

## MINIREVIEW

**Molecular control of cold acclimation in trees**Annikki Welling<sup>a,b,\*</sup> and E. Tapio Palva<sup>b</sup><sup>a</sup>Division of Plant Biology, Department of Biological and Environmental Sciences, Viikki Biocentre, University of Helsinki, FIN-00014 Helsinki, Finland<sup>b</sup>Division of Genetics, Department of Biological and Environmental Sciences, Viikki Biocentre, University of Helsinki, FIN-00014 Helsinki, Finland**Correspondence**\*Corresponding author,  
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November 2005

doi: 10.1111/j.1399-3054.2006.00672.x

Frost tolerance is an acquired characteristic of plants that is induced in response to environmental cues preceding the onset of freezing temperatures and activation of a cold acclimation program. In addition to transient acclimation to low non-freezing temperatures and enhancing survival to short frost episodes during the growth season, perennial woody plants need additionally to survive the cold winter months. Trees have evolved a complex dynamic process controlling the development of dormancy and freezing tolerance that secures accurate initiation and termination of the overwintering process. Although the phenology of overwintering has been known for decades, only recently has there been progress in elucidating the molecular mechanisms of dormancy and freezing tolerance development in perennial plants. Current molecular and genomic studies indicate that herbaceous annual and woody perennial plants share similar cold acclimation mechanisms. Both the signal processes controlling cold acclimation and the cold-regulated target genes appear to be shared by herbaceous and woody plants. However, the dormancy development during overwintering brings new players in the molecular control of seasonal cold acclimation of woody perennials.

**Introduction**

Plants native to boreal and temperate zones are characterized by their ability to cope with low and freezing temperatures. Freezing tolerance is an acquired characteristic that is induced in response to various environmental cues preceding frost by the process of cold acclimation (Thomashow 1999). Plants are able to cold acclimate very rapidly in response to low, non-freezing temperatures (LT) and thus survive transient cold periods during their growing season. As cold acclimation is counteracting growth, plants are also able to de-acclimate rapidly during a subsequent temperature increase. This type of cold acclimation has been

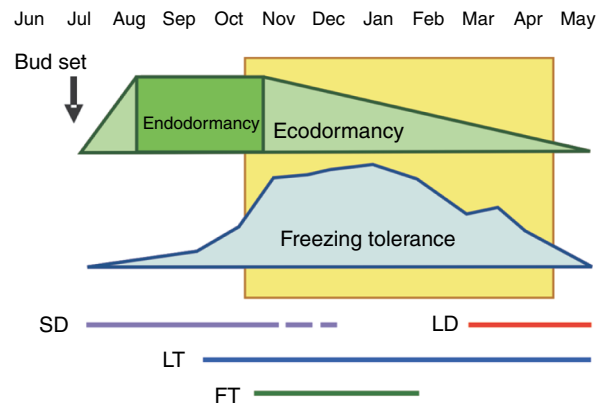
extensively studied in the model plant *Arabidopsis thaliana*, and some of the molecular mechanisms involved are well characterized (see for example Thomashow 1999, Heino and Palva 2003, Sung et al. 2003). Perennial plants need additionally to survive the cold winter months. They have evolved a complex dynamic process controlling the development of dormancy and freezing tolerance that secures accurate initiation and termination of overwintering. Mechanisms involved in overwintering of trees have interested researches for decades, and particularly phenological and physiological aspects, such as environmental control of

**Abbreviations** – ABA, abscisic acid; AP2/EREB, APETALA2/ethylene responsive element binding protein; BSP, bark storage protein; CBF, c-repeat-binding factor; DREB, dehydration-responsive element-binding factor; ELIP, early light-inducible protein; ET, ethylene; EST, expressed sequence tags; FA, fatty acid; FR, far red light; GA, gibberellic acid; HSP, heat-shock proteins; LT, low, non-freezing temperature; LTRE/DRE/CRT, low temperature-responsive element/drought-responsive element/c-repeat; PhyA, phytochrome A; PR, pathogenesis related; R, red light; SD, short day length.

dormancy and freezing tolerance, are well characterized (for review, see Weiser 1970, Sakai and Larcher 1987). More recent reviews update the present knowledge in genetics and molecular regulation of dormancy and freezing tolerance in woody perennials (Rowland and Arora 1997, Rohde et al. 2000, Arora et al. 2003, Wisniewski et al. 2003b). Currently, an increasing numbers of studies have focused on elucidating the molecular mechanisms involved in the control of dormancy and freezing tolerance in perennial plants. These processes are often discussed separately in the literature due to their complexity and distinct regulation. However, as they are parallel processes regulated by common environmental cues and as dormancy has a clear impact on the freezing tolerance of trees, it is important to take both into account when trying to understand overwintering of trees.

### Phenology of overwintering

After an active growth period in early summer, trees begin to prepare for winter well in advance of the adverse conditions in the fall. Activity of the apical bud meristem ceases in response to shortening day length culminating in growth cessation and development of dormancy (Rinne et al. 2001). A few weeks after growth cessation both apical and axillary buds have entered into endodormancy (Fig. 1). Endodormancy refers to a state of buds regulated by internal factors where growth is possible only after plants have been exposed to sufficient amount of chilling (Lang et al. 1987). Parallel to growth cessation in response to short day (SD) photoperiod, the freezing tolerance of trees starts to increase (Fig. 1) (Fuchigami et al. 1971, Junttila and Kaurin 1990, Welling et al. 1997, 2002). Prolonged SD exposure can lead to a substantial increase in freezing tolerance, up to a LT<sub>50</sub> of  $-40^{\circ}\text{C}$ . However, subsequent exposure to low and freezing temperatures enhance freezing tolerance rapidly (Junttila and Kaurin 1990) and are needed for the development of maximum frost hardiness (Fig. 1) (Weiser 1970, Greer and Warrington 1982). In addition to the hardiness increase, low and subzero temperatures simultaneously release buds from endodormancy (Rinne et al. 1997, Cox and Stushnoff 2001) resulting in buds that are hardened and ecodormant (Fig. 1) (Lang et al. 1987). Ecodormant buds maintain a hardened resting state until growth conditions turn favorable in spring. Increasing mean temperatures will also result in a gradual decrease in freezing tolerance (Fig. 1). However, significant fluctuations in temperature in spring can also stop such de-acclimation and lead to a transient increase in freezing tolerance (Welling et al. 2004).



**Fig. 1.** Annual development of dormancy and freezing tolerance in adult, field-grown birch during overwintering in northern Finland. After growth cessation and bud set in July, buds gradually enter into endodormancy, in which they are incapable of bud break until they have received sufficient chilling treatment. Endodormancy is released by November, and ecodormant buds are able to burst under favorable conditions. The more the buds have experienced chilling, the less time they need to start growth under favorable conditions, that is, their thermal time decreases, so that in May they are able to burst within a day. Freezing tolerance starts to increase during autumn in response to SD. Subsequent low and freezing temperatures increase freezing tolerance to its maximum values. February onwards, trees start to de-acclimate and their freezing tolerance decreases with increasing mean temperature. If temperature decreases transiently, trees are able to reacclimate. The yellow box refers to time of the year when mean temperature remains below zero. Horizontal bars denote time of the year when different environmental factors are most likely to have greatest impact on growth of the trees. LD, long day length; LT, low, non-freezing temperature; FT, freezing temperature; SD, short day length (Welling et al. 1997, 2004, Rinne et al. 1998).

### Physiology of overwintering

Frost hardy woody plants can cold acclimate at least to the temperature of liquid nitrogen ( $-196^{\circ}\text{C}$ , Rinne et al. 1998), whereas frost tolerance of annual herbaceous plants rarely exceeds  $-25^{\circ}\text{C}$ . The determinants of extreme tolerance in woody perennials have not been fully elucidated, but most likely the cellular changes associated with the development of dormancy play an important role. Osmotic adjustment, i.e. changes in water content and water and osmotic potentials (Skriver and Mundy 1990) are essential events in the overwintering process of woody plants. Water content as well as water and osmotic potential of the buds and stem has been shown to decrease in autumn or in response to SD (Welling et al. 1997, 2004, Rinne et al. 1998). This programmed dehydration is characteristic to overwintering tissues and may at least partly contribute to their extreme hardiness by preventing ice formation and ameliorating the effects of freeze-induced cellular dehydration. Although water content of the leaves decrease under

water stress similar to buds, under SD conditions water content of the leaves remain essentially unchanged (Welling et al. 1997). Similarly, cold acclimation of herbaceous plants appears to result only in a modest downregulation of tissue water potential (Lång et al. 1994). The observed decrease in water content during fall in overwintering organs might largely result from an increase in dry matter including accumulation of storage proteins (Coleman et al. 1991) as well as sugars and starch (Kuroda and Sagisaka 1993, Rinne et al. 1994b, Sauter and Wellenkamp 1998). This accumulation of proteins and carbohydrates is so intensive that it will alter the ultrastructure of the cells; the large central vacuole which is characteristic for plant cells during growth is displaced in autumn with numerous small vacuolar compartments filling up the cells such as amyloplasts, protein bodies and oleosomes, storing starch, proteins, and fat, respectively (Sauter et al. 1996). These cellular changes are triggered by alteration of source–sink relationships after growth cessation (Zhu and Coleman 2001), indicating that growth cessation and dormancy development are required for osmotic adjustment.

Sugars appear essential in plant cold acclimation, as shown for example by the inability of an *Arabidopsis* sucrose synthase mutant to cold acclimate (Uemura et al. 2003) or the requirement for light in LT-induced cold acclimation connected to sugar accumulation (Wanner and Junttila 1999). Precise function of sugars is not known, but their high abundance in cold acclimated plants suggests a role in osmoregulation, and less abundant sugars might also have a role in cryoprotection or as signaling molecules (Stitt and Hurry 2002). One of the unique characteristics of woody plants is to form metastable cell solutions known as glasses that make cells extremely stable and relatively unaffected by the stresses associated with low temperature and the presence of ice. Aqueous glasses are highly viscous and brought about by a high solute concentration of, for example, sugars at a sufficiently low temperature (for review, see Wisniewski et al. 2003b). Correlation with sucrose accumulation and increase in freezing tolerance has been shown also in woody plants (Sauter et al. 1996). SD 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 (Kuroda and Sagisaka 1993, Rinne et al. 1994b). Starch is converted to maltose and then to sucrose and its galactosides, raffinose, and stachyose, in response to low and freezing temperatures (Rinne et al. 1994b, Sauter et al. 1996). Large starch storages provide sugar supply for trees needed for cold acclimation at the time of the year when deciduous trees have already shed their leaves (Sauter and Wellenkamp 1998).

## Phytochrome photoreceptors in cold acclimation of woody plants

Plants measure day length by measuring duration of the night. If it exceeds the genetically determined ‘critical day length’ it is sensed as SD triggering growth cessation and development of dormancy in trees. Williams et al. (1972) showed that interruption of a long dark period by red (R) light in the middle of the night prevents perception of SD and thus cold acclimation of trees. The effect of R night break treatment is reversed with far-red light (FR), suggesting involvement of R and FR light-responsive phytochrome (phy) photoreceptors in day length perception of trees (Williams et al. 1972, Howe et al. 1996). Recent results suggest that especially phytochrome A has an important role in this perception. Overproduction of PhyA in hybrid aspen renders plants less sensitive to the SD signal and consequently prevents SD-induced growth cessation and cold acclimation (Olsen et al. 1997, Welling et al. 2002). In agreement with the central role of PhyA in photoperiod perception, reduction of transcript level of *PHYA* in transgenic hybrid aspen leads to increased sensitivity to SD photoperiod (Eriksson 2000).

## Hormonal control of overwintering

The mechanism by which plants perceive environmental cues, relay this information to the cellular machinery, and trigger adaptive responses is mediated by several signal transduction pathways. Plant hormones, especially abscisic acid (ABA) and ethylene (ET), have been shown to play an essential role in plant stress signaling (McCourt 1999). Hormonal signaling involves changes both in the hormone levels and in the sensitivity of plant tissues to hormones through, for example, modulating the number of active hormone receptors. Hormonal perception in turn triggers downstream signaling events that subsequently lead to induction of stress-responsive genes and the physiological responses (Xiong et al. 2002).

The level of ABA increases transiently in buds of birch and poplar seedlings under SD conditions (Rinne et al. 1994a, Welling et al. 1997, 2002, Li et al. 2002, Rohde et al. 2002, Ruonala et al. 2006) and in the buds (Rinne et al. 1994b, 1998) and xylem sap (Bertrand et al. 1997) of field-grown adult trees during autumn. Rohde et al. (2002) suggested that a transient increase in bud ABA content under SD conditions could be involved in growth cessation. However, endogenous ABA levels do not always correlate with growth cessation, and altering of ABA levels does not appear to influence growth cessation or dormancy development (Welling

et al. 1997). Interestingly, sensitivity of willow buds to applied ABA has been shown to increase during SD and decrease during subsequent chilling (Barros and Neill 1986). Similar alterations were seen in overwintering birch buds; ABA sensitivity of buds decreased during spring, as buds received an increasing amount of chilling (Rinne et al. 1998). ET has also been shown to have a role in dormancy induction and bud identity in birch. Ethylene-insensitive birch expressing *Arabidopsis* ET receptor gene carrying the dominant *etr1-1* mutation exhibited delayed dormancy development under SD conditions (Ruonala et al. 2006). ABA was not able to prevent bud bursting in ethylene-insensitive birches, suggesting interaction between ABA and ET in regulating bud growth (Ruonala et al. 2006). In conclusion, although the changes in ABA levels in buds do not seem to play a clear role in dormancy development, changes in sensitivity to ABA may contribute to growth cessation and dormancy in trees.

A more direct correlation is evident between the levels of ABA and increased freezing tolerance. Similar to herbaceous species (Chen et al. 1983, Lång et al. 1994), ABA level has been shown to increase in woody plants under conditions that lead to increased freezing tolerance (Rinne et al. 1997, 1998, Welling et al. 1997, 2002, Li et al. 2002). Exogenous ABA can increase freezing tolerance without LT exposure in both woody and herbaceous plants (Chen et al. 1983, Heino et al. 1990, Lång et al. 1994, Mäntylä et al. 1995, Welling et al. 1997, Rinne et al. 1998, Li et al. 2002). ABA-deficient and -insensitive mutants of *Arabidopsis* (*aba1* and *abi1*, respectively) are impaired in cold acclimation (Heino et al. 1990, Mäntylä et al. 1995), but the cold acclimation capacity of *aba1* mutant can be restored by exogenous ABA (Heino et al. 1990). A decrease of ABA content by the ABA biosynthesis inhibitor, fluridone, leads to impaired cold acclimation in birch (Welling et al. 1997). Transgenic hybrid aspen overexpressing oat *PHYA*, deficient in SD-induced cold acclimation, was shown to lack photoperiodic control of the ABA level. However, these trees were still able to respond to LT stimulus and increase their freezing tolerance and ABA content similar to wild-type (Welling et al. 2002). This suggests that ABA levels might be controlled by different mechanisms during SD- and LT-induced acclimation and an increase in ABA content preceding acclimation may be a general scheme by which environmental factors trigger an increase in freezing tolerance.

Gibberellic acid (GA) has been suggested to function as an ABA antagonist in growth processes, accelerating growth. GA<sub>1</sub>, that is one of the bioactive gibberellins, controls cell divisions in the subapical meristem of *Salix*

in response to SD and LD, leading to growth cessation and growth initiation, respectively (Hansen et al. 1999). Photoperiodic control of elongation growth is mediated by regulation of GA biosynthesis, the levels being higher under LD than under SD conditions (Jackson and Thomas 1997). SD perceived by *phyA* has been shown to block certain steps in biosynthesis of GA<sub>1</sub> leading to growth cessation in poplar trees (Olsen et al. 1995, 1997, Eriksson 2000).

Auxin and cytokinins are less studied in relation to overwintering, but they have been shown to be involved in apical dominance (Rohde et al. 2000), also referred to as one form of paradormancy (Lang et al. 1987). Auxin is required for maintaining the cambium in a meristematic state, as decrease in auxin level leads to differentiation of cambium cells to axial parenchyma (Schrader et al. 2003). In addition, during endodormancy, polar auxin transport as well as sensitivity to auxin is reduced in poplar (Schrader et al. 2003). Besides its role in dormancy regulation, ET appears to be involved in cold acclimation in some plants. This is suggested by an increase in ET during cold acclimation and ability of endogenous ethylene to induce number of antifreeze proteins (Yu et al. 2001).

## Gene expression

### Genes and molecular processes in cold acclimation of woody plants

Many of the cellular changes taking place during cold acclimation can be associated with alterations in gene expression (Guy 1990, Thomashow 1999). The major stress encountered by cells during freezing is believed to be the severe freeze-induced dehydration resulting from cell-water potential coming into equilibrium with the vapor pressure of extracellular ice and leading to concomitant cellular changes, such as perturbations in membrane structure, protein denaturation, and increased levels of toxic solutes (Wisniewski et al. 2003a). In addition, cold-induced production of reactive oxygen species especially in combination with high light contributes to damage to membranes (Wang et al. 2003). Consequently, induction of genes controlling membrane stability, biosynthesis of molecular chaperones, enhancement of antioxidative mechanisms, and accumulation of sugars and compatible solutes take part in protecting plant cells against freezing stress.

Microarray technology has enabled the study of the expression of thousands of genes simultaneously, and has been instrumental in demonstrating the numerous changes in gene expression taking place during cold acclimation (Fowler and Thomashow 2002, Seki et al.

2002), and has defined the cold-responsive transcriptome in herbaceous plants (Vogel et al. 2005). In general, stress-responsive 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, Heino and Palva 2003, Van Buskirk and Thomashow 2006). Logically, genes encoding transcriptional activators are among the first ones that are upregulated in response to cold, followed by their respective target genes (Fowler and Thomashow 2002, Seki et al. 2002, Vogel et al. 2005). 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. It is notable that there is also extensive downregulation of gene expression during cold acclimation: Fowler and Thomashow (2002) showed that in *Arabidopsis* nearly 30% of cold-regulated genes were downregulated during acclimation, encoding proteins involved in transcription, signaling, cell-wall biogenesis, and defense. One important group of downregulated genes were those involved in photosynthesis (Fowler and Thomashow 2002).

The novel tools of functional genomics, widely used in cold acclimation studies of herbaceous species (see de la Fuente van Bentem et al. 2006, Renaut et al. 2006,

Wang et al. 2006), have been applied also to studies of dormancy and freezing tolerance development in trees. These include analysis of the transcriptome or proteome or even metabolome of acclimating woody species (see Table 1). The functional genomics approach based on analysis of expressed sequence tags (ESTs) have been used to study cold acclimation of blueberry (Dhanaraj et al. 2004), *Rhododendron* (Wei et al. 2005), and birch (K Ojala et al. unpublished data), dormancy of poplar (Schrader et al. 2004), and various stresses in poplar (Nanjo et al. 2004) (Table 1). The EST approach is especially useful in species whose genome has not been sequenced, revealing new candidate genes associated with cold acclimation and giving rough estimation of the expression level of the genes under given condition. Several hundred unique transcripts were found for both cold-acclimated and -non-acclimated EST libraries of blueberry (Dhanaraj et al. 2004) and rhododendron (Wei et al. 2005). Less than 7% of ESTs were shared between the cold-acclimated and -non-acclimated libraries in both studies, showing that there can be extensive remodeling of the transcriptome during cold acclimation of woody plants. Large-scale EST sequencing of dormant and active cambium showed reduced complexity of the dormant cambium cDNA libraries (Schrader et al. 2004). EST libraries generated from poplar leaves exposed to different abiotic stresses were enriched for genes that were shown to be stress inducible also in *Arabidopsis*. Further studies showed that

**Table 1.** Functional genomics studies in woody plants related to overwintering.

Method	Number of ESTs/genes/and part of the plant	Treatment(s) if specified	Species	Reference
EST sequencing	4406 and 3649 EST sequences from active and dormant cambium, respectively	Summer/autumn (active growth/ dormancy)	Poplar ( <i>Populus tremula</i> )	Schrader et al. (2004)
EST sequencing	4500 full-length EST clones from leaves	Dehydration, high salinity, chilling, heat, ABA, H <sub>2</sub> O <sub>2</sub>	Poplar ( <i>Populus nigra</i> var. <i>italica</i> )	Nanjo et al. (2004)
EST sequencing	430 and 483 EST clones from cold acclimated and non-acclimated floral buds, respectively	Non-acclimated and after 600 chill units	Blueberry ( <i>Vaccinium corymbosum</i> L.)	Dhanaraj et al. (2004)
EST sequencing	862 ESTs from summer- and winter-collected leaves		Rhododendron ( <i>Rhododendron catawbiense</i> Michx)	Wei et al. (2005)
EST sequencing	15 574 contigs and 6804 singlet	16 different libraries	Poplar	Sterky et al. (2004)
EST sequencing	17 500 unigenes	12 different libraries	Birch ( <i>Betula pendula</i> )	K. Ojala et al. Unpublished data
Microarray	POP1 array containing 33 000 ESTs	Summer/autumn (active growth/ dormancy)	Poplar	Schrader et al. (2004)
Microarray	2171 ESTs from xylem and shoot tip library	Seasonal changes in earlywood-latewood	Loblolly pine ( <i>Pinus taeda</i> )	Yang and Loopstra (2005)

similar genes were induced in poplar (Nanjo et al. 2004) and birch (K. Ojala et al. Unpublished data) by the same abiotic factors as in *Arabidopsis*, indicating similarities in stress responses between herbaceous and woody plant species. Recent transcriptome analysis of poplar, overexpressing *Arabidopsis CBF1* gene, reveal strong similarity between poplar and *Arabidopsis* CBF regulons, suggesting a conservation in cold regulation between herbaceous and woody plants (Benedict et al. 2006).

### Genes involved in membrane alterations

Expression profiling as well as analysis of individually isolated genes have identified specific types of genes and processes associated with cold acclimation of woody perennials. Genes encoding early light-inducible protein (ELIP) were found to be the most highly upregulated in cold-acclimated blueberry (Dhanaraj et al. 2004) and rhododendron (Wei et al. 2005). *ELIPs* were also one of the most highly expressed categories of genes during cold acclimation of *Arabidopsis* (Fowler and Thomashow 2002) and in dormant cambium of poplar (Schrader et al. 2004). Photosynthesis is very sensitive to environmental changes, and decrease in temperature causes imbalance between the source of energy and metabolic sink (Ensminger et al. 2006). *ELIPs* are nuclear-encoded thylakoid membrane proteins that are speculated to function as photoprotective pigment carrier or chlorophyll exchange proteins protecting chloroplasts from light-induced damage (Adamska et al. 1992). They might also provide protection against photooxidative damage through the dissipation of excessive light energy (Hutin et al. 2003).

Also, the plasma membrane is a target of a number of changes in its composition during cold acclimation. One of the central metabolic consequences of cold acclimation is an increase in fatty acid (FA) desaturation in membrane lipids, which allows maintaining functional membrane fluidity at LT. Expression of fatty acid desaturase (*FAD*) genes is induced in response to low temperature (Berberich et al. 1998). Martz et al. (2006) cloned three *FAD* genes of birch, *BpFAD3*, *BpFAD7*, and *BpFAD8*, as well as a gene for 3-ketoacyl-ACP synthase II (*BpKASII*) (Table 2). These genes were regulated differentially in response to SD and LT, showing that genes for microsomal (*BpFAD3*) and chloroplast enzymes (*BpFAD7* and *BpFAD8*) act in concert in determining the level of FA desaturation in extra-chloroplast membranes (Martz et al. 2006). The levels of several plasma membrane proteins, especially glycoproteins, exhibit seasonal fluctuations (Yoshida 1984). In addition, there are fluctuations in activity, for example, plasma membrane ATPase activity increases during the

fall due to more efficient ATP catalysis and increased substance-binding capacity during cold acclimation (Mattheis and Ketchie 1990). On the other hand, expression of the genes encoding H<sup>+</sup>ATPase in endodormant buds is low but increases after chilling when buds are in ecodormant state (Gévaudant et al. 2001).

### Genes involved in sugar metabolism

Accumulation of sucrose and starch in fall and mobilization of starch in response to low and freezing temperatures is also reflected at level of gene expression. Transcript levels of genes, such as sucrose synthase, that are involved in the generation of sugars like sucrose and raffinose, are upregulated in poplar cambium in response to SD during autumn. Under the same conditions, starch-degrading enzymes, such as  $\beta$ -amylase and starch phosphorylase as well as R1 protein involved in starch breakdown, are also significantly upregulated (Schrader et al. 2004). Similarly, a high number of EST clones encoding  $\alpha$ -amylase were found in cold-acclimated blueberry flower library (Dhanaraj et al. 2004).

### Molecular chaperones and proteins with cryoprotective activity

Renaut et al. (2004) used proteomic tools to analyze changes in protein levels during chilling of poplar (Table 1). Most prominent changes were associated with deactivation of reactive oxygen species, accumulation of dehydrins and chaperon-like proteins, and transcription factors involved in cold signaling (Renaut et al. 2004, 2006). Molecular chaperones, especially small heat shock proteins (sHSP) are upregulated in response to both high and low temperature extremes, preventing irreversible protein inactivation and aggregation under these conditions (Wang et al. 2004). Ukaji et al. (1999) showed that sHSP WAP20 (Table 2) accumulated in endoplasmic reticulum of cortical parenchyma cells in mulberry during winter and suggested that WAP20 might act as molecular chaperone to stabilize proteins involved in cold acclimation (Ukaji et al. 1999). In chestnut, an sHSP, CsHSP17.5, showed seasonal variation, the level being highest during winter. It was rapidly upregulated by LT and showed cryoprotective activity in vitro (Lopez-Matas et al. 2004). Wang et al. (2002) isolated a novel, boiling soluble, hydrophilic stress-responsive protein SP1 distinct from HSPs that showed molecular chaperone activity (Table 2).

Some of the pathogenesis-related proteins (PR proteins) show seasonal variation, the level being the highest during cold winter months. These proteins may protect plants against diverse stresses encountered

**Table 2.** Genes and proteins implicated in cold tolerance and dormancy development in woody plants.

Mechanism	Genes/proteins	Species	Putative function	Reference
Transcriptional control	<i>PcCBF1</i>	Sour cherry ( <i>Prunus cerasus</i> L.)		Owens et al. (2002)
	<i>CIG-B</i> (CBF)	Sweet cherry ( <i>Prunus avium</i> )		Kitashiba et al. (2004)
	<i>PtCBF1-4</i>	Populus sp.		Benedict et al. (2006)
	<i>BpCBF</i>	Birch ( <i>Betula pendula</i> )		Welling A and Palva ET, unpublished
Signaling	<i>PtABI3</i>	Poplar ( <i>Populus trichocarpa</i> )	Bud morphology	Rohde et al. (2002)
HSPs and molecular chaperones	WAP20 (HSP)	Mulberry ( <i>Morus bombycis</i> Koidz.)		Ukaji et al. (1999)
	CsHSP17.5	Sweet chestnut ( <i>Castanea sativa</i> )	Cryoprotective activity	Lopez-Matas et al. (2004)
PR proteins	<i>Pin m III</i> (PR-10 protein)	Western white pine ( <i>Pinus monticola</i> Dougl. ex D. Don)	Antifreeze protein	Yu et al. (2000)
	<i>PmPR10</i>	Western white pine ( <i>Pinus monticola</i> Dougl. ex D. Don)		Liu et al. (2003)
	<i>Picg5</i> (PR10 protein)	White spruce ( <i>Picea glauca</i> )		Liu et al. (2004)
Proteins against ROS	WAP18 (PR-10 protein)	Mulberry ( <i>Morus bombycis</i> Koidz.)	Cryoprotective activity	Ukaji et al. (2004)
	GR-1NH, GR-1H	Red spruce ( <i>Picea rubens</i> Sarg.)	Antioxidant	Hausladen and Alscher (1994)
LEA-type proteins	<i>PbCORc115</i> , <i>PbCORc119</i>	Trifoliolate orange ( <i>Poncirus trifoliata</i> [L.] Raf.)		Cai et al. (1995)
	<i>ppdhn1</i> , PCA60	Peach ( <i>Prunus persica</i> [L.] Batsch)	Cryoprotective and antifreeze activity	Artlip et al. (1997), Wisniewski et al. (1999)
	<i>CuCOR19</i>	( <i>Citrus unshiu</i> Marcov.)	Cyoprotective activity, radical-scavenging activity	Hara et al. (1999, 2001, 2003, 2004)
	60 kDa dehydrin	Blueberry ( <i>Vaccinium corymbosum</i> [L.])		Levi et al. (1999), Dhanaraj et al. (2005),
	14 kDa dehydrin			Campalans et al. (2000)
	<i>Parab21</i>	Almond ( <i>Prunus amygdalus</i> )		Richard et al. (2000)
	<i>PgDhn1</i>	White spruce ( <i>Picea glauca</i> )		Ukaji et al. (2001)
	<i>WAP27</i> (LEAIII)	Mulberry tree ( <i>Morus bombycis</i> Koidz.)		
	<i>cor1ss5</i>	Grapefruit ( <i>Citrus paradisi</i> )		Porat et al. (2002, 2004)
	<i>ROD60</i> , <i>ROD44</i> , <i>ROD48</i>	Red-osier dogwood ( <i>Cornus sericea</i> L.)		Sarnighausen et al. (2004)
	<i>BpuDhn1</i> , <i>BpuDhn2</i>	Birch ( <i>Betula pubescens</i> )		Welling et al. (2004)
	<i>Bplti36</i>	Birch ( <i>Betula pendula</i> )		Puhakainen et al. (2004b)
	24 kDa dehydrin protein	Red-osier dogwood ( <i>Cornus sericea</i> L.)		Sarnighausen et al. (2002), Karlson (2003a, 2003b)
	<i>csDHN</i>	Orange ( <i>Citrus sinensis</i> )		Porat et al. (2004)
	<i>cpDHN</i>	Grapefruit ( <i>Citrus paradisi</i> )		Porat et al. (2004)
<i>PV-dhn</i>	Pistachio ( <i>Pistacia vera</i> [L.])		Yakubov et al. (2005)	
Cell cycle	PttCDKA, PttCDKB	Poplar ( <i>Populus tremula</i> × <i>P. tremuloides</i> )		Espinosa-Ruiz et al. (2004)
	CsTOC1, CsLHY	Chestnut ( <i>Castanea sativa</i> )		Ramos et al. (2005)
Membrane alterations	<i>BpFAD3</i> , <i>BpFAD7</i> , <i>BpFAD8</i> , <i>BpKASII</i>	Birch ( <i>Betula pendula</i> )	Desaturation of the membranes	Martz et al. (2006)
	<i>PPA1-4</i>	Peach ( <i>Prunus persica</i> )	H <sup>+</sup> ATPase	Gévaudant et al. (2001)
Storage proteins	<i>bspA</i>	Poplar ( <i>Populus deltoides</i> )		Coleman et al. (1992)
Novel proteins	sp1	Poplar ( <i>Populus tremula</i> )	Chaperone activity	Wang et al. (2002), Dgany et al. (2004)
	Defensin <i>CLTa</i>	Peach ( <i>Prunus persica</i> [L.] Batsch) Trifoliolate orange ( <i>P. trifoliata</i> )	Antimicrobial activity	Wisniewski et al. (2003a) Robbins and Louzada (2005)

during winter, as some have been shown to have anti-freeze activity, cryoprotective activity, or antifungal activity (see Table 2) (Hon et al. 1995). Ukaji et al. (2004) identified WAP18 protein from mulberry that was shown to belong to the PR-10 protein family. WAP18 levels were highest during winter, and it was shown to have cryoprotective activity in vitro (Table 2). A related PR-10 class of protein, Pin m III from western white pine, showed increased accumulation during winter months. It was additionally induced by blister rust, suggesting an interaction between the cold and pathogen-regulated plant defense systems (Yu et al. 2000).

## Dehydrins

Due to their abundance and predicted role in protection against cellular dehydration, dehydrins are by far the most extensively studied proteins in relation to cold acclimation and dormancy in woody plants. Several dehydrin genes contain the LTRE/DRE/CRT (low temperature response element, drought responsive element, or c-repeat) in their promoter (Svensson et al. 2002) that is recognized by CBF transcription factors and are thus part of the CBF regulon of a given plant. 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). In addition, some of the dehydrins accumulate in response to ABA, whose level increases in response to osmotic stresses (Skriver and Mundy 1990). Although the precise function of dehydrins 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 encounter dehydration for two different reasons: first, SD-induced growth cessation and development of dormancy is accompanied by a decrease in water content and osmotic potential of the overwintering organs (Junttila and Kaurin 1990, Welling et al. 1997, 2002, Rinne et al. 1998, Kontunen-Soppela and Laine 2001) and, second, freezing temperatures cause freeze-induced cellular dehydration, as water is moving from the cytoplasm to form extracellular ice (Pearce 2001).

Several studies have demonstrated seasonal variation in dehydrin gene expression and protein content in various woody plants, the level being high during winter and low during the active growth period (Table 2) (e.g. Arora et al. 1992, Wisniewski et al. 1996, 1999, Artlip et al. 1997, Rinne et al. 1998, Kontunen-Soppela and Laine 2001, Karlson et al. 2003b, Sarnighausen et al. 2004, Welling et al. 2004). To dissect the factors

regulating dehydrin appearance during overwintering, dehydrin gene expression has been studied in response to various environmental conditions. The majority of woody plant dehydrins are induced in response to LT, and a few have been shown to be induced in response to SD (Karlson et al. 2003b, Welling et al. 2004) or SD followed by LT (Welling et al. 2004, Puhakainen et al. 2004b). Additionally, dehydrins have been shown to be induced in dormant tissue in response to freezing temperatures, although only after thawing of the tissue (A. Welling and E.T. Palva, unpublished). In birch, distinct dehydrins were expressed, in fall, in response to SD, and in midwinter, in response to low and freezing temperatures (Welling et al. 2004). This correlative data suggests that in trees, dehydrins participate in protection against both types of cellular dehydration during the overwintering process.

In peach, dehydrins were shown to be located in the cytoplasm, plastids, and nucleus of bark and xylem parenchyma cells (Wisniewski et al. 1999). Puhakainen et al. (2004a) showed that in *Arabidopsis*, dehydrins were located mostly in cytoplasm in normal growth temperatures but they were relocated into plasma membranes in response to LT. In addition, dehydrins have been localized to plasmodesmata in *Cornus* (Karlson et al. 2003a). Dehydrins have been proposed to have in vitro cryoprotective (Close 1996, Rinne et al. 1999, Wisniewski et al. 1999, Hara et al. 2001) and antifreeze activity (Wisniewski et al. 1999), to function as possible osmoregulators (Nylander et al. 2001) or function as radical scavengers (Hara et al. 2004). Recent results suggest that dehydrins may also have calcium-binding activity (Alsheikh et al. 2005). Additionally, there is some recent, direct evidence for the protective role of dehydrins. Simultaneous overexpression of two dehydrin genes in *Arabidopsis* resulted in increased freezing tolerance in non-acclimating conditions (Puhakainen et al. 2004a). Similarly, ectopic expression of citrus dehydrin in tobacco resulted in increased tolerance against freezing and lipid peroxidation (Hara et al. 2003).

## Bark storage proteins

During autumnal leaf senescence, leaf proteins in deciduous trees are hydrolyzed and resulting amino acids are translocated in phloem sap to overwintering organs, where they are converted to storage proteins. These bark storage proteins (BSP) accumulate in large amounts in autumn and are degraded at the onset of spring growth (Gomez and Faurobert 2002). Trees accumulate BSPs during fall to levels, which account from 30 to 50% of soluble bark proteins during winter (Coleman et al. 1991). Therefore, it is not surprising that nearly

20% of the ESTs of dormant cambium were shown to account for BSPs (Schrader et al. 2004). Poplar BSPs are encoded by a small multigene family (Coleman et al. 1992). One of the genes, *bspA*, has been cloned (Table 2) (Coleman et al. 1992) and its expression has been shown to be responsive to photoperiod (Coleman et al. 1991, 1992), nitrogen availability (van Cleve and Apel 1993), temperature (van Cleve and Apel 1993), and wounding (Davis et al. 1993). Although *bspA* 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).

### Biosynthesis of hormones and hormone receptors

During endodormancy, polar auxin transport as well as sensitivity to auxin is reduced (Schrader et al. 2003). However, genes for only two out of four efflux carriers are downregulated during dormancy, while the transcript level of others remaining unchanged (Schrader et al. 2004). Schrader et al. (2004) also found that transcript level of the poplar gene *PttRGA1*, which is highly similar to *RGA1*, a repressor of gibberellin response, is highly upregulated in dormant cambium. This indicates that not only the level of gibberellins is altered during dormancy but also the GA signaling is modulated in overwintering tissues. There was no difference in the level of transcription of ABA biosynthesis gene, *9-cis-epoxycarotenoid dioxygenase*, between dormant and active cambium (supplemental data from Schrader et al. 2004).

### Regulation of gene expression

#### CBFs

The best understood regulatory pathway in cold acclimation is the CBF/DREB1 cold response pathway, which has been extensively characterized in *Arabidopsis* (see Nakashima and Yamaguchi-Shinozaki 2006, Van Buskirk and Thomashow 2006). *Arabidopsis* encodes a small family of cold-responsive transcription factors known as CBF1-3 (Stockinger et al. 1997, Gilmour et al. 1998) or DREB1b, c, and a, respectively (Liu et al. 1998, Kasuga et al. 1999). The *CBF* genes are induced within 15 min of plant exposure to cold, followed by induction of CBF target genes constituting the so-called ‘CBF regulon’. CBFs activate cold-regulated genes by binding to LTRE/DRE/CRT-element of their promoters. The contribution of the CBF regulon in cold acclimation is demonstrated by studies where overexpression of *CBFs* leads to constitutive expression

of CBF target genes, resulting not only in increased freezing tolerance of the plants in non-inductive conditions but also in increased drought and salt tolerance (Jaglo-Ottosen et al. 1998, Kasuga et al. 1999, Gilmour et al. 2000).

The CBF protein family appears to be conserved among plant species. CBF protein sequences of different plant species are variable, but they can be distinguished from other AP2/EREb transcription factors by the CBF signature sequence flanking the conserved AP2/EREb domain (Jaglo et al. 2001). In spite of the differences in protein sequence, CBF proteins are recognized between species, demonstrated by several studies showing that ectopic expression of *CBF* genes improves freezing tolerance of non-acclimated plants (Jaglo-Ottosen et al. 1998, Gilmour et al. 2000, Jaglo et al. 2001, Haake et al. 2002, Gilmour et al. 2004, Benedict et al. 2006, A Welling and ET Palva, unpublished). The CBF regulon is also present and functional in plant species incapable of cold acclimation such as tomato (Zhang et al. 2004). It was shown that tomato contains the CBF cold response pathways but that the CBF regulon is much smaller and does not contain tomato orthologs of *Arabidopsis* CBF-controlled genes, partly explaining the inability of tomato to tolerate cold (Zhang et al. 2004).

Although the CBF pathway encompasses only part of the cold acclimation capacity of plants (Fowler and Thomashow 2002), it is of interest to study whether the extreme cold acclimation capacity of boreal and temperate zone woody plants is reflected in function or size of their CBF regulons. CBF orthologs have been cloned from several woody species, and overexpression studies have shown that they are able to convey increased tolerance to freezing and other abiotic stresses (Table 2) (Owens et al. 2002, Kitashiba et al. 2004, Benedict et al. 2006, A Welling and ET Palva, unpublished). Similar to *Arabidopsis*, the woody plant *CBFs* are induced by LT during growing season (Benedict et al. 2006, A Welling and ET Palva, unpublished), but also in dormant cambium in autumn (Schrader et al. 2004) and in response to low and freezing temperatures after SD exposure (A Welling and ET Palva, unpublished), suggesting that they participate in both seasonal cold acclimation and acclimation to periodic frost episodes during the growing season (Table 2).

Comparison of transcriptomes of the wild-type plants after cold exposure and CBF overexpressors has identified which of the cold-regulated genes are under CBF regulation (Fowler and Thomashow 2002, Maruyama et al. 2004, Vogel et al. 2005). By this approach, Benedict et al. (2006) were able to show that functional

distribution of genes affected by cold in wild-type poplar leaves was rather similar to that of transgenic poplar expressing *AtCBF1*. A number of these cold-regulated genes encoded orthologs of *Arabidopsis CBF3* regulon genes. Ectopic expression of *AtCBF1* in poplar also resulted in increased freezing tolerance. Interestingly, in stem tissues, overexpression of *AtCBF1* resulted in a different set of genes compared with that of cold-treated stem and leaves, suggesting different functional roles for the CBFs in perennial and annual tissues. This was supported by the fact that two of the four poplar *CBF* orthologs show differential expression in annual and perennial tissues (Benedict et al. 2006). Several putative *CBF* orthologs have been identified in birch EST libraries (Table 1) of which two full-length EST clones were used for functional studies. Overexpression of birch *CBF* orthologs in *Arabidopsis* lead to increased freezing tolerance and induction of *CBF* target genes in non-inductive conditions, demonstrating functional similarity between birch and *Arabidopsis CBF* genes. Birch *CBFs* were induced by LT in both leaf and stem tissues; however, in dormant plants, LT-induced *CBF* expression was delayed compared with that of actively growing plants (A Welling and ET Palva, unpublished). Expression of the *CBF* target gene, *Bplti36*, encoding a birch dehydrin (Puhakainen et al. 2004b) was sequential to birch *CBFs* (A Welling and ET Palva, unpublished). Interestingly, *CBFs* were also induced in plants frozen to  $-12^{\circ}\text{C}$ , but only after the plants were thawed. Earlier studies have shown that some of the birch dehydrins are upregulated in midwinter, during the coldest months when minimum temperatures may drop down to  $-30^{\circ}\text{C}$ , but are also fluctuating rapidly with non-freezing temperatures (Welling et al. 2004). These results suggest that plants have some kind of a priming mechanism to provide memory of preceding frost and to activate gene expression when conditions become permissive.

These novel findings of *CBF* expression in woody plants illuminate the complex regulation of *CBFs* additionally to LT. Schrader et al. (2004) showed that *CBFs* are upregulated in field-grown poplar trees in response to SD. Although there was no significant increase in *CBF* expression in birch stems or buds under controlled SD conditions (A Welling and ET Palva, unpublished), SD might indirectly affect *CBF* expression, by responding to SD-induced osmotic stress as suggested by Schrader et al. (2004). SD has also direct effect on LT-induced genes, as SD has been shown to enhance LT induction of birch dehydrin gene *Bplti36*, which is under *CBF* regulation (Puhakainen et al. 2004b). Increasing evidence suggests that light modulates *CBF* expression also in herbaceous plants (Fowler et al.

2005). *CBF1-3* in *Arabidopsis* is gated by the circadian clock, the induction of the *CBF* genes by LT being highest during dawn (Fowler et al. 2005). The molecular mechanism or biological significance of involvement of light in regulation of cold-inducible genes is not currently known. However, studies with woody plants having strong response to combination of SD and LT should bring new insights into regulation of *CBF* cold-responsive pathway in response to light.

The recent molecular and genomic studies clearly suggest that herbaceous annual and woody perennial plants share similar mechanisms of cold acclimation. Decrease in temperature leads to activation of similar signaling process and expression of orthologous target genes in both herbaceous and woody plants. These similarities are particularly evident in acclimation to short frost episodes during growth but are also partly shared by seasonal cold acclimation of woody perennials. However, the overwintering process is more complex due to strong photoperiodic regulation and dormancy development. Therefore, in order to understand regulation of the extreme cold tolerance of woody plants during overwintering, it is essential to elucidate the complex interplay between dormancy and cold tolerance. Such studies in woody plants have been hampered by the long generation time and slow growth; therefore, the number of studies elucidating molecular regulation of woody plant cold acclimation is still minuscule compared with that of herbaceous plants. However, the situation is rapidly changing due to the novel and efficient approaches of functional genomics that are now applied to woody plant research, and it should not be too optimistic to expect rapid progress in understanding the molecular control of cold acclimation and development of winter hardiness in woody plants.

*Acknowledgements* – The work in the authors' laboratory is supported by Academy of Finland, Biocentrum Helsinki and the National Technology Agency of Finland.

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