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Climatic adaptation in *Picea abies* progenies is affected by the temperature during zygotic embryogenesis and seed maturation

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ABSTRACT

The temperature during maternal reproduction affects adaptive traits in progenies of Norway spruce (*Picea abies* (L) Karst.). Seed production in a cold environment advances bud set and cold acclimation in the autumn and dehardening and flushing in spring, whereas a warm reproductive environment delays timing of these traits. We repeated crosses between the same parents and produced seeds under contrasting temperatures. Elevated temperatures were applied at different time points from female meiosis to embryogenesis, followed by full-sib progeny tests in common environments. We measured timing of terminal bud formation, cold acclimation in the autumn and transcription levels of conifer phytochromes *PhyO, PhyN*, *PhyP*, and the class IV chitinase *PaChi4* in these tests. No progeny differences were found that could be related to temperature differences during prezygotic stages and fertilization. In contrast, progeny performance was strongly associated with the degree-days from proembryo to mature seeds. Progenies with a warm embryonic history formed terminal buds later, were less hardy and expressed lower transcription levels of the *Phy* and *PaChi4* genes. We hypothesize that temperature during zygotic embryogenesis and seed maturation regulates an 'epigenetic memory' in the progeny, involving differential expression of genes that may regulate bud phenology, cold acclimation and embryogenesis in Norway spruce.

Key-words: *Picea abies*; adaptive traits; after effects; chitinases; climate change; epigenetic memory; maternal effects; phytochromes.

INTRODUCTION

Survival and competitive successes of boreal forest trees species depend on a balance between exploiting the full growing season and minimizing frost injury through proper timing of hardening in autumn and dehardening in spring. As sessile organisms they use temperature and night length to regulate physiological changes between active growth and dormancy in synchrony with seasonal change in temperature and day length (Bigras & Colombo 2001). Extreme temperature is probably a major selecting agent causing population differentiation along latitudinal and altitudinal gradients for such adaptive traits (Saxe *et al*. 2001). The anticipated change in global climate may be so fast that the species will have too little time to adapt, productivity will be affected, and the persistence of many species may even be threatened (Davis & Shaw 2001; Rehfeldt *et al*. 2002). Compensating aid through forest management practice may become complex and expensive to run (see Discussions in Rehfeldt, Spittlehouse & Hamilton 1999; Hänninen *et al*. 2001; Saxe *et al*. 2001).

However, conifer trees may respond faster to change in temperature than expected. During the last 20 years, independent tests in several conifer tree species have shown that growth, bud phenology and frost hardiness of progenies are influenced by the climatic conditions during sexual reproduction (Skrøppa & Johnsen 2000; Johnsen & Skrøppa 2001). We have been working particularly with Norway spruce (*Picea abies*), and found that the female flowering environment affects the progenies in a rather predictable manner (hereafter called memory effects for simplicity). The timing of frost hardiness losses and bud burst in spring, leader shoot cessation in summer, bud set, lignification of the annual ring and increase of frost hardiness in the autumn are all processes that are advanced or delayed according to temperature during female reproduction (Bjørnstad 1981; Johnsen 1989a, b; Johnsen, Dietrichson & Skaret 1989; Johnsen & Østreng 1994; Kohmann & Johnsen 1994; Skrøppa 1994; Skrøppa *et al*. 1994; Johnsen *et al*. 1995, 1996). The memory effects seem to endure for many years in the filial generation (Johnsen 1989a, b; Johnsen *et al*. 1989; Skrøppa 1994; Edvardsen, Johnsen & Dietrichson 1996). Differences in temperature and photoperiod during male meiosis and microsporogenesis produce no change in progeny performance (Johnsen *et al*. 1996), indicating that there are processes in the female flower that might be sensitive to environmental cues, manifested as altered progeny performance. These findings have changed

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our view of the underlying factors influencing the observed clinal variation in Norway spruce. It cannot solely be a product of directional, natural selection among genotypes within a generation, but is also directed by the weather and climate during which the seeds are produced (Hänninen *et al*. 2001).

The long-lasting memory effect may be the result of genetic and/or epigenetic changes. Genetic changes includes among others; gametophytic selection, preferential mating and post-zygotic selection (Mulcahy 1979; Hormaza & Herero 1992; Ottaviano, Sari-Gorla & Frova 1988). The extensive overlap between genes expressed during pollen germination and growth of the sporophyte makes influences on sporophytic fitness possible through gametophytic selection (Tanksley, Zamir & Rick 1981; Sari-Gorla *et al*. 1986; Honys & Twell 2003). Epigenetic changes in gene expression, on the other hand, do occur without change in nucleotide sequence (Wolffe & Matzke 1999; Meyer 2001; see several papers in *Science*, volume 293 no. 5532, 2001). The epigenetic regulation of vernalization in *Arabidopsis* is a well-described example (Sung & Amasino 2004a, b; Bastow *et al*. 2004). Epigenetic change may also be transmitted from one generation to the next (Jablonka & Lamb 1995, 2002; Kalisz & Purugganan 2004). The two major types of methylation associated with epigenetic regulation in plants; methylation of cytosine in DNA and histone H3 lysine methylation, seem to be dependent of each other (Johnson, Xiaofeng & Jacobsen 2002; Gendrel *et al*. 2002).

The reproductive process in Norway spruce offers some possibilities for directional selection to occur (Sarvas 1968; Owens & Blake 1985). Within each female flower, three out of four genetically different megaspores degenerate, three to seven pollen grains compete during tube growth, and two to four genetically different embryos compete within each developing seed, only one of them becomes the mature embryo. The selection potential is rather small, however, and can hardly explain the large phenotypic change observed, unless selection operates in the same direction at all prezygotic (megaspore formation, pollen tube growth) and postzygotic (embryo competition) stages. If, for example, temperature treatments at the two prezygotic stages have no impact on progeny performance, it would be unlikely that postzygotic selection alone could account for the memory effects reported earlier (Johnsen & Skrøppa 2001). Thus, we wanted to distinguish between sensitive and possibly non-sensitive stages in the reproductive process in Norway spruce, related to the presence or absence of temperature induced memory effects expressed in the progenies.

If the memory effect is regulated at the epigenetic level, then transcription of regulatory genes should be differentially expressed in the progeny, related to the prevailing temperature during reproduction. Moreover, the transcriptional difference should be associated with the phenotypic difference in common progeny test environments. In the present paper, we report results from five experiments with Norway spruce, conducted in the period from 1993 to 2004. We made identical pair crosses and produced seeds under contrasting temperatures. Elevated temperatures were timed to different reproductive stages in the female flower, based on known relationships between the degree-days (Sarvas 1968) accumulated under the experimental conditions and the reproductive events in the female flower (Owens *et al*. 2001). Progenies from these crosses were tested in phytotron, greenhouse and growth chambers, and were compared for bud set, frost hardiness and transcription of genes with putative regulatory function in adaptive traits. We included three known coniferous phytochrome genes *PhyO, PhyN* and *PhyP* (Clapham *et al*. 1999; Schmidt & Schneider-Poetsch 2002). Analogue genes and their products seem to play an important role in the regulation of growth, growth cessation and frost hardiness in angiosperm trees (Olsen *et al*. 1997; Eriksson 2000), and are suspected to be involved in photoperiodic control of growth and bud phenology in conifers (Clapham *et al*. 2001). Moreover, we also quantified transcription of a family 19 glycohydrolase (E.C. 3.2.1.14) class IV chitinase gene (*PaChi4*) from Norway spruce. *PaChi4 has* been implicated in programmed cell death and zygotic and somatic embryo development in Norway spruce (Wiweger *et al*. 2003), and have been proposed to hydrolyse endogenous *N*-acetylglucosamine containing substrates such as arabinogalactan proteins. Programmed cell death should be involved in the formation of bud scales during growth cessation. We asked three questions. (1) During which stage(s) in the reproductive process does elevated temperature exerts its effect on the progenies? (2) Is the response to temperature related to short-term or long-term periods of elevated temperature? (3) Is the transcription level of genes with putative regulatory functions in growth cessation, cold acclimation and regulation of zygotic embryogenesis related to the temperature given during seed production?

MATERIALS AND METHODS

Genetic material

Four groups of experimental material were raised in the period from 1993 to 1998 (Table 1; Figs 1 & 2). The clonal parents originated from south-eastern Norway and Sweden (58–61°N, 100–300 m above see level). Experiment 1 consisted of two independent full-sib families including their reciprocals, with all four parents from southern Sweden (described by Owens *et al*. 2001), and crosses were performed in 1995. Experiment 2 comprised four full-sib families organized as two independent pair of half-sib families; two of the clones were used as a father and a mother, all six parents were from south-eastern Norway and crosses were performed in 1997. Experiment 3 included eight full-sib families, using eight mothers and seven fathers, thus two of the full-sib families were actually half-sibs. All the 15 parents were from south-eastern Norway, and crosses were performed in 1998. Experiment 4 used crosses done in 1993, but with full-sib families that had not been tested before (see Johnsen *et al*. 1996). Nine single pair crosses with unrelated parents originating from Norway and Sweden were

Table 1. A list of the different experiments included, categorized by the type of elevated maternal temperature given in contrast to the cold maternal control, progeny test environment given, traits measured and where the growth conditions are described in detail

Experiment	Maternal heat treatment	Progeny test environment	Progeny trait measured	Growth condition described
1	Short heat treatments from pollination to proembryo formation (Fig. 1)	Phytotron	Freezing injury	In Johnsen *et al*. (1995), 1996)
2	Short heat treatments from female meiosis to proembryo formation (Fig. 1)	Phytotron	Freezing injury	In Johnsen *et al*. (1995), 1996)
3	Long heat treatments from female meiosis to mature seeds (Fig. 2)	Greenhouse	Bud set and freezing injury	In text
4	Long heat treatments from female meiosis to mature seeds (Fig. 2)	Greenhouse	Bud set	In text
5	The longest heat treatment versus the cold controlin	Growth chambers	Gene transcription	In Kohmann & Johnsen (1994) and text

included. In experiment 5, we used three full-sib family pairs, one from a cold and a warm cross in 1993, and two from a cold and a warm cross in 1998.

Cultivation of grafts and flowering induction

Grafts of Norway spruce clones were grown in Biri Nursery and Seed Improvement Centre as described earlier (Johnsen *et al*. 1994; Owens *et al*. 2001). Ramets of each clone were 7–10 years from grafting, 2.5–4 m tall at time of flower induction treatments, and were grown in 50 L pots that could be moved in and out of a heated greenhouse for specific timing of heat treatment during cone bud induction as well as during sexual reproduction. Male and female buds were induced on separate grafts (ramets of a clone) the year before seeds were produced. Flowering was induced as described in Johnsen *et al*. (1994) and Owens *et al*. (2001).

Pollen forcing, extraction, pollination and maternal heat treatment

Pollen cones for experiment 4 was carefully forced on grafts placed inside the heated greenhouse in the beginning of February, 1993, as described earlier (Johnsen *et al*. 1996). No artificial lights were used. In all the other experiments male cones were forced in the spring, pollen extracted and cleaned, and pollination performed as described by Owens *et al*. (2001). In all the experiments maternal temperatures in the greenhouse varied between 20 and 30 °C during day and 18–20 °C during night (6 h). Timing of the maternal heat treatments was done by moving grafts inside a heated greenhouse, and difference between treatments is explained in Figs 1 and 2. In experiments 1 and 2 the treatments lasted for short periods from female meiosis to proembryo formation, related to degree-days (Fig. 1; $dd = \Sigma(T_i - 5)$ where T_i = mean temperature of day i, and 5 °C the threshold temperature; Sarvas 1968). In experiments 3 and 4, most of the treatments lasted for relatively longer periods, timed to specific dates (Fig. 2). In experiment 4, five full-sib families had members from treatment 2 and 3 (late inside versus outside control), and four independent full-sib families had members from treatment 1 and 2 (early inside versus late inside).

Testing progenies

Full-sib progenies from different maternal treatments were tested for difference in bud set, frost hardiness and transcription of genes. The common test environments were a phytotron, a greenhouse and growth chambers, depending on the experiment (Table 1). The cultivation and cold-acclimation treatment in the phytotron environment has been described in detail elsewhere (Johnsen 1989c; Johnsen *et al*. 1995, 1996). In our greenhouse, plants were grown under natural days (60°N), without supplementary lights, and they were cold acclimated inside the greenhouse during the autumn of 1999 (experiment 3) and 2002 (experiment 4). Night temperatures were kept at 15 °C during cold acclimation in the greenhouse, and the ceiling wickets were programmed to open when temperature reached 20 °C during the days. Plants were grown in multipot containers with 95 pots, each pot with a root volume of 50 mL. The root medium was peat with 0.5 kg lime and 0.5 kg dolomite per m^3 (Närkes, Svartå, Sweden) and perlite (crude 65; Ticon, Drammen, Norway) in a 3 : 1 ration. Seedlings were irrigated and fertilized three times per week with a mixture of 0.7‰ Red Superba (Hydro Agri, Landskrona, Sweden) and 0.8‰ $(NH_4)_2SO_4$ and were additionally watered without fertilizer when needed (Johnsen 1989c). Cultivation regimes in the growth chamber (experiment 5; Table 1) have earlier been described in detail by Kohmann & Johnsen (1994). Plants were grown in four growth chambers in small multipot containers (Vefi 240 12×20 pots). The root medium and fertilizer were the same as were used in the greenhouse. After 8 weeks growth in continuous light, two of the chambers were used for short day treatment of 12 h darkness per day. The two other chambers

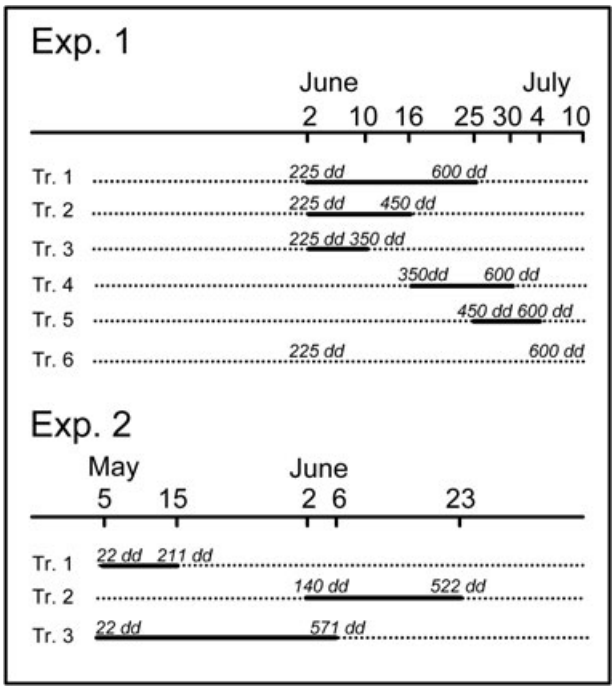

Figure 1. Short heat treatments in experiment 1 (upper part) and experiment 2 (lower part) were timed according to degree-days reached at start and end of treatment (Sarvas 1968; Owens *et al*. 2001; degree-days = dd), using 5 °C as the threshold temperature. Dotted lines indicate periods outside, and solid line periods inside the greenhouse. Dates at start and end are indicated on axis. In experiment 1 grafts were treated equally until pollination ended at 225 dd. In treatment 1, grafts were inside from 225 to 600 dd (2 June to 25 June), including elevated temperatures during pollen tube growth, megagametophyte development, fertilization and proembryo formation. Treatment 2 included the same stages as treatment 1, except proembryo formation. Treatment 3, included pollen tube growth and megagametophyte development at elevated temperatures, treatment 4 included fertilization and proembryo, and treatment 5 included elevated temperatures during late fertilization and proembryo. Treatment 6, which was the outside control, reached 600 dd on 10 July. Experiment 2 included three treatments in the early stages of the reproductive phase (lower part). Treatment 1 started by moving grafts inside at 22 dd and moving them outside at 211 dd (2–15 May), from female meiosis until pollination ended. Treatment 2 started at 140 dd when pollination was proceeding and ended at 522 dd during proembryo (2–23 June), and treatment 3 was timed from 22 dd to 571 dd, from female meiosis until proembryo.

were used as a long day control (continuous light). Epicotyls were collected and immediately frozen in liquid nitrogen at day 6 and day 20 from onset of short day treatment, using sampling from 0930 to 1030 h every time of collection.

Plants were recorded as having set bud when a pale, white developing bud became visible at the shoot apex. Bud set was recorded once a week during August and beginning of September in experiments 3 and 4. Whole plant freezing tests were performed as outlined in Johnsen (1989c) (see also Johnsen *et al*. 1995, 1996; Dalen, Johnsen & Ogner 2001), starting and ending at 5 °C, using the same freezing and thawing rate set at 2 °C/ h, and 4 h at set test temperature. Test temperatures from –11 to –15 °C gave 30–75% needle injury with variation among experimental entries, and these hardiness levels were attained in the phytotron experiments when nights had reached 12–13 h, and during the last week of September 1999 in the greenhouse. Seedling injury was scored as browning and discoloration of needles 3 weeks after freezing, according to categories from 0 to 11, 0 = no visible injury, 11 = 100% complete browning and death, and 1–10 = 10% increments of needle injury.

RNA isolation and cDNA synthesis

Epicotyls of 10 individuals from each experimental entry were processed for RNA isolation and real-time polymerase chain reaction (PCR). The samples were frozen immediately in liquid N_2 and later ground to fine powder in liquid N_2-chilled mortars for addition of RNA extraction buffer. RNA was isolated by using an RNAqueous medium Scale Phenol-Free Total RNA Isolation Kit and Plant RNA Isolation Aid (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Contaminating DNA was removed by using DNA-FreeTM (Ambion) with 2 U DNase I per sample and 30 min incubation at 37 °C. Total RNA was quantified with a VersaFluor Fluorometer (Bio-Rad, Hercules, CA, USA) and a RiboGreen RNA Quantification Kit (Molecular Probes, Eugene, OR, USA) accord-

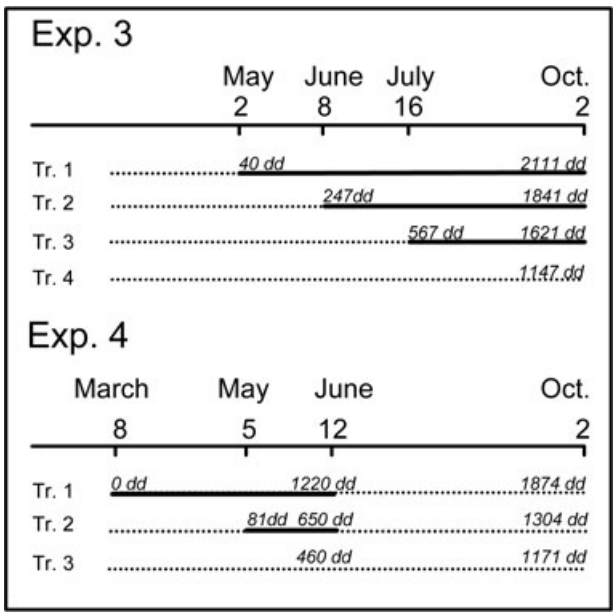

Figure 2. Heat treatments in experiment 3 (upper part) and experiment 4 (lower part) were timed according to dates at start and end of treatment (upper axis), and degree-days (dd) at start and end of treatments are indicated on the solid line (inside the greenhouse). Start of embryogenesis was assumed to occur at 500 dd (Owens *et al*. 2001). Experiment 3 (1998) was designed to study the effect of long heat treatment over the entire reproductive period in the female flower (upper part). The treatments in experiment 4 (lower part) (1993) have been described in detail by Johnsen *et al*. (1996), and created differences during embryogenesis and seed maturation as well.

ing to the manufacturer's instructions. Total RNA was normalized between samples: 400 ng total RNA was reverse transcribed to cDNA with Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions with 1.25 U reverse transcriptase per sample and 2.5 μM polydT primers in a 50 μL reaction volume.

Design of oligonucleotide primers for quantification of gene expression

For multiplex real-time PCR quantification, the primers and probes for *PhyO, PhyN, PhyP* and the endogenous reference *αTubulin* were designed with the Primer Express® software 1.5a provided with Applied Biosystems Real Time Quantitative PCR systems. Phytochrome genes show a significant degree of similarity at the nucleotide level, therefore alignment of the known cDNA regions of the *PhyO, PhyN and PhyP* sequences was performed to select primers from regions with the low degree of similarity, possible to enable specific amplification (alignments not shown), and labelled TaqMan® probes were included to ensure specificity during amplification. For the singleplexing real-time PCR quantification with SYBR Green, we used primers for the chitinase sequences *PaChi4* designed with the same Primer Express® software 1.5a, using the *αTubulin* as the endogenous control. All primers and probes (Table 2) were ordered from ABI PRISM® Primers & TaqMan® Probes Synthesis Service (Applied Biosystems).

PCR conditions and optimization of primer and probe concentrations

For multiplex real-time PCR quantification of *PhyO, PhyN*, and *PhyP* each 25 μL PCR reaction was performed in TaqMan® Universal PCR Master Mix (P/N 4304437, Applied Biosystems). The level of *αTubulin* was co-amplified as an endogenous reference in each case. The mix is optimized for real-time PCR assay using TaqMan® probes and contains AmpliTaq Gold® DNA polymerase, AmpErase® UNG, dNTPs with dUTP, passive reference 1 and optimized buffer components. The AmpErase® uracil-*N*-glycosylase prevents the re-amplification of any carryover-PCR products containing uracil. Optimization of Phytochrome N, O and P primer and VIC-labelled probe in combination with *αTubulin* primer and FAM-labelled probe concentrations were performed in order to avoid the interference between the two competing amplicons during multiplex PCR. The linearity and specificity of the primer probe sets were verified using known concentrations of each of the phytochromes and for *αTubulin*. No cross amplifications were detected, demonstrating that the primer probe pairs was specific in each case. Initial testing showed that the reference transcript of *αTubulin* was the more abundant mRNA species present in the cDNA templates, and care was taken to ensure that the *αTubulin* primer concentrations were kept at a low to avoid interference with the phytochrome amplification reactions during multiplex PCR. Thus, combinations of primer concentrations of 900, 600, 500, 450 and 300 nM and probe concentrations of 900, 600 and 450 nM were tested for each of the phytochrome targets, in combination with *αTubulin* 300, 150, 70 and 50 nM primer and 600, 450 and 300 nM probe concentrations. The primer concentrations chosen were those that gave the lowest ΔRn values while the ΔCt remained constant (Hietala *et al*. 2003). From these tests, the following optimal reaction parameters were chosen for analysing the experimental samples: 500 nM of the phytochrome probes, and 300 nM of each phytochrome primer, 450 nM of the *αTubulin* probe and 50 nM of each *αTubulin* primer. These primer/probe concentrations were applied both in multiplex PCR. PCR cycling parameters were 50 °C for 3 min for UNG enzyme activity, 95 °C for 10 min to denature the UNG enzyme and to activate the polymerase, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Real-time detection of fluorescence emissions was performed on the

Table 2. Nucleotide sequence (GENBANK accession no.) of forward (F) and reverse (R) primers and labelled TaqMan® probes (T) for detecting mRNA transcripts in Norway spruce

Transcript target	Nucleotid sequence
PhyN	(F) 5′-TCTTCCATGGGAGCATTGC-3′
(U38363)	(R) 5′-CCCACTAGGGATCGATTGCA-3′
	(T) 5′-TATAAGCTTGCTGCAAAGGCCATATCACGG-3′ (VIC)
PhyO	(F) 5′-AATAGATGCAGGGTTGGTCATAGAC-3′
(U60264)	(R) 5′-GCACCAGCGGCAGATACC-3′
	(T) 5′-ACATCGGAAGGCATCACAGGCTCAA-3′ (VIC)
PhyP	(F) 5′-GCGGCTGGTGCAGTACAA-3′
(U38368)	(R) 5′-GGAAGTGACTGCAGACGAGAAA-3′
	(T) 5′-CGCCCGCACAGCAAGCTTCTG –3′ (VIC)
αTubulin	(F) 5′-GGCATACCGGCAGCTCTTC-3′
(X57980)	(R) 5′-AAGTTGTTGGCGGCGTCTT-3′
	(T) 5′-ATCCTGAACAGCTGATCTCGGGCAAA-3′ (FAM)
PaChi4	(F) 5′-GCGAGGGCAAGGGATTCTAC-3′
(AY450924)	(R) 5′- GGTGGTGCCAAATCCAGAAA-3′

FAM or VIC was used as fluorescent reporters and TAMRA as a quencher for multiplexing. SYBR Green was used as reporters for singleplexing.

ABI PRISM® 7700 (Applied Biosystems). Data acquisition and analysis were performed with Sequence Detection System 1.7a Software Package (Applied Biosystems).

For *PaChi4* only primers and no probes were used for singleplex quantification and each 25 μL PCR reaction was performed in SYBR Green PCR Mastermix (P/N 4309155; Applied Biosystems) and the *αTubulin* PCR reaction was used as an endogenous reference in a separate reaction tube. A primer concentration of 50 nM was chosen for the *PaChi4* and for the *αTubulin* assayed. As template, 5 μL of the cDNA solution described above was used for each reaction. Each reaction was repeated twice for each sample. PCR cycling parameters were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Fluorescence emissions were detected with an ABI PRISM 7700 (Applied Biosystems). Data acquisition and analysis were performed with Sequence Detection System, version 1.7a, Software Package (Applied Biosystems). The target specificity of each primer set was examined in a melting point analysis. The melting point analysis was performed after the real-time reverse transcriptase (RT)-PCR program described above by using the thermal profile of 95 °C for 15 s, 60 °C for 20 s followed by the melting step in which the temperature was raised linearly from 60 °C to 95 °C in a ramping 10 time of 19 min 59 s.

Experimental design and statistics

Plants were grown in contiguous split-plots in all experiments, using full-sib family as main plots and maternal heat treatment within family as subplots, replicated eight times in experiment 1, 12 times in experiment 2 and 3, four times in experiment 4, and four times in experiment 5. Each subplot comprised 20 (experiments 2 and 3) or 30 (experiments 2 and 4) plants. Bud set was recorded on plants growing in the original containers. We used the transformation arcsine $\sqrt{p}$ to homogenize variances between groups as well as direct analyses of proportions (p). Separate analyses were run for each date buds were recorded, using the Mixed procedure in SAS (SAS Institute Inc. 1997), regarding maternal temperature as a fixed effect, and families and replicate as random components. Significant difference between maternal temperature treatment means was tested with least significant difference (LSD with $\alpha = 0.05$). We found no difference between the analyses of transformed and non-transformed variables, and decided to present results with non-transformed bud set data.

For every freezing test occasion plants were lifted from the growth containers, sorted in test containers, and put into freezing test chambers within one day. They were sorted in such way that plants from a full sib family main plot, using one seedling for each maternal treatment, were placed adjacent to each other in the test containers. The roots were insulated; whole plants subjected to freezing, and were then placed in the greenhouse for developing visible injury at high humidity (misting operating below 95% relative humidity, Johnsen 1989c). Injury data were transformed to normal scores by assigning midpoint *x*-values of cumulative frequency distributions of plants within each test temperature and replicate, based the original liability classes of injury, to the individual plants (Gianola & Norton 1981; Falconer 1989; Eriksson 1994). The transformed single seedling data formed approximate normal distributions with a mean = 0 and variance ranging from 0.9 to 0.95, and assigned seedling values within the range ±2.45. Negative values represent hardier, and positive values less hardy seedlings than average.

The normal score data from experiments 2 and 3 were analysed using the Mixed procedure in SAS (SAS Institute Inc. 1997). Maternal treatment was regarded as fixed and family as random, and differences between maternal temperature treatment means were tested with LSD as above.

We made the raw data from real time PCR by obtaining the critical threshold cycle value (dCt) for the endogenous transcript of *αTubulin* (AT) and the corresponding threshold value for the transcript to be quantified (TARGET). The higher the transcription, the lower are the dCt values; number of cycles needed to reach the thresholds are low when the abundance of the specific transcripts are high. The threshold level of *αTubulin* was stable and independent of experimental entries (verified with analyses of variance; all $F < 1$; Fig. 7). This fact validated the use of AT as an internal reference (see Brunner, Yakovlev & Strauss 2004), and a subtraction variable $X = AT - TARGET$ was made for each sample and each target gene. However, this variable was negative because the transcript of the target genes *PhyO, PhyN*, *PhyP* and *PaChi4* was always lower (5–10 more PCR-cycles for the phytochrome genes and 2–5 PCR-cycles more for the chitinase gene) than the internal reference *αTubulin* in each sample. To obtain a better presentation of up- and down-regulation of transcriptions, the raw data X was transformed. First, a mean value (Θ) was calculated from all experimental observations of X for a specific target gene. Then a new transformed variable was made by the formula $Y = X - \Theta$ for each sample. Note that Θ is a scalar specific for each target gene, and Y and X represents variables (varying by samples) specific for each target gene. Y is symmetric around its mean value, which is 0 for each target gene. Positive values represent higher and negative values lower transcription than average, and the unit is number of PCR cycles above or below zero (see Fig. 7). The new variable was analysed with the GLM procedure in SAS (SAS Institute Inc. 1997). Family, maternal treatment, the effect of day length and the effect of days after onset of short day treatment were regarded as fixed. We could not find any evidence for change in transcripts from 6 to 20 short days and no effects of time in long days ($0.71 < P < 0.85$), and decided to pool the results from the two time points (Fig. 7). The effect of the maternal environment and day length were tested using the residual as the error term.

RESULTS

The timing of the various temperature treatments is shown in Figs 1 and 2 for four of the experiments (experiments 1–

Table 3. Freezing injury expressed as needle injury class means (least squares) of progenies from the short maternal heat treatments in experiments 1 and 2

	Treatments					
	1	2	3	4	5	6
Experiment 1	6.3	6.7	6.5	6.3	6.3	6.8
Experiment 2	5.0	5.2	5.1	–	–	–

Numbers of observations per mean value were 94 and 113 for experiments 1 and 2, respectively. See Fig. 1 for treatment description.

4). In experiment 1, we applied short heat treatments in a period when the degree-days increased from 225 to 600 dd (Fig. 1 upper part). However, we found no significant differences in freezing injury that could be related to the short maternal heat treatments during pollen tube growth, syngamy and proembryo (Table 3, $P = 0.056$). We then tested the effects of short heat treatments applied at earlier stages in the female flower including female meiosis, megaspore development, archegonia formation and pollination (experiment 2, 1997; Fig. 1 lower part). There were no significant differences in freezing injury between progenies, which could be related to the different short maternal heat treatments during the early events in the female flower (Table 3, $P = 0.45$), and we conclude that elevated heat applied during a short period from female meiosis to proembryo does not induce memory effects in the progeny.

Next, we asked if the effect of heat treatment applied over the longer reproductive period in the female flower could induce change in progeny performance (experiment 3; Fig. 2 upper part). Figure 3 shows that the long -lasting heat treatment produced a significant difference ($P < 0.01$) in bud set (at days 42–61) in comparison with the outside control. No difference between elevated temperature treatments given from May versus from June was observed. This confirms results from experiments 1 and 2; difference in temperature during the prezygotic stages and fertilization seems to be of minor importance. We decided to set the value for start of embryogenesis to 500 dd (Owens *et al.* 2001), calculated the degree-days of the various long heat treatments from start of embryogenesis until cones were collected on 2 October, and plotted the calculated degree-days data against the mean freezing injury data from the progeny test of experiment 3. The freezing injury increased with increasing amount of degree-days during embryogenesis and seed maturation (Fig. 4), and reached a saturated response at about 1350 dd. The significant difference between the outdoor control and elevated temperature starting on 16 July (Figs 3 & 4) is of particular importance, because the temperature difference was introduced from 560 dd, in the period from proembryo to mature seeds.

The proven relationship between the degree-days and frost hardiness (Fig. 4) prompted us to investigate seedlings from non-tested seed lots of an experiment conducted in 1993. We tested the differences in bud set in the greenhouse in 2002 (experiment 4). Figure 5 shows that maternal treatment significantly ($P < 0.01$) affected the bud set of the full sib progenies. The most pronounced difference was found between the early indoor (March) versus the late indoor treatment (May). The bud set difference from experiment 3 (Fig. 3; 23 August, indicated by the arrow) and experiment 4 (Fig. 5; 26 August, arrow) was plotted against to the degree-days differences during embryogenesis and seed maturation. The relationship shown in Fig. 6 indicates that a difference in progeny bud set is positively associated to the degree-days differences from embryogenesis to matured seeds, probably reaching a saturated response at about 600 dd difference between the cold and the warm treatments.

In experiment 5, we asked if the transcription levels of the three phytochrome genes *PhyO, PhyP, PhyN* and the extracellular class IV chitinase *PaChi4* in the growing seedlings were affected by the temperature during their embryo development and seed maturation, and how the transcription changed after onset of short day treatments during progeny growth. The chosen genes showed interesting dif-

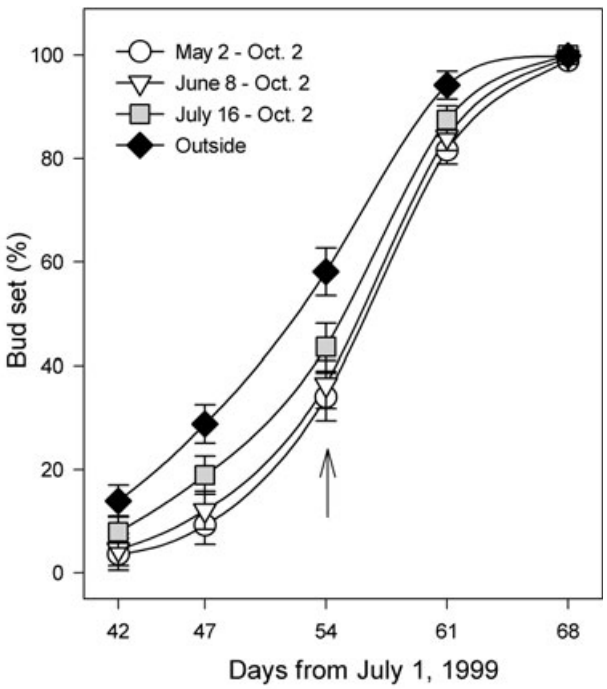

Figure 3. Formation of visible terminal buds in experiment 3. Full-sibs from the contrasting maternal treatments were grown the first season from seeds in a greenhouse and formed terminal buds during August and September of 1999. The legends for the four maternal heat treatments are explained in the figure. Each point is a mean of eight families and 12 replicates, where each replicate comprised 20 seedlings per family, maternal treatment and replicate. Thus, each mean is based on 96 estimated proportions per maternal environment per recording date. Standard errors of means are estimated from the random interactions between family and maternal treatment for each recording date from the analyses of variances.

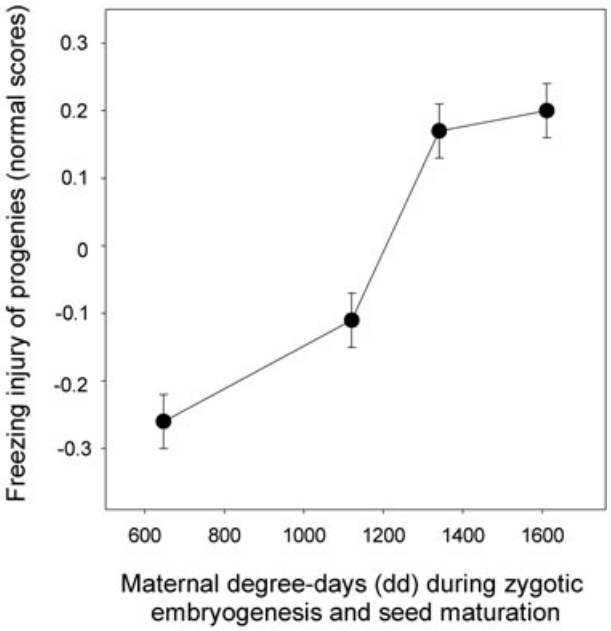

Figure 4. Freezing injury expressed as least square means of normal scores in relation to the amount of degree-days from embryogenesis to seed maturation in experiment 3. Number of estimated normal scores per mean value on *y*-axis is 504 (eight families, three test temperatures and 21 seedlings). Standard errors of means are estimated from the residual component of the analyses of variance of normal scores.

ferences in transcription patterns, while the internal reference remained stable (Fig. 7). *PhyN* was significantly ($P < 0.01$) more transcribed when progenies originated from a cold maternal environment and were tested in both long days and short days, but the averaged difference between short days and long days was not significant ($P = 0.09$). *PhyO* transcription was higher in seedlings from the cold embryogenesis in short day treatment only ($P < 0.01$), and this seems to be the result of a down-regulation from long days to short days in seedlings from the warm embryogenesis ($P = 0.02$), and a non-significant up-regulation ($P = 0.15$) in seedling from the cold embryogenesis. *PhyP* transcription was down-regulated in short days ($P < 0.001$), and transcription was significantly lower for seedling from the warm embryogenesis in short days only ($P = 0.001$). *PaChi4* showed significantly higher transcription levels in seedlings from the cold embryogenesis ($P < 0.01$) in both long and short days, and a substantial and consistent up-regulation appeared in short days ($P < 0.001$).

DISCUSSION

Embryo development and seed maturation is the sensitive period

Our timing experiments with elevated temperature during female flowering revealed that the period from embryogenesis through seed maturation is the most likely sensitive period, and that a sort of a 'memory' persists, which depends on long-term exposure of temperature levels rather than short-term heat shock. The relationship found between degree-days during embryogenesis and differences in progeny performance (Figs 4 & 6), is rather convincing, and the finding of no responses to short-term treatment during female meiosis, megaspore development, pollen tube growth and fertilization (Table 3; Figs 3 & 4), underline that selection during female gamete formation and competition among growing pollen grains seem to be of little importance. No difference was even found between elevated temperature from early May and elevated heat from early June in experiment 3 (Figs 3 & 4), which excludes that degree-days differences and/or abrupt changes in temperatures during early stages are important.

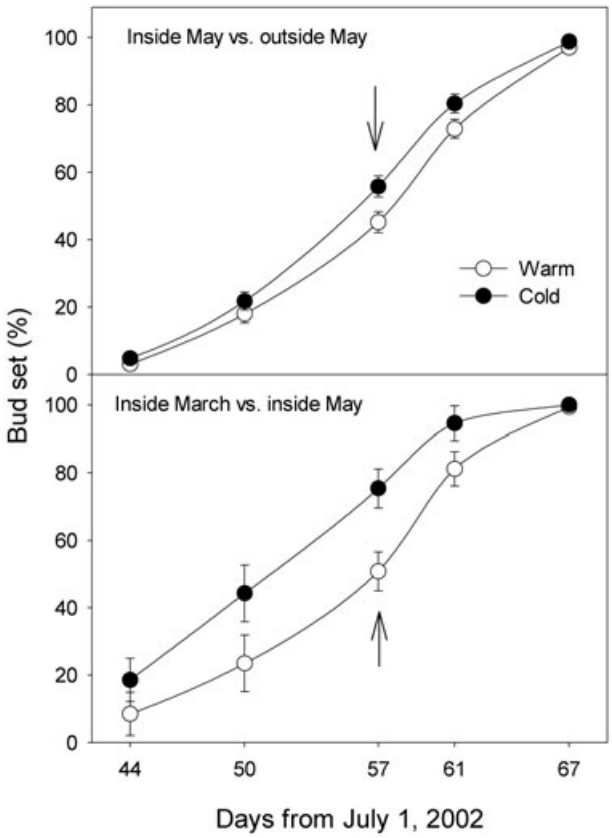

Figure 5. Formation of visible terminal buds in experiment 4. Full-sibs from the contrasting maternal treatments were grown the first season from seeds in a greenhouse and formed terminal buds during August and September of 2002. Upper part shows the mean difference between the cold outside control and warm inside during 5 May to 12 June, and the lower part shows the mean difference between the warm inside during 8 March to 12 June and the cold (relatively speaking) inside during 5 May to 12 June. Numbers of estimated percentages for each mean were 20 (five families, four replicates) in the upper subfigure, and 16 (four families and four replicates) in the lower part. Each percentage was based on 30 seedlings. Standard errors of means were estimated from the random interaction between family and maternal temperature from the analyses of variances of the proportions.

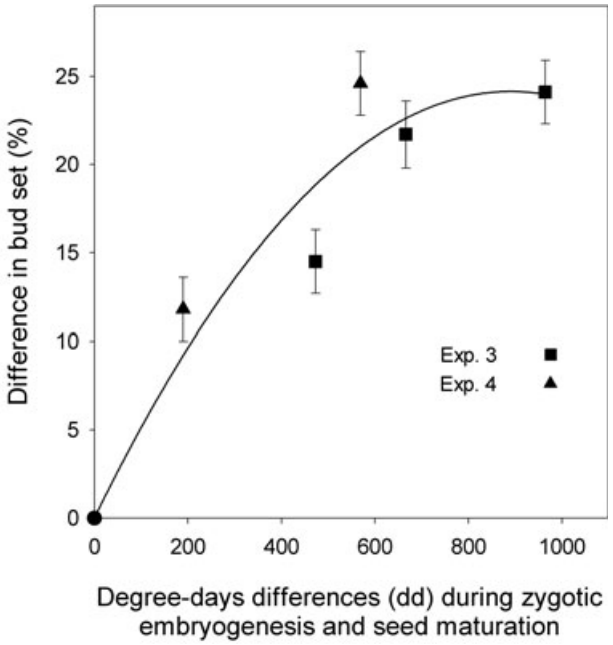

Figure 6. The relationship between degree-days differences of the warm and the cold embryo development and the maximal differences in bud set of the progenies from the contrasting maternal temperatures grown in a greenhouse environment. Different symbols denote the two experiments, 3 and 4. Standard errors of means were estimated from the residuals of the analyses of variances of the proportions. Numbers of estimated proportions for each mean are 96 for experiment 3, 20 and 16 for the low and the high values, respectively, in experiment 4.

Elevated temperatures at early stages speed up the processes, however (Owens *et al*. 2001), advancing the development to early summer, leaving the embryos to form under a longer period during summer and early autumn, and thereby the embryos are exposed to more heat. There seems to be very little differences in time when seeds become mature and dry in late September/early October This fact probably explains why we observed effects of heat treatment during May compared to outside control in earlier studies (Johnsen *et al*. 1995, 1996), because grafts where moved out of the greenhouse in the beginning of June after heat treatment in March to May, at more advanced reproductive stages, leaving developing embryos to receive higher degree-days during June, July and August than in the control cones attached to grafts standing outside. The data thus corroborate what was found in herbaceous perennial *Plantago lanceolata*, where the postzygotic environment more strongly influenced the offspring phenotype than did the prezygotic environment (Lacey 1996; Lacey & Herr 2000).

Possible mechanisms

Our findings weaken the hypothesis that directional selection among haplotypes and genotypes inside the female flower could be the main explanation of the observed phenomenon in Norway spruce. A maximum of four genetically different, competing and developing embryos inside a seed (Sarvas 1968; Owens & Blake 1985) would only create a minimum proportion selected of 0.25. This theoretical selection intensity of 1.27 (Falconer 1989) is too low to explain the difference found in the present paper. Moreover, in experiment 1 we found higher incidence of megagametophyte, egg or embryo abortions at elevated temperatures inside the greenhouse (Owens *et al*. 2001). Total abortion rate was estimated to 13% in the outside control (treatment 6; Fig. 1 upper part) versus 44% in treatment 2 (elevated temperatures from 225 to 450 dd; Fig. 1), but the phenotypic performance of the progenies from these two treatments were not different (Table 3). Thus, possible changes from directional selection during reproduction, can hardly explain the observed results in the present study. Schmidtling & Hipkins (2004) used allozymes to tests for segregation ratio change between two contrasting reproductive environments. They documented significant differences between the two maternal environments in segregation ratios for many cross/loci combinations. However, the environmental irregularities in allozyme alleles did not relate well to differences in

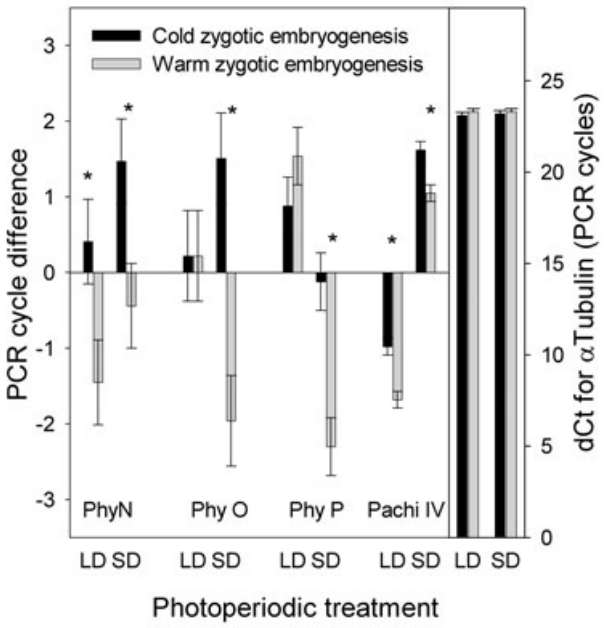

Figure 7. The relative transcript level (number of PCR cycles above or below 0) of *PhyO*, *PhyP*, *PhyN* and *PaChi4* in identical full-sib family pairs (cold and warm embryonic history) in long days (LD = continuous light) and short days (SD = 12 h night). Transcript level is measured as the difference between *αTubulin* (endogenous control showing no response to treatment) and the chosen transcripts relative to the mean value for the gene targets. Bars indicate standard error of means. Each mean is based on four replicates and 10 pooled plants per replicate. Significant differences between cold and warm embryogenesis indicated with *. The right-hand part of the figure shows the threshold (dCt) of the internal reference gene *αTubulin* in relation to the treatments given.

progeny performance induced by the crossing environments.

An alternative hypothesis may be that temperature regulates the level of gene expression in the developing embryos, creating some sort of an 'epigenetic memory', which is expressed as a long-lasting effect on phenology traits in the progenies. The likely candidate for such memory would be methylation of cytosine in genomic DNA (Wolffe & Matzke 1999; Matzke, Matzke & Kooter 2001; Meyer 2001; Paszkowski & Whitham 2001). If cytosine is more methylated in the coding regions of genomic DNA, and specifically in the promoters, gene transcription is anticipated to be down-regulated. We have, as an initial step, measured the proportion of methylated cytosine in total DNA extracts from two of the three full sib families of Norway spruce included in Fig. 7, and found that DNA is much more methylated in seedlings originating from a warm embryonic environment than their full-sibs with a cold embryonic history (Baumann *et al*. unpublished). We have no information about the presence/absence of methylated cytosine in the coding region, let alone if the specific genes shown in Fig. 7 are methylated. However, the transcription of the internal reference *αTubulin* was not affected at all (Fig. 7), while the transcripts of the four target genes were reduced in progenies from the warm embryogenesis. In future research we need to test for possible promoter methylation and other epigenetic causes (Kalisz & Purugganan 2004), and whether the degree of specific methylation would correlate with the temperature during zygotic embryogenesis and with phenotypic performance in adaptive traits. In addition, more knowledge about synthesis and breakdown of regulatory proteins, and how such gene products may cross talk in Norway spruce is needed.

The phytochrome genes are hard to study for many reasons, their low transcription levels in Norway spruce is one of them. Moreover, we are aware of the complexity of photosensory perception and signalling in plant cells (Quail 2002; Eriksson & Millar 2003; Kevei & Nagy 2003), and because we have been studying a restricted set of five genes only, we should not over-emphasize their importance. Nevertheless, we hypothesize that phytochrome transcription may be among one of several early regulatory steps upstream in the cascade of events that control photoperiodic responses in trees (D'Aust & Hubac 1986; Howe *et al*. 1996; Clapham *et al*. 1998; Clapham *et al*. 2002). In future research we intend to use microarrays to get a broader picture of up- and down-regulated genes related to bud phenology and cold acclimation. However, for some regulatory elements with low transcription levels, like the phytochromes, RealTime PCR may be the only current technique that is sensitive enough to be used in quantitative studies of their transcripts.

The expression pattern of *PaChi4* is interesting (Fig. 7). Among other functions, class IV chitinases are extracellular family 19 glycohydrolases that have been implicated in programmed cell death and can stimulate embryo development in Norway spruce (Wiweger *et al*. 2003). This functionally coincide with the finding that embryo development and seed maturation is the sensitive stage in reproduction (Figs 4 & 6) that leads to the altered phenotypic performance. However, the most pronounced increase in transcription occurs after short day treatment (Fig. 7), and this suggest that the gene may be involved in the process of growth cessation as well, perhaps playing a regulatory role due to strong expression as early as 6 d after short days, before any visible change can be observed on the phenotype. During bud set, bud scales are being formed, and after shoot growth cessation, plants still continues with radial growth, form secondary walls, and xylem. Development of bud scales and xylem formation must be accompanied with programmed cell death. Class IV chitinases genes have been proposed to be involved in programmed cell death in carrot and *Arabidopsis*, possibly by acting on arabinogalactan proteins (van Hengel *et al*. 1998; Passarinho *et al*. 2001), and might also be involved in transition from polyembryonal masses to somatic embryo transition by direct or indirect activation of programmed cell death (Wiweger *et al*. 2003). Our data certainly calls for further studies related to photoperiodic control of growth cessation as well as studies on interactions between cold acclimation and defense reactions to pathogens (Hon *et al*. 1995; Griffith & Yaish 2004).

Ecological and practical significance

The reported difference (Figs 3–6) is comparable to a 3° latitudinal difference in provenance performance measured with Norwegian provenances from latitude 59–66°N (Dæhlen, Johnsen & Kohmann 1995; Kohmann 1996). Moreover, the performance of seedling from seeds produced on the same population of trees may depend on the temperature in the year in which the seeds are produced. A warm and a cold seed year gave rise to seedlings differences which was comparable to 4° difference in latitudinal origin of provenances (Kohmann & Johnsen 1994). By comparison, the latitudinal range of the natural geographic distribution of the species in Norway is 9° (58–67°N), thus this memory effect could account for 30–40% of the total range of provenance variation in Norway.

Seeds of Norway spruce are produced in untested Norwegian seed orchards. The parental clones have been phenotypically selected (so-called plus trees), propagated as grafts, and sometimes planted in warm areas south of parental origin or at low elevation. The memory effect is expressed in progenies from these orchards (Skrøppa & Johnsen 2000). From data presented in Figs 3–6 we can conclude that the longer the climatic distance between clonal origin and seed orchard environment is, the larger will the difference in progeny performance be expressed. We have observed most beneficial and some adverse effects of using seeds from these orchards (Johnsen 1989a, b; Johnsen *et al*. 1989; Skrøppa, unpublished). In comparison with local provenances, the seed orchard seedlings have a delayed bud burst in spring, and are less injured by late spring frosts. This is an important ability, as spring frost damage often is detrimental (Aitken & Hannerz 2001). On the other hand, the delayed growth cessation and cold accli-

mation in the autumn occasionally make the plants from these seed orchards more injured by autumn frosts. However, seedlings often recover well from autumn frost injury (Johnsen *et al*. 1989; Skrøppa unpublished). If no frost injuries occur, the seed orchard progenies grow taller (up to 15%; Johnsen 1989b; Skrøppa unpublished). This difference can hardly be explained as an effect of phenotypic plus-tree selection (Johnsen & Østreng 1994).

The memory effect is also present in other coniferous species (reviewed by Skrøppa & Johnsen 2000), but not as clear-cut, important and long lasting as reported for Norway spruce (Dormling & Johnsen 1992; Stoehr *et al*. 1998; Schmidtling & Hipkins 2004). This is probably due to the longer distance transfer along climatic gradients in the Norwegian seed orchards, and because Norway spruce is particularly responsive to the temperature during seed production. In conifer breeding in general, parents are ranked according to family performance in field trials. If such performances are assessed at young ages (e.g. 5 years), care should be taken by using seeds produced in a single seed year at a specific site, or at least check if different seed years have similar temperature conditions. Breeding values could be biased if genetic performance and memory effects are being confounded. Moreover, significant interactions between family and reproductive environment may occur (e.g. Schmidtling & Hipkins 2004), and should be checked if families in test site are made from crosses at different crossing environments. In Norway spruce, however, such interactions are not important (Johnsen *et al*. 1996).

We have recently discussed how the memory effects may influence the adaptive potential of Norway spruce under global warming (Hänninen *et al*. 2001). Contemporary populations of Norway spruce that are growing in milder, southern areas may produce better adapted progenies in the next generation than anticipated, because of the long-term impact of temperature during embryo development on progeny performance. However, the data in the present paper indicate that the memory effects of temperature may become saturated at the upper temperature level (Figs 4 & 6), thus bringing stronger beneficial effects on the productivity in the colder geographic regions than in the warmer regions under raised temperatures. The phenomenon may increase the competitive ability of progenies in the cool regions, because they grow taller and thus increase their competitive ability. If the condition becomes more unstable in the future, however, with rapid changes between temperature extremes in spring and autumn, the memory effects may reduce risk of spring frost injury followed by increase in productivity, but increase risk of autumn frost injury with reduced productivity as a consequence.

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