

Progress Report Nr. 2

1 March 2001 – 1 March 2003

COLDTREE

The application of cDNA microarray technology for unravelling molecular events underlying dormancy and cold hardiness in forest tree seedlings.

A first step towards the development of molecular diagnostic tests for cost efficient reforestation and nursery logistics.

- Agrotechnological Research Institute
- Plant Research International
- Dalarna University
- Danish Institute of Agricultural Sciences
- Applied Plant Research
- Hedeselskabets Planteskole
- Forestry Commission Research Agency
- Alba Trees



Progress Report

Title of the project: The application of cDNA microarray technology for unravelling molecular events underlying dormancy and cold hardiness in forest tree seedlings. <i>A first step towards the development of molecular diagnostic tests for cost efficient reforestation and nursery logistics.</i>		
Acronym of the project: COLDTREE		
Type of contract : RTD		Total project cost (in euro) 2,616,949 €
Contract number QLK5-2001-00135	Duration (in months) 48 Months	EU contribution (in euro) 1,308,464 €
Commencement date 1 March 2001		Period covered by the progress report 1 March 2001 – 1 March 2003
PROJECT COORDINATOR		
Name: Monique F. van Wordragen	Title: Dr.	Address: ATO BV. PO-box 17, 6700 AA Wageningen, The Netherlands
Telephone: +31-317-475114/5000	Telefax: +31-317-475347	E-mail address: Monique.vanwordragen@wur.nl
Key words (5 maximum – Please include specific keywords that best describe the project.). CDNA microarrays, forestry, cold hardiness, dormancy		
World wide web address (the project's www address)		

List of participants Provide all partners' details including their legal status in the contract i.e., contractor, assistant contractor (to which contractor?).

Nr	Name	Legal status	Contact	Address
1	Agrotechnological Research Institute (ATO)	Co-ordinator	Dr. M.F. van Wordragen Monique.vanwordragen@wur.nl	PO-box 17, 6700 AA Wageningen, The Netherlands. Tel: +31.317.475000 Fax: +31.317.475347
2	Plant Research International (PRI)	Contractor	Dr. A.H.M. van der Geest Lonneke.vandergeest@wur.nl	PO-box 16, 6700 AA Wageningen, The Netherlands. Tel: +31.317.476990 Fax: +31.317.423110
3	Dalarna University (DU)	Contractor	Dr. E. Stattin esa@du.se	Herrgårdsvägen 122, 776 98 Garpenberg, Sweden. Tel: +46.225.26000 Fax: +46.225.26100
4	Danish Institute of Agricultural Sciences (DIAS)	Contractor	Dr. P. Brønnum p.bronnum@agrsci.dk	Kirstinebjergvej 10, DK-5792 Aarslev, Denmark. Tel: +45.63.904343 Fax: +45.63.904393
5	Praktijkonderzoek Plant en Omgeving (PPONL)	Assistant contractor to partner 4	Dr. H. Franssen Hanneke.franssen@wur.nl	PO-box 118, 2770 AC Boskoop, The Netherlands. Tel: +31.172.236700 Fax: +31.172.236710
6	Hedeselskabets Planteskole (HSP)	Assistant contractor to partner 4	Mr. B. Karlsson blk@hedeselskabet.dk	Brøndlundgaard, Brøndlundvej 2, Gabøl, DK-6500 Vojens, Denmark. Tel: +45.74.871600 Fax: +45. 74871543
7	Forestry Commission Research Agency (FC(FR))	Contractor	Dr. M. Perks mike.perks@forestry.gsi.gov.uk	Northern Research Station, EH25 9SY Roslin, Midlothian, United Kingdom. Tel: +44.131.4452176 Fax: +44.131.4455124
8	Alba Trees (ALBA)	Assistant contractor to partner 7	Mr. J. Hepburne-Scott Sales@alba-trees.co.uk	Lower Winton, EH33 2AL Gladsmuir, East Lothian, United Kingdom. Tel: +44.1620.825058 Fax: +44.1620.825316

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1. Objectives and expected achievements

1.1 Objectives

Sustained yield from Europe's commercially exploited forests requires a supply of millions of seedlings annually. The planting stock for reforestation and urban horticulture, almost 1.7 billion tree seedlings and ornamental woody plants comprising a total value of about 2 billion Euro, is mainly produced by European forest tree nurseries. These nurseries rely on a tight scheduling of operations, to be able to deliver vital seedlings to the planting site. A critical step in a modern nursery production chain is the transfer of seedlings to cold or frozen storage. Cold storage is required to prevent winter damage, especially in containerised seedlings, to maintain planting stock in an inactive condition, and to ensure plant supply for geographically distinct planting sites, a requirement for large scaled or internationally operating nurseries.

Indoor storage has therefore become common practice, but poses a new dilemma for nurserymen. 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 be able to determine accurately the peak physiological condition for lifting or transfer.

Despite the economic importance of such decisions, nurserymen still predominantly rely on traditional (morphological) methods to identify this moment. Recently, several physiological measurement techniques have been proposed, and some of them have been used operationally. However, the number of nurseries in Europe utilising these techniques is limited, because the methods are either unreliable, labour 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.

In addition to the onset of dormancy, dormancy release is also of economic importance. If plants growing out in the nurseries are put into cold storage too late in spring, they show evidence of damage, particularly to the root system. In spring, plants start reversing the processes that protect them during winter before there is a visible sign of regrowth.

So, efficient forestation and cost-effective nursery management require tools for rapid and reliable determination of the physiological condition of forest tree seedlings. To develop such tools, a thorough understanding of the cellular and molecular processes underlying cold hardiness is required. Unravelling the gene expression pattern as a seedling acquires the hardened state will reveal key processes that can be used as landmarks to describe the physiological condition of the tree. Eventually, this will result in molecular tests based on the presence or absence of specific messenger RNA's or proteins, that will allow a rapid evaluation of the physiological state and will facilitate forestation logistics. Such 'techniques of tomorrow' are not yet available to forest tree nurseries and in this respect the forestry sector is lagging behind on horticulture and agriculture.

Winter hardiness is closely correlated to endodormancy, the state of inactivity in which plants spend the winter. Not much is known on the molecular nature of the intertwined processes of dormancy and hardiness in woody species. In order to identify molecular mechanisms involved in winter hardening in woody plants the project participants will employ the powerful technology of cDNA micro-arrays, also known as DNA chips, which has recently been developed and allows the monitoring of thousands of mRNA's simultaneously. The technique will be used to detect transcripts characteristic for the dormant or active phase in Scots pine (*Pinus sylvestris*) and common beech (*Fagus sylvatica*). These two economically important forest trees were chosen as model species to represent the coniferous and deciduous European trees. Relevant mRNAs will be selected and characterised to unravel molecular pathways involved in the process of hardening. Seedlings will be grown in climate-controlled environments for the initial identification of relevant genes. Outdoor trials will be performed to detect the effect of climatic conditions, geographical position and provenance on the underlying molecular processes. Plant material (buds, roots, needles) collected during these trials will be analysed physiologically and for gene expression, employing cDNA microarrays and PCR technology. Together, these data will allow the creation of a detailed picture of molecular events involved in the onset and release of cold hardiness and dormancy and the effect of environmental factors on these processes. Furthermore, the research will result in a selected set of genes with a strong predictive value for cold acclimation in the tested plant species.

These genes can be used for the future development of a rapid hardiness test that will support nursery management decisions and facilitate forestation logistics.

Summarising, the objectives are:

To identify genes and molecular pathways involved in the onset and release of winter hardiness and dormancy in woody species using cDNA microarrays and to postulate a conceptual model describing the molecular events underlying these processes.

To select a set of key genes, of which the expression patterns can be used to describe the various stages of dormancy and hardiness,

To evaluate the merits of these key genes as molecular diagnostic tool for nursery practice and improved forestation.

1.2 Expected achievements

Molecular monitoring of forest tree seedling quality will aid in the development of improved forestation and planning techniques. Adequate tools for predicting and measuring the physiological condition of tree seedlings will facilitate the management of forest tree nurseries. Efficiently operating nurseries will ensure a steady supply of high quality forest tree seedlings, which is required for the sustained-yield use of commercially exploited forests and cost-effective reforestation. At present, it is not uncommon for 25% of the seedlings in new plantations to die. Poor establishment is often caused by frost damage or desiccation, during storage of insufficiently hardened plants. Therefore, better characterisation of the seedling level of cold hardiness will also ensure an enhanced quality of planting stock. The European forestry sector lags behind agriculture and horticulture in the application of new technologies for solving practical problems. This project has been orchestrated in order to obtain a better insight into the relevant molecular and cellular processes underlying these problems. Furthermore, the project results will also enhance knowledge of the fundamental cellular and molecular processes underlying winter dormancy and cold hardiness development in woody species. The cDNA microarray technology provides a new experimental tool for global searches on the function of genes, and is just starting to penetrate the field of plant molecular biology. The technique is pre-eminently suited for the unravelling of functional networks and interlinked molecular pathways that determine complex physiological processes. The present project will allow for the correlation of functional and genomic data concerning bud dormancy and cold tolerance.

2. PROJECT WORKPLAN

2.1 Introduction

To allow for the identification of key genes and pathways involved in the onset, development and release of winter dormancy and stress tolerance in economically and ecologically important woody species, the extremely powerful technology of cDNA micro-arrays will be employed. The arrays enable the monitoring of thousands of mRNA species simultaneously. The technique will be used to detect mRNAs characteristic for dormancy or hardiness in pine and beech. Relevant mRNAs will be selected and characterised to reveal molecular pathways involved in the process of hardening.

Outline

The workplan is designed around two economically important forest tree species: *Pinus sylvestris*, the primary model, and *Fagus sylvatica*, the secondary model. Two models have been chosen to have both coniferous and broad-leaved species represented. Pine mRNA will be used for screening a cDNA microarray that carries clones from three cDNA expression libraries, made from dormant pine buds. The array was supplemented with pine homologues of conserved genes from other species that are expected to be of importance in the development of bud dormancy such as cell cycle genes and dehydrin genes. To identify relevant clones, the expression information will be compared with data derived from thorough physiological and morphological analysis of the seedlings. For the selected pine genes, homologous genes from beech will be isolated and checked against beech mRNA, also derived from trees cultured in controlled environments. For rapid detection of the selected genes RT-PCR assays will be developed. Employing quantitative detection (via real-time monitoring of accumulating fluorescence) these assays will be used to obtain detailed information on the expression profile of the selected genes in field conditions.

The work in this project can be divided into three phases, and was preceded by a kick-off meeting and a Molecular Analysis Workshop in which all partners were trained in RNA-isolation and RT-PCR, according to standard protocols. Also, standard protocols for sampling the plant material and for physiological analyses were agreed upon during this workshop.

Phase 1

In the first two years climate room experiments were conducted by DIAS and FC(FR), aimed at the production of pine and beech seedlings in which the processes of dormancy and cold tolerance development were separated as far as possible. To this end three climate regimes were used:

- constant daylength and decreasing temperature
- constant temperature and decreasing daylength
- constant growth-permissive temperature and constant daylength (control)

DIAS focusses on beech and FC(FR) on pine, but in both cases the experimental set-up was the same, except for the daylength and temperature values that differ for pine and beech. In the second trial season climate room experiments will be used for assessing the combined effect of decreasing and fluctuating temperature and decreasing daylength. In a series of regimes, natural occurring fluctuations in temperature will be mimicked. Each partner will do at least one dormancy test (RGP and/or terminal budbreak) and one hardiness test. (frost tolerance, REL/SEL of excised root and shoot parts). The assays will be performed with 4 replicates, each sample consisting of material from 5 different plants. Thus, at each sampling date 20 different plants will be analysed. Regrowth will be monitored in randomised trials consisting of 5 plots, of 10 plants each. Results will be analysed statistically using analysis of variance calculations. For analysis of gene expression DIAS and FC(FR) will prepare total RNA from bud and root samples taken at each time point, according to a common protocol. The RNA will be shipped to PRI, who will use it for the preparation of microarray probe.

Concurrently, ATO pre-selected 2000 putative dormancy related genes from several pine cDNA expression libraries and isolated the corresponding inserts. PRI sequenced the 2000 cDNAs, and selected a subset to ensure the highest number of unique genes. Supplemented with several known conserved genes and a set of controls, these were used to construct a cDNA microarray, for the analysis of gene expression in pine seedlings. Independent pine samples will be used to challenge the microarray. In addition, 5 beech mRNA samples will be analysed to test homology, with respect to sequence and expression pattern.

From each sample two probes will be made, using different fluorescent dyes. The screenings data will be analysed in comparison to the physiological data obtained in the climate room trials to identify a set of 20 - 30 transcripts presumably involved in either dormancy, frost hardiness, or both. The selected pine transcripts will be characterised and the information will be used to isolate homologous genes from the beech genome. This task will be performed co-operatively by ATO and Plant RI.

Phase 2

As soon as sequence information from the selected pine and beech transcripts comes available, it will be used to design PCR primers for the development of RT-PCR assays with fluorescent detection markers (Molecular Beacon, Taqman). The PCR-based assays allow for the rapid and reliable detection of specific gene expression and will be carried out by all partners to gain in-depth information on the expressional regulation of the selected genes in plants grown in a variety of environmental conditions. For these analyses field trials, using multiple provenances from pine and beech, will be performed in three subsequent trial seasons, by DIAS and FC(FR) and their assistants HSP and ALBA. The replication (both in time and in location) is necessary to be able to identify genes whose expression is correlated to the traits of interest, independent of climatic variance, environmental conditions or geographic position. Field trials will mostly be done in the second and third trial season, but some initial experiments will already start in year 1. Outdoor trials on pine will be carried out using containerised seedlings to allow comparison with the controlled environment experiments. In beech this is not possible due to technical limitations and therefore bare-rooted seedlings will be used. Assessment of dormancy and hardiness development will start in September and proceed until January. Individual experiments may proceed until spring to evaluate dormancy release as well. In general, samples will be taken every two weeks for physiological analyses and regrowth assessment as described for the climate room trials, and for RNA isolation. In RNA samples the presence of relevant transcripts will be monitored using PCR assays. Part of the RNA will be used for detailed expression studies using quantitative PCR. Eventually, these data will aid in the selection of approximately ten key genes that are descriptive of the physiological condition of pine and beech with respect to dormancy and cold hardiness. Additionally, the development of PCR-assays is a first step towards implementation of the knowledge gained within the project in the development of an operational test.

Phase 3

The key genes selected in phase 2 will be evaluated in the practical setting of forest tree nurseries. This task will be performed by DU and PPO, for pine and beech respectively. In addition, DU will investigate the predictive value of the selected genes for cold storage performance. These trials will reveal whether the future development of molecular diagnostic tests for hardiness and dormancy, based on the expression of these genes, is feasible.

All data derived from the physiological and morphological analysis of the seedlings will be collected by FC(FR) and combined in one database. The expression information for each spotted clone, derived from the screenings of the microarray, will be stored and made accessible using specialised software. Specific adaptations will be made to allow linkage of physiological, expressional, functional, and sequence information into an integrated and searchable database. This will be carried out by the bio-informatics department of Plant RI.

This combination of data in the final project phase will lead to a profound insight in the molecular pathways involved in the onset and release of winter hardening in *Pinus sylvestris* and *Fagus sylvatica*. Information on the influence of climate, environment and provenance on the expression of the genes concerned will become available, and will contribute to the definition of a general hypothesis on the molecular events underlying the onset and development of dormancy and cold hardiness in woody species.

Finally, the third phase will also be used to communicate the project results to the sector, via a demonstration workshop for nurserymen. In this workshop the main focus will be on the significance of the results for facilitating nursery management.

2.2 Project structure, planning and timetable

2.2.1 Progress during the first reporting period

The first project year started with a kick-off meeting that was combined with a Molecular Analysis Workshop in which all partners were trained in RNA-isolation and RT-PCR, according to standard protocols. Also, standard protocols for sampling and analysing the plant material and procedures for shipping seed and RNA samples were agreed upon during this workshop.

The research conducted in the first year can be divided in two main parts:

- experiments set up to produce physiologically extensively characterised plant material from pine and beech
- work directed at the production of a dedicated pine cDNA microarray carrying genes that are likely to be involved in dormancy and/or cold tolerance

In this section an overview will be given of the progress made along these two main research lines. For more details and information on additional work please consult chapter 3.

Production of characterised plant material

Controlled environment experiments

With respect to the first topic climate room experiments were conducted by DIAS and FC(FR), aimed at the production of pine and beech seedlings in which the processes of dormancy and cold tolerance development are separated as far as possible.

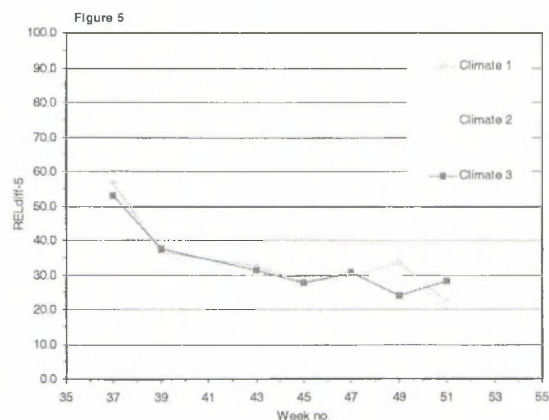
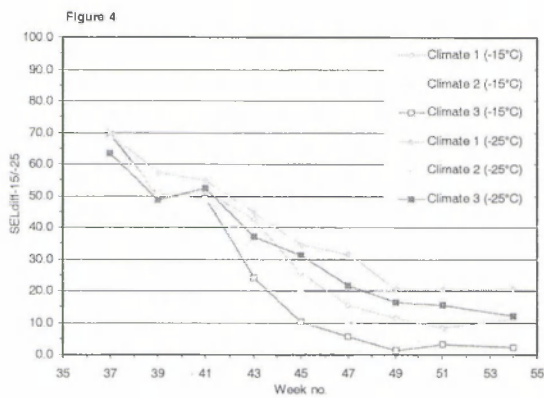
DIAS focused on beech and partner FC(FR) on pine, but in both cases the experimental set-up was the same, except for the daylength and temperature values that differ for pine and beech.

In week no. 19 DIAS sowed pre-germinated seeds of the standard beech provenance 'Bregentved' (origin Denmark) in trays placed outside and raised approximately 3500 seedlings. In week no. 33 approximately 3000 of these seedlings were transferred to three climate rooms, where they were subjected to three different climate regimes until week no 2 in 2002:

- Climate 1: day length (DL) = 15h, temperatures $T_{day} = 15^{\circ}C$ and $T_{night} = 13^{\circ}C$
- Climate 2: as climate 1, but DL reduced by 1h every two weeks until 8.5 h
- Climate 3: as climate 1, but T_{day} and T_{night} reduced by $2^{\circ}C$ every two weeks until $+1^{\circ}C/+1^{\circ}C$.

Seedling samples from each climate regime were collected in week no. 37, 39, 41, 43, 45, 47, 49, 51 in 2001 and in week no. 2 in 2002. In each sampling week seedlings were tested for shoot and root frost tolerance and dormancy status. In addition each sampling week 50 terminal buds and 50 fine roots were collected and immediately frozen in liquid nitrogen for isolation of RNA.

$SEL_{diff-15}$ and $sel_{diff-25}$ decreased during autumn for growth room beech seedlings in all three climate regimes (figure 4). The results show that shoot became frost tolerant to $-15^{\circ}C$ first, and this occurred in the order climate 3, climate 2 and climate 1. It is not likely that shoots became tolerant to $-25^{\circ}C$, except perhaps for climate 3. The order in which beech of different climates developed frost tolerance appears to be the same as mentioned for $-15^{\circ}C$.



This was expected, except for the fact that seedlings in climate 1 (long day/high temperature) also attained some frost tolerance, which we can not explain yet. The precise relationship between sel_{diff} and frost tolerance or storability/field performance for beech still remains to be determined (1-year performance of bare root beech will be ready in the first months of 2003) and also the linkage between RNA and acclimation parameters (sel_{diffs}).

No development of root frost tolerance to -5°C was observed in beech (figure 5).

Beech seedlings from all three climate regimes entered almost equal levels of dormancy (number of days to terminal bud break under favourable growth conditions), although climate 3 seedlings, for a relatively short period, seemed to be more dormant. Dormancy is normally induced by shorter day lengths and is independent of f.ex. temperature and it was therefore surprising that dormancy was induced in both climate 1 and climate 3.

FC(FR) raised containerised seedlings of *Pinus sylvestris* [Scots pine] (standard provenance A70) at the Research Station in Scotland and transferred them to growth rooms in the middle of August (Week 33) where they were subjected to three different climate regimes until Week 2 (2002):

Climate 1 (control): long day (15h), high temperature (15/13 $^{\circ}\text{C}$)

Climate 2: decreasing daylength (15h \rightarrow 7h), high temperature (15 $^{\circ}\text{C}$)

Climate 3: decreasing temperature (15/13 $^{\circ}\text{C}$ \rightarrow 5 $^{\circ}\text{C}$), long day (15h).

Every second week, starting in week 37 and until week 4 (2002), all experimental plant types were tested for shoot and root frost tolerance ($SEL_{diff-25^{\circ}\text{C}}$ / $SEL_{diff-15^{\circ}\text{C}}$ / $REL_{diff-5^{\circ}\text{C}}$), dormancy (days to terminal bud break) and material for mRNA analysis was collected from terminal buds and fine roots. Pine seedlings from all controlled environment climate regimes showed development of root frost tolerance assessed at -5°C , and shoot frost tolerance as assessed by $SEL_{diff-15^{\circ}\text{C}}$, but this was not evident at the $SEL_{diff-25^{\circ}\text{C}}$ assessment temperature (Figure 6). This indicates that plants had not developed hardiness to this level under the controlled environment conditions. Shoot frost hardiness assessed at -15°C indicated that there was a lower level of induced hardiness in climate 1 seedlings over the last four weeks of the trial.

All experimental plant types had a more or less distinct dormancy period, even the climate 1 seedlings. Under controlled conditions declining temperature led to a decrease in dormancy levels, no clear influence of daylength was evident, and the lack of response was compounded by poor plant response to the conditions favourable for budbreak.

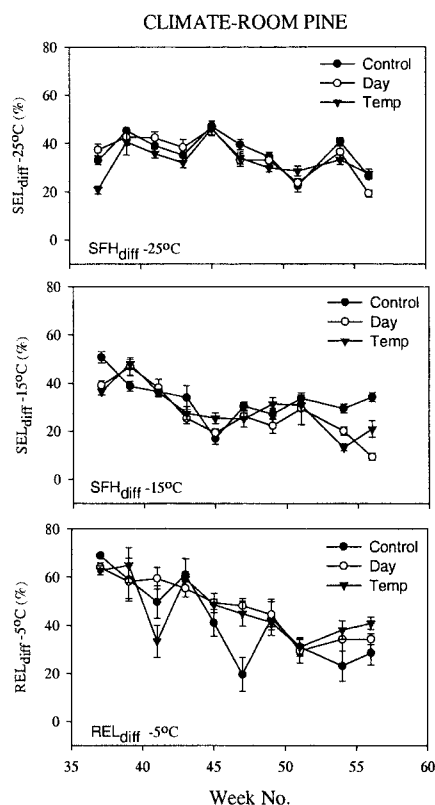


Figure 6. Physiological response of A70 pine under controlled environment conditions, in year 1 (2001-2).

Outdoor experiments

As to the field trials DIAS sowed the standard Scots pine provenance A70 (origin UK) and the provenance 'Lindås' (origin Norway) in week nr 20 in trays placed in a greenhouse. Approximately 1500 seedlings of each provenance were produced in accordance with normal nursery practice for containerised stock. Seedlings were transferred from the greenhouse to an outdoor container cultivation area in week 32 where they remained during the entire sampling period. The standard provenance 'Bregentved' (Czech origin, Danish seed source) and the local provenance 'Gråsten' (Danish origin) were sown in week no. 15 and cultivated in accordance with normal nursery practice for bare rooted stock by Hedeselskabets Planteskole. Pine seedlings were sampled and tested using the same procedures as for the climate room experiments.

The bare-rooted beech seedlings of both provenances were lifted in the nursery in week 37, 39, 41, 43, 45, 47, 49, 51 in 2001 and in week 2 and 4 in 2002. (in week 2 shoots only, because of frozen nursery soils). The lifted seedlings were subjected to the same tests, following the same procedures, as described for WP 2 and 3, except for REL and dormancy test in week 2. In each lifting week 75 seedlings were cold stored at 4°C for field planting in April 2002 (storage temperature lowered to -1°C in December), in order to study the relationship between pre-storage physiological condition (frost tolerance, dormancy and gene expression) and storability (assessed as survival and growth).

Both provenances of containerised Scots pine seedlings grown outdoors during autumn developed shoot frost tolerance. Previous studies have shown that conifer shoots are frost tolerant at a given temperature when sel_{diff} is lower than 5% and storable when they are frost tolerant to -25°C (i.e. $sel_{diff-25} < 5\%$). Both provenances were frost tolerant to -15°C around week 43-44. The provenance 'Lindås' acclimatised faster and was frost tolerant to -25°C by week 47, approximately two weeks before the standard provenance 'A70'. Contrary to beech, Scots pine seedlings developed root frost tolerance at least to -5°C and starting around week 43. The same provenance difference was observed for root frost tolerance, but not as clear as for shoots. Pine seedlings developed dormancy during autumn and again there seemed to be a difference between provenances, as 'Lindås' appeared to be more dormant than 'A70' during the period from week 43 to week 4.

Like in pine, the bare rooted beech seedlings developed shoot frost tolerance, although acclimation in beech occurred later in autumn than in pine. Assuming a sel_{diff} -threshold of 5% for frost tolerance of broadleaves as well, beech seedlings would be frost tolerant to -15°C by week 47, and frost tolerant to -25°C between week 51 and 2 (2002), i.e. in the second half of December. As with the growth room beech seedlings, no root acclimation was observed. The bare rooted beech seedlings entered dormancy around week 41 and were released, or at least back to the initial level by week no. 4 (maybe even earlier, but due to difficulties with frozen nursery soils in week no. 2, it was not possible to lift seedlings for dormancy test in this week). There were no clear physiological differences between the standard provenance 'Bregentved' and the local provenance 'Gråsten'.

The overall shoot frost tolerance development in bare rooted beech seedlings grown outside was greater than in the growth room seedlings. Dormancy in beech was considerably deeper than in pines, and more distinct in the bare-rooted than the growth room seedlings.

At the Research Station in Scotland containerised seedlings of *Pinus sylvestris* [Scots pine] (standard provenance A70 and local provenance N401 'Abernethy') and *Fagus sylvatica* [beech] (standard provenance Bregentved) seedlings were raised during summer and autumn. Beech (local provenance Cirencester) seedlings were provided from the nursery of ALBA Trees.

Every second week, starting in week 37 and until week 4 (2002), all experimental plant types were tested for shoot and root frost tolerance ($SEL_{diff-25^{\circ}\text{C}} / SEL_{diff-15^{\circ}\text{C}} / REL_{diff-5^{\circ}\text{C}}$), dormancy (days to terminal bud break) and material for mRNA analysis was collected from terminal buds and fine roots. Samples of containerised 'field-grown' beech were cold stored (at 1°C) on alternate sampling weeks to be outplanted at the end of April 2002.

Shoots of 'field-grown' pine were significantly more frost tolerant than the controlled environment pine seedlings with effects apparent at all freeze test temperatures. Provenance differences were not observed. 'Field-grown' containerised Scots pine roots developed frost tolerance and there was evidence of occasional response differences between provenances.

All experimental plant types had a more or less distinct dormancy period. Dormancy in beech was considerably deeper than in pines, and the ontogenetic pattern of dormancy development was later in the UK provenance than the standard Danish provenance. Dormancy in the field-grown pine provenances showed almost identical seasonal variation with the native Abernethy (N401) provenance showing periodically deeper dormancy levels than in the provenance A70 over the second half of the testing period (from Week 45). Dormancy development appeared to be correlated with soil temperature.

Development of root frost tolerance (-5°C) in 'field-grown' beech was absent, while frost tolerance of beech shoots from the outdoor trial showed distinct seasonality, though no provenance effects were found.

Production of a pine cDNA microarray

Constructing and characterising pine cDNA libraries.

To enhance the chance for relevant genes to be present on the array, three different cDNA libraries and a set of specific gene fragments were used for the selection of clones. One of the cDNA libraries was a previously constructed library, based on dormant pine bud tissue harvested in early December. This library was designated FULL and 700 clones were randomly selected, amplified and purified and transferred to partner 2, for sequence-analysis and subsequent array-construction. The two others were specifically made for this project and are enhanced for genes related to dormancy and cold tolerance, respectively. These libraries were made from apical buds taken from two-year-old *Pinus sylvestris* trees, obtained from a local grower, at the beginning of February (i.e. dormant sample). Some of the trees were cold treated (20°C to -10°C , overnight in a controlled cold cell by Partner 5 (PPO) and an apical bud sample was taken (cold sample). At the beginning of April an apical bud sample was taken from the same batch of trees (release sample). These samples were used to construct the subtracted cDNA-libraries. One of the libraries is enriched for genes that are cold induced (COLD-library) and the second library contains material that is enriched for genes that are related to dormancy development of the apical bud (DOR-library). From each of these libraries approximately 400 clones were sent to Partner 2 (PRI) for sequence-analysis and subsequent array-construction. Furthermore, a set of specific cDNA-clones was delivered to PRI so they might also be included onto the array.

These clones represent genes that are expected to have impact on either dormancy or cold tolerance based on literature data or previous research. The clones were isolated using a PCR based approach and degenerate primers, or via differential expression analysis.

Analysis of the selected clones

Approximately 3000 individual cDNA clones from the libraries were checked by PCR for insert size, and approximately 2000 clones of sufficient length were selected for further analysis. These clones were subjected to DNA sequence analysis, and the resulting sequence data were used to compare the sequences to GenBank in order to predict gene function. In addition, a contig analysis was performed to align the different sequences to each other and determine the number of unique clones and the number of genes that was found multiple times. Contig analysis revealed a total of 1080 contigs, representing different DNA sequences. Some sequences were found very frequently in the different libraries, resulting in relatively large contigs for the following gene families: A large number of dehydrin genes were found, that were divided into 5 different contigs, containing between 12 and 27 genes. Three different contigs (containing 14-17 sequences) were found encoding pathogenesis related proteins, and one contig of 13 genes represented a stress/ABA related protein gene. The fact that these genes occurred frequently in these libraries indicates that their expression level in the tissues from which the libraries were made was relatively high, suggesting that they may be involved in such processes. Indeed, for dehydrins reports exists for a role in protection against drought stress, and possible links with cold stress. This gene family had also been identified in a previous project, and might be a good marker for stress tolerance. Most other genes were found only once in the sequence set, and are represented by the 961 contigs containing only 1 or 2 genes. Some genes of intermediate abundance are represented by 52 contigs containing three sequences, 44 contigs containing 4 or 5 sequences, and 14 contigs containing 6 to 10 sequences (see table 1).

No. of contigs	Genes per contig	Types of genes
961	1-2	Mainly housekeeping genes, some stress related genes and ESTs
52	3	Mainly housekeeping genes, some stress related genes, unknown genes and ESTs
44	4-5	Stress related genes, seed maturation related genes, translation genes, pine ESTs, housekeeping genes
14	6-10	Common pine ESTs, putative proteins, dehydrins, etc
5	11-15	Dehydrins, pathogenesis related proteins, ABA/stress related proteins
4	>15	Dehydrins, pathogenesis related proteins

Table 1: Distribution of the gene sequences over the contigs.

Production of cDNA microarray

The sequence information from blast and contig analysis was used to make a selection of genes to be spotted. From the 2000 clones 1451 genes were selected for spotting on the array, ensuring the maximum number of different genes, or different fragments from the same gene. Additional genes were added to a total of 153 genes. These included fragments of genes that were differentially present in a PCR assay performed during the previous project (amplified fragment length polymorphisms: AFLP fragments), and some genes that were described in the plant literature to be associated with stress or dormancy. ATO provided the 31 characterized AFLP and PCR fragments ready for spotting on the array. Additionally a number of control genes were spotted on the array to allow data normalisation. In order to increase the reliability of the produced data, all clones were spotted onto the microarray in duplo, on different regions of the microarray.

Initial hybridisation experiments

In order to assess the usefulness of the cDNA microarray for detection of differential gene expression during dormancy, cold stress and active growth, RNA from samples treated at Applied Plant Research in Boskoop was used for labelled cDNA production. Five different samples (3 that had received some kind of cold stress, 1 that was fully dormant and one that was harvested in late spring) were compared to the same reference sample.

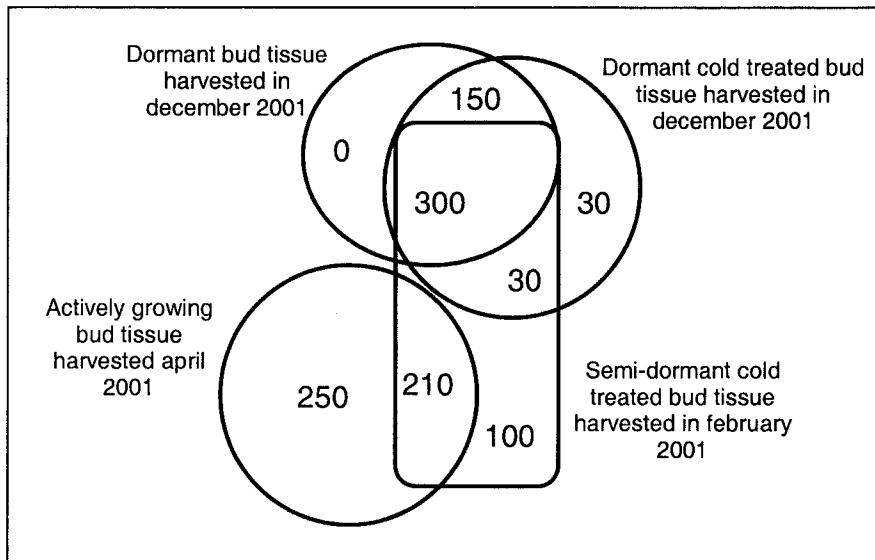


Figure 1. Gene expression in 4 different conditions was measured relative to a mixture of these conditions. Groups of genes that show expression levels that are higher than the mixture are indicated in this Venn diagram. Circles represent samples, numbers in the circles indicate the number of genes that are upregulated in the various samples. Overlapping regions indicate genes that are upregulated in more than one sample.

The samples that were used represent the extremes from the physiological spectrum that will be analysed in the COLDTREE program, and should therefore generate clearly distinguishable gene expression patterns. Analysis of the hybridisation results revealed dramatic differences in gene expression indeed. Different groups of genes could be identified, some associated with cold stress, some associated with dormancy, and some specific to actively growing buds (after dormancy breaking). Using the samples from the growth room and field experiments with pine we will be able to determine which of these genes is truly predictive of dormancy and/or cold tolerance, and can be further developed as a marker. Fig 1 shows a diagram of the different types of gene classes based on their expression in the cold treated, dormant or non-dormant samples.

2.2.1.1 Discussion-Conclusion

In this first project year the work has been conducted according to the planning, which means that all necessary preparations have now been made to start the first large-scale transcriptional analyses. At this moment no major scientific breakthroughs have been achieved yet.

Outdoor and indoor trials have resulted in the production of large series of physiologically carefully analysed and described plant material that is perfectly suited as test panel for the selection of genes whose expression profile relates to either dormancy or cold hardiness. The climate room trials using beech or pine have been performed in Denmark and Scotland respectively, whereas outdoor trials were done at both locations with both species. Trials were performed using an identical set-up and evaluation of dormancy and cold tolerance was done using the same standard operating procedures. This made it possible to compare the development of cold hardiness and dormancy on geographically different locations and yielded plant material that can be used for the selection of genes that relate to the main physiological events, while expressional responses to locally occurring climatic changes can be sorted out.

Since it turned out that the outdoor trials were very comparable in the two locations we decided during the first annual meeting to use that material for the initial challenging of the array. This is a deviation from the technical annex, in which is stated that samples taken from the seedlings grown under controlled conditions will be used for the first selection of genes. However, the climate room grown pine plants did not develop a very pronounced dormancy or frost tolerance. Furthermore, even the control plants that were kept under constant day length and temperature, did show a moderate level of dormancy. Because of this it was decided that this plant material was less likely to reveal the genes that are most prominently involved in normal development of dormancy or cold hardiness. On the other hand the samples taken from climate room grown plants will be very useful to select subsets of genes that are either involved in pre-programmed dormancy development or triggered by cold or day length reduction.

Another significant deviation from the technical annex relates to the way the cDNA microarray has been constructed. This results from the continuously changing insights, in this very young area of research, in how to make an optimal cDNA microarray. Whereas the original plan was to construct an array consisting of up to 500 unknown gene fragments, pre-selected from just one cDNA library, we finally decided on using several cDNA libraries as a source for 2000 clones. The libraries are enriched for genes relating to specific characteristics and thereby the risk of important genes not being present on the final array, is spread. Also we decided to sequence all clones before putting them on the array. Results from other projects revealed that a black-box array, however carefully composed, often still has a high level of redundancy, especially in the subset of main responsive genes. Initial costs for making the array are now higher than originally planned, but each hybridisation will yield more information, which means that we may be able to reduce the number of hybridisations.

The expectation that this approach would yield in a highly specific and dedicated array was confirmed by the results of the first test hybridisations. These revealed dramatic differences in gene expression of the genes positioned at the array. Different groups of genes could be identified, some associated with cold stress, some associated with dormancy, and some specific to actively growing buds.

Together with the sets of well-defined plant material we now possess all tools necessary to start the transcriptional profiling of dormancy development in pine buds.

2.2.1.2 Future action

As all work to date has been completed on schedule and with no major deviations from the workplan, we will be able to continue our research along the lines set in the technical annex. This means that the array will be hybridised with samples derived from the first trial season and the results will be analysed. These results for pine will be translated to beech, for which cDNA libraries will be made. Efforts will be made to develop a rapid PCR based assay for selected genes. At the same time preparations for the second trial season will commence and these trials will start in September 2002. Climate room experiments will focus on the effects of a sudden drop in temperature and field trials will be aimed at resolving the effect of climatic conditions, geographical position and provenances on the development of dormancy and hardiness and the expression of associated genes.

2.2.1.3 Action requested from the Commission

No specific action from the Commission is required.

2.2.2 Progress during the second reporting period

The second year showed the first results and proved the feasibility of our approach. At the same time it became clear that some adaptations of the original workplan would make it possible to reach our goals more efficiently. Below, the results of year 2 are followed by a discussion on how to proceed in the second half of the projects lifetime.

The research conducted in the second year can be divided in two main parts:

- experiments set up to produce physiologically extensively characterised plant material from pine and beech
- screening of the pine cDNA microarray and analysing the expression profiles of genes that are likely to be involved in dormancy and/or cold tolerance
- development of rapid test systems as a first step towards implementation of the results in practice

Production of characterised plant material

Climate room experiments

Both beech and pine seedlings were subjected to 4 climate regimes in an attempt to induce deviations from gradual dormancy/frost hardiness development. Differential gene expression analysis in these seedlings might point to genes involved specifically in adaptation to varying temperature conditions.

Both for beech and pine the 4 regimes were:

- Climate 1 (CONTROL): long day, high temperature
- Climate 2 (DECLINE) decreasing daylength, decreasing temperature
- Climate 3 (SCOLD): removal from Climate 2 about halfway the experiment to lower day/night temperature and equivalent light regime
- Climate 4 (SWARM): removal from Climate 2 about halfway the experiment to Climate 1 regime.

The specific conditions varied for pine and beech. The general conclusions from these experiments is that half-time cold or warm shocks do not significantly interfere with dormancy and frost tolerance development as it is apparent in the DECLINE climate. This conclusion holds for both pine and beech and it suggests that the biological processes related to protection against winter conditions are pre-programmed to a much larger extent than we assumed on forehand. This idea is reinforced by the fact that, just as in the previous season, plants in the constant control climate still developed a certain level of dormancy and hardiness.

The fact that the initial rate of frost tolerance acclimation (week 37 - 45) was the same over a large range different of environments, suggests that a common mechanism is active in these seedlings. The aim should now be to make use of these physiologically equivalent seedlings to identify the molecular markers linked to this process.

Field experiments

Dormancy and hardiness was followed in field grown pine and beech plants of 1 and 2 years of age and for 2 provenances. The older plants showed more pronounced effects in both pine and beech. In pine the provenance effect, as reported in the previous season was much less pronounced, but still present in the root frost tolerance values from Danish plants. As in the previous season, the physiological performance of beech and pine was very similar at both geographical locations. In general, outdoors grown plants became significantly more frost tolerant compared to plants from controlled environments.

In the second project year containerized pine seedlings from various provenances were produced for long term frozen storage experiments. The level of frost tolerance that was measured correlated with the geographical origin of the provenances. The northern provenance was cold hardened earlier than the southern provenances. Tissue samples taken from these provenances will be analysed for differences at RNA level correlated to this variation.

Expression profiling

22 samples from first-season fieldgrown pine, from both Danish and Scottish trials were analysed using the cDNA microarray. Based on the results the genes on the array could be divided in three major classes. The first class contains genes involved in active metabolism that are most strongly expressed in the early weeks of the trial. The second group represents genes that are strongly induced during the onset of dormancy and cold hardiness (week 41/43).

The third group of genes is also induced during onset of dormancy, but reaches the highest expression level at a later stage of development. Clustering analysis confirmed the physiological observation that pine plants grown at different geographical locations are remarkably similar. Provenance differences are identified but here comparison with physiological observations is not straightforward. Further analysis using samples from the second growing season is needed to be able to draw conclusions.

In general, a set of some 300 genes could be directly correlated to the process of dormancy/hardiness in pine. Out of these an initial selection of 30 most promising potential markers has been made. However, this selection will have to be validated with samples from the second trial season before we will start developing PCR assays based on these genes.

The pine microarray has also been used to compare early and late samples from field grown beech, but differences in gene sequence between pine and beech proved to be too large to allow analysis of beech mRNA on the pine array. For this reason we decided to make a small beech array to directly identify relevant beech genes and work in parallel with pine. Additional considerations were: the improved cost-efficiency of microarray analysis, compared to the time when the proposal was written and the fact that dormancy development in beech is more pronounced than in pine. As a first step towards this beech array a representative cDNA library has been made of beech buds harvested at various stages of dormancy development.

Assay development

The dehydrin gene family has been used as a model for the development of markers using RT-PCR. Several members of two dehydrin gene-classes are present on the array. The two classes show distinct expression patterns: the K2 genes are induced upon dormancy induction (starting from week 47) whereas expression of SK genes decreases in dormant buds. For both classes specific primers have been made. Expression varies, but in all samples the ratio K2/SK is below 1 in non-dormant buds and above 1 in dormant tissue. Therefore these primer pairs are a promising candidate for a dormancy indicator assay. A straightforward PCR based assay was developed for these markers, that can be used as a dormancy test. This assay was practised in a workshop that was organised during the second annual meeting. In this workshop it was shown that persons without molecular expertise were able to handle the test.

2.2.2.1 Discussion, conclusions and future plans

In the second project year the first Milestone, initial selection of relevant pine genes, was delivered. We generated large datasets containing expressional, physiological and experimental (climate, time) data that will be extended, linked and analysed in the forthcoming years and will point towards the most important key events in dormancy and hardiness development.

The research in the second year also led to interesting and partly unexpected results that justify (and in fact demand!) some alterations of the original workplan.

- Variation in geographical location has a much smaller impact on development of dormancy than originally assumed.
- To some extent (especially for beech) this also holds true for different provenances
- The process is much more preprogrammed than we expected.
- Separation of the processes of dormancy and cold hardiness in controlled environments is only partly possible
- Beech seedlings develop real dormancy while pine seedlings (especially 1-year old) are only quiescent

It is necessary to explore variation in gene-expression in order to make a robust selection of key genes for the processes studied. The different geographical locations did not yield this variation. For this reason we decided to extend the number of microarray hybridisations. To stay within the budget this means we will put less focus on RT-PCR analysis.

As to beech, it seems that this species is more suitable to study dormancy, compared to pine. Therefore it seems no longer wise to rely on the results from the pine array to identify key genes for dormancy in beech. To allow independent study of expressional regulation during dormancy development in beech we decided to make a small beech array and hybridise it with carefully selected samples from field and climate room trials. The results will be compared with the results from the pine array.

For both these decisions an important factor is that the costs of applying microarray-technology are now less high compared to the period before the start of this project. Cost-efficiency was one of the main reasons to switch to RT-PCR halfway the project. RT-PCR will still be employed to

- translate lab results to practical assays
- validate microarray results

2.2.2.2 Significant difficulties and delays

There have only been some delays in the molecular work. The analysis of the pine array was delayed because of a shift in persons at PRI and consequently the selection of beech genes had to be postponed as well. The pine data of 2001/2002 field samples have been analysed in the first months of 2003 and

Final selection of key genes still awaits analysis of field samples from 2002/2003 and climate room samples. In the second half of 2002 we decided to start in advance with the construction of a beech cDNA library, but the first attempt resulted in a low quality library. The second attempt in March 2003 did result in a good library that will now be used as a basis for a small array. The hybridisations on that array will yield an initial selection of prominent beech genes (Milestone 2, due in month 23) in the second half of 2003 (around month 30). However, the use of a beech array will facilitate the validation of the key genes and ensure a more representative selection than would have been possible in the original planning.

2.2.2.3 Future action requested from the Commission

From the commission we request consent with the amendments to the technical annex that are proposed and described in the sections 2.2.2, 2.2.2.1 and 2.2.2.2

Lists of workpackages, milestones and deliverables, updated for project year 2

Workpackage list							
Number	Workpackage title	WP leader	Person months		Start month	End month	Deliverables
			total	Already devoted			
WP1	Climate chamber experiments pine	7	22	9,5	0	24	DL1, DL2, DL4, DL24, DL25
WP2	Climate chamber experiments beech	4	24	10,8	0	24	DL3, DL4, DL24, DL25
WP3	Field experiments pine	7	35	4,5	0	38	DL4, DL5, DL24 DL25
WP4	Field experiments beech	4	26	4,5	0	38	DL4, DL6, DL24, DL25
WP5	Production and screening of a pine cDNA microarray	2	28	26,7	0	20	DL7, DL8, DL9, DL10, DL24, DL25
WP6	Isolation of beech homologues to the selected pine genes	1	23	0	12	23	DL11, DL12, DL24, DL25
WP7	Development and application of RT-PCR assays	1	42	6	20	38	DL13, DL14, DL15, DL24, DL25
WP8	The effect of provenance, climate and age	5	23	1	0	43	DL16, DL18, DL23, DL24, DL25
WP9	Prediction of storage performance	3	12	3,5	18	43	DL17, DL18, DL23, DL24, DL25
WP10	Set up of an integrated physiological and molecular database	2	20	0	39	46	DL19, DL20, DL21, DL22, DL24, DL25
	TOTAL		255	66,5			

List of milestones			
Nr	Name	Due date	Status
M1	Initial set of relevant pine genes selected	Month 20	Delivered
M2	Initial set of relevant beech genes selected	Month 23	In progress
M3	Subset of pine and beech key genes selected	Month 38	Not started
M4	Usefulness of key genes as molecular diagnostic test evaluated	Month 43	Not started
M5	Integrated database available	Month 43	Not started
M6	Conceptual model on molecular processes underlying dormancy/hardiness defined	Month 46	Not started

List of deliverables					
Nº	Deliverable title	WPs	Participant	Delivery status	Confidential nature
DL1	RNA for preparing microarray probe	1,2	4,7	Delivered	CO
DL2	Physiological data on dormancy and hardiness development in pine in controlled environments	1	7	Delivered	CO
DL3	Physiological data on dormancy and hardiness development in beech in controlled environments	2	4	Delivered	CO
DL4	RNA for RT-PCR	1,2,3,4	4,6,7	Delivered	CO
DL5	Physiological data on dormancy and hardiness development in pine in outdoor situations	3	4,7	Delivered	CO
DL6	Physiological data on dormancy and hardiness development in beech in outdoor situations	4	4,6,7	Delivered	CO
DL7	CDNA microarray carrying dormancy related pine cDNAs and relevant conserved genes from other species	5	1,2	Delivered	CO
DL8	Expression profiles during dormancy and hardiness development of the clones on the microarray	5	1,2	Delivered	CO
DL9	Selected genes whose expression is indicative for dormancy or hardiness in pine	5	1,2	Delivered	CO
DL10	Primers for PCR amplification of selected genes whose expression is indicative for dormancy or hardiness in pine	5	1,2	Delivered	CO
DL11	Selected genes whose expression is indicative for dormancy or hardiness in beech	6	1,2	Delayed to month 30	CO
DL12	Primers for PCR amplification of selected genes whose expression is indicative for dormancy or hardiness in beech	6	1,2	Delayed to month 30	CO
DL13	RT-PCR protocols for the amplification of dormancy/hardiness related genes from pine or beech	7	1,2	Delivered	CO
DL14	In depth expressional information on selected genes from pine and beech, in relation to dormancy and hardiness development in varying environmental conditions	7	1,2,4,7	Due in month 38	CO
DL15	A subset of ca. 15 highly informative key genes, descriptive of the physiological state of the tree seedling with respect to dormancy and frost hardiness	7	1,2,4,7	Due in month 38	CO
DL16	Information on the influence of provenance, climatic conditions and age on the expression of dormancy/hardiness related genes in pine and beech	8	3,5	Due in month 43	CO
DL17	An assessment of the usefulness of the selected key genes as predictors for performance of pine seedlings during cold storage.	9	3	Due in month 43	CO

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DL18	An assessment of the use of the selected key genes as molecular diagnostic tool to predict the physiological state of tree seedlings in a commercial setting	7,8,9	3,4,5,6,7,8,9	Due in month 43	CO
DL19	A physiological database combining all information on growth conditions and physiological parameters obtained during the project	10	3,4,5,6,7,8,9	Due in month 41	CO
DL20	A molecular database combining all expressional and sequence data, including PCR primers, obtained during the project	10	1,2, 4, 7	Due in month 41	CO
DL21	An integrated searchable database combining the physiological and molecular databases	10	2	Due in month 43	CO
DL22	A conceptual model describing the molecular events and pathways underlying the development of winter dormancy and frost hardiness in pine and beech seedlings.	10	All	Due in month 46	CO
DL23	Trade demonstration workshop aimed at communication of the results to the forestry sector (nurserymen, foresters)	8,9	All	Due in month 46	PU
DL24	Scientific publications in peer-reviewed journals	All	1,2,3,4,5,7	In progress	PU
DL25	Publications in trade journals	All	1,2,3,4,5,7	Delivered	PU
DL26	Annual reports	All	All	Delivered year 1 and 2	PU
DL27	Final report	All	All	Due in month 48	PU

2.3 Description of the workpackages

Workpackage number: WP1	
Workpackage title:	Climate room experiments using pine
Start date or starting event:	Start of project
Completion date:	Month 24
Partners responsible:	FC(FR)
Personmonths per partner	
Already devoted(total):	FC(FR): 16 (22) months
Total person months	22 months

<p>Objectives</p> <ul style="list-style-type: none"> - To assess seedling response to the two prime environmental factors of daylength and temperature, particularly fluctuating temperature - To produce, by manipulating daylength and temperature in an otherwise constant environment, pine seedlings at various stages of dormancy and frost hardiness. The plant material will be subjected to thorough physiological analyses and will be used for preparing microarray probe and RT-PCR template.
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<p>Methodology</p> <p>Climate chamber experiments in the first trial season were designed to separate the processes of dormancy and frost hardiness development as far as possible, to allow for the selection of genes specific for the two phenomena. The experiments are performed with the <i>P. sylvestris</i> provenance A70, derived from a British seed orchard which is composed of tested clones with good progeny performance. This provenance will serve as a standard in all pine experiments performed within this project.</p> <p><i>Set-up:</i> Three climate regimes will be applied:</p> <ol style="list-style-type: none"> 1. Fixed daylength: 16 h. Decreasing temp: 15 -> 5 °C 2. Fixed temperature 15°C,. Decreasing daylength: 16 h -> 6 h 3. Fixed daylength: 16 h. Fixed temperature: 15 °C. <p>The second trial season will be devoted to the interaction of temperature and daylength.</p> <p><i>Set-up:</i> Five climate regimes will be applied:</p> <ol style="list-style-type: none"> 1. Fixed daylength: 16 h. Fixed temperature: 15 °C. 2. Decreasing daylength: 16 h -> 6 h. Decreasing temp: 15 -> 5 °C 3. As 1, but with an intermittent cold spell of 2 weeks starting week 40 <p>Sampling dates will be in week number 34, 36, 40, 41,42, 43, 46,47, 48, 49 and 52. Physiological tests are aimed at evaluating dormancy depth (RGP and/or terminal budbreak) and hardiness status (frost tolerance, REL/SEL of excised root and shoot parts). The assays will be performed with 4 replicates, each sample consisting of material from 5 different plants. Thus, at each sampling date 20 different plants will be analysed. Regrowth will be monitored in randomized trials consisting of 5 plots, of 10 plants each. Results will be analysed statistically using analysis of variance calculations. For analysis of gene expression the partners will prepare total RNA from bud and root samples taken at each time point, according to a common protocol.</p>

Progress during the first reporting period:

Trials were carried out according to the workplan using the following regimes:

Climate 1 (control): long day (15h), high temperature (15/13°C)

Climate 2: decreasing daylength (15h → 7h), high temperature (15°C)

Climate 3: decreasing temperature (15/13°C → 5°C), long day (15h).

The decision to use a separate night temperature was taken by the management team, to exclude artefacts to constant temperature.

Pine seedlings from all controlled environment climate regimes showed development of root frost tolerance assessed at -5 °C, and shoot frost tolerance as assessed by SEL_{diff-15°C}, but this was not evident at the SEL_{diff-25°C} assessment temperature. This indicates that plants had not developed hardiness to this level under the controlled environment conditions. Shoot frost hardiness assessed at -15°C indicated that there was a lower level of induced hardiness in climate 1 seedlings over the last four weeks of the trial.

Progress during the second reporting period

The regimes for the second season deviate from the workplan in a response to the results of year 1. With these regimes we hoped to induce alterations in dormancy development. The regimes were:

Climate 1 (control): long day (17h, high temperature (15/13°C)

Climate 2: decreasing daylength (17h → 8h), decreasing temperature (15/13°C → 5°C).

Climate 3: 'SCOLD': removal from Climate 2 at week 40 to 6°C day/night and equivalent light regime, tested week 41 & 42.

Climate 4: 'SWARM': removal from Climate 2 at week 46 to Climate 1 regime, tested week 47.

Pine seedlings from the controlled environment climate regimes showed no significant development of root frost tolerance, assessed at -5 °C, in year 2. Both temperature assessments of shoot frost tolerance, by SEL_{diff-15°C} and SEL_{diff-25°C}, showed seasonal patterns of cold tolerance development for seedlings treated in the declining temperature environment. The lack of any clear picture for the root based assessment indicates that plants did not develop root hardiness in response to the controlled environment conditions. This is not unexpected for shoots which are already cold tolerant but only semi-dormant, as is the case with this species. Clear differences in shoot frost were evident, in comparison with Climate 1 (control) seedlings over the last four weeks of the trial. There was no evidence of changes in shoot physiology resulting from cold or warm 'shock' periods (SCOLD & SWARM). Root based assessments did show effects of SCOLD and SWARM treatments, and we hope to repeat this during the coming year on field-grown material.

MRNA was isolated from the first seasons trial and stored at -80 until it will be analysed. MRNA from second season samples will be isolated shortly.

Deliverables	Status
DL1 RNA for preparing microarray probe	Delivered for season 1 and 2
DL2 Physiological data on dormancy and hardiness development in pine in controlled environments	Delivered for season 1 and 2
DL4 RNA for RT-PCR	Delivered for season 1 and 2
DL24 Scientific publications in peer-reviewed journals	-
DL25 Publications in trade journals	-
DL26 Annual reports	Delivered for year 1 and 2
DL27 Final report	-
Relevant milestones	Status
M 1 Initial set of relevant pine genes selected	completed

} → ?

Workpackage number: WP2

Workpackage title: Climate room experiments using beech

Start date or starting event: Start of project

Completion date: Month 24

Partners responsible: DIAS

Person months per partner

already devoted(total): DIAS: 23,8 (24) months

Total person months: 24

Objectives

- To assess seedling response to the two prime environmental factors of daylength and temperature, particularly fluctuating temperature
- To produce, by manipulating daylength and temperature in an otherwise constant environment, beech seedlings at various stages of dormancy and frost hardiness. The plant material will be subjected to thorough physiological analyses and will be used for preparing microarray probe and RT-PCR template.

Description of work

Climate chamber experiments in the first trial season were designed to separate the processes of dormancy and frost hardiness development as far as possible, to allow for the selection of genes specific for the two phenomena. The experiments make use of a selected *F.sylvatica* provenance, Bregentved, that will serve as a standard in all beech experiments performed within this project.

Set-up: Three climate regimes will be applied:

4. Fixed daylength: 14 h. Decreasing temp: 15 -> 0 °C
5. Fixed temperature 15°C,. Decreasing daylength: 14 h -> 6 h
6. Fixed daylength: 14 h. Fixed temperature: 15 °C.

The second trial season will be devoted to the interaction of temperature and daylength.

Set-up: Three climate regimes will be applied:

1. Fixed daylength: 14 h. Fixed temperature: 15 °C
2. Decreasing daylength: 14 h -> 6 h. Decreasing temp: 15 -> 0 °C
3. As 1, but with an intermittent cold spell of 2 weeks starting week 40

Sampling dates will be in week number 34, 36, 40, 41, 42, 43, 46, 47, 48, 49, and 52. Physiological tests will be aimed at evaluating dormancy depth (RGP and/or terminal budbreak) and hardiness status (frost tolerance, SEL of excised root and shoot parts). The assays will be performed with 4 replicates, each sample consisting of material from 5 different plants. Thus, at each sampling date 20 different plants will be analysed. Regrowth will be monitored in randomized trials consisting of 5 plots, of 10 plants each. Results will be analysed statistically using analysis of variance calculations. For analysis of gene expression the partners will prepare total RNA from bud and root samples taken at each time point, according to a common protocol.

Progress during the first reporting period

During summer approximately 3500 seedlings were raised in accordance with normal nursery practice. In week no. 33 approximately 3000 of these seedlings were transferred to three climate rooms, where they were subjected to three different climate regimes until week no 2 in 2002:

Climate 1: day length (DL) =15h, temperatures $T_{day} = 15^{\circ}C$ and $T_{night} =13^{\circ}C$

Climate 2: as climate 1, but DL reduced by 1h every two weeks until 8.5 h

Climate 3: as climate 1, but T_{day} and T_{night} reduced by $2^{\circ}C$ every two weeks until $+1^{\circ}C/+1^{\circ}C$.

The deviations from the workplan are made to optimize the regimes with respect to beech seedling physiology.

$sel_{diff-15}$ and $sel_{diff-25}$ decreased during autumn for growth room beech seedlings in all three climate regimes. The results show that shoot became frost tolerant to $-15^{\circ}C$ first, and this occurred in the order climate 3, climate 2 and climate 1. No development of root frost tolerance to $-5^{\circ}C$ was observed. Beech seedlings from all three climate regimes entered almost equal levels of dormancy (number of days to terminal bud break under favourable growth conditions), although climate 3 seedlings, for a relatively short period, seemed to be more dormant (figure 6). Dormancy is normally induced by shorter day lengths and is independent of f.ex. Temperature and it was therefore surprising that dormancy was induced in both climate 1 and climate 3.

Progress during the second reporting period

In concordance with WP1, we deviated from the workplan and used alternative climate regimes for the second season in the hope to induce an effect on dormancy hardiness development. The regimes are comparable to those used in WP1, but optimized for beech. The exact conditions were:

Climate 1 (CONSTANT): day length (DL) =17h, temperatures $T_{day} = 15^{\circ}C$ and $T_{night} =13^{\circ}C$

Climate 2 (DECLINING): initially as climate 1, but DL reduced by 1h and T by $2^{\circ}C$ every two weeks until DL=8.5 h and $T_{day}/T_{night} =1/1^{\circ}C$.

Climate 3 (SCOLD): as climate 2. Seedlings tested after being subjected to a short cold period in week 39 and 40 and subsequently after a 2 week 'recovery' period (week 41 and 42) in climate 2.

Climate 4 (SWARM): as climate 2. Seedlings tested after being subjected to a short warm period in week 45 and 46 and subsequently after a 2 week 'recovery' period (week 47 and 48) in climate 2.

Beech seedlings from all controlled environment climate regimes developed shoot frost tolerance, but CONTROL seedlings seemed less hardy than DECLINE seedlings. However, long days and high temperature did not prevent the onset of frost tolerance and dormancy development in CONTROL, even though DL had been extended by two hours since the first season (2001-2002). The 2-week cold and warm periods did not affect the development of shoot frost tolerance compared to the development in the parent climate (DECLINE). All experimental plant types had a dormancy period, even the CONTROL seedlings. Dormancy developed earlier in CONTROL than in DECLINE seedlings. Dormancy in beech was considerably deeper than in pines.

Deliverables	Status
DL1 RNA for preparing microarray probe	Delivered for year 1 and 2
DL3 Physiological data on dormancy and hardiness development in beech in controlled environments	Delivered for year 1 and 2
DL4 RNA for RT-PCR	Delivered for year 1 and 2
DL24 Scientific publications in peer-reviewed journals	-
DL25 Publications in trade journals	-
DL26 Annual reports	Delivered for year 1 and 2
DL27 Final report	-
Relevant milestones	Status
M2 Initial set of relevant beech genes selected	In progress

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Workpackage number:	WP3
Workpackage title:	Field trials using pine
Start date or starting event:	Start of project
Completion date:	Month 38
Partners responsible:	FC(FR), DIAS
Person months per partner	
Already devoted(total):	FC(FR): 4.8 (26) months, DIAS: 4 (9) months
Total person months:	35
Objectives	
<ul style="list-style-type: none"> - To assess, in field trials, the effect of climatic conditions, geographical position and provenances on the development of dormancy and hardiness - To produce <i>P. sylvestris</i> plant material for studying the expression of genes involved in these processes 	
Methodology	
<p>General trials, to be performed by both FC(FR) and DIAS: Containerized pine seedlings, 1 or 2 year old, are grown in field trials at two different locations in North-Western Europe. Both a standard provenance (A70) and a local one are used. Samples are lifted in bi-weekly intervals starting from September 1 until January. The samples are subjected to physiological analyses, comparable to the analyses in WP1 and results will be processed using statistical methods. Additionally, the material is used for the isolation of mRNA. Part of the RNA will be shipped to partner 1 and 2, who will use it for quantitative PCR. Partly the RNA will be used as a template for RT-PCR assays. The trials will be repeated three times to capture the effect of year to year climatic variation and to allow for the selection of genes that are least influenced by these fluctuations.</p> <p>Specific trials, to be performed by FC(FR): Several other parameters will be assayed in additional small-scale field trials by FC(FR), to which pine is assigned as a primary target species. The trials are aimed at assessing the predictive value of selected genes in circumstances occurring in nursery practice, not covered by the general trial. Topics to be evaluated are: containerized vs. bare-root plants, effect of plant age on key gene expression, assessment of dormancy release, the effect of cold storage parameters, etc. The set-up of these trials, with respect to sampling and performed tests will be the same as for the general trials.</p>	

Progress during the first reporting period

Field trials were performed according to the planning in Scotland and Denmark. In Denmark the standard Scots pine provenance A70 (origin UK) and the provenance 'Lindås' (origin Norway) were produced in the greenhouse in accordance with normal nursery practice for containerised stock. In Scotland seedlings of *Pinus sylvestris* [Scots pine] (standard provenance A70 and local provenance N401 'Abernethy' were produced. The seedlings were transferred from the greenhouse to an outdoor container cultivation area in week 32 where they remained during the entire sampling period. Samples were taken every two weeks for REL, SEL and DBB assays and buds were sampled and stored at -80 for future molecular analysis. All provenances of containerised Scots pine seedlings at both locations developed shoot frost tolerance and dormancy. In Denmark the provenance 'Lindås' acclimatised faster and was frost tolerant to -25°C by week 47, approximately two weeks before the standard provenance 'A70'. The same provenance difference was observed for root frost tolerance and dormancy, but not as clear as for shoot frost tolerance. In Scotland dormancy in the field-grown pine provenances showed almost identical seasonal variation with the native Abernethy (N401) provenance showing periodically deeper dormancy levels than in the provenance A70 over the second half of the testing period (from Week 45).

Progress during the second reporting period

MRNA was isolated from samples from the previous season and sent to partner 2, who used it for hybridisation of the microarray and to partner 1, who used it for Real Time PCR analysis. In the second reporting period the field trials were repeated, but now also 2-year old seedlings were studied. Both provenances of containerised Scots pine seedlings developed SFT, showing a fast increase in SFT between week 41 and 43 in both provenances. This is in contrast with observations made in the previous year where 'Lindås' acclimated earlier than A70. A provenance difference was, however, observed for RFT. 'Lindås' developed dormancy during autumn, although it occurred earlier and the level was more shallow than in the previous season. The dormancy level in A70 was very shallow, and seemed slightly delayed as compared to 'Lindås'. In Scotland provenance differences were not observed, with respect to frost tolerance. The rate of cold tolerance development was quicker (steeper response slope) than that noted in the first year of field trials.

Deliverables		Status
DL4	RNA for RT-PCR	Delivered for year 1 and 2
DL5	Physiological data on dormancy and hardiness development in pine in outdoor situations	Delivered for year 1 and 2
DL24	Scientific publications in peer-reviewed journals	-
DL25	Publications in trade journals	-
DL26	Annual reports	Delivered for year 1 and 2
DL27	Final report	-
Relevant milestones		Status
M3	Subset of pine and beech key genes selected	Not started

Workpackage number:	WP4
Workpackage title:	Field trials using beech
Start date or starting event:	Start of project
Completion date:	Month 38
Partners responsible:	DIAS, FC(FR), HSP
Person months per partner	
Already devoted(total):	DIAS 5,4 (15) months, FC(FR): 1,7 (9) months, HSP: 0,3 (2) months
Total person months:	26
Objectives	
<ul style="list-style-type: none"> - To assess, in field trials, the effect of climatic conditions, geographical position and provenances on the development of dormancy and hardiness. - To produce <i>F. sylvatica</i> plant material for studying the expression of genes involved in these 	
Methodology	
<p>General trials, to be performed by both DIAS and FC(FR): Bare-rooted beech seedlings, 1 or 2 year old, are grown in field trials at two different locations in North-Western Europe. Both a standard provenance (the same as used in WP2) and a local one will be used. Samples will be lifted in bi-weekly intervals starting from September 1 until January. The samples will be subjected to physiological analyses, comparable to the analyses in WP1 and results will be processed using statistical methods. Additionally, the material will be used for the isolation of mRNA. Part of the RNA will be shipped to partner 1 and 2, who will use it for quantitative PCR. Partly the RNA will be used as a template for RT-PCR assays. The trials will be repeated three times to capture the effect of year to year climatic variation and to allow for the selection of genes that are least influenced by these fluctuations.</p> <p>Specific trials, to be performed by DIAS: Several other parameters will be assayed in additional small-scale field trials by DIAS, to which beech is assigned as a primary target species. The trials are aimed at assessing the predictive value of selected genes in circumstances occurring in nursery practice, not covered by the general trial. Topics to be evaluated are: containerized vs. bare-root plants, effect of plant age on key gene expression, assessment of dormancy release, the effect of cold storage parameters, etc. The set-up of these trials, with respect to sampling and performed tests will be the same as for the general trials.</p>	

Progress in first reporting period

The standard provenance 'Bregentved' (Czech origin, Danish seed source) and the local provenance 'Gråsten' (Danish origin) were sown in week no. 15 and cultivated in accordance with normal nursery practice for bare rooted stock by Hedeselskabets Planteskole. In Scotland standard provenance Bregentved seedlings were raised at the Research Station and local provenance Cirencester was provided by partner 8.

The seedlings were transferred from the greenhouse to an outdoor container cultivation area in week 32 where they remained during the entire sampling period. Samples were taken every two weeks for REL, SEL and DBB assays and buds were sampled and stored at -80 for future molecular analysis. In Denmark the bare rooted beech seedlings entered dormancy around week 41 and was released, or at least back to the initial level by week no. 4 (maybe even earlier, but due to difficulties with frozen nursery soils in week no. 2, it was not possible to lift seedlings for dormancy test in this week). There were no clear physiological differences between the standard provenance 'Bregentved' and the local provenance 'Gråsten'.

Progress during the second reporting period

MRNA was isolated from samples from the previous season and sent to partner 2, who used it for hybridisation of the microarray and to partner 1, who used it for constructing a beech cDNA library. Bare rooted beech seedlings of both ages developed SFT. During this period 2-year old seedlings were ahead of 1-year olds by approximately 2 weeks. The older seedlings reached the 10%-threshold for $SEL_{diff-25}$ in week 46 and the 1-year old seedlings in week 48-49. SFT(-25C) of 1-year old seedlings was reached at approximately the same time as in the previous season. Acclimation in beech started earlier than in pine and was, contrary to pine, linear over most of the period until week 47-49.

Development of bud dormancy was earlier and consistently deeper in 2-year old than in 1-year old seedlings, and both had a maximum level of approximately 60 DBB in week 47. From this week dormancy level of both ages decreased concurrently until the end of the experimental period.

Development of root frost tolerance (-2°C) in 'field-grown' beech was absent, while frost tolerance of beech shoots from the outdoor trial showed distinct seasonality. In Scotland, no provenance effects were found. The rate of cold tolerance development was quicker (steeper response slope) than that noted in the first year of field trials.

Deliverables

Deliverables		Status
DL4	RNA for RT-PCR	Delivered for year 1 and 2
DL6	Physiological data on dormancy and hardiness development in beech in outdoor situations	Delivered for year 1 and 2
DL24	Scientific publications in peer-reviewed journals	-
DL25	Publications in trade journals	-
DL26	Annual reports	Delivered for year 1 and 2
DL27	Final report	-

Relevant milestones

Relevant milestones		Status
M3	Subset of pine and beech key genes selected	Not started

Workpackage number: WP5	
Workpackage title:	Production and screening of a pine cDNA microarray
Start date or starting event:	Start of project
Completion date:	Month 20
Partners responsible:	Plant RI, ATO
Person months per partner	
Already devoted(total):	Plant RI: 28 (21) months, ATO: 12,7 (7) months
Total person months:	28
Objectives	
<ul style="list-style-type: none"> - Production and screening of a cDNA microarray carrying dormancy/hardiness related genes from <i>P. sylvestris</i>. - Selection of genes involved in dormancy/frost hardiness development in pine. 	
Description of work	
<p>ATO has selected 2000 clones from one previously constructed and two newly prepared cDNA expression libraries from dormant pine buds. PRI constructed the array, that was be supplemented with a number of pine genes known in other species to be involved in dormancy and hardiness. Examples are: cell-cycle genes, dehydrins, ABA-regulated genes</p> <p>The arrays will be screened with probes derived from pine seedlings grown under controlled conditions (WP1) to reduce the number of positives, due to non-specific variation. A total of 200 screenings will be performed, and each probe will be analysed twice, alternating the fluorescent dies. This results in the analysis of 100 independent samples, divided amongst the three climate chamber regimes and the 8 sampling moments. In addition five probes from beech will be used, to analyse homology and cloning feasibility.</p> <p>In combination with the physiological data obtained in WP1, the expression analyses will be used to identify genes putatively involved in the processes of dormancy and cold hardiness in <i>Pinus sylvestris</i> seedlings, using statistically sound correlations.</p> <p>Selected genes will be sequenced and characterized.</p>	

Progress during the first reporting period

A selection was made of 1536 clones from three different cDNA libraries, which were made by Partner 1 (ATO). The libraries contained genes that were expressed in either dormant tree buds (no selection: full library), or was made using an enrichment strategy to increase the percentage of genes specific for cold hardiness (cold library) or dormancy (dormancy library). From these libraries we selected 730 full-length genes and about 360 gene fragments from each of the subtracted libraries. Together with some eDNA AFLP genes, some genes highlighted from a literature survey and approximately 50 reference spots (positive for normalizing dye intensity and negative for calculating background hybridisation) we arrived at 1536 genes which were spotted with 3 pins in duplo. Each spot represented an individual gene for which gene expression could be measured in pine bud RNA. We experimentally tested five hybridisations using RNA prepared by ATO from either dormant, cold induced or metabolically active buds that gave rise to promising results. It therefore appears that the chip will be useful for analysis of gene expression in the samples provided by the other partners.

Progress during the second reporting period

Hybridisations were performed with over 20 samples obtained from partners 4 and 7, covering three different provenances and two locations. Based on the hybridisation profiles of the different samples, genes on the microarray could be divided into distinct classes. A group of genes whose RNA levels declined over time was designated “active genes”. A second group of genes (represented by 113 spots on the microarray and named “early cold tolerance genes”) increase in mRNA levels as the season progresses. Expression of these genes rises sharply between week 41 and 45; thus these genes may be correlated to cold tolerance or dormancy. A third category of genes, the “late cold tolerance genes”, represented by about 50 spots on the microarray, show a more gradual increase in gene expression during the early weeks, and reach their maximum levels between week 49 and 51. From these groups of genes 36 representative genes have been selected that can be further tested for their usefulness as marker. These results are derived from samples from year 1 only. The WP was delayed because the responsible technician left and it took some time to fill the vacancy. It was decided to first carry out additional microarray experiments, using year 2 samples, to validate the results, before RT-PCR assays will be developed. In addition a general shift of focus from RT-PCR to more microarray work was decided. This WP will therefore be extended into the 3rd project year. Partner 2 will devote more months to this WP and less to WP7

Deliverables		Status
DL7	cDNA microarray carrying dormancy related pine cDNAs	Delivered
DL8	Expression profiles during dormancy and hardiness development of the clones on the microarray	Delivered
DL9	Set of ca. 30 genes whose expression is indicative for dormancy or hardiness in pine	In progress
DL10	Primers for PCR amplification of ca. 30 genes whose expression is indicative for dormancy or hardiness in pine	In progress
DL24	Scientific publications in peer-reviewed journals	-
DL25	Publications in trade journals	-
DL26	Annual reports	Delivered for year 1 and 2
DL27	Final report	-
Relevant milestones:		
M 1	Initial set of relevant pine genes selected	Completed

Workpackage number: WP6	
Workpackage title:	Identification and isolation of beech homologues
Start date or starting event:	Identification of relevant pine genes
Completion date:	Month 23
Partners responsible:	ATO, Plant RI
Person months per partner	
Already devoted(total):	ATO: 6(14) months, Plant RI: 1,5(9) months
Total person months:	23
Objectives	
To identify <i>F. sylvatica</i> genes that are involved in dormancy and/or frost hardiness development by isolating homologues to the selected pine genes (M1) and verifying their expression pattern in RNA isolated from controlled environment grown beech seedlings.	
Description of work	
To allow for the isolation of beech genes, homologous to the pine genes selected in WP5, a cDNA library will be made from <i>F. sylvatica</i> dormant buds. Via non-stringent hybridisation, the library will be screened for the presence of homologues to the pine genes selected as relevant for dormancy and hardiness development.	
In addition, PCR based strategies will be used when feasible. These will employ degenerate primers, based on the <i>Pinus</i> sequence to amplify the corresponding beech gene fragment. Isolated genes will be sequenced and characterized.	
The expression pattern of the identified genes in beech will be checked against cDNA derived from the climate room experiments from WP2. This will reveal whether the isolated homologue is also relevant for the processes under investigation in beech. The experiments will start when the first results from the cDNA microarray screenings are available.	

Progress during the first reporting period

This WP was not active in the first project year

Progress during the second reporting period

Based on the test hybridisations of the pine array a list of putatively relevant genes has been made as an initial guide for the selection of beech genes. The genes present on this list are consistent with the hypothesis that lower temperature results in more rigid membranes, which become more permeable for calcium. The calcium influx triggers several expression cascades.

A cDNA library of beech bud RNA, sampled in week 37 through week 4, provenance Graasten, has been made. The beech library resulted in the selection of several putatively interesting genes such as an ethylene receptor, a metallothionin, an alternative oxidase, a histon 4 and a serine/threonine protein phosphatase. However, the library turned out to be not representative and had a low titer of 1.2×10^4 pfu/ml. Therefore a new library has been made from buds from two-year-old beech trees from the provenance's BRE, GR and NL (Boskoop) from week 35 until week 06, covering the early (eco-dormant) and mid-dormant (endo-dormant) phases. The titer of the primary library was $5.10e05$ pfu/ml. 200.000 pfu were used for subsequent amplification. Preliminary results indicate that the sizes of the cloned cDNA-molecules range from 0.3 until 2.0 kB. Because we had to wait for the results of the pine array, this workpackage has been delayed and will be extended into the third project year. In the mean time it became clear that beech develops stronger dormancy than pine. Therefore it was decided to analyse beech gene expression independent from the pine results. A small beech array will be made and analysed using samples from field and climate room trials. This work will replace the search for beech homologues using a PCR approach.

Deliverables

Deliverables		Status
DL11	Set of ca. 30 genes whose expression is indicative for dormancy or hardiness in beech	In progress
DL12	Primers for PCR amplification of ca. 30 genes whose expression is indicative for dormancy or hardiness in beech	In progress
DL24	Scientific publications in peer-reviewed journals	-
DL25	Publications in trade journals	-
DL26	Annual reports	Delivered for year 1 and 2
DL27	Final report	-

Relevant milestones

Relevant milestones		Status
M2	Initial set of relevant beech genes selected	In progress

Workpackage number: WP7	
Workpackage title:	Development and application of RT-PCR assays
Start date or starting event:	Identification of relevant pine and beech genes
Completion date:	Month 38
Partners responsible:	ATO, Plant RI, DIAS, HSP, FC(FR), FE Nurseries, ALBA
Person months per partner:	ATO: 11,2(25) months, Plant RI: 0(8) months, DIAS: 0(3) months,
(already devoted)(total):	HSP: 0(1) month, FC(FR): 0(4) months, ALBA: 0(1) month
Total person months:	42
Objectives	
<ul style="list-style-type: none"> - To develop straightforward and rapid tests for the presence of dormancy/hardiness keygenes - To use those tests for detailed analysis of the expression profiles of the selected genes. - To assess the diagnostic value of the tests in a commercial setting 	
Description of work	
<p>ATO and Plant RI will design RT-PCR primers, based on the sequence of the selected keygenes. The primers will be used by both participants in a quantitative PCR assay to refine the expression profile of the selected genes using previously untested material from the climate room experiments (WP1 and WP2) and material from the WP3 and WP4 outdoor trials, which will reveal the effects of annual and geographical climatic variation, growth conditions and provenance. These experiments will start as soon as the first relevant genes have been selected and will continue to the end of the project. They will result in the selection of a sub-set of highly informative key genes, descriptive and predictive of dormancy and frost hardiness state.</p> <p>DIAS and FC(FR) will evaluate this sub-set of primers in specific field trials and, aided by HSP, FE Nurseries and ALBA, in a commercial setting. Trials will be designed to test whether the PCR assays can be used as diagnostic tests to predict the level of hardiness or dormancy. Primers showing optimal predictive properties will be tested in a commercial setting to evaluate their usefulness as practical diagnostic test for forest tree nurseries.</p>	

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Progress during the first reporting period

In deviation from the technical annex we already started to do some initial experiments related to WP 7. In order to be able to quantify gene-expression of selected markers in the future, we started analyzing mRNA-content of a specific dehydrin in bud tissue of pine trees, using real-time PCR. This analysis will serve as a testing model to optimize protocols and procedures. In addition dehydrin is an important marker of dormancy so the results will in itself be useful to the project as well.

Progress during the second reporting period

The expression pattern of representatives of three dehydrin classes (SK2, SK4 and K2) has been analysed in detail and revealed that in particular SK2 and K2 are responsive to dormancy development. K2 shows a sharp increase starting from the onset of endodormancy and SK2 reacts to the same event by decreasing its expression. The ratio between both genes is therefore a good indication for the dormancy status. A straightforward PCR based assay was developed for these markers, that can be used as a dormancy test.

The expression analysis of dehydrin genes and a tubulin gene was also used to validate the array results. Microarray based expression profiles for these genes matched very well with the I-Cycler (realtime PCR) results.

Development of PCR tests for other genes awaits the final selection of key indicator genes for beech and pine.

Deliverables

		Status
DL13	RT-PCR protocols for the amplification of dormancy/hardiness related genes from pine or beech	Delivered for dehydrin
DL14	In depth expressional information on ca. 30 genes from pine and beech, in relation to dormancy and hardiness development in varying environmental conditions	In progress
DL15	A subset of ca. 15 highly informative key genes, descriptive of the physiological state of the tree seedling with respect to dormancy and frost hardiness	-
DL18	An assessment of the use of the selected key genes as molecular diagnostic tool to predict the physiological state of tree seedlings in a commercial setting	-
DL24	Scientific publications in peer-reviewed journals	-
DL25	Publications in trade journals	-
DL26	Annual reports	Delivered for year 2
DL27	Final report	-

Relevant milestones

		Status
M3	Subset of pine and beech key genes selected	Not started
M4	Usefulness of key genes as molecular diagnostic tests evaluated	Not started

Workpackage number: WP8	
Workpackage title:	Provenance effect on gene expression patterns
Start date or starting event:	Start of the project
Completion date:	Month 43
Partners responsible:	PPO, DU, ATO
Person months per partner	
Already devoted(total):	PPO: 7,8 (11) months, DU: 2(10) months; ATO: 0(2) months
Total person months:	23
Objectives	
To evaluate the correlation of key gene expression and dormancy/hardiness status, under conditions were the hardening process might interfere with plant specific genetic properties and with changes in physiological conditions caused by age and climatic conditions.	
Description of work	
<p>The aim of this trial is to evaluate the expression of the selected genes under conditions were the hardening process might interfere with plant specific genetic properties and with changes in physiological conditions caused by age and climatic conditions. These are situations that are common in commercial situations. In controlled trials the effect of provenance, age, and climate conditions on the predictive value of marker gene expression will be evaluated. In addition the diagnostic value of key gene expression will be studied in material from commercial nurseries. Gene expression profiles will be compared with relative root and shoot freezing tolerance ($REL_{diff-10}$ and $SEL_{diff-10}$) and with stem conductance (EC). The trials will involve beech (RSNS) and pine (DU).</p> <p><i>Set-up provenance effect:</i></p> <p>Seeds of 3 geographic provenances will be sown in three subsequent years, starting in 2001. One of the provenances will be identical to the standard provenance used in WP3 and WP4, respectively. In trial season 2002/2003 $REL_{diff-10}$, $SEL_{diff-10}$ and EC of 0.5 and 1.5 year old seedlings of the 3-4 provenances will be measured bi-weekly from autumn to mid winter (6 sampling dates). Material will be collected for mRNA analysis and send to partner 1. In the season 2003/2004 this experiment will be repeated with seedlings from all three sowing seasons (0.5, 1,5 and 2,5 years old). At each sampling date 40 plants per provenance-age combination are used for $REL_{diff-10}$, $SEL_{diff-10}$ and EC analyses (4 replicates of 5 seedlings at the two temperatures). For mRNA analysis 50 plants are used.</p> <p><i>SET-UP COMMERCIAL TESTING:</i></p> <p>In year 2003 samples of beech and pine seedlings will be taken tree times from the onset of hardening in September to completely hardened in December from 3 commercial nurseries. The root and shoot freezing tolerance will be measured as $REL_{diff-10}$ and $SEL_{diff-10}$ using 40 seedlings (4 replicates with 5 seedlings in each for each temperature) at each sampling date. Material for mRNA analyses will be collected at the same occasions and sent to ATO. The results will be compared with that of the provenance trial.</p>	

Progress during the first reporting period

During the first project year plants have been produced for provenance trials planned in autumn 2002. Three different provenances of beech have been sown: NL2.1, Greenhill and Bregentved.

Progress during the second reporting period

The bare-rooted seedlings of all provenances and two ages were planted at the trial field and lifted 8 times during the season, in week 33, 35, 40, 42, 44, 46, 48 2002 and in week 6 2003, 20 shoots of each age/provenance combination. Apical buds of 20 plants were sampled (3 buds/plant on average) and stored immediately in liquid nitrogen. The buds were stored at –80°C until mRNA analysis. For mRNA analysis week 33 is missing. The seedlings were subjected to measurements in length, shoot electrolytes leakage ($Se_{diff-25}$) and electric conductivity of the shoot (EC). Results indicated that the bare rooted beech seedlings all developed frost tolerance, but no provenance differences were detected. Samples derived from these trials have been used by partner 1 to construct a beech cDNA library.

Deliverables

		Status
DL16	Information on the influence of provenance, climatic conditions and age on the expression of dormancy/hardiness related genes in pine and beech	In progress
DL18	An assessment of the use of the selected key genes as molecular diagnostic tool to predict the physiological state of tree seedlings in a commercial setting	-
DL23	Trade demonstration workshop	-
DL24	Scientific publications in peer-reviewed journals	-
DL25	Publications in trade journals	-
DL26	Annual reports	Delivered for year 2
DL27	Final report	-

Relevant milestones

M4	Usefulness of key genes as molecular diagnostic tests evaluated
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Workpackage number:	WP9
Workpackage title:	Relating storage performance to key gene expression
Start date or starting event:	Month 18
Completion date:	Month 43
Partners responsible:	DU, ATO
Person months per partner	
Already devoted(total):	DU: 5.8(10) months; ATO: 0(2) months
Total person months:	12

Objectives

To study the expression of selected markers in relation to storage performance of pine and to evaluate their predictive value for this application

Description of work

The first storage trial (season 2001-2002) will focus on frozen (below 0°C) storage. One and two-year old seedlings of three different provenances, one of which is the standard provenance used in WP1 and WP3 will be stored in frozen storage when physiological freezing tolerance tests show that the seedlings are storable (approx in September-October). The root and shoot freezing tolerance will be evaluated 6 times during storage (at transfer to storage, mid-Oct, mid-Dec, mid-March and early in May). At these occasions, tissue samples will be taken and stored at -80°C for future mRNA screening by ATO.

The second trial (season 2002-2003) will be focused on cool (above 0°C) storage and the results will be compared to the frozen storage. One- and 3-year-old seedlings of three different provenances will be used to be able to evaluate the effect of seedlings age and provenance. The root and shoot freezing tolerance will be evaluated 6 times during storage (at transfer to storage, mid-Oct, mid-Dec, mid-March and early in May). At these occasions, tissue samples will be taken and stored at -80°C for future mRNA screening by ATO. These screenings will be performed once the key genes have been selected.

Progress during the first reporting period

Apart from growing plant material for future experiments, this WP has not yet started.

Progress during the second reporting period

During autumn 1 and 2-year-old containerised seedlings of *Pinus sylvestris* (Scots pine) (standard provenance "A 70", local provenance "Sollerö" and northern provenance "Åmsele") seedlings were raised and nursed at the Research Station in Garpenberg. Every second week, starting in week 36 and until week 48 all experimental plant types were tested for shoot and root frost tolerance ($SEL_{diff-25^{\circ}C}$ / $SEL_{diff-15^{\circ}C}$ / $REL_{diff-5^{\circ}C}$), rest/"dormancy" (days to bud break) and material for mRNA analysis was collected from terminal buds. The seedlings of northern provenance became freezing tolerant at an earlier time and their shoots did achieve a higher degree of freezing tolerance. There was also a clear age effect on root freezing tolerance, since roots of the 2-year-old seedlings became freezing tolerant earlier than the roots of 1-year-old seedlings. The freezing tolerance needed for long term frozen storage ($SEL_{diff-25} \leq 5$ and $REL_{diff-25} \leq 7$) was, with one exception, reached for all experimental plant types. Days to budburst (DBB) decreased from early September to late November for all seedling types. In September there was a clear provenance effect on days to budburst where seedlings from northern provenances displayed a deeper rest or "dormancy" than seedlings of southern origin. Seedlings have been transferred to frozen storage (at $-5^{\circ}C$) in week 40 and 48 and then tested for freezing tolerance and vitality in week 6 2003. Freezing tolerance and vitality will be checked again at the end of storage in late spring 2003. The freezer in which the seedlings are stored has accidentally had a failure which may lead to a second experiment in frozen storage in next winter instead of the planned experiment concerning cold storage

Deliverables		Status
DL17	Evaluation of the predictive value of the selected keygenes with respect to storage performance of pine	In progress
DL18	An assessment of the use of the selected key genes as molecular diagnostic tool to predict the physiological state of tree seedlings in a commercial setting	-
DL23	Trade demonstration workshop	-
DL24	Scientific publications in peer-reviewed journals	-
DL25	Publications in trade journals	-
DL26	Annual reports	Delivered for year 2
DL27	Final report	-
Relevant milestones		Status
M4	Usefulness of key genes as molecular diagnostic tests evaluated	-

Workpackage number: WP10	
Workpackage title:	Development of an integrated database
Start date or starting event:	Month 39
Completion date:	Month 46
Partners responsible:	Plant RI, FC(FR), ATO, DIAS
Person months per partner	
Already devoted(total):	Plant RI: 0(7) months, FC(FR): 0(5) months, ATO: 0(4) months, DIAS 0(4) months
Total person months:	20
Objectives	
<ul style="list-style-type: none"> - To develop an integrated searchable, cross-referenced database that combines all data obtained in this project. For each clone on the microarray physiological, morphological, expressional and sequence information should be accessible. - To define an hypothesis describing the molecular pathways involved in dormancy and hardiness in Scots pine and common beech. - To communicate these results to the sector in a demonstration work shop 	
Description of work	
<p>FC(FR) will combine all physiological and morphological data obtained during the project in one database that includes information on growth conditions. Plant RI will purchase specific software for storing and manipulating microarray data and will adapt this to allow linkage with the physiological database. The database will be used to reveal coordinated expression patterns linked to the development of dormancy and cold tolerance in pine and beech and will thus aid in the definition of an hypothesis or conceptual model on the molecular events underlying these processes. In addition the database can be used for the selection of keygenes whose expression pattern is tightly linked to the development of hardiness or dormancy and not subject to aspecific fluctuations. These genes will be a good basis for the future develeopment of a diagnostic test. The information available in the integrated database and the conclusions drawn from it will be presented to the sector in a demonstration workshop that will be organized at the end of the project. This workshop will be focused on the significance and implementation of the results in forest tree nurseries.</p>	

Progress during the first reporting period

This WP is not active yet

Progress during the second reporting period

A common Excell datasheet has been designed for field/cliamte room trial results. Partners 3,4,5 and 7 have entered their data in this sheet. All data, including gene expression data, will be collected by the co-ordinator as a initial step in the construction and analysis of a common database

Deliverables		Status
DL19	A physiological database combining all information on growth conditions and physiological parameters obtained during the project	-
DL20	A molecular database combining all expressional and sequence data, including PCR primers, obtained during the project	-
DL21	An integrated searchable database combining the physiological and molecular databases	-
DL22	A conceptual model describing the molecular events and pathways underlying the development of winter dormancy and frost hardiness in pine and beech seedlings.	-
DL23	Trade demonstration workshop aimed at communication of the results to the forestry sector (nurserymen, foresteers)	-
DL24	Scientific publications in peer-reviewed journals	-
DL25	Publications in trade journals	-
DL26	Annual reports	-
DL27	Final report	-
Relevant milestones		Status
M5	Integrated database available	not started
M6	Conceptual model defined	not started

3. ROLE OF PARTICIPANTS

3.1 PARTNER 1, CO-ORDINATOR: ATO

Agrotechnological Research Institute
PO-box 17,
6700 AA Wageningen,
The Netherlands.
Tel: +31.317.475000
Fax: +31.317.475347

Scientific team

Dr. Monique F. van Wordragen – co-ordinator and project leader for ATO
Ing. Peter A. Balk – Junior Scientist
Ing. Brigitte Verkerk-Bakker – Technician. has filled the vacancy mentioned in the technical annex

Contractual links to other participants

None

Objectives

The Agrotechnological Research Institute has ample experience in the molecular handling of woody species and will focus on isolation and characterisation of relevant genes and expressional analysis. ATO is experienced in the application of molecular diagnostics for quality and vitality assessment in plants, including woody species.

In close co-operation with PRI, ATO will be involved in the selection and characterisation of genes relevant to dormancy and hardiness in Scots pine and common beech; the institute will supply a pine cDNA expression library and several previously selected genes, whose expression is correlated with dormancy development. ATO will focus on the development of RT-PCR assays, the first step towards the development of molecular diagnostic tests for forestry practice.

Next to the scientific work, ATO will be responsible for the overall administrative, financial and scientific co-ordination of the project.

Workplan

At the kick-off of the project ATO organised a general meeting in which detailed agreements were made for the organisation of the work in the first 6 months. The meeting was also used for the presentation and discussion of a draft version of the consortium agreement. The kick-off meeting included a molecular biology workshop, organised by ATO and Plant RI. The workshop was aimed at the introduction and training of standard RNA isolation and handling protocols, to ensure uniform RNA quality.

In the first project year ATO was involved in the construction of a cDNA microarray carrying pine genes (WP5).

In the next year, as the first selected pine genes come available, ATO will start with the selection of beech homologues to these genes using PCR based approaches as well as cDNA library screening (WP6). Also a start will be made with the design of specific primers for the selected genes and the development of RT-PCR assays (WP7). This work will continue into the third project year. The RT-PCR assays will be used during the rest of the project for the production of detailed expressional profiles of the selected genes (WP7). This will be done in co-operation with DIAS, FC(FR) and the participating nurseries. ATO will participate in the analysis of the effect on gene expression of provenance climate and age, directed by PPO, and in the evaluation of the use of the selected genes as marker for storage performance of pine, directed by DU. In both work packages (WP8 and WP9) ATO will perform the molecular analysis. Data obtained from WP7, WP8 and WP9 will be communicated to the sector via a demonstration workshop. In this workshop nurserymen and foresters will be shown how to apply the selected genes as management tools in practice. ATO will feed all gene expression information obtained, into the molecular database, managed by Plant RI and will use the data derived from the integrated database for the conception of a working hypothesis on the molecular mechanisms underlying dormancy and hardiness development in woody plants (WP10).

Deliverables

The contribution of ATO to the project deliverables is specified in the scheme below

Deliverable	WPs	Confidential nature	Delivery month											
			12	20	24	30	36	38	41	43	46	48		
DL7 - cDNA microarray	5	CO	✓											
DL8 - Expression profiles	5	CO		✓										
DL9 - Set of 30 pine genes	5	CO												
DL10 - Primers for PCR on pine genes	5	CO		✓										
DL11 - Set of 30 beech genes	6	CO												
DL12 - Primers for PCR on beech genes	6	CO												
DL13 - RT-PCR protocols	7	CO			✓									
DL14 - In depth expressional information	7	CO												
DL15 - A subset of 15 key genes,	7	CO												
DL20 - A molecular database	10	CO												
DL22 – Conceptual model describing molecular events underlying winter dormancy and frost hardiness.	10	CO												
DL23 - Trade demonstration workshop	8,9	PU												
DL24 – Scientific publications	All	PU												
DL25 – Publications in trade journals	All	PU												
DL26 - Annual reports	All	PU	✓											
DL27 - Final report	All	PU												

Grey field: date when deliverable is due according to the technical annex. ✓ = delivered

Research activities during the first reporting period**Activities related to WP5**

The first year of the project for ATO, was devoted to the construction of the cDNA microarray. The array itself is printed by partner 2, Plant Research International, but the clones of which the array was constructed have been prepared and selected by ATO. For this we used a three-way approach in order to enhance the chance for relevant genes to be present on the array.

- characterise a previously constructed cDNA library, based on dormant pine bud tissue. The tissue was harvested in early december. This library was designated FULL and 700 clones were randomly selected, amplified and purified and transferred to partner 2, for sequence-analysis and subsequent array-construction
- construct two specific cDNA libraries enhanced respectively for genes related to dormancy and cold tolerance. These libraries were made from apical buds taken from two-year-old *Pinus sylvestris* trees, obtained from a local grower, at the beginning of February (i.e. dormant sample). Some of the trees were cold treated (20°C to -10°C, overnight in a controlled cold cel by Partner 5 (PPO) and an apical bud sample was taken (cold sample). At the beginning of April an apical bud sample was taken from the same batch of trees (release sample). These samples were used to construct so-called subtracted cDNA-libraries. One of the libraries is enriched for genes that are cold induced (COLD-library) and the second library contains material that is enriched for genes related to dormancy development of the apical bud (DOR-library). From each of these libraries approximately 400 clones were sent to Partner 2 (PRI) for sequence-analysis and subsequent array-construction.
- Furthermore, a set of specific cDNA-clones was delivered to PRI so they might also be included onto the array. These clones represent pine genes that are expected to have impact on either dormancy or cold tolerance based on literature data or previous research. The clones include
 - 20 AFLP fragments related to dormancy development
 - Dehydrins
 - Abscisic acid (ABI1 and ABI3)
 - Gibberellin (CPS and GA 20-oxidase)
 - Enzymes involved in proline accumulation (P5CS and P5CR)
 - Genes involved in cell cycle control (CDC2 and tubulin)
 - a dormancy related marker (DRM1)
 - transcription factor CBF/DREB1 proteins
 - superoxide dismutases – both CuZn-SOD, Mn-SOD and Fe-SOD

In addition a lot of effort was put in isolating a pine homologue of the CBF transcriptionfactor, which functions as a master switch for cold regulated genes in *Arabidopsis*. (RT)-PCR was performed with primer sets based on sequences from *Arabidopsis* (CBF1, 2, 3), *Oryza*, *Hordeum*, and an EST homologue from *Pinus taeda*. A colony hybridization of the COLD cDNA-library was performed with the *Arabidopsis* fragment. And hybridizations of Northern-blot made from time-series of cold treated pine with the *Arabidopsis* CBF2 fragment and the *Pinus taeda* homologue. All without success, which indicates that either *P. sylvestris* does not possess a homologue of CBF (very unlikely since close relative *P. taeda* does have the gene) or the gene is not expressed in bud tissue. This will be resolved in the coming months.

Activities related to WP7

In deviation from the technical annex we already started to do some initial experiments related to WP 7. In order to be able to quantify gene-expression of selected markers in the future, we started analyzing mRNA-content of a specific dehydrin in bud tissue of pine trees, using real-time PCR. This analysis will serve as a testing model to optimize protocols and procedures (see figure 1). In addition dehydrin is an important marker of dormancy so the results will in itself be useful to the project as well.

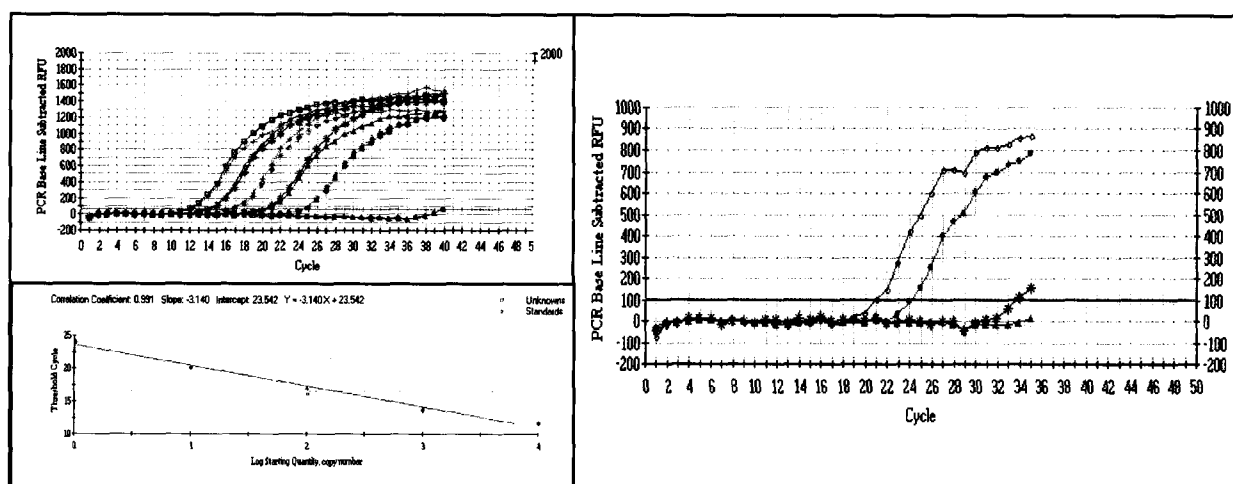


Figure 1. Upper left panel: RT-PCR amplification of standard amounts of, in-vitro generated, dehydrin RNA molecules. This shows that the reaction is linear over a wide range and reproducible (duplicates). With this result it is clear that accurate measurements of specific mRNA contents are possible. The threshold crossing (lower left panel) is taken as a measure for initial RNA content. Right panel: Amplification of a specific dehydrin mRNA (different dilutions) from a pine bud sample. The threshold crossing is taken as a measure for initial RNA content.

Significant difficulties or delays experienced during the first reporting period

Apart from the fact that we were thus far not able to isolate a pine CBF gene, no difficulties or delays were experienced. The CBF gene is therefore not present on the array, but is also not indispensable. We will look for the gene at the DNA level and in other tissue than buds and will analyse its expression profile separately.

Sub-contracted work during the first reporting period

Identify the sub-contractor. Report on the actual sub-contracted work for the reporting period and, where appropriate, present interim or final results and conclusions.

Research activities during the second reporting period

Scientific team year 2

Dr. Monique F. van Wordragen – co-ordinator and project leader for ATO

Ing. Peter A. Balk – Junior Scientist

Ing. Brigitte Verkerk-Bakker – Technician.

Research related to WP5:

RNA of cold-stressed and dormant pine buds was extracted and delivered to PRI for use in test hybridisations. A pine glucan-synthase gene fragment was picked up using a PCR-based approach.

The enzyme is known to be involved in blocking cellular communication, one of the key-events in dormancy induction (ATO, unpublished data). Based on (partial) sequence information, from *Arabidopsis thaliana*, *Populus* spp., *Brassica* sp., *Nicotiana tabacum* and *Pinus taeda*, primers were selected to isolate a glucan-synthase fragment.

The sequences were:

Forward primer1: 5'TGATTGGCCAGATTGGAACAAGTG
 Forward primer2: 5'ATCGTGGAGGGATTGGTGTACC
 Reverse primer1: 5'TTTCCACCAAGATTCCCAAC

Two highly homologous fragments were isolated using primerset Forward1 and Reverse1. The size of both fragments is 88 basepairs and the homology on the nucleotide level was 85%. Both sequences show high homology with a glucan synthase fragment from *Pinus pinaster* (AL750522), 96% on the nucleotide level. On the amino acid level both sequences are highly homologous to glucan synthase from *Gossypium hirsutum* (AF085717). The consensus sequences are as follows;

DW_DWNKWIN__GGIGV__EKSEWESWWE and DW_DWNKWINN_GGIGVP_EKSWESWWE respectively.

Homology is high because this fragment is part of the catalytic sequence.

We performed 5-prime RACE reactions using specific primers for both fragments. The GeneRacer Kit from Invitrogen was used and reactions were performed according to manufacturer instructions. One 1.5 kb fragment was isolated and sequenced. This fragment is, on the amino acid level, slightly homologous to glucan synthases from *Filobasidiella neoformans* (At3g07160) and *Arabidopsis thaliana* (AC006921). Based on this sequence a primerset was synthesised in order to study expression using Realtime RT-PCR.

Primer sequences were:

PgsF: 5'TGCAAAGCATGTGTGGTCAGTTC
 PgsR: 5'CAGCTCTGAGATCTTGCAGTTCCTTG

Amplicon size was 162 base pairs, optimal for Realtime analysis. Reaction conditions were optimised and RT-PCR was performed on field grown Lindas pine RNA from apical buds (season 2001/2002). RNA from week 39 until week 04 was used. Results indicate that there is no significant variation in expression during this period. Because of the fact that it is expected that glucan synthases be represented by large gene-families, it is difficult to draw any conclusion from this. We should at least pay much more attention to the outcome of the RACE reaction and the expression of a certain set of isoforms should be studied before any clear conclusions can be drawn. This is however beyond the scope of this project so therefore we do not extend our study of this gene.

Research related to WP 6:

As a first step towards the identification of beech homologues a cDNA library of beech bud RNA, sampled in week 37 through week 4, provenance Graasten, has been made using the λ ZAP II system (Stratagene) following manufacturer instructions. Depending on the availability of material we will also make subtracted libraries. The beech library resulted in the selection of several putatively interesting genes such as an ethylene receptor, a methallothionin, an alternative oxidase, a histon 4 and a serine/threonine protein phosphatase. However, the library turned out to be not representative and had a low titer of 1.2×10^4 pfu/ml. Therefore a new library has been made that is being characterised at the moment. Buds from two-year-old beech trees from the provenance's BRE, GR and NL (Boskoop) were mixed and grinded. RNA was isolated from this material from week 35 until week 06, covering the early (eco-dormant) and mid-dormant (endo-dormant) phases. Total RNA from the separate weeks was mixed in equal amounts and used for isolation of mRNA using the Oligotex kit (Qiagen). 5 Microgram of mRNA was used for the library. The cDNA Expression library was made using the λ ZAP II system (Stratagene) following manufacturer instructions. The titer of the primary library was $5.10e05$ pfu/ml. 200.000 pfu were used for subsequent amplification. Preliminary results indicate that the sizes of the cloned cDNA-molecules range from 0.3 until 2.0 kB.

Based on the test hybridisations of the pine array a list of putatively relevant genes has been made as an initial guide for the selection of beech genes. The genes present on this list are consistent with the hypothesis that lower temperature results in more rigid membranes, which become more permeable for calcium. The calcium influx triggers several expression cascades.

Research related to WP 7.

Dehydrin gene family has been used as a model for the development of markers using RT-PCR. Several members of three dehydrin gene-classes are present on the array. The expression pattern of representatives of three dehydrin classes (SK2, SK4 and K2) has been analysed in detail and revealed that in particular SK2 and K2 are responsive to dormancy development. Differential expression of the different dehydrins was studied based on the data presented in the next table. Primer sequences that were used for the Real-time RT-PCR analysis were also indicated in the table.

cDNA-clone	conserved domains		Acc.no.	Real-time PCR primer
	S	K		
<i>PsDhn1</i>	1	4	AJ289610	Forward 5'CACACGGGTTTGATAGG Reverse 5'TGATCTGAAGAATGCTGTCC
<i>PsDhn2</i>	1	2	AJ512361	Forward 5'TGAGAATAATGGTACGTGCGTGTTG Reverse 5'CTGAAGGTAAGCTCGTACCGAAACC
<i>PsDhn3</i>	-	2	AJ512362	Forward 5'GGAAGAAACCGGGAATGG Reverse 5'TTTTGTGTCCCGGCAGC
<i>PsDhn4</i>	-	2	AJ512363	
<i>PsDhn5</i>	-	2	AJ512364	
<i>PsDhn6</i>	-	2	AJ512365	
<i>PsDhn7</i>	-	2	AJ512366	
<i>PsDhn8</i>	-	2	AJ512367	

Because of the high homology of *PsDhn3-8*, one primerset was used that amplified the mRNA-molecules from all genes simultaneously.

Real-time RT-PCR protocol

A real time PCR protocol was developed for the dehydrin genes as a first step to a easy-to-use assay and as a validation for the microarray results. For Realtime RT-PCR, total RNA was isolated and RNA preparations were treated with DNaseI (AP Biotech) and purified using the RNeasy system (Qiagen) following manufacturer instructions. One microgram of pure total RNA was used for the synthesis of cDNA. Anchored oligo (dT)₂₃ from SIGMA was used together with M-MLV Reverse Transcriptase (Life Technologies). This cDNA was used for real-time PCR using the qPCR Core Kit (Eurogentec) in the iCycler system (BIORAD Laboratories). Reaction conditions were carefully optimised for each primerset. PCR product formation was detected directly by monitoring the increase in fluorescence caused by Sybr Green I intercalation. A threshold was set, which intersected the amplification curves in the linear region of the semi-log plot. The point at which the curve crosses the threshold, the so-called threshold-value (C_T), is taken as a measure for the original amount of target that is present in a certain sample. The original amount of cDNA in an unknown sample is measured by interpolation from a standard curve of C_T -values generated from known starting concentrations of target.

These analyses revealed that K2 shows a sharp increase starting from the onset of endodormancy and SK2 reacts to the same event by decreasing its expression. The ratio between both genes is therefore a good indication for the dormancy status. For both classes specific primers have been made. Expression varies, but in all samples the ratio K2/SK is below 1 in non-dormant buds and above 1 in dormant tissue. Therefore this primer pair is a promising candidate for a dormancy indicator assay. A straightforward PCR based assay was developed for these markers, that can be used as a dormancy test. The expression analysis of dehydrin genes and a tubulin gene was also used to validate the array results. Microarray based expression profiles for these genes matched very well with the I-Cycler (realtime PCR) results.

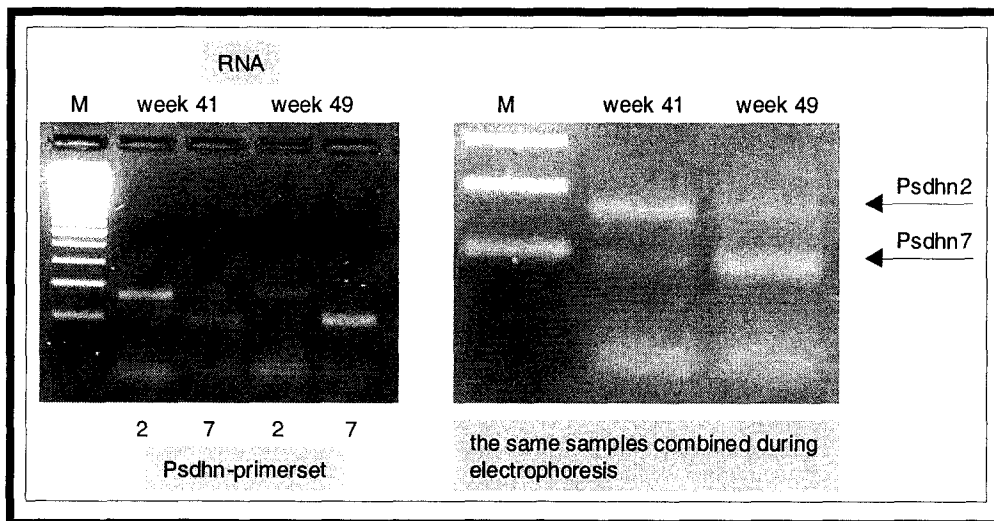
Determination of dormancy stages using dehydrins as markers

Based on the difference in expression-patterns of the dehydrins *PsDhn2* and *PsDhn3-8*, a protocol was made that makes it possible to discriminate between the two dormancy stage described. This test protocol is easy to perform and needs only a PCR machine and gel-electrophoresis equipment. The method was successfully applied by non-experienced participants in a workshop during the second annual meeting. The manual to this workshop is attached as Annex 6. RNA isolated from small amounts (100mg) of grinded buds using the RNeasy Kit from Qiagen can be used.

SuperScript One-Step RT-PCR with Platinum Taq (Life Technologies) was used for subsequent RT-PCR using a primerset specific for Psdhn2 (Table) and Psdhn7 (or Psdhn3-8, Table). The sequences of the Psdhn7 primers are as follows:

Psdhn7F 5'AGAGCTTGGGTGAGGAGCAT
 Psdhn7R 5'TTGTACCCGAAGTCCCATT

After different numbers of cycles samples were taken and analysed using agarose electrophoresis. A typical result of this is shown below.



Significant difficulties or delays experienced during the second reporting period

The selection of beech genes had to be postponed as a result of the fact that the analysis of the pine array was delayed because of a shift in persons at PRI. In the second half of 2002 we decided to start in advance with the construction of a beech cDNA library, but the first attempt resulted in a low quality library. The second attempt in March 2003 did result in a good library that will now be used as a basis for a small array. The hybridisations on that array will yield an initial selection of prominent beech genes (Milestone 2, due in month 23) in the second half of 2003 (around month 30). However, the use of a beech array will facilitate the validation of the key genes and ensure a more representative selection than would have been possible in the original planning.

Sub-contracted work during the second reporting period

During the second reporting period sequencing of selected cDNA clones was subcontracted to Baseclear. Production of specific primers was subcontracted to Eurogentec and Biologio.

3.2 Partner 2 – contractor: PRI

Agricultural Research Department Plant Research International (PRI)
PO-box 16,
6700 AA Wageningen,
The Netherlands.
Tel: +31.317.477001
Fax: +31.317.418094

Scientific team

Dr. Ir. A. (Lonneke) H.M. van der Geest - project-leader Plant RI tasks.

Dr. Ir. Mark G.M. Aarts - scientist, has left Plant Research International. His tasks were taken over by Dr. van der Geest.

Dr. J. (Hans) M. Sandbrink - Database development support.

Ing. Maurice C.J.M. Konings - technician experienced in cDNA micro array technology.

Contractual links to other participants

Link with ATO (partner 1), another Research Institute member of the foundation DLO.

Objectives

Plant Research International is experienced in the molecular handling of woody species and will focus on isolation and characterisation of relevant genes and expressional analysis. Plant RI will provide the cDNA chip technology to the project and its expertise related to bioinformatics and database managing. A cDNA microarray facility is operational within Plant Research International and will be used in the frame of this project to produce the DNA chip. Large numbers of identical chips can be produced and screened with different probes, allowing in depth expression analysis of the genes on the chip

In close co-operation with ATO, PRI will be involved in the selection of relevant pine genes and the isolation of beech homologues. The institute will participate in the assembly of detailed expressional profiles of the selected genes and will be responsible for managing the molecular database. PRI Bio-informatics department will merge this database with the physiological data into an integrated searchable database.

Workplan

PRI will assist the co-ordinator in organising the molecular handling workshop during the kick-off meeting. In the first year the institute will assemble a pine microarray, using cDNA clones provided by ATO.

The microarray will be screened in the first and second project year using RNA derived from experiments in controlled conditions (**WP5**). Data analysis will be performed by PRI and used for the selection of relevant pine genes. In co-operation with ATO, PRI will search for beech homologues to these genes using PCR based approaches (**WP6**) and develop RT-PCR assays for detailed expression analysis (**WP7**). This work will extend into the third and fourth project year. In the final project year Plant RI will be responsible for constituting the molecular database using data from all partners. The institutes bio-informatics department will construct an integrated and searchable database by combining the molecular and physiological information resulting from the project. The integrated database will be used for the conception of a working hypothesis on the molecular mechanisms underlying dormancy and hardiness development in woody plants (**WP10**).

Deliverables

The contribution of PRI to the project deliverables is specified in the scheme below

Deliverable	WPs	Confidential nature	Delivery month											
			12	20	24	30	36	38	41	43	46	48		
DL7 - cDNA microarray	5	CO	✓											
DL8 - Expression profiles	5	CO		✓										
DL9 - Set of 30 pine genes	5	CO												
DL10 - Primers for PCR on pine genes	5	CO		✓										
DL11 - Set of 30 beech genes	6	CO												
DL12 - Primers for PCR on beech genes	6	CO												
DL13 - RT-PCR protocols	7	CO												
DL14 - In depth expressional information	7	CO												
DL15 - A subset of 15 key genes,	7	CO												
DL20 - A molecular database	10	CO												
DL21 - An integrated searchable database	10	CO												
DL22 - Conceptual model describing molecular events underlying winter dormancy and frost hardiness.	10	CO												
DL23 - Trade demonstration workshop	8,9	PU												
DL24 - Scientific publications	All	PU												
DL25 - Publications in trade journals	All	PU												
DL26 - Annual reports	All	PU	X		X									
DL27 - Final report	All	PU												

Grey field: date when deliverable is due according to the technical annex. ✓ = delivered

Research activities during the first year

Characterisation of cDNA libraries provided by ATO

ATO produced 3 different cDNA libraries, one with random genes expressed in dormant pine buds (FULL), one enriched for genes that were preferentially expressed in dormant pine buds (compared to cold shocked or non-dormant pine buds: DOR) and one library enriched for genes that were preferentially expressed in cold treated pine buds (compared to dormant or non-dormant pine buds: COLD). Bacterial colonies from these cDNA libraries were provided to us on agar plates. Colony PCR with universal primers (M13 forward and reverse) was performed, in order to generate a PCR product containing the cDNA insert. This allowed testing for gene (cDNA) insert presence and size. Approximately 3000 individual cDNA clones from these libraries were checked by PCR for insert size, and approximately 2000 clones of sufficient length were selected for further analysis. These clones were subjected to DNA sequence analysis, and the resulting sequence data were used to compare the sequences to GenBank in order to predict gene function. In addition, a contig analysis was performed to align the different sequences to each other and determine the number of unique clones and the number of genes that was found multiple times. Contig analysis revealed a total of 1080 contigs, representing different DNA sequences. Some sequences were found very frequently in the different libraries, resulting in relatively large contigs for the following gene families: A large number of dehydrin genes were found, that were divided into 5 different contigs, containing between 12 and 27 genes. Three different contigs (containing 14-17 sequences) were found encoding pathogenesis related proteins, and one contig of 13 genes represented a stress/ABA related protein gene. The fact that these genes occurred frequently in these libraries indicates that their expression level in the tissues from which the libraries were made was relatively high, suggesting that they may be involved in such processes. Indeed, for dehydrins reports exists for a role in protection against drought stress, and possible links with cold stress. This gene family had also been identified in a previous project, and might be a good marker for stress tolerance. Most other genes were found only once in the sequence set, and are represented by the 961 contigs containing only 1 or 2 genes.

Some genes of intermediate abundance are represented by 52 contigs containing three sequences, 44 contigs containing 4 or 5 sequences, and 14 contigs containing 6 to 10 sequences (see table 1).

Nr of contigs	Genes per contig	Types of genes
961	1-2	Mainly housekeeping genes, some stress related genes and ESTs
52	3	Mainly housekeeping genes, some stress related genes, unknown genes and ESTs
44	4-5	Stress related genes, seed maturation related genes, translation genes, pine ESTs, housekeeping genes
14	6-10	Common pine ESTs, putative proteins, dehydrins, etc
5	11-15	Dehydrins, pathogenesis related proteins, ABA/stress related proteins
4	>15	Dehydrins, pathogenesis related proteins

Table 1: Distribution of the gene sequences over the contigs.

Production of cDNA microarray

The sequence information from blast and contig analysis was used to make a selection of genes to be spotted. From the 2000 clones 1451 genes were selected for spotting on the array, ensuring the maximum number of different genes, or different fragments from the same gene. Additional genes were added to a total of 153 genes. These included fragments of genes that were differentially present in a PCR assay performed during the previous project (amplified fragment length polymorphisms: AFLP fragments), and some genes that were described in the plant literature to be associated with stress or dormancy. ATO provided the 31 characterized AFLP and PCR fragments ready for spotting on the array. Additionally a number of control genes were spotted on the array to allow data normalisation. These included 4 different yeast genes that are known not to be present in most plants, to allow correction for background hybridisation, and different sized fragments of the firefly luciferase gene. The luciferase gene serves as a positive control during hybridisation experiments, since a known amount of luciferase mRNA is added during the labelling of the RNA to be tested. Based on the level of luciferase fluorescence, the labelling efficiency of the different RNA samples can be calculated, and data can be normalized. Table 2 shows the composition of the cDNA microarray.

Clone origin	Nr. clones
FULL cDNA library	729
DOR cDNA library	362
COLD cDNA library	360
AFLP fragments (ATO)	20
Other genes (ATO)	11
Controls (positive and negative)	54

Table 2: Origin of cDNA clones that were spotted on the cDNA microarray.

In order to increase the reliability of the produced data, all clones were spotted onto the microarray in duplo, on different regions of the microarray.

Initial hybridisation experiments

In order to assess the usefulness of the cDNA microarray for detection of differential gene expression during dormancy, cold stress and active growth, RNA from samples treated at Applied Plant Research in Boskoop was used for labelled cDNA production. Five different samples (3 that

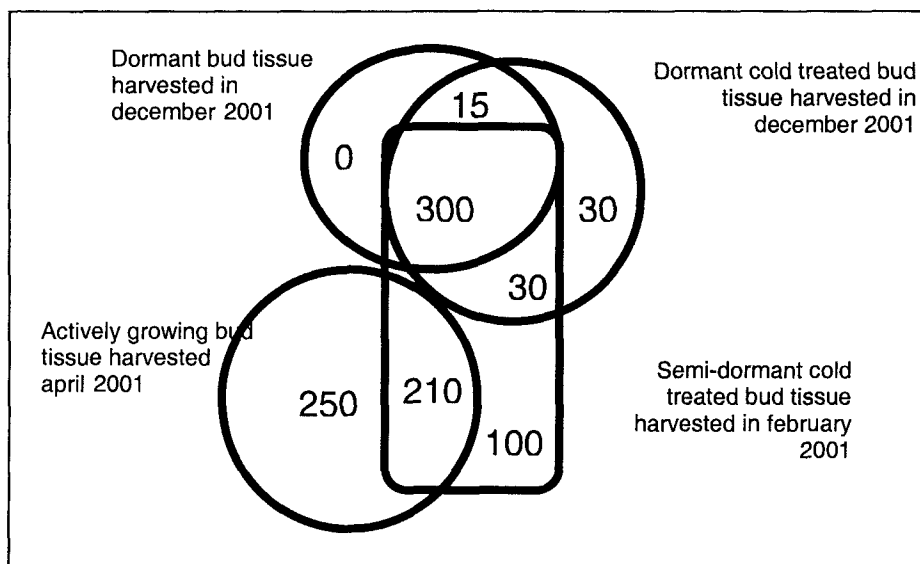


Figure 1. Gene expression during 4 different conditions was measured relative to a mixture of these conditions. Groups of genes that show expression levels that are higher than the mixture are indicated in this Venn diagram. Circles represent samples, numbers in the circles indicate the number of genes that are upregulated in the various samples. Overlapping regions indicate genes that are upregulated in more than one sample.

had received some kind of cold stress, 1 that was fully dormant and one that was harvested in late spring) were compared to the same reference sample. The samples that were used represent the extremes from the physiological spectrum that will be analysed in the COLDTREE program, and should therefore generate clearly distinguishable gene expression patterns. Analysis of the hybridisation results revealed dramatic differences in gene expression indeed. Different groups of genes could be identified, some associated with cold stress, some associated with dormancy, and some specific to actively growing buds (after dormancy breaking). Using the samples from the growth room and field experiments with pine we will be able to determine which of these genes is truly predictive of dormancy and/or cold tolerance, and can be further developed as a marker. Fig 1 shows a diagram of the different types of gene classes based on their expression in the cold treated, dormant or non-dormant samples.

Significant difficulties or delays experienced during the first year

None

Sub-contracted work during the first year

None

Research activities during the second year

Scientific team year 2

Dr. Ir. A. (Lonneke) H.M. van der Geest - project-leader Plant RI tasks.

Ing. Maurice C.J.M. Konings - technician experienced in cDNA micro array technology

Ing. Michiel Lammers, technician experienced in cDNA micro array technology

Ing. Paul Dijkhuis, technician experienced in plant handling and physiology

The second year was devoted to the analysis of gene expression in the physiologically characterised pine bud samples from the 2001/2002 season of partners 4 (DIAS) and 7 (FCFR). The original plan was to start with the climate room experiments, but since the physiological response of the climate room material was disappointing (see report year 1), it was decided to use the samples from the field trials. The partners performing the physiological experiments isolated RNA from 25 buds. The RNA samples were shipped frozen to PRI and the RNA concentration and purity was measured by PRI. Table 3 shows the total amount of RNA in each sample; a minimum of 40 ug is needed for each hybridisation. The boldfaced samples were selected for microarray hybridisations.

Partner	4	4	7	7
Provenance	A70	Lindas	A70	N410
Week 39 (2001)	45	203	61	78
Week 41 (2001)	53	70	63	124
Week 43 (2001)	90	50	20	NA
Week 45 (2001)	29	39	82	NA
Week 47 (2001)	27	46	81	NA
Week 49 (2001)	64	69	NA	146
Week 51 (2001)	50	82	130	176
Week 02 (2002)	110	66	126	83
Week 04 (2002)	37	126	10	NA

Table 3: Total RNA amounts in micrograms of the samples received from the partners (extracted from 25 buds). Concentrations in bold type represent samples that were used for hybridisations. NA=not available.

In order to be able to compare expression of all 1500 genes in the different samples, hybridisations were performed using two different fluorescent labels (Cy5 and Cy3). The samples from the partners were labelled with Cy5, and mixed with a reference sample isolated from Dutch and Danish buds by PRI (1800 ug RNA was prepared to provide sufficient reference) that was labelled with Cy3. Based on the hybridisation profiles of the different samples, genes on the microarray could be divided into distinct classes. A group of genes whose RNA levels declined over time was designated “active genes”. This group contained approximately 100 genes, including ribosomal subunits (involved in protein synthesis), ubiquitin related genes (involved in protein degradation), tubulin (involved in spindle formation during cell division), metabolic enzymes, etc. Expression of these genes drops between week 41 and week 45, when cold tolerance and dormancy are on the increase. Since measurements of gene expression only start in week 39 (late fall), expression of these genes is expected to be much higher in late spring and summer, as they are representative of actively growing and dividing cells. A second group of genes (represented by 113 spots on the microarray) increase in mRNA levels as the season progresses. Expression of these genes rises sharply between week 41 and 45; thus these genes may be correlated to cold tolerance or dormancy. This category, named “early cold tolerance genes” contains a number of stress related genes, such as dehydrins, ABA and stress induced proteins, pathogenesis related genes, etc. Additionally this category contains a number of genes that lack homology to any known genes, transcription factor genes, etc. A third category of genes, the “late cold tolerance genes”, represented by about 50 spots on the microarray, show a more gradual increase in gene expression during the early weeks, and reach their maximum levels between week 49 and 51. This class contains genes like embryogenesis abundant protein (involved in desiccation tolerance during late seed development), and many hypothetical or unknown proteins. Interestingly, also a histone H2A subunit belongs to this class of genes. Normally histones are highly expressed in actively dividing cells, since they are needed to package the newly replicated DNA into nucleosomes. This particular gene might represent an isoform that allows a better packaging of the DNA, giving the DNA to achieve a more stable, stress tolerant conformation. Figure 2 shows the average expression profile of these groups of genes in the various samples. The expression is given as a two-log ratio, relative to a common sample that was used as a reference for all hybridisations. This way the samples can be accurately compared. A change of the cold tolerance genes from -1 to $+2.5$ over the season therefore represents a more than 10-fold difference in expression of the average of 100 genes!

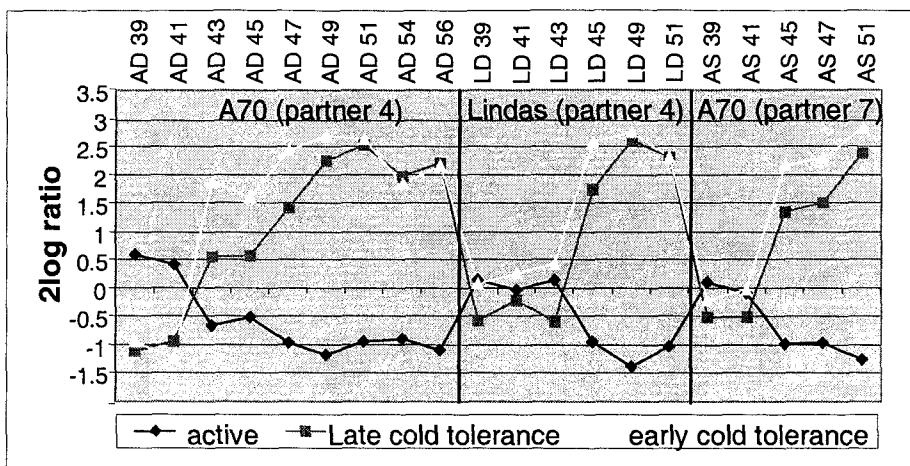


Figure 2. Average expression profile of the selected genes from each category. Relative expression (2-log ratio) is given to a common reference sample. The lines represent 108 genes (“active”), 113 genes (“early cold tolerance”) and 51 genes (“late cold tolerance”).

It can be seen in figure 2 that in Lindas (the harder provenance) the level of mRNA of the cold tolerance related genes is higher than that in A70 grown in the same location. Also, A70 grown in Scotland (Partner 7), in its native environment seems to have higher levels of mRNA compared to A70 grown in Denmark (partner 4). On the other hand, early cold tolerance genes go up in A70 earlier than in Lindas in Scotland, even though Lindas is physiologically faster in becoming cold tolerant. It will be interesting to look at the results from A70 grown in Sweden next season. Comparing the sequences from these 273 spots on the microarray revealed that several genes were represented by a number of individual spots (cDNAs), so that the 273 spots represented 189 unique genes.

For example, one of the dehydrin genes was represented by 15 spots, all of which gave the same expression ratios in a number of samples from A70 from partner 4 (figure 3). This confirms the reliability of the gene expression data obtained using individual spots.

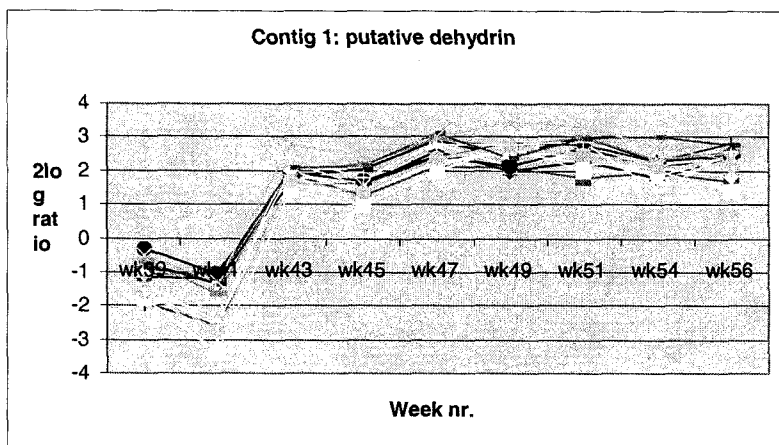


Figure 3. Expression ratios of 15 individual dehydrin spots on the microarray (representing replicate measurements of the same dehydrin gene).

From these 273 cDNAs, 36 were selected for further study, based on their expression over time, absolute expression level, and gene homology. Representative genes were chosen from each class. These genes are good examples of the general pattern changes in gene expression as buds become quiescent and cold tolerant. The true challenge lies in the precise matching of expression patterns of individual genes to the physiological changes in the different provenances at the different locations.

For example, comparing A70 and Lindas grown in Denmark, physiologically Lindas is quicker to respond to the fall climate by showing a drop in SEL –25 between week 39 and 41, while A70 shows this drop between week 41 and 43. For SEL –15, both provenances respond about the same. Genes that match this exact pattern (bigger change in expression for Lindas between 39 and 41 than for A70) have not yet been found. This may be due to the fact that only one sample (REL –25 for Lindas in week 41) marks the difference in response between the provenances. It will be necessary to analyse samples representing this shift from all three locations from the 2002 season in order to identify additional genes that can be used as a reliable marker for this subtle difference. The 36 genes now selected should prove reliable markers for the physiological state during the season since their expression changes quite reproducibly for different locations and provenances.

One hybridisation of the pine microarray has also been performed using beech RNA. An early sample (week 39) was compared to a late sample (week 51) to assess whether 1) there is sufficient homology in gene sequence between beech and pine (this can be deduced from the absolute fluorescence levels of the spots when hybridising with labelled beech cDNA) and 2) whether it is possible to find differences in gene expression between week 39 and 51 that match the differences seen in pine. The results from this hybridisation confirmed that the differences in gene sequence between pine and beech are too large to allow use of the pine array for beech samples. More than half the genes did not hybridise at all, and for those that did give a hybridisation signal, no correlation between gene expression and dormancy level could be demonstrated. In order to clone beech homologues there are two options currently open: 1) Use the pine data and the selected pine genes, and check whether these gave a hybridisation signal on the array using beech samples (this will indicate roughly how easy/difficult cloning the beech homologue will be). Then for each gene try to clone the beech homologue. 2) Make a small beech array to directly identify relevant beech genes and work in parallel with pine. Currently the second option is preferred, especially given the physiological differences observed related to dormancy in beech and pine. This may indicate that some different genes play a role in beech than in pine. ATO will prepare an array with approximately 500 beech genes. It is expected that this will yield sufficient marker genes (300 from the 1500 on the pine array proved to be useful as a potential marker).

Significant difficulties or delays experienced during the second year

A set of 30 pine genes is available and ATO has started some PCR experiments with primers based on these gene sequences. However, we have decided that one season of samples was insufficient to identify those genes that reflect subtle differences between locations and provenances, and that instead of starting with RT-PCR it will be more useful to perform additional microarray hybridisations. This will delay the PCR work by one year, but will improve the reliability of the markers we find. This also results in a delay for the beech work, as a different approach will be taken to find relevant beech genes.

Sub-contracted work during the second year

None

3.3 Partner 3 – Contractor: DU

Dalarna University,
Herrgårdsvägen 122,
776 98 Garpenberg,
Sweden.
Tel: +46.225.26000
Fax: +46.225.26100

Scientific team

Dr E Stattin – project leader
Assoc Prof A Lindström – Head of the department.
L Håkansson – Technical engineer
M Vemhäll – Technical engineer
C Hellqvist – Technical engineer

Objectives

The research at the Dalarna University Department of Forest Industries comprise disciplines such as stress and post-harvest physiology, quality and vitality testing, development of planting and nursery techniques. This research is conducted in close contact with Swedish nursery companies and the major forest companies in Sweden. This comprehensive extension network makes the department well suited for the technology transfer that constitutes one of the main objectives of this proposal.

In the project DU, in co-operation with PPO, will be charged with the first steps towards implementation of the results in forestry practice. A putative limiting factor for the general application of marker genes is the provenance effect. This climate-induced intraspecific physiological variation is one of the hindrances in international trade. Therefore DU and PPO will evaluate the expression of the key-genes in a series of provenances derived from geographically distinct commercial nurseries and from controlled field trials, in which Dalarna University will focus on pine. These experiments will aid in the selection of key genes that are of general importance in large parts of Europe.

In addition to these questions, Dalarna University will pay special interest to the practical problems experienced in nursery operations. Timing of storage and storage conditions are essential for successful overwintering of such seedlings. Therefore Dalarna University will study the correlation of key-gene expression with performance during cold storage of pine seedlings. For the economically important Swedish and UK nurseries this is of vital interest and it will provide the proposers with an application example, that will encourage interest from commercial partners to participate in future implementation projects.

Workplan

In the first project year Dalarna University started a series of experiments aimed at the evaluation of the effect of provenance, age and climatic conditions on pine gene expression (WP8). Seeds of 3 geographic pine provenances will be sown. One of the provenances will be identical to the standard provenance used in WP1 and WP3. The storage trial in the second project year (season 2002-2003) will be focused on cool (above 0°C) storage and the results will be compared to the frozen storage. Also, in this year a second sowing will be done for evaluating provenance and age. In addition physiological parameters will be measured on the 0.5 and 1.5 year old seedlings of the 3 provenances. In the next year, season 2003/2004, this experiment will be repeated with seedlings from all three sowing seasons (0.5, 1.5 and 2.5 years old). Also, in 2003 samples of pine seedlings at the onset of hardening to completely hardened will be taken from 3 commercial nurseries.

In all these experiments tissue samples will be taken and stored at -80°C for mRNA screening by ATO. Physiological data will be send to FC(FR) for integration into the physiological database.

Deliverables

The contribution of DU to the project deliverables is specified in the scheme below

Deliverable	WPs	Confidential nature	Delivery month											
			12	20	24	30	36	38	41	43	46	48		
DL16 – Information on the influence of provenance, climatic conditions and age on key gene expression	8	CO												
DL17 – Assessment of key genes as predictors for cold storage performance.	9	CO												
DL18 – Assessment of key genes as molecular diagnostic tool	7,8,9	CO												
DL19 - A physiological database	10	CO												
DL22 – Conceptual model describing molecular events underlying winter dormancy and frost hardiness.	10	CO												
DL23 – Trade demonstration workshop	8,9	PU												
DL24 – Scientific publications	All	PU												
DL25 – Publications in trade journals	All	PU												
DL26 – Annual reports	All	PU			✓									
DL27 - Final report	All	PU												

Grey field: date when deliverable is due according to the technical annex. ✓ = delivered

Research activities during the first year

Apart from growing plant material for future experiments, Partner 3 has not yet started active research.

Significant difficulties or delays experienced during the first year

None

Sub-contracted work during the first year

None

Research activities during the second year

Scientific team in the second year

Dr E Stattin – project leader

L Håkansson – Technical engineer

M Vemhäll – Technical engineer

At Garpenbergs Research Station in Sweden 1 and 2-year-old containerised *Pinus sylvestris* (Scots pine) seedlings of three provenances (standard provenance “A 70”, local provenance “Sollerö” and northern provenance “Åmsele”) have been raised according to standard nursery protocol.

Every second week starting in week 36 and until week 48 (2002) all experimental plant types were tested for shoot and root freezing tolerance ($SEL_{diff-25^{\circ}C}$ and $REL_{diff-5^{\circ}C}$) and rest/dormancy (bud break test) and material for mRNA analysis was collected from terminal buds (WP8). Seedlings were freeze-stored ($-5^{\circ}C$) in week 40 and 48 and in week 6 (2003) material for mRNA analysis was collected and the shoot and root freezing tolerance was determined (WP 9).

Shoot (Figure 1) and root (Figure 2) freezing tolerance increased in all seedling types from early September to early November. In November there were no major changes in the freezing tolerance of the seedlings. There was a clear provenance effect on both shoot and root freezing tolerance. The seedlings of northern provenance became freezing tolerant at an earlier time and their shoots did also achieve a somewhat higher degree of freezing tolerance. There was also a clear age effect on root freezing tolerance. The roots of the 2-year-old seedlings became freezing tolerant earlier than the roots of 1-year-old seedlings. On the other hand there was a tendency that the shoots of the 1-year-old seedlings hardened earlier than the shoots of the 2-year-old seedlings.

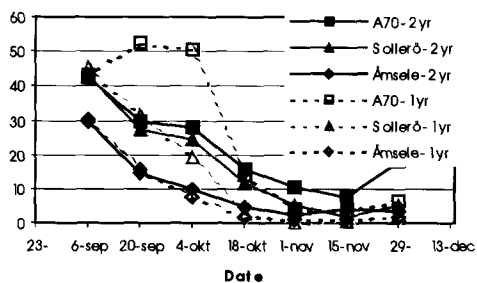


Figure 1. Shoot freezing tolerance, measured as $SEL_{diff-25}$, in 1- and 2-year-old Scots pine seedlings of three different provenances. N = 15

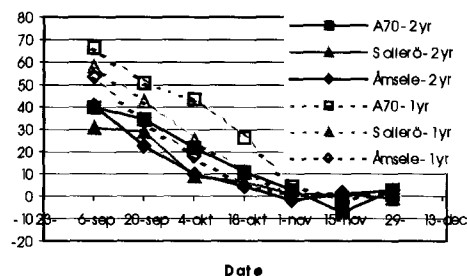


Figure 2. Root freezing tolerance, measured as REL_{diff-5} , in 1- and 2-years-old Scots pine seedlings of three different provenances. N = 5

The freezing tolerance needed for long term frozen storage ($SEL_{diff-25} \leq 5$ and $REL_{diff-25} \leq 7$) was, with one exception, reached for all experimental plant types (Fig 1 and 2). Åmsele seedlings were according to this standard ready for frozen storage in week 42 and Sollerö seedlings in week 43. The A70 seedlings never reached the limit set for shoots while the limit for roots was reached in week 44. In the end of November, three of the experimental plant types (2-year-old Sollerö/shoots, A70/roots and 1-year-old A70/shoots) lost freezing tolerance to the extent that they did not have recommended freezing tolerance when they were stored in week 48. None of the seedlings were storable according to these recommendations in week 40. Storage will be evaluated in April 2003.

All 2-year-old seedlings had set bud when the seedlings were taken from outdoor conditions into the favourable growing conditions in the greenhouse for the bud break test. For many of the 1-year-old seedlings there was no bud visible when the bud break test started (Figure 3). Hardly any of the 1-year-old A70 seedlings did even have a visible bud in late November.

Days to budburst (DBB) decreased from early September to late November for all seedling types (Figure 4). In September there was a clear provenance effect on days to budburst where seedlings from northern provenances displayed a deeper rest or “dormancy” than seedlings of southern origin. The rest in the seedlings, especially the 2-year-old, of northern origin was so deep in early September that most of them did not break bud within 90 days (Table 1). (Even today -April 2003 – after 7 months most of these seedlings have not broken their buds). There was also clear age effect on DBB in September, which revealed that the rest in 2-year-old seedlings was deeper than in 1-year-old seedlings.

Table 1. Number of 1 and 2-year-old seedlings of three different provenances that died or did not burst in 90-day long budburst tests. 15 seedlings of each provenance and age were planted at seven occasions from early September to the end of November 2001.

Plant ed in week	A70 1-year		A70 2-year		Sollerö 1-year		Sollerö 2-year		Åmsele 1-year		Åmsele 2-year	
	No burst	Dead	No burst	Dead	No burst	Dead	No burst	Dead	No burst	Dead	No burst	Dead
36	2		2		4		2		4		13	
38	1				1		11		7		14	
40			1		3		11		1		11	
42												
44				2								
46				2								
48	1			3								

A plot of the shoot freezing tolerance (hardiness) versus days to budburst (rest/dormancy) reveals that release of dormancy precedes achievement of frost hardiness (Figure 5.). This is clearly shown for the northern provenances (Åmsele $r^2 = 0,9357$; Sollerön $r^2 = 0,9122$). This is not shown for the southern provenances, which can be due to the fact that they, in these tests, have not displayed a deep rest. An mRNA analysis of the buds from the 2-year-old seedlings of the northern provenances could perhaps make it possible to discriminate between the genes responsible for rest/dormancy and those responsible for hardening.

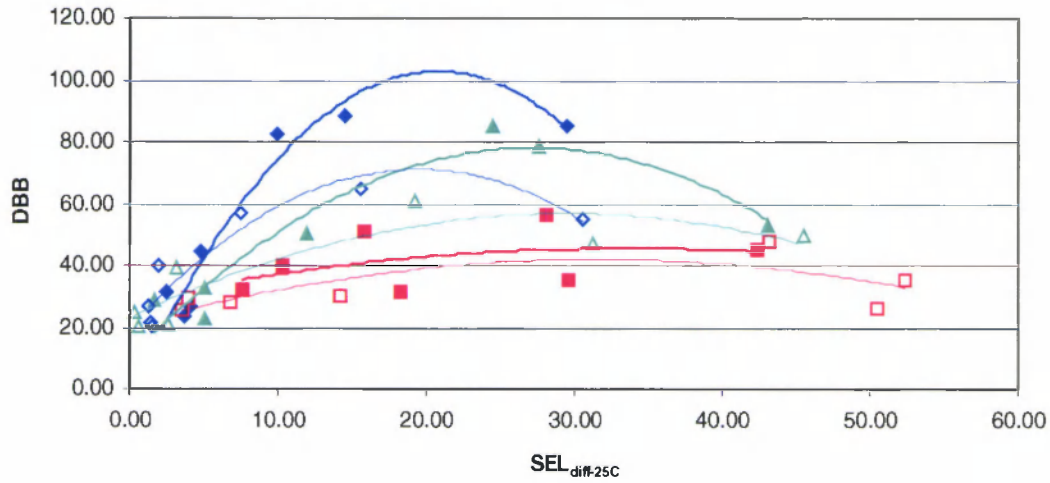


Figure 5. Shoot freezing tolerance measured as SELdiff-25oC versus days to budburst (DBB).

Significant difficulties or delays experienced during the first year

None

Sub-contracted work during the first year

None

3.4 Partner 4 - Contractor: DIAS

Danish Institute of Agricultural Sciences,
Kirstinebjergvej 10,
DK-5792 Aarslev,
Denmark.
Tel: +45.63.904343
Fax: +45.63.904393

Scientific team

Scientist Peter Brønnum, project leader, plant physiology
Senior scientist Per Hove Andreassen, molecular biology*
Senior scientist Poul Erik Brander, head of research unit
*Laboratory technician Annette S. Larsen**
*Laboratory technician Nina Eggers**
*Research technician Connie Damgaard**
Nursery gardener Erling Hyldig
*Nursery gardener Alis Rasmussen**
*Nursery gardener Elin Rosenstrøm**
* not mentioned in the technical annex

Objectives

For almost sixty years DIAS has conducted applied research in nursery production of plants for forestry and the open landscape and has very close relations to the nursery business. The department is experienced in research in disciplines such as stress- and post-harvest physiology, quality and vitality testing, vegetative propagation and –development and seed physiology. Plant breeding programs employ tissue physiology, gene technology and micropropagation. DIAS will study the effects of different climate regimes on tree seedling physiology and corresponding molecular markers during the period of winterhardening (autumn). Besides the tasks of preparing and providing plant material for the identification of molecular markers by partner 1 and 2, DIAS will perform a number of physiological tests (e.g. frost hardiness, root growth potential, root electrolyte leakage) of the climate treated plant material as well as field performance trials that will work as references to the identified markers.

DIAS will investigate the effect of various environmental conditions on the physiology of *F. sylvatica* seedlings and will produce plant material, which is well defined in terms of dormancy and hardiness status. The first two seasons will be used for experiments in controlled environments. This will be done in close contact with FC(FR), who will perform the same type experiments, but using *P. sylvestris*. The material derived from these trials will be used to make the initial selection of relevant genes. Next to the climate room trials, field trials will be performed using both pine and beech. The information on seedling response to the two prime environmental factors of daylength and temperature, particularly fluctuating temperature, will be of immense practical value and will be incorporated into advice to the forestry industry. Plant material derived from these trials will be used for detailed expression analysis of the selected genes aimed at the identification of a subset of key genes.

DIAS will co-operate closely with two assistant contractors: Hedeselskabets Planteskole and PPO.

Workplan

In the first year both climate room experiments (WP2) and field experiments (WP3, WP4) took a start, but the main focus was on the controlled environment trials. Seedlings were followed through the process of hardening and at various time points physiological analyses were performed and samples for RNA analysis were taken. In this first trial season the aim was to separate the processes of dormancy and frost hardiness development as far as possible. Both field and climate room trials will proceed into the next project year. In the second year climate room experiments will be devoted to the interaction of temperature and daylength. Field trials will be designed to assess the effect of climatic conditions, geographical position and provenances on the development of dormancy and hardiness. In the third year field trials will proceed. By that time RT-PCR assays for several key genes will be available.

DIAS will use these assays in co-operation with HSP, to obtain detailed expression information using material from the field trials (WP7). This work will continue into the fourth year and the results will be used for the organisation of a demonstration workshop aimed at the implementation of the RT-PCR assays in practice. All physiological data will be sent to FC(FR) for the physiological database. The gene-expression data will be forwarded to Plant RI for building the molecular database. (WP10)

Deliverables

The contribution of DIAS to the project deliverables is specified in the scheme below

Deliverable	WPs	Confidential nature	Delivery month													
			12	20	24	30	36	38	41	43	46	48				
DL1 - RNA for preparing microarray probe	1,2	CO	✓		✓											
DL3 - Physiological data on dormancy in beech in controlled environments	2	CO	✓		✓											
DL4 - RNA for RT-PCR	1,2,3,4	CO	✓		✓											
DL5 - Physiological data on dormancy in pine in outdoor situations	3	CO	✓		✓											
DL6 - Physiological data on dormancy in beech in outdoor situations	4	CO	✓		✓											
DL14 - In depth expressional information	7	CO														
DL15 - A subset of 15 key genes,	7	CO														
DL18 - Assessment of key genes as molecular diagnostic tool	7,8,9	CO														
DL19 - A physiological database	10	CO														
DL20 - A molecular database	10	CO														
DL22 - Conceptual model describing molecular events underlying winter dormancy and frost hardiness.	10	CO														
DL23 - Trade demonstration workshop	8,9	PU														
DL24 - Scientific publications	All	PU														
DL25 - Publications in trade journals	All	PU														
DL26 - Annual reports	All	PU	✓		✓											
DL27 - Final report	All	PU														

Grey field: date when deliverable is due according to the technical annex. ✓ = delivered

Research activities during the first reporting period

Research related to WP2

Objectives

- To assess seedling response to the two prime environmental factors of daylength and temperature, particularly fluctuating temperature
- To produce, by manipulating daylength and temperature in an otherwise constant environment, beech seedlings at various stages of dormancy and frost hardiness. The plant material will be subjected to thorough physiological analyses and will be used for preparing microarray probe and RT-PCR template.

Work description

Production of plant material:

In week no. 19 pre-germinated seeds of the standard beech provenance 'Bregentved' (origin Denmark) was sown in HIKO 265 trays placed outside. During summer approximately 3500 seedlings were raised in accordance with normal nursery practice. In week no. 33 approximately 3000 of these seedlings were transferred to three climate rooms, where they were subjected to three different climate regimes until week no 2 in 2002:

Climate 1: day length (DL) =15h, temperatures $T_{\text{day}} = 15^{\circ}\text{C}$ and $T_{\text{night}} = 13^{\circ}\text{C}$

Climate 2: as climate 1, but DL reduced by 1h every two weeks until 8.5 h

Climate 3: as climate 1, but T_{day} and T_{night} reduced by 2°C every two weeks until $+1^{\circ}\text{C}/+1^{\circ}\text{C}$.

Sampling and testing of plant material:

Seedling samples from each climate regime were collected in week no. 37, 39, 41, 43, 45, 47, 49, 51 in 2001 and in week no. 2 in 2002. In each sampling week seedlings were tested for shoot and root frost tolerance and dormancy status.

A. Each sampling week, 2-cm shoot tip sections of 15 beech seedlings per climate were excised and frozen to -15°C and -25°C and 15 fine roots samples from the same seedlings were frozen to -5°C . 15 shoot and root control samples were held at $+4^{\circ}\text{C}$. Shoot and root frost injury were evaluated using the conductivity method described in the common protocol, and denominated SEL and REL, respectively*. Shoot frost tolerance was quantified as the difference in SEL(-25C) and control SEL($+4\text{C}$), and hence denominated $\text{sel}_{\text{diff}-25}$. Similarly, root frost tolerance was calculated as $\text{rel}_{\text{diff}-5}$.

B. Each sampling week 15 beech seedlings per climate were transferred to a greenhouse with favourable growth conditions (DL= 18h, T= 20°C) for testing of bud dormancy status. The number of days to terminal bud break (DDB) of each seedling was recorded and dormancy status was calculated as the average DBB for each sampling week.

C. Each sampling week 50 terminal buds and 50 fine roots were collected and immediately frozen in liquid nitrogen for isolation of RNA. The method of isolation is described in the common protocol.

*Due to too high conductivity of the deionised water used for electrolyte leakage measurements in week no. 37, conductivity results from this week should not be considered reliable. The week 37 data is, however, presented in all figures.

Research related to WP 3.**Objectives**

To assess, in field trials, the effect of climatic conditions, geographical position and provenances on the development of dormancy and hardiness.

To produce Scots pine (*Pinus sylvestris*) plant material for studying the expression of genes involved in these processes

Work description**Production of plant material:**

In week no. 20 the standard Scots pine provenance A70 (origin UK) and the provenance 'Lindås' (origin Norway) were sown in HIKO120ss trays placed in a greenhouse. Approximately 1500 seedlings of each provenance were produced in accordance with normal nursery practice for containerised stock. Seedlings were transferred from the greenhouse to an outdoor container cultivation area in week 32 where they remained during the entire sampling period.

Sampling and testing of plant material:

Same procedures as described for WP2.

Research related to WP4**Objectives**

To assess, in field trials, the effect of climatic conditions, geographical position and provenances on the development of dormancy and hardiness.

To produce beech (*Fagus sylvatica*) plant material for studying the expression of genes involved in these processes

Work description**Production of plant material:**

The standard provenance 'Bregentved' (Czech origin, Danish seed source) and the local provenance 'Gråsten' (Danish origin) were sown in week no. 15 and cultivated in accordance with normal nursery practice for bare rooted stock by Hedeselskabets Planteskole (participant 6).

Sampling and testing of plant material:

The bare-rooted beech seedlings of both provenances were lifted in the nursery in week 37, 39, 41, 43, 45, 47, 49, 51 in 2001 and in week 2 and 4 in 2002. (in week 2 shoots only, because of frozen nursery soils). The lifted seedlings were subjected to the same tests, following the same procedures, as described for WP 2 and 3, except for REL and dormancy test in week 2.

In each lifting week 75 seedlings were cold stored at 4°C for field planting in April 2002 (storage temperature lowered to –1°C in December), in order to study the relationship between pre-storage physiological condition (frost tolerance, dormancy and gene expression) and storability (assessed as survival and growth).

Deliverables

Isolation of RNA (DL1)

WP1, WP2 and WP3. The isolated RNA from terminal buds and roots was undegraded as judged by agarose-gel-electrophoresis. The yield of RNA is sufficient for micro-array analysis ($\geq 40\mu\text{g}$) except for some preparations from root samples of beech grown in growth rooms. The specific RNA yields for WP 2, WP 3 and WP 4 are presented in figures 1-3 (please note that week no. 2 and 4 in 2002, for practical reasons, are numbered week 54 and 56 resp.)

Physiological parameters (DL2)

WP 2. $\text{sel}_{\text{diff}-15}$ and $\text{sel}_{\text{diff}-25}$ decreased during autumn for growth room beech seedlings in all three climate regimes (figure 4). The results show that shoot became frost tolerant to –15°C first, and this occurred in the order climate 3, climate 2 and climate 1. It is not likely that shoots became tolerant to –25°C, except perhaps for climate 3. The order in which beech of different climates developed frost tolerance appears to be the same as mentioned for –15°C. This was expected, except for the fact that seedlings in climate 1 (long day/high temperature) also attained some frost tolerance, which we can not explain yet. The precise relationship between sel_{diff} and frost tolerance or storability/field performance for beech still remains to be determined (1-year performance of bare root beech will be ready in the first months of 2003) and also the linkage between RNA and acclimation parameters ($\text{sel}_{\text{diffs}}$).

No development of root frost tolerance to –5°C was observed in beech (figure 5).

Beech seedlings from all three climate regimes entered almost equal levels of dormancy (number of days to terminal bud break under favourable growth conditions), although climate 3 seedlings, for a relatively short period, seemed to be more dormant (figure 6). Dormancy is normally induced by shorter day lengths and is independent of f.ex. Temperature and it was therefore surprising that dormancy was induced in both climate 1 and climate 3.

WP 3. Both provenances of containerised Scots pine seedlings grown outdoors during autumn developed shoot frost tolerance. Previous studies have shown that conifer shoots are frost tolerant at a given temperature when sel_{diff} is lower than 5% and storable when they are frost tolerant to –25°C (i.e. $\text{sel}_{\text{diff}-25} < 5\%$). Figure 7 shows that both provenances were frost tolerant to –15°C around week 43-44. The provenance 'Lindås' acclimatised faster and was frost tolerant to –25°C by week 47, approximately two weeks before the standard provenance 'A70'. Contrary to beech, Scots pine seedlings developed root frost tolerance at least to –5°C and starting around week 43 (figure 8). The same provenance difference was observed for root frost tolerance, but not as clear as for shoots (figure 5). Pine seedlings developed dormancy during autumn (figure 9) and again there seemed to be a difference between provenances, as 'Lindås' appeared to be more dormant than 'A70' during the period from week 43 to week 4.

WP 4. Like in pine, the bare rooted beech seedlings developed shoot frost tolerance (figure 10), although acclimation in beech occurred later in autumn than in pine. Assuming a sel_{diff} -threshold of 5% for frost tolerance of broadleaves as well, beech seedlings would be frost tolerant to –15°C by week 47, and frost tolerant to –25°C between week 51 and 2 (2002), i.e. In the second half of December. As with the growth room beech seedlings, no root acclimation was observed (figure 11). The bare rooted beech seedlings entered dormancy around week 41 and was released, or at least back to the initial level by week no. 4 (maybe even earlier, but due to difficulties with frozen nursery soils in week no. 2, it was not possible to lift seedlings for dormancy test in this week) (figure 12). There were no clear physiological differences between the standard provenance 'Bregentved' and the local provenance 'Gråsten' (figure 10, 11 and 12).

The overall shoot frost tolerance development in bare rooted beech seedlings grown outside was greater than in the growth room seedlings (figure 4 and figure 10). Dormancy in beech was considerably deeper than in pines, and more distinct in the bare-rooted than the growth room seedlings (figure 6 and 12).

Significant difficulties or delays experienced during the first reporting period

Control seedlings (climate 1) in WP2 still developed a moderate level of frost tolerance as described above. In WP2, climate 1 seedlings were consistently less frost tolerant at both -15°C and -25°C than climate 2 and climate 3 seedlings. The climate room material is therefore less suited for use in the primary selection of important genes. However, the field trials were very consistent also compared to the results of partner 7. Therefore the field-grown pine material will be used for initial selection. The climate room grown trees will be highly informative in filling in details of function and regulation for specific genes and processes.

We have good descriptions of the physiological development during autumn in all almost all experimental plant types, particularly the seedlings grown outside under ambient conditions. We will obtain, after the first growing season, information on the relationship between storability (cold storage tolerance = strain resistance) and the corresponding physiological condition and gene expression pattern at all lifting time points.

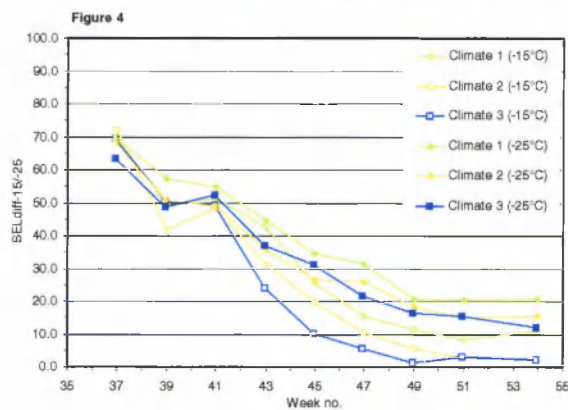
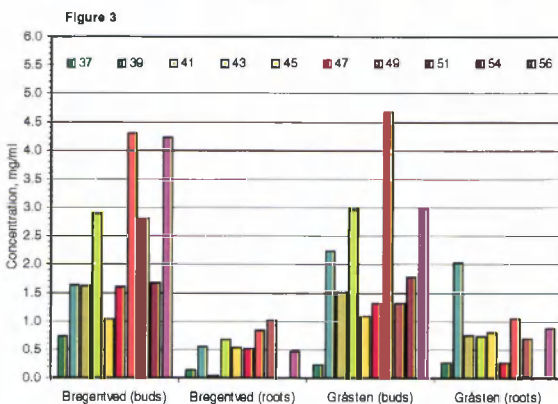
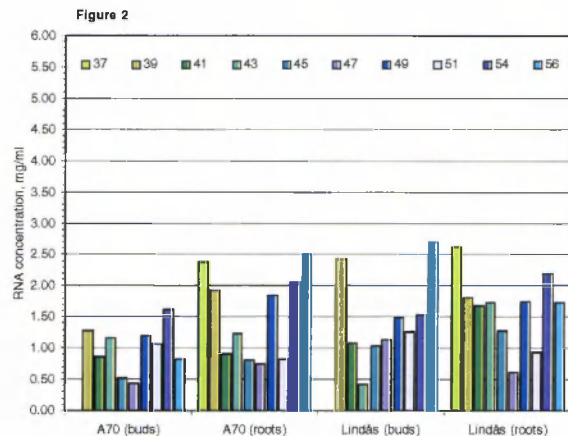
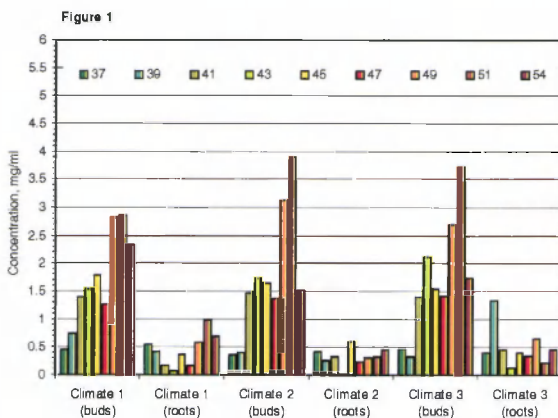
Due to the consistent absence of root acclimation in beech it seems pointless to continue the search of relevant genes in these, and consequently beech root frost testing and RNA-isolation will stop in the next period. More emphasis will be put into the study of effects of periodic cold spells during the acclimation process in growth rooms.

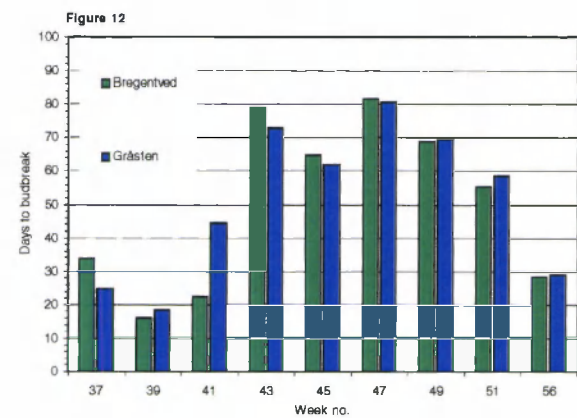
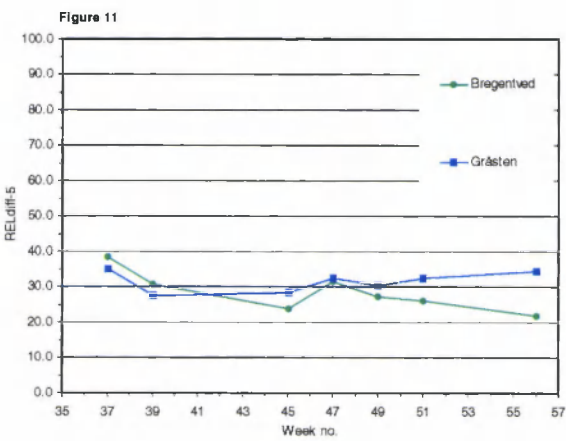
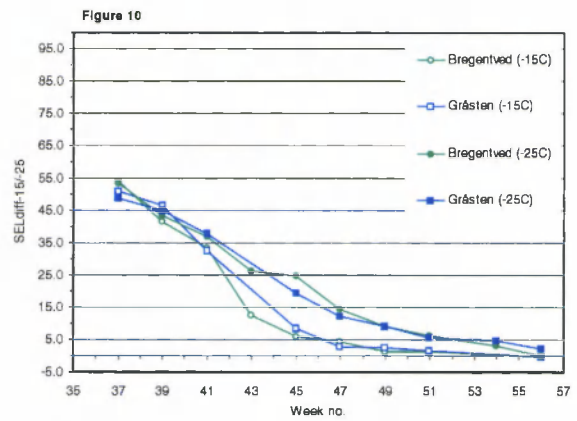
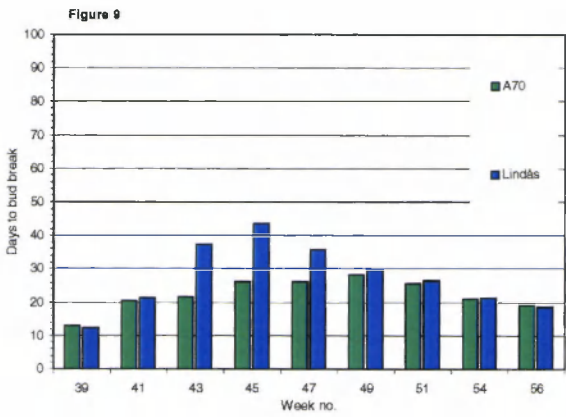
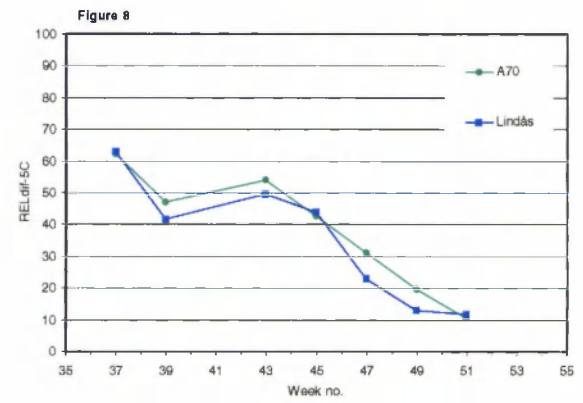
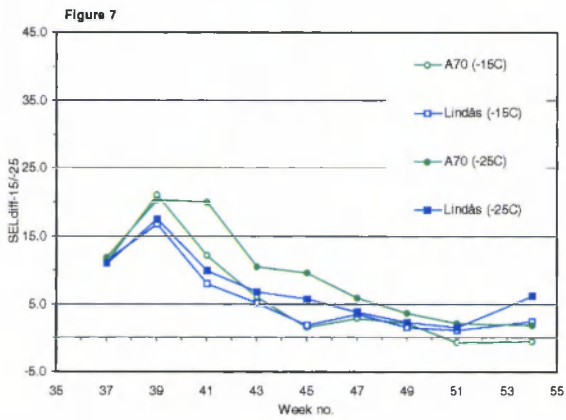
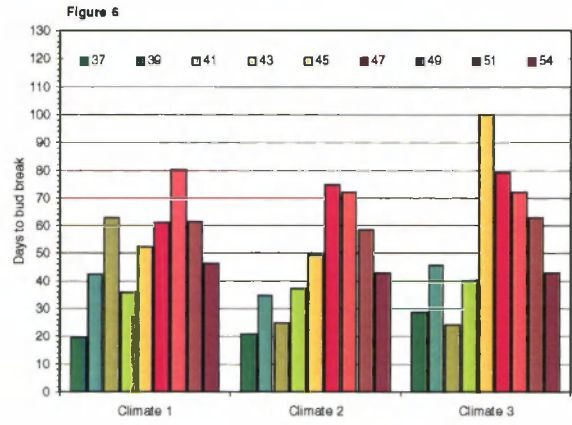
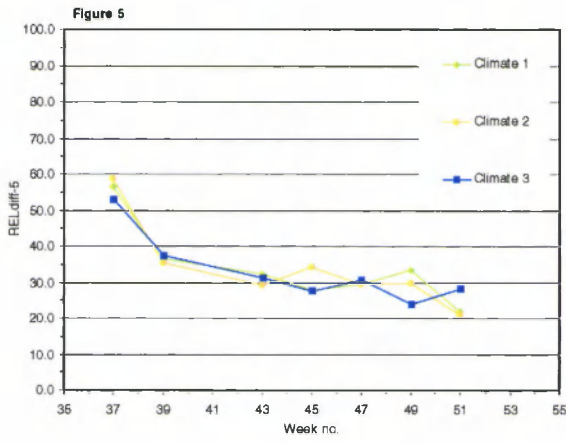
Furthermore, because of the lack of physiological differences between the two beech provenances, we will stop testing the local provenance 'Gråsten' and instead study differences between 1- and 2-year old seedlings using the standard provenance 'Bregentved' only.

Sub-contracted work during the first reporting period

None

Figures relating to the first reporting period





Research activities during the second reporting period*Scientific team in year 2**Scientist Peter Brønnum, project leader, plant physiology**Senior scientist Poul Erik Brander, head of research unit**Laboratory technician Annette S. Larsen**Laboratory technician Nina Eggers**Research technician Connie Damgaard**Nursery gardener Erling Hyldig**Nursery gardener Elin Rosenstrøm***Research related to WP2***Production of plant material:*

In week no. 19 pre-germinated seeds of the standard beech (*Fagus sylvatica* L.) provenance 'Bregentved' (Czech origin, Danish seed source) were sown in HIKO 265 trays placed outside. 2000 seedlings were raised in accordance with normal nursery practice. In week no. 33 these seedlings were transferred to climate rooms, where they were subjected to 4 different climate regimes until week no 2 in 2002:

- Climate 1 (CONSTANT):** day length (DL) = 17h, temperatures $T_{\text{day}} = 15^{\circ}\text{C}$ and $T_{\text{night}} = 13^{\circ}\text{C}$
Climate 2 (DECLINING): initially as climate 1, but DL reduced by 1h and T by 2°C every two weeks until DL=8.5 h and $T_{\text{day}}/T_{\text{night}} = 1/1^{\circ}\text{C}$.
Climate 3 (SCOLD): as climate 2. Seedlings tested after being subjected to a short cold period in week 39 and 40 and subsequently after a 2 week 'recovery' period (week 41 and 42) in climate 2.
Climate 4 (SWARM): as climate 2. Seedlings tested after being subjected to a short warm period in week 45 and 46 and subsequently after a 2 week 'recovery' period (week 47 and 48) in climate 2.

Sampling and testing of plant material:

Seedling samples from each climate regime were collected in week no. 37, 39, 41, 43, 45, 47, 49, 51 in 2002 and in week no. 2 in 2003. In each sampling week seedlings were tested for shoot frost tolerance (SFT) and dormancy status.

Each sampling week, 2-cm shoot tip sections of 15 beech seedlings per climate were excised and frozen to -15°C and -25°C . 15 shoot control samples were held at $+4^{\circ}\text{C}$. Shoot frost injury was evaluated using the conductivity method described in the common protocol. SFT was quantified as the difference in shoot electrolyte leakage SEL(-15°C) or SEL (-25°C) and unfrozen control SEL($+4^{\circ}\text{C}$), and hence denominated $\text{SEL}_{\text{diff-15}}$ and $\text{SEL}_{\text{diff-25}}$, respectively.

Each sampling week 15 beech seedlings per climate were transferred to a greenhouse with favourable growth conditions (DL= 18h, $T=20^{\circ}\text{C}$) for testing of bud dormancy status. The number of days to terminal bud break (DDB) of each seedling was recorded and dormancy status was calculated as the average DDB for each sampling week.

Each sampling week 50 terminal buds, collected for isolation of RNA were immediately frozen in liquid nitrogen and kept at -80°C .

$\text{SEL}_{\text{diff-15}}$ and $\text{SEL}_{\text{diff-25}}$ decreased during autumn for growth room beech seedlings in both climate regimes (figure 1). Shoots became frost tolerant to -15°C first, and this happened faster in climate 2 than in climate 1.

In climate 2 beech shoots was freezing tolerant at -15°C (i.e. $\text{SEL}_{\text{diff}} < 5\text{-}10\%$) between week 47 and 49 and almost tolerant to -25°C in week no. 2 (2003). In climate 1 shoots were almost tolerant to -15°C , but never reached -25°C . Development of frost tolerance at -15°C was delayed by 5 weeks when compared to climate 1 in 2001 (1st Annual Report, p 40). This could be an effect of the 2 h longer days used in the 2002 experiments. However, long days and high temperatures, did not prevent the development of SFT in any year.

Short cold and warm periods (SCOLD and SWARM resp.) had no influence on the further development of hardiness (figure 2).

Beech seedlings from both climate regimes entered almost equal levels of dormancy (number of days to terminal bud break under favourable growth conditions, DDB) (figure 3).

Seedlings in both climate regimes entered a dormant state beginning in week 41 (climate 1) and in week 43 (climate 2). The positive effect of high temperatures on dormancy development has been demonstrated in e.g. Norway maple (*Acer platanoides* L.) (Westergaard, L. and Eriksen, E.N. *Scand. J. For. Res.* 12: 11-16, 1997). Seedlings from climate 2 tended to remain dormant, while climate 2 seedlings started to release from dormancy from week 47.

Research related to WP 3

Production of plant material:

In week no. 20 the standard Scots pine provenance A70 (origin UK) and the provenance 'Lindås' (origin Norway) were sown in HIKO120ss trays placed in a greenhouse. Approximately 1500 seedlings of each provenance were produced in accordance with normal nursery practice for containerised stock. Seedlings were transferred from the greenhouse to an outdoor container cultivation area in week 32 where they remained during the entire sampling period.

Sampling and testing of plant material:

Seedling samples from each provenance were collected in week no. 37, 39, 41, 43, 45, 47, 49, 51 in 2002 and in week no. 2 and 4 in 2003. In each sampling week seedlings were tested for shoot and root frost tolerance and dormancy status.

Each sampling week, 2-cm shoot tip sections of 15 pine seedlings per provenance were excised and frozen to -15°C and -25°C and 15 fine roots samples from the same seedlings were frozen to -5°C . 15 shoot and root control samples were held at $+4^{\circ}\text{C}$. Shoot and root frost injury were evaluated using the conductivity method described in the common protocol. SFT was quantified as described above. Similarly, RFT was calculated as $\text{REL}_{\text{diff-5}}$.

Each sampling week 15 beech seedlings per climate were transferred to a greenhouse with favourable growth conditions (DL= 18h, T= 20°C) for testing of bud dormancy status. The number of days to terminal bud break (DDB) of each seedling was recorded and dormancy status was calculated as the average DDB for each sampling week.

Each sampling week 50 terminal buds, collected for isolation of RNA were immediately frozen in liquid nitrogen and kept at -80°C .

Both provenances of containerised Scots pine seedlings developed SFT. Previous studies have shown that conifer shoots are frost tolerant at a given temperature when SEL_{diff} is lower than 5% and storable when they are frost tolerant to -25°C (i.e. $\text{SEL}_{\text{diff-25}} < 5\%$). Figure 4 shows a fast increase in SFT between week 41 and 43 in both provenances. This is in contrast with observations made in the previous year where 'Lindås' acclimated earlier than A70. A provenance difference was, however, observed for RFT (figure 5).

'Lindås' developed dormancy during autumn (figure 6), although it occurred earlier and the level was more shallow than in the previous season. The dormancy level in A70 was very shallow, and seemed slightly delayed as compared to 'Lindås'

Research related to WP 4

Production of plant material:

The standard provenance 'Bregentved' were sown in week 15 and cultivated in accordance with normal nursery practice for bare rooted stock by Hedeselskabets Planteskole (participant 6). In addition, the remaining seedlings of last year's production (now 2 year old) were used.

Sampling and testing of plant material:

The bare-rooted beech seedlings of both ages were lifted in the nursery in week 37, 39, 41, 43, 45, 47, 49, 51 in 2002 and in week 2 and 4 in 2003. (in week 51 and 2 shoots only, because of frozen nursery soils). The lifted seedlings were subjected to the same tests, following the same procedures, as described for WP 2, except for the dormancy tests in week 51 and 2.

In each lifting week (except 51 and 2) 75 seedlings were cold stored at 4°C (storage temperature lowered to -1°C in December) for field planting in April 2003, in order to study the relationship between pre-storage physiological condition (frost tolerance, dormancy and gene expression) and storability (assessed as survival and growth).

Bare rooted beech seedlings of both ages developed SFT (figure 7). During this period 2-year old seedlings were ahead of 1-year olds by approximately 2 weeks. The older seedlings reached the 10%-threshold for $SEL_{diff-25}$ in week 46 and the 1-year old seedlings in week 48-49. SFT(-25C) of 1-year old seedlings was reached at approximately the same time as in the previous season. Acclimation in beech started earlier than in pine and was, contrary to pine, linear over most of the period until week 47-49.

Development of bud dormancy was earlier and consistently deeper in 2-year old than in 1-year old seedlings, and both had a maximum level of approximately 60 DBB in week 47 (figure 8). From this week dormancy level of both ages decreased concurrently until the end of the experimental period.

Discussion and conclusions

Beech: effects of environment and age.

The results of WP2 and WP4 allows us to separate the effects of different environments and seedling age on the physiological development in beech. Climate 2 simulated quite well the climatic conditions in the nursery, with respect to average temperature and daylength. The major difference between controlled and ambient climates were the shifts between day and night temperature in the nursery (around 5°C) and in the climate rooms (2°C) and, hence, the earlier development of SFT in the nursery. On the other hand, the results shows that other parameters, than the well known temperature and daylength, may contribute to development SFT, as seen in climate 1. It is also clear that seedling age has an effect on the development of SFT.

The fact that the initial rate of acclimation (week 37 - 45) was the same over a large range different of environments (figure 9: climate 1, 2, 3, 4 and nursery), suggests that a common set of possible molecular markers could be found in seedlings in the same physiological state. The aim should now be to identify the presence of these markers in physiologically equivalent seedlings (e.g. $SEL_{diff-25} = 10\%$; marked with arrows in figure 10). To evaluate these as markers for storability the further selection should also be based on the relationship found between physiological parameters and field performance, which is currently being analysed.

Pine: effects of provenance

No clear provenance effect on SFT was found which is in contrast to last years observations. However, we still found an effect of provenance on RFT, and a less distinct effect on bud dormancy. For A70 the development of dormancy was particularly weak this year.

Significant difficulties or delays experienced during the second reporting period

None. Each sampling week 50 terminal buds of both species and pine roots, collected for isolation of RNA were immediately frozen in liquid nitrogen and kept at -80°C. However, decision about isolation of specific samples currently awaits the analysis of physiological response and field performance.

Sub-contracted work during the second reporting period

None

Figures relating to research activities in the second reporting period

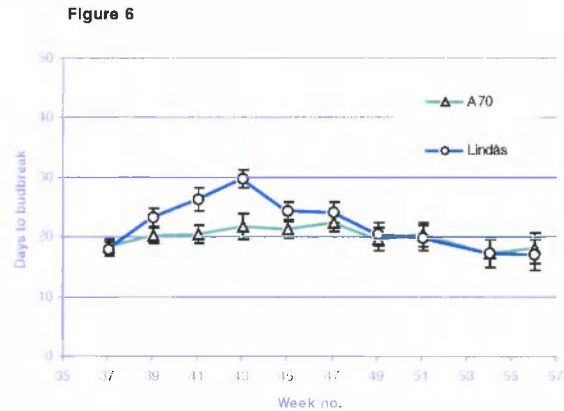
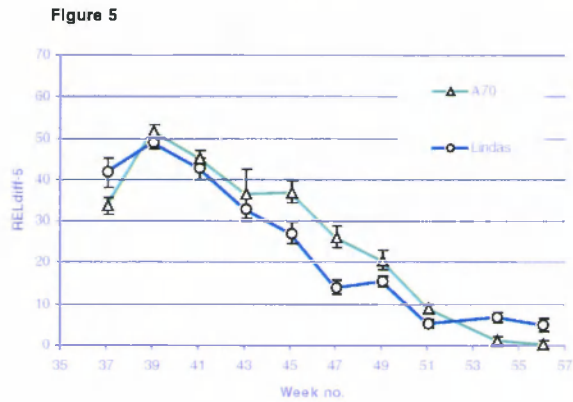
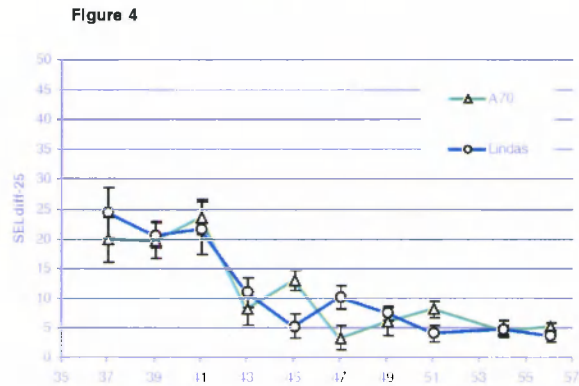
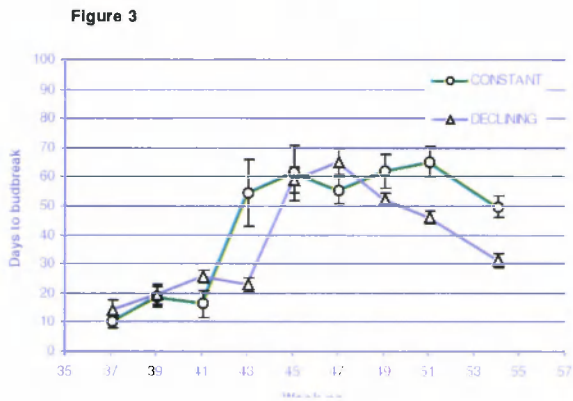
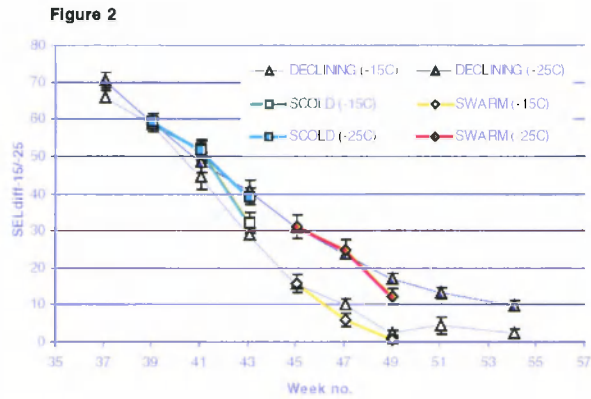
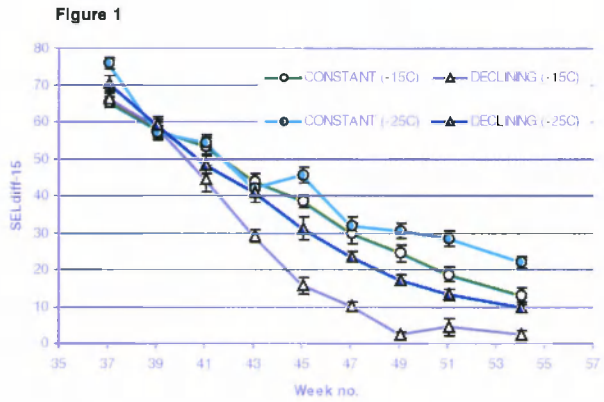


Figure 1. Development of SFT of beech grown in the constant and declining climate regimes during autumn. SFT is expressed as SEL_{diff} at -15°C and -25°C.

Figure 2. The effect of a short cold period (SCOLD) in week 39 and 40 and a short warm period (SWARM) in week 45 and 46 on SFT of beech seedlings from the declining climate. SCOLD and SWARM were each time followed by a 2-week 'recovery' period after returning to the declining climate.

Figure 3. State of dormancy of beech seedlings grown in the constant and declining climate regimes. Dormancy level was assessed as number of days to terminal bud break (DBB) under favourable growth conditions.

Figure 4. SFT (expressed as SEL_{diff}) at -25°C of Scots pine seedlings grown outside during autumn.

Figure 5. RFT (expressed as REL_{diff}) at -5°C of Scots pine seedlings grown outside during autumn.

Figure 6. State of dormancy of Scots pine seedlings grown outside during autumn. Dormancy level was assessed as number of days to terminal bud break (DBB) under favourable growth conditions (greenhouse).

Figure 7

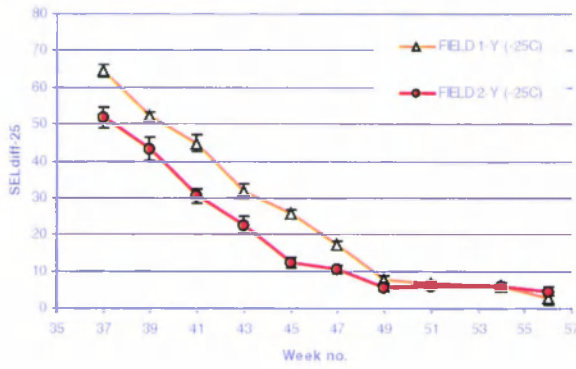


Figure 8

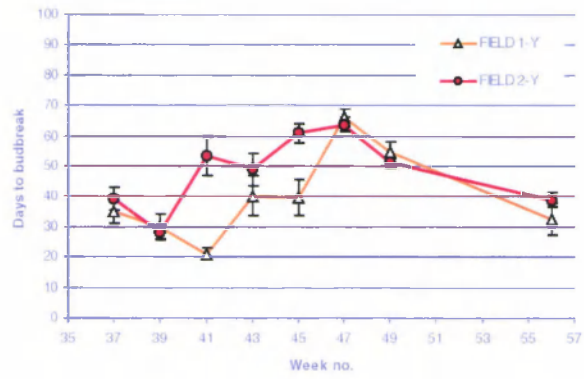


Figure 9

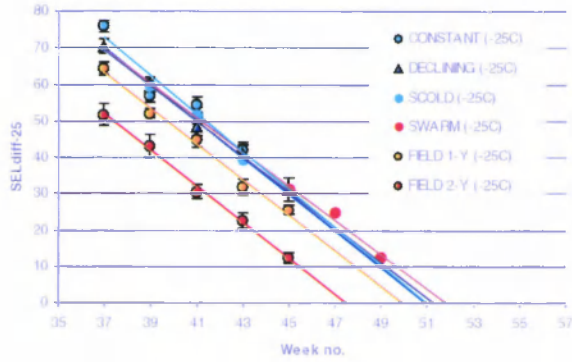


Figure 10

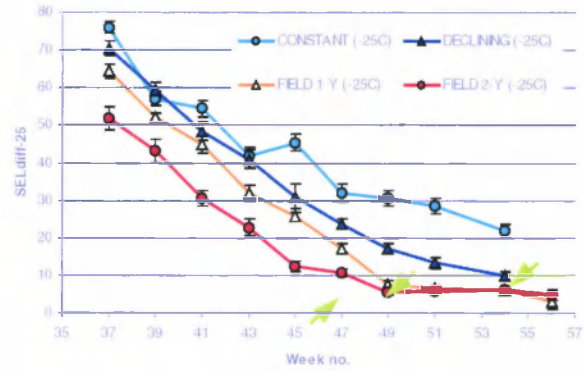


Figure 7. SFT (expressed as SEL_{diff}) at -25°C of beech seedlings grown outside during autumn.

Figure 8. State of dormancy of beech seedlings grown outside during autumn. Dormancy level was assessed as number of days to terminal bud break (DBB) under favourable growth conditions (greenhouse).

Figure 9. Linear regressions of SEL_{diff-25} during the acclimation phase of beech seedlings grown under different environmental conditions.

Figure 10. Developments in SFT of beech seedlings grown in different environments during autumn. Arrows show the time points where possible molecular markers indicating the start of storability should be searched for.

3.5 Partner 5 - Assistant Contractor to DIAS - PPO

Applied Plant Research
PO-box 118,
2770 AC Boskoop,
The Netherlands.
Tel: +31.172.236700
Fax: +31.172.236710

Scientific team

Drs. M.B.M. Ravesloot - Project leader for the PPO tasks. Replaces dr. Kunneman who left the organisation.

J. Brouwer – scientist, fills in the vacancy mentioned in the TA

M. Ijspeert. - Technician responsible for tending field trials, replaces *A.J van Fulpen*

Objectives

Applied Plant Research division Woody Plants is a main intermediary between fundamental research and practical implications in the Dutch forestry sector. PPO operates in close co-operation with the nursery industry. The research station has a lot of experience on cultivation, storage, plant quality and physiological research and has good laboratory facilities for this research including a range of temperature controlled (storage) chambers and incubators. In this project the role of the research station is to evaluate key gene expression under practical nursery conditions. For this PPO will co-operate closely with the Dutch Nursery Association (NBVB), who will have an advisory role. Individual growers will play a role in plant supply and in evaluation of key gene expression under commercial conditions.

In co-operation with DU, PPO will be charged with the first steps towards implementation of the results in forestry practice. A putative limiting factor for the general application of marker genes is the provenance effect. This climate-induced intraspecific physiological variation is one of the hindrances in international trade. Therefore PPO and DU will evaluate the expression of the key-genes in a series of provenances derived from geographically distinct commercial nurseries and from controlled field trials. This set-up guarantees that the final key gene selection will be of general importance in large parts of Europe. PPO will focus on beech, and will therefore co-operate closely with DIAS, that is responsible for the field and climate room trials concerning beech.

Workplan

In the first project year PPO started a series of experiments aimed at the evaluation of the effect of provenance, age and climatic conditions on beech gene expression (**WP8**). Seeds of 3 geographic beech provenances were sown. One of the provenances is Bregentved, the standard provenance used by DIAS in WP2 and WP4. In the second project year a second sowing will be done resulting in seedlings of 0.5 and 1,5 year age in the winter of 2002/2003. Physiological parameters will be measured on these seedlings of the 3 provenances. In the next year, season 2003/2004, this experiment will be repeated with seedlings from all three sowing seasons (0.5, 1,5 and 2,5 years old). Also, in 2003 samples of beech seedlings at the onset of hardening to completely hardened will be taken from 3 commercial nurseries.

In all these experiments tissue samples will be taken and stored at -80°C for mRNA screening by ATO. Physiological data will be send to FC(FR) for integration into the physiological database.

Deliverables

The contribution of PPO to the project deliverables is specified in the scheme below

Deliverable	WPs	Confidential nature	Delivery month											
			12	20	24	30	36	38	41	43	46	48		
DL16 - Information on the influence of provenance, climatic conditions and age on key gene expression	8	CO												
DL18 - Assessment of key genes as molecular diagnostic tool	7,8,9	CO												
DL19 - A physiological database	10	CO												
DL22 - Conceptual model describing molecular events underlying winter dormancy and frost hardiness.	10	CO												
DL23 - Trade demonstration workshop	8,9	PU												
DL24 - Scientific publications	All	PU												
DL25 - Publications in trade journals	All	PU			✓									
DL26 - Annual reports	All	PU			✓									
DL27 - Final report	All	PU												

Grey field: date when deliverable is due according to the technical annex. ✓ = delivered

Research activities during the first reporting period

In the first reporting period PPO has worked on plant production for provenance trials planned to in autumn 2002. 3 different provenances of beech have been sown: NL2.1, Greenhill and Bregentved.

Significant difficulties or delays experienced during the first reporting period

Field germination was variable, and rather low for 'Greenhill' (44%). However, the expectation is that there will still be enough plants for the trials.

Sub-contracted work during the first reporting period

None

Research activities during the second reporting period

Scientific team in year 2

Nico Dolmans - Senior scientist

Marc Ravesloot - project leader, plant physiologist

Hanneke Franssen - plant physiologist

Maarten IJspeert - Laboratory technician

Gosia de Winter - (student)

Jan van Leijden - Laboratory technician

Jan Brouwer - Nursery gardener

Bram Buitenwerf - Nursery gardener

Henry Drent - Nursery gardener

Marian de Beuze – communication specialist

Jan Sieverink - statistician

PPO activities are all within WP 8

Production of plant material:

The standard provenance 'Bregentved', obtained from the Danish Institute of Agricultural Sciences (participant 4), and the provenances NL2.1, 'Greenhill', and 'Het Loo' were treated for stratification and sown on June 10th, May 7th, May 21st and May 7th respectively and cultivated in accordance to standard nursery practice. The germination percentages were 45, 80, 67 and 68% respectively.

Also material sown in 2001 was replanted for practical reasons and cultivated according to standard nursery practice.

Sampling and testing of plant material:

The bare-rooted seedlings of all provenances and the two ages were lifted 8 times during the season, in week 33, 35, 40, 42, 44, 46, 48 2002 and in week 6 2003, 20 shoots of each age/provenance combination. Apical buds of 20 plants were sampled (3 buds/plant on average) and stored immediately in liquid nitrogen. The buds were stored at -80°C until mRNA analysis. For mRNA analysis week 33 is missing.

The lifted seedlings were at random divided in two groups of 10 and subjected to measurements in

- length
- shoot electrolytes leakage ($\text{Sel}_{\text{diff-25}}$).
- electric conductivity of the shoot (EC)

at room temperature (approximately 20°C) and after treatment at -25°C .

*Physiological parameters**- Length*

The length of the seedlings was rather variable (figure 1). Although the seedlings sown in 2001 were taller than those sown in 2002, the differences were quite small and the variation between the separate seedlings large.

- Sel

The bare rooted beech seedlings all developed frost tolerance (-25°C). No differences between the provenances were found (figure 2A and 2B).

Seedlings germinated in 2001 (figure 2A) stopped acclimatizing around the end of November (week 48), reaching the assumed threshold $\text{sel}_{\text{diff-25}}$ of about 5%.

The seedlings sown in 2002 also showed no differences in the development of frost tolerance between the provenances (figure 2B), but the acclimation was slower than in seedlings one year older, and seemed to continue between week 48 2002 and week 6 2003. To visualize the difference in acclimatizing between the two sowing years in figure 2C shows the averaged values of all provenances.

- EC

The EC showed a tendency similar to sel. Again, no differences between the provenances were found (see figure 3A and 3B), although the variation was slightly larger. The development of frost acclimation was different for the two groups of seedlings sown in 2001 and 2002, and seemed to stop at around week 45 and week 48 respectively. Again (figure 3C) the younger seedlings showed the tendency to acclimatize slower. More data are necessary to compare EC and Sel in terms of hardiness.

Significant difficulties or delays experienced during the second reporting period

The germination percentage of some provenances was low, possibly resulting in a shortage of plant material. If adaptations in the experimental design are necessary, DIAS will be consulted.

Sub-contracted work during the second reporting period

None

3.6 Partner 6 - Assistant Contractor to DIAS: HSP

Hedeselskabets Planteskole Brøndlundgaard,
Brøndlundvej 2, Gabøl,
DK-6500 Vojens,
Denmark.
Tel: +45.74.871600
Fax: +45. 74871543

Scientific team

Bent Karlsson - Project leader for HSP tasks
Leo Thybo Møller - Nursery Director, advisor.

Objectives

Hedeselskabets Planteskole is a department of Hedeselskabets (Danish Land Development Service), which comprises forestry activities and advisory services for private forest owners. Hedeselskabets Planteskole is one of the leading Danish nurseries with respect to plant production and innovative activities within seedling quality control. The participation of HSP and other nurseries will enhance dissemination of the project results to the sector. HSP will participate in the field trials of DIAS, and supply plant material and trial fields. In addition, the nursery will aid in the evaluation of molecular marker utilisation in commercial situations.

Workplan

In the first two project years HSP will be involved in field trials concerning beech, aimed at the evaluation of the effect of climatic conditions, geographical position and provenances on the development of dormancy and hardiness (WP4). In the third and fourth project year the nursery will also participate in the application of RT-PCR assays and thus evaluate molecular marker utilisation in commercial situations (WP7).

Deliverables

The contribution of HSP to the project deliverables is specified in the scheme below

Deliverable	WPs	Confidential nature	Delivery month											
			12	20	24	30	36	38	41	43	46	48		
DL4 - RNA for RT-PCR	1,2,3,4	CO			✓									
DL6 - Physiological data on dormancy in beech in outdoor situations	4	CO	✓		✓									
DL18 – Assessment of key genes as molecular diagnostic tool	7,8,9	CO												
DL19 - A physiological database	10	CO												
DL22 - Conceptual model describing molecular events underlying winter dormancy and frost hardiness.	10	CO												
DL23 – Trade demonstration workshop	8,9	PU												
DL26 – Annual reports	All	PU	✓		✓									
DL27 – Final report	All	PU												

Grey field: date when deliverable is due according to the technical annex. ✓ = delivered

Research activities during the first reporting period

During the first reporting period HSP has mainly been involved in production of plant material (bare rooted beech seedlings) to be used by DIAS in WP 4 as described above.

Significant difficulties or delays experienced during the first reporting period

Field germination was variable, and rather low for 'Greenhill' (44%). However, the expectation is that there will still be enough plants for the trials.

Sub-contracted work during the first reporting period

None

Research activities during the second reporting period*Scientific team in year 2**Bent Karlsson - Project leader for HSP tasks*

During the second reporting period HSP has mainly been involved in production of plant material (bare rooted beech seedlings) to be used by DIAS in WP 4 as described above.

Significant difficulties or delays experienced during the second reporting period

None

Sub-contracted work during the second reporting period

None

3.7 Partner 7 - Contractor: FC(FR)

Forestry Commission Research Agency,
Northern Research Station,
EH25 9SY Roslin, Midlothian,
United Kingdom.
Tel: +44.131.4452176
Fax: +44.131.4455124

Scientific team

William Mason - Project leader for FC(FR) tasks, senior scientist and head of department.
Dr. Mike Perks - Scientist (Appointed 01/04/01)
C. McEvoy - technician, has been responsible for laboratory determinations and data collation for 6 years.

Objectives

FC(FR) is the research arm of the government Department of Forestry in the UK. Nursery and establishment research in general is done on behalf of the forest industry in the UK. There has been a major project on nursery stock quality for 20 years with an emphasis on physiological quality in the past 10 years. FC(FR) is well informed on the local growth conditions, climatic variation and provenance characteristics and maintain close contacts with the forestry sector. FC(FR) offers a non-profit making Plant Quality Testing Service for public sector and private nurseries and forest managers which has operated for the past 5 years.

Within the present project FC(FR) will study the frost hardiness response in forest tree seedlings with the aim to obtain a better understanding of the roots response to their winter environment in terms of a range of interrelated attributes. FC(FR) will focus on pine. The first two seasons will be used for experiments in controlled environments. The material derived from these trials will be used to make the initial selection of relevant genes. Next to the climate room trials, field trials will be performed. The information on seedling response to the two prime environmental factors of daylength and temperature, particularly fluctuating temperature, will be of immense practical value and will be incorporated into advice to the forestry industry.

Plant material derived from these trials will be used for detailed expression analysis of the selected genes aimed at the identification of a subset of key genes in close co-operation with ATO and Plant RI.

FC(FR) will co-operate closely with its assistant contractor Alba Trees.

Workplan

In the first year both climate room experiments (**WP1**) and field experiments (**WP3, WP4**) took a start, but the main focus was on the controlled environment trials. Pine seedlings were followed through the process of hardening and at various time points physiological analyses were performed and samples for RNA analysis were taken. In this first trial season the aim was to separate the processes of dormancy and frost hardiness development as far as possible. Both field and climate room trials will proceed into the next project year. In the second year climate room experiments will be devoted to the interaction of temperature and daylength. Field trials, using both beech and pine, will be designed to assess the effect of climatic conditions, geographical position and provenances on the development of dormancy and hardiness. In the third year field trials will proceed. By that time RT-PCR assays for several key genes will be available. These assays will be used by FC(FR), in co-operation with the nurseries ALBA and FE-Nurseries, to obtain detailed expression information using material from the field trials (**WP7**). This work will continue into the fourth year and the results will be used for the organisation of a demonstration workshop aimed at the implementation of the RT-PCR assays in practice. During the course of project FC(FR) will collect physiological data from all partners, for the construction of a physiological database. This database will be merged to the molecular database into an integrated and searchable information tool. (**WP10**)

Deliverables

The contribution of FC(FR) to the project deliverables is specified in the scheme below

Deliverable	WPs	Confidential nature	Delivery month											
			12	20	24	30	36	38	41	43	46	48		
DL1 - RNA for preparing microarray probe	1,2	CO	✓		✓									
DL2 - Physiological data on dormancy in pine in controlled environments	1	CO	✓		✓									
DL4 - RNA for RT-PCR	1,2,3,4	CO	✓		✓									
DL5 - Physiological data on dormancy in pine in outdoor situations	3	CO	✓		✓									
DL6 - Physiological data on dormancy in beech in outdoor situations	4	CO	✓		✓									
DL14 - In depth expressional information	7	CO												
DL15 - A subset of 15 key genes,	7	CO												
DL18 – Assessment of key genes as molecular diagnostic tool	7,8,9	CO												
DL19 - A physiological database	10	CO												
DL20 - A molecular database	10	CO												
DL22 – Conceptual model describing molecular events underlying winter dormancy and frost hardiness.	10	CO												
DL23 – Trade demonstration workshop	8,9	PU												
DL24 – Scientific publications	All	PU												
DL25 – Publications in trade journals	All	PU												
DL26 – Annual reports	All	PU	✓		✓									
DL27 – Final report	All	PU												

Grey field: date when deliverable is due according to the technical annex. ✓ = delivered

Research activities during the first reporting period

The work packages (WP1, 3 & 4) of Partner 7 are continuing, with the first years trials having been successfully implemented without significant technical difficulty:

During summer and autumn containerised seedlings of *Pinus sylvestris* [Scots pine] (standard provenance A70 and local provenance N401 'Abernethy') and *Fagus sylvatica* [beech] (standard provenance Bregentved) seedlings were raised at the Research Station in Scotland and Beech (local provenance Cirencester) seedlings were provided from the nursery of Partner 9. Some of the containerised pine seedlings of provenance A70 were transferred to growth rooms in the middle of August (Week 33) and subjected to three different climate regimes until Week 2 (2002):

Climate 1 (control): long day (15h), high temperature (15/13°C)

Climate 2: decreasing daylength (15h → 7h), high temperature (15°C)

Climate 3: decreasing temperature (15/13°C → 5°C), long day (15h).

Every second week, starting in week 37 and until week 4 (2002), all experimental plant types were tested for shoot and root frost tolerance ($SEL_{diff-25^{\circ}C}$ / $SEL_{diff-15^{\circ}C}$ / $REL_{diff-5^{\circ}C}$), dormancy (days to terminal bud break) and material for mRNA analysis was collected from terminal buds and fine roots. Samples of containerised 'field-grown' beech were cold stored (at 1°C) on alternate sampling weeks to be outplanted at the end of April 2002.

Pine seedlings from all controlled environment climate regimes showed development of root frost tolerance assessed at -5°C, and shoot frost tolerance as assessed by $SEL_{diff-15^{\circ}C}$, but this was not evident at the $SEL_{diff-25^{\circ}C}$ assessment temperature. This indicates that plants had not developed hardiness to this level under the controlled environment conditions. Shoot frost hardiness assessed at -15°C indicated that there was a lower level of induced hardiness in climate 1 seedlings over the last four weeks of the trial (figure 1 and figure 2).

Shoots of 'field-grown' pine were significantly more frost tolerant than the controlled environment pine seedlings with effects apparent at all freeze test temperatures.

Provenance differences were not observed. 'Field-grown' containerised Scots pine roots developed frost tolerance and there was evidence of occasional response differences between provenances (figure 3).

Development of root frost tolerance (-5°C) in 'field-grown' beech was absent, while frost tolerance of beech shoots from the outdoor trial showed distinct seasonality, though no provenance effects were found (figure 4).

All experimental plant types had a more or less distinct dormancy period, even the climate 1 seedlings. Dormancy in beech was considerably deeper than in pines, and the ontogenetic pattern of dormancy development was later in the UK provenance than the standard Danish provenance (figure 5). Dormancy in the field-grown pine provenances showed almost identical seasonal variation with the native Abernethy (N401) provenance showing periodically deeper dormancy levels than in the provenance A70 over the second half of the testing period (from Week 45). Dormancy development appeared to be correlated with soil temperature. Under controlled conditions declining temperature led to a decrease in dormancy levels, no clear influence of daylength was evident, and the lack of response was compounded by poor plant response to the conditions favourable for budbreak.

The material collected for RNA analysis, from terminal buds and roots, will be isolated in April 2002. There has been a small delay in this portion of the workpackages whilst a suitable facility for processing and analysis of the tissues was sought, which was further compounded by infrastructure changes to the relevant laboratories.

Plans for 2002

In 2002 containerised pine seedlings will be transferred to growth rooms in August and cultivated under decreasing day lengths and temperature. In week 40 a group of these seedlings will be subjected to one- and two-week cold spells ($T \rightarrow +5^{\circ}\text{C}$). The field trial experiment with Scots pine seedlings will be repeated and the field trial with beech also. The beech trials will not test root material or root RNA as these did not exhibit any relevant physiological information in the first trial year. Additional studies of storage-induced declines in plant health and the potential for the use of fluorescence will be undertaken on an ad-hoc basis.

The same tests will be conducted as in 2001, but because of the poor frost development and RNA yield in roots of beech in 2001-2, we also suggest (as does Partner 4) to cancel root frost testing and root RNA isolation, at least in this species.

Significant difficulties or delays experienced during the first reporting period

We have had some difficulty in securing the necessary lab facilities for processing and analysis of collected RNA samples. This has been compounded by the infrastructure changes at the University. An arrangement has been made to analyse the samples in April-May 2002. The management group have now confirmed which array samples are required in the first instance.

Sub-contracted work during the first reporting period

Sub-contractor 8 . The Alba nursery group have provided us with local provenance beech material. This is additional to their specified remit and will occur again in 2002 and 2003.

Next page: Figures relating to the work of partner 7

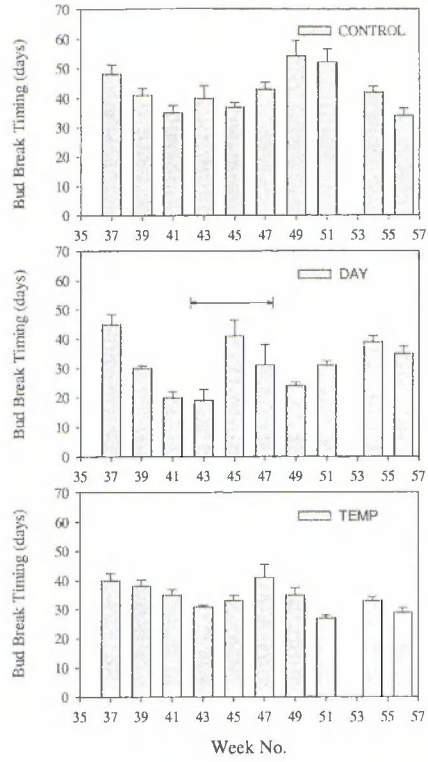
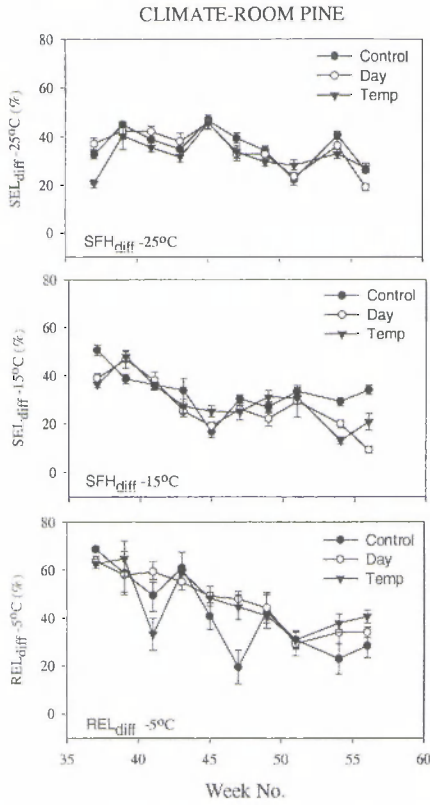


Figure 1. Physiological response of A70 pine under controlled environment conditions, in year 1 (2001-2).

Figure 2. Days to budbreak in climate grown A70 pine seedlings assessed in 2001-2 (Year 1).

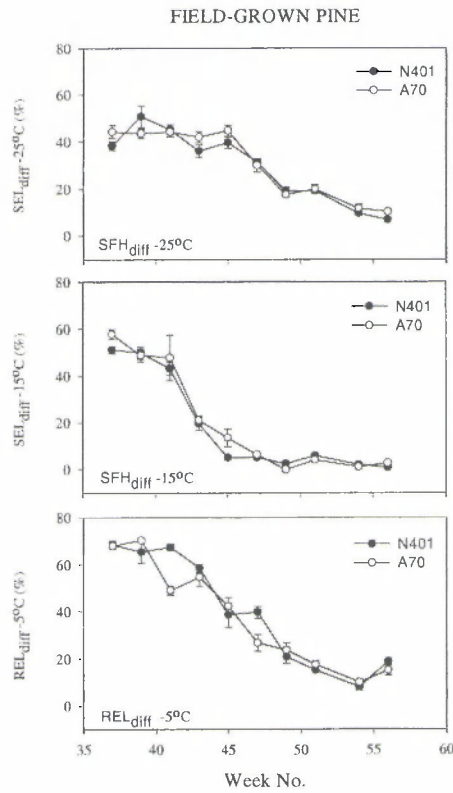


Figure 3. Development of cold tolerance in roots and shoots of Scots pine, taken from two proveniences (A70 and 'native' N401), in the UK, assessed in 2001-2 (Year 1).

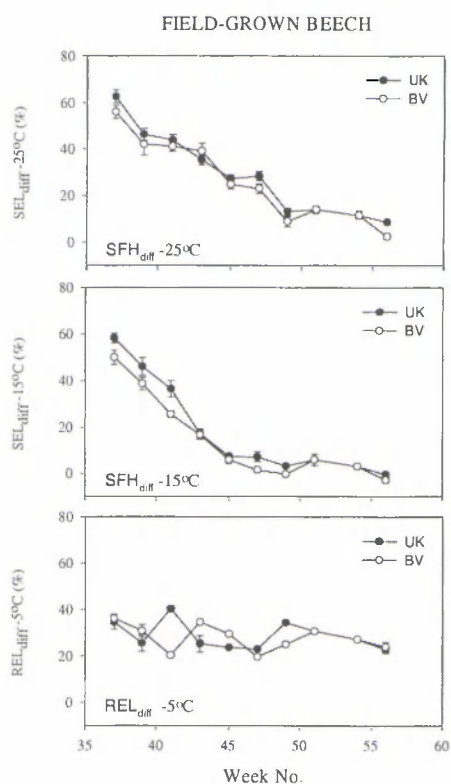


Figure 4. Cold tolerance development in field-grown Beech, in the UK, 2001-2 (Year 1).

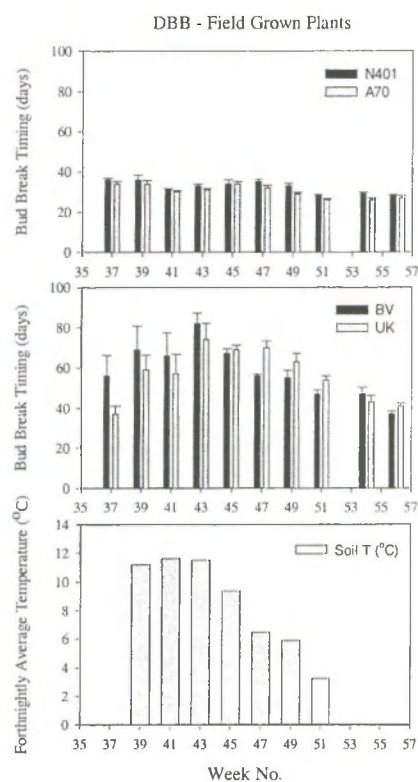


Figure 5. Days to budbreak (dormancy testing) for field grown provenances of pine and beech, in the UK, assessed in 2001-2 (Year 1).

Research activities during the second reporting period

Scientific team for year 2

William Mason - Project leader for FC(FR) tasks, senior scientist and head of department.

Dr. Mike Perks - Scientist (Appointed 01/04/01)

C. McEvoy - technician, has been responsible for laboratory determinations and data collation for 6 years.

S Smith – project leader now left FR

A Milner – Statistician now left FR

D Brooks – Technical support (growth room maintenance)

D Clark – Technical support (nursery operations)

A Harrison – technical support (nursery operations)

T Connolly – Statistician (taken over responsibilities from A Milner)

A Lovat – Student working on laboratory analyses (Aug 2002-3)

A Blain – Student working on laboratory analyses (Aug 2001-2)

Various – Technical support (plant watering etc)

J Nicholl – technical support (electrician – growth room maintenance)

L Rooney – data preparation dept (data organisation from field experiments)

J Strachan – technical support

The work packages (WP1, 3 & 4) of Partner 7 are continuing, with the second years trials having been successfully implemented without significant technical difficulty:

During summer and autumn containerised seedlings of *Pinus sylvestris* [Scots pine] (standard provenance A70 and local provenance N401 'Abernethy') and *Fagus sylvatica* [beech] (standard provenance Bregentved) seedlings were raised at the Research Station in Scotland and Beech (local provenance Cirencester) seedlings were provided from the nursery of Partner 9. Some of the containerised pine seedlings of provenance A70 were transferred to growth rooms in the August (Week 33) and subjected to one of four different climate regimes until Week 2 (2002):

Climate 1 (control): long day (17h), high temperature (15/13°C)

Climate 2: decreasing daylength (17h → 8h), decreasing temperature (15/13°C → 5°C).

Climate 3: 'SCOLD': removal from Climate 2 at week 40 to 6°C day/night and equivalent light regime, tested week 41 & 42.

Climate 4: 'SWARM': removal from Climate 2 at week 46 to Climate 1 regime, tested week 47.

Every second week, starting in week 39 and until week 51, with additional tests in week 02 (2002) for SELdiff-25°C testing procedure. All experimental plant types were tested for shoot and root frost tolerance (SEL diff-25°C / SEL diff-15°C / REL diff-5°C *Pine* or REL diff-2°C *Beech*), dormancy (days to terminal bud break) and material for mRNA analysis was collected from terminal buds and fine roots. Samples of containerised 'field-grown' beech and pine were cold stored (at 1°C) on alternate sampling weeks to be outplanted during April 2002, which has now been completed. It is intended to assess shoot and root vitality post-storage (Year 2 & 3) and relate these measurement to establishment success.

Pine seedlings from the controlled environment climate regimes showed no significant development of root frost tolerance, assessed at -5°C, in year 2 (Figure 2.1). Both temperature assessments of shoot frost tolerance, by SELdiff-15°C and SELdiff-25°C, showed seasonal patterns of cold tolerance development for seedlings treated in the declining temperature environment (Figure 2.1). The lack of any clear picture for the root based assessment indicates that plants did not develop root hardiness in response to the controlled environment conditions. This is not unexpected for shoots which are already cold tolerant but only semi-cormant, as is the case with this species. Clear differences in shoot frost were evident, in comparison with Climate 1 (control) seedlings over the last four weeks of the trial. There was no evidence of changes in shoot physiology resulting from cold or warm 'shock' periods (SCOLD & SWARM (Figure 2.1). Root based assessments did show effects of SCOLD and SWARM treatments, and we hope to repeat this during the coming year on field-grown material.

Shoots of 'field-grown' pine were significantly more frost tolerant than the controlled environment pine seedlings with effects apparent at all freeze test temperatures (Figure 2.2). Provenance differences were not observed. 'Field-grown' containerised Scots pine roots also developed frost tolerance (after initial 'noise' in the data which requires further investigation). The rate of cold tolerance development was quicker (steeper response slope) than that noted in the first year of field trials (compare with Figure 1. 2).

Development of root frost tolerance (-2°C) in 'field-grown' beech was absent, while frost tolerance of beech shoots from the outdoor trial showed distinct seasonality, though no provenance effects were found (Figure 2.3). Again the rate of cold tolerance development was quicker (steeper response slope) than that noted in the first year of field trials (compare Figure 1.4).

All experimental plant types had a distinct dormancy period (see Figures 2.4, 2.5) with the exception of plants in the control climate treatment which did not develop any dormancy during the second year (Figure 2.5). It should be noted that a new analysis protocol has been adopted (after Heide 1993a¹, b²) where any plant that has not broken dormancy (burst bud) after 90 days has a dormancy value assigned of 90 days. Previous results *may* need to be reanalysed in the light of this decision. Dormancy levels in field-grown beech was similar to that found for field grown pine provenances in the second season of assessment, and the ontogenetic pattern of dormancy onset was later in the UK provenance than the standard Dutch provenance (Figure 2.4). Dormancy in the field-grown pine provenances showed almost identical seasonal variation with the native Abernethy (N401) provenance preceding the A70 material on four dates.

Dormancy development appeared to be correlated with soil temperature. Under controlled conditions declining temperature led to a decrease in dormancy levels which was not evident for control climate treatment material (Figure 2.5). This suggests the 5°C day/night temperature difference is 'critical' in (pseudo)dormancy development in this species.

The material collected for RNA analysis, from terminal buds and roots, will be isolated in April 2003. The same tests were conducted as in 2001, but because of the poor frost development and low RNA yield in roots of beech in 2001-2, we attempted a RELdif-2°C to try to capture a physiologically relevant assessment protocol.

¹ Heide Om (1993). Dormancy Release In Beech Buds (*Fagus-Sylvatica*) Requires Both Chilling And Long Days
Physiol Plantarum 89: 187-191.

² Heide Om (1993). Daylength And Thermal Time Responses Of Budburst During Dormancy Release In Some Northern Deciduous Trees *Physiol Plantarum* 88: 531-540.

As was evident in 2001/2 this procedure does not appear to have provided any improvement and we suggest that the 'physiological' partners cancel root frost testing and root RNA isolation, at least for this species.

In 2003/4 containerised pine and beech seedlings of 1 and 2 year-old field-grown stock (all provenances) will be assessed. Additional studies of storage-induced declines in plant health and the potential for the use of fluorescence will be undertaken on an ad-hoc basis. If sufficient plant numbers are available then SCOLD & SWARM treatments may be repeated using material removed from the field trials. Furthermore RT-PCR will be assessed as a tool for the nursery industry, in collaboration with Subcontractors (Partner 8 & 9).

Significant difficulties or delays experienced during the second reporting period

An arrangement has been made to analyse the samples in April-May 2003. The management group have now confirmed which array samples are required in the first instance.

Sub-contracted work during the second reporting period

Sub-contractor 8 . The Alba nursery group have provided us with local provenance beech material. This is additional to their specified remit.

Next page: Figures relating to the work of partner 7

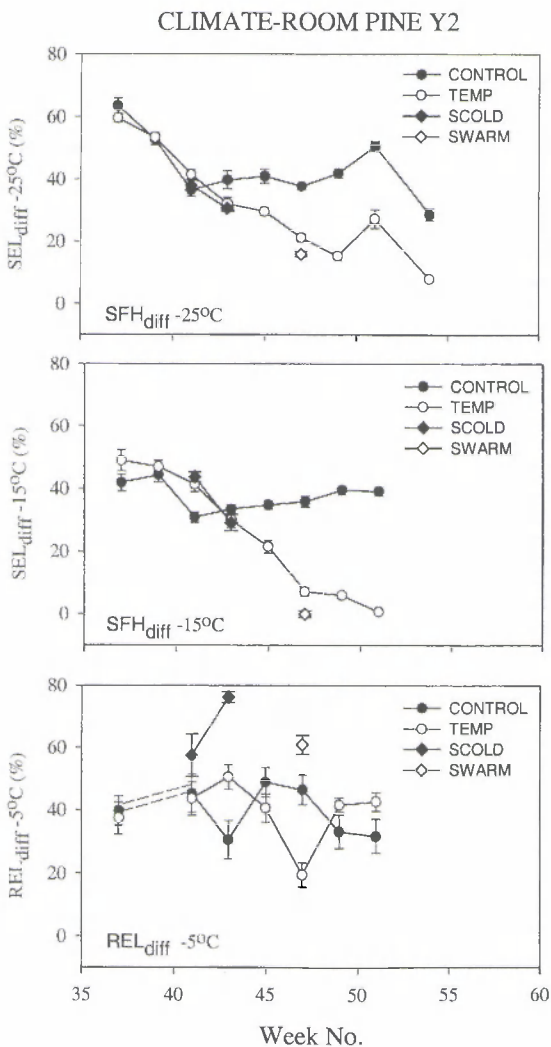


Figure 2.1. Physiological response of A70 pine under field conditions in year 2

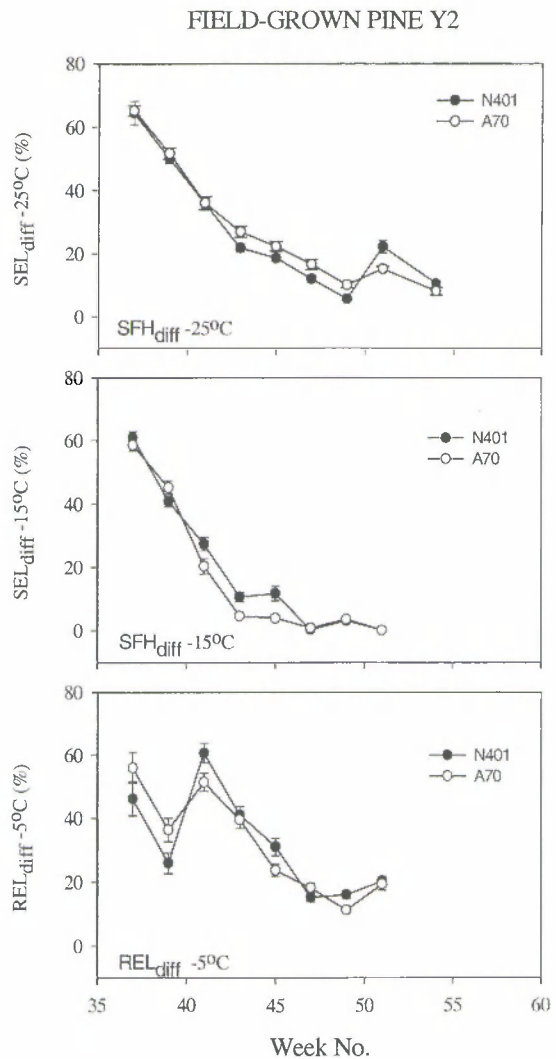


Figure 2.2. Physiological response of A70 pine under controlled environment

3.8 Partner 8 - Assistant contractor to FC(FR) - ALBA

Alba Trees,
Lower Winton,
EH33 2AL Gladsmuir, East Lothian,
United Kingdom.
Tel: +44.1620.825058
Fax: +44.1620.825316

Scientific team

John Hepburne-Scott - Project leader for ALBA tasks

Objectives

Alba Trees is the largest producer of cell-grown stock in the UK producing 8m seedlings. One of their main products is Scots pine of native seed origin. The participation of ALBA and other nurseries will enhance dissemination of the project results to the sector. ALBA will be involved in the evaluation of molecular marker utilisation in commercial situations. The company will co-operate closely with FC(FR)

Workplan

In the third project years ALBA will participate in the application of RT-PCR assays and thus evaluate molecular marker utilisation in commercial situations (**WP7**).

Deliverables

The contribution of ALBA to the project deliverables is specified in the scheme below

Deliverable	WPs	Confidential nature	Delivery month											
			12	20	24	30	36	38	41	43	46	48		
DL18 - Assessment of key genes as molecular diagnostic tool	7,8,9	CO												
DL19 - A physiological database	10	CO												
DL22 - Conceptual model describing molecular events underlying winter dormancy and frost hardiness.	10	CO												
DL23 - Trade demonstration workshop	8,9	PU												
DL26 - Annual reports	All	PU												
DL27 - Final report	All	PU												

Grey field: date when deliverable is due according to the technical annex. ✓ = delivered

Research activities during the first reporting period

Partner 8 has not yet started active research.

Significant difficulties or delays experienced during the first reporting period

None

Sub-contracted work during the first reporting period

None

Research activities during the second reporting period

Partner 8 has not yet started active research.

None

Sub-contracted work during the second reporting period

None

4. PROJECT MANAGEMENT AND CO-ORDINATION

4.1 First reporting period

Project meetings

The project started in March 2001 with a plenary kick-off meeting in Wageningen, The Netherlands, organised by the co-ordinator and PRI. During this meeting the details of the workplan for the first project year were worked out. The meeting was combined with a Molecular Analysis Workshop in which all partners were trained in RNA-isolation and RT-PCR, according to standard protocols. Also, standard operating procedures were agreed upon for

- sampling the plant material
- physiological assays
- shipping seed
- shipping RNA samples.
- Recording data

The management team was installed and discussed a first version of the consortium agreement, that was drafted by the co-ordinator. The minutes for this meeting are attached as Annex 1.

In October the management team met again in Aarslev, Denmark, hosted by DIAS. Standard operating procedures were discussed again and several adjustments were made. Initial results of the first trial season were debated and the format of the common database was discussed. Shipping of seeds for the next trial season was arranged. The final version of the consortium agreement was discussed and adopted. The minutes and a progress update were spread to all participants as E-Update 1, and are included as Annex 2a and 2b.

In March 2002 the first annual meeting took place in Edinburgh, Scotland, hosted by the Forestry Commission. All partners presented progress reports on the first year and results were discussed. The participants visited ALBA nurseries, partner in the project, and were introduced into daily practice of a nursery that uses a containerized growth system.

Linked to the general meeting was the second Management Team Meeting, in which the workplan for the coming year was discussed, with emphasis on the deviations of the technical annex mentioned above. The minutes for both meetings and a summary progress update in the form of E-Update 2 were spread to all participants and are attached to this annual report as Annex 3a and 3b.

Other co-ordination tasks during the first reporting period

The co-ordinator has constituted the consortium agreement and discussed amendments with all partners and legal advisors. The final version of the CA was adopted by the management team in October 2001 and duly signed by all participating organisations in February 2002.

Scheduled project meetings for the second project year

- | | |
|---|--|
| - 3 rd Management Team Meeting | 11 October 2002, Garpenberg, Sweden |
| - 2 nd Annual Meeting | 24/25 March 2003, Boskoop, The Netherlands |

4.2 Second reporting period

Project meetings

In March 2002 the first annual meeting took place in Edinburgh, Scotland, hosted by the Forestry Commission. All partners presented progress reports on the first year and results were discussed. The participants visited ALBA nurseries, partner in the project, and were introduced into daily practice of a nursery that uses a containerized growth system.

Linked to the general meeting was the second Management Team Meeting, in which the workplan for the coming year was discussed, with emphasis on the deviations of the technical annex mentioned above. The minutes for both meetings and a summary progress update in the form of E-Update 2 were spread to all participants and are attached to this annual report as Annex 3a and 3b.

In October 2002 the management team met in Garpenberg, Sweden, hosted by DU. Standard operating procedures were discussed again and several adjustments were made. Details for the climate room trials of the second season were debated and the format of the common database was discussed. Co-operation with other European research groups in the field of forestry were discussed and in principle the MT agreed upon participation in a Network of Excellence, EVOLTREE, co-ordinated by Antoine Kremer (INRA, Bordeaux). The minutes and a progress update were spread to all participants as E-Update 3, and are included as Annex 4.

In March 2003 the second annual meeting and 4th management team meeting were organised by PPO, Boskoop, Netherlands and attended by 15 COLDTREE participants. Since we were now halfway the project, results were evaluated thoroughly and several changes with respect to the technical annex were suggested and discussed. It was decided to spread all databases (physiological and molecular) to all partners. The MT formally agreed upon participation in the NoE EVOLTREE and adopted rules for co-authorship.

Other co-ordination tasks during the first reporting period

The co-ordinator has represented the COLDTREE consortium during the synthesis of the EVOLTREE NoE, that was submitted in April 2003. The co-ordinator will assemble all collected data and spread them to all partners in the form of a CD-ROM. Also, the co-ordinating institute has started additional analyses of these data that will lead to visualisation of common trends in physiological and molecular data and will facilitate selection of predictive indicators.

Scheduled project meetings for the third project year

- 5th Management Team Meeting 10 October 2002, Edinburgh, Scotland
- 3rd Annual Meeting 22/23 March 2003, Aarslev, Denmark

5. EXPLOITATION AND DISSEMINATION ACTIVITIES

5.1 First reporting period

- Thusfar no dissemination of scientific results has taken place.
- The project has been introduced to the Dutch forestry sector via
 - Interview with a journalist from 'De Boomkwekerij', a Dutch tradejournal for nurserymen
 - Publication in Resource, the corporate magazine of Wageningen University and Research Centre that is spread to main customers
 - Publication in 'Handelswijzer'. nr. 12, December 2001 (Dutch trade journal) by A. Laffort. 'DNA-chip brengt kwaliteitsgarantie sierteeltproduct binnen handbereik (interview)'.
- The project has been introduced to the Swedish forestry sector via a publication in "Plantnytt", a trade journal that covers forest trees seedling production, reforestation and tree-improvement. In number 2 in 2001, an article was published under the title "Gene technology takes the step into forest tree nurseries". In Swedish.

5.2 Second reporting period

COLDTREE results have been communicated in:

- Scientific lectures
 - By Lonneke van der Geest: - Forest Biotechnnology course, Crete, September 2002
 - By Monique van Wordragen - Plant and Animal Genomics conference, San Diego, January 2003
- Posters at:
 - BIOFOR conference, November 2002, Valencia, Spain
 - Plant and Animal Genomics conference, San Diego, January 2003
 - Experimental School of Plant Sciences conference, Lunteren, Netherlands

Several scientific papers are in preparation (leading author between brackets):

- Dehydrin paper (Peter Balk)
- Physiological comparison in different geographical locations pine (Mike Perks)
- Physiological comparison in different geographical locations beech (Peter Brønnum)
- Microarray results season 2001/2002 (Lonneke)

One paper aimed at the forestry sector is in preparation by Hanneke Franssen.

6. ETHICAL ASPECTS AND SAFETY PROVISIONS

Not relevant

ANNEX 1**MINUTES COLDTREE KICK-OFF MEETING**

Wageningen, The Netherlands, 19 and 20 April 2001.

Thursday April 19.

Present: Per Hove Andreasen, Peter Balk, Peter Brønnum, Lonneke v.d. Geest, James Hepburne-Scott, Maurice Konings, Bernard Kunneman, Marc Ravesloot, Michael Perks, Stephen Smith, Eva Stattin, Marianne Vernhäll, Brigitte Verkerk-Bakker, Monique van Wordragen.

MORNING

- Word of welcome by Toine Timmermans and Monique van Wordragen
- Peter Brønnum: outline of DIAS plans
 - Beech standard will be Bregentveld, a Danish provenance from Czech origin. This leads to some discussion of origin vs. provenance. Conclusion: for our goal the difference is not really important as long as the geographical location to which the trees have adapted is known. A local provenance may, therefore, be derived from a neighbouring country.
 - Germination of the seed has to be synchronized. Peter will send a standard operating procedure to other partners. **ACTION Peter**
 - Local beech provenance will be Graasten
 - Local pine provenance will be derived from southern Sweden
 - Several questions arise regarding the physiological tests that have to be performed. They will be discussed on Friday
- Stephen Smith gives an overview of the Forestry Commission organisation and the position of the Research Agency
- Mike Perks: outline of FCFR plans
 - Standard pine provenance will be A70
 - Local pine provenance will be Abernethy
 - Local beech provenance will be derived from northern France
 - Suggested temp regime for climate room trials: start decline at week 36, end at 5 °C
 - Some discussion follows on numbers of plants needed for the various assays. 50 plants are needed for molecular analyses per time-point.
 - Mike suggests a terminal budbreak assay, based on degree of lignification as a dormancy test. This discussion is postponed till Friday afternoon
- James Hepburne-Scott shows an interesting presentation, on management and logistics in ALBA nursery. ALBA, leading nursery in the UK, is very progressive in automation, tracking and tracing of plant material flows.
- Eva Stattin presents a general overview of Swedish seedling production, since she will not perform any experiments in the first trial season
- Marc Ravesloot will succeed Bernard Kunneman. He presents measurement and sampling schedules for Boskoop. This first season Boskoop will perform no experiments
- Lonneke van der Geest explains the technology behind cDNA microarrays
- Monique van Wordragen discusses the selection of genes that should be part of the cDNA microarray. PRI will produce this chip during the first project year, based on ATO cDNA libraries

AFTERNOON

- Peter Balk gives an introduction to the workshop and explains the protocol. The need for accurate and clean laboratory practice (RNA degrading enzymes are omnipresent) is stressed
- Workshop

Friday, April 20

MORNING

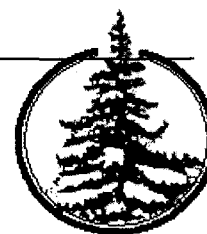
- Workshop is continued; all participants perform adequately.
- Consortium agreement is being discussed. Tony Bouten, legal adviser of ATO is present to answer juridical questions. Several amendments will be made to the draft version
 - A division will be made between research partners and end-user partners, the latter being ALBA and Hedeselskabet, since FE nurseries is part of Forestry Commission. The meaning of those terms will be made clear in article 2. **ACTION Monique and Tony**
 - Article 8.2 and 8.3 will be rewritten in such a way that research partners will obtain ownership of the results, whereas end-user partners will have all access rights and the right to a first offer for license agreements on favourable conditions. Benefits of commercial exploitation of project results, obtained via efforts of more than one partner will be divided based on relative investments. In general the overall budget will be the measure for this. **ACTION Monique and Tony**
 - All partners will produce a short description of relevant pre-existing, private knowledge. **ACTION All**
- Lonneke has arranged a tour around PRI genomics facilities such as the cDNA micro-arrayer and proteomics equipment.

AFTERNOON

- Decisions are made on testing procedures
 - Dormancy will be evaluated using a bud break test, evaluated 3 times/week. This is the only truly reliable assay. Peter writes standard procedure. **ACTION Peter**
 - Cold tolerance will be measured using a freezing test and SELdiff/REldiff. Two testing temperatures will be used (-25 °C and -15 °C), particular for the climate room experiments. Top sections will be used for pine and a nodal section will be used for beech. Autoclaving will be done for 30 min. minimum. Mike will write a standard operating procedure. **ACTION Mike**
 - For each sampling point 15 plants will be used
 - For molecular sampling 2 x 25 plants will be needed for each sampling point. Even if this results in too much material still, top buds from 25 plants will be mixed for each RNA isolation.
 - Standard cold storage will be at +3 °C in autumn and -1 °C, starting from December 1.
 - Morphological assays (if applicable) will comprise measurements of height, root collar diameter, shoot collar diameter and dry weight.
 - Data will be recorded in Excell files: dates vertical, treatments horizontal
 - Mike suggests the use of Sigma Plot for graphic representations.
 - A general protocol will be made for growing conditions; Marc will write this. Peter will use a different protocol because of his specific situation. **ACTION Marc**
 - Seeds of standard provenances for use in 2002 will be shipped in January, before pre-treatment
 - Shipping of molecular samples: samples will be shipped to PRI, following the instructions in the manual
 - Labeling: Samples for molecular analysis will be labeled using a standard format. Experiments will be numbered using 3 digits, of which the first is the partner number. Eg: Eva's first trial will be numbered 301. For each sample this is followed by a low-key letter for tissue type (b = (top) bud, r = (lateral) root, s = stem, l = leaf or needle) and a 4-digit code for sampling week and year, eg: 4801 is week 48 in 2001. Bud tissue from Eva's first trial harvested in this week will thus be labeled: **301b4801**
 - Data storage. A common format is needed which allows for cross reference between molecular and physiological data. Monique made a set-up in MS-Access, but this will probably not be adequate. Monique will investigate whether an IT professional should be asked to make the format. **ACTION Monique**
 - Next general meeting will be organised by Mike. The date depends on the UK Easter holidays break and will be communicated by Mike **ACTION Mike**
 - The first management meeting will be organised by Peter, probably on Friday October 12. This will be confirmed by Peter **ACTION Peter**

ANNEX 2a**COLDTREE**

The application of cDNA microarray technology for unraveling molecular events underlying dormancy and cold hardiness in forest tree seedlings

**E-Update Nr. 1**

22 October 2001

Progress report concerning the period 1 March 2001 – 1 October 2001

General

The project officially started 1 March 2001 and in August this was confirmed by signing the contract with the EC. The kick-off meeting was held in Wageningen, 19 – 20 April. The minutes of this meeting have been distributed to all partners and the responsible EC official. On October 12, DIAS hosted the first COLDTREE management meeting. The report on that meeting accompanies this E-Update.

The schedule below lists the active workpackages of the first 7 months of the project. Further down the activities carried out by each participant are explained in more detail.

WP number	WP titel	Partners involved
WP1	Climate chamber experiments pine	FC
WP2	Climate chamber experiments beech	DIAS
WP3	Field experiments pine	FC, DIAS
WP4	Field experiments beech	DIAS, FC
WP5	Production and screening of a pine cDNA microarray	ATO, PRI
WP8	The effect of provenance, climate and age	PPO (formerly RSNS), DU

Agrotechnological Research Institute – WP5*Progress towards the production of a cDNA microarray:*

ATO has been active in collecting the clones that will constitute the cDNA microarray. For this several sources were used

- An already existing cDNA library made from dormant pine buds (FULL library) was characterised in further detail and 7 x 96 clones were transferred to PRI
- A subtracted cDNA library enriched for cold tolerance genes (COLD library) was made from pine buds and passed over to PRI for characterisation and selection of 4 x 96 clones
- A subtracted cDNA library enriched for dormancy related genes (DOR library) was made from pine buds and passed over to PRI for characterisation and selection of 4 x 96 clones
- Previously identified differential genes and gene fragments both derived from ATO research (cDNA AFLPs, dehydrins) and public databases (Cell cycle gene *cdc2*, Proline acc. P5CS, Proline acc. P5CR, Dorm. Rel. marker DRM1) were selected, characterised and transferred to PRI for spotting on the array
- For several other genes (CBF, Gibb. CPS, Gibb. GA20 oxidase, SOD, ABI1) isolation is in progress

Plant Research International – WP5*Progress towards the production of a cDNA microarray:*

Three cDNA libraries (cold, dor and full) were produced by ATO, and transferred to PRI. Using PCR individual cDNA clones from these libraries are isolated and purified. Instead of sequencing only a few of the clones we have decided to sequence all clones before spotting them on the array, to ensure the high quality and low redundancy of the genes on the chip. We plan to have 375 cold, 375 dor and 650 full clones on the chip. Other clones from ATO (29 AFLP fragments and 20 other selected clones), and control clones (luciferase and yeast genes) will bring the total number of spots to 1536.

The current status of the cDNA clone production is:

Cold: 300 clones isolated, 120 clones sequenced, 110 clones selected for the chip.

Dor: 300 clones isolated, 120 clones sequenced, 110 clones selected for the chip.

Full: 200 clones isolated, 90 clones sequenced, 85 clones selected for the chip.

We plan to print the chip in December, and do some initial hybridisations (to check the quality of the chip etc) early in 2002. The first samples from the other partners should start arriving in April of 2002.

Dalarna University – WP8

In the first week of June, seed of three origins,

- A70 (lat. 56°32'N, Long. 03°32'W, alt 160 m) – seed orchard
- Sollerö (lat. 61°00'N) – seed orchard
- Åmsele (lat. 64°24'N, alt 200 m) – natural stand

were sown in multipot containers (Plantek, Sweden, 805 seedlings · m²) filled with peat substrate. The seedlings were germinated and grown for the first seven weeks in a greenhouse. During germination the relative air humidity was 85 - 95 % and the air temperature was 20°C. After 11 days the air humidity was lowered to 40-60 % and the night temperature was lowered to 15°C after one month. Fertilizing, which is made by adding a complete mineral nutrient solution to the irrigation water twice a week, started 2 weeks after sowing. The seedlings were moved outdoors and placed on elevated pallets 15 cm above ground in mid-July. In late September, when the two most northern provenances already had set bud, the first frost for the season (-1.5°C) came. The seedlings were watered when air temperature fell below -1°C to avoid freezing damages.

Danish Institute of Agricultural Sciences – WP2, WP3, WP4*WP2: Climate room experiments using beech*Production of plant material:

In week no. 19 pre-germinated seeds of the standard beech provenance 'Bregentved' (orig. DK) was sown in HIKO 265 trays placed outside. During summer approximately 3500 seedlings were raised in accordance with normal nursery practice.

In week no. 33 approx. 3000 of these seedlings were transferred to three climate rooms, where they are being subjected to three different climate regimes.

1. Constant day length (15h), constant temperature (T_{day}=15°C, T_{night}=13°C)
2. As in climate 1, but day length reduced by 1h every two weeks
3. As in climate 1, but temperatures (day and night) reduced by 2°C every two weeks

Sampling and testing of plant material:

Seedling samples from each climate regime have been collected in week 37, 39 and 41. In each sampling week seedlings were tested for shoot and root frost hardiness and dormancy status (bud break test). Besides terminal buds and fine roots have been collected for isolation of mRNA. Preliminary results show that it is possible to obtain mRNA from the material.

WP3 and WP4: Field trials using pine and beech, respectively.

Production of plant material:

Pine: In week no. 20 the standard provenance A70 (orig. UK) and the 'local' provenance 'Lindås' (orig. Norway) were sown in HIKO120ss trays placed in a greenhouse. Some 1500 seedlings of each provenance were produced.

Seedlings were transferred from the greenhouse to an outdoor container cultivation area in week 32 where they will remain during the sampling period. After this autumn's sampling has stopped the remaining seedlings will be grown on until next year's autumn sampling.

Beech: In week no. 15 the standard provenance 'Bregentved' and the local provenance 'Graasten' (orig. DK) were sown by Hedeselskabets Planteskole (partner 6) and cultivated as bare-rooted stock in accordance with normal nursery practice.

Sampling and testing of plant material:

Same procedures as described for WP2.

Results

Until now results are very limited. We have measured shoot and root frost hardiness of all plant types in week 37, 39, and 41 and isolated mRNA from terminal buds and fine roots in these weeks.

Hedeselskabets Planteskole – WP4

Production of plant material:

The activities during this period are described within the report of partner 4.

Applied Plant Research (PPO, formerly RSNS) – WP8

Production of plant material :

3 different provenances of beech have been sown

1. NL2.1
2. Greenhill
3. Bregentved

We tested the vitality of the seeds first. Results were resp. 96%, 44% and 78% germination. We used 40 ton/ha so called "naturecompost". The first chemical weed control took place on 17 May using 1,5 l/ha Simasin. We saw 150 plants per meter bed. Sowing took place on the 14th of May 2001. The results of Greenhill are poor. We hope to have enough plants for the coming years.

Forestry Commission Forestry Research – WP1, WP3, WP4

WP1: Climate chamber experiments using pine

Experiments have been set-up in fully randomised trial looking at interactions between daylength and temperature for dormancy and hardiness development in 1 year old *Pinus sylvestris* L.

WP3 & 4: Field trials using pine and beech

Experiments replicated and randomised for both *Fagus* and *Pinus* species, in the nursery, and include both standard and local provenances.

OVERALL:

FR is currently on the fourth lifting date for all of the aforementioned WP's. No significant difficulty has been met and all assessments continue without delay. At the recent management meeting fine-tuning of protocols was agreed and work continues. Material is being collected for molecular sample preparation and subsequent analysis by partners 1 and 2.

ANNEX 2b**MINUTES COLDTREE MANAGEMENT TEAM MEETING***Årslev, Denmark, 12 October 2001.***Present:**

Per Hove Andreasen, Peter Balk, Peter Brønnum, Lonneke v.d. Geest, Michael Perks, Eva Stattin, Monique van Wordragen.

Opening

- Word of welcome by Dr. Ole Callesen and Peter Brønnum
- Minutes of kick-off meeting are adopted
- Partners agree to send each management team member a cc of bilateral e-mails concerning subjects of general interest, such as methodology or deviations from standard procedures.

Progress

- Progress reports are given by all participants. These reports are represented in the first COLDTREE e-Update, attached to these minutes.
- The seed supply for the season 2002/2003 is arranged. Seeds will be sent in January, before pre-treatment. Amounts will be communicated bilaterally, via e-mail.
- For the next season beech will be cultured bare-root, since this is common practice and according to the technical annex.

Inspection of experiments

- The DIAS Coldtree experiments are inspected. The climate room beech trials already show differential morphology due to the various light/temperature regimes. Also outdoor trials are inspected.

Standard Operating Procedures

- There is discussion on how to interpret bud break in pine in the bud break assay, since at this stage there is no real dormancy. FC has a standard protocol for this based on an accurate description of sequential morphological stages. Mike will distribute this protocol to all partners.
> ACTION MIKE
- The freezing process in the cold tolerance assay varies between FC and DIAS. The main difference is that at DIAS all samples are frozen simultaneously and when the respective required temperatures have been reached parts of the root and shoot samples are taken out. Thawing is not controlled. At FC root and shoot freezing tests are done separately and thawing is controlled as well. Since there is only one controllable freezer, some trees have to be kept at 4 degrees for two days prior to the SEL test. There was some concern of the effect of this storage, especially since the buds have been taken off and a wound response may take place. The general feeling is that these differences will probably have no effect on the outcome of the experiments and in any case be insignificant compared to the physiological variation induced in the experiment. Still Mike will look at the effects of this storage by switching the order of the experiments and letting other samples wait for 2 days..
> ACTION MIKE
- Freezing test samples will be analysed separately and the average will be calculated. Lonneke proposes a calculation method that allows including the experimental variation.
 - First: average controls - this results in a blank for the experiment
 - Next: subtract blank from each separate measurement
 - Finally: average resulting values and calculate standard deviation
 This method is accepted. Per will consult a statistician on the merits of this method compared to other available methods and inform the others of the outcome. **->ACTION PER**
- Samples will be autoclaved for 60 minutes
- All SOPs will be updated by the responsible partners and distributed to the project leaders of all participants. **-> ACTION ALL**

Consortium agreement

- The final alterations of the CA are being discussed. Most important changes concern:
 - an additional section that deals with a situation in which a partner does not want to be involved in a patent that is (partly) based on his results
 - the period of time during which protection of intellectual property rights will have to be maintained after the completion of the project
 - a statement that arranges the PCC to be effective during a certain period after the project ends.
 - Addition of pre-existing know-how for a number of partners.
- Monique will include these changes and will arrange for the CA to be signed. -

> ACTION MONIQUE

- Lonneke has started with setting up the database that will include experimental, physiological, and gene expression data. To prevent confusion all partners will have to use the trial code that was agreed upon during the kick-off meeting
- Partners will send a list of experiments including experimental conditions to Lonneke. ->

ACTION ALL

E.g. Peter will have experiments:

401 - climate room, 6 month old beech, fixed day length at ..hr., fixed temperature at... C.

402 - climate room, 6 month old beech, fixed day length at ..hr., decreasing tempetc...

- Bud break and freezing test results will be sent as a single value + SD per sample point and tissue type.

New EU-initiative

- Monique informs the PCC members of a new initiative towards an EU project that has been started by Reza Yezdani last summer and in which COLDTREE has been asked to participate. The new project should include the main European actors in the field of 'molecular forestry' and would focus on environmental genomics. The aim would be to identify and correlate specific genes and QTLs related to stress. Malcolm Campbell is the intended co-ordinator. Eva has visited Inger Ekberg and David Clapham, putative partners, and discussed the possibilities of co-operation.
- Since the new project is still in the conception stage there is no need to take a decision on participation now. The general feeling is that participation would be beneficial for COLDTREE since it would add value to our results in the form of linked QTLs and expressional information in more species than will be investigated in COLDTREE. However, protection of COLDTREE results will have to be guaranteed properly.
- Monique will keep everyone informed of further developments -> **ACTION MONIQUE**

Next meeting

- Next meeting will be combined with the general annual meeting. This meeting will be organised by FCFR in March/April 2002. Mike will decide on the date for this meeting soon. -> **ACTION MIKE**

ANNEX 3a**COLDTREE**

The application of cDNA microarray technology for unraveling molecular events underlying dormancy and cold hardiness in forest tree seedlings

**E-Update Nr. 2**

2 May 2002

Progress report concerning the period 1 October 2001 – 1 March 2002

General

In January 2002 the Consortium Agreement was adopted and signed by all partners. The first annual meeting and the second management team meeting, taking place on 20-21 March 2002, were hosted by FCFR in Roslin, Scotland. The minutes of these meetings accompany this E-Update. The first annual report and cost summary have been prepared and sent to the responsible EU-official in Brussels.

Forthcoming project meetings

- 3rd management team meeting 11 October, 2002. (Dalarna University, Garpenberg.)
- 2nd Annual meeting 24 and 25 March 2003. (PPO, Boskoop)

The next E-Update (Nr. 3) can be expected in October 2002.

Progress

The schedule below lists the active workpackages of the previous period. Further down the activities carried out by each participant are explained in more detail.

<i>WP number</i>	<i>WP titel</i>	<i>Partners involved</i>
WP1	Climate chamber experiments pine	FC
WP2	Climate chamber experiments beech	DIAS
WP3	Field experiments pine	FC, DIAS
WP4	Field experiments beech	DIAS, FC
WP5	Production and screening of a pine cDNA microarray	ATO, PRI
WP7	Development and application of RT-PCR assays	ATO
WP8	The effect of provenance, climate and age	PPO
WP9	Prediction of storage performance	DU

Individual Progress Summary Reports**Partner 1. Agrotechnological Research Institute (ATO).**

Monique van Wordragen, Peter Balk & Brigitte Verkerk

WP5: Apical bud material was taken from two-year-old *Pinus sylvestris* trees, obtained from a local grower, at the beginning of February (i.e. dormant sample). Some of the trees were cold treated (20°C to -10°C, overnight in a climate room by Partner 5 (PPO formerly RSNS) and an apical bud sample was taken (cold sample). At the beginning of April an apical bud sample was taken from the same batch of trees (release sample).

These samples were used to construct so-called subtracted cDNA-libraries. One of the libraries is enriched for genes that are cold induced (COLD-library) and the second library contains material that is enriched for genes that are related to dormancy development of the apical bud (DOR-library).

From each of these libraries approximately 400 clones were sent to Partner 2 (PRI) for sequence-analysis and subsequent array-construction. Together with this material, 700 clones from a formerly generated cDNA-library (FULL-library), representing genes that are active in dormant bud tissue, were also processed by PRI. Furthermore, a set of cDNA-clones representing AFLP fragments related to dormancy development, dehydrins and superoxide dismutases, were delivered to PRI so they might also be included (spotted) onto the array.

WP 7: In order to be able to quantify gene-expression of selected markers in the future, we have already started analyzing mRNA-content of a specific dehydrin in bud tissue of pine trees, using real-time PCR.

In the forthcoming year, together with PRI, marker genes will be selected, based on results from array hybridisations, and validated (WP5). Furthermore, a cDNA-library will be constructed using *Fagus sylvatica* bud material, preferably from Partner 4 (DIAS). This library will be used to identify beech homologues for the selected markers (WP6).

Partner 2. Plant Research international (PRI).

Lonneke van der Geest and Maurice Konings

A selection was made of 1536 clones from three different cDNA libraries, which were made by Partner 1 (ATO). The libraries contained genes that were expressed in either dormant tree buds (no selection: full library), or was made using an enrichment strategy to increase the percentage of genes specific for cold hardiness (cold library) or dormancy (dormancy library). From these libraries we selected 730 full-length genes and about 360 gene fragments from each of the subtracted libraries. Together with some eDNA AFLP genes, some genes highlighted from a literature survey and approximately 50 reference spots (positive for normalizing dye intensity and negative for calculating background hybridisation) we arrived at 1536 genes which were spotted with 3 pins in duplo. Each spot represented an individual gene for which gene expression could be measured in pine bud RNA. We experimentally tested five hybridisations using RNA prepared by ATO from either dormant, cold induced or metabolically active buds that gave rise to promising results. It therefore appears that the chip will be useful for analysis of gene expression in the samples provided by the other partners.

In the next year the array will be hybridised with RNA samples delivered by partners 4 and 7. Results will be analysed using dedicated software and a initial selection of putatively interesting pine genes will be made.

Partner 4. Danish Institute of Agricultural Sciences (DIAS).

Peter Brønnum and Per Hove Andreasen

During summer and autumn containerised Scots pine (standard prov. A70 and local prov. Lindås) and beech (standard prov. Bregentved) seedlings were raised at the Research Station in Aarslev and bare-rooted beech (Bregentved and local prov. Gråsten) seedlings in a nursery by partner 6. The containerised beech seedlings were transferred to growth rooms in the middle of August (week 33) and subjected to three different climate regimes until week 2 (2002):

Climate 1 (control): long day (15h), high temperature (15/13 °C)

Climate 2: decreasing daylength (15h - 8.5h), high temperature (15°C)

Climate 3: decreasing temperature (15/13°C - 1/1°C), long day (15h).

Every second week, starting in week 37 and until week 2 or 4 (2002), all experimental plant types were tested for shoot and root frost tolerance (SELdiff-25°C/RELDiff-5°C), dormancy (days to terminal bud break) and RNA was isolated from terminal buds and fine roots. Samples of bare-root beech were cold stored each sampling week until planting in April 2002. Beech seedlings from all controlled environment climate regimes developed shoot frost tolerance, but climate 1 seedlings seemed less hardy than climate 2 and 3 seedlings.

Shoots of bare-rooted beech were more frost tolerant than growth room beech seedlings. Provenance differences were not observed. Scots pine shoot developed frost tolerance too and apparently earlier in the "local" provenance Lindås. Development of root frost tolerance (-5°C) in beech was very moderate (or absent), while frost tolerance of pine roots clearly increased from week 43. All experimental plant types had a more or less distinct dormancy period, even the climate 1 seedlings. Dormancy in beech was considerably deeper than in pines, and more distinct in the bare-rooted than the growth room seedlings. Dormancy in the pine provenance Lindås was periodically deeper than in the provenance A70.

The isolated RNA from terminal buds and roots has not degraded, as judged by agarose-gel-electrophoresis. The yield of RNA is sufficient for micro-array analysis (> 40[µg]) except for some preparations from root samples of beech grown in climate chambers.

In 2002 containerised beech seedlings will be transferred to growth rooms in August and cultivated under decreasing day lengths and temperature. The field trial experiment with Scots pine seedlings will be repeated and the field trial with beech also. However, the local beech provenance Gråsten will be skipped and we will study differences between one- and two-year old beech seedlings instead.

The same tests will be conducted as in 2001, but because of the poor frost development and RNA yield in beech roots, we suggest to cancel root frost test and root RNA isolation, at least in this species.

Partner 7. Forest Research (FR).

Mike Perks et al.

During summer and autumn containerised seedlings of *Pinus sylvestris* (Scots pine) (standard provenance A70 and local provenance N401 'Abernethy') and *Fagus sylvatica* [beech] (standard provenance Bregentved) seedlings were raised at the Research Station in Scotland and beech (local provenance Cirencester) seedlings were provided from the nursery of partner 9. Some of the containerised pine seedlings of provenance A70 were transferred to growth rooms in the middle of August (Week 33) and subjected to three different climate regimes until Week 2 (2002):

Climate 1 (control): long day (15h), high temperature (15/13°C)

Climate 2: decreasing daylength (15h → 7h), high temperature (15°C)

Climate 3: decreasing temperature (15/13°C → 5°C), long day (15h).

Every second week, starting in week 37 and until week 4 (2002), all experimental plant types were tested for shoot and root frost tolerance (SELdiff-25°C / SELdiff-15°C / RELdiff-5°C), dormancy (days to terminal bud break) and material for mRNA analysis was collected from terminal buds and fine roots. Samples of containerised 'field-grown' beech were cold stored (at 1 °C) on alternate sampling weeks to be outplanted at the end of April 2002.

Pine seedlings from all controlled environment climate regimes showed development of root frost tolerance assessed at -5 °C, and shoot frost tolerance as assessed by SELdiff at -15°C, but this was not evident at the -25°C assessment temperature. This indicates that plants had not developed hardiness to this level under the controlled environment conditions. Shoot frost hardiness assessed at -15°C indicated that there was a lower level of induced hardiness in climate 1 seedlings over the last four weeks of the trial. Shoots of 'field-grown' pine were significantly more frost tolerant than the controlled environment pine seedlings with effects apparent at all freeze test temperatures. Provenance differences were not observed. 'Field-grown' containerised Scots pine roots developed frost tolerance and there was evidence of occasional response differences between provenances.

Development of root frost tolerance (-5°C) in 'field-grown' beech was absent, while frost tolerance of beech shoots from the outdoor trial showed distinct seasonality, though no provenance effects were found.

All experimental plant types had a more or less distinct dormancy period, even the climate 1 seedlings. Dormancy in beech was considerably deeper than in pines, and the ontogenic pattern of dormancy development was later in the UK provenance than the standard Dutch provenance.

Dormancy in the field-grown pine provenances showed almost identical seasonal variation with the native Abernethy (N401) provenance showing periodically deeper dormancy levels than in the provenance A70 over the second half of the testing period (from Week 45). Dormancy development appeared to be correlated with soil temperature. Under controlled conditions declining temperature led to a decrease in dormancy levels, no clear influence of daylength was evident, and the lack of response was compounded by poor plant response to the conditions favourable for budbreak.

The material collected for RNA analysis, from terminal buds and roots, will be isolated in April 2002.

In 2002 containerised pine seedlings will be transferred to growth rooms in August and cultivated under decreasing day lengths and temperature. The field trial experiment with Scots pine seedlings will be repeated and the field trial with beech also. Additional studies of storage-induced declines in plant health and the potential for the use of fluorescence will be undertaken on an ad-hoc basis

The same tests will be conducted as in 2001, but because of the poor frost development and RNA yield in roots of beech in 2001-2, we also suggest (as does Partner 4) to cancel root frost testing and root RNA isolation, at least in this species.

ANNEX 3b**MINUTES of COLDTREE FIRST ANNUAL MEETING and
SECOND MANAGEMENT TEAM MEETING***Roslin, Scotland, 20 - 22 March 2002.*

Present: Per Hove Andreasen, Peter Balk, Peter Brønnum, Lonneke v.d. Geest, James Hepburne-Scott, Maarten IJspeert, Bent Karlsson, Maurice Konings, Marc Ravesloot, Michael Perks, Colin McEvoy, Stephen Smith, Eva Stattin, Brigitte Verkerk-Bakker, Monique van Wordragen.

Wednesday April 20.**Morning – progress updates**

- Word of welcome by Barry Gardiner and Mike Perks
- Opening address by Monique van Wordragen
- Progress reports from Peter Balk (ATO), Maurice de Koning (PRI), Peter Brønnum (DIAS) and Mike Perks (FCFR). Progress summaries are enclosed in the accompanying E-update 2. No major deviations from the planning, as outlined in the technical annex were reported. Results were discussed, with special emphasis on the climate room experiments since those results were somewhat deviant from the expectation. Pine seedlings hardly became dormant. Beech seedlings showed moderate dormancy development, but this was also true for control seedlings, kept at constant temperature and daylength.
- Bent Karlson presents Hedeselskabets Planteskole. The main business of the nursery is forestry, hedge and shelter plants in a wide range of species and provenances. The nursery has a large own production but is also working as a trade centre with import from and export to most of Northern Europe. HSP invests in product development aimed at improving quality, flexibility and reducing environmental impact.
- Research facilities at the Northern Research Station are inspected, including the climate rooms. Outdoor plots are visited including on-going COLDTREE experiments.

Afternoon – plans for next year

- Eva Stattin (DU) describes preparations for the first experiments regarding frozen storage, that will commence the coming season. She will use three pine provenances including the standard A70. SEL₂₅, REL₅ and budbreak assays will be performed. There is some discussion on how many separate samples are needed to obtain a reliable REL figure. 15 (as directed in the SOP) is not feasible because of the workload.
- DIAS will start the next climate room experiment in August. Since the results from year 1 showed that plants in controlled environment develop only moderate dormancy and cold tolerance the discussion focussed on whether or not to hold on to the experimental scheme as described in the TA. This discussion was postponed until the management meeting. Field trials will be repeated but only the standard beech provenance will be used since the two provenances behave identical. Instead differences between 1- and 2-year old seedlings will be studied. Also, in beech no REL will be performed since the species does not develop root frost tolerance.
- Marc Ravesloot describes PPO plans for next season. A second sowing of 3 beech provenances will be done in addition to the first sowing in the previous year, resulting in seedlings of 0.5 and 1,5 year age in the winter of 2002/2003. Physiological parameters will be measured on these seedlings of the 3 provenances.
- Mike Perks informs the group on FCFR plans for the coming season. First priority is RNA isolation from samples taken during the past season. Final decision on the climate room trials will be taken during the management meeting. The field trials with beech and pine will be repeated. Additional studies on storage induced declines in plant health will be undertaken. In beech no REL will be performed since the species does not develop root frost tolerance.

- Peter Balk explains the ATO workplan for the next year. ATO will be involved in the analysis of array results and the selection and further characterisation of putatively interesting pine genes. In addition work on beech cDNA libraries will start and initial selection of beech homologues for selected pine genes will be undertaken. Also, ATO will continue to work on fast and reliable RT-PCR tests for selected genes.
- The workplan of PRI is explained by Maurice Konings. PRI will start hybridisations of the array. The decision on which samples to take, will be made during the management meeting. Data will be analysed using specialized software and in close consultation with other partners a selection of most interesting genes will be made. PRI will participate in the characterisation of those genes. In addition PRI will make a database format and integrate trial data and experimental test values of DIAS and FCFR.

Closing of the annual meeting research forum. Monique thanks Mike, Colin and Stephen and FCFR for their hospitality

Thursday April 21.

Morning

The morning was devoted to a visit to Alba Trees, one of the two nurseries that participate in the project. Alba is a young, but very successful nursery that has fully adopted the containerised growth method. Alba is unique for this in Scotland. The nursery cultures a wide variety of species and provenances. The COLDTREE group enjoyed a full tour around the nursery facilities and received detailed explanations on the growing schedules and logistics. Alba uses sophisticated, in house developed software for tracking and tracing of seedlings and stock management.

Afternoon

The afternoon was used for the second Coldtree Management Team Meeting.

Present: Peter Brønnum, Lonneke van der Geest, Mike Perks, Marc Ravesloot (guest), Eva Stattin and Monique van Wordragen

- The minutes from the previous meeting were adopted
- The workplan for the next 6 months was discussed in detail and decisions were taken on the following subjects:
 - **climate room experiments coming season**
 Since dormancy development was very poor in seedlings grown in controlled environment it was decided to deviate slightly from the technical annex in order to ensure the production of dormant and cold tolerant plant material. 3 climate regimes (for both beech and pine) will be applied in the next season:
 - constant daylength and temperature (control 1)
 - decreasing daylength and temperature (control 2)
 - decreasing daylength and temperature but with a sharp drop in temperature introduced in week 40. Keep constant for 2 weeks and revert to normal scheme (as in control 2)
 This schedule will allow for the identification of genes involved in pre-programmed dormancy (control1) and genes triggered by external conditions ((control 2) – (control 1)). The cold shocked plants will reveal genes that are important in environmentally induced cold tolerance development. **➔ ACTION PETER AND MIKE**
 - **selection of samples for the array**
 It was decided not to use the pine samples from the climate room experiments for initial hybridisations of the array. These plants did not develop normal dormancy or cold hardiness and are thus not suited to reveal the genes involved in those processes. However the pine field trials were very successful. There was a consistent development in dormancy and hardiness, which was highly reproducible in Scotland and Denmark.

It was decided to use the full range of samples from the A70 field grown pine trees from both Scotland and Denmark for initial hybridisations. This will make it possible to eliminate local influences and select only those genes that are consistently related to dormancy in both locations. Mike and Peter will send the RNA from those samples to Lonneke. ➔

ACTION PETER AND MIKE

- **reference material for hybridisations**

A large amount of reference RNA is needed for the array hybridisations. Each hybridisation will be done with two separately labeled probes: one from the sample of interest and one common reference. This will allow mutual comparison of the samples. The reference should preferentially be made of pine bud tissue in various stages of dormancy and hardiness.

- **Database**

Lonneke will make an Access format for the database and based on that an Excel format for data transfer. The Excell format will be send to all. ➔ **ACTION LONNEKE**

Mike and Peter will send details on experimental set-up and physiological data to Lonneke.

Chillsum will be defined as Nr of hours below 5 °C. ➔ **ACTION MIKE AND PETER**

- **Seeds**

Marc did not receive Bregentved beech seeds. Bent will be able to supply them. Mike will send A70 seeds to Eva and Peter. ➔ **ACTION BENT AND MIKE**

- **EU matters**

Cost statements and individual progress reports will have to be prepared and sent to Monique in the coming two weeks. Monique will make the consolidated report and send it to Brussels. Several initiatives have been started in the field of forestry genomics in relation to FP6. It is important to be involved. Monique will contact the initiators and will try to link the Coldtree group to relevant actions. ➔ **ACTION MONIQUE**

- **Other agreements**

Mike will update the various SOPs and send them to all. ➔ **ACTION MIKE**

- **Next meetings**

- Next management team meeting will be hosted by Eva Stattin and the Dalarna University and is scheduled for 11 October, 2002.
- The second Annual meeting will be hosted by Marc Ravesloot and PPO-Boskoop and is scheduled for 24 and 25 March 2003.

ANNEX 4**COLDTREE**

The application of cDNA microarray technology for unraveling molecular events underlying dormancy and cold hardiness in forest tree seedlings

**E-Update Nr. 3**

28 October 2002

Progress report concerning the period 1 March 2002 – 1 October 2002

General

The third management team meeting, has taken place on 11 October 2002, hosted by Eva Stattin and Dalarna University in Garpenberg, Sweden. The minutes of this meeting accompany this E-Update. The second trial season has started and the first array results are available. The project is rolling!

Forthcoming project meetings

- 2nd Annual meeting 17 and 18 March 2003. (PPO, Boskoop)

The next E-Update (nr 4) can be expected in April 2003, after the annual meeting.

Progress

The schedule below lists the active workpackages of the previous period. Further down the activities carried out by each participant are explained in more detail.

WP number	WP titel	Partners involved
WP1	Climate chamber experiments pine	FC
WP2	Climate chamber experiments beech	DIAS
WP3	Field experiments pine	FC, DIAS
WP4	Field experiments beech	DIAS, FC
WP5	Production and screening of a pine cDNA microarray	ATO, PRI
WP6	Isolation of beech homologues to selected pine genes	ATO
WP7	Development and application of RT-PCR assays	ATO
WP8	The effect of provenance, climate and age	PPO, DU
WP9	Prediction of storage performance	DU

*Individual Progress Summary Reports***Partner 1. Agrotechnological Research Institute (ATO).**

Monique van Wordragen, Peter Balk & Brigitte Verkerk

WP5: RNA of cold-stressed and dormant pine buds was extracted and delivered to PRI for use in test hybridisations. A pine glucan-synthase gene fragment was picked up using a PCR-based approach. The enzyme is known to be involved in blocking cellular communication, one of the key-events in dormancy induction (ATO, unpublished data).

Using the RACE method the fragment was extended to 1,5 kb, but is not full length yet. An expression profile of this gene will be made using realtime PCR on samples from DIAS and FCFR.

WP 6: As a first step towards the identification of beech homologues a cDNA library of beech bud RNA, sampled in week 37 through week 4, provenance Graasten, has been made. Depending on the availability of material we will also make subtracted libraries.

Based on the test hybridisations of the pine array a list of putatively relevant genes has been made as an initial guide for the selection of beech genes. The genes present on this list are consistent with the hypothesis that lower temperature results in more rigid membranes, which become more permeable for calcium. The calcium influx triggers several expression cascades.

WP 7: Dehydrin gene family has been used as a model for the development of markers using RT-PCR. Several members of two dehydrin gene-classes are present on the array. The two classes show distinct expression patterns: the K2 genes are induced upon dormancy induction (starting from week 47) whereas expression of SK genes decreases in dormant buds. For both classes specific primers have been made. Expression varies, but in all samples the ratio K2/SK is below 1 in non-dormant buds and above 1 in dormant tissue. Therefore this primer pair is a promising candidate for a dormancy indicator assay.

In the next 6 months, together with PRI, marker genes will be selected, based on results from array hybridisations, and validated (WP5). Based on this result, beech genes will be selected from the cDNA libraries, either or not via a beech cDNA array (WP6). The dehydrin primer pair will be developed into a assay that can be used by DIAS, FCFR, DU and PPO in there own experiments (WP7).

Partner 2. Plant Research International (PRI).

Lonneke van der Geest and Maurice Konings

In order to produce sufficient tree bud RNA to use as reference in the microarray hybridisations 5000 buds were harvested April 2002 from three year old trees from the Netherlands. Additionally 3000 buds were harvested by partner 4 and sent to PRI. From samples of these buds RNA was isolated and expression patterns were compared on the microarray. Expression profiles in the Dutch buds had gene expression profiles in common with the "release" sample tested before, while the Danish buds had a more dormant gene expression profile. Mixed together, these two RNA batches should prove to be a well-balanced reference sample. In total 8000 μg RNA was isolated for use as reference (representing >1000 buds).

RNA samples from field trials 2001 were received from partners 4 and 7. These were analyzed for quality and quantified using RiboGreen dye. For most of the samples sufficient RNA was provided to do a single hybridisation experiment using 40 μg RNA. In order to perform a swapped dye experiment, 80 μg additional RNA is required, which is available for only a limited number of samples. For selected samples a swapped dye will be performed, while for most timepoints only a single hybridisation can be performed. Four different samples (week 39 and 51 from A70 from partners 4 and 7) were labeled and hybridised onto the microarray. Week 39 buds from both partners were not yet cold tolerant or dormant, while in week 51 on both locations buds were fully cold tolerant. Expression profiles from the two samples that were harvested in the same week on different locations were almost identical, indicating that gene expression can be used to measure gross physiological state. Now that it is clear that RNA from buds grown at different locations and isolated by different scientists can be used for successful hybridisations, additional samples will be analysed to measure gene expression in samples that have more subtle differences in physiological state. For partner 4 the full range of samples from both provenances (which differ physiologically) will be analysed on the microarray. For partner 7 three to five time points of different physiological status will be selected in order to correlate expression pattern to the full set of data from partner 4. In addition an experiment will be performed comparing an RNA sample from dormant beech buds with a sample from non-dormant beech buds to assess whether the microarray can be used for beech.

Partner 3. Dalarna University (DU)*Eva Stättin*

In the end of May, seed of three origins;

- A70 (lat. 56°32'N, Long. 03°32'W, alt 160 m) – seed orchard
- Sollerö (lat. 61°00'N) – seed orchard
- Åmsele (lat. 64°24'N, alt 200 m) – natural stand

was sown in multipot containers (Plantek, Sweden, 805 seedlings/m²) filled with peat substrate. The seedlings were germinated and grown in a greenhouse for the first five weeks. During germination the relative air humidity was 85 - 95 % and the air temperature was 20°C. The air humidity was lowered after 11 days to 40-60 % and the night temperature was lowered to 15°C after one month. Fertilizing, which is made by adding a complete mineral nutrient solution to the irrigation water twice a week, started 2 weeks after sowing. The seedlings were moved outdoors in early July and placed on elevated pallets 15 cm above ground.

The seedlings of the same provenances that were sown in 2001 and over-wintered outdoors have been cultivated outdoors according to standard nursery protocol during this summer. In early June, all seedlings were spaced out - still in the original Plantek container - to 400 seedlings/m². In early August, the seedlings to be tested as 3-year-old seedlings in 2003, were transplanted into larger pots.

In week 36, the biweekly testing of freezing tolerance and dormancy in 1- and 2-year-old Scots pine seedlings started (WP 8). Every second week until week 48 the following tests will be made:

- assessment of shoot and root freezing tolerance through the freeze-induced electrolyte leakage method
- collection of buds for later isolation of mRNA
- budbreak-test

In week 40, 1- and 2-year-old seedlings of all three provenances were transferred into frozen storage. The root and shoot freezing tolerance will be assessed, bud-break tests will be performed and mRNA will be collected from these seedlings during and after storage (WP 9).

Preliminary results from week 36, 38 and 40 are summarized below.

Freezing tolerance:

In early October, none of the tested seedlings had reached the shoot (SFT) or root (RFT) freezing tolerance needed for long term frozen storage. The limit set is $SEL_{diff-25} \leq 5$ and $REL_{diff-25} \leq 7$.

Provenance effects:

There was a clear difference in both RFT and SFT, especially for 1-year-old seedlings, between the provenances with northern provenances being more freezing tolerant compared to southern.

The rate with which the SFT and RFT have increased was almost equal for all provenances

Age effects:

The 1-year-old seedlings had a distinctly lower RFT than the 2-year-old seedlings. This age effect was also demonstrated in SFT but not as distinctly for the two northern provenances.

Budbreak and budset:

In early September when seedlings were taken from outdoors into the greenhouse for assessment of budbreak all 2-year-old seedlings had set bud while the 1-year-old seedlings still had not reached this stage. Still in early October, only 6 of 15 of the 1-year-old seedlings of the most southern provenance had set bud.

Provenance effects- budbreak:

Seedlings of southern origin broke their buds earlier (2-year-old) or after a shorter rest (1-year-old) compared to seedlings of more northern origin.

Age effects - budbreak:

It took longer time for 2-year-old seedlings to break the bud compared to 1-year-old seedlings.

Partner 4: Danish Institute of Agricultural Sciences*Peter Brønnum**Summary of activities in WP2, WP3 and WP4:*

Due to difficulties with obtaining dormant Scots pine material in Holland to be used as reference RNA in the array hybridisations, approx. 2000 pine buds, provided by HSP, were collected and frozen in week 14 and shipped to PRI on dry ice together with RNA collected during autumn 2001.

In week 19 approx. 2000 beech and 2000 Scots pine seedlings were sown in containers. Beech were cultivated outside and pine in a greenhouse until the middle of August.

In week 33 the Scots pine seedlings were transferred from greenhouse to open air to be grown ambient climate conditions. In the same week beech seedlings were transferred to climate rooms for further cultivation under two different climate regimes:

- climate 1: constant temperature and daylength: 15C/13C (day/night); 17 h day.
- climate 2: decreasing temperature and daylength (15C/13C → 1C/1C; 16 h → 8.5 h day).

The biweekly sampling of shoot and roots (pine only) freezing tests sampling of buds for RNA-isolation commenced in week 37.

In week 39 a two weeks cold spell treatment (6C/4C; 13 h day) of climate 2 beech seedling was started. Half of the seedlings will be tested in week 41 while the other half will be put back in climate 2 for two weeks (41 and 42) and tested in week 43.

Partner 6. Applied Plant Research (PPO, formerly RSNS)*M. Ravesloot, M. IJspeert, Jan Brouwer, Gosja de Winter**Provenance effect (WP8):*

The aim of this trial was to evaluate the performance of the DNA test under conditions where the hardening process might interfere with plant specific genetic properties and with changes in physiological conditions caused by age and climatic conditions.

Sowing

To the 3 different provenances of beech we decided to add an extra provenance, NL 't Loo. This was done because the germination of the standard provenance, Bregnevet was poor. The risk of not having a good standard would cause difficulties in comparing results.

So eventually 4 different provenances of beech have been sown.

4. NL2.1
5. Greenhill
6. Bregentved
7. NL 't Loo (extra)

Weed control

In 2002 weed control was carried out mechanically, and two times by hand.

Fertilization

- 40 t/ha naturecompost was added before sowing and transplanting the 1a1 plants.
- 200 kg/ha K₂O MgO (patentkali) standard
- Extra N supply NH₄NO₃ CaCO₃ (KAS) up to max. 80 kg/ha after soil N measurements.

Local field damage

On the field we had some damage (bad growth) caused by two times 40 mm heavy rainfall in a very short period time.

Planning measurements

At each sampling date 20 plants per provenance-age combination are used for REL_{diff-25} SEL_{diff-25} and EC analyses (4 replicates of 10 seedlings at the two temperatures (room and frost). For mRNA analysis 20-25 plants will be sampled. Foto's will be taken at each sampling moment (visual control of the bud stadium)

Field climate

By loggers registered, microclimate between plants at 10 cm above soil surface (t, RH), and 10 cm below soil surface (t).

Results

Not after measurements in the autumn and begin wintertime.

Partner 7. Forest Research (FR).

Mike Perks et al.

During summer and autumn containerised seedlings of *Pinus sylvestris* (Scots pine) (standard provenance A70 and local provenance N401 'Abernethy') and *Fagus sylvatica* [beech] (standard provenance Bregentved) seedlings were raised at the Research Station in Scotland and beech (local provenance Cirencester) seedlings were provided from the nursery of partner 9. Some of the containerised pine seedlings of provenance A70 were transferred to growth rooms in August (Week 35) and subjected to one of two different climate regimes until Week 51 (2003) at which time a review of the data will determine if further evaluation in 2004 is necessary:

Climate 1 (CONTROL): long day (15h), high temperature (15/13°C)

Climate 2 (DECLINE): decreasing daylength (15h → 8h), decreasing temperature (15/10°C → 5°C). The day/night temperature difference has been altered (relative to the Technical annex) in response to the Year 1 results and literature information on seasonal development in Scots pine.

Two other 'short shock' treatments are intended for this season:

Climate 3 (SCOLD): Plants previously held under the DECLINE regime are transferred to 14h daylength and low temperature (6°C day & night). Standard analysis protocol assessments (*see below*) will be carried out after 1 and 2 weeks duration under SCOLD treatment. Some extra plants will be returned to DECLINE to allow the time at which treatments merge (in physiological terms) to be evaluated.

Climate 4 (SWARM): Plants previously held under the DECLINE regime are transferred to long daylength and high temperature (*ie.* CONTROL). Standard analysis protocol assessments (*see below*) will be carried out after 1 weeks duration under SWARM treatment.

Every second week, starting in week 37 and until week 51 all experimental plant types were tested for shoot and root frost tolerance (SEL_{diff-25°C} / SEL_{diff-15°C} / REL_{diff-5°C}), dormancy (days to terminal bud break) and material for mRNA analysis was collected from terminal buds and fine roots. Samples of containerised 'field-grown' beech and pine are being removed to cold storage (at 1 °C) on alternate sampling weeks to be outplanted at the end of April 2003. Declines in plant vitality in storage will be assessed using both chlorophyll fluorescence and electrolyte leakage.

The use of fluorescence based assessments of cold tolerance are also in place, from week 43, for field-grown pine material.

In Year 1 development of root frost tolerance (-5°C) in 'field-grown' beech was absent, so assessments of a -2°C trial are being conducted instead.

The material collected for RNA analysis, from terminal buds and roots, was isolated in June 2002.

MINUTES COLDTREE MANAGEMENT TEAM MEETING



Garpenberg, Sweden, 11 October 2002.

Present:

Peter Brønnum, Lonneke v.d. Geest, Michael Perks, Eva Stattin, Monique van Wordragen.

Opening

- Word of welcome by Dr. Anders Lindstroem
- No comments on minutes of second management meeting (march 2002)

Progress

- Progress reports are given by all participants. These reports are represented in the third COLDTREE e-Update, attached to these minutes. Field and climate-room trials are on schedule. Array analyses and consequently beech gene isolation is a bit delayed because of a personnel-change at PRI.
- Mike introduces a new analyses method for budbreak data. The Kaplan-Meyer method was developed in the medical sector but is very well suited for DBB data. Mike will send details of this method to all PI's, and all will analyse their data in this way. **-> ACTION MIKE**
- The seed supply for the season 2002/2003 is arranged. Seeds will be sent before pre-treatment. Amounts will be communicated bilaterally, via e-mail. Sendings will be announced and a confirmation of receipt will be sent when the seeds arrive.

Inspection of experiments

- The Dalarna Coldtree experiments are inspected. Clear differences between A70 and local provenances are visible in the outdoor pine plots. A70 shows more vigorous growth but is more effected by Swedish winter conditions.

Database

- The data generated will initially be combined in two types of database: physiological database and gene-expression database. Both will use input in excell format.
- A summary of physiological data will be sent to Lonneke for incorporation in gene-expression database
- Mike did an Access course and will evaluate the usefulness of this program
- Monique is exploring a possibility for using strategic budget for the development of a software tool for linking both datasets
- Lonneke already designed a format for a standard Excell datasheet. This has been sent to Mike only. Mike would look at it and include comments and send to all PI's.
PI's will respond/comment before October 30 **-> ACTION ALL**
- Lonneke will desing final format by November 15 **-> ACTION LONNEKE**
- Mike will start using coldtree nomenclature for RNA samples also send experiment nrs to Lonneke **-> ACTION MIKE**
- All data will be combined and compiled before the next management meeting. All will send their data to Monique (including pictures) **-> ACTION ALL**
- Monique will combine them in one dataset and make CD's for all **-> ACTION MONIQUE**

Consortium agreement

- Partners did not receive each others signed signature pages. Monique will look into the problem and arrange for the pages to be sent. **-> ACTION MONIQUE**

Collaborations

- Monique updates MT on two EU-initiative in which COLDTREE participates as a group. Both are Networks of Excellence:
 - GenoSilva on Tree genomics, co-ordinated by Malcolm Campbell.
 - EVOLTREE on ecophysiology and genetic diversity, co-ordinated by Antoine Kremer.
- Christophe Plomion and Malcolm Campbell have asked, via Lonneke, to be allowed to use our data. It's agreed to co-operate but make data available in phases. We will be allowed to use their clones and data in return. These co-operation can be highly valuable for the Coldtree consortium

Dissemination

- Lonneke plans a small paper on test hybridisation results
- ATO is working on a paper on dehydrin gene expression
- Mike and Peter will write two combined paper (one on pine, one on beech) in which the geographic effect on dormancy/cold hardiness development is discussed
- Mike plans a paper on use of fluorescence as a vitality measure
- Lonneke has given a lecture on the COLDTREE approach in a Forest Biotechnology course
- Monique will present COLDTREE at the forestry workshop in the Plant and Animal Genomics conference in San Diego (January 2003)
- ATO (Peter Balk) will attend the BIOFOR conference in November, in Spain, and present COLDTREE results
- Mike considers to attend the SEB conference and present COLDTREE.

Next meeting

- Next meeting will be combined with the general annual meeting. This meeting will be organised by PPO in 24/25 March, 2003.

Monique van Wordragen

ANNEX 5**COLDTREE**

The application of cDNA microarray technology for unraveling molecular events underlying dormancy and cold hardiness in forest tree seedlings

**E-Update Nr. 4**

11 April 2003

Progress report concerning the period 1 October 2002 - 1 March 2003

The 2nd annual meeting

The second annual meeting, combined with the fourth management team meeting, has taken place on 24 and 25 March 2003, hosted by PPO – Tree cluster, in Boskoop, the Netherlands. The meeting was an inspiring event, organised by Hanneke Franssen and Marc Ravesloot. The results from the second trial season were discussed and we evaluated the consequences in relation to the workprogramme for the second half of the project. Also, at the first day we had a guided tour around the assortment garden of the research centre in Boskoop. The second day was devoted to a PCR workshop, in which the first Coldtree dormancy status test was practised. Parallel to the workshop was the 4th management team meeting. The minutes of this meeting accompany this E-Update. In the afternoon we made a tour around Boskoop and visited a specialized ornamental tree auction. Next we were guided around a Dutch nursery, that had automated the operating procedures to a large extent. The meeting ended with a farewell drink at the Research Centre.

Forthcoming events

- The second annual report accompanied by the cost statements, will be presented to Brussels in the last week of april.
- Forthcoming project meetings
 - 5th Management Team Meeting: 10 or 17 October 2003. (FCFR, Edinburgh)
 - 3rd Annual Meeting (provisional date): 22-23 March 2004 (DIAS, Arsliev) *DIAS*
- The next E-Update (nr. 5) can be expected in October 2003, after the Management Team meeting.

Progress

The progress of all participating groups was discussed during the annual meeting. A summary of the presented results can be found below.

In the table the active workpackages of the previous period are depicted.

WP number	WP titel	Partners involved
WP1	Climate chamber experiments pine	FC
WP2	Climate chamber experiments beech	DIAS
WP3	Field experiments pine	FC, DIAS
WP4	Field experiments beech	DIAS, FC
WP5	Production and screening of a pine cDNA microarray	ATO, PRI
WP6	Isolation of beech homologues to selected pine genes	ATO
WP7	Development and application of RT-PCR assays	ATO
WP8	The effect of provenance, climate and age	PPO, DU
WP9	Prediction of storage performance	DU

*Individual Progress Summary Reports***Partner 1. Agrotechnological Research Institute (ATO).***Monique van Wordragen, Peter Balk & Brigitte Verkerk*

WP5: The expression profile of a pine glucan-synthase gene fragment was made using realtime PCR on samples from DIAS and FCFR. The mRNA coding for the enzyme, which was expected to be involved in dormancy, based on its role in blocking cellular communication, was not regulated in response to dormancy or cold hardiness development. We therefore did not extend our study of this gene.

WP 6: The beech library resulted in the selection of several putatively interesting genes such as an ethylene receptor, a methallothionin, an alternative oxidase, a histon 4 and a serine/threonine protein phosphatase. However, the library turned out to be not representative. Therefore a new library has been made that is being characterised at the moment.

WP 7: The expression pattern of representatives of three dehydrin classes (SK2, SK4 and K2) has been analysed in detail and revealed that in particular SK2 and K2 are responsive to dormancy development. K2 shows a sharp increase starting from the onset of endodormancy and SK2 reacts to the same event by decreasing its expression. The ratio between both genes is therefore a good indication for the dormancy status. A straightforward PCR based assay was developed for these markers, that can be used as a dormancy test.

The expression analysis of dehydrin genes and a tubulin gene was also used to validate the array results. Microarray based expression profiles for these genes matched very well with the I-Cycler (realtime PCR) results.

Partner 2. Plant Research international (PRI).*Lonneke van der Geest and Michiel Lammers*

Hybridisations were performed with over 20 samples obtained from the other partners, covering three different provenances and two locations. In order to be able to compare expression of all 1500 genes in the different samples, hybridisations were performed using two different fluorescent labels (Cy5 and Cy3). The samples from the partners were labelled with Cy5, and mixed with a reference sample isolated from Dutch and Danish buds by PRI (1800 ug RNA was prepared to provide sufficient reference) that was labelled with Cy3. Based on the hybridisation profiles of the different samples, genes on the microarray could be divided into distinct classes. A group of genes whose RNA levels declined over time was designated "active genes". This group contained approximately 100 genes, including ribosomal subunits (involved in protein synthesis), ubiquitin related genes (involved in protein degradation), tubulin (involved in spindle formation during cell division), metabolic enzymes, etc. A second group of genes (represented by 113 spots on the microarray) increase in mRNA levels as the season progresses. Expression of these genes rises sharply between week 41 and 45; thus these genes may be correlated to cold tolerance or dormancy. This category, named "early cold tolerance genes" contains a number of stress related genes, such as dehydrins, ABA and stress induced proteins, pathogenesis related genes, etc. Additionally this category contains a number of genes that lack homology to any known genes, transcription factor genes, etc. A third category of genes, the "late cold tolerance genes", represented by about 50 spots on the microarray, show a more gradual increase in gene expression during the early weeks, and reach their maximum levels between week 49 and 51. This class contains genes like embryogenesis abundant protein (involved in desiccation tolerance during late seed development), and many hypothetical or unknown proteins. From these groups of genes 36 representative genes have been selected that can be further tested for their usefulness as marker. It was decided to first carry out additional microarray experiments to validate the results, before RT-PCR assays will be developed.

Partner 3. Dalarna University.*Eva Stattin and Marianne Vernhäll*

During autumn 1 and 2-year-old containerised seedlings of *Pinus sylvestris* (Scots pine) (standard provenance "A 70", local provenance "Sollerö" and northern provenance "Åmsele") seedlings were raised and nursed at the Research Station in Garpenberg .

Every second week, starting in week 36 and until week 48 all experimental plant types were tested for shoot and root frost tolerance ($SEL_{diff-25^{\circ}C}$ / $SEL_{diff-15^{\circ}C}$ / $REL_{diff-5^{\circ}C}$), rest/dormancy" (days to bud break) and material for mRNA analysis was collected from terminal buds. Clear differences between provenances and seedling ages in development of freezing tolerance and rest/dormancy have been established.

Seedlings have been transferred to frozen storage (at $-5^{\circ}C$) in week 40 and 48 and then tested for freezing tolerance and vitality in week 6 2003. Freezing tolerance and vitality will be checked again at the end of storage in late spring 2003. The freezer in which the seedlings are stored has accidentally had a failure which may lead to a second experiment in frozen storage in next winter instead of the planned experiment concerning cold storage.

Partner 4. Danish Institute of Agricultural Sciences (DIAS).*Peter Brønnum*

During summer and autumn 2002 containerised Scots pine (standard provenance A70 and local provenance Lindås) and beech (standard provenance Bregentved) seedlings were raised at the Research Station in Aarslev. Besides, 1- and 2-year old bare-rooted beech (Bregentved) seedlings were raised by partner 6 in a nursery. The containerised beech seedlings were transferred to growth rooms in the middle of August (week 33) and subjected to four different climate regimes until week 2 (2002):

- Climate 1 (CONTROL): day length (DL) =17h, temperatures $T_{day} = 15^{\circ}C$ and $T_{night} = 13^{\circ}C$
- Climate 2 (DECLINE): initially as climate 1, but DL reduced by 1h and T by $2^{\circ}C$ every two weeks until $DL=8.5$ h and $T_{day}/T_{night} = 1/1^{\circ}C$.
- Climate 3: (SCOLD) as climate 2, but seedlings tested after being subjected to a short cold period in week 39 and 40 and subsequently after a 2 week 'recovery' period (week 41 and 42) in climate 2.
- Climate 4 (SWARM): as climate 2, but seedlings tested after being subjected to a short warm period in week 45 and 46 and subsequently after a 2 week 'recovery' period (week 47 and 48) in climate 2.

Beech seedlings from all controlled environment climate regimes developed shoot frost tolerance, but CONTROL seedlings seemed less hardy than DECLINE seedlings. However, long days and high temperature did not prevent the onset of frost tolerance and dormancy development in CONTROL, even though DL had been extended by two hours since the first season (2001-2002). The 2-week cold and warm periods did not affect the development of shoot frost tolerance compared to the development in the parent climate (DECLINE).

Shoots of bare-rooted beech were more frost tolerant than growth room beech seedlings, and 2-year old seedlings developed frost tolerance earlier than 1-year olds. Scots pine shoot developed frost tolerance as well, but contrary to the previous season there was no provenance effect on shoot frost tolerance. Development of root frost tolerance was however earlier in the provenance Lindås.

All experimental plant types had a dormancy period, even the CONTROL seedlings. Dormancy developed earlier in CONTROL than in DECLINE seedlings and was also earlier in 2-year old compared to 1-year olds. Dormancy in beech was considerably deeper than in pines. For Scots pine, dormancy was almost absent in A70, and in Lindås more shallow compared to the previous year.

The initial rate of acclimation (week 37 - 45) was the same over a large range of different environments (climate 1, 2, 3, 4 and nursery), which suggests that a common set of possible molecular markers could be found in seedlings in the same physiological state. The aim should now be to identify the presence of these markers in physiologically equivalent seedlings.

To evaluate these as markers for storability, a further selection should also be based on the relationships found between physiological parameters and field performance, which is currently being analysed.

Isolation of RNA from bud samples collected during autumn awaits the analysis of the physiological data and field performance results of planted beech seedlings.

The field trials of pine and beech will be repeated in a third year (WP3 and 4). Furthermore, potential markers will be evaluated using RT-PCR (WP7), in collaboration with the involved nursery partner (partner 6).

Partner 7. Forest Research (FR).

Mike Perks et al.

We have successfully completed physiological analysis and RNA material collection for containerised pine and beech seedlings. Some seedlings of provenance A70 were transferred to growth rooms in August (Week 35) and subjected to one of two different climate regimes until Week 51 (2003) at which time a review of the data will determine if further evaluation in 2004 is necessary:

- Climate 1 (CONTROL): long day (15h), high temperature (15/13°C)
- Climate 2 (DECLINE): decreasing daylength (15h → 8h), decreasing temperature (15/10°C → 5°C). The day/night temperature difference has been altered (relative to the Technical annex) in response to the Year 1 results and literature information on seasonal development in Scots pine.

Two other short 'shock' treatments were included for this season:

- Climate 3 (SCOLD): Plants previously held under the DECLINE regime are transferred to 14h daylength and low temperature (6°C day & night). Standard analysis protocol assessments (*see below*) will be carried out after 1 and 2 weeks duration under SCOLD treatment. Some extra plants will be returned to DECLINE to allow the time at which treatments merge (in physiological terms) to be evaluated.
- Climate 4 (SWARM): Plants previously held under the DECLINE regime are transferred to long daylength and high temperature (*ie.* CONTROL). Standard analysis protocol assessments (*see below*) will be carried out after 1-week duration under SWARM treatment.

Every second week, starting in week 37 and until week 51 all experimental plant types were tested for shoot and root frost tolerance (SELdiff-25°C / SELdiff-15°C / RELdiff-5°C), dormancy (days to terminal bud break) and material for mRNA analysis was collected from terminal buds and fine roots. Samples of containerised 'field-grown' beech and pine were removed to cold storage (at 1 °C) on alternate sampling weeks (*ie* monthly) to be outplanted at the end of April 2003 (in hand – plants have just been delivered to the experimental site). Declines in plant vitality in storage will be assessed using both chlorophyll fluorescence (pine only) and root electrolyte leakage.

The use of fluorescence based assessments of field-grown pine material for cold tolerance prediction was not possible for technical reasons, in year 2 but will be included in the coming years workpackage. In Year 1 development of root frost tolerance (-5°C) in 'field-grown' beech was absent, so assessments of a -2°C trial were conducted. Again this test did not provide physiologically relevant information so will be omitted in the final year of field testing.

The material collected for RNA analysis, from terminal buds and roots, will be isolated in April / May 2002.

MINUTES COLDTREE MANAGEMENT TEAM MEETING



Boskoop, Netherlands, 25 March 2003.

Present:

Peter Brønnum, Lonneke v.d. Geest, Michael Perks, Eva Stattin, Marc Ravesloot (guest), Hanneke Franssen (guest), Monique van Wordragen.

Opening by Monique

Minutes 3rd MTM

- The minutes of the third management meeting (october 2002) are adopted
- Mike will continue on Kaplan Meyer method for analysis of bud break data. It is decided to set an end-date of 90 days for bud break
-> **ACTION MIKE**

Data analysis

- A standard Excell format is developed by Lonneke, Mike, Eva and Peter. All will paste their data into the file and send it to Monique. Monique will combine the data with expression data and PCR primer info on a cd for each partner.
-> **ACTION ALL**
- Hanneke did not receive the Excell format. Lonneke will send it to her asap.
-> **ACTION LONNEKE**
- Colin developed a key to the Excel format and the abbreviations used. Monique will add this key to the data cd.
-> **ACTION MIKE**
- Monique will discuss the data with ATO bio-informatic specialist and try to have them analysed for common trends
-> **ACTION MONIQUE**

SEL –25

- There is uncertainty on the threshold level for frost tolerance in beech. Decided is that pine is considered frost tolerant at SELdiff-25 <5% and beech at SELdiff-25 <10%.

How to proceed?

- The arrayhybridisations resulted in very clear profiles that are consistent across geographical positions and provenances. Real time PCR confirmed the patterns for a few selected genes. However, the PCR approach is time consuming and less informative. PCR is necessary to make the translation into a practical test, but is considered less suitable for solving the molecular mechanisms underlying dormancy.
- Microarray experiments are nowadays less expensive then during the conception of the project.
- It is therefore decided to deviate from the technical annex and perform more arrayhybridisations and less realtime PCR experiments.
- This decision also affects the work on beech. Beech keygenes will be selected from a small array (ca. 500 clones) and not by searching beech homologues to selected pine genes.

Selection of indicator genes

- We are a bit behind schedule in selecting the pine key genes. Lonneke made a initial selection based on the results from season 2001/2002. This is a good start but the selection should be made more robust by including additional hybridisations.
- Mike, Peter and Eva will analyse their (pine) data and select additional datapoints for microarray analysis. In particular they will look for samples with various backgrounds that still fit the same physiological characteristics, for instance with respect to storability. In addition they will look for samples at and flanking the steepest points in the frost tolerance and dormancy curves.
-> **ACTION MIKE, PETER, EVA**

- Time path:
 - end of April: sets of 2001 sample points are send to Lonneke
 - end of May: 2002 samples are added
 - June: selected beech samples are send to Monique
 - Summer: microarray hybridisation of pine samples
 - September: microarray hybridisations of beech samples
- Lonneke needs samples from non-dormant tissue for comparison. Mike will send samples A70-week 30 and A70-week 14. **-> ACTION MIKE**
- Eva is not able to do RNA isolation and should send bud samples to Monique. It is unclear how to do this safely. Initial ideas (RNAlater) did not result in high quality RNA. Monique will ask Peter (Balk) to search for a safe method. **-> ACTION MONIQUE**

Dissemination

- To avoid confusion on co-authorship the MT adopts the criteria for co-authorship that are used by DIAS. Co-authors should be involved in at least three of the following five criteria.
 1. Significant contribution to creation of the original idea/hypothesis or writing of the project proposal
 2. Significant contribution to designing the specific experiment (plant material, treatments, assessments)
 3. Significant contribution to conducting the experiment, management, plant cultivation and/or data collection
 4. Data processing/analysis and statistics
 5. Writing the paper
- Papers in preparation (leading author):
 - Dehydrin paper (Peter Balk)
 - Physiological comparison in different geographical locations pine (Mike)
 - Physiological comparison in different geographical locations beech (Peter Brønnum)
 - Microarray results season 2001/2002 (Lonneke)
- Forthcoming papers
 - Selection of indicator genes (all)
 - Fluorescence as assay method (Mike)
 - Growth room experiments combined with microarray analysis pine
 - Growth room experiments combined with microarray analysis beech
- Monique has presented COLDTREE at Plant and Animal Genomics conference in January 2003.
- Lonneke will present COLDTREE in Umea
- PPO published Coldtree results in Dutch trade Journal and is preparing another publication
- Mike, Peter and Eva will communicate the existence and results of the project to their respective national forestry sectors.

EU

- Coldtree participates in EVOLTREE, a network of excellence submitted in FP6. This will create opportunities for new collaborations and spin-off projects. Part of the integration activities of the network is meta-analysis on existing datasets, such as the data obtained in COLDTREE.
- The MT decides to offer the COLDTREE data to this effort, under the condition of prior publication by COLDTREE participants.

Next meeting

- Next MT meeting will be in Edinburgh on October 10 or October 17. Mike will decide on the final date

Monique van Wordragen

ANNEX 6

MANUAL

Detection of specific mRNA molecules using RT-PCR

Workshop during the second annual meeting

25 March 2003

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INFORMATION AND SUPPORT

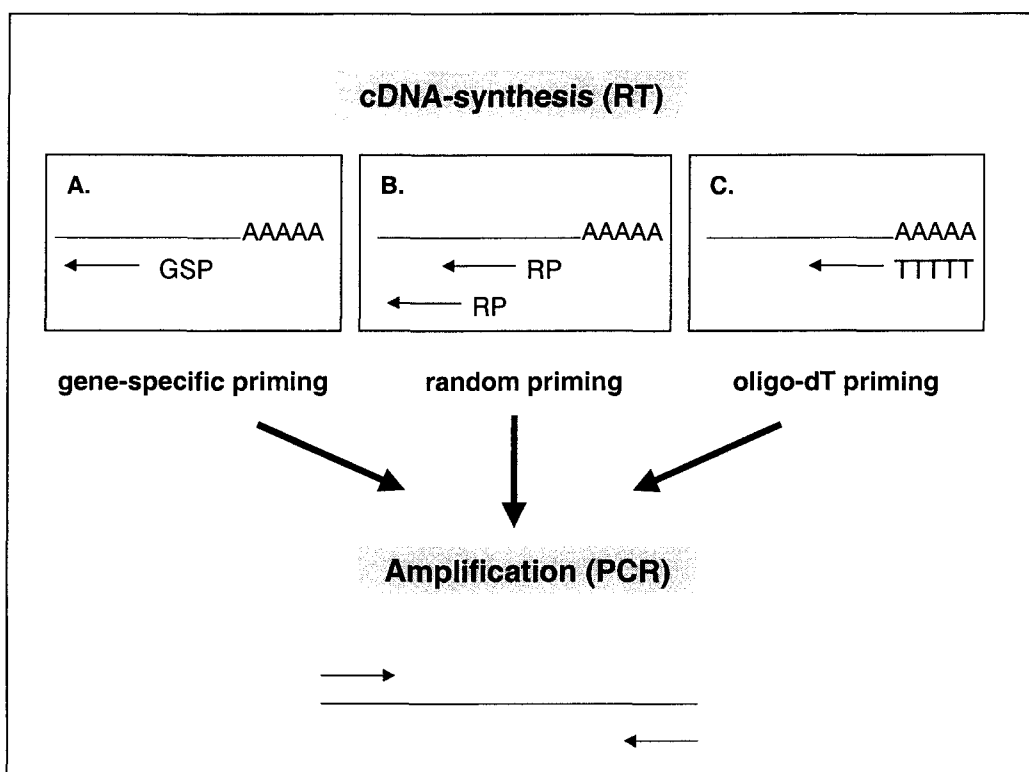
P.A. BALK, E-MAIL P.A.BALK@ATO.WAG-UR.NL

INTRODUCTION

During this workshop we will focus on the detection of specific mRNA molecules using RT-PCR (Reverse Transcription – Polymerase Chain Reaction). The principle of this method is depicted in the next figure. RNA molecules are reverse-transcribed into cDNA that serves as a template for the PCR.

cDNA can be produced using different kinds of primers:

- A. Gene-specific primers. In this case only specific mRNA molecules are being transcribed.
 - B. Random primers. These are mixtures of randomly synthesised oligo-nucleotides. In this case you will end up with a complex mixture of cDNA molecules, originating from all RNA molecules present.
 - C. Oligo-dT primers. In this case cDNA is produced from RNA molecules that carry a polyA-tail.
- In the next step, a specific cDNA is being amplified using PCR. The amount of product that is being formed in early cycles is a measure for the original amount of mRNA molecules present in a specific biological sample.



1.1. Selected Markers

During this workshop we will analyse the expression of two different pine dehydrin genes, Psdhn2 and Psdhn7. The expression level of both genes is indicative for certain stages of dormancy. In early stages the level of Psdhn2 mRNA molecules is relatively high in comparison with the level at later stages. The opposite is true for Psdhn7. In early stages the level is relatively low while in later stages the level is elevated. As long as the difference in mRNA level for each gene is high, at least 10 times, it is detectable.

We will work with two RNA preparations (provided), one from an early stage (week 41 2001) and the other one from a later stage (week 49 2001), both from provenance Lindås.

This set-up is to be considered as a first step towards development of test systems that can be used to specify certain stages of dormancy. Although these two dehydrin representatives can be used, additional markers are needed in order to give more certainty.

1.2. RNA isolation

When using RT-PCR to analyse specific mRNA molecules in your RNA preparations you have to consider the following.

- RNA concentration

Because of the high sensitivity of the method you need only small amounts of total RNA. With the RT-PCR system that we are using it is possible to use as little as 1 µg per reaction. Therefore you can use only small amounts of tissue for the isolation. In this workshop we will take 100 mg of bud material. This will result in enough RNA to perform several RT-PCRs.

- RNA quality

RT-PCR requires a high degree of purity of your RNA preparations. This quality can be achieved using the RNeasy Kit from Qiagen, at least for pine bud material. RNA that is isolated by the CTAB method (Chang, 1993) should also be purified using the RNeasy Kit. It is advisable to check the integrity of the RNA using agarose gel-electrophoresis.

- Uniformity between samples

When several samples have to be analysed for certain mRNA molecules in (semi) quantitative procedures, uniformity of isolation is very important. When you take care of that, your RNA preparations will have comparable yields and quality. Especially when yields are expected to be unequal, quantification of RNA content is advisable before starting the RT-PCR.

- DNA contamination

Although, with the RNeasy kit for instance, the isolation procedure is RNA-specific, chromosomal DNA contamination can occur. This DNA can cause problems during PCR because, in general, primers don't discriminate between cDNA (that originates from your mRNA) and chromosomal DNA. You have to do a control reaction in order to analyse whether your PCR product originates from cDNA or from chromosomal DNA. You have to do this once for every primerset and for every RNA preparation. When you are using a RT-PCR system in which the Reverse Transcription is separated from the PCR you simply use your RNA preparation directly for the PCR. When you then get PCR products you know that they originated from DNA. When DNA gives serious problems than it is advisable to treat your RNA preparation with DNase I, according to the instructions of the manufacturer. After DNase I treatment, it is advisable to purify your RNA preparation (again) using the RNeasy system (Qiagen).

During this workshop we will exercise with the RNeasy Kit as a first step towards preparation of high quality RNA that can be used in RT-PCR. At this moment Qiagen is the only company that sells a kit that can be used for plant material.

1.3. RT-PCR

High quality RNA preparations can be used in RT-PCRs. There are lots of companies that sell user-friendly kits. The two main choices that you have are One-step or Two-step reactions. In One-step systems the Reverse Transcription and the PCR are combined in the same tube whereas in the Two-step systems they are separated. Besides, certain companies work with master-mixes of components that are needed for your reactions while others prefer to deliver the components separated. This gives you the chance to fully optimise your reaction yourself. Using master-mixes is of course very convenient and they guarantee uniformity.

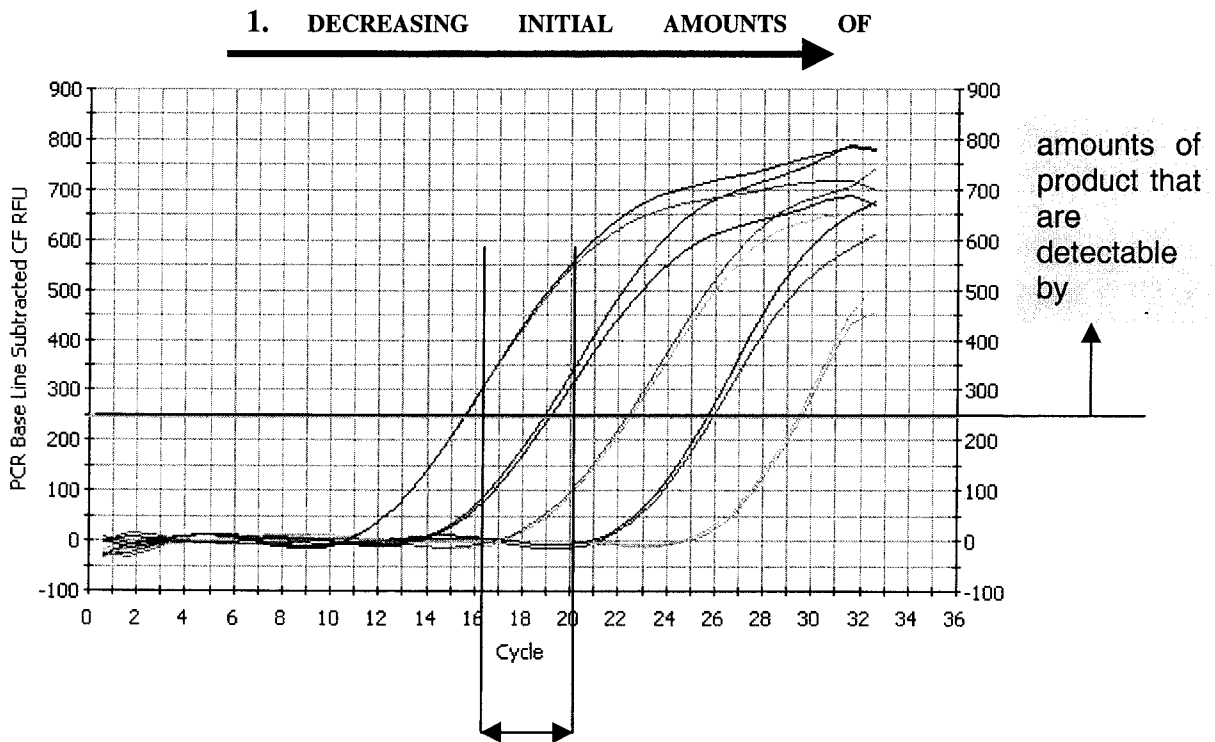
One-step mixes are easy to handle but they have certain drawbacks. It is difficult to control for chromosomal DNA contamination of your RNA preparations. Besides, you can only use a Gene-specific primer for the Reverse Transcription. When you work with a Two-step system you can perform cDNA synthesis using oligodT or random primer mixtures to initiate the transcription. In that case you can use the same cDNA sample for PCRs with various primersets in order to detect different mRNA molecules.

It is also advisable to use so-called hot-start enzymes in the PCR. In general they reduce formation of by-products. You also have the possibility to use thermostable Reverse Transcriptases, which will also increase specificity of the reaction.

During this workshop we will use the One-step RT-PCR system from Invitrogen (Life Technologies) to detect mRNA molecules.

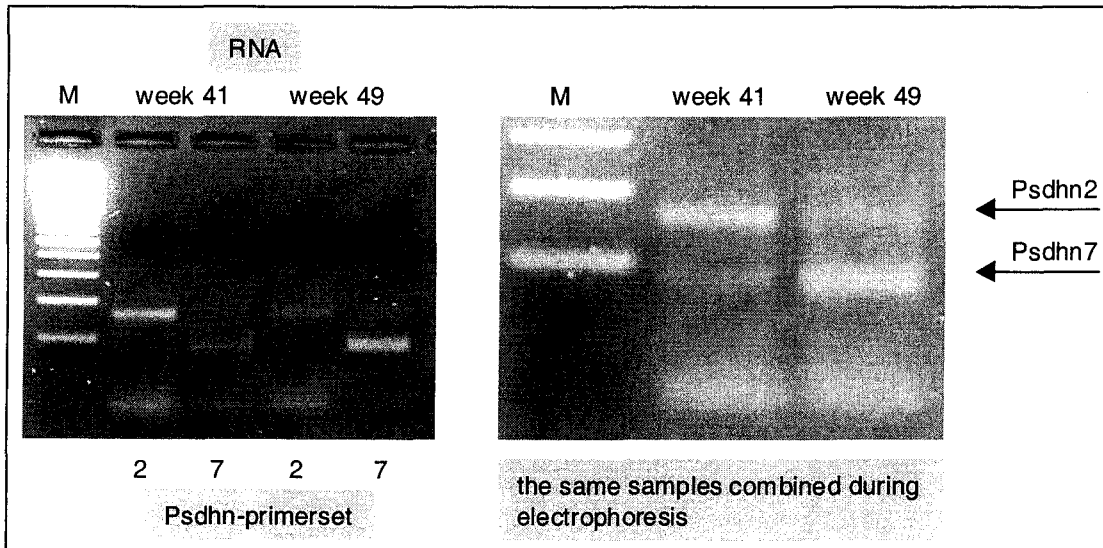
1.4. Analyses of the PCR product

In the picture below you will find typical amplification profiles of a serial dilution (factor 10) of a specific DNA-template. The amount of product that is formed during the PCR is monitored online using Real-time PCR equipment from BIORAD. This technique makes it possible to measure differences in original amounts of templates in several samples over a wide range. In most cases it is not possible to use this equipment. Conventional methods, such as Ethidium-Bromide stained agarose-gels, are to be used to analyse the products that are formed during the PCR. From the profiles below it is clear that the window in which you can measure differences accurately is restricted. For instance, in order to measure differences between the first and the second sample you have to take a sample from the amplification-mixture after cycle 17, 18, 19 or 20. This is mainly because of the fact that at higher cycle numbers the linear amplification stops. The size of this window is also restricted by the sensitivity differences between the methods that are used for the detection of the products (depicted by the horizontal line).



2. ACCURACY WINDOW FOR DIFFERENTIAL ANALYSIS USING CONVENTIONAL METHODS

During this workshop we will compare the expression-levels of Psdhn2 and Psdhn7 in two different RNA preparations from pine apical buds. PCR products will be detected using Ethidium-Bromide stained agarose-gels. In order to catch the described window we have to take samples at several time-points during the PCR. Below you will find a result from a One-step RT-PCR using RNA preparations from week 41 and week 49. In this case the optimal amount of cycles was 25 for both templates. You have to consider that this amount of cycles varies from situation to situation.



2. WORKPLAN TUESDAY 25 MARCH 2003

We will start with the RT-PCR in order to detect two different dehydrin mRNA molecules in provided RNA preparations. Samples will be taken after different rounds of PCR and analysed on agarose-gel. During the run we will isolate RNA from frozen pine bud material using the Qiagen RNeasy system. The general time schedule will be as follows.

- Short introduction
- Thawing the reaction components
- Programming the PCR machine
- Preparation of primer mixtures
- Starting the RT-PCR
- Weighing the frozen bud material
- Preparation of the RLT-buffer
- Performing the purification
- Sampling the RT-PCR and continue amplification
- Preparation of the agarose gel
- Prepare RNA and PCR samples for loading the agarose gel
- Electrophoresis
- Viewing the results

3. PROTOCOLS

Manuals of the manufacturers (Qiagen and Invitrogen) are added as appendices. They form the bases for the protocols that are described here. In general every component that is needed is included in the purchased kits unless stated otherwise.

3.1. RNA isolation using RNeasy (Qiagen)

3.1.1.1. Extraction

- Prepare the RLT buffer. Take 500µl buffer and add 5µl β-Mercaptoethanol (not included) for every isolation.
- Weigh approximately 100 mg frozen bud material in an Eppendorf vial. The tissue must remain frozen during handling (use liquid nitrogen).
- Add 450 µl RLT to the vial containing the frozen material and mix vigorously. From now on you can keep the vial at room temperature.
- Incubate for 3 minutes at 56 °C.
- Centrifuge the vial for 10 seconds at maximum speed in an Eppendorf centrifuge.
- Transfer the supernatant to the provided lilac column and centrifuge again for 2 minutes at maximum speed.
- Carefully, without disturbing the pellet, transfer the flow-through to a new Eppendorf vial.
- Add 0.5 volume 96% ethanol and mix by pipetting.
- Apply this sample to the provided pink column.
- Centrifuge for 15 seconds, maximum speed and discard the flow-through.

3.1.1.2. Washing

- Add 700µl RW1 buffer to the column.
- Centrifuge for 15 seconds, maximum speed and discard the flow-through.
- Transfer the column to a new collection tube.
- Add 500µl RPE buffer to the column (make sure that ethanol is added to this buffer according to the description on the bottle).
- Centrifuge for 15 seconds, maximum speed and discard the flow-through.
- Add 500µl RPE buffer to the column.
- Centrifuge for 15 seconds, maximum speed and discard the flow-through.
- Transfer the column to a new collection tube.
- Centrifuge for 1 minute, maximum speed.

3.1.1.3. Elution

- Transfer the column to a new collection tube.
- Add 50µl sterile water to the column and incubate for 1 minute.
- Centrifuge for 1 minute, maximum speed.
- Analyze 5 µl using agarose-electrophoresis (1.2% agarose) according to standard procedures, stain with Ethidium-Bromide and visualize using UV-irradiation.
- Store the RNA preparation at -20°C.

3.2. One-step RT-PCR

Preparations

Make sure that the PCR machine is programmed so that you can start immediately after combination of the reaction components.

3.2.1.1.

3.2.1.2. Amplification program

50°C for 30 minutes

94°C for 2 minutes

20 cycles of Denature 94°C for 10 seconds
 Anneal 55°C for 10 seconds (primer specific)
 Extend 70°C for 15 seconds

take a sample of 5 µl, mix with loading solution and place on ice

perform 5 identical cycles

take a sample of 5 µl, mix with loading solution and place on ice

perform 5 identical cycles

stop the reaction

take a sample of 5 µl, mix with loading solution and place on ice

Make sure that every solution that is needed is thawed.

Use the following table to determine what reactions have to be done. Add a code to every reaction (rxn) that can be used to label the vial.

template(RNA)	µl	primerset1	primerset2	primerset3	rxns/template
rxns/primerset					

3.2.1.3. Primer mixtures

In every RT-PCR 10 pmol of each primer will be used.
 For every dehydrin that will be detected we need one combination of primers.

Combine the following (for 10 rxns):

- 1µl 100µM forward primer
- 1µl 100µM reverse primer
- 9µl sterile MQ

3.2.1.4. Final Reaction mixture

COMBINE THE FOLLOWING COMPONENTS IN A THIN-WALLED PCR VIAL (ON ICE!)

- 1 μ l primer mixture
- X μ l RNA preparation
- (9-X) μ l sterile MQ

Mix the following components in a separate tube. Multiply the indicated amounts by the number of reactions that you need

- 25 μ l 2X Reaction Mix
- 14 μ l sterile MQ
- 1 μ l RT/Platinum *Taq* Mix

Prepare vials to take samples after the indicated amounts of cycles. They contain 1 μ l of a 6x concentrated loading solution. It is convenient to use a microtiterplate for collection of the samples.

After the last samples have been collected analyze the fragments using agarose-electrophoresis (3% agarose). Stain the gel with Ethidium-Bromide and visualize the result using UV-irradiation.