Cold Hardening and Dehardening in *Salix*

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Abstract

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The variation in cold hardiness in *Salix* in the autumn was investigated using clones of different geographic origins. In late growing season, the variation was small and inversely related to a phenotypic variation in potential growth rate. When growth had stopped in response to the reduction in daylength, however, large differences in cold hardiness developed. Northern/continental clones started cold hardening up to two months earlier and showed up to three times higher inherent rates of cold hardening than the southern/maritime ones. The two components of cold hardening, the timing of onset and the inherent rate, seemed to be separately inherited traits, as judged from analyses of the prodigy of a crossing between an early-and-rapidly hardening clone and a late-and-slowly hardening one. This suggests that cold hardiness can be improved without adversely affecting growth by selecting for a late onset of cold hardening combined with a rapid rate. Also, in the early stages, cold hardening was more sensitive to low, non-freezing temperatures in the southern/maritime clones than in the northern/continental ones.

Cold hardening of stems in the autumn could be monitored from the accumulation of sugars, most predominantly sucrose, raffinose and stachyose. The accumulation of sucrose started already with the cessation of growth, whilst the accumulation of raffinose and stachyose started later and was stimulated by cool temperatures. Multivariate models using sugar data could explain 76% of the variation in cold hardiness in the early stages of hardening. Changes in levels of sugars and other compounds during cold hardening could be assessed non-intrusively from the visible and infrared reflectance spectra of stems. Multivariate models using spectral data could predict up to 96% of the variation in cold hardiness. This technique is expected to greatly facilitate breeding for improved cold hardiness by allowing rapid screening of large populations.

The variation in cold hardiness in spring was also investigated. Loss of cold hardiness in spring was closely related to the bursting of buds. A relatively large genetic variation in the temperature requirement for bud burst was demonstrated indicating that this might be modified in sensitive clones to improve their cold hardiness in spring.

Key words: cold hardiness, dehardening, sugars, NIR-spectroscopy, *Salix*, breeding

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“But, what did you feel during this time?”

“Fear. Fear and discipline, Sir.”

Soldier, Warner Bros (1988)

Contents

Appendix

List of papers

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Lennartsson, M. & Ögren, E. 2002. Causes of variation in cold hardiness among fast-growing willows (*Salix* spp.) with particular reference to their inherent rate of cold hardening. *Plant Cell and Environment 25*, 1279-1288.

II. Lennartsson, M. & Ögren, E. Predicting the cold hardiness of willow stems using visible and near-infrared spectra and sugar concentrations. *Trees – Structure and Function*, Accepted

III. Lennartsson, M. & Ögren, E. Selecting for efficient cold hardening in a breeding population of *Salix* using near infrared spectroscopy. *Submitted*.

IV. Lennartsson, M. & Ögren, E. Genetic variation in temperature requirements for bud burst and dehardening in *Salix*. *Manuscript*.

Introduction

Plants are subjected to, and must adapt to, many different abiotic stresses. While shortage of water may be the most limiting factor for plant productivity, low temperature may be the most critical determinant of plant distributional limits (Salisbury & Ross, 1992). In fact, global vegetation patterns can be closely correlated with temperature zones. Figure 1 shows such patterns, ranging from the warm tropics to the extremely cold polar regions. However, even in the tropics some plants, which inhabit high altitude sites, must be able to tolerate frosts. On Mount Kenya, at altitudes of about 4000 m, the giant *Senecio* plants encounter night temperatures as low as -5 °C. In order to protect their frost-sensitive parts, the dead leaves are retained to form a dense, insulating covering (Sakai & Larcher, 1987; Attenborough, 1995). A further means whereby plants have adapted to survive frosts by avoiding freezing is to protect their vital organs in the ground or the snow pack. This provides a basis for classifying plants into different 'life forms' (Fig 2). Another example of an adaptive trait for avoiding freezing is the formation of night buds, via leaves curling around each other, and still another is the formation of water-repellent leaf hairs to avoid wetting of the leaf surface, which could initiate freezing (Sakai & Larcher, 1987).

In addition, many plants have evolved the ability to acclimate to low temperature by metabolic adjustments that give a limited degree of cold hardiness. This can be seen in various annuals of cold climates that can prolong their growth by such adjustments into the autumn. Perennial herbs and grasses, for instance winter cereals and cabbage, have the ability to undergo somewhat more extensive cold hardening. After growth has stopped in response to low temperatures, they can undergo cold hardening to resist temperatures down to about -25 °C. Although air temperatures may fall lower than this in winter, the over-wintering parts are protected by insulating soil and/or snow (Burke *et al*., 1976). The woody plants of the boreal forests can also develop this type of low-temperature-induced dormancy (ecodormancy) and associated, limited cold hardening. However, responding to reductions in temperatures per se is not a sufficiently reliable strategy for acclimating to low winter temperatures for a tall tree that is not protected by snow, and may have a life span lasting several hundreds of years, as temperatures are too unpredictable to serve as the triggering cue. A much more reliable annual event is the reduction in daylength in the autumn which, accordingly, serves as the triggering cue for deep cold hardening.

In order to undergo deep cold hardening, all growth must first be terminated before the true dormant state can be attained. This state can only be broken by another environmental cue that announces the arrival of spring. In annuals, the true dormant state is only exhibited by the seeds, and their dormancy can only be broken, in many species, by exposure to chilling (stratification; Fitter & Hay, 1987). In herbaceous perennials, dormancy also develops in the buds of the over-wintering organs, i.e. the bulbs, corms, rhizomes and tubers (Fitter & Hay, 1987). For many of these organs, chilling is required to release dormancy and to re-start growth (Larcher, 1995). In northern boreal trees, the acquisition of dormancy,

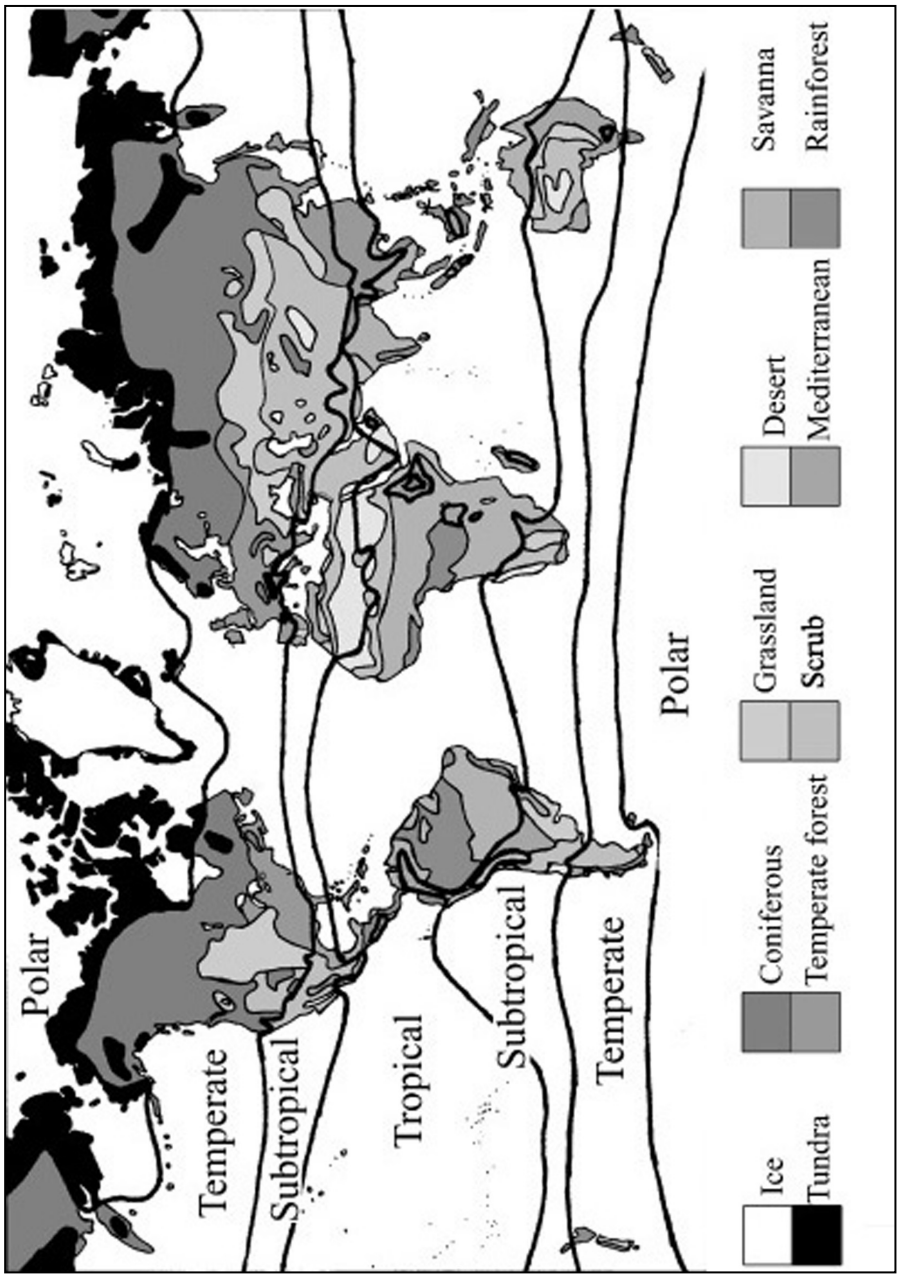

Figure 1. Global vegetation patterns and temperature zones.

triggered by the reduction in daylength, is a prerequisite for deep cold hardening. Induction of dormancy involves the cessation of growth, the build-up of cryoprotective substances (e.g. sugars and proteins), the down-regulation of photosynthesis and respiration, the shedding of leaves, and the development of dormant buds. These active physiological changes in the tree are fundamentally important for the subsequent phase of physical cold hardening that completes the cold hardening process, and allows survival at the very low temperatures encountered in northern areas. Breaking of dormancy and the commencement of growth can only occur after extensive periods of chilling. So, even if there is an extended thaw in early winter, the trees will not start growing. (Lambers *et al*., 1998).

Some trees can survive temperatures as low as that of liquid nitrogen when in the deep cold hardy stage (Sakai & Larcher, 1987), but will be killed at temperatures as high as -2 to -5 °C while actively growing. Perhaps the most hazardous times of year, with respect to frost injuries, are the autumn (when cold hardening has not been fully completed) and the spring (when dehardening may have started). At both of these times, frosts can be severe and frequent, as illustrated in Figure 3, showing the average and minimum temperatures during a single year in Vindeln, northern Sweden.

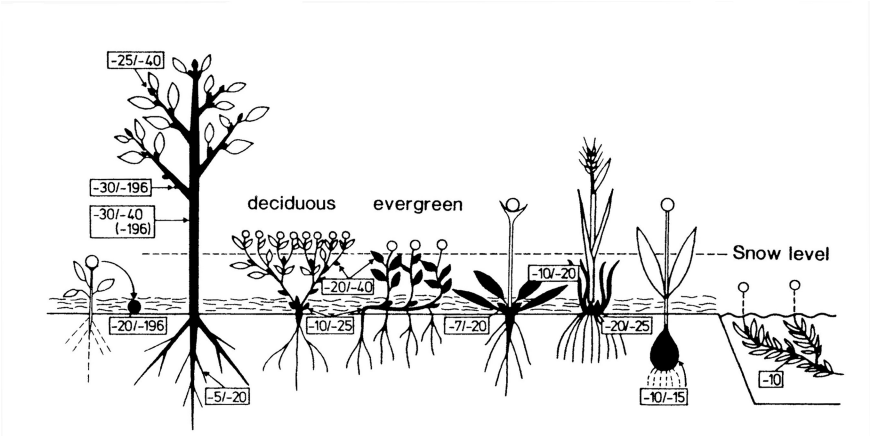

Figure 2. Adaptations of plants to freezing stress according to the life-form terminology of Raunkier. Over-wintering parts are shown in black with their typical ranges of cold hardiness shown. After Sakai & Larcher (1987).

There is currently much concern that the global climate will become warmer due to the anthropological emission of greenhouse gases, particularly CO_2. Prognoses

predict that not only will the global mean temperature rise, but the temperatures will also start to fluctuate more widely, with increased risks of extreme weather (SWECLIM, 2002). How trees will respond to these changes is of great interest, particularly with respect to their annual cycles of growth and hardening. To reduce our dependence on fossil fuels, there is interest in replacing them with renewable energy sources. In the early 1980's an association of companies, governmental authorities, and organizations was formed in Sweden to promote the increased use of bioenergy, including biomass plantations of fast-growing *Salix*. In 1998, these plantations covered an area of 16 000 ha with average yields of 7-9 tons dry matter ha-1 y-1 (Svebio faktablad 5/98). The hope is that the results of the studies described in this thesis will help to increase these production levels by supplying knowledge that will facilitate the breeding of more frost resistant Salix cultivars. Frost injuries are considered a major limiting factor for today's *Salix* plantations (Larsson, 1998).

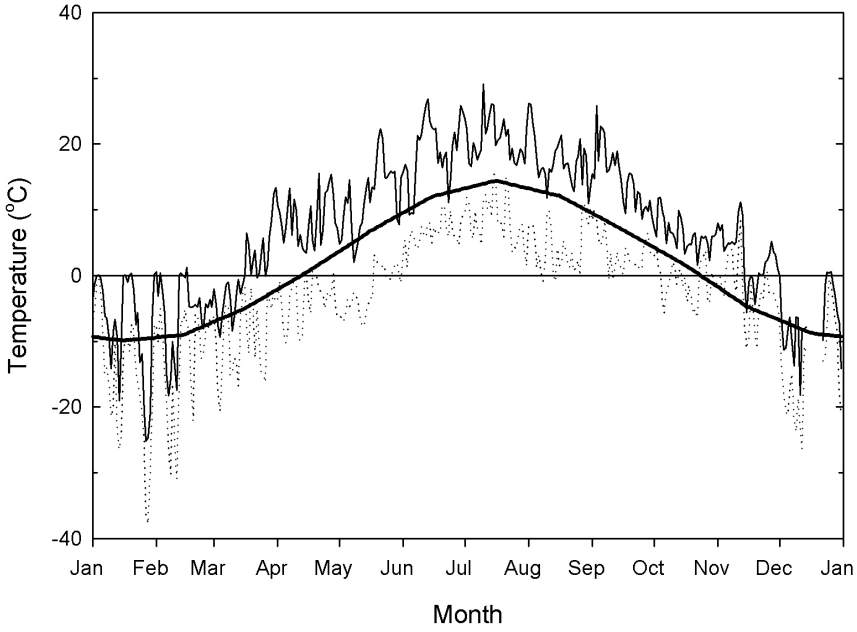

Figure 3. Annual temperature profile of an inland location of northern Sweden (latitude 64.2 °N, longitude 19.6 °E): the daily maximum (solid line) and minimum (dotted line) temperatures during 1999 and the daily average temperature during 1980-2002 (bell-shaped, solid line). Only the short summer period from June to August was frost-free. Data from the climate monitoring program at Vindeln Experimental Forests.

Aims

Frosts can cause extensive losses of biomass production in *Salix* plantations (von Fircks, 1992). Thus, improvement of our knowledge of cold hardening in the genus should facilitate breeding programs to improve the cold hardiness of *Salix*. Knowledge of the physiological components that contribute to cold hardiness, and how they can be quantified in breeding programmes, would be especially valuable.

Three major traits identified by Ögren (1999a) as determining the cold hardiness of *Salix* in the autumn were the pre-hardening level of cold hardiness while the trees are still growing, the timing of growth cessation (which marks the onset of cold hardening) and the inherent rate of cold hardening.

A major objective of the studies presented here was to investigate the degree of genetic variation in *Salix* with respect to these hardening components. Another objective was to investigate whether late growth cessation and rapid hardening traits can be combined to improve autumn cold hardiness without adversely affecting growth rates.

During spring, the loss of cold hardiness is closely linked to budburst and the commencement of growth (Dormling, 1982; Stevenson *et al*., 1999). Knowledge of the variation in temperature requirements for budburst is therefore important in order to breed and select for timely budburst to avoid freezing injuries due to premature burst.

There are two possible means of indirectly monitoring the development of cold hardiness for large-scale quantification and screening programs. One alternative is to measure the biochemical changes that occur during cold hardening, particularly the build-up of dry matter content and cryoprotective sugars, such as sucrose, raffinose and stachyose (Calmé *et al*., 1995; Leborgne *et al*., 1995; Ögren, 1999b). The other is to indirectly follow the biochemical and physiological changes that occur during cold hardening by scanning the visible and near infrared (NIR) reflectance spectra (Sundblad *et al*., 2001). The latter approach involves constructing multivariate statistical models linking spectral data to cold hardiness levels.

Paper I examines genetic variation in the components of cold hardiness, particularly the inherent rate of cold hardening, and its relations to the rate of growth and the timing of its cessation. The studies described in this Paper also investigated whether cold hardiness can be can be monitored by measuring increases in dry matter and sucrose contents during cold hardening.

The studies described in **Paper II** evaluated the possibility of using spectral information from visible and near-infrared regions, and information on the content of various sugars, for predicting the cold hardiness of *Salix* stems. Furthermore, it was investigated whether there was any variation in temperature sensitivity of cold hardening among clones of different geographical origins.

Paper III describes the use of NIR for selecting lines with rapid cold hardening in a breeding population of *Salix*. Of particular interest was to investigate whether the timing of the onset and the rate of cold hardening are independently inherited traits, allowing breeding for the desirable combination of a late cessation of growth and a rapid rate of cold hardening.

Finally, **Paper IV** examines the differences in temperature requirements for budburst and dehardening in spring among different *Salix* clones.

Freezing in plants

Water is the universal solvent for living organisms. If the water of the body fluids freezes this usually leads to death. Organisms that are cooled to subfreezing temperatures must either prevent or become tolerant to the freezing of their body water in order to survive (Zachariassen & Kristansen, 2000). Plants have evolved two main strategies that alleviate or avoid freezing damage. The first is to avoid freezing by supercooling water, and the other is to allow ice formation, but only in extracellular spaces. The main adaptive process in the latter strategy comprises changes in cells that allow them to tolerate the associated cellular dehydration (Sakai & Larcher, 1987).

Pure water has an equilibrium freezing point of 0 °C, but will usually supercool to a lower temperature before it freezes, and can supercool down to -38.1 °C (Rasmussen & MacKenzie, 1972). In plant cells the water may supercool to even lower temperatures due to the presence of cell solutes (Sakai & Larcher, 1987). Freezing of water is initiated at certain sites, so-called nuclei, where the water molecules are organized into an ice crystal lattice. With cooling, the movements of the water molecules slow down and they aggregate into clusters that spontaneously undergo homogenous ice nucleation when the temperature becomes sufficiently low. Aggregation of water can also be catalyzed by non-aqueous ice nuclei agents (INA), a process referred to as heterogeneous nucleation (Zachariassen & Kristiansen, 2000). In plants these INAs may include, *inter alia*, bacteria (Sakai & Larcher, 1987). It should be noted that the intracellular spaces lack effective ice nucleators (Steponkus, 1984). At the point of freezing heat is released and the temperature rises to a maximum of 0 °C (pure water), or less, depending on the degree of freezing depression due to the presence of solutes. This so-called exothermic peak can be used to detect the freezing point in samples (Burke *et al*., 1976; Sakai & Larcher, 1987).

Since the water vapour pressure is lower above ice than above a solution of water, the formation of ice in the extracellular spaces creates a driving force that promotes the movement of water out of cells. The intact plasma membrane allows this movement, but acts as a barrier against growth of ice into the cells (Steponkus, 1984). The rate of water efflux from cells is determined by the permeability of the membrane, the surface area of the cell and the difference in water vapour pressure

between the ice and the cytoplasm at the given temperature (Sakai & Larcher, 1987). If the cooling rate is slow, so-called equilibrium freezing occurs, whereby water leaves the cells to equilibrate the difference in water vapour pressure. As a result, the solute concentration of the cytoplasm is increased, further reducing the risk of intracellular ice formation. If the cooling rate is rapid, however, equilibrium freezing is precluded, the cytoplasm is supercooled and the risk of intracellular ice formation is increased (Steponkus, 1984).

The lowest temperatures that actively growing, unhardened plant cells can normally survive by means of supercooling are only a few degrees below 0 °C (Salisbury & Ross, 1992). Plant cells with particularly high concentrations of cell solutes can depress this point another few degrees (Burke *et al*., 1976). Herbaceous plants that are capable of over-wintering, like winter cereals and cabbage, can cold harden to survive winter temperatures of around -25 °C. These plants rely, instead, on the ability to tolerate ice formation in the extracellular spaces. The temperature limit for survival is then determined by the level of cellular dehydration, caused by extracellular freezing, that can be tolerated (Burke *et al*., 1976). This ability to tolerate freezing-induced dehydration when properly hardened is also displayed by most, but not all, woody species.

Some plants and tissues rely heavily on the ability to avoid freezing by deep supercooling. Such tissues include flower buds and xylem parenchyma cells, which can deep supercool to temperatures of around -40 °C (Burke *et al*., 1976). According to Sakai & Larcher (1987) effective supercooling is promoted by a range of factors, including small cell sizes, minimisation of intercellular spaces for nucleation, relatively low water contents, absence of internal nucleators, barriers against external nucleators and separation of cells into independently freezing units. In addition, anti-nucleating substances may be present that oppose the initiation of nucleation. Some hardwood deciduous trees (e.g. the genera *Fraxinus*, *Quercus*, *Ulmus* and *Malus*) rely on the supercooling of their xylem parenchyma cells; which are therefore the most vulnerable parts, and set the northern limits for the distribution of these species (Sakai & Larcher, 1987).

By contrast, boreal conifers and species of the *Salix*, *Betula* and *Populus* genera can develop almost limitless cold hardiness, allowing them to live much further north. The wood of these species is softer and the parenchyma cells have thinner and more elastic cell walls, which impose no restrictions on the extracellular ice formation (Sakai & Larcher, 1987). The very deep cold hardiness presumably involves formation of the aqueous glass state of cells. This state restricts the amount of water lost to extracellular ice growth at low temperatures (Hirsh, 1985). The role of sugars is further discussed in the context of cold hardening below.

Freezing usually starts in the water conducting system and rapidly spreads throughout the plant. Large vessels do not supercool, and the diluted sap has a high freezing point. Freezing injuries can be manifested in various ways, depending on the organ or tissue concerned the cold hardiness level, and the severity of the freezing. Discoloration of plant tissues is caused by the disruption of plasma membranes, allowing previously compartmentalized cell contents to

come into contact with each other and oxygen. The reaction products give a brownish or blackish coloration that can be seen, for example, in freeze-damaged *Salix* stems (Fig 4). Due to the leakage of cell solutes, tissues can also acquire a water-soaked appearance. Green parts of the plant may bleach and turn pale green, yellow or white. If the injury is severe and cannot be repaired, tissues will shrink and die back (for typical symptoms, see Fig 4). Some freezing damage is specific for trees. Strong sunshine during winter can lead to alternating freeze-thaw cycles in the stem, and thus de-hardening in bark tissue and cambium. This 'sunscald' is often restricted to the lower part of the stem, with the lower limit being set by the snow depth. If the injury is serious enough, the inner bark may become separated from the wood. Another tree-specific type of injury is the radially oriented frost splitting of stems, which may occur when wood with high moisture content ('wetwood') freezes during severe frosts. The frequency of such frost-splitting seems to increase near the northern or altitudinal distribution limits for tree species. A more detailed description of freeze injuries in plants is given in Sakai & Larcher (1987).

In an extensive review, Steponkus (1984) argued that the primary cause of freezing injury during a freeze-thaw cycle is the disruption of the plasma membrane. Although most data related to this process have been acquired using isolated protoplasts, they still provide an idea of how the plasma membrane behaves during freezing and thawing, and how injuries may arise.

Figure 4. Frost injuries of *Salix* stems in the field (left) and in closer view (middle) with unfrozen stems shown for comparison (right). The tissues of the stem become brownish or blackish in coloration, which is especially evident in the cambium. The injured shoot then shrinks and dies back and auxiliary buds beneath the damaged section start to flush.

The predominant form of injury in non-hardened plant cells is lysis due to the re-entry of water into them when they thaw. During freezing, membrane material is lost because of the contraction of the cell. In cold hardened cells, this is prevented by the formation of extrusions, still attached to the plasma membrane, which are therefore not lost on thawing. Instead, the cold hardened cells may suffer injuries, but at much lower temperatures, due to the plasma membrane losing semi-permeability in the contracted state. There are probably several causes for this, such as the formation of a more concentrated electrolyte solution upon freezing, and the removal of water from the surface of the plasma membrane causing a phase transition of the plasma membrane. The effect of these changes can be counteracted by the build-up of sugars, organic acids, amino acids and proteins: compounds that may dilute the electrolytes/toxic substances, and/or stabilize the membranes by direct interaction. Also, changes in fatty acid unsaturation and lipid content increase the membrane fluidity and contribute to the plant's ability to function at low temperature (Burke *et al*., 1976). However, the increased ability to function at low above-freezing temperatures may have little connection with the ability to survive low, sub-freezing temperatures. In addition, the injury may be reversible, and has been related to the loss and restoration of plasma membrane ATPase activity (*e.g.* Palta *et al*., 1977; Iswari & Palta, 1989; Arora & Palta, 1991). Finally, intracellular freezing may occur. For isolated protoplasts, this requires cooling rates >3 °C min^{-1}, regardless of whether the protoplasts were isolated from non-hardened or hardened cells (Steponkus, 1984). Given the very high cooling rate required, it seems more likely that the intracellular ice formation is an artefact resulting from the rupture of the plasma membrane allowing intrusion of the extracellular ice.

Dynamics of cold hardening

The annual cycle of cold hardening and dehardening is closely synchronized to the annual temperature cycle. Several factors may affect the level of cold hardiness attained, e.g. nutritional status, and the occurrence of pests, diseases, and drought, but the two most important factors are light and temperature (Sakai & Larcher, 1987). In northern boreal trees, the acquisition of cold hardiness is closely related to the acquisition of winter dormancy.

In many northern deciduous species, such as *Salix*, the maximum level of cold hardiness attained in winter is more than sufficient to tolerate prevailing winter temperature minima (Sakai & Larcher, 1987; Junttila & Kaurin, 1990). The most critical periods for the survival of the plants are, rather, the spring and autumn periods when cold hardiness levels are low, but frosts are frequent.

State of active growth

While plants of the temperate and boreal zones are actively growing they are not particularly cold hardy (Fitter & Hay, 1987). In a study by Christersson (1985), for

instance, growing shoots of *Salix* spp, *Picea abies*, *Alnus glutinosa* and *Betula verrucosa* were injured when subjected to temperatures between -3 and -5 °C. The damage occurred when ice crystals were formed in the tissues. Needles of *Pinus sylvestris*, by contrast, could survive temperatures down to -8 °C, even while they were elongating, despite the fact that ice crystals were formed in the tissues at temperatures as high as ca. -2 °C. This observation that variations in the basal level of cold hardiness is greater between than within tree genera was also reported in a comparison of species of the *Picea*, *Larix* and *Pinus* genera (Christersson *et al*., 1987).

When the basal level of cold hardiness was assessed in five species of Salix, some differences between species were found, although differences were generally small, with LT50 values of shoots ranging from -1 to -3 °C (von Fircks, 1985). Although the injured shoots eventually died back, the axillary buds beneath the dead section quickly flushed soon afterwards. Differences in the basal level of cold hardiness among *Salix* clones have also been reported by Ögren (1999a). However, in a more thorough study (Paper I) it was demonstrated that the clonal differences in the basal level of cold hardiness are phenotypic rather than genetic in origin. The basal levels of cold hardiness were generally weak, ranging from -1 to -4 °C. Although the basal level of cold hardiness was essentially the same across the different temperature regimes tested, it was inversely related to the potential rate of growth at the time of testing. Thus, there seems to be little potential for improving the basal level of cold hardiness by breeding and selection. This type of reciprocal relationship between active growth and cold hardiness has also been reported by other workers (Cooper, 1964; Pollock & Eagles, 1988; Greer *et al*., 2000).

As mentioned in the introduction, herbs and grasses have the ability to cold acclimate in response to low temperatures per se. This response involves the acclimation of processes of growth and photosynthesis, as well as the accumulation of cellular sugars (Hurry *et al*., 1995). Although boreal trees can undergo a similar type of cold acclimation, this should not be confused with dormancy-linked cold hardening (discussed below). In trees, this cold acclimation in response to low temperatures per se is slower, and does not lead to the same level of cold hardiness as cold hardening in response to reductions in daylength. Also, it does not involve the acquisition of tolerance to extensive freeze-induced cellular dehydration, which is of great importance for the development of deep cold hardiness. However, *Pinus sylvestris* and *Picea abies* could still attain cold hardiness levels corresponding to LT50 values of -19 and -16 °C, respectively, by exposure to low temperature (2 °C) without a shift in daylength (Christersson, 1978). Hybrid aspen (*Populus tremula* x *Populus tremuloides*) attained a LT50 value of about -9 °C after ten weeks at low temperature (0.5 °C), compared to -14 °C when low temperature was combined with a short day treatment in an investigation by Welling *et al*. (2002).

State of dormancy

The development of dormant winter buds and meristems in boreal trees represents a temporary interruption of growth that is endogenously regulated by phytohormones and triggered by environmental cues. Mitotic activity of meristems is greatly reduced or stops altogether at this time, and the cell nuclei remain in the G1 phase of the cell cycle (Larcher, 1995). This set of changes is typically triggered by photoperiodic cues, detected by the phytochrome system (Williams *et al*., 1972; McKenzie *et al*., 1974). There are, however, exceptions to this rule in that extremely northern ecotypes can enter dormancy in response to fluctuating temperatures in late summer while the days are still 24 h long (Junttila, 1980; Junttila, 1982).

Phytochrome, the pigment that detects the shift in daylength, occurs in two forms: P_r and P_{fr}), with absorption peaks of 660 nm (red) and 730 nm (far red), respectively. Upon absorption of red light, the inactive form P_r is transformed into the active form P_{fr}: a process that is reversed by the absorption of far red light and occurs spontaneously in the dark by thermal processes. In bright sunlight, the red light is more prominent than far red, leading to the equilibrium being shifted towards the active form. The active form P_{fr} is known to affect protein biosynthesis by regulating the transcription of DNA. The major form of phytochrome in leaves and shoots is phytochrome A (Heldt, 1997), which is responsible for detecting the critical reduction in daylength that induces the state of dormancy. This has been proved by overexpression of the phytochrome A gene, *PHYA*, in hybrid aspen: the modified plants showed reduced sensitivity to daylength reduction and this prevented them from undergoing cold hardening (Olsen *et al*., 1997). By contrast, plants with underexpressed *PHYA* levels showed increased sensitivity to daylength (Eriksson, 2000).

The differentiation of plants into latitudinal and altitudinal ecotypes with respect to critical daylength responses has been observed in many species (Heide, 1974; Juntilla, 1980; Kuser & Ching, 1980; Hurme *et al*., 1997). In addition, there is a longitudinal differentiation along coast-to-continental transects (Ögren, 1999a), which also reflects climatic influences on growth. Furthermore, variation in growth cessation occurs among coexisting genotypes (Aitken & Adams, 1996; Hurme *et al*., 1997). Besides the differences in critical daylength requirements, variation in the sensitivity to the absolute light level may be present. In latitudinal ecotypes of *Salix pentandra* studied by Junttila (1982), low levels of irradiance inhibited apical growth cessation more in southern than in northern ecotypes. In addition, genetic variation may occur in the rate of dormancy induction. In *Salix pentandra* there were significant differences in the time to apical growth cessation between three latitudinal ecotypes examined by Junttila (1982), with the one of most northerly origin being the fastest. The same trends have been observed in *Populus trichocarpa* ecotypes of different latitudinal origins (Howe *et al*., 1995).

Dormancy develops gradually through different stages, starting with the pre-dormancy state and ending with the true dormant state, in which plants can no longer be activated by temporary warming, a state that is attained in November-December (Larcher, 1995). In *Betula pubescens*, the attainment of the final state coincides with the shedding of the leaves (Myking, 1997). The degree to which dormancy has been developed can usually be tested by probing the plant's re-growth potential following transfer back to favourable temperatures: a high stimulatory effect of temperature suggests an early stage of dormancy. Although the induction of dormancy is triggered by the reduction in the critical daylength, temperature is important because it governs the speed at which terminal buds are formed. At temperatures between 18 and 24 °C, seedlings of *Picea abies* formed terminal buds within two weeks, but at temperatures between 12 and 15 °C the process was longer in investigations by Heide (1974). If the seedlings were treated with short days for up to 16 days at 12 and 24 °C, they resumed growth when transferred back to long days, suggesting that the temperature was too high for dormancy to develop completely. Similarly, in our studies, *Salix* plants kept at 15 °C eventually flushed even when kept in short day conditions (data not shown). In both *Picea sitchensis* and *Pseudotsuga menziesii*, height growth ceased in September, but buds did not become truly dormant until shoot apical mitotic activity had ceased completely, in connection with the first frosts in November (Cannell *et al*., 1985).

The critical daylength for cambial growth is shorter than the corresponding period for apical growth (Håbjörg, 1972; Heide, 1974). The transition from early to late wood tracheids, with the latter having thicker cell walls and narrower lumens than the former, is also induced by reductions in daylength (Heide, 1974). By contrast, root growth is not directly affected by daylength (Heide, 1974). Prominent cytological changes occurring when dormancy develops are the reductions in the number and size of cellular membranes and organelles (Larcher, 1995).

Throughout the summer period levels of sugars in the wood are low, while levels of starch start to rise in late summer (Lambers *et al*., 1998). With the onset of autumn, starch is broken down, while at the same time there is a build-up of sugars, predominantly sucrose, raffinose and stachyose, peaking in mid-winter. In addition, there is a build-up of storage proteins at the time of yellowing and shedding of leaves. In early spring, with the blossoming of catkins, the sugar and starch contents decrease (Witt & Sauter, 1994; Sauter & Wellenkamp, 1998). However, levels of glucose and fructose do not show such seasonal variations (Aronsson, 1976; Sauter & Wellenkamp, 1998; Ögren 1999b; Paper II). The break down of starch in autumn is reflected in the occurrence of a transient maltose pool (Sauter & van Cleve, 1993, Witt & Sauter, 1994). We also observed the increase in maltose in *Salix* plants treated to short days, but the subsequent decline that must occur was not observed, presumably because the plants were not studied for sufficiently long (Paper II).

Several studies have shown that the major sugars that build up during the development of dormancy are sucrose, raffinose and stachyose. However, the accumulation of these sugars occurs at different times, and temperature has strong

effects on the kinetics of these changes, which differ between different sugars. The rise in sucrose levels occurs in direct response to short days in both conifers (Aronsson *et al*., 1976) and *Salix* (Ögren, 1999b), while raffinose and stachyose levels rise later in autumn, in response to the drop in temperature (Hinesley *et al*., 1992; Stushnoff *et al*., 1998; Ögren 1999b; Cox & Stushnoff, 2001). The levels of raffinose and stachyose also seem to be more stable during the winter, compared to those of sucrose, which oscillate from month to month (Stushnoff *et al*., 1998).
Several proteins accumulate in the wood in response to short days (Coleman *et al*., 1991). Some of these are storage proteins (Sauter & Wellenkamp, 1998) but others, the so-called dehydrins, are involved in the cold hardening process (Artlip *et al*., 1997; Welling *et al*., 2002). In conjunction with the down-regulation of photosynthesis there is an accumulation of anthocyanin (Howe *et al*., 1995; Chalker-Scott, 1999; Merzlyak & Chivkunova, 2000) and a partial degradation of chlorophyll (Ottander *et al*., 1995). In *Salix* stems, this is manifested as a change of colour from green to reddish hues.

Cold hardening

From studies of apple trees, Howell & Weiser (1970) concluded that short days per se can trigger cold hardening down to a certain plateau, even when temperatures remain ≥15 °C. However, the temperature still affects the rate of cold hardening, and the level of cold hardening that can be attained, as occasional frosts trigger a deeper cold hardiness, and temperatures between -15 and –50 °C trigger the very deep cold hardiness seen in boreal trees in winter (Glerum, 1985).

Variations in the timing of growth cessation have been considered the primary cause of the genetic variations in cold hardiness in autumn, and they provide the most obvious differences between and within species (Nilsson & Walfridsson, 1995; Aitken & Adams, 1996; Deans & Harvey, 1996; Hurme *et al*., 1997; Hawkins & Shewan, 2000). The other important component, the rate at which cold hardening proceeds has mainly been considered in relation to the effect of temperature (Howell & Weiser, 1970; Aronsson, 1975; Christersson, 1978; Leinonen 1995), but seldom in the context of possible genetic variation. Some findings from field experiments suggest that such variation exists, for example in *Acer rubrum*, in which northern genotypes start hardening earlier and have a higher rate of cold hardening than southern genotypes (Lindstrom & Dirr, 1989). Similar trends have been observed in the field in *Pinus sylvestris* (Nilsson & Walfridsson, 1995). However, in order to be able to properly separate the rate of cold hardening from its timing, studies must be done in controlled environments to rule out temperature effects. Incidentally, in a study of three *Alnus* species, differences in the rate of cold hardening were seen both between and within the species (Tremblay & Lalonde, 1987), and a specific study on the inherent rate of cold hardening has shown that genetic variation for the trait exists among *Solanum* species (Vega *et al*., 2000).

The findings of Ögren (1999a) that *Salix* clones with similar timing could have different rates of cold hardening, led to a more extensive study in which eight

clones covering a wide geographic distribution were investigated (Paper I). From the results it can be concluded that northern/continental clones not only started cold hardening up to seven weeks earlier than the southern/maritime clones, but their inherent rates of hardening were up to three times higher (Fig 5 and Table 2, Paper I). The genetic differences were consistent across temperatures, with a lowering of temperature stimulating the rate of cold hardening in all clones. One clone of southern/maritime origin was particularly strongly stimulated by the falling temperature (Table 2, Paper I), although this was not reflected in greater cold hardiness as compared to other clones of similar origin, as cold hardening was initially delayed in this clone. The modulating effect of temperature on cold hardening is well known (Tremblay & Lalonde, 1987; Leinonen *et al*., 1995), especially in later stages of the process (Leinonen *et al*., 1995), when frosts become stimulatory (Junttila & Kaurin, 1990). Fluctuating temperatures may also be stimulatory (Junttila & Kaurin, 1990). Intra-specific differences in temperature sensitivity have been observed in *Salix pentandra*, where northern ecotypes were found to be less sensitive than southern ones (Junttila & Kaurin, 1990). Similarly, using other *Salix* species we showed that the northern/continental clones were less sensitive to low temperature than the southern/maritime clones. In a cool regime with day, night and average temperatures of 8, 10 and 3 °C, respectively, the northern/continental clones were able to cold harden continuously throughout the eleven-week experiment, whereas the southern/maritime clones did not attain further cold hardiness after four weeks of hardening. Only the northernmost clone was able to attain equally deep cold hardiness in this cool regime as in the mild regime, with day, night and average temperatures of 14, 5 and 8 °C, respectively. Another conclusion from our studies is that the optimum temperature for the initial phase of cold hardening for *Salix* is above 3 °C (Paper II), but below 11 °C (Paper I).

Leaves of deciduous species are also able to undergo a certain degree of cold hardening in parallel with stems, in both *Salix* (Tsarouhas, 2002; Paper I) and *Populus* (Welling *et al*., 2002). This does not seem to involve the accumulation of leaf sugars (Ögren, 1999b). The experimental removal of leaves reduces the ability of the remaining plant to cold harden, suggesting that leaves play a vital role during hardening and must, therefore, be maintained during the early autumn: this may explain why their retention during autumn frosts is assured by their cold hardening (Howell & Stockhouse, 1973). Cold hardening was faster in needles than stems of *Pinus sylvestris* studied by Hurme *et al*. (1997).

Several attempts have been made to develop models for cold hardening in order to predict cold hardiness and to better understand the process. Anisko *et al*. (1994) modelled cold hardening for several taxa of deciduous woody plants, using various temperature parameters as input variables (minimum, maximum, average, chill and heat accumulation), together with daylength and the time of year. Their major finding was that cold hardiness involves two processes: one that is internally regulated and independent of temperature and another that is externally regulated and dependent on the accumulated chill or heat. It seems from the discussion above that the first process is related to the initiation of the cold hardening process induced by growth cessation and dormancy, and the second to

the modulatory effect of temperature. Another attempt to model the process was based on cold hardening data derived from seedlings of *Psedotsuga menziesii* var. *glauca* tested at three different temperatures (Leinonen *et al*., 1995). At each temperature, a certain level of cold hardiness was attained and maintained if the temperature was held constant. The rate of change in cold hardiness was dependent on the temperature and on the prevailing level of cold hardiness.

The relationship between the development of cold hardiness and the build-up of sugars is well established (Aronsson *et al*., 1976; Ögren 1999b), and a mechanistic explanation has been proposed. First, there is the osmotic effect, whereby a high sugar level reduces the amount of cellular dehydration that follows freezing. Second, there is a metabolic effect, since sugars are needed for the production of other protective substances. Third, there is the direct cryoprotective effect of sugars on cellular structures (Sakai & Larcher, 1987). However, different sugars show different patterns of correlation with cold hardiness. In the initial stages of cold hardening, the hardiness levels are correlated best with the sucrose level (Ögren 1999b; Paper I and II). In later stages, oligosaccharides, such as raffinose and stachyose, become more important (Hinesley *et al*., 1992; Stushnoff *et al*., 1998; Ögren, 1999b; Cox & Stushnoff, 2001). Using controlled regimes we observed essentially the same patterns of sugar variation at 3 and 8 °C, but there may have been a slightly greater increase of raffinose and stachyose at the lower temperature (Paper II).

As for the cryoprotective effect, disaccharides, such as sucrose, stabilize plasma membranes and proteins during freezing (Crowe *et al*., 1990). As water is withdrawn from the membrane, its lamellar liquid crystalline structure is lost. Sucrose can replace water and interact with phospholipids so as to maintain the membrane structure (Strauss & Hauser, 1986; Caffrey *et al*., 1988). Sucrose has a strong tendency to crystallize at high concentrations, but this can be suppressed by the presence of raffinose (Caffrey *et al*., 1988).

Regarding the osmotic effect, the build-up of compatible solutes, sugars and certain proteins is thought to protect cell structures by binding water molecules and thus limiting dehydration. Freezing is often compared to dehydration (Ingram & Bartels, 1996), but there are important differences between the two processes, in that larger amounts of water can be removed from cells by dehydration than by freezing (Crowe *et al*., 1990). Cells of cold hardy plants have higher amounts of non-freezeable water and retain more water by osmotic action, and thus lose less water during freeze dehydration (Hodge & Weir, 1993). Loss of water to the extracellular ice is also prevented by intracellular glass formation, which occurs at temperatures at or below -30 °C (Hirsh, 1985). A glass is a liquid with the viscosity of a solid. Sugars in general are known to be excellent glass formers (Stushnoff *et al*., 1998). From studies of poplar wood, Hirsch (1987) concludes that the primary glass-forming solutes are complexes of special proteins binding to raffinose, stachyose and KCl.

Recently, much interest in cold hardiness research has been focused on the accumulation of certain proteins, the so-called dehydrins, and associated genes.

The dehydrin gene DHN is responsive to the phytohormone abscisic acid (ABA), which is known to promote dormancy (Cambell & Close, 1997; Welling *et al*., 1997). Dehydrins have been proposed to stabilize both macromolecules (Cambell & Close, 1997) and membranes during dehydration (Hincha *et al*., 1989; Hincha *et al*., 1990; Danyluk *et al*., 1998; Ismail *et al*., 1999). There is also evidence suggesting that they may have cryoprotective and antifreeze functions (Wiesniewski *et al*., 1999).

Breaking of dormancy

Breaking of dormancy is triggered when a certain degree of chilling has been experienced. Chilling has two critical components: temperature and duration. Chilling thus has a cumulative effect, increasing up to a threshold level that triggers full release from dormancy (Samish, 1954). Dormancy is broken at temperatures between -3 and 12 °C, with an apparent optimum at about 3-5 °C (Sarvas, 1972, 1974; Hänninen, 1990; Myking & Heide, 1995; Perry, 1971). It has also been suggested that species differ in their lower limit for dormancy release (Myking, 1998). The mechanisms responsible for chilling-induced release from dormancy are not fully understood, but Erez (2000) suggests that the effect is related to the activity of certain enzymes bound to membranes that affect their specific fatty acid contents.

Genetic variation in the earliness of dormancy release has been observed in *Betula* and *Alnus* (Heide, 1993a). Myking & Heide (1995) found a clinal variation in the chilling requirement among latitudinal ecotypes of *Betula pendula* and *B. pubescens*, with the southern ecotypes having greater chilling requirements than the northern ones. The difference was due to a difference in the duration of the chilling, rather than the temperature per se. Although the chilling exposure is the major factor responsible for the release of dormancy, it can ultimately be replaced by exposure to long days, if they are sufficiently long (Downs & Borthwick, 1956; Wareing, 1969; Heide, 1993a; Myking and Heide, 1995). In *Fagus sylvatica*, long days are in fact a prerequisite for dormancy release and budburst, in addition to chilling (Heide, 1993b).

Budburst

The period between release from dormancy and budburst can be predicted from the accumulated heat sum, calculated in terms of day-degrees (d °C), above a certain threshold temperature. A higher temperature promotes faster budburst due to the more rapid accumulation of the heat sum. As the temperature ranges for chilling and budburst overlap, it can be difficult to separate their influence (Hänninen, 1990; Chuine, 2000). Nevertheless, Heide (1993a) was able to demonstrate, by comparing different species, that there is no direct relationship between the chilling and bud burst parameters, as release from dormancy can be early, but budburst late, i.e. chilling requirements can be low, while the temperature sum required for budburst can be large. For calculating the temperature sum, the use of an appropriate threshold temperature is important. The

use of 5 °C as a threshold temperature has often been proposed (Cannell & Smith, 1983; Sarvas, 1972; Hannerz, 1999), but has been questioned as being too high by Heide (1993a), who instead recommended 0 °C.
Different species differ in their timing of budburst, attributed to their differences in temperature sum requirements for budburst (Heide, 1993a). This parameter can also vary extensively among genotypes of the same species. Among *Salix* clones, we found genetic variation in both the threshold temperature and the temperature sum requirement (Paper IV). The variation in the threshold temperature, however, was quite small, in the range of 1.0 to 1.2 °C, with the only exception being a hybrid between a Siberian and a Western Europe clone that had a threshold as low as 0.4 °C. The variation in timing of budburst was therefore largely explained by the variation in the temperature sum requirement, which ranged from 110 to 183 d °C. The temperature sum requirement for bud burst was only weakly linked to their geographical origin (Table 2, Paper IV). In other species, for instance *Betula pendula*, *B. pubescens* and *Picea abies*, however, latitudinal ecotypes of the northern ecotypes were shown to require lower temperature sums and to have lower threshold temperatures (Worall, 1975; Myking & Heide, 1995). Also, threshold temperature decreased with increased elevation of alpine conifers (Worrall, 1983, 1993). It is tempting to suggest that the northern ecotypes can afford to have lower temperature requirements, as they are less likely to encounter prolonged spells of mild temperatures in winter that could trigger premature dehardening. The southern ecotypes may have an adaptive need for a more conservative strategy. Dehardening in spring is a much faster process than cold hardening in autumn (Nilsson & Walfridsson, 1995), and seems to be closely related to the initiation of growth processes in buds (Dormling, 1982; Stevenson *et al*., 1999). In *Salix*, stems could still resist temperatures of about -15 °C or lower as late as the beginning of May and we found no apparent differences in the rate of dehardening among genotypes (Fig 2, Paper IV), as previously noted for *Pinus sylvestris* (Nilsson & Walfridsson, 1995).

Because there are genetic variations in the chilling requirement for dormancy release, in both the threshold temperature and the temperature sum for budburst, there is scope for breeding and selection with respect to these important parameters (Heide, 1993a; Myking & Heide, 1995; Paper IV).

Material and Methods

Freeze tests typically play a critical role in studies of cold hardiness, such as the investigations reported in this thesis. A freeze test has two parts: a controlled freeze-thaw-cycle, and the subsequent evaluation of freeze injuries. There are several alternative approaches, especially for the evaluation, which will be briefly reviewed below. Also, the possibility of predicting cold hardiness using different methods will be discussed. For a full description of the methods involved, the reader is referred to Papers I, II and III, and the references therein.

The Plant Material

All of the studied clones are included in the Svalöf Weibull breeding program, primarily chosen for their fast and erect growth characteristics. The *Salix* clones we studied were representatives of the species *S. viminalis*, *S. dasyclados*, and *S. schwerinii* x *S. viminalis*. However, determination of species is in many cases difficult as hybridization occurs naturally amongst these species. In our selection, clones were also chosen to represent as much as possible of the geographical variation, and thus (hopefully) the overall genetic variation of this group of species. The geographical origin of the clones is shown in Fig. 5, together with their commercial names. Two further points should be noted. Firstly, *Salix* is a cultural plant that has been used for basket production and house building for thousands of years and has been widely spread for these purposes. So, the genotypes may have evolved originally in different environmental settings, although some selection might have occurred in their new settings. Secondly, the studied genotypes represent individuals of populations, and thus cannot display the full variation present at the population level.

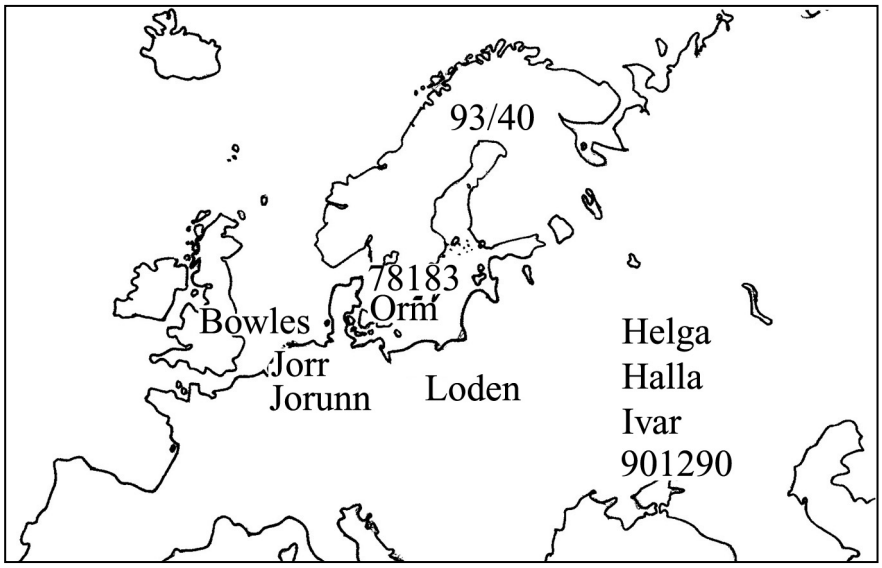

Figure 5. Geographical origin of clones studied using their commercial names (Svalöf Weibull AB).

A critical element of our studies was determination of the point of growth cessation. This can be done by monitoring the rate of growth in the autumn. However, under natural conditions this requires a means of separating the influence of the intrinsic factors affecting growth from that of the temperature

drop in the autumn. This can be done by relating the observed growth to the potential growth at the prevailing temperature (Ögren, 1999a). However, a less time-consuming alternative for assessing the timing of growth cessation is to monitor changes in the appearance of the shoot apex. *Salix* does not form a terminal bud, but as growth is reduced the shoot apex is reduced in size and eventually aborted, as shown in Fig 6. To reduce the level of subjectivity, the point of growth cessation was defined as when the length of the shoot apex was reduced to half of the length of the closest leaf with a width of 3 mm (Paper III). As discussed above, the development of dormancy may be slower in genotypes of southern/maritime origin than in northern/continental genotypes, and thus the reduction in the size of the shoot apex may also be slower. This might complicate determination of the timing of growth cessation by visual inspection.

Figure 6. Growth cessation at the shoot apex for *Salix*. Left, the growth cessation has been initiated, middle, the apex is reduced and withered and right, the apex has been abscised. Point of growth cessation was defined as when the length of the shoot apex was reduced to half of the length of the closest leaf with a width of 3 mm.

In order to assess the rate of cold hardening, independently of the variation in the timing of growth cessation, an abrupt shift in daylength was applied to ensure there was a common point of growth cessation. This procedure has several advantages. Firstly, all clones, irrespective of their geographical origin, can be compared on the same time scale. Secondly, the risk that clones will start cold hardening at different temperatures is eliminated and, thirdly, the time requirement is greatly reduced. A potential risk is that the responses of the clones will differ according to the size of the divergence between the photoperiod applied and their respective critical photoperiods that trigger growth cessation and dormancy

development in the field. In *Populus trichocarpa* the greater this divergence, the faster the response is in terms of variables such as the number of days to bud set (Howe *et al*., 1995). Moreover, in the cited study, the authors suggested that northern ecotypes have greater daylength sensitivity, i.e. show a greater change per unit change in daylength, compared to southern ecotypes. Furthermore, in *Picea glauca* given a two week treatment at different daylengths, ranging from 8 to 14 hours, the shorter the daylength, the faster the cold hardening was when the plants were subsequently switched to a 13 h photoperiod (Bigras & D'Aoust, 1993). This implies that when subjected to the same short-day, dormancy-triggering treatment, the northern/continental ecotypes might have a relatively rapid response in comparison to the southern/maritime ecotypes, as the effective change is greater for the former than the latter, due to the differences in critical daylength values. More studies are needed to determine if this is indeed the case. However, we have reason to believe that the method of abrupt daylength reduction is valid, at least, for the *Salix* clones studied here, because there was close agreement between rates of cold hardening found in the natural and abrupt daylength reduction regimes (Paper I, Fig 5b). The small deviation from the one-to-one ratio that was detected was presumably due to a small difference in temperature, as discussed in Paper I. It should also be noted that if daylength is reduced too much, the rate of cold hardening might be slowed down. In *Picea abies* and *Pinus sylvestris* cold hardening was much slower at daylengths of 2-4 h than 6-12 h in a study by Aronsson (1975).

Freeze tests

The same basic procedure for the freeze test (detailed in Paper I) was used in all studies included in this thesis. The most crucial aspects of freeze tests are to ensure good conductivity between the samples and the surrounding cooling medium, to initiate ice formation at -2 °C to avoid supercooling, to apply slow cooling (3 °C h-1) to ensure equilibrium freezing, and finally to apply slow thawing to avoid expansion-induced lysis. Generally, the initial freezing damage may either progress into secondary, more severe damage or be repaired. This general rule also applies to *Salix* stems (Ögren, 1999a). The initial responses have been related to the behaviour of the membrane-bound ATPase (Iswari & Palta, 1989; Arora & Palta, 1991). The more severe the primary injury, the more susceptible the stems become to infection by either bacteria or moulds causing secondary injury. In freeze tests conducted in mid winter (Paper II) when concentrations of mould spores were low, the samples were infected by bacteria instead, in a few cases, resulting in the failure of the fluorescence technique for assessing LT50 values. A standardized inoculation treatment is perhaps the most convenient way to ensure that the outcome of freeze tests is consistently based on the variation in the ability to withstand freezing, rather than the random variation in the amount of microorganisms present.

Evaluation

Freezing tests can be evaluated by assessing the plant's capacity for regrowth after freezing and the extent of visible injuries, e.g. browning of the cambium and the inner bark (Stergios & Howell, 1973; Calkin & Swanson, 1990). The drawbacks of these techniques are the long incubation periods and large amounts of plant material required. In addition, the assessment of tissue discoloration is prone to subjective errors (Ögren, 1999a). However, because these techniques can provide reliable data on the survival of plants they serve as a reference for other methods.

To attain more quantitative data, the electrolyte leakage method is often used (Palta *et al*., 1977; Paper 1). Briefly, this method involves estimating the extent of injury by measuring the leakage of electrolytes, using a conductivity probe, before and after freezing and relating this to the maximal possible leakage following total cell disruption (caused, for instance, by boiling). Electrolytic leakage shows a good correlation with visual injury, but reliance on this technique may lead to underestimation of the true cold hardiness below -80 °C (Sutinen *et al*., 1992). The method usually provides a measure of the primary injury, but can be modified to allow the post-thaw processes to be taken into account (Ögren, 1996).

Chlorophyll fluorescence

The rapidity, reproducibility, ease of use and the scope it provides for quantifying secondary damage makes chlorophyll fluorescence an attractive variable to measure in order to evaluate damage in freeze tests. The method shows good correlation with visual assessments of injury in *Pinus* needles (Lindgren & Hällgren, 1993), Douglas-fir seedlings (Fisker *et al*., 1995) and *Salix* stems (Ögren, 1999a).

The test relies on the fact that the maximum efficiency of photosynthesis, using dark-adapted material, can be assessed from the relationship $(F_m-F_o)/F_m = F_v/F_m$, where F_o is the minimum fluorescence yield when all reactions centres of photosystem II are open, F_m is the maximum fluorescence yield in response to a saturating flash of light which closes all reaction centres, and F_v is the variable fluorescence (Shreiber, Bilger & Neubauer, 1994). The ratio in light-adapted material is usually correlated with the quantum yield of electron transport of photosystem II and the quantum yield of carbon dioxide assimilation (Genty *et al*., 1989). Control values of F_v/F_m for dark-adapted material are typically in the range of 0.8-0.83 (Shreiber, Bilger & Neubauer, 1994).

Like many other biological stress responses, the development of freezing injury follows a typical sigmoid function (Figure 7), where the inflection point can be taken to indicate the LT50 value (von Fircks, 1993). As the control value for F_v/F_m declines from 0.8 as cold hardiness develops during the fall, a sigmoid function with a variable maximal value is best suited for fitting the regression.

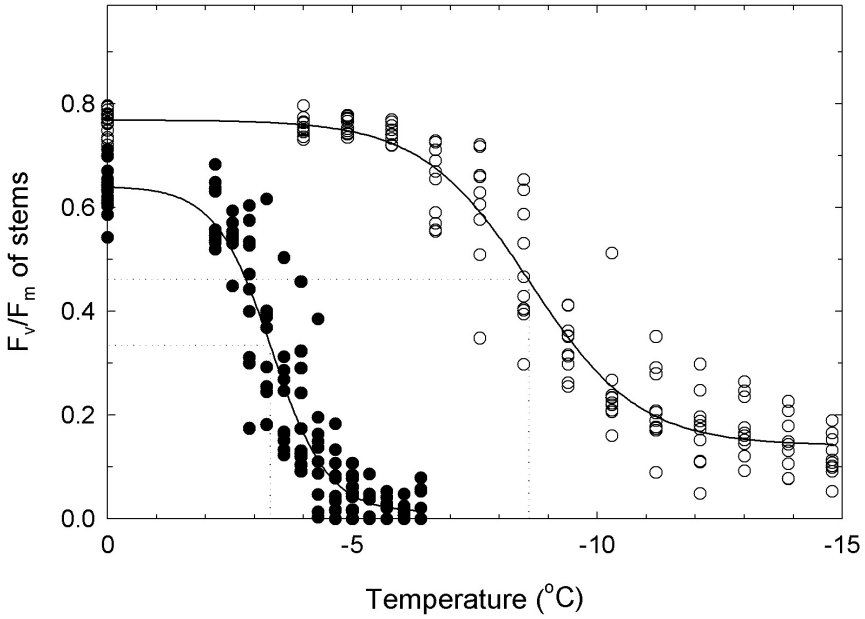

Figure 7. Evaluation of freeze test assessed by the chlorophyll fluorescence method. Filled symbols represent actively growing plants, and open symbols plants that had been cold hardened for about four weeks. Curves were fitted by regression analysis to a sigmoid function. Dotted lines represent the LT50 values. From Paper I, (Lennartsson & Ögren, 2002).

Prediction

As freeze tests are, inevitably, laborious and require large amounts of plant material, an alternative method for screening cold hardiness is needed. Several methods have been suggested for this that is briefly described below.

Sugar contents

The total content of sugars, including glucose, fructose, sucrose and raffinose, has been shown to correlate strongly with cold hardiness levels down to LT50 values of -20 °C (Ögren, 1999b). In addition, the ratio of sucrose-to-glucose contents correlates with LT50 values down to -12 °C (Paper I). The advantage of using sucrose-to-glucose ratios instead of the absolute amounts as a marker of cold hardiness is that much smaller amounts of plant material are needed for the analysis. Thus, the method is almost non-destructive, allowing the same plants to be tested repeatedly. Dry matter content is well known to increase with cold

hardening (see, for instance, Calmé *et al*., 1995; Pellet & White, 1969; Ögren 1999b; Paper I). Expressing the amount of sugars present on a dry matter basis might, therefore, be misleading. In Paper II, this was clearly illustrated by the changes in sucrose levels per unit dry matter, which rose as LT50 declined to about -11 °C. However, beyond this point the levels fell, due to a major rise in dry matter content. Thus, the decline in sucrose contents was only apparent, reflecting a cessation or slow down in sucrose accumulation. Alternatively, sucrose can be related to a neutral sugar, such as glucose, which is maintained at constant levels during hardening (Ögren 1999b; Papers I and II). Similarly, the amounts of raffinose and stachyose have been related to sucrose levels, to follow the later stages of cold hardening (Stushnoff *et al*., 1998).

When a multivariate model for predicting cold hardiness in *Salix* stems based on sugar levels was developed and tested, the predictive ability (Q^2) was in the range of 0.62 to 0.73 and the fraction of the explained variation (R^2) was between 0.65 to 0.75 (the higher values being observed when the model was applied solely to one temperature regime: see Paper II for details). In a similar attempt to model cold hardiness levels of conifer seedlings from the levels of sugars, lignification and dry matter, the explained variation was 80-85% (Sundblad *et al*., 2001).

Electrical impedance analysis

Intracellular resistance, measured by electrical impedance spectroscopy, reflects cellular changes that occur during cold hardening (Repo *et al*., 2000). When LT50 values of *Salix* stems were determined by visual inspection and by impedance spectroscopy there was good agreement between the methods down to LT50 values of about -10 °C (Repo *et al*., 1997).

Spectral reflective analysis

Near infrared (NIR) spectroscopy has the potential to replace more traditional 'wet chemistry' techniques for assessing the chemical content of plants that may be more expensive, hazardous and laborious (Osborne *et al*., 1993). The method has proven to be useful for determining the content of a wide range of plant cellular components (Gillon *et al*., 1999) and for the analysis of wood properties (Baillères *et al*., 2002). It is therefore not surprising that it could also be used for predicting autumn dormancy in alfalfa (Kallenbach *et al*., 2001), and cold hardiness in both conifer seedlings (Sundblad *et al*., 2001) and *Salix* stems (Papers II and III). Briefly, NIR spectra can provide information about chemical composition by detecting the overtones and combinations of vibrational modes of chemical bonds (Osborne *et al*., 1993). This information can then be calibrated against the reference method by means of a multivariate statistics approach called partial least squares projection to latent structures (PLS). The method has been reviewed (Martens & Naes, 1989; Eriksson *et al*., 1999), and packages for its implementation are included in the SIMCA software (Umetrics, Umeå, Sweden). A short description is provided in Paper II. In addition, orthogonal signal correction (OSC) was used to remove irrelevant systematic data (light scattering) not related to cold hardiness (Wold *et al*., 1998).

In Paper II we examined the spectral properties of intact stems in the range 410 to 2450 nm, thus including information in the visible region. In studies described in Paper III we probed dried and homogenised stems in the more limited spectral range of 1100 to 2500 nm. The difference in findings between the two studies allows some mechanistic interpretations to be postulated. In theory, the use of homogenised samples instead of heterogeneous, intact stems should result in less light scattering and thus, provide better estimates. However, the use of intact stems resulted in a model that could predict as much as 96% of the variation in cold hardiness (Paper II), while the corresponding value for dried and homogenised samples was 73% (Paper III): presumably because the intact samples provided more hardening-related information. The additional information found in the visible range included spectral changes reflecting reductions in the chlorophyll content and increases in the anthocyan content that accompany cold hardening (for details see Paper II). In addition, the use of dried samples excluded information on reductions in relative water content, which are well correlated with cold hardening (discussed above). Furthermore, the use of intact samples instead of dried and homogenised samples is more attractive, because it is less laborious and non-intrusive, allowing the same individual to be monitored throughout the cold hardening process.

Genetic markers

One way of improving cold hardiness through breeding is to identify and localize genes controlling this trait. A genetic marker has a specific sequence that is linked to one or several genes influencing a phenotypic trait, e.g. cold hardiness. Traits with a continuous variation are referred to as quantitative or polygenic, in contrast to traits where the variation is due to allelic differences at just one or a couple of genes. Quantitative trait loci (QTL) are genetic markers that are linked to such quantitatively inherited traits. To be able to find useful QTLs a population is studied phenotypically and associations between markers and phenotypes are sought for statistically. Identifying such loci requires the development of a genetic linkage map (Howe *et al*., 1999). Statistically, for QTL analysis to be efficient, the number of plants that are tested is more important than the number of markers. With small sample sizes, minor genes are not detected, leading to an underestimation of the number of genes involved (Lercetau *et al*., 2000).

A genetic linkage map for detecting QTLs related to cold hardiness in *Salix* was recently developed (Tsarouhas, 2002). Ten genomic regions for controlling cold hardening were found, each explaining 3-45% of the phenotypic variation.

Studies have shown that QTLs hold considerable promise for the study of dormancy-related traits (Chen *et al*., 2002; van Buijtenen, 2001). Currently, however, they do not provide the necessary precision of predictions (Byrne *et al*., 1997; Chen *et al*., 2002).

Breeding and Selection

Cold hardening in plants is a quantitative trait, involving the action of many genes with small additive effects (Stushnoff *et al*., 1985; Aho, 1994; Thomasshow, 1999). Cold hardiness levels during autumn, winter and spring, all seem to be under separate genetic control (Nilsson & Walfridsson, 1995; Aitken & Adams, 1996; Anekonda *et al*., 2000). Furthermore, there is generally greater genetic variation in cold hardiness in the autumn than in mid-winter (Aitken & Adams, 1996; Deans & Harvey, 1996; Suojala & Linden, 1997). Winter survival shows a relatively low heritability compared to other dormancy-related traits (Chen *et al*., 2002). In some studies variability has been found to be greater among clones within provenances than between provenances (Hawkins, 1993).

Traditionally, breeding for increased growth has led to extensions of the growth period and, thus, increased risks of injuries from autumn frosts (Mikola, 1982; Rehfeldt, 1992; Stevenson *et al*., 1999). Autumn frosts also cause significant biomass losses in *Salix* plantations (von Fircks, 1992): a problem that is being addressed in ongoing breeding programmes (Gullberg, 1993; Larsson 1998). In Papers I and II it was shown that autumn cold hardiness was affected not only by the timing, but also by the inherent rate of cold hardening. The main objective of the study described in Paper III was to investigate whether or not these two traits were independently inherited. In other studies it has been generally observed that inter-provenance crosses show intermediate responses with respect to the timing of the onset of cold hardening (Rehfeldt, 1979; Mikola, 1982; Junttila & Kaurin, 1985; Junttila & Kaurin, 1990). This was not found in the study presented in Paper III, where timing in the F2 population was strongly skewed towards the male grandparent (Paper III, Fig 2c): a peculiarity that was explained by this grandparent being hexaploid, whereas the female grandparent was diploid. However, the rate of cold hardening was normally distributed and only slightly skewed towards the hexaploid grandparent (Fig 2b, Paper III), suggesting that this trait is controlled by a larger number of genes than the timing of the onset of cold hardening. The main conclusion from Paper III is that the inherent rate and timing of cold hardening are independently inherited traits in *Salix*. However, the most desirable combination, i.e. late start and rapid hardening, was not found, probably because the number of clones displaying late hardening characteristics was too small (Fig 2a, Paper III).

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