

Innovative tests for nursery management Unravelling molecular events in dormancy and cold hardiness of tree seedlings to inform operational practice

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THE COLDTREE PROJECT: INTRODUCTION

The COLDTREE project is a first step towards the development of molecular diagnostic tests for cost efficient reforestation and nursery logistics. Sustained yield from Europe's commercially exploited forests requires a supply of millions of seedlings annually (see Figure 1). The planting stock requirement for reforestation, new woodland creation and urban forestry projects is almost 1.7 billion tree seedlings and ornamental woody plants annually in the EU, comprising a total value of £1.4 billion (€2 billion), the bulk of which is produced by European forest tree nurseries. Nursery logistics require a tight scheduling of operations to be able to deliver healthy seedlings to the planting site. A critical step in a modern nursery production chain is the transfer of seedlings to cool or frozen storage. Storage is required to prevent winter damage, to maintain planting stock in an inactive condition for extended periods, and to ensure plant quality where supply is for geographically distinct planting sites. Efficient management requires that operational handling of seedlings, including transfer to cold storage, is implemented at a time that ensures continued plant health while maximising operational flexibility. 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 particular importance that nursery managers are able to determine accurately the peak physiological condition of seedling trees for lifting or transfer.

Assessing seedling quality

Despite the economic importance of such decisions, nursery managers still often rely on traditional (morphological) methods to identify 'plant movement windows' (Mohammed, 1997), i.e. the start of operational practices in winter, cold storage etc.



Figure 1

Aerial view of containerised Scots pine (*Pinus sylvestris* L.) seedlings.

Recently, several physiological measurement techniques have been proposed, some of which are 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 (dependent on specific test) can vary from 2 to over 14 days (Puttonen, 1997). Furthermore techniques developed and routinely applied in UK bare-root nurseries (e.g. root electrolyte leakage, REL; McKay, 1992) are unreliable when applied to containerised production systems. In nursery practice, where lifting opportunities can be severely limited by rainfall, frost and snow, such delays can significantly reduce the number of plants lifted at peak physiological condition. In spring, plants start reversing the processes that protect them during winter before there is a visible sign of regrowth; consequently if they are put into cold storage too late in spring, they experience damage, particularly to the root system. Efficient post-planting establishment and cost-effective nursery management therefore require tools for rapid and reliable determination of the physiological condition of forest tree seedlings such as those shown in Figure 1. Ideally these test procedures should not require high levels of investment or technical knowledge.

Molecular diagnostic tools

To develop such tools a detailed understanding of the cellular and molecular processes underlying cold hardiness and dormancy processes is required (Li *et al.*, 2004). Unravelling the gene expression pattern as a seedling acquires the hardened state will reveal key processes that can be used as indicators to describe the physiological condition of the tree (cf. Pearce, 1999; Thomashow, 1999).

Investigation of these processes and identification of candidate genes was the primary goal of the COLDTREE project (Box 1). Eventually, this effort could result in molecular tests based on the presence or absence of specific messenger RNAs or proteins, that will allow a rapid evaluation of the physiological state of the seedling and will facilitate afforestation and reforestation logistics. Such 'techniques of tomorrow' are not yet available to forest tree nurseries. Little knowledge of the molecular nature of the intertwined processes of dormancy and hardiness in woody species exists. In order to identify molecular mechanisms involved in winter hardening in woody plants the COLDTREE project participants employed the powerful cutting-edge technology of cDNA microarrays, also known as DNA chips, which allows the monitoring of thousands of mRNAs simultaneously (see Figure 2).

Box 1

COLDTREE project: summary of objectives.

To identify novel physiological and genetic techniques indicative of the onset of winter hardiness and dormancy in woody species and, using cDNA microarrays, 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 stages of dormancy and hardiness.

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

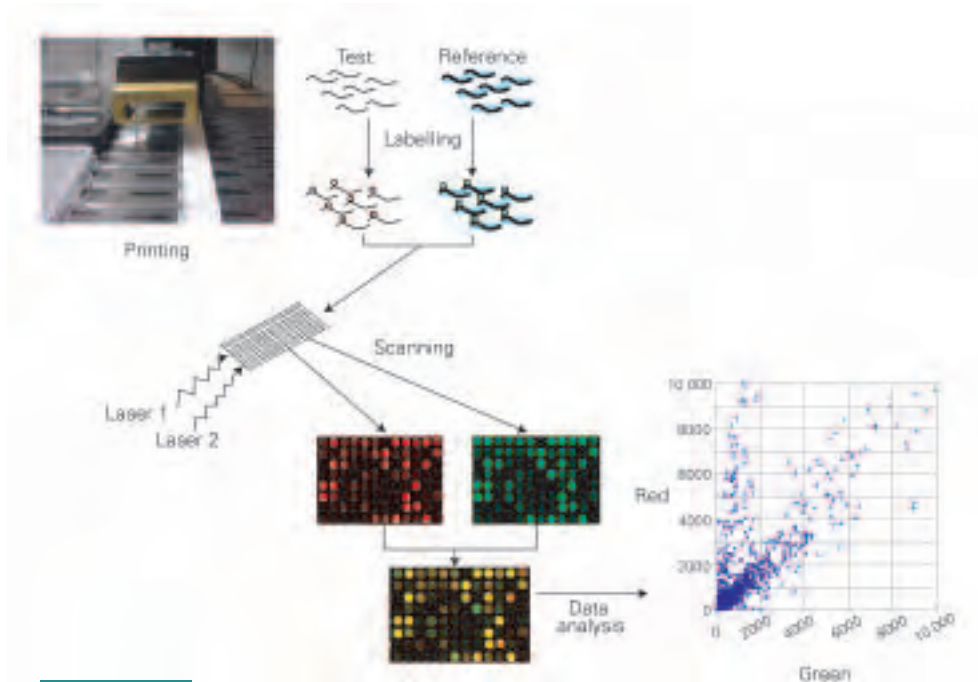


Figure 2
 Schematic of the microarray probe technology. A ‘chip’ with 1500 wells has target genes inserted. The plant tissue extract is introduced to each well and if the target gene is functioning (upregulated) or switched off (downregulated) fluorescence occurs when laser-scanned.

This technique was used to detect gene transcripts characteristic for the dormant or active phases in Scots pine (*Pinus sylvestris*) and common beech (*Fagus sylvatica*). These two economically important forest trees were chosen as model species to represent coniferous and deciduous European trees. Relevant mRNAs were selected and characterised to unravel molecular pathways involved in the process of winter hardening. Seedlings were grown in climate-controlled environments for the initial identification of relevant genes. Outdoor trials were also performed to detect the effect of climatic conditions, geographical location (across a north European ecocline) and provenance on the underlying molecular processes. Plant materials (buds, shoots and roots) collected during these trials were analysed both physiologically, employing physiological assessments of cell damage, and for gene expression, by cDNA microarrays and polymerase chain reaction (PCR) technology. Together, this data allowed the creation of a detailed picture of physiological and molecular events characteristic of the onset and release of cold hardiness and dormancy, and the effect of environmental factors on these processes.

Furthermore, the research resulted in a set of selected genes with a strong predictive value for cold acclimation and dormancy in the tested plant species. These genes can be used for the future development of an ‘off-the-shelf’ plant hardiness test-kit that will support nursery management decisions and facilitate forestation logistics.

cDNA microarray

Several cDNA libraries, either enriched for cold tolerance or dormancy related genes, were used to construct a cDNA microarray for pine bud tissue. Fifteen hundred clones were sequenced and blasted and contig-analyses were made to ensure the maximum number of different genes.

To test the cDNA microarray five preliminary hybridisations were performed using samples that represent the extremes from the physiological spectrum that will be analysed in the COLDTREE programme (Figure 3). These tests resulted in clearly distinguishable gene expression patterns. Different groups of genes could be identified, some associated with cold stress, some associated with dormancy, and some specific to release of dormancy.

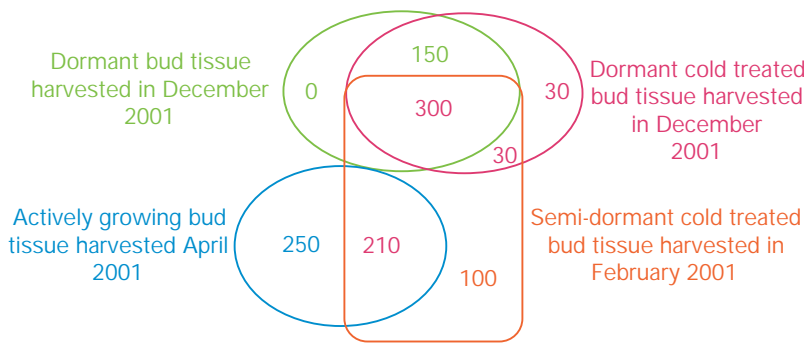


Figure 3

Gene expression collected from plants obtained under four different conditions was measured relative to a reference sample. Groups of genes that show expression levels that are higher than the reference are indicated in this Venn diagram. Circles represent samples, numbers in circles indicate the number of genes that are upregulated. Overlapping regions indicate genes that are upregulated in more than one sample.

COLDTREE achievements

Molecular monitoring of forest tree seedling quality will aid in the development of improved planning in the forestry sector. At present, it is not uncommon for 10–20% of seedlings in new plantations to die. Frost damage or desiccation during storage of insufficiently hardened plants often causes poor establishment. Therefore, better characterisation of seedling cold hardiness will ensure an enhanced quality of planting stock. This project enhanced existing knowledge of the fundamental cellular and molecular processes underlying winter dormancy and cold hardiness development in woody species. The cDNA microarray technology provided a new experimental tool enabling global searches on the function of genes, a capability which is only just starting to penetrate the field of plant molecular biology. The techniques used were eminently suited for the unravelling of functional gene networks and interlinked molecular pathways that determine the complex physiological processes involved.

COLDTREE project outline

The COLDTREE work plan used two model species for the development of the cDNA microarray. To identify relevant clones, the expression information was compared with data derived from thorough physiological analysis of the seedlings. For rapid

detection of the selected genes RT-PCR assays were developed. Employing quantitative detection (via real-time monitoring of accumulating fluorescence) these assays can be used to obtain detailed information on the expression profile of the selected genes, collected from seedlings produced under operational conditions.

The work of the COLDTREE project was divided into three phases.

Phase 1

In the first two years climate room experiments were conducted by Forest Research (FR) and the Danish Institute of Agricultural Sciences (DIAS) in order to produce 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 (i.e. control).

DIAS focused on beech and FR on pine, but in both cases the experimental set-up was the same, except for the daylength and temperature cues which differ for pine and beech. In the second trial season climate room experiments assessed the

combined effect of decreasing temperature and decreasing daylength with each partner assessing dormancy (by assessment of days to budbreak, DBB) and cold hardiness test (by assessment of root and shoot electrolyte leakage, REL/SEL) of excised root and shoot parts. To enable molecular investigation of the developmental processes, RNA from bud and root samples was taken according to a common protocol. The RNA was shipped to Plant Research International (PRI, University of Wageningen, Netherlands), for the preparation of the microarray probe.

Concurrently, the Agrotechnological Research Institute (ATO, Netherlands) pre-selected 1500 putative dormancy related genes from several pine cDNA expression libraries and isolated the corresponding inserts. PRI sequenced the 1500 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 independent samples were used to challenge the microarray.

From each sample two probes were constructed, using different fluorescent dyes. The screenings data were analysed in comparison to the physiological data obtained from experimental trials to identify a set of 20–30 transcripts involved in either dormancy, cold hardiness, or both.

Phase 2

Once information from selected transcripts became available, these were used to design PCR primers for the development of reverse transcription–polymerase chain reaction (RT–PCR) assays with fluorescent detection markers. The PCR-based assays enable rapid and reliable detection of specific gene expression to be performed. Assessments of dormancy and hardiness development were conducted from September to December/January. In RNA samples the presence of relevant transcripts were monitored using PCR assays. This data aided

the selection of six key genes which were descriptive of the physiological condition of pine and beech with respect to dormancy and cold hardiness. Additionally, the development of the PCR assays is a first step towards implementation of the knowledge gained within the project for the development of an operational test.

Phase 3

The key genes selected in phase 2 were evaluated in forest tree nurseries in the UK, Denmark, Sweden and The Netherlands. The trials identified the potential future benefit of molecular diagnostic tests for hardiness and dormancy, based on gene expression.

All data derived from the physiological and molecular analyses have been combined in a single searchable database allowing linkage of physiological, expressional, functional and sequence information. This combination of data offers a profound insight into 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 is now available, and continues to contribute to the definition of those molecular events underlying the onset and development of dormancy and cold hardiness in woody species.

We have communicated the project results to the sector, via a UK demonstration workshop for nurserymen. The main focus of this workshop was the significance of the results for facilitating nursery management by highlighting the potential of RT-PCR assays, and newly developed shoot-based physiological assessments of cold tolerance, for informing operational decision-making.

Project proposals have been implemented with the specific aim of developing ‘off the shelf’ molecular diagnostic tests for use in nurseries, based on the results from the COLDTREE project.

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