Exposure to 0.5ºC resulted in a transient increase in ABA levels both in the WT and the line 22, showing that photoperiod and temperature regulate ABA levels differently. Line 22 was not able to accumulate DHN proteins under SD conditions, but at LT the accumulation was similar to WT. The line 22 was, however, not able to cold acclimate similarly to WT under these conditions. It was suggested that cellular changes under SD conditions are essential for increased freezing tolerance during subsequent LT.

4.4. Birch dehydrins are expressed sequentially during overwintering (IV)

As latitudinal ecotypes of trees show clinal order in responses that are under photoperiodic control, we investigated the physiological and genetic factors involved in overwintering by comparing three latitudinal ecotypes of birch growing in a common garden experiment at the same location. In the autumn each ecotype responded to the shortening daylength according to its critical daylength resulting in induction of freezing tolerance and decrease in water content first in the northern ecotype, followed by central and southern ecotypes, respectively. However, during spring no clinal order could be observed among the ecotypes in deacclimation, dormancy release or increase in water content suggesting that these traits were controlled by temperature. Therefore, trees seem to be sensitive to photoperiod during autumn, but are responsive to ambient temperatures later in the winter. To obtain molecular markers for cold acclimation or dormancy development, two genomic clones from birch were isolated and expression of the corresponding genes were studied in the field and controlled conditions. Both genes showed seasonal variation in their expression, the level being high during winter and low during the active growth period. However, the timing of the highest expression was different for these dehydrins and it was shown to result in their different responses to environmental conditions. *BpuDHN1* was induced during autumn, in response to SD and low, non-freezing temperature, while *BpuDHN2* was upregulated later, in response to freezing temperatures. *BpuDHN2* was also induced in response to LT under LD conditions, suggesting that photoperiod has influence on DHN gene expression in woody plants. It was concluded that these DHNs are part of the overwintering strategy of birch so that *BpuDHN1* is induced well before the stressfull conditions, preparing the cells to tolerate the freezing temperatures while *BpuDHN2* represents those genes that are needed during the most severe cellular dehydration.

5. DISCUSSION

All four studies forming this thesis are discussed in detail in their relevant scientific context in the corresponding articles. Therefore, only selected conclusions of involvement of ABA and dehydrins in overwintering of woody plants are discussed in this chapter.

5.1. Involvement of ABA in overwintering of woody plants

5.1.1. Involvement of ABA in growth cessation and dormancy development

Short daylength (SD) led to cessation of growth and induction of dormancy and freezing tolerance in birch (I, II) and hybrid aspen (III). Abscisic acid (ABA) as a growth inhibiting substance has been the natural candidate to regulate these phenomena. Indeed, the level of ABA increased transiently in buds of birch seedlings under SD conditions (I, II) and in the buds of field grown adult birches during autumn (II) simultaneously as dormancy and freezing tolerance developed (II). Similar seasonal changes in ABA level have been observed in other woody species (Barros and Neill 1987, Rinne et al. 1994b, Bertrand et al. 1997), and in seedlings under controlled SD conditions (Rinne et al. 1994a, Rohde et al. 2002), although the time of the maximum levels varies among the species. Rohde et al. (2002) suggested that a transient increase in bud ABA content under SD conditions could be involved in growth cessation. Our results indicate that if ABA has such a function, it is indirect, as endogenous ABA levels did not always correlate with growth cessation. Applied ABA alone could not induce growth cessation under LD conditions in birch (I). In addition, fluridone treatment led to a gradual decrease in ABA level in birch buds under SD conditions, but did not prevent growth cessation or dormancy development (I). Similarly, water stress led to very high endogenous ABA levels, and to decreased growth, but was not able to induce growth cessation in birch (I). However, according to Trewavas (1991), not only the increased level of ABA is important for the regulation of cellular metabolism, but changes in the sensitivity may also have an impact. Imai et al. (1995) have shown that plants are less sensitive to exogenous ABA under normal conditions than to endogenous ABA during stress. Sensitivity of willow buds to applied ABA has been shown to increase during SD and decrease during chilling (Barros and Neill 1986). In addition, when birch seedlings were grown near their critical daylength they responded to applied ABA by growth cessation and dormancy development, although under LD conditions ABA application led only to decreased growth (Li et al. 2003a). We were able to demonstrate also that sensitivity of birch buds changed during overwintering. Ecodormant buds of birch were most sensitive to applied ABA in autumn and winter just after the endodormancy was broken (II). Later in the spring the same amount of ABA was not able to prevent bud bursting (II). Therefore, although the changes in ABA level

in buds do not seem to play a role in dormancy development, changes in sensitivity to ABA may contribute to growth cessation and dormancy in birch. It is not completely understood how growth arrest is regulated, but ABA may be involved in growth cessation through regulation of the cell cycle. In rose buds, alleviation of endodormancy by fluridone treatment was accompanied with a decrease in ABA content and a change in cell cycle from G_2 to M-phase, suggesting involvement of ABA in dormancy through cell cycle regulation (Le Bris et al. 1999). Cyclin-dependent kinase inhibitor, (ICK1) has been shown to be responsive to ABA (Wang et al. 1998). Changes in ABA level or responsiveness during SD may function as an input signal in cell cycle regulation, leading to growth cessation (Anderson et al. 2001).

5.1.2. Involvement of ABA in freezing tolerance

Growth cessation induced by SD, and thus dormancy development, seemed to be regulated in a complex way and involvement of ABA, if any, was not dependent on changes in ABA content. However, a more direct connection could be seen between the levels of ABA and increased freezing tolerance. Both water stress and exogenous application of ABA increased freezing tolerance of birch seedlings under LD conditions (I, II). Inability of fluridone treated seedlings to increase freezing tolerance in response to SD (I) and impaired cold acclimation of ABA deficient birch in response to SD or water stress (II) was probably due to their impaired capacity to accumulate ABA under these conditions (I, II). Although the level of ABA was shown to be controlled by different mechanism during SD and LT induced acclimation (III), an increase in ABA content preceding acclimation may be a general scheme with all of the environmental factors triggering an increase in freezing tolerance. Several studies have shown an increase in ABA level in the early stage of cold acclimation at LT (Chen et al. 1983, Lång et al. 1994, Li et al. 2002) and in response to water stress (Lång et al. 1994, Li et al. 2002). Transgenic hybrid aspen line 22, that was impaired in cold acclimation under SD conditions, and did not show any photoperiodic control of ABA level, was, however, able to respond to LT by cold acclimation and showed also a transient increase in ABA content similarly to wild type (III). After plants have received the SD signal, increase in ABA level might be a trigger in a signalling cascade, mediating changes in the environment to a mechanism that leads to metabolic and molecular changes resulting in increased freezing tolerance. The response to short photoperiod is quantitative: the shorter the photoperiod is the faster is the increase in freezing tolerance. In other words, the longer the critical photoperiod of the tree is, the quicker is the increase in freezing tolerance if trees are grown under the same short photoperiod, as demonstrated in Li et al. (2002). In their experiment, ABA level of the buds of birch latitudinal ecotypes increased during the first week under SD conditions, the increase being most rapid in the northern ecotype, followed by central and southern ecotypes, respectively (Li et al. 2002). Trees might respond to a changing environment, such as change in daylength, by an increase in ABA level. The speed of ABA accumulation may depend on the difference between the critical daylength of the ecotype and the daylength the same tree is exposed to. Thus, the greater the difference is, the more rapid is the increase in ABA level and, consequently, also induction of the freezing tolerance.

5.1.3. ABA independent pathway in freezing tolerance

Changes in freezing tolerance under SD conditions were not solely regulated by ABA. Although ABA content increased to the same (II) or even higher level (I) under water stress (WS) conditions as compared to SD, freezing tolerance under WS remained lower than under SD (I, II). In addition, plants grown under 95% RH under SD conditions had no transient increase in ABA content, but otherwise they developed dormancy and freezing tolerance

similarly to SD control plants (I). Moreover, in spite of the fact that in ABA deficient birch (*B. pubescens* f. *hibernifolia*) ABA content did not increase under SD conditions or during autumn in the field, it was able to develop some freezing tolerance under SD conditions and similar hardiness during winter as the WT birch (II). Chen and Li (1978) have suggested that WS, LT and SD each trigger independent cold acclimation mechanism in red osier dogwood. Consequently, when combined together, increased hardiness was the sum of the effects of individual factors, and no interactions were observed (Chen and Li 1978). Li et al. (2002) confirmed these results in birch, and showed also that ABA have an additive effect on freezing tolerance under SD, LT and in combinations of these treatments (Li et al. 2003a). It is well documented that LT triggers both ABA dependent and ABA independent cold acclimation mechanisms in herbaceous plants (Gilmour and Thomashow 1991, Nordin et al. 1991, Shinozaki and Yamaguchi-Shinozaki 2000). Although ABA deficient birch exhibited delayed freezing tolerance in the field during autumn, during first frosts the measured freezing tolerance was the same in ABA deficient birch and in the WT (II), supporting the idea that LT induced freezing tolerance has developed normally despite the ABA deficiency of the mutant birch. It could be that in addition to LT, also SD induces both ABA dependent and ABA independent cold acclimation pathways in woody plants. Thus, some acclimation is possible without any increase in ABA, but activation of both pathways results in higher freezing tolerance under SD conditions (I, II) or more rapid acclimation during overwintering (II). Although Shinozaki and Yamaguchi-Shinozaki (2000) have suggested that drought induces an ABA independent and ABA dependent signalling pathway leading to increased stress tolerance, the complete inability of the ABA deficient birch to cold acclimate in response to WS (II) suggests that water stress induces cold acclimation in birch only through the ABA dependent pathway.

5.2. Role of dehydrins in overwintering of woody plants

5.2.1. Seasonal variation in dehydrin levels

Although the precise function of DHNs has not been elucidated, their consistent accumulation in various plant groups during conditions that cause cellular dehydration, and presence in tissues that have low water content, strongly supports their role in dehydration tolerance of plants (Ingram and Bartels 1996). Cells of overwintering trees are encountering dehydration for two different reasons: Firstly, SD induced growth cessation and dormancy development is accompanied with a decrease in water content or osmotic potential of the overwintering organs (I, II, III) (Junttila and Kaurin 1990, Imanishi et al. 1998, Kontunen-Soppela and Laine 2001, Li et al. 2003b). Secondly, freezing temperatures cause cellular dehydration as water is moved from the cytoplasm to form extracellular ice (Pearce 2001, Ashworth and Pearce 2002). Several studies have demonstrated that there is seasonal variation in dehydrin gene expression and protein content in various woody plants, the level being high during winter and low during active growth period (Arora et al. 1992, 1996, Wisniewski et al. 1996, 1999, Artlip et al. 1997, Sauter et al. 1999, Kontunen-Soppela and Laine 2001, Sarnighausen et al. 2002, Karlson et al. 2003), suggesting that dehydrins participate in overwintering of woody plants. We showed similar seasonal variation in both transcript (IV) and protein levels (II) of birch dehydrins. Distinct *DHN*s were expressed in birch during SD and freezing temperatures (IV), suggesting that in trees DHNs participate in protection against both types of cellular dehydration during the overwintering process. Although the accumulation of different dehydrins varied in different months, their combined highest levels coincided with the lowest temperatures in the field (II, IV), thereby providing

the maximum protection against freeze-induced dehydration. The differential accumulation and degradation of distinct DHN family members during overwintering (II, IV) demonstrate that trees have sensitive systems to regulate the protection of tissues according to the severity of the stress.

5.2.2. Regulation of dehydrin accumulation in woody plants

Nylander et al. (2001) have suggested that dehydrins could principally carry out the same functions in different tissues. Their regulation would therefore be based on the differential accumulation in response to various stresses. Two types of dehydrins were accumulating in birch during overwintering, those that were responsive to SD and subsequent LT, and those that responded to freezing temperatures. The *BpuDHN1* gene (IV), and the 24 kD and 33 kD RAB-16 like DHNs (II) represent those dehydrins that were responsive to SD and subsequent LT, while the *BpuDHN2* gene (IV) and 30 kD RAB-16 like DHN (II) represent freeze-induced dehydrins. The correlation between the expression of the *BpuDHN1* (IV) and accumulation of the 24 kD DHN (II) and expression of the *BpuDHN2* (IV) with accumulation of the 30 kD DHN (II) in the field, suggests that they might represent the corresponding birch genes and proteins, respectively. However, to confirm this, amino acid sequence of the proteins should be obtained. The transcript level of the *BpuDHN1* (IV) and the level of 24 kD DHN (II) started to increase in the field grown birch during autumn, preceding any freezing temperatures. Transcript level of the other birch DHN, *BpuDHN2*, and the level of a 30 kD DHN were increased in the field in mid-winter, when trees were encountered to freezing temperatures (II, IV). *BpuDHN2* was also expressed in response to drought and LT stress under LD conditions (IV). It seems therefore that birch accumulates at least two types of dehydrins. The other ones are mainly under developmental regulation and play a role in anticipation of stress, especially during overwintering. On the contrary, the other ones are induced rapidly during the actual experience of stress. Similarly, Zhu et al. (2000) showed that in barley, part of the *DHN*s are induced in low, non-freezing temperatures while some respond only to freezing temperatures. They suggested that DHNs that accumulate at low temperatures prime cells for more severe conditions. In woody plants not only the low, non-freezing temperature, but also the SD functions as an early warning signal.

Our results suggest involvement of phytochrome A (PhyA) in photoperiodic regulation of dehydrins, as transgenic hybrid aspen line 22, overproducing oat *PHYA* gene was impaired in DHN accumulation under SD conditions (III). It is likely that photoperiodic regulation of dehydrins is not directly under PhyA control, but DHNs are responsive to cellular changes during dormancy development. Photoperiodic induction of dehydrins was not very rapid, but took several weeks both in hybrid aspen (III) and in birch (II, IV). Similarly, photoperiod was shown to control accumulation of a 24 kD dehydrin like protein in *Cornus sericea*, but the accumulation was not evident until after 8 weeks in SD conditions (Karlson et al. 2003). It was suggested that photoperiod-induced water deficit is the stimulus for the 24 kD DHN accumulation (Karlson et al. 2003). Our studies show similar correlation between the decrease in water content under SD conditions and the increase in dehydrin levels (I, II, III). This was particularly clear in transgenic hybrid aspen line 22, that did not have decreased water content under SD conditions similarly to WT and was also impaired in DHN accumulation (III). However, unlike the 24 kD protein in *Cornus* (Karlson et al. 2003), none of the proteins in our studies that were shown to be responsive to SD, accumulated to a very high level in response to WS (I, II, IV), although the water content in WS conditions were lower (I), or the same (II), as under SD conditions. Therefore, it is not likely that merely the decrease in water content under SD conditions is the determining factor in DHN accumulation, but other, yet unidentified, factors are also involved.

Our results suggest that in addition to dehydrins that are regulated in ABA independent pathway, and are accumulating in ABA deficient birch similarly to WT (II), some of the SD induced proteins require ABA for their accumulation. In contrast to WT, ABA deficient birch was neither accumulating the 24 kD and the 30 kD DHNs in the field, nor in response to SD and chilling (II). This suggests their accumulation to be dependent on ABA. Exogenous ABA did not trigger the accumulation of the 24 kD DHN in ABA deficient birch under LD conditions. However, ABA application combined with SD and subsequent chilling treatments both triggered accumulation of the 24 kD DHN, suggesting that ABA is important, yet another SD-induced factor is needed for full expression of the 24kD DHN (II). This type of regulation that requires both SD and ABA can be specific to overwintering organs of woody plants. For example expression of ABA dependent dehydrin, *RAB18* in *Arabidopsis* leaves (Lång and Palva 1992) can be restored by exogenous ABA in ABA deficient *aba* mutant (Lång and Palva 1992, Lång et al. 1994, Mäntylä et al. 1995). Similarly, a number of drought responsive proteins in ABA-deficient *viviparous* mutant in maize leaves could be restored by exogenous ABA (Pla et al. 1989). As the immunoblot of birch proteins against dehydrin consensus K-segment suggests birch genome to contain several DHNs (I), their characterization would help to elucidate if birch also contains DHNs that are directly regulated by ABA. In addition, as the same environmental factors affected *DHN* expression differently in leaves and stem (IV), it is possible that exogenous ABA alone is able to induce accumulation of the 24 kD DHN in birch leaves.

5.2.3. Involvement of dehydrins in freezing tolerance and dormancy

Several lines of evidence suggest that dehydrins participate in the development of freezing tolerance in woody plants. Correlation with dehydrin accumulation and increased freezing tolerance was shown at LT under LD (III), and freezing conditions (IV), both of which have been shown to lead to increased freezing tolerance and increase in dehydrins in herbaceous and woody plants (Lång et al. 1994, Mäntylä et al. 1995, Rinne et al. 1999, Zhu et al. 2000). In addition, treatments that exclusively increase freezing tolerance in woody plants, such as SD (II, III) and SD followed by LT (II, III) both correlated positively with dehydrin accumulation and the increase in freezing tolerance. Inability of the transgenic line 22 to acclimate under SD conditions correlated with its impaired DHN expression under these conditions (III). Moreover, delayed freezing tolerance of the ABA deficient birch in the field (II), coincided with delayed DHN protein accumulation, further suggesting that DHNs play an important role in cold acclimation in woody plants. However, proper functioning of DHNs during cellular dehydration might involve a concerted action of a group of DHNs or interaction of other protective molecules such as other LEA proteins or compatible solutes (Svensson et al. 2002). Indeed, in addition to dehydrins, also other LEA proteins have been shown to participate in the protection of membranes in dehydrative conditions (Wolkers et al. 2001). Seasonal accumulation of the 19 kD LEA protein in ABA deficient birch was similar to WT (II) and might have contributed to freezing tolerance in the field conditions (II). Sugars have been shown to play an important role in cold acclimation (Sakai and Larcher 1987, Wanner and Junttila 1999, Greer et al. 2000, Uemura et al. 2003). Although *Arabidopsis* is able to express a number of cold induced genes at LT in darkness, it fails to acclimate, as it is unable to accumulate sucrose under these conditions (Wanner and Junttila 1999). During overwintering, the sugar source for woody plants is starch which accumulates during SD and is converted to sucrose at LT (Sauter and van Cleve 1991). Impaired cold acclimation of the transgenic hybrid aspen line 22 at LT under SD conditions (III) is most likely a consequence of its inability to accumulate starch under previous SD conditions. Therefore, although line 22

accumulates DHNs at LT under SD, one reason for its impaired cold acclimation under these conditions results from the shortage of its sucrose reserves.

5.3. Relationship between dormancy and freezing tolerance

Osmotic adjustment *i.e.* changes in water content and water and osmotic potentials (Skriver and Mundy 1990), play an important part in overwintering process of woody plants. Water content of the buds and stem decreased in autumn (II, IV) or in response to SD (I, II, III). In addition, both water potential and osmotic potential of the stem decreased under SD conditions and during autumn (II). This programmed dehydration was characteristic to overwintering tissues, since although water content of the leaves decreased under water stress similarly to buds (I), under SD conditions water content of the leaves remained unchanged (I). As mentioned above, dehydration of overwintering tissues during winter is characteristic to boreal and temperate zone woody plants. This decease in water content might largely result from an increase in dry matter. Ultrastructural and metabolic studies show that cells of woody plants undergo massive cellular changes during SD, augmentation of vacuole and accumulation of membranes (Wisniewski and Ashworth 1985, 1986), storage proteins (Wetzel et al. 1989, Coleman et al. 1991) and sugars and starch (Nelson and Dickson 1981, Fege and Brown 1984, Kuroda and Sagisaka 1993, Rinne et al. 1994b, Imanishi et al. 1998) are evident and might contribute to dry matter accumulation. These cellular changes are triggered by alteration of source-sink relationship after growth cessation (Nelson and Dickson 1981, Zhu and Coleman 2001), demonstrating that growth cessation and dormancy development are required for osmotic adjustment. PhyA is involved indirectly in osmotic adjustment, as it participates for receiving the SD signal that leads to growth cessation and dormancy development (III) (Olsen et al. 1997). Transgenic hybrid aspen line 22, overproducing oat PhyA did not respond to SD by growth cessation, nor were the stem or buds dehydrating under these conditions (III). In addition, low temperature prevented this decrease in water content, demonstrating its most efficiently function at relatively high temperature. Our results are in accordance with previous studies that show correlation with dehydration of the tissues and increase in freezing tolerance (Imanishi et al. 1998). Clinal order in the decrease of bud water content in latitudinal birch ecotypes during autumn and rehydration of the buds during spring correlated with the increase and decrease in freezing tolerance, respectively (IV). Similarly, part of the poor acclimation capacity of the line 22 or ABA deficient birch under SD and WS conditions may result from their impaired osmotic adjustment of the buds and stem under these conditions (II, III). These results demonstrate that osmotic adjustment is an essential part of freezing tolerance, which, consequently, is dependent on dormancy development during overwintering.

Generally, drought is thought to stimulate ABA biosynthesis and the increased ABA level in turn triggers expression of genes that participate in the protection against desiccation (Skriver and Mundy 1990). According to our results, ABA might be part of the osmotic adjustment also in woody plants. Increase in ABA level under SD conditions could be seen only in buds, which dehydrated under these conditions (I), while under WS conditions increase in ABA level could be seen both in the buds and leaves that showed a similar decrease in water content (I). A similar discrepancy in osmotic adjustment in buds and leaves under SD conditions, and correlation of this osmotic adjustment and ABA level, has been shown earlier (Rinne et al. 1994a). Involvement of ABA in the osmotic adjustment was further supported by impaired ability of ABA deficient birch for increased ABA content and osmotic adjustment under SD and WS conditions, and in the field (II). Genes that have been shown to participate in osmotic adjustment include enzymes involved in the synthesis of

compatible solutes, such as proline and glycine betaine, together with enzymes of sugar metabolism (Ingram and Bartels 1996). One large group of proteins that are accumulating during drought stress are DHNs and other LEA proteins (Skriver and Mundy 1990, Ingram and Bartels 1996). Accumulation of DHNs in birch coincided with low water content of the buds in the field (II, IV) and most probably participated in the protection against drought during this time. As long as buds are either endo- or ecodormant, their water content remains low (IV). Although none of the dehydrins was shown to be exclusively connected to dormancy, some of the birch DHNs were present in buds as long as water content was low (II, IV). Therefore, although DHNs are not likely to participate in the maintenance of dormancy, they are part of a program that protects cellular structures during low water content characterizing a dormant structure.

5.4. Concluding remarks

This study demonstrates that both abscisic acid (ABA) and dehydrin proteins (DHN) are involved in the overwintering process in woody plants. Short daylength in the autumn functions as a reliable warning signal for the forthcoming unfavourable season. In trees of the boreal and temperate zone this results in a change of vegetative growth to dormancy and accumulation of reserves. Photoreceptor PhyA plays a role in perceiving the short daylength signal that initiates growth cessation and dormancy development in woody plants. ABA may participate in the signalling cascade leading to growth cessation and dormancy, but indirectly, through changes in sensitivity to ABA under SD conditions. Growth cessation under SD conditions is an essential step in the overwintering of trees, as it enables resource allocation to storages that are needed during cold acclimation at freezing temperatures. Similar to herbaceous species, woody plants have both an ABA dependent and ABA independent pathway for cold acclimation. These functions both during the SD induced first stage of acclimation and during the subsequent stages of acclimation, and are induced by low and freezing temperatures, respectively. The ABA dependent and independent pathways may also converge. This was demonstrated under SD conditions, resulting quicker freezing tolerance development in the field or higher freezing tolerance under SD conditions. ABA was shown to participate in the regulation of some of the dehydrins in birch. In this study, two types of dehydrins were characterized in birch. Some dehydrins were mainly under developmental regulation and played a role in anticipation of stress, especially during overwintering, and others were induced rapidly during the actual experience of stress. These distinct types of *DHN*s were expressed in a sequential order during overwintering, in response to SD and freezing temperatures, suggesting that in trees DHNs participate in protection against both types of cellular dehydration during the overwintering process. However, DHNs alone are not able to protect the cellular structures against freezing stress, but they function together *e.g.* sugars and other proteins.

This study shows that cold acclimation of woody plants during overwintering shares some common components with LT induced cold acclimation of herbaceous species. However, the requirement of dormancy development is unique for woody plants and cellular changes during dormancy development under SD conditions may account for the extreme tolerance against freezing in boreal and temperate zone woody plants. The classical way to study the physiological processes in plants is to change one parameter involved and observe the changes it brings to this particular process. As mutant trees are not readily available, transgenic approach is the only reasonable way to obtain trees that have altered function in a specific character. Transgenic hybrid aspen overproducing oat *PHYA* is impaired at the very beginning of the signalling cascade in dormancy development, leading to numerous

physiological alterations, of which all may participate in cold acclimation under SD conditions. Therefore, more subtle changes in parameters involved in photoperiod signalling or target genes would be instrumental in elucidating the crucial components in photoperiodic acclimation. The ABA deficient birch, unable to increase ABA levels in response to stress, warrants more research for example in regard of the defects that cause the ABA deficiency. In addition, increase or decrease in ABA biosynthesis at certain developmental stages would give more than correlative data about the involvement of ABA in these processes.

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