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RESEARCH PAPER

Photoperiod and temperature differentially regulate the expression of two dehydrin genes during overwintering of birch (*Betula pubescens* Ehrh.)

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Abstract

The overwintering of trees in northern areas depends on processes regulated by photoperiod and temperature. To identify the physiological and genetic factors involved in this environmental control, three latitudinal ecotypes of pubescent birch (*Betula pubescens* Ehrh.) growing in a common garden experiment were used. Each ecotype responded to the shortening of the photoperiod according to its specific critical daylength, resulting in the induction of freezing tolerance and dehydration of buds first in the northern ecotype, followed by the central and southern ecotypes, respectively. By contrast, there was no clear difference in the timing of dormancy release, bud rehydration, and deacclimation in the spring, suggesting that these traits were controlled mainly by temperature. To elucidate the role of dehydrins (DHN) in the overwintering process, two *DHN* genomic clones were isolated from pubescent birch and expression of the corresponding genes, both in field and under controlled conditions, was characterized. *BpuDhn1* was found to encode an Y_nK_n-type of basic DHN, while *BpuDhn2* encoded an acidic, SK_n-type of DHN. In field-grown trees the level of *BpuDhn1* increased in buds during the autumn, while the level of *BpuDhn2* was highest during the coldest winter months. Under controlled conditions *BpuDhn1* increased in response to the combined effect of short daylength and low, non-freezing temperatures whereas the expression of *BpuDhn2* was mainly controlled by low temperature while photoperiod had less effect on its expression. These results suggest that DHNs participate in the sensitive environmental regulation of the overwintering process in birch.

Key words: Birch, dehydrins, dormancy, ecotype, freezing tolerance.

Introduction

To cope with the extreme temperature and light conditions during winter, trees in the northern latitudes have evolved mechanisms that allow the timely acclimation to these adverse conditions. Acclimation proceeds sequentially in response to short daylength (SD), low non-freezing temperatures (LT), and then freezing temperatures. Trees respond to the shortening of daylength beyond a certain critical value by ceasing growth and by developing endodormancy and freezing

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Abbreviations: ABA, abscisic acid; ANOVA, analysis of variance; DHN, dehydrin protein; *DHN*, dehydrin gene; DW, dry weight; FW, fresh weight; HSD, honestly significant difference; LEA, late embryogenesis abundant; LD, long daylength; LT, low temperature; LT_{50}, temperature of 50% lethality; SD, short daylength; WS, water stress.

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tolerance (Weiser, 1970; Welling *et al.*, 1997). Endodormancy is a state of buds in which internal factors prevent growth (Lang *et al.*, 1987; Rinne *et al.*, 2001). Subsequent exposure to low and freezing temperatures result in the development of full winter hardiness (Howell and Weiser, 1970; Christersson, 1978; Greer and Warrington, 1982). In addition to increasing hardiness, low and subzero temperatures simultaneously release buds from endodormancy (Noodén and Weber, 1978; Fuchigami *et al.*, 1982; Rinne *et al.*, 1997) resulting in buds that are hardened and ecodormant. These buds maintain a hardened resting state ready for proliferation when the conditions become supportive for growth in spring. Bud bursting during ecodormancy is thus prevented by unfavourable external conditions, and promoted by conditions that support growth (Lang *et al.*, 1987; Rinne *et al.*, 2001). The development of hardiness is so crucial for survival that many boreal and temperate zone woody species have evolved latitudinal ecotypes, differing in their timing of growth cessation and cold acclimation. For example, northern ecotypes have a longer critical day-length and they cease growth earlier during autumn than southern ecotypes (Håbjørg, 1978; Junttila, 1980; Li *et al.*, 2002). The response to photoperiod shows a clinal pattern, i.e. a gradual change of genetic variation, which is associated with both latitudinal and elevation gradients (Håbjørg, 1972*a*, *b*, 1978).

The extreme freezing tolerance of woody plants is based on their ability to tolerate cellular dehydration (Vertucci and Stushnoff, 1992). Dehydration results from the withdrawal of water from the cytoplasm by ice formation in the extracellular space (Steponkus, 1984). To cope with dehydrative stress, tolerant plants accumulate compatible solutes and sugars that contribute to the stabilization of native protein structure and maintenance of the membrane bilayer (Hoekstra *et al.*, 2001; Oliver *et al.*, 2002). However, it has been suggested that special proteins, called LEA-proteins (late embryogenesis abundant) function together with sugars in dehydration tolerance (Wolkers *et al.*, 2001). Dehydrins (DHN) are a group of LEA proteins that accumulate in plant tissues under conditions that lead to increased desiccation tolerance, such as high salinity, water deficit, low temperature, or ABA treatment (Close, 1996; Ingram and Bartels, 1996; Svensson *et al.*, 2002). In orthodox seeds, the production of DHNs together with the desiccation of the embryo is part of the maturation process (Ingram and Bartels, 1996). Furthermore, the programmed dehydration of, for example, birch buds during dormancy induction coincides with the accumulation of LEA proteins (Welling *et al.*, 1997; Rinne *et al.*, 1998). The central role of DHNs in cold acclimation is supported by a recent study with *Arabidopsis* showing that among the several hundred genes that are modified in response to cold the most abundant group of long-term up-regulated genes is encoding DHNs or other LEA-proteins (Fowler and Thomashow, 2002). In a previous study it was shown that an antibody raised against a drought-specific DHN from *Craterostigma plantagineum* detects a number of DHNs whose level show annual variation in birch buds (Rinne *et al.*, 1998). The accumulation patterns in buds and stems differ in response to SD, LT, water stress, and exogenous ABA, and correspond to the elevation of freezing tolerance (Rinne *et al.*, 1998).

In several plant species DHNs are encoded by a multigene family and can be classified into sub-groups by using the numbers of conserved Y-, S-, and K- segments (Campbell and Close, 1997). The variation in spatial distribution and stress specificity of different DHNs suggests a functional specialization of different sub-families (Choi *et al.*, 1999; Nylander *et al.*, 2001). For example, most of the YSK_2-type DHNs in barley are alkaline and they accumulate in response to dehydration and ABA, but not low temperature. By contrast, acidic or neutral SK-type DHNs are regulated by low temperature (Choi *et al.*, 1999; Zhu *et al.*, 2000). However, it has been suggested that all the dehydrins could, in principle, carry out the same functions in different tissues. Their regulation would be based on differential accumulation in response to various stresses (Nylander *et al.*, 2001).

To understand how winter hardiness develops in trees, it is necessary to elucidate how the perception of changes in photoperiod and temperature leads to the cessation of growth, the onset of dormancy, and the development of hardiness. In addition, it is necessary to identify the underlying mechanisms that bring about these distinct and sequential responses. The physiological and genetic factors that are directly regulated by photoperiod have been investigated here by comparing three latitudinal origins of pubescent birch (*Betula pubescens* Ehrh.), grown in a common garden experiment under natural photoperiod and temperature regimes. It was assumed that factors that are under photoperiodic control would show a clinal order among the ecotypes. Water content, level of freezing tolerance, and dormancy were measured throughout the experimental periods. The regulation of *DHN* genes during the acclimation process in field conditions was investigated by assessing the expression patterns of two *DHN* genes of birch. The involvement of photoperiod and temperature in *DHN* expression was confirmed under controlled growth conditions. It is shown here that, during autumn, the photoperiod has a determining role in the initiation of acclimation and the induction of dormancy, while deacclimation and release of dormancy in spring are regulated by temperature. The sequential pattern of expression of the two birch dehydrins in response to photoperiod and temperature suggests that they have a central role in the overwintering process of birch.

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Materials and methods

Experimental design

The study was conducted with 12–16-year-old latitudinal ecotypes of pubescent birch (*Betula pubescens* Ehrh.) growing in a common garden experiment at Muhos in central Finland (64°53′ N, 26°09′ E). Trees of each ecotype were offspring of one open-pollinated mother tree from Kittilä (North, 67°40′ N), Pyhäjärvi (Central, 63°40′ N), and Kangasala (South, 61°20′ N). The reported approximate critical daylengths for these latitudinal ecotypes are N, 20–24 h; C, 16–18 h; S, 14–16 h (Håbjørg, 1972*b*). Samples were collected every month except for December and June, starting from November 1996. The monthly mean-, maximum- and minimum temperatures preceding the sample collection day and the daylength of the collection day are described in Fig. 1.

In addition, a central ecotype of birch from Oulu (65°05′ N) was used to study the effect of photoperiod, temperature and water stress (WS) on the expression of dehydrin genes (*DHN*). Plants were micropropagated with standard procedures and then transferred to 10 cm pots in peat:sand:vermiculite (6:2:1, by vol.) mixture. Plants were grown in the greenhouse under a long daylength (LD) (22 h) at 18 °C and fertilized with commercial fertilizer once a week. At the beginning of the experiments, plants were 2 months old and approximately 50 cm tall. Some of the plants were transferred to short daylength (SD) conditions (12 h day, 18 °C) for 9 weeks and subsequently given a low temperature (LT) treatment (12 h, 4 °C) for 2 weeks. Some of the plants received the LT treatment under LD conditions (22 h, 4 °C) for 2 weeks. Water stress (WS) treatment was done under LD conditions by withholding water for 2 d.

Sample collection

In outdoor experiment twigs were collected at monthly intervals from five individual trees of each ecotype. The collections were repeated three times from the same tree except in the case of the northern ecotype where the trees were small and could have been stressed by successive sampling. One twig from each tree, with 4–12 buds, was used for dormancy testing and one twig from each tree was collected for each temperature in the freezing-tolerance test. From the rest of the twigs approximately 0.5 g and 1 g (FW) of buds were collected for water content measurements and RNA analyses, respectively. Buds collected for RNA analyses, were pooled from five trees, frozen in liquid N_2 and stored at –70 °C. In addition, from the seedlings grown in controlled conditions the upper 10 cm of the stem and three uppermost fully expanded leaves were collected for RNA analyses. The samples from two plants were pooled, frozen in liquid N_2 and stored at –70 °C.

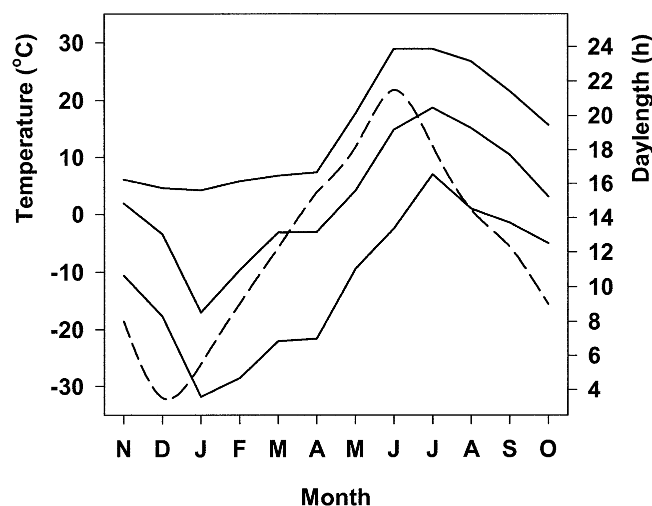

Fig. 1. Mean, minimum and maximum monthly temperatures at Muhos (64°53′ N, 26°09′ E) preceding the day of sample collection and the daylength (dashed line) on the day of sample collection. The Finnish Meteorological Institute has provided the data.

Dormancy tests

Bud dormancy was estimated indirectly by scoring bud bursting of single-node cuttings in water culture under forcing conditions (24 h day, 18 °C), as described previously (Rinne *et al.*, 1998). Bud bursting was monitored every second or third day for 3 weeks, after which the bud burst percentage was calculated. Data from five trees was pooled each month for calculating the mean day of bud bursting and the bud bursting percentage.

Water content measurement

The water content of buds from field-grown trees was measured by weighing the samples immediately after collection (fresh weight, FW). The dry weight (DW) was measured after freeze-drying the buds for 2 d. Water content was calculated from the formula $[(FW-DW)/FW]\times 100\%$.

Freezing tests

Freezing tests were performed in a controlled freezer as described earlier (Welling *et al.*, 1997). The lowest temperature used was –70 °C and injuries were scored visually by estimating the browning of the tissue according to Ritchie (1991). Injuries were estimated both for the stem tissue and the buds. For the stem, the outermost green tissue, including both phloem and cambium were estimated. Buds were scored as non-injured if they were able to burst in water culture, after the freezing test (Rinne *et al.*, 1998). Buds that did not burst were also scored visually for damage. Freezing tolerance is expressed as an LT_{50} value, which denotes the temperature at which 50% of the samples were killed.

PCR and cloning techniques

Genomic DNA from birch leaves was isolated according to Lodhi *et al.* (1994). Birch *DHN*-fragments were amplified with PCR using genomic DNA as a template. Degenerated primers for the amplification of different *DHN* sequences were designed by using conserved amino-acid sequences of known DHNs of *Arabidopsis thaliana*. The first 5′-primer was 5′-GAT/C GAA/G TAC/T GGI AAT/C CC-3′ corresponding to the amino acid sequence DEYGN and the second was 5′-GAT/C AGA/G GGI GTA/C/G/T TTT/C GAT/C TT-3′, corresponding to the amino acid sequence DRGVFD. The 3′- primer, 5′-CC IGG IAG C/TTT T/CTC C/TTT T/G/CAT-3′ corresponding to the amino acid sequence IKEKLP was used in both PCR reactions. The PCR conditions were 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 30 s for 34 cycles followed by a final elongation cycle of 30 min. An initial 7 min denaturation at 96 °C was carried out before adding *Taq* DNA polymerase (Promega). The gel-purified PCR product was cloned into pGEM-T-easy Vector (Promega) and sequenced.

RNA isolation and hybridization analysis

Total RNA from buds, stems and leaves was isolated according to Chang *et al.* (1993). Northern analysis was carried out as described previously (Welling *et al.*, 2002) except that the temperature of the hybridizations and washes was 68 °C. The cloned PCR fragments corresponding to birch *DHN* genes were used as probes.

cDNA synthesis

For cDNA synthesis, the total RNA of both SD- and LD-grown, 14 d LT-treated stem samples were combined and Poly (A) RNA was

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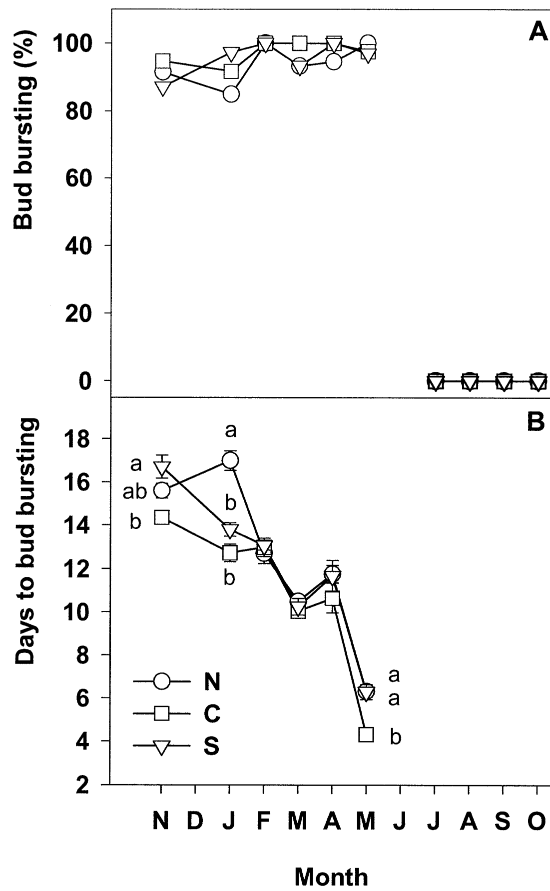

Fig. 2. The percentage of bud bursting (A) and the speed of bud bursting (B) in three latitudinal ecotypes of pubescent birch during the year. Bud dormancy was estimated by scoring bud bursting of single-node cuttings in water culture under forcing conditions (24 h day, 18 °C). Values are means (±SE for speed of bud bursting) of pooled data from five trees. N refers to northern (67°40′ N), C to central (63°40′ N), and S to the southern ecotype (61°20′ N). Speed of bud bursting between the three ecotypes in each month was analysed with Tukey's HSD test after ANOVA. Significant differences at the 0.05 confidence level are denoted with different letters.

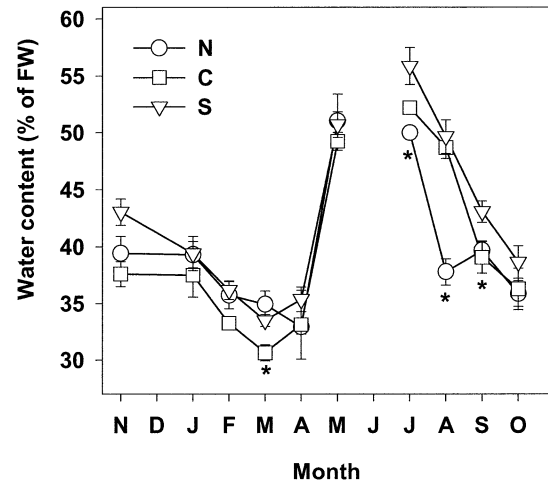

Fig. 3. Water content of the buds of three latitudinal ecotypes of pubescent birch during the year. Values are means (±SE) of data from five trees. N refers to northern (67°40′ N), C to central (63°40′ N), and S to the southern ecotype (61°20′ N). Significant differences in water content between different ecotypes in each month were tested by the non-parametric Kruskal–Wallis test. Months with significant differences at the 0.05 confidence level are denoted with an asterisk.

purified from total RNA with DynaBeads (Dynal AS, Oslo). cDNA was synthesized with SuperScript™ II RT (Invitrogen) according to the manufacturer's instructions and was used as a template for PCR as described above.

Statistical analyses

One-way analysis of variance (ANOVA) was used to test whether differences in speed of bud bursting between the ecotypes in each month were statistically significant and Tukey's honestly significant difference (HSD) test was used for *post hoc* comparisons. The Kruskal–Wallis test was used to analyse whether differences in the water content between different ecotypes in each month were statistically significant.

Results

Bud dormancy

To study the role of photoperiod and temperature on dormancy status of buds, bud bursting ability and the speed of bud bursting was measured in three latitudinal ecotypes of birch growing in the field. Buds of each ecotype were unable to burst between July and October. In November, the high bud bursting ability (85–100%) indicated a complete release of endodormancy at about the same time in all ecotypes (Fig. 2A). The speed of bursting of these ecodormant buds increased during the spring in rather similar way in the three latitudinal ecotypes (Fig. 2B) and it was not possible to establish any clear order among them. Interestingly, the speed of bud bursting in all ecotypes decreased transiently in April (Fig. 2B), coinciding with an exceptionally cold period (Fig. 1). Since there was no apparent clinal order in the percentage or speed of bud bursting, and since birch ecotypes responded similarly to naturally fluctuating temperatures, the results suggest that temperature is the primary determinant of dormancy release in birch.

Water content of the buds

As tissue desiccation is implicated in short day (SD)-induced dormancy development (Faust *et al.*, 1991; Welling *et al.*, 1997; Rinne *et al.*, 1998), the impact of photoperiod and temperature on the water content of the buds during overwintering was characterized. In July, the water content of buds was lowest in the northern ecotype, followed by the central and southern ecotypes, respectively (Fig. 3). The decrease in bud water content followed a clinal pattern until October, after which there were no statistically significant differences in bud water content between the ecotypes until March (Fig. 3). Throughout the winter, water content decreased further in all ecotypes,

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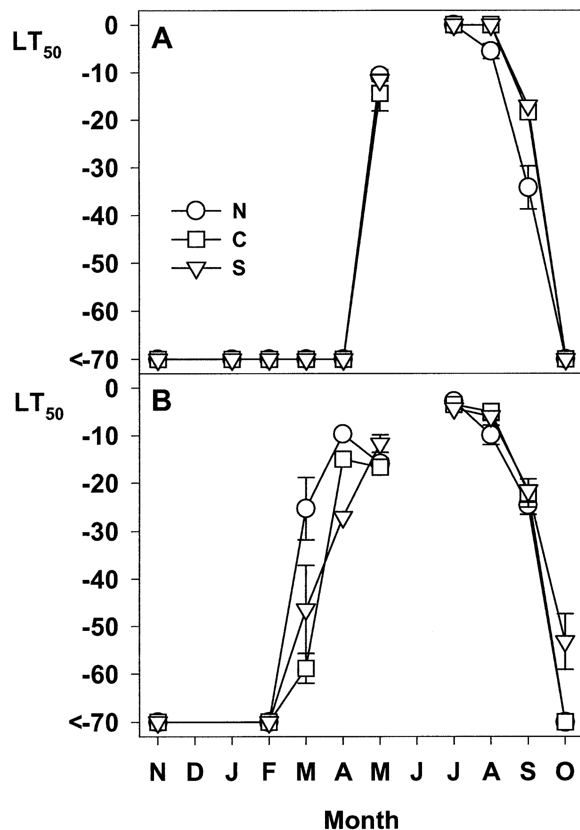

Fig. 4. Seasonal changes in freezing tolerance of the buds (A) and stem (B) of the three latitudinal ecotypes of pubescent birch. N refers to northern (67°40′ N), C to central (63°40′ N), and S to the southern ecotype (61°20′ N). Stem samples with buds were frozen to different temperatures in a controlled freezer and thawed overnight. Stems were cut into single-node cuttings and grown thereafter in water culture under forcing conditions (24 h day, 18 °C) for 3 weeks, after which injuries were estimated visually. Bud freezing tolerance was estimated as bud-bursting ability. During endodormancy when buds were not able to burst, freezing injury was estimated visually. The LT_{50} value denotes the temperature at which 50% of the samples show injury. Values are means (±SE) of five different samples. The lowest temperature used for freezing was –70 °C and in mid-winter plants were not injured at this temperature. Therefore, no SE values are shown in these time points.

being lowest in the central ecotype. In May, just prior to bud burst, there was a rapid but similar increase in water content in all three ecotypes (Fig. 3).

Freezing tolerance

To study the impact of photoperiod and temperature on the freezing tolerance of various tissues of birch, the degree of bud and stem freezing tolerance was measured once a month throughout the year from field-grown latitudinal ecotypes of birch. The initial non-acclimated freezing tolerance of all the tissues varied between 0 °C and –5 °C in all three ecotypes in July (Fig. 4). Subsequently, from July onwards, freezing tolerance increased gradually in all tissues. The increase followed a clinal order, especially in stem tissue, being fastest in the northern ecotype, followed by the central and, subsequently, the southern ecotypes. All birch ecotypes showed a very high cold acclimation capacity. Although freezing tolerance of the buds of the central and southern ecotypes only started to develop after August, by November their freezing tolerance was –70 °C or more (Fig. 4A). This was the lowest freezing temperature used in this study and after November no injury was seen at –70 °C in any of the tissues in any of the ecotypes. In the stem tissue, freezing tolerance started to decrease after February. The decrease did not show a clinal order, but tolerance decreased first in the northern ecotype, followed by the southern and the central ecotypes, respectively. This deacclimation was not linear, but it included periods during which freezing tolerance was maintaining the same level, or even increased (Fig. 4B). By contrast, buds retained their extremely high level of freezing tolerance throughout the winter until May. At this point, the decrease in freezing tolerance coincided with the increase in water content, just prior to bud burst (Fig. 3). Taken together, these results demonstrate that photoperiod plays an important role in the induction of freezing tolerance in the autumn, while deacclimation in spring is controlled mainly by temperature. In addition, the results suggest that the maintenance of freezing tolerance is differently regulated in stems and buds.

Sequence analysis of birch DHN *genes*

To study the regulation of *DHN* gene expression during the acclimation process, PCR with degenerative primers were used to isolate two genomic *DHN* fragments from pubescent birch and they were used as probes in northern blotting. The genes corresponding to the cloned fragments were named as *Betula pubescens* dehydrin 1, and 2, *BpuDhn1* [AJ555331] and *BpuDhn2* [AJ555332]. The deduced amino acid sequences corresponding to the partial genomic fragments were similar to previously reported DHNs. *BpuDhn1* contains three DEYGNP-motifs (Y-segment) in the N-terminus and has two Lys-rich regions at the C-terminus of the protein (K-segments). However, it lacks the Ser-repeat, common to this type of proteins (Fig. 5A). Since the clone is incomplete, *BpuDhn1* could be identified as a Y_nK_n-type of DHN (Close, 1996). According to the deduced amino-acid sequence, *BpuDhn1* encodes a basic DHN protein. A BLAST search against protein databases suggests that the *BpuDhn1* is most closely related to cold-induced dehydrin from *Cornus sericea* [AF345989] and *Solanum commersonii* [X83596] (Baudo *et al.*, 1996). The polypeptide predicted from another *B. pubescens DHN* clone, *BpuDhn2*, encodes an SK_n-type acidic DHN protein (Fig. 5B). It shows considerable similarity to the *B. pendula* dehydrin *Bplti36*, which represents the SK_2-type of DHN (T Puhakainen *et al.*, personal communication) and a drought-induced putative dehydrin from *Populus euramericana* [AJ300524].

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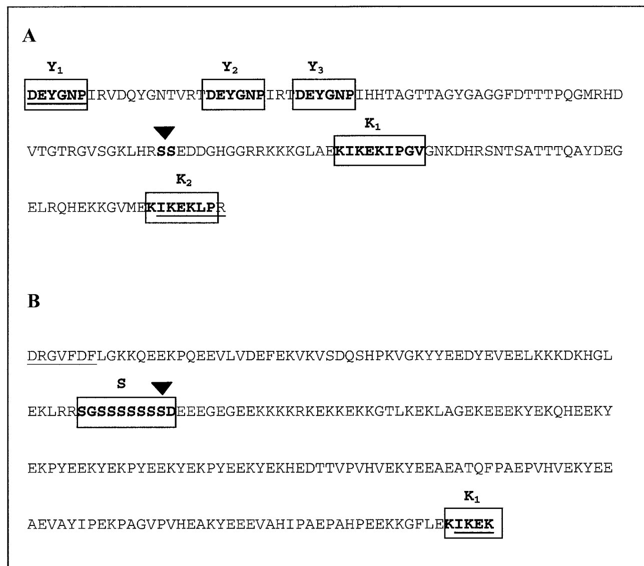

Fig. 5. Deduced amino acid sequences of pubescent birch genomic clones *BpuDhn1* (A) and *BpuDhn2* (B). Both clones contain an intron, the location of which is marked by an arrow. The consensus dehydrin Y-, S-, and K-segments are indicated in bold and boxed. Underlining shows the places of the degenerative primers used.

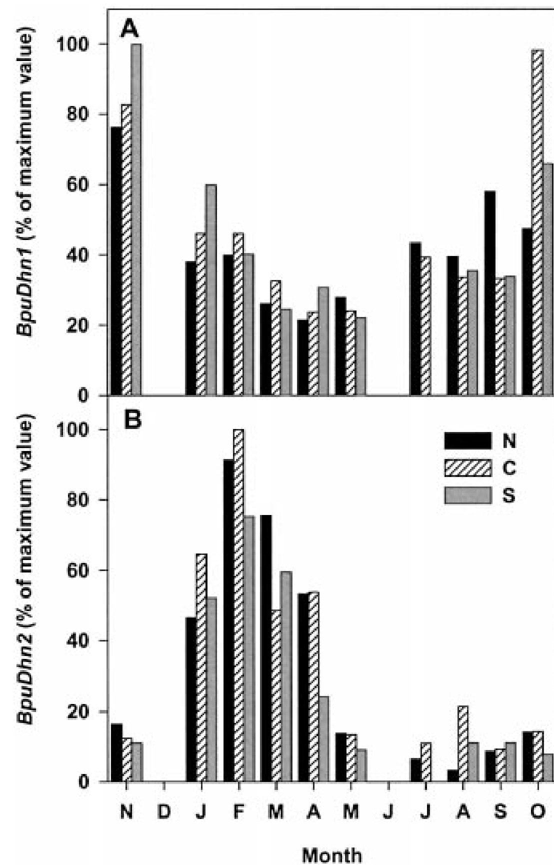

Fig. 6. Annual variation in the level of *BpuDhn1* (A) and *BpuDhn2* (B) transcripts in the buds of three latitudinal ecotypes of pubescent birch grown in a common garden experiment at Muhos, in central Finland (64°53′ N, 26°09′ E). N refers to northern (67°40′ N), C to central (63°40′ N), and S to the southern ecotype (61°20′ N). ^{32}P-radiolabelled genomic fragments of *BpuDhn1* and *BpuDhn2* were used as probes in northern hybridization. The histogram shows the normalized values of *BpuDhn1* and *BpuDhn2* after standardization to ribosomal signal intensities presented as a percentage of the highest value.

Between the S- and K-repeats, *BpuDhn2* has nine glutamate-rich repeats, that are also characteristic of *Bplti36*. The predicted coding sequence of both *B. pubescens DHN* genes appeared to be interrupted by one short intron (Fig. 5). The location of the intron was verified by characterization of the corresponding cDNA sequence.

Expression of DHN *genes of birch*

To elucidate the role of *DHN* genes in overwintering, the annual variation of the *BpuDhn1* and *BpuDhn2* transcript levels was measured in the buds of the three latitudinal ecotypes of birch grown in the field. Both *DHNs* showed an annual variation in their transcript levels, but the timing was different for *BpuDhn1* and *BpuDhn2*. In the case of *BpuDhn1*, the transcript started to accumulate first in the northern ecotype, in September, followed by the central and southern ecotypes in October (Fig. 6A). Transcript levels were highest in October and November and decreased gradually after that (Fig. 6A). Transcript levels of *BpuDhn2* were low during the autumn and started to increase in January (Fig. 6B). There was no clear clinal variation in the transcript levels of *BpuDhn2*, and all the ecotypes exhibited the maximal transcript levels in February, decreasing back to the basal level in May. These results suggest that the distinct difference between the pattern of expression of the birch *DHN*s *BpuDhn1* and *BpuDhn2* in the field are due to their different responses to the environmental factors that trees encounter during overwintering.

To dissect the environmental factors that induce expression of *BpuDhn1* and *BpuDhn2* the transcript levels were also analysed under controlled conditions. Birch seedlings of a central ecotype were grown either under SD or LD conditions after which they were exposed to low temperature (LT). Seedlings grown under LD conditions were also water-stressed (WS). The levels of *BpuDhn1* and *BpuDhn2* mRNAs were analysed from stem and leaf samples. Since SD eventually led to growth cessation and senescence of leaves, leaf samples were not collected after 3 weeks. The results indicate that *BpuDhn1* and *BpuDhn2* are differentially regulated under these conditions. SD treatment led to a slight increase in the level of *BpuDhn1* mRNA in the stem after 9 weeks under SD conditions, and subsequent LT treatment increased the level tremendously in stem tissue (Fig. 7B). By contrast, LT under LD conditions or WS had only a minor effect on the level of

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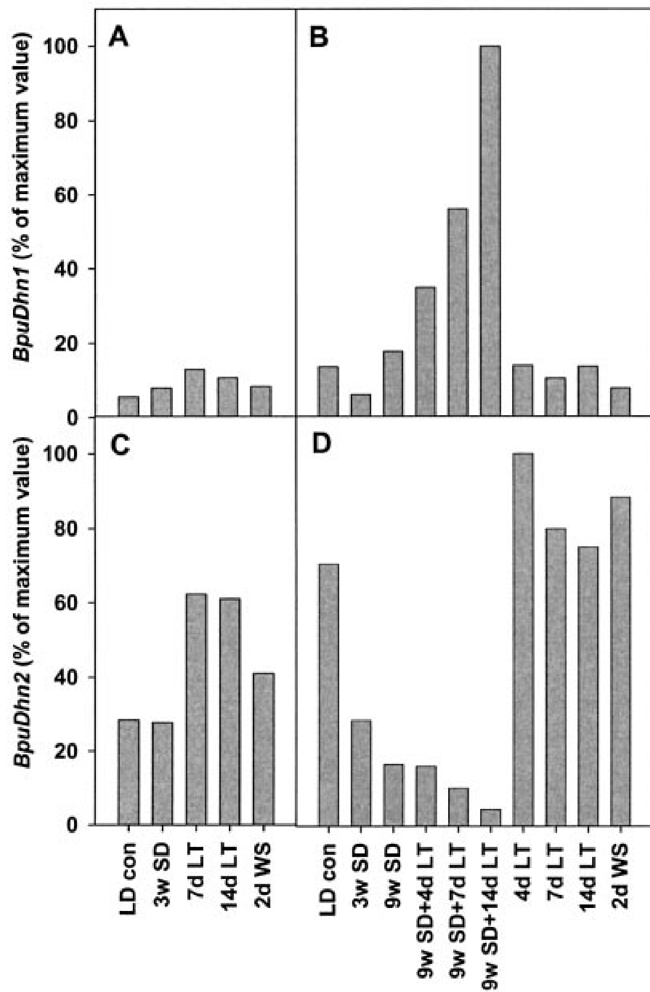

Fig. 7. Transcript level of *BpuDhn1* in the leaves (A) and stem (B) and the transcript level of *BpuDhn2* in the leaves (C) and stem (D) of the central ecotype of pubescent birch from Oulu (65°05′ N). Plants were grown under LD conditions (LD con; 22 h day, 18 °C), after which they were exposed either to low temperature LT (22 h day, 4 °C), water stress (WS; water was withheld for 2 d), short daylength (SD; 12 h day, 18 °C), or SD followed by low temperature (12 h day, 4 °C) treatment. Numbers refer to the duration of the treatment in days (d) or weeks (w). ^{32}P-radiolabelled genomic fragments of *BpuDhn1* and *BpuDhn2* were used as probes in northern hybridization. The histogram shows the normalized values of *BpuDhn1* and *BpuDhn2* after standardization to ribosomal signal intensities presented as a percentage of the highest value.

BpuDhn1 transcripts in the stem or leaves (Fig. 7A, B). *BpuDhn2* showed a very different pattern of expression under these conditions. The level of *BpuDhn2* transcripts was relatively high in control stem samples, but the level decreased during the 9 weeks of SD treatment and continued to decrease during the subsequent LT treatment (Fig. 7D). In the leaves, the level of *BpuDhn2* was lower than in the stem and the SD treatment did not decrease the level (Fig. 7C). LT and WS under LD conditions increased the level of *BpuDhn2* slightly, both in the stem and leaf tissues (Fig. 7C, D).

Discussion

At the phenological level it has been well established that latitudinal ecotypes of boreal and temperate zone trees follow a clinal pattern in response to short photoperiod, with the northernmost ecotypes responding first to the shortening daylength in autumn (Håbjørg, 1972*b*). This feature was used to study whether some of the central physiological and genetic factors known to be involved in overwintering (Welling *et al*., 1997; Rinne *et al*., 1998) followed a clinal pattern, i.e. indicating photoperiod control. To establish this, three latitudinal ecotypes of pubescent birch, growing in a common garden experiment in the same location and exposed to natural changes in photoperiod and temperature, were used. By using this approach, it was possible to show that birch trees, like many other temperate and boreal zone trees species, have evolved a strategy where photoperiod functions as an accurate and unquestionable signal to initiate overwintering. During winter, birch becomes insensitive to photoperiod and the overwintering process is mainly regulated by ambient temperature. It has been shown here that this pattern was evident both at the physiological and the molecular level.

The decrease of bud water content has been shown to be a characteristic event during dormancy development (Faust *et al*., 1991; Welling *et al*., 2002). In support of this, it was also found that, in the latitudinal birch ecotypes, water content typically decreased to 35–40% of FW in a clear clinal pattern during the autumn (Fig. 3). Even though a decrease in water content followed clinal pattern and has been shown to correlate with dormancy initiation (Welling *et al*., 1997), it is not an exclusive feature of endodormancy, as water content remained low and even decreased during the phase when endodormancy was removed (Fig. 2). This indicates that low water content reflects the non-proliferating status of the plants, regardless of whether the buds are endo- or ecodormant.

Buds of each ecotype were unable to burst from July to October (Fig. 2A). In July, the high water content (Fig. 3) and small size of the buds, as well as the green colour of the bud scales, suggested that ontogenesis of the buds was still unfinished. Therefore, it is likely that immaturity of the buds prevented bud bursting in July, as shown earlier (Rinne *et al*., 1994), and later on the endodormant state is the main reason for the inability of the buds to burst. Endodormancy was broken by November in all ecotypes (Fig. 2A) and there were no clear clinal differences between the ecotypes in the speed of bud bursting during the spring (Fig. 2B). In general, the different ecotypes responded in the same way, even to an exceptionally cold period in April (Fig. 1) as they all showed the same delay in bud bursting (Fig. 2B). Earlier studies have reported that seedlings of northern birch ecotypes have shorter chilling requirements for dormancy release compared with the

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southern ecotypes (Hänninen, 1990; Myking and Heide, 1995; Leinonen, 1996; Li *et al*., 2003). In this study, bud bursting of adult, field-grown trees was measured using single node cuttings under forcing conditions, which emphasizes the ability of bud bursting without correlative inhibition by the apical bud or other plant parts (Crabbé and Barnola, 1996). On the other hand, the low ambient temperatures during the study period might have resulted in the fulfilment of the chilling at an early phase. Whether or not the chilling requirements differ between ecotypes, it seems clear, that in the case of birch, dormancy release is not dependent on photoperiod (Heide, 1993). This is in accordance with the recent findings that breaking of dormancy involves the action of hydrolytic enzymes, the intracellular trafficking of which is responsive to chilling (Rinne *et al*., 2001).

Freezing tolerance induction during the autumn followed a clinal order in all tissues of the latitudinal ecotypes (Fig. 4), indicating that it is controlled by photoperiod. On the other hand, the maintenance of freezing tolerance of the stem was controlled by temperature only, since increasing temperatures tended to decrease freezing tolerance and a subsequent lowering of the temperature induced reacclimation (Figs 1, 4). This is in accordance with Sauter *et al*. (1996), who showed that freezing tolerance of poplar stems follows temperature changes during winter. By contrast, freezing tolerance of the buds did not decrease until May, just prior to bud burst (Fig. 4), demonstrating the relationship between dormancy and freezing tolerance. As long as buds were either endo- or ecodormant, their water content remained low, and they maintained high freezing tolerance. Since cold acclimation is accompanied by cellular changes that are reversed during deacclimation (Sauter *et al*., 1996) and probably counteracting the growth processes, a timely dehardening of stem tissues may be advantageous for the rapid initiation of growth after bud burst. Thus, the rapid deacclimation of the northern ecotype may reflect its adaptation to a short growing season. On the other hand the higher resistance of the buds to dehardening may safeguard them from freezing damage in case of fluctuating spring temperatures. The fact that a frost-hardened state can continue independently from endodormancy, in birch as well as many woody plants (Sakai and Larcher, 1987), suggests that bud dormancy and freezing tolerance are differentially maintained. Nonetheless, some relationship between dormancy and freezing tolerance exists, as freezing tolerance is lost more easily after the release from endodormancy (Junttila and Kaurin, 1989; Leinonen *et al*., 1997).

Molecular changes underlying the overwintering process of birch were examined by using two partial genomic clones encoding dehydrins (DHN) (Fig. 5). These results are in accordance with earlier studies showing that dehydrins in woody plants display seasonal expression patterns. Consistently, the levels of dehydrins are lowest during the active growth period and highest during the winter months (Wisniewski *et al*., 1996; Artlip *et al*., 1997; Rinne *et al*., 1998; Kontunen-Soppela and Laine, 2001). However, the specific expression patterns of the two birch *DHN*s are different. In field-grown birch, the level of *BpuDhn1* increased in the buds during the autumn, most likely in response to short daylength (SD) and to low, non-freezing temperatures (LT) (Figs 1, 6A), as these were the main factors inducing expression of *BpuDhn1* in controlled conditions (Fig. 7B). The sequential transient increase of *BpuDhn1* in different ecotypes in the autumn confirms the importance of photoperiod on its regulation. By contrast, the level of *BpuDhn2* was low in the autumn (Fig. 6B), in the same way as it was low at LT under SD conditions (Fig. 7D). The level of *BpuDhn2* was high between January and April (Fig. 6B). During this period the mean temperature was the lowest, but trees were also exposed to freeze–thaw cycles, with temperatures fluctuating between +5 °C and –30 °C (Fig. 1). Zhu *et al*. (2000) showed that, in barley, part of the *DHN*s are induced by low, non-freezing temperatures while some of the *DHN*s respond only to freezing temperatures, which is also the prerequisite for maximum hardiness. They suggested that low-temperature induced DHNs ‘prime’ cells for more severe cold. In birch and other temperate and boreal zone woody plants SDs function as an early warning signal for freezing conditions. It is suggested that *BpuDhn1* is one of the genes that are increased in response to SD, preparing the cells for subsequent steps in the acclimation process.

Distinct types of the *DHN* gene family have been shown to respond differentially to low and freezing temperatures, water deficit, and ABA treatment (Choi *et al*., 1999; Zhu *et al*., 2000; Nylander *et al*., 2001). In addition, photoperiodically regulated *DHN*s, which might be characteristic for deciduous woody plants, are demonstrated here. *BpuDhn1* encodes a basic, Y_nK_n-type DHN, which in stems was induced in response to SD and enhanced by subsequent LT (Fig. 7B). LT alone, under LD conditions, was not able to induce expression of *BpuDhn1* (Fig. 7A, B), suggesting that SD, either directly or via cellular changes, enables birch to respond to LT and trigger the expression of *BpuDhn1*. In hybrid aspen, the transcript levels of the homologous *DSP16* gene, encoding basic YSK_2-type DHN, increased in response to SD and was up-regulated during subsequent LT. However, temperature was shown to trigger the induction of *DSP16* independently from photoperiod (Welling *et al*., 2002). The other birch *DHN* studied here, *BpuDhn2*, had a high expression level in stems under LD control conditions (Fig. 7D). High basal levels have been reported earlier for pea B61 (Robertson and Chandler, 1994) and *Arabidopsis* ERD14 (Nylander *et al*., 2001), which both represent acidic, SK_n-type DHNs, similar to the protein encoded by *BpuDhn2* (Fig. 5). Constitutively expressed DHNs have been

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suggested to function, for example, as water attractants in water transport (Nylander *et al*., 2001). The constitutive expression of *BpuDhn2* in the tip of actively growing birch seedlings and down-regulation during SD supports the idea that *BpuDhn2* could have a role as supporting water transport to the tip of the growing plant. As SD induces growth cessation (data not shown), the need for water transport is diminished and *BpuDhn2* is down-regulated (Fig. 7D). The differential expression of *BpuDhn2* in leaves under LD and SD conditions supports the idea that it might have special functions in different tissues (Fig. 7C). In general, *BpuDhn2* was expressed in response to drought or low temperature stress when birch was either growing or in the ecodormant stage, i.e. able to grow under favourable conditions. In conclusion, birch seems to accumulate at least two types of DHNs. *BpuDhn1* is able to respond to stress at certain developmental stages, while *BpuDhn2* represents DHNs that are induced rapidly during the actual experience of the stress.

By using heterologous antibodies, it has previously been shown that a number of DHN proteins show both qualitative and quantitative annual variation in birch buds (Rinne *et al*., 1998). The initiation of expression of *BpuDhn1* and *BpuDhn2* in the field (Fig. 6) correlates with the initiation of accumulation of previously described 24 kDa and 30 kDa DHN proteins, respectively. However, during the annual cycle, the level of these proteins remains high for longer (Rinne *et al*., 1998) than the level of the corresponding transcripts (Fig. 6). The uncoupling of dehydrin mRNA and protein accumulation has been shown earlier (Artlip *et al*., 1997; Zhu *et al*., 2000) and may be accounted for by a stabilizing factor of protein or the regulation of translation. Therefore, the examination of the protein levels of the birch dehydrins is necessary to corroborate the role of different DHNs in overwintering. For example, although the 24 kDa and 30 kDa proteins were induced at different times during overwintering, their combined level was highest during the coldest months, presumably providing maximum protection under these conditions (Rinne *et al*., 1998).

Participation of several DHNs for the overwintering process of birch may arise from the need to control their expression accurately. On the other hand, as most DHNs are degraded rapidly after the stress treatment (Lång *et al*., 1994; Nylander *et al*., 2001), regulation of DHN degradation might be as important as their induction. The water-binding capacity of DHNs might, potentially, cause water deprivation in active cells, thereby necessitating rapid degradation of DHNs after removal of stress. The present work supports the possibility that the differential accumulation and degradation of *BpuDhn1* and *BpuDhn2* constitutes a sensitive framework for the regulated protection of tissues until the conditions return that are supportive for growth and development.

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