

Innovative Cold Tolerance Test for Conifer Seedlings

Peter A. Balk

Peter Brønnum

Mike Perks

Eva Stattin

Lonneke H.M. van der Geest

Monique F. van Wordragen

Peter A. Balk is with NSure, P.O. Box 14, 6700 AA, Wageningen, The Netherlands. **Peter Brønnum** is with the Danish Institute of Agricultural Sciences, Aarslev, Denmark. **Mike Perks** is with the Forestry Commission Research Agency Northern Research Station, Roslin, Midlothian, United Kingdom. **Eva Stattin** is with Dalarna University, Garpenberg, Sweden. **Lonneke H. M. van der Geest** is with Plant Research International, Wageningen. **Monique F. van Wordragen** (corresponding author) is with NSure, P.O. Box 14, 6700 AA, Wageningen, The Netherlands; Tel: 31.317.475114; E-mail: monique.vanwordragen@nsure.nl.

In: Riley, L. E.; Dumroese, R. K.; Landis, T. D., tech. coords. 2007. National proceedings: Forest and Conservation Nursery Associations—2006. Proc. RMRS-P-50. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. Online: <http://www.rmnr.net/nurseries/publications/proceedings>

Keywords: genomics, dormancy, cold acclimation

Introduction

Forest tree nurseries rely on tight scheduling of operations to deliver vital seedlings to the planting site. Cold storage is required to: (1) prevent winter damage, especially in container seedlings; (2) to maintain planting stock in an inactive condition; and (3) to ensure plant supply for geographically distinct planting sites, a definite requirement for large-scale or internationally operating nurseries.

Cooler storage has become common practice, but poses a dilemma for nursery managers. Efficient management requires that the handling of seedlings, such as transfer to cold storage, be carried out at the earliest possible time. However, lifting and storage of insufficiently hardened plants reduces vitality and may lead to cold damage, dehydration, and fungal infection. To prevent this kind of damage and its adverse economic effects on nurseries and end-users, it is of vital importance to accurately determine the peak physiological condition for lifting or transfer.

Despite the economic importance of such decisions, European nurseries still predominantly rely on traditional (morphological) methods to identify peak physiological condition. Recently, several physiological measurement techniques have been proposed, and some of them have been used operationally. However, the number of nurseries utilizing these techniques is limited because the methods are either unreliable, labor intensive, or technically demanding, and the minimum test period (test dependent) can vary from 3 days to over 14 days. In nursery practice, where lifting opportunities can be severely limited by rainfall, frost, and snow, such a delay may significantly reduce the number of plants lifted at peak physiological condition.

At present, a robust, fast and quantitative method to support this or other important nursery management decisions is not available, yet. With this in mind, a European research project was initiated to develop functional monitoring tools for nurseries based on direct analysis of the biological processes that control dormancy and cold tolerance using genomics technology.

Genomics Diagnostics

Applied genomics is an innovative field of research that aims at the application, in practice, of the tremendous amounts of data generated within a variety of genomics disciplines. These include large-scale analysis of gene expression, protein profiles, and metabolite composition, supported by bioinformatics, data management, and statistics. In the coming decade,

this type of research will prove to have a large economic impact on agribusiness.

Brought back to its essence, genomics technology provides the tools to understand life in all its complicated details. Genomics diagnostics can facilitate the implementation of precisely controlled agroproduction and distribution chains. In the present paper, gene-expression analysis is proposed as the most effective technology for determining cold hardiness. In living organisms, each developmental step and every interaction with the environment is orchestrated by DNA encoded genes. The history and actual condition of a plant, animal, or microorganism is accurately reflected in the activity profile of its genes. Gene expression analysis, or transcriptomics, is performed using microarrays or biochips. On these microarrays, several thousands of copied genes are present on a glass slide of a few square centimeters. The array is used to detect the level of activity of each of the represented genes simultaneously. Thus, the responses of the plant on any environmental trigger can be followed in a direct way. The information is used to select those genes that are most involved in controlling the trait of interest, in this case cold hardiness. The selected set of indicator genes is translated into a reliable and robust assay for use in practice.

Cold Acclimation

Cold acclimation is a complex process comprised of several more-or-less discrete sub-processes, which are triggered by different environmental cues (Sakai and Larcher 1987). Trees native to northern latitudes grow under conditions where marked annual temperature changes are observed, and have developed physiological mechanisms that result in strong periodicity in cold hardiness in phase with the climate (Bigras and others 2001). Although cold acclimation and dormancy onset are influenced by temperature, the primary environmental signal for most northern latitude native tree species for induction of the cessation of shoot elongation and some degree of enhanced frost hardiness is photoperiod, that is, the attainment of critical night lengths (Wareing 1951; Thomas and Vince-Prue 1997). The critical night length in plants is under strong genetic control and varies according to latitude and elevation (Aitken and Hannerz 2001). The attainment of tolerance to freezing temperatures occurs in response to near-zero thermal exposure; to achieve maximal hardiness, temperatures below freezing are usually required (Burr and others 1989). The maximal capacity to cold acclimate then varies according to organ/tissue type, species, provenance, and exposure temperature.

When plants are exposed to cold, a number of events at the molecular level take place. At lower temperatures, membrane fluidity decreases, causing membrane rigidification. This triggers a signal transduction cascade involving structural rearrangement of the cell, followed by a transient cytoplasmic calcium increase, which in turn triggers gene expression (reviewed by Chinnusamy and others 2004). Changes in the expression of specific genes are thus an accurate and early indicator for the development of cold tolerance.

Prominent gene classes that were found to be long-term cold-induced include transcription factors (including CBF and

downstream targets), COR/LEA proteins (hydrophilic “anti-freeze” polypeptides thought to play roles in cryoprotection) (Close 1997), and proteins with roles in sugar accumulation (Fowler and Thomashow 2002; Seki and others 2002). However, most molecular research has been done in herbaceous model plants. Much less is known on the molecular basis of cold acclimation in trees.

Molecular Diagnostic Cold Tolerance Testing

In order to gain a better understanding of cold tolerance development in trees, a comprehensive study was performed to correlate physiological and molecular responses in Scots pine (*Pinus sylvestris* L.) and common beech (*Fagus sylvatica* L.) seedlings. Pine and beech represent gymnosperm and broadleaved trees and differ in dormancy profile. The study was performed in four countries (Denmark, Netherlands, Scotland, and Sweden), and involved both research institutes and nurseries. The main objective in the project was to link physiological features of shoot cold tolerance and bud dormancy in Scots pine and common beech seedlings with vitality during and after cold and frozen storage, and to correlate this with gene expression by utilizing genomics technology. The results led to the development of a rapid, predictive molecular diagnostic test.

Research Approach

During climate room and field trials at different locations, samples were simultaneously analysed for cold hardiness, dormancy, and gene expression programs, while photoperiod and ambient temperature were also recorded. Using these parameters, the relation among climate, physiological response, and gene expression program could be quantitatively determined, and potential marker genes for cold hardiness could be identified. Seedlings were grown in climate-controlled environments for the initial identification of relevant genes, followed by outdoor trials that were used to detect the effect of climatic conditions, geographical position, and provenance on the underlying molecular processes. All trials were done for Scots pine and common beech. Later, results were extended to include white spruce (*Picea abies* [L.] Karst.). Table 1 summarizes the provenances used in this study. The present paper will focus on the results for pine.

Field trials were performed according to the same experimental protocol at the various locations. Various local provenances were grown at each location along with a standard lot from a particular provenance. This allowed comparisons for most parameters that are known to influence dormancy and cold hardiness development, such as geographic location (day length), genetic background, and adaptation history. The field experiments resulted in data covering the variation in dormancy/hardiness encountered in practice to a large extent. Samples for molecular analyses, taken from the field-grown trees, were eminently suited for studying the differential gene expression accompanying this biological variation.

Table 1—Plant material used to correlate frost tolerance with gene expression and establish the role of genes with diagnostic potential.

Species	Provenance	Origin	Test location
<i>Pinus sylvestris</i>	A70	Scotland	Scotland, Denmark, Sweden
	Lindås	Denmark	Scotland, Denmark
	Sollerön	Sweden	Sweden
	Åmsele	Sweden	Sweden
	Local Dutch	Netherlands	Netherlands
<i>Picea abies</i>	Witspar	Netherlands	Netherlands
<i>Fagus sylvatica</i>	Bregentved	Denmark	Scotland, Denmark, Netherlands
	Cirencester	Scotland	Scotland
	Greenhill	Netherlands	Netherlands
	NL2.1	Netherlands	Netherlands

Results

The general approach taken in transcriptional profiling was aimed towards finding the major shifts in gene expression accompanying generally occurring physiological changes. A dedicated microarray was used carrying preselected genes. This cDNA microarray was constructed using cDNA clones from three different *Pinus sylvestris* bud cDNA libraries. One library was prepared using mRNA from buds that were harvested in December, and two libraries were prepared using subtraction in order to enrich for cDNAs that were up-regulated in cold-treated or quiescent buds. In total, about 1,450 ESTs were spotted on the microarray.

The field sample series turned out to be excellent material to reconstruct the general order of molecular events. Comparison between different provenances and geographic locations allowed distinguishing between differential expression related to the physiological developments of interest and noise effects induced by local environment. Subsequent analysis of climate room-grown samples allowed further correlation of the gene expression events to physiological developments (detailed results are described in Joossen and others 2006).

Figure 1 shows a typical set of results, combining environmental, physiological, and molecular data. The comparison of field-grown and climate room-grown plants enabled us to differentiate between genes involved in the early or pre-programmed part of hardiness development and genes that are more responsive to actual environmental conditions.

Using Pearson clustering, three different gene groups were identified that correlated to physiological parameters. In samples from different locations and provenances, genes from each group display a characteristic gene expression profile during the acquisition of frost hardiness:

- Group 1: Increase in expression during the acquisition of frost tolerance, indicating a potential role of these genes in the hardening process.
- Group 2: Decrease in expression during the acquisition

of frost hardiness. Genes from group 2 switched off as early as week 39 of the field samples, correlating their expression to active growth in the summer.

- Group 3: Decrease in expression during the acquisition of frost hardiness, but were active longer into the fall season than group 2 genes, and might play a role in initiating the response to short day length and decreasing temperatures.

Based on their expression pattern, these groups of genes can be linked to physiological events that occur during acquisition of frost hardiness. cDNAs from groups 2 and 3, which RNA levels decline during hardening, represent genes that are involved in active growth and that are shut down during the quiescent winter period. Many cDNAs from these groups encode genes that are involved in translation, primary and secondary metabolism, and cell wall biosynthesis. A relatively small number of stress-related genes fall within this group. Genes from group 1, however, increase in expression in parallel with the increase in cold tolerance, implicating a role in the cold response, adaptation, and hardening process. This group contains a remarkably high number of stress-related genes (induced by various stresses), as well as genes that are involved in biosynthesis of cellular components that can protect against stress (osmotics, antimicrobial or antifungal compounds, LEA proteins, and so on). Additionally a number of primary and secondary metabolic genes, as well as transporter proteins are induced. Figure 2 shows an overview of the physiological and molecular processes and corresponding gene expression programs that take place during the transition from active growth to quiescence and frost hardiness.

The Cold Hardiness Test

The data obtained enabled the selection of a set of genes that can be used as indicators for either the hardening stage or the fully hardened stage. Selection was based on correlation with shoot electrolyte leakage (SEL) measurements (determined by subjecting shoot tips to a freeze temperature of -15 or -25 °C [5 to -13 °F] in a programmable freezing cabinet and comparison against a baseline control value, obtained after a $+4$ °C [39 °F] treatment) and validated against regrowth potential after storage.

It was possible to bring the selection down to three indicator genes that together provide enough information to give an accurate estimate of the cold hardiness stage of the seedling. The corresponding genes dominate the process of hardiness development in all provenances studied and have a strong correlation with SEL values (fig. 3). The activity of three of these indicators, two differentially regulated dehydrin gene family members and one control gene, is measured in the cold hardiness test implemented by the company NSure. The dehydrin genes have different biological roles. One gene is involved in general drought and cold resistance and is active during growth and initial stages of hardening. The other dehydrin gene is highly specific for the fully hardened bud, and the activity of the corresponding gene peaks when maximum cold hardiness has been attained. The assay is based on the relative activity of these genes and the control gene.

The test was validated in multiple *Pinus sylvestris* provenances grown at different geographical locations in Europe

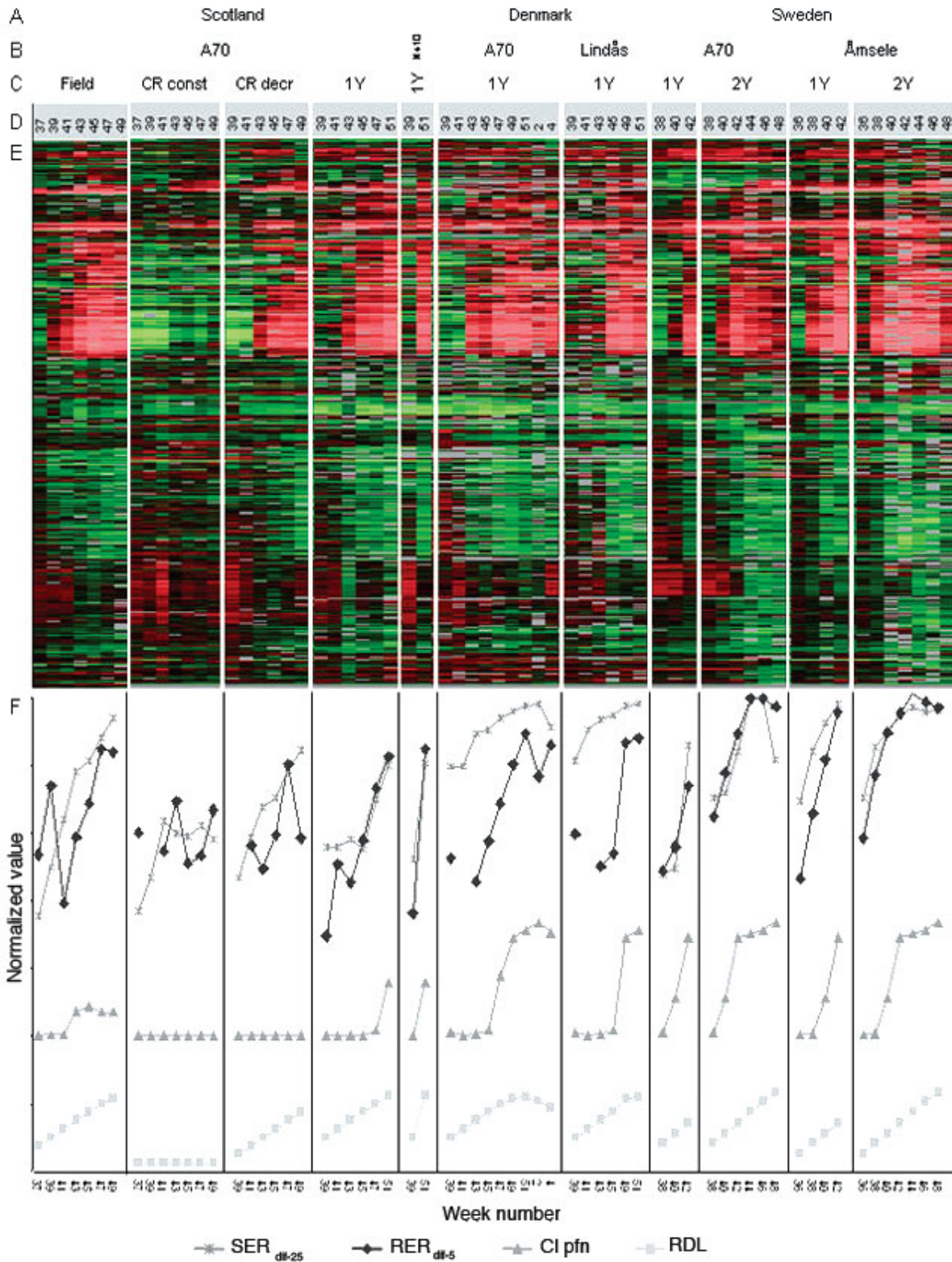


Figure 1—Overview of gene expression patterns and physiological characteristics of all pine samples analyzed using the cDNA microarray. A) Location of the experiment. B) Provenance used. C) Field or climate room experiment (1-year-old seedlings), or seedling age (field experiment). D) Sample week number. E) Gene expression in all samples (relative to a common reference) shown after hierarchical clustering of the expression patterns. Each row represents a gene. Red is high relative to the common reference, while green is low relative to the common reference. Shifts from green to red indicate increasing gene activity, while changes from red to green indicate declining gene activity. F) Physiological characteristics of the samples analyzed using the microarray. The transformed physiological characteristics are shown: asterisks represent $SER_{diff-25} = (100 - SEL_{diff-25})/10$; diamonds represent $RER_{diff-5} = (100 - REL RER_{diff-5})/10$; triangles represent $CI pfn = (\text{hours below } 4^\circ C \text{ previous 2 weeks})/100$; squares represent $RDL = \text{day length}/4$.

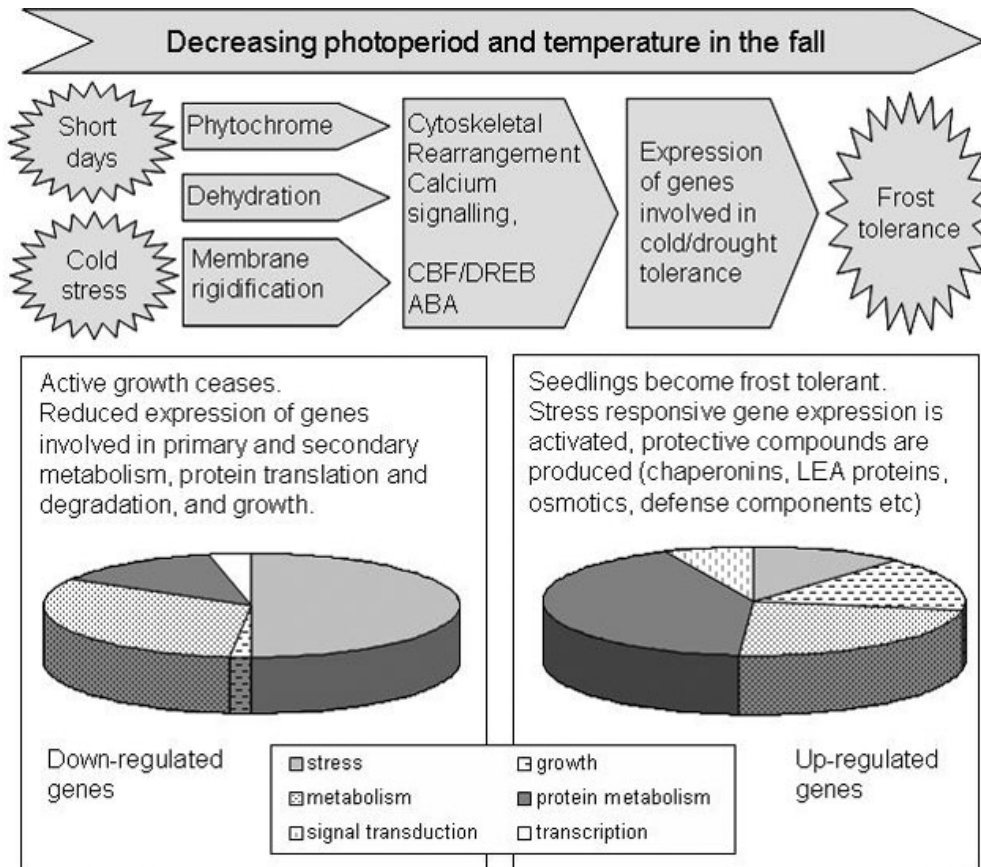


Figure 2—Molecular and physiological events in the transition from active growth to quiescence and frost hardness. Shortening photoperiod and reducing temperatures trigger physiological (stress) responses leading to quiescence and frost tolerance. Lower panels show the functional classification of the most highly up-regulated (60) or down-regulated (43) genes on the microarray.

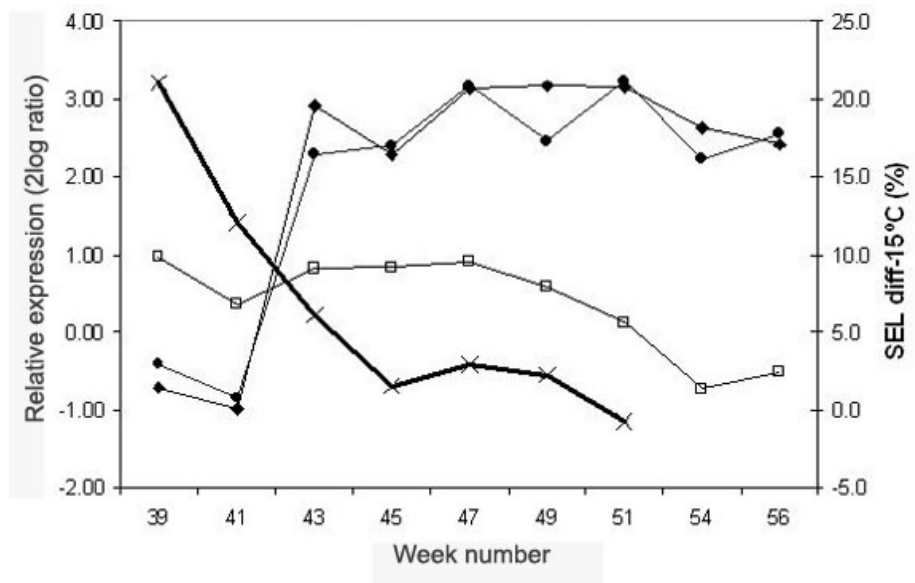


Figure 3—Expression profiles of 2 dehydrin types, measured in apical buds of Scots pine, which form the basis for frost tolerance testing. As soon as the shoot electrolyte leakage (SEL) diff-15 °C (%) value drops below 10, which indicates full hardiness, the expression ratio of the 2 dehydrin types is inverted. Closed symbols represent 2 different genes from dehydrin type I. Open symbols represent a single dehydrin type II. Frost tolerance data are indicated by crosses.

under various nursery regimes, and has proven to be highly consistent. The reason for this is that a biological process of crucial importance for hardiness is monitored. Recently, the test has been adapted for use in *Picea abies* as well.

Conclusions

Gene expression analysis is a highly accurate and reliable means for determining cold tolerance status in pine seedlings. Combined with the short analysis time, this makes it highly suitable for implementation as a nursery management decision support tool.

Acknowledgments

The authors thank Ronny Joossen, Michiel Lammers, and Brigitte Verkerk for their contribution to the work described in this paper.

References

- Aitken SN, Hannerz M. 2001. Genecology and gene resource management strategies for conifer cold hardiness. In: Bigras FJ, Colombo SJ, editors. Conifer cold hardiness. Boston (MA): Kluwer Academic Publisher. p 23–53.
- Bigras FJ, Ryyppö M, Lindström A, Stattin E. 2001. Cold acclimation and deacclimation of shoots and roots of conifer seedlings. In: Bigras FJ, Colombo SJ, editors. Conifer cold hardiness. Boston (MA): Kluwer Academic Publisher. p 57–88.
- Burr KE, Tinus RW, Wallner SJ, King RM. 1989. Relationships between cold hardiness, root growth potential and bud dormancy in three conifers. *Tree Physiology* 5:291–306.
- Chinnusamy V, Schumaker K, Zhu JK. 2004. Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. *Journal of Experimental Botany* 55:225–36.
- Close TJ. 1997. Dehydrins: a commonality in the response of plant to dehydration and low temperature. *Physiologia Plantarum* 100:291–296.
- Fowler S, Thomashow MF. 2002. Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *Plant Cell* 14:1675–90.
- Joosen RVL, Lammers M, Balk PA, Brønnum P, Konings MCJM, Perks M, Stattin E, Van Wordragen MF, Van der Geest LHM. 2006. Correlating gene expression programs to physiological parameters and environmental conditions during cold acclimation of pine (*Pinus sylvestris*). *Tree Physiology*. Forthcoming.
- Sakai A, Larcher W. 1987. Frost survival of plants—responses and adaptation to freezing stress. *Ecological Studies* 62. New York (NY): Springer-Verlag. 321 p.
- Seki M, Narusaka M, Abe H, Kasuga M, Yamaguchi-Shinozaki K, Carninci P, Hayashizaki Y, Shinozaki K. 2002. Monitoring the expression pattern of 1300 Arabidopsis genes under drought and cold stresses by using a full-length cDNA microarray. *The Plant Cell* 13:61–72.
- Thomas B, Vince-Prue D. 1997. Photoperiodism in plants. 2nd ed. London (United Kingdom): Academic Press Ltd. 428 p.
- Wareing PF. 1951. Growth studies in woody species. III. Further photoperiodic effects in *Pinus sylvestris*. *Physiologia Plantarum* 4:41–56.