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Plants in northern latitudes are adapted to cold, however climate change increases the risk of frost and soil freezing, exposing non-acclimated tissues to stress. This PhD thesis investigates transcriptional regulation and responses to low temperature in boreal tree species and evaluates the potential of genomic selection to predict spring frost tolerance in Norway spruce.

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DOCTORAL THESIS NO. 2025:72 • Global warming challenges low temperature resilience of boreal trees • Tuuli Aro

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FACULTY OF FOREST SCIENCES

Global warming challenges low temperature resilience of boreal trees

Colder in a warming world

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Colder in a warming world

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Cover: Acclimated Norway spruce needles under freezing temperatures (Photo by Tuuli Aro, 2025)

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Colder in a warming world - global warming challenges low temperature resilience of boreal trees

Abstract

Plants in northern latitudes are well adapted to low temperatures, yet anthropogenic climate change increases the risk of frost and cold soil events, exposing non-acclimated tissues to novel stress conditions. This thesis investigates transcriptional responses to low temperature in boreal tree species and evaluates the genomic selection models to enhance spring frost tolerance in Norway spruce. The results show that plant tissues, as well as different species, exhibit distinct low temperature responses, with roots facing a potential trade-off between sustaining growth and activating stress responses. This thesis also demonstrates that genomic prediction models incorporating bud burst as an assisting trait show great potential for predicting spring frost tolerance in Norway spruce. These findings provide new insight into the molecular regulation of cold stress in boreal trees and identify fine roots as particularly vulnerable to low soil temperatures, potentially impairing whole-tree growth in the future. Results underscore the need for further research on species-level root responses to cold, while also emphasizing genomic selection as a promising approach to enhance tree resilience under climate change.

Keywords: transcriptomics, Arabidopsis, *Picea abies*, Scots pine, silver birch, aspen, needle, *CBF*, co-expression network, needle

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Kallare I en varmande värld – global uppvärmning utmanar boreala träds köldtålighet

Sammanfattning

Växter i nordliga breddgrader är väl anpassade till låga temperaturer, men de antropogena klimatförändringarna ökar risken för frost och kall jord, vilket utsätter icke-acklimatiserade växtvävnader för nya stressförhållanden. Denna avhandling undersöker transkriptionella responser på låga temperaturer hos boreala trädarter och utvärderar potentialen hos genomisk selektion för att förbättra vårfrosttolerans i gran (*Picea abies*). Resultaten visar att växtvävnader, liksom olika arter, uppvisar distinkta responser på låga temperaturer, där rötter står inför en potentiell avvägning mellan att upprätthålla tillväxt och att aktivera stressresponser. Avhandlingen demonstrerar även de framgångsrik användning av genomiska prediktionsmodeller som inkluderar skottskjutning som en assisterande egenskap för att förutsäga vårfrosttolerans i gran. Dessa resultat ger ny kunskap om den molekylära regleringen av köldstress i boreala träd och identifierar finrötter som särskilt sårbara för låga jordtemperaturer, vilket potentiellt kan försämra hela trädets tillväxt i framtiden. Resultaten understryker behovet av ytterligare forskning om rotresponser på kyla på artnivå, samtidigt som de framhäver genomisk selektion som en lovande metod för att stärka trädens resiliens under klimatförändringar.

Nyckelord: transkription, Arabidopsis, *Picea abies*, tall, björk, asp, barr, *CBF*

Hyisempää kuumenevalla planeetalla – ilmastonmuutos haastaa boreaalisten puiden kylmänkestävyyden

Tiivistelmä

Pohjoisten leveysasteiden kasvit ovat sopeutuneet alhaisiin kasvulämpötiloihin, mutta ilmastonmuutoksen lisäämät hallan ja kylmän maaperän riskit altistavat kasvit uusille stressitekijöille. Tutkin tässä väitöskirjassa boreaalisten puulajien transkriptionaalisia vasteita kylmästressiin, keskittyen erityisesti juuriin, sekä arvioin genomivalinnan mahdollisuuksia metsäkuusen keväthallan sietokyvyn parantamiseen. Tulokset osoittavat että juuret ja maanpäälliset kasvukudokset reagoivat kylmään eri tavoin, erityisesti juuret joutuvat tasapainoilemaan kasvun ylläpitämisen ja stressivasteiden aktivoimisen välillä. Vertailuanalyysi boreaalisten puulajien välillä osoittaa myös että juurten kylmävaste on voimakkaasti lajikohtainen. Nämä havainnot tuovat uutta tietoa kylmästressin yksityiskohtaisesta molekulaarisesta säätelystä boreaalisilla puilla ja korostavat hienojuurten erityistä haavoittuvuutta ilmastonmuutoksen voimistamille kylmille maaperäolosuhteille. Tämä haavoittuvuus voi tulevaisuudessa heikentää puiden kasvua, korostaen lisätutkimuksen tarvetta lajikohtaisesta hienojuurten kylmänkestävyydestä. Tulokset osoittavat myös että genomisia ennustemalleja, joissa silmun puhkeaminen sisällytetään avustavaksi geneettiseksi ominaisuudeksi, voidaan hyödyntää kuusipopulaatioiden keväthallan sietokyvyn ennustamisessa.

Avainsanat: metsäkuusi, mänty, koivu, haapa,

Heljä-mummolle, joka ratkeaisi ylpeydestä.

Contents

List of publications

This thesis is based on the work of the following papers, referred to by Roman numerals in the text:

I. Vergara A., Haas C.J., **Aro T**., Stachula P., Street N.R. & Hurry V. (2022). Norway spruce deploys tissue-specific responses during acclimation to cold. Plant Cell Environ. 2022; 45:427–445. https://doi.org/10.1111/pce.14241

II. **Aro T.,** Tan B., Chen Z-Q., Hallinbäck H., Suontama M., Westin J., Wu H. & Hurry V. Multivariate models improve accuracy of genomic prediction for spring frost tolerance in Norway spruce. (submitted)

III. **Aro T**., van Zalen E., Vergara A., Canovi C.,Kumar, V., Street R.N. & Hurry V. Comparative transcriptional analysis of cold temperature regulation and responses in Boreal tree roots (manuscript)

Paper I is reproduced with the permission of the publisher.

The contribution of Tuuli Aro to the papers included in this thesis was as follows:

I. Performing experiment. Data preparation, analysis and visualization. Manuscript editing.

II. Planning, sampling and performing experiment. Data preparation analysis and visualization. Manuscript writing and editing.

III. Planning, sampling and performing experiment. Data preparation, analysis and visualization. Manuscript writing and editing.

List of figures

1. Introduction

This thesis investigates the physiological and transcriptional responses of boreal forest tree species to low temperature. The experimental material includes key species representative of northern European forests: Norway spruce (*Picea abies*), Scots pine (*Pinus sylvestris*), common aspen (*Populus tremula*) and silver birch (*Betula pendula*). Cold temperature is one of the main factors limiting plant distribution and productivity worldwide, with its effects most pronounced in boreal and Arctic regions (Pearce 2001; Körner 2021). As a result, plants in these ecosystems have evolved diverse physiological and molecular adaptations to survive the short growing season and prolonged exposure to low temperatures (Sakai 1983; Howe *et al.* 2003). These cold acclimation and adaptation mechanisms are increasingly challenged by ongoing anthropogenic climate warming (Gauthier *et al.* 2014; Stubbs *et al.* 2018; Reich *et al.* 2022; Lee *et al.* 2023), increasing the pressure for more careful species and genotype selection to maintain forest growth and production in more uncertain future (Callahan 2025).

Understanding how boreal tree species respond to low temperature stress is crucial for anticipating and managing the impacts of climate change on this ecosystem. The aim of this thesis is to outline these responses at both the physiological and molecular levels, with a particular focus on transcriptional responses and regulation of low temperature under warming world. The introduction begins by reviewing the physiological effects of low temperature in plants, followed by an overview of the molecular mechanisms underlying their cold stress responses and regulation. Focus then shifts to boreal tree species, exploring how climate change is reshaping their growth conditions, followed by the future challenges these changes pose to forestry. The section concludes with a brighter note, highlighting emerging strategies for enhancing climate resilience in tree breeding and forestry practises. The results and discussion of the thesis present original research conducted on cold responses in boreal trees, offering new insights into species-specific and conserved transcriptional mechanisms in both above- and below-ground tissues.

1.1 Low temperature stress in plants

I. Defining low temperature stress

The biological significance of low temperatures to plants lies in their impact on chemical processes and equilibria, as well as their alteration of biological processes, such as cell membrane composition (Levitt 1980; Sakai & Larcher 1987). In general, low temperatures are divided into chilling temperatures (>0°C) and freezing temperatures (<0°C). Frost occurs when the temperature drops below 0°C, while freezing refers specifically to the solidification of water into ice in aqueous solutions (Levitt 1980). In plants these terms are not synonyms, since damage is not inevitable when temperature drops below 0°C. Initiation of freezing can be referred to as ice nucleation, when a nucleating particle under freezing conditions promotes organization of water molecules into ice crystals. Ice nucleation can be heterogenous, when ice is promoted by an inorganic or organic ice nucleating agent (INA) and it occurs at higher temperatures (-1°C to -2°C) than homogenous nucleation (< -2°C), which happens spontaneously between water molecules without external nucleators (Zachariassen & Kristiansen 2000).

Defining plant responses to low temperature stress can be complex with multiple definitions presented in literature. In this thesis, I categorize them into two main groups*: freezing mitigation*, which encompasses phenological and environmental strategies to avoid frost, and *frost resistance*, which refers to physiological mechanisms that enable survival under subfreezing temperatures. Frost resistance mechanisms can be further divided into two subcategories:

1. Avoidance mechanisms, which involve structural adaptations in plant cells that control the timing and location of ice formation, such as supercooling, and annual growth habits.
2. Tolerance mechanisms, which include biochemical mechanisms that control the formation, location and growth of ice, but also processes that prevent, minimize or repair the ice crystallization and dehydration damage (Levitt 1980; Gusta & Wisniewski 2013).

Two key tolerance strategies are basal low temperature tolerance and cold acclimation. Basal low temperature tolerance refers to the inherent ability of an organism to withstand freezing temperatures without prior exposure to cold, while cold acclimation is enhanced tolerance to freezing temperatures that develops following exposure to cold conditions (Horton *et al.* 2016; Hoermiller *et al.* 2022; Zhao *et al.* 2023b). Cold acclimation ensures the survival of an organism during long periods of freezing temperatures.

Freezing avoidance and tolerance mechanisms are employed simultaneously in plants during cold. This dual utilization highlights the complexity of plant responses to low temperature and their capacity to adapt to extreme temperature conditions (Gusta &

Wisniewski 2013). In addition, term “hardiness” refers to the robustness of an organism to endure stressful environmental conditions (Wisniewski *et al.* 2003). It can also be used as a scale to cold tolerance, covering both avoidance and tolerance mechanisms.

1.1.1 Freezing stress in plants

Low-temperature damage in plants is not uniform; it varies depending on the climatic conditions to which the plant is adapted, its developmental stage, tissue type and prior exposure to low temperatures. Additionally, the intensity, duration and frequency of freezing exposure are crucial factors influencing the extent of cellular damage.

The plant cell wall and plasma membrane serves as the primary interface that separates and connects the cell to its external environment (extracellular space), making it one of the first sites to experience damage under external stress conditions (Steponkus 1984). During freezing, damage occurs through ice nucleation and the subsequent growth of ice crystals in the extracellular space, which can physically disrupt the membranes (Wisniewski *et al.* 1997). In addition to physical damage, extracellular ice crystals draw water molecules from the surrounding area, creating an osmotic imbalance between the intra- and extracellular spaces. This imbalance causes water to flow out of the cell, increasing the risk of cellular dehydration (Steponkus 1984; Steponkus & Lynch 1989; Pearce 2001) Under severe freezing stress, ice crystallization can impair cell metabolism through protein inactivation and the overproduction of reactive oxygen species (ROS), followed by oxidative stress, collapse of cellular structures and eventual rupture of the cell (Thomashow 1998). Such cellular damage exacerbates tissue injury, slows overall growth and, in extreme cases, results in plant death.

Plants have evolved diverse strategies to respond to low temperatures, employing a multitude of mechanisms occurring simultaneously in different parts of the plant. Among these, avoidance mechanisms are preventing ice nucleation and controlling its formation. One key avoidance strategy is supercooling, a metastable state in which water remains in liquid form even at subfreezing temperatures (Wilson 2012). The capacity of supercooling is species-specific and often occurs in flowering organs, such as buds (George *et al.* 1974; Ashworth 1982; Wisniewski *et al.* 2004). Plants can have structural barriers within their tissues to inhibit or redirect the ice propagation or utilize ice-nucleating-active bacteria to regulate the formation of ice to protect sensitive parts of the tissues (Krog *et al.* 1979; Wisniewski *et al.* 1997).

Avoidance mechanisms are particularly advantageous for plants in environments with frequent temperature fluctuation. By avoiding ice damage through supercooling, plants can survive repeated freezing events without relying on the resource-intense process of cold acclimation, thereby maintaining active growth, such as the tropical high latitude genus *Espeletia* (Compositae) in the Venezuelan Andes (Goldstein *et al.* 1985). Overall, relying on both avoidance and tolerance mechanism is suggested to be most beneficial for plants, since avoidance mechanisms last only a few hours, making relying on this methods risky during prolonged freezing events (Squeo *et al.* 1991).

1.1.2 Plant physiology changes during cold acclimation

An important environmental trigger for plants to endure winter's low temperatures is the exposure to non-freezing temperatures, a process known as cold acclimation (Thomashow 1999). Cold acclimation encompasses multiple physiological changes in plants to achieve sufficient protection against freezing, for example alterations of cell wall and plasma membrane, solute and protein accumulation and photosynthetic adjustments (Ruelland *et al.* 2009).

Cell walls are the outermost layer of plant cells and during cold acclimation, plants strengthen their structure and change properties to mitigate the adverse effects of low temperatures (Stefanowska *et al.* 1999; Solecka *et al.* 2008; Liu *et al.* 2022). Specifically, accumulation of pectins enhance the rigidity by modifying the mechanical properties of cell walls (Takahashi *et al.* 2024). This increased rigidity in mesophyll cells reduces freezing-induced dehydration and improves supercooling capacity (Griffith & Brown 1982). Thickening cell walls decrease the low relative area of intercellular spaces, slowing the progress of dehydration by lowering the turgor pressure in the cell (Stegner *et al.* 2022). Cell walls mitigate the cold damage to plasma membranes by protection from extracellular ice and resisting cellular collapse during plasmolysis, specifically in cold-resistant plant cells (Yamada *et al.* 2002; Panter *et al.* 2020).

During cold acclimation the production of solutes, such as sugars, amino acids and specialized proteins, increase in cells. Accumulation of these solutes play a vital role in stabilizing membranes against denaturation and structural disruption caused by freezing (Koster & Lynch 1992; Wilson *et al.* 2003; Kosová *et al.* 2008). The accumulation of sugars, such as sucrose and trehalose provide physical protection against dehydration by stabilizing the bilayer of the membranes (Strauss & Hauser 1986; Savitch *et al.* 2000). Also, amino acids, such as proline and specialized proteins, protects membranes during low temperature stress (Lalk & Dörffling 1985; Rudolph & Crowe 1985; Griffith *et al.* 1992). Dehydrins are a group of Late Embryogenesis Abundant (LEA) proteins, that are important in both membrane stabilization and prevention of protein aggregation (Hundertmark & Hincha 2008), their accumulation is directly involved in both frost responses and acquisition of cold tolerance (Kosová *et al.* 2007; Kosová *et al.* 2014). Several heat shock proteins (HSP) function in membrane stabilization by maintaining protein integrity and as chaperons, preventing aggregation or misfolding of proteins during cold stress (Timperio *et al.* 2008; ul Haq *et al.* 2019), also several pathogen resistant (PR) proteins, such as chitinases and β-1,3-glucanase, can inhibit intracellular ice formation (Yeh *et al.* 2000).

Plants require a functional photosynthetic machinery or stored carbon reserves to cover the energy cost for cold acclimation (Dexter 1933). Although photosynthetic systems exhibit a high level of plasticity to the low temperature (Hüner *et al.* 2013), in plants' non-acclimated state cold can disrupt all major components of this machinery (Berry & Bjorkman 1980). Specifically, the combination of cold temperature with high-light exposes plants to the risk of photoinhibition (Powles *et al.* 1983; Greer *et al.* 1986). Low temperature reduces the photosynthetic reaction rates limiting the energy sinks of CO_2 fixation and photorespiration (Huner *et al.* 1998). This increased excitation energy (due to reduced energy consumption) creates an energy imbalance and exposes plants for the accumulation of reactive oxygen species (ROS) and oxidative damage (Goh *et al.* 2012; Li *et al.* 2018). ROS act as important signalling molecules while having a high cell damaging potential (Demmig-Adams & Adams 2006; Tikkanen *et al.* 2012; Foyer & Noctor 2016). During cold acclimation plants increase their capacity to tolerate photoinhibition (Somersalo & Krause 1989) through, for example, with increase of photosynthetic enzymes, particularly those involved in the Calvin-Benson-Bassham (CBB) cycle and sucrose synthesis (Hurry *et al.* 1994; Strand *et al.* 1999; Yamori *et al.* 2005). In addition, the adjustments of membrane fatty acid composition stabilizes the cellular functions and accumulation of photosynthetic proteins enhance photosystem protection (Ivanov *et al.* 2012; Li *et al.* 2018).

A large number of physiological changes during cold acclimation enhance cell membranes (Murata *et al.* 1982; Uemura & Steponkus 1999), highlighting the indispensable nature of functional cell membranes in plant survival. Freezing damage can be lethal, but often cell damage is only partial and recoverable, and an essential trigger for a cascade of stress responses to enhance cellular cold protection (Palta 1990).

1.1.3 Molecular regulation of cold responses

I. Low temperature signalling

Plasma membranes serve as vital sites for low-temperature perception and the subsequent signal transduction pathways, ultimately leading to transcriptional responses and cold acclimation (Örvar *et al.* 2000). Cold changes the physical properties of the membranes altering their rigidity (Uemura *et al.* 1995; Zheng *et al.* 2011). Together with microtubules, membranes regulate cold signalling (Abdrakhamanova *et al.* 2003; Wang *et al.* 2020) by controlling calcium (Ca^{2+}) transport. Ca^{2+} acts as an important cellular signalling molecule during stress (Wilkins *et al.* 2016; Mohanta *et al.* 2018; Wdowiak *et al.* 2024) and transient cytosolic Ca^{2+} levels regulate expression of several cold responsive genes (Örvar *et al.* 2000; Sangwan *et al.* 2001). Plants have several groups of gene regulators that possess Ca^{2+} sensors, such as cold responsive CaM-binding (*CAMTA*) transcription factors (TFs), Ca^{2+}-dependent protein kinases (CDPKs) and calmodulin (caM) proteins (Saijo *et al.* 2000; Townley & Knight 2002; Doherty *et al.* 2009). Specifically, *CAMTAs* are considered to be closely involved in cold perception due to their Ca^{2+} motifs (Liu *et al.* 2015) and they also regulate the downstream core cold transcription factors, *CBFs*, with circadian clock-related MYB-like TFs (Dong *et al.* 2011; Kidokoro *et al.* 2021).

II. CBF-dependent cold regulation and COR-genes

The most studied cold response pathway is regulated by *CBF/DREB1* (C-repeat binding factor/dehydration-response element-binding protein 1) TF family, that were identified as a key transcription factors controlling cold-responsive gene expression in *Arabidopsis thaliana* (Shinozaki & Yamaguchi-Shinozaki 1996). In the Arabidopsis genome, *CBF1-3* genes are located in a tandem array, rapidly activated by cold and inducing the cold acclimation process (Gilmour *et al.* 1998b). They recognise the *CRT/DRE* regulatory region (Baker *et al.* 1994; Yamaguchi-Shinozaki & Shinozaki 1994; Stockinger *et al.* 1997) present in the promoters of many cold-inducible genes (Fowler & Thomashow 2002). These >4000 genes comprise group of cold acclimation activators in Arabidopsis (*COR*-genes), of which the *CBF*s are regulating approximately 10% (*COR/CBF*-regulon) (Seki *et al.* 2001; Maruyama *et al.* 2004; Park *et al.* 2015). The *CBF*-regulon is also involved in freezing tolerance, which was discovered by constitutively overexpressed *CBF*s in Arabidopsis tolerating freezing temperatures without a cold acclimation period (Jaglo-Ottosen *et al.* 1998; Liu *et al.* 1998; Gilmour *et al.* 2004). Overexpressing *CBF*s increases the basal freezing tolerance, but also affects the development of the plants; slower growth, delayed flowering and dwarfed stature in Arabidopsis and other plant species (Liu *et al.* 1998; Jaglo *et al.* 2001; Choi *et al.* 2002; Dubouzet *et al.* 2003; Gilmour *et al.* 2004). Forcing plants to grow with transcriptomes and metabolomes designed for cold temperature survival is a potential explanation of these growth abnormalities.

The regulation of *CBFs* during cold are controlled by several upstream transcription factors, such as Inducer of CBF expression 1 (*ICE1*), *CAMTA* family TFs and TFs utilizing circadian-clock regulation (Doherty *et al.* 2009; Dong *et al.* 2011; Kim *et al.* 2017; Chao *et al.* 2022). The *ICE1* TF, also known as *SCREAM* (SCRM), was the first identified positive regulator of *CBFs* and many published papers have supported its role as a major regulator in cold stress response (Chinnusamy *et al.* 2003; Lee *et al.* 2005; Dong *et al.* 2006). The original study by Chinnusamy *et al.* (2003) showed a reduced cold acclimation in dominant *ice1-1* mutants by lowered *CBF3* levels, while overexpression of wild-type *ICE1* increased levels of both *CBF1* and *CBF3*, followed by increased freezing tolerance and indication of the role of *ICE1* in cold regulation of *CBFs*. Later, a high expression of osmotically responsive gene 1 (HOS1) was shown to negatively regulate *CBFs* with *ICE1* (Dong *et al.* 2006). However, controversies have recently arisen regarding the functional role of *ICE1* and *ICE2* in cold stress, questioning the role of the used *ice-1-1* mutant in *ICE1-2* studies (Kidokoro *et al.* 2020; Thomashow & Torii 2020; Park & Jung 2024). Due to these opposing results of *HOS1-ICE1* in cold regulatory model, the role of this regulatory pathway requires more investigation. However, the role of *ICE1* in cold acclimation in several other species indicates the importance its role in low temperature stress regulation (Dong *et al.* 2013; Huang *et al.* 2015; Deng *et al.* 2017; Vergara *et al.* 2022; Lin *et al.* 2023).

In addition to the *CBF*-regulon, multiple other cold acclimation pathways are present in Arabidopsis (Fowler & Thomashow 2002). *COR*-gene expression is regulated by both *CBF*-dependent and *CBF*-independent pathways, by having extensive crosstalk and co-regulation, these pathways create a complex cold regulatory and response network (Park *et al.* 2015; Zhao *et al.* 2016; Park *et al.* 2018). Induction of the *COR*-regulon improves cold tolerance processes, such as increased cellular defences against freezing damage, accumulation of dehydrins and heat shock proteins for membrane and protein stabilization, and PR proteins to inhibit intracellular ice formation (Yeh *et al.* 2000; Hundertmark & Hincha 2008; Timperio *et al.* 2008).

III. Hormonal and post-transcriptional regulation

Plant hormones (phytohormones) are small molecules regulating every aspect of plant's growth and development. These compounds are highly responsive to changing environment, making them important during abiotic and biotic stress (Santner *et al.* 2009; EL Sabagh *et al.* 2022). Various plant hormones have independent and interconnected links to cold regulatory pathways, and some constitute a central hub with *CBF-COR* pathway during cold stress (Eremina *et al.* 2016a). ETHYLENE INSENSITIVE3 (*EIN3*) is a key TF in ethylene signalling that negatively regulates *CBF*-expression (Shi *et al.* 2012), while both jasmonate and brassinosteroids (BR) function as inducers of the *CBF*-dependent pathway increasing freezing and cold tolerance, respectively (Hu *et al.* 2013; Eremina *et al.* 2016b; Li *et al.* 2017a). Lastly, abscisic acid (ABA) is induced by low temperature dehydration and osmotic stress to induce antioxidant ROS scavenging (Gusta *et al.* 2005).

Epigenetic as well as post-transcriptional and post-translational regulation contribute to plant's phenotype by regulating gene expression in response to stress (Mazzucotelli *et al.* 2008; Grativol *et al.* 2012; Barrero-Gil & Salinas 2013). Epigenetic modifications, primarily involving histone modification and DNA methylation change the chromatin structure of DNA without altering the DNA sequence

(Kim *et al.* 2015). Post-transcriptional regulation targets mRNA, through processes such as alternative splicing of mRNA (Calixto *et al.* 2018). Both microRNA (Dong & Pei 2014) and long noncoding RNA (Kindgren *et al.* 2018) participate in cold post-transcriptional regulation. Post-translational modification alters proteins during cold stress, through processes such as phosphorylation (Monroy *et al.* 1993; Furuya *et al.* 2013), ubiquitination (Wang *et al.* 2023) and sumoylation (Miura & Hasegawa 2008).

1.1.4 Do roots cold acclimate?

Roots are generally more susceptible to freezing injury than above-ground tissues (Stier *et al.* 2003). This increased vulnerability is often attributed to the insulating properties of soil and snow, which has protected roots from direct selective pressures of low temperatures, leading to inherently low basal freezing tolerance. Although roots can employ supercooling to avoid freezing injury (Fiorino & Mancuso 2000), this avoidance mechanisms is often inefficient because soils and their associated microorganisms are rich in ice-nucleating agents (INAs), such as salts and colloids, which facilitate ice formation (Hill *et al.* 2016). These factors highlighting the importance of understanding root-specific tolerance mechanisms under low temperature stress.

Despite the evident (discussed in depth later in the thesis) ecological and physiological importance of roots to plant growth and increasing risks of root damage in the future, their cold acclimation capacity is still a mystery. Moreover, the variety of methods used to assess root cold tolerance complicates synthesis across the existing studies. In cold-hardy crop species, evidence for root acclimation is scarce, for example, a study in wheat showed no evidence for root acclimation (*Triticum aestivum L.*) (Perras & Sarhan 1989). In contrast, studies in temperate and boreal trees report widely variable and sometimes contradictory results (Johnson 1976; Smit-Spinks *et al.* 1985; Coleman *et al.* 1992; Ryyppö *et al.* 1998b). Where acclimation occurs, roots exhibit tissue-specific responses, with each tissue responding to distinct environmental cues (Smit-Spinks *et al.* 1985). Soil temperature appears to be the principal environmental trigger for cold acclimation in roots, potentially explaining the weaker cold hardening observed in root tissues relative to above-ground tissues (Smit-Spinks *et al.* 1985; Sakai & Larcher 1987; Ryyppö *et al.* 1998b). Since roots are not directly exposed to photoperiodic cues, key regulators of cold acclimation in above-ground tissues of many boreal and temperate species, further contributing to their reduced cold acclimation capacity (Smit-Spinks *et al.* 1985; Li *et al.* 2003). Nonetheless, photoperiod appears to have a role in root acclimation; Norway spruce roots acclimate under long-day conditions when exposed to low temperatures, but maximum acclimation is only achieved under combined cold and short days (Johnson 1976). In contrast, Scots pine roots appear to reach the full acclimation under long days with only cold treatment (Smit-Spinks *et al.* 1985).

Root cold acclimation shows high interspecific variation; tree species inhabiting similar environments often display distinct root tolerance profiles (Johnson 1976; Coleman *et al.* 1992). Variation also occurs within the root system itself: woody (lignified) roots typically acquire tolerance at mild, non-freezing temperatures, whereas fine roots exhibit limited acclimation capacity (Ryyppö *et al.* 1998b). This may reflect the short-lived nature and high turnover of fine roots (Yuan & Chen 2010; Solly *et al.* 2018) which reduces the evolutionary advantage of investing in acclimation. Furthermore, the temperature thresholds and duration of cold periods required for acclimation vary by species and latitudes of the origin (Bannister & Neuner 2001). The origin of the genotype has been shown to influence the photoperiod requirement of cold acclimation in above-ground tissues (Howe *et al.* 2003; Zuther *et al.* 2012).

In conclusion, available evidence indicate that root acclimation capacity is highly species-dependent, shaped by interactions among minimum temperature thresholds, duration of cold period, genotypic origin and possible signalling requirements from above-ground tissues, including photoperiod sensing.

I. Molecular responses in roots during cold stress

Despite the recognized importance of the roots in global systems regulating water and nutrient cycling between the soil and plant (McCormack *et al.* 2015), their molecular responses to low temperatures remain relatively unexplored. While above- and below-ground tissues share broad physiological mechanisms under cold stress, fine-scale responses are often tissue-specific. For instance, barley (*Hordeum vulgare*) and cucumber (*Cucumis sativus*) roots exhibit distinct cold-induced adjustments in bioactive lipids and respiration, respectively (Hu *et al.* 2006; Margutti *et al.* 2018). Similarly, roots and shoots of strawberry (*Fragaria* x *ananassa* 'Korona'), rice (oryza sativa) and wheat (*Triticum aestivum)* exhibit divergent polyamide accumulation patterns under cold stress (Racz *et al.* 1996; Lee *et al.* 1997; Koehler *et al.* 2015).

Studies on Arabidopsis suggest that Ca^{2+} signalling plays a key role in triggering cold responses in roots, similar to leaves (Sulaiman *et al.* 2012; Wilkins *et al.* 2016). Cold also promotes membrane reorganization (Smolénska & Kuipier 1977; Iivonen *et al.* 2004), and upregulation of aquaporins, thereby improving water transport and reducing dehydration damage (Aroca *et al.* 2005). Roots, like above-ground tissues, accumulate cryoprotective solutes during cold, including dehydrins (Danyluk *et al.* 1998; Nylander *et al.* 2001) and antifreeze proteins (AFPs), that inhibit ice formation during cold exposure (Antikainen *et al.* 1996; Chew *et al.* 2012). Extensive production of polyamides and specialized amino acids, such as HSPs supports the maintenance of root growth and development during cold (Tang & Newton 2005; Sasaki *et al.* 2007). Cold stress also induces the accumulation of ROS in roots (Heidarvand *et al.* 2017; Margutti *et al.* 2018), while elevated proline levels provide ROS scavenging during freeze-thaw cycles (Yin *et al.* 2017; Margutti *et al.* 2018).

While temperature appears to be the primary cue for cold acclimation in roots, photoperiod may indirectly influence responses by altering carbohydrate translocation between above- and below-ground tissues (Smit-Spinks *et al.* 1985; Tinus *et al.* 2000). Cold strongly affects sugar metabolism (Cunningham *et al.* 2003), though patterns vary across species: in both aspen and Norway spruce, raffinose accumulates during root frost hardening (Wiemken & Ineichen 1993; Regier *et al.* 2010), whereas in strawberry roots, raffinose with sucrose predominate this process (Koehler *et al.* 2015). Moreover, pectin methylesterases in root cell walls also contribute to the balancing trade-off between growth and freezing tolerance (Thonar *et al.* 2006; Chen *et al.* 2018).

Despite broad physiological and metabolomic overlaps between roots and shoots, transcriptional responses appear to show tissue-specificity. Norway spruce and sugar beet (*Beta vulgaris*), for example, display distinct tissue-level cold responses and regulatory networks (Moliterni *et al.* 2015; Vergara *et al.* 2022). Furthermore, Zhao *et al.* (2021) reported cold-responsive membrane lipid transcriptional profiles unique to roots in maize. These findings emphasise the need for root-focused studies, as both tissue- and species-specific differences complicate generalization about low temperature responses.

1.2 Boreal forests

The circumboreal forests encompass approximately 14 million km^2, accounting for 32% of global forests and covering the colder regions of Europe, Asia and North America (Burton *et al.* 2003). Boreal ecosystems provide essential habitats for a diverse set of species and contribute significantly to ecosystem services; offering recreational value and providing natural resources such as wood, berries, mushrooms and herbs (Vanhanen *et al.* 2012; Gauthier *et al.* 2015; Holmberg *et al.* 2019). The defining character of boreal forests is its harsh climate, which imposes constraints on species distribution and growth, with short growing season, low solar radiation and severe winters (Larsen 1980). During boreal winters, temperatures drop below 0°C and snow covers the landscape for several months (Gauthier *et al.* 2015). These environmental stressors have driven all boreal species to evolve specialized mechanisms to either avoid or tolerate such extreme conditions.

Trees are the cornerstone of boreal forests. With their long lifespan and wide habitation area, boreal trees are important drivers of the global carbon (Malhi *et al.* 1999; Magnani *et al.* 2007) and nutrient cycles (Melvin *et al.* 2015; Sheil 2018). A range of tree species have adapted to the harsh northern environmental conditions: many gymnosperm species, such as spruces, pines and larches, and angiosperm tree species, such as birches and aspens (Davis *et al.* 2005; Klisz *et al.* 2023).

Despite the ongoing anthropogenic climate warming, boreal forests appear to maintain their resilience to changing habitat conditions. In northern Europe, these ecosystems have repeatedly experienced continental wide climatic-fluctuations, forcing populations to migrate in response to shifting environmental patterns (Taggart & Cross 2009). Following the most recent glaciation, which ended approximately 9,000 years ago, boreal trees species such as birch, spruce and pine successfully recolonized areas previously covered by ice, establishing themselves across northern Europe in a relatively short timeframe (Huntley & Birks 1983; Bergman *et al.* 2005; Kullman 2008; Parducci *et al.* 2012). Evolutionary studies further highlight the adaptive potential of boreal tree species. For example, Li *et al.* (2022) demonstrated that Norway spruce in Fennoscandia underwent rapid local adaptation within only ~100 generations – an exceptionally short period in the evolutionary history of conifers. This capacity for shift adaptation, combined with extensive gene flow facilitated by long-distance pollen dispersal (Petit *et al.* 2004; Savolainen *et al.* 2007; Kremer *et al.* 2012), underpins the resilience of boreal tree populations. Spanning broad climatic gradients, these species maintain high levels of genetic diversity, which enhances their potential to adapt to new conditions. While geographic distributions are projected to shift under future climate scenarios, many populations are nevertheless expected to retain substantial adaptative capacity (Petit & Hampe 2006; Crawford 2008; Kremer *et al.* 2024; Saikkonen *et al.* 2025), as indicated in case of Norway spruce (Li *et al.* 2022).

However, the genetic resilience does not imply immunity to climate change. Although the high genetic diversity provides a higher gene pool for selecting more climate resilience Norway spruce genotypes (Ingvarsson & Dahlberg 2019), changing climate is projected to alter forest dynamics and reduce their utility for production forestry. Increasing frequency of extreme climatic events, such as drought, forest fires, and late spring frost events, together with rising precipitation, higher vapour-pressure deficits, and changing snow conditions, will exert both individual and interactive stresses on tree growth and survival (Hänninen 2006; Randerson *et al.* 2006; Peng *et al.* 2011; Price *et al.* 2013; Novick *et al.* 2024). Furthermore, stress resilience in boreal trees is strongly developmental stage dependent and tightly linked to seasonal cycles. In northern latitudes, phenological periodicity plays a central role in synchronizing development with environmental conditions (Kramer *et al.* 2000), meaning that altered seasonal cues may profoundly disrupt the adaptive strategies of boreal species.

1.2.1 Seasonality of low temperature resilience

Seasonal dynamics shape the distribution of plant species (Chuine 2010). At the colder end of a species' distribution range, maximum cold hardiness becomes the key limiting factor. Conversely, at the warmer edge of the range, insufficient chilling accumulation can disrupt dormancy release, thereby constraining distribution (Chuine 2010; Körner 2021; Jones *et al.* 2024).

Boreal trees are adapted to the seasonal climate fluctuations. In autumn, declining temperature and shortening daylength initiate physiological processes that enhance cold tolerance and prepare trees for winter dormancy. In spring, rising temperature and longer photoperiod initiate the reactivation of bud burst and overall growth. A precise synchronization of these phenological transitions is critical for maximizing the short growth season while minimizing the risk of cold damage during the shifts between winter and summer. These phenological events are tightly regulated by environmental cues, primarily temperature and photoperiodicity (Lang *et al.* 1987; Delpierre *et al.* 2016; Kovaleski 2022; North *et al.* 2024).

1.2.2 Autumn phenology in boreal trees

Boreal conifers, such as Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*), are evergreen species, whose needles require hardening to withstand subzero winter temperatures (Chang *et al.* 2021). In contrast, all boreal angiosperm tree species, including birches (*Betula* spp.) and aspens (*Populus* spp.), are deciduous and shed their leaves in autumn through a process known as senescence (Fracheboud *et al.* 2009; Chen *et al.* 2020). All boreal trees enter dormancy in autumn, during which vegetative growth ceases and cellular activity is significantly reduced (Perry 1971; Kovaleski 2024). The current definition of dormancy progression, focusing on bud and vegetative above-ground tissues, would benefit from revision. While the winter phenology of leaves and buds has been extensively studied; emerging evidence suggests that other tree organs, such as fine roots and woody tissue, also participate in dormancy processes. However, the phenological progress of these organs remain poorly understood (Barbaroux *et al.* 2003; Nord & Lynch 2009; Delpierre *et al.* 2016; Ding *et al.* 2024). Dormancy is not a singular event but rather a progression of developmental stages. It begins with the growth cessation and the formation of protective buds that cover sensitive meristematic tissues (bud set), followed by dormancy maintenance, and finally ends with dormancy release and resumption of growth (bud burst) (Ding *et al.* 2024). Shortening days in late summer induce the cessation of apical meristem growth, initiating the dormancy maintenance stage. This phase, which reaches its depth in late autumn and early winter, is referred as endodormancy. (Rinne et al. 2001). During this period, internal physiological controls prevent bud reactivation, even under unseasonably warm temperatures, thus protecting the tree from premature dehardening and bud flush (Lang *et al.* 1987). Simultaneously, overwintering tree species begin the process of cold acclimation, developing tolerance to cold and freezing temperatures (Sakai & Larcher 1987; Öquist & Huner 2003; Li *et al.* 2004). Although the development of freezing tolerance coincides with dormancy induction, they are interconnected yet independent processes. Both are triggered by the combined environmental cues of decreasing temperature and shortening photoperiod, but regulated by separate molecular mechanisms (Arora *et al.* 1992; Rinne *et al.* 2001). Same mechanisms are expected to regulate also root acclimation, due to the independent regulation of low temperature and daylength to root hardiness.

1.2.3 Cold avoidance and tolerance mechanisms in trees

The critical role of photoperiod in initiating winter preparation at northern latitudes is showcased by the heightened sensitivity of northern ecotypes to changes in daylength, in contrast to the weaker photoperiodic responses observed in southern ecotypes (Welling *et al.* 2002; Li *et al.* 2003; Holliday *et al.* 2008). In addition to photoperiod, falling temperatures also initiate cold acclimation (Senser & Beck 1984). While the processes appear to be independent, for the full hardiness changes in both photoperiod and cold are required (Welling *et al.* 2002). Once cold acclimation is initiated, boreal trees undergo physiological adjustments similar to those observed in other cold-adapted plant species, such as changes in membrane lipid composition (Siminovitch *et al.* 1968; Senser & Beck 1984; DeHayes *et al.* 1997; Wellburn 1997; Martz *et al.* 2006), accumulation of soluble sugar and other cryoprotective solutes (Angelcheva *et al.* 2014) and calcium-related cold signalling (Holliday *et al.* 2008; Chen *et al.* 2013; Li *et al.* 2024)

The *ICE1-CBF*-pathway appears to be also regulating cold responses in several tree species. *ICE*-like TFs function in cold response during cold hardiness in the conifer species Sitka spruce (*Picea sitchensis*) (Reid *et al.* 2013) and in Japanese cedar (*Cryptomeria japonica*) (Mishima *et al.* 2014). *CBF* activation have been shown to regulate cold stress in deciduous trees, such as aspen, birch, maple and walnut (Puhakainen *et al.* 2004; Benedict *et al.* 2006; Welling & Palva 2008; Li *et al.* 2017b; Zhao *et al.* 2023a; Zhou *et al.* 2023) and evergreen angiosperms, such as Eucalyptus (Kayal *et al.* 2006; Navarro *et al.* 2009). *CBF* orthologs increased the freezing tolerance of birch and Arabidopsis in overexpression studies, showing functional similarities of *CBFs* between angiosperm trees and herbaceous plants (Benedict *et al.* 2006; Welling & Palva 2008). On the contrary, while there are indications of existing *CBFs* in conifer genomes, such as in Norway spruce (Vergara *et al.* 2022), white spruce (*Picea glauca*) and Sitka spruce (*Picea sitchensis*) (Rigault *et al.* 2011; Reid *et al.* 2013), their role in the *CBF*-related cold acclimation pathway remain unidentified.

The cessation of growth downregulates photosynthetic activity in conifers during their cold acclimation (Huner *et al.* 1998; Öquist & Huner 2003; Ensminger *et al.* 2006), unlike in overwintering annuals, such as winter wheat (*Triticum aestivum* L.) and rye (*Secale cereale* L.) that maintain photosynthesis during winter (Huner *et al.* 1986; Hurry & Huner 1991). Carbohydrate metabolism changes during cold to balance the decreased carbon assimilation (Ensminger *et al.* 2006) and to support the carbon transport between tissues in preparation of freezing temperatures (Oleksyn *et al.* 2000; Hoch *et al.* 2003). Genes inducing sucrose synthesis and degradation of starch are upregulated, as shown in common aspen (*Populus tremula*) (Schrader *et al.* 2004). In addition, cold upregulates the production of osmo- and cryoprotective carbohydrates, such as sucrose, glucose and raffinose family oligosaccharides (Hinesley et al. 1992; Schaberg et al. 2000; Joosen et al. 2006), as well as LEA proteins, in overwintering trees. Dehydration in trees arise in two occasions: first the growth cessation by short daylength in autumn decreases water content in the tissues (Welling *et al.* 1997) and second, freeze-induced cell damage induces dehydration (Pearce 2001). To protect cells against dehydration, LEA proteins (Schrader *et al.* 2004; Gao & Lan 2016; Zhou *et al.* 2023), and specifically dehydrins, that are specialized LEA proteins, are produced during the overwintering process to protect cell membranes (Richard *et al.* 2000; Puhakainen *et al.* 2004; Renaut *et al.* 2004; Welling *et al.* 2004; Kjellsen *et al.* 2013).

Throughout winter, boreal trees also confront other cold-related challenges. Freezing temperatures with high light levels challenge the photosynthetic apparatus in overwintering needles and major reorganization of photosynthetic apparatus is required to protect chloroplast from photooxidative damage (Ottander *et al.* 1995; Chang *et al.* 2016). In addition, trees have adapted to combat winter embolism, the disruption of long-distance water transport in the xylem, that is caused by freeze-thaw cycles in winter (Sperry *et al.* 1994). Woody plants have an ability to inhibit the formation of ice crystals by supercooling and deep supercooling by preventing the intercellular water to transit from a liquid to a solid state, which is regulated by the structural reorganization of cell wall (Wisniewski *et al.* 2004).

1.2.4 Spring phenology and cold deacclimation

Spring is the time for regrowth for temperate trees, but to overcome dormancy related growth cessation, both cold and warm temperatures are required. The chilling requirement, a cold period required for trees to break dormancy, allows trees to estimate when the unfavourable winter period is over and when the environmental conditions will be again suitable for growth (Knight 1801; Doorenbos 1953). After adequate chilling, bud break is initiated in spring by warm temperatures and increasing day length, but its timing is highly interconnected to the temperature during bud set and the duration and depth of chilling exposure. High temperatures in late autumn can postpone bud break, while in late winter the effect of warming accelerates the initiation of bud development (Malyshev 2020). Furthermore, increased duration and depth of chilling exposure can lead to a reduced length of heat requirements before bud break (Nanninga *et al.* 2017). Moreover, temperature is not only essential for timing of spring growth initiation but plays a part also in cold deacclimation process.

Cold acclimation against freezing temperatures is essential for the trees' survival. However, the timing of loss of cold hardiness (deacclimation) in spring is crucial for frost avoidance and the optimization of growth initiation. Deacclimation is a dynamic process that starts when the trees are still in dormancy (Kovaleski *et al.* 2018). Rising temperature in spring is the main cue for the start of deacclimation (Pagter & Arora 2013), while the deacclimation rate is influenced both by spring temperature and photoperiod (Jönsson & Bärring 2011; Basler & Körner 2014). Interestingly, the chilling accumulation required for the maximum rate of deacclimation seems to be similar across woody plant species (Kovaleski 2022). As in cold acclimation, deacclimation also shows genotypic variance (Rowland *et al.* 2005; Wisniewski *et al.* 2014)

In general, deacclimation is characterized by physiological processes that allow the return to active growth and development:

water content of the tissues increase (Rapacz 2002; Webster & Ebdon 2005) and abundance of stress-related proteins decrease (Pagter *et al.* 2014). In addition, the photosynthetic capacity recovers to initiate the growth and development (Repo *et al.* 2006; Yang *et al.* 2020); the levels of biochemical agents, such as soluble sugars, decreases during spring and starch re-synthesis increases to support the energy requirements of bud flushing (Ögren 1997; Poirier et al. 2010; Shin et al. 2015; Liu et al. 2017). All these processes initiate growth by reducing the hardiness state, maximizing the utilization of the (often) short growth season.

In spring, during cold deacclimation and bud break, trees are generally most vulnerable to low-temperature damage (Kalberer *et al.* 2006). For example, Scots pine needles can suffer severe frost damage at temperatures as mild as -4°C (Ryyppö *et al.* 1998b), yet survive freezing temperatures as low as -80°C when cooling occurs gradually prior to freezing (Strimbeck & Schaberg 2009). This contrast highlights both the tolerance capacity of boreal conifers under adapted and optimal conditions and the heightened risk they face during sensitive developmental stages.

1.3 Climate change in boreal forests

I. Boreal forests are intensively managed in Northern Europe

Two-thirds of forested boreal regions in Northern Europe are managed, providing material for lumber and paper production (Vanhanen *et al.* 2012). Intensification of modern forestry in the Nordic countries started with the introduction of even-aged stands in the early 20th century. The new intense regeneration method by planting seedlings on clearcuts in the mid-20th century greatly increased the biomass volumes and started modern intensive forestry (Östlund *et al.* 1997; Lier & Schuck 2020). This intensification of tree regeneration and growth was backed up with the contemporary view of boreal forests as a simple systems, where tree growth can easily be managed (Kuuluvainen & Siitonen 2013). However, nowadays it is known that despite the low species diversity, boreal forests are ecologically complex systems (Messier *et al.* 2013), and optimal growth and stress responsiveness of trees reflect their surrounding growth conditions.

The intensification of forest management has led to the domination of Norway spruce and Scots pine monocultures in Northern Europe, resulting in reduced species diversity and increased susceptibility to abiotic and biotic damage (Knoke & Seifert 2008; Nevalainen 2017). Seedlings, and later mature trees, growing in open clear-cuts experience microclimate conditions that differ substantially from their natural establishment environment under cover from close-by vegetation. For example, the open clear-cut is challenging the growth of Norway spruce seedlings that are late-successional species with slow early growth (Engelmark & Hytteborn 1999), making spruce vulnerable to abiotic and biotic stress factors during the first years of its growth. Studies have demonstrated that maintaining high species abundance and diversity enhances the resiliency of forest ecosystems to disturbances (Holling & Meffe 1996; Drever *et al.* 2006). Transitioning towards more sustainable forest management practices, such as continuous cover forestry and mixed-species stands, is therefore essential for improving the resilience of trees to climate change (Puettmann *et al.* 2013). In parallel, advancing genetic improvement by molecular breeding (Cortés *et al.* 2020; Feng *et al.* 2024) and applying modern molecular technologies to enhance stress tolerance in tree species will complement management practises and could secure both the adaptability of forests and ensuring their utilization in the future.

1.3.1 Warming increases low temperature stress in boreal trees

Breeding and intensive forest management have significantly enhanced tree growth rates in northern Europe over recent decades (Nilsson *et al.* 2011). However, despite sustained research efforts and the implementations of advanced silvicultural and breeding programs, growth trends in managed forests have begun to show decline in past 15 years (Henttonen *et al.* 2024; Korhonen *et al.* 2024). This decline in growth, or declining trend of the growth in the future, is expected to be largely attributed to the impacts of climate change (Laudon *et al.* 2024), increasing uncertainty over the continued productivity of Northern European forests, specifically at its southern-most edge. In particular, Norway spruce stands have shown increasing vulnerability to environmental stressors, including drought and biotic attacks (Mensah *et al.* 2023). Increasing number of studies are expecting decline in forest resiliency in the future, which has been reflected in the future plans of breeding programs in Fennoscandia (Haapanen & Mikola 2008; Mullin & Lee 2013).

In boreal trees, frost hardiness follows the annual cycle of mean temperatures by staying lower than the threshold of damaging low air temperatures (Sakai & Larcher 1987; Repo 1992). The ability to withstand cool temperatures and freezing events, while maximizing resource utilisation, is one of the key adaptations that has allowed trees to dominate boreal forest in northern hemisphere (Sakai & Larcher 1987; Bannister & Neuner 2001). In the future, this key adaptation will be challenged, when the optimal time window for balancing resource utilization and frost avoidance in spring and autumn, is expected to shift outside the species adaptive capacity (Schwartz *et al.* 2006; Bennie *et al.* 2010; Marquis *et al.* 2022). In the context of low-temperature stress, boreal forests are expected to face growing challenges related to the early onset of spring growth and increased frost risk. In addition, changes in snowpack dynamics may increase ground ice cover and reduce soil insulation, exposing soils to cold temperatures during winter (Marquis *et al.* 2022), which has been shown to be important in determining tree growth and in setting the limits of species distribution (Körner & Hoch 2006; Körner 2021; Wang & Hoch 2022).

I. Longer growth season exposes trees to both cold and frost

Spring onset is advancing in the northern hemisphere as temperatures continue to increase (Menzel *et al.* 2006). This rapid warming extends the potential growing season while simultaneously altering conditions at both its onset and termination (Mikkonen *et al.* 2015; Pörtner *et al.* 2021). In Northern Europe, spring is advancing faster than the last frost date, thereby exposing plants to an elevated risk of spring frost damage (Jönsson *et al.* 2004; Schwartz *et al.* 2006). The risk is further amplified by plant dehardening in spring, which reduces tissue tolerance to low temperatures. Because deacclimation is primarily triggered by rising temperatures, earlier spring onset increases frost damage risk, especially in frost-prone growth sites (Aronsson 1975; Leinonen 1996). For instance, the frost sensitivity of Norway spruce may reflect its preference for frost-prone habitats and its comparatively vulnerable reactivation of photosynthesis under rising temperatures, relative to Scots pine (Linkosalo *et al.* 2014). Similar patterns have been observed in temperate forests, where increased heat accumulation prior to leaf-out in deciduous trees has heightened the frequency and severity of damaging spring frosts (Augspurger 2009; Hufkens *et al.* 2012; Sangüesa-Barreda *et al.* 2021).

II. Altered snowpack

Boreal flora is well adapted to cold climates, yet the rapid warming in the boreal and Arctic regions (Rantanen *et al.* 2022) is reshaping the conditions the species of these areas are adapted to. The rising temperature influences not only the growing season length but also the depth, persistence, and physical properties of the snowpack. Because snow acts as a critical insulator against temperature fluctuations, changes in its structure and timing of melt will have effects on boreal ecosystems (Vuosku *et al.* 2022). For plants, the snowpack protects both above- and below-ground tissues from frost damage; its reduction increases the risk of soil freezing, which can injure fine roots and the understorey vegetation (Blume-Werry *et al.* 2016).

Growth and survival of conifer seedlings are dependent on the insulating cover of the snow pack during winter (Ensminger *et al.* 2009). Conifer seedlings, such as those of Norway spruce and Scots pine, are mainly planted in spring and summer to ensure adequate rooting before the onset of winter (Luoranen *et al.* 2018). The success of their establishment depends less on summer temperatures than on the duration of snow cover, which protects seedlings from frost and desiccation (Renard *et al.* 2016). Snow also stabilizes underground temperatures; in winter, soil surface temperatures are more dependent on the snowpack thickness than air temperature. As a result, milder but less snowy winters can paradoxically increase the likelihood of deep soil frost, with soils freezing more extensively and thawing later in spring (Eurola 1975; Richardson *et al.* 2024).

The vulnerability of all major boreal trees to reduced snow cover is further amplified by their root architecture. The majority of fine roots are concentrated in the uppermost soil layer, with nearly half located in the top 10 cm to maximize the water and nutrient uptake (Yuan & Chen 2010). This shallow distribution, combined with limited cold acclimation capacity in fine roots, makes them highly susceptible to freeze-thaw cycles when snow insulation is insufficient (Roitto *et al.* 2019; Vuosku *et al.* 2022; Richardson *et al.* 2024).

Besides direct short-term effects, low soil temperature have enduring consequences for whole tree physiology and productivity (Repo *et al.* 2021). Boreal tree roots follow a distinct phenological rhythm, with growth peaks in spring and dieback in autumn (Ritchie & Dunlap 1980; Teskey & Hinckley 1981; Pregitzer *et al.* 2000; Quan *et al.* 2010; McCormack *et al.* 2014). Low soil temperature, as a consequence of the deep soil frost and delayed soil thawing, may suppress or postpone this growth peak, thereby reducing overall tree growth (Repo *et al.* 2014; Li & Hoch 2025). When soils remain frozen while air temperatures rise, water movement from soils to the above ground tissues becomes restricted, increasing the risk of dehydration and limited shoot development (Ryyppö *et al.* 1998a; Hoch 2013). Cold soil altered both short- and long-term radial growth in Norway spruce (Repo et al. 2021), limit water uptake during spring in Scots pine (Mellander et al. 2006), delayed spring photosynthetic recovery in black spruce (*Picea mariana*) (Fréchette et al. 2011), and altered carbohydrate levels and decreased whole tree biomass in birch (*Betula pubescens*) and Scots Pine (Domisch *et al.* 2017; Domisch *et al.* 2018). Also deepening soil frost can decrease fine root biomass the following year (Kreyling *et al.* 2012). Collectively, these findings underscore the central role of roots in whole-plant fitness, linking low soil temperature to above-ground vitality, biomass accumulation and even the frost tolerance of shoots (Sutinen *et al.* 2015; Sun *et al.* 2018)

Climate change exposes roots not only to individual stressors, but to complex, multi-stress situations. For example, excess winter precipitation (as water instead of snow) combined with frost can intensify fine root damage in both crop and tree species (Stone *et al.* 1999; Bélanger *et al.* 2006; Soualiou *et al.* 2022; Vuosku *et al.* 2022), while drought in combination with low soil temperature exerts stronger adverse effect that either factor alone (Sánchez-Bermúdez *et al.* 2022). Given that fine roots are essential part of carbon flux in forests (Gower *et al.* 1996; Gower *et al.* 2001), their vulnerability has implications to global carbon budgets. Altered snow cover in Arctic-boreal region have already linked to altered carbon assimilation, decreased storage capacity and accelerated carbon losses (Lyr 1996; Iivonen *et al.* 1999; Repo *et al.* 2004; Pongracz *et al.* 2024). Thus, understanding how root systems respond to changing winter conditions is not only essential for predicting tree productivity but also a matter of global concern.

1.3.2 Towards climate resilient boreal trees

I. Forest tree breeding

Tree breeding relies on the selection of superior tree material with enhanced growth and wood properties – traits that natural selection alone has not optimized for human needs. Conventional tree breeding in Northern Europe began in the late 1940s, when phenotypically superior trees were first selected from natural forests and propagated through grafting in seed orchards (Haapanen & Mikola 2008; Stener *et al.* 2015). A defining characteristic of tree breeding in northern latitudes is the long rotation periods, which complicates the long-term planning of breeding objectives (Rosvall *et al.* 2011). Furthermore, due to the significant role of the forest industry in northern economies, breeding programs are designed to align with goals for high biomass production and superior tree quality (Mullin & Lee 2013). Intense breeding programs for Norway spruce and Scots pine in Sweden are expected to increase growth and simultaneously improve wood quality (Rosvall 2001). However, these potential gains may not be realized if the selected tree material lacks the capacity to thrive under changing climate conditions. To address this challenge, tree breeding can enhance the plasticity and adaptability of trees by leveraging the genetic variation within existing populations, while ensuring that genetic diversity is maintained (Rosvall *et al.* 2011). The broad geographic distribution of boreal tree species is crucial for their climatic adaptation, as it facilitates strong local adaptation (Rehfeldt *et al.* 1999; Aitken & Hannerz 2001; Savolainen *et al.* 2007). When combined with high genetic diversity, this distribution provides a foundation for adaptive capacity, enabling tree populations to withstand environmental fluctuations and respond to ongoing change in climate (Aitken *et al.* 2008). Molecular breeding methods, such as genomic selection, can utilize this genetic diversity to accelerate the development of traits required for trees to overcome and manage challenges of ongoing climate change.

II. Genomic selection

Traditional breeding is based on genetic evaluation utilizing phenotypic measurements and pedigrees, with the help of statistical models (e.g. BLUP) that estimate breeding values, which represent the heritable component of an individual’s performance that can be passed on to its offspring. The access to genomic information has enhanced efficiency of breeding by greater accuracy and shorter generation intervals (Crossa *et al.* 2017). The use of genome-wide single nucleotide polymorphism (SNP) data enables the estimation of realized relatedness instead of recorded pedigrees among individual trees, thereby improving relationship estimates to enhanced accuracy and greater genetic gains (Meuwissen *et al.* 2001).

The introduction of molecular markers in the 1980s represented the first major step towards accelerating selection speed and accuracy (Lande & Thompson 1990). This development was further advanced by next-generation sequencing (NGS) technologies, which provided rapid, cost-effective and high-throughput genotyping, thereby facilitating the widespread use of SNP markers (Poland & Rife 2012; He *et al.* 2014). Building on these advances, genomic selection (GS) methods emerged as an approach that integrates molecular and phenotypic data through advanced computational and statistical approaches (Meuwissen *et al.* 2001). GS employs genome-wide markers to predict the genetic potential of individuals (i.e. their breeding values), thereby guiding selection decisions and accelerating genetic gain in breeding programs (Crossa *et al.* 2017). In GS models, a training population (TP) (Figure 1a) combines genotypic and phenotypic data to estimate genomic estimated breeding values for a validation population (VP), which has been only genotyped but not phenotyped (Figure 1b).

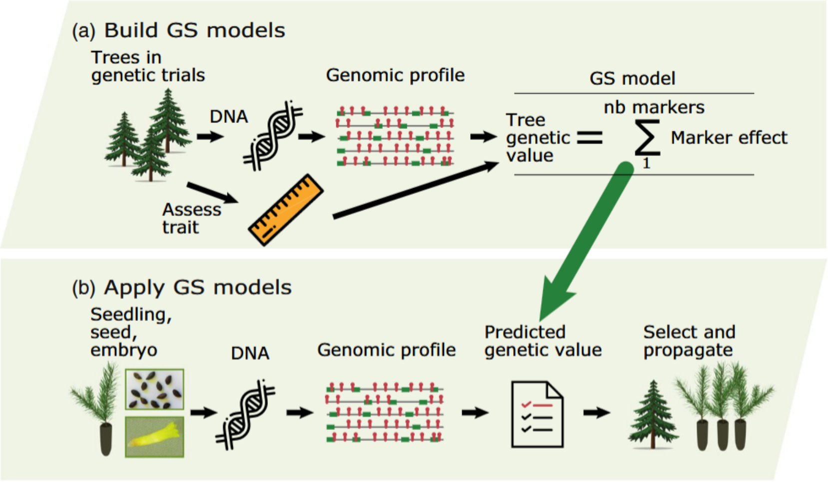

Figure 1 Genomic selection modelling and integration. Figure by Lenz *et al.* (2020)

The primary advantages of GS are its potential cost-effectiveness of genotyping compared to phenotyping, as well as the potential to shorten breeding cycles by enabling early selection based on genotyping data (Crossa *et al.* 2017). In forest tree breeding, where shortening the breeding cycles is a key objective, the ability to genotype seedlings or even embryos has the potential to markedly increase efficiency (Resende *et al.* 2012), this would benefit specifically Norway spruce with its late and uneven flowering (Nikkanen & Ruotsalainen 2000). Genomic approaches can also improve estimates of genetic gain by increasing accuracy through the capture of Mendelian segregation (Visscher et al. 2006; Lee et al. 2010; Muñoz et al. 2014), reducing the generation interval (Li & Dungey 2018; Cobb *et al.* 2019), and enabling larger populations while maintaining the genetic diversity (Meuwissen & Oldenbroek 2017). While tree breeding is constantly advancing, it remains constrained particularly in the Northern Europe by long generation cycles, low flowering rates and challenges in early phenotyping of economically important traits. Moreover, the validation of GS across generations, environments and developmental stages is challenged by the long life cycles of coniferous trees important in Fennoscandia (Hayatgheibi *et al.* 2025).

Genomic prediction is not limited to single traits. Multivariate GS models allow the simultaneous inclusion of several traits, thereby potentially increasing selection efficiency, specifically of low heritable traits (Thompson & Meyer 1986; Guo *et al.* 2014; Klápště *et al.* 2020). The improved accuracy of multivariate models over univariate models arises from utilizing high genetic correlations between traits (Thompson & Meyer 1986). In tree breeding, multivariate GS studies have primarily been applied to growth and wood quality traits (Rambolarimanana *et al.* 2018; Klápště *et al.* 2020). However more recently, GS models have expanded to include biotic and abiotic stress-related traits, with the aim of enhancing climate resilience through genomic prediction (Lenz *et al.* 2020; Laverdière *et al.* 2022). Such traits are highly polygenic, controlled by hundreds or thousands of genes, making genomic approaches such as GS or genome-wide association studies (GWAS) great alternatives for uncovering their genetic architecture and applying this knowledge in breeding. Future progress in forest tree breeding will likely incorporate the integration of GS with advances in artificial intelligence, big data analytics, and multi-omics approaches to further enhance predictive power and breeding efficiency (Xu *et al.* 2022; Escamilla *et al.* 2025).

III. Genomic tools to enhance low temperature resilience

a. Whole-genome sequencing allows regulatory analysis

Molecular methods require knowledge of the genetic structure of individuals, and nowadays whole genome annotations are available for a wide range of tree species (Nystedt *et al.* 2013; Salojärvi *et al.* 2017; Robinson *et al.* 2024; Nilsson *et al.* 2025). The availability of complete genome sequences has enabled the application of high-throughput sequencing for transcriptome profiling (such as RNA-seq), the use of targeted genomic editing tools such as CRISPR-Cas9 (Fan *et al.* 2015; Dai *et al.* 2020), and the development of efficient genotyping tools such as SNP arrays for genomic prediction and GWAS (Bernhardsson *et al.* 2021). Furthermore, the increased number of annotated genomes facilitate comparative approaches across both within and across plant species. Such single- and multi-omics studies can provide deeper insight into the conservation of molecular pathways as well as the evolutionary diversification of gene functions across the plant kingdom (Feller *et al.* 2011; Yang *et al.* 2021; Roychowdhury *et al.* 2023).

Tree genomes, however, present specific challenges compared to many herbaceous plants. Gymnosperms in particular typically have very large genome sizes (Pellicer *et al.* 2018). This complexity is largely attributed to the extensive amplification of long terminal repeat (LTR) retrotransposons (Morse *et al.* 2009; Kovach *et al.* 2010). As a results, tree genomes are not only massive but also highly repetitive, making their sequencing, assembly and annotation more demanding. These features complicate downstream analysis and developing advanced molecular tools, thereby slowing progress compared to model plants with small and less repetitive genomes.

b. Genome editing and genetic transformation in trees

Genetic methods allow deep dive into key molecular regulatory systems and the in-depth knowledge on genes and transcription factors responsible for certain biological processes. One promising, although politically controversial, method is the alteration of genes or gene functions in genome. Nowadays, these methods are divided into genome editing: changing the innate characteristics of the native genome (Strauss *et al.* 2022), and genetic transformation: inserting new genes to gain new traits in the genome. In transgenic approaches with coniferous species, genes are introduced with biolistic methods with the assistance of *Agrobacterium tumefacience* (Sederoff *et al.* 1986; Bekkaoui *et al.* 1988; Huang *et al.* 1991). However, *Agrobacterium*-mediated transformation is more popular due to more stable transgenic expression and lower risk of gene silencing (Hadi *et al.* 1996).

The development of genetic engineering of important boreal coniferous species has been slow, and issues to produce stable and effective transformants still remain. Legislation uncertainties in EU are hindering development of the transformation tools. However recently there has been a proposal of a new EU's regulatory framework towards plant generated by recent genomic techniques (Gao *et al.* 2022; Purnhagen *et al.* 2023). This update would apply for New Genomic Techniques (NGTs), allowing to produce plants with tools that create targeted changes in plant genomes without the use of exogenous material, such as CRISPR-Cas9. Successful implementation of CRISPR-technology in coniferous species have been achieved with Japanese cedar (*Cryptomeria japonica* D. Don) and *Pinus radiata*, although the edited plants contained random insertions, deletions and replacements in the target regions (Nanasato *et al.* 2021; Poovaiah *et al.* 2021). This, once more, highlights the complexity of conifers and how the application of advanced molecular methods require still plenty of development, although the technology is progressing. However, when reproducible, these techniques have the potential to mitigate the effects of climate change by enhancing carbon sequestration by trees (Jansson *et al.* 2010), and by enhancing adaptation to altered growth conditions, such as increased drought resiliency (Zilberman *et al.* 2018; Cao *et al.* 2024).

2. Research aims and objectives

As growth conditions for boreal trees are changing rapidly and low-temperature stress events are expected to increase, it is essential to understand their core responses to cold and freezing stress to assess impacts on growth and productivity and to develop methods for mitigating these effects.

The aim of my thesis was to establish a comprehensive understanding of core basal responses and regulation of low temperature stress in boreal trees, by using both experimental approaches such as RNA-sequencing and applied approaches, such as genomic prediction models. Specific objectives to achieve this aim are listed below and discussed further in this thesis:

1. Comparative assessment of low temperature transcriptional responses of coniferous and herbaceous Arabidopsis above- and below-ground tissues.
2. Determine the core transcriptional cold temperature response and regulatory mechanisms of boreal tree roots and their conserved nature across tree species.

3. Assess basal freezing stress variation in Norway spruce population and its connection to growth and phenological traits and their implications to molecular forest breeding.

This thesis provides comprehensive research on physiological and transcriptional responses of low temperature stress on boreal tree species and links these effects to the ongoing anthropogenic global warming effects on boreal forests.

3. Material and methods

Publish or perish.

Publishing in peer-reviewed journals maintains the quality of the work of scientist. But sometimes there is a great idea, method or a protocol that, for one reason or another, does not end up published. That is why I have included here an ion-leakage method specifically for roots, to quantify cold or freezing tolerance in root tissues. The method has been published in **Paper I,** but here is the version that has been tested with success with root of Arabidopsis Col-0 and Ost-0 (Appendix 4), Norway spruce, Scots pine, aspen and silver birch (Appendix 3).

3.1 Freezing tolerance in roots

Tree seedlings were sown in individual 1 l pots with mix of soil and perlite and grown at 23°C until roots had explored the soil volume, followed by the shift to 5°C. Samples for ion leakage assay were collected after 0 h, 3 days and 10 days at 5°C. Roots were rinsed with MQ water before cutting the active growing fine roots (~1 mm diameter) in three 1 cm sized pieces and moving them to a glass vial with 500 ul MQ water. Each time point was replicated by root samples from five replicate trees. All samples were kept on ice before moving them into the freezing bath. Samples were incubated at 2 °C in programmable bath (Julabo FP45) for 15 min before starting the cooling program. Vials with root samples were subjected to a decreased temperature at cooling rate of -1°C/h. Ice formation was initiated at -2°C using a metal probe cooled by liquid nitrogen. At designated intervals, 0, -1, -2, -3, -4, -5 and -6°C, samples were removed from the bath and kept at 4°C overnight. Control vials were kept at 4°C. On the following day, 2 ml of MQ water was added to the vials and placed on a shaker at room temperature overnight. Initial electrolyte leakage (C_b) was measured using a conductivity meter (CDM210, Radiometer), followed by flash freezing the samples and controls in liquid nitrogen, and shaking overnight at room temperature. The final conductivity (C_a) was then determined. Electrolyte leakage (EL) was determined as EL = $(R_1 + R_2)/2$, R_1 representing leakage from control tissue and R_2 representing leakage from sample tissue. Leakage percentage for R_1 and R_2 were determined using the formula, with C_b as the initial conductivity before flash freezing and C_a as the final conductivity after flash freezing.

Arabidopsis seeds were sown in individual pots and grown for 32 days until shift to 5°C and samples for ion leakage assay were collected after 0 h, 3 days and 10 days in cold treatment. Roots of three individual plants were collected in a glass tube with 500 ul MQ water to represent one replicate samples. Five replicates for each time point were collected and the ion leakage method was followed as with tree roots above.

4. Results and discussion

The results of the included papers are presented within the broader context of low temperature responses in boreal trees, with particular attention to their implications for enhancing climate resilience of boreal forests. The warming climate is expected to increase the growth-reducing effects of low-temperature stress, especially during the non-acclimated developmental stages of trees. This thesis provides insight into the transcriptional regulation and responses of boreal tree species, while also exploring the potential application of genomic selection to improve spring frost tolerance. **Paper I** and **Paper II** provide comprehensive insights into transcriptional responses to cold and freezing stress in boreal tree species, whereas **Paper III** examines the application of genomic selection methods to predict spring frost tolerance in Norway spruce.

4.1 Transcriptional low temperature stress responses in spruce

The release of the Norway spruce whole-genome sequence by Nystedt *et al.* (2013) began a new era of exploring the complex transcriptional responses of this extremely important coniferous species. The updated annotation by Nilsson *et al.* (2025) moved Norway spruce one step closer as a model for woody tree species, which will help to clarify the detailed mechanisms behind growth and development, but also the complex regulation and response networks behind abiotic and biotic stress events.

4.1.1 Cold response and regulation of needles and leaves

Needles of coniferous species are specialized leaf structures that have evolved to withstand harsh environments. In **paper I** and **II**, we compared transcriptional and physiological cold responses of Norway spruce needles with Arabidopsis Col-0 leaves during a 10-day cold exposure. While both herbaceous and woody perennials are known to employ broadly similar mechanisms in coping with cold stress, detailed comparative analyses between leaves of an herbaceous model and a coniferous species have been lacking.

Despite major morphological and evolutionary divergence, both Norway spruce and Arabidopsis exhibited significant Gene ontology (GO) enrichment of genes in the “response to stress” category, along with terms such as “signal transduction”, “transport”, “plasma membrane”, and “carbohydrate metabolic processes” (**Paper I**, Figure 2; **Paper II**, Figure S4, S7), all indicating stress related responses. Moreover, both species shared a conserved orthologous set of cold-responsive differentially expressed genes (**Paper** I, Figure 1C). As orthologs generally retain comparable biological functions across species (Fitch 1970), this conservation points to shared cold-response mechanisms. GO enrichment analysis revealed overlapping categories such as “stress responses” and” ion binding” (**Paper I**, Table S3), further supporting their functional parallels.

Although core cold responses were found, above-ground tissues of these species are adapted into highly different climatic conditions, which was reflected in the GO enrichment analyses results in **paper I** and **II**. Arabidopsis displayed the broadest range of cold-responsive GO terms and maintained relatively stable expression dynamics throughout the 10-day treatment (**Paper II**, Figure S1A, S4). In contrast, spruce responded with a slower transcriptional response and exhibited the highest expression levels at 10 days (**Paper I**, Figure 1B and **Paper II**, Figure S7). GO analysis based on the updated spruce genome assembly revealed cold-induced enrichment linked to biotic defence (“defence respond to insects”, “response to wounding” and “respond to oomycetes”), indicating overlapping crosstalk between abiotic and biotic stress pathways, as reported previously in European ash (Fujita *et al.* 2006; Lane *et al.* 2016). However, these terms may also be linked to oxidative stress, since NUCLEOSIDE DIPHOSPHATE KINASE 2 (*NDPK2)* was identified as an Arabidopsis ortholog of the term "defence response to insect" (GO:0002213), which has shown to be part of both oxidative stress and light signalling in aspen and Arabidopsis (Shen *et al.* 2005; Kim *et al.* 2011) The enrichment of "photoprotection" highlights a needle-specific adaptation to mitigate photoinhibition during cold periods with high irradiance (Krivosheeva *et al.* 1996; Linkosalo *et al.* 2014). In addition, terms such as "cell division", "response to far-red light" and "response to blue light" may further suggest that needles integrate photoperiod cues to sustain growth despite low temperatures. Far-red light is growth-promoting in long-day conditions particularly for northern-origin Norway spruce (Junttila & Kaurin 1990; Clapham *et al.* 1998), and cell division is a fundamental process during growth and development.

Transcription factors differentially expressed by cold (TF-DEC) were rapidly upregulated in Arabidopsis, peaking at 6 hours and declining by 24 hours (Figure 2), consistent with the temporal behaviour of known cold-related TFs (Chinnusamy *et al.* 2003; Kim *et al.* 2013). In Norway spruce needles, the expression of TF-DECs was delayed, with highest expression level at day 10 (Figure 2). Although overall delayed, a small subset of early-responding regulators in spruce was induced between 6 and 24 hours expressing homologs (ORA47, AP2, MYB33, ERF53, NAC025 and TINY2) (Figure 2), which are previously known stress or cold TFs in Arabidopsis (Xie et al. 2019; Ritonga et al. 2021; Wang et al. 2021; Jiang et al. 2022; Zeng et al. 2022; Xu et al. 2024). This suggests that at least a partial conservation of early cold regulators between species. Conversely, spruce-specific upregulation of TFs, such as ZFP4, MYB123, MYB101 and WRKY20-like homologs point to unique late cold regulatory pathways. This suggests that Norway spruce needles display not only a temporally delayed response compared to Arabidopsis, but also a unique late cold regulation response. However, given that the main expression response in Norway spruce was not initiated until day 10, the distinction between “early” and “late” responses between these species remains somewhat artificial. A more meaningful comparison would require a longer cold treatment period in Norway spruce to determine whether, and what stage, expression levels would decline. Interestingly, while *ICE1* transcription factor – a central *CBF* regulator in Arabidopsis (Chinnusamy et al. 2003) – emerged as a hub in roots and was differentially expressed in needles (**Paper I**, Figure 7C), no cold-responsive CBF-like regulators were identified in spruce needles in **Paper I.**

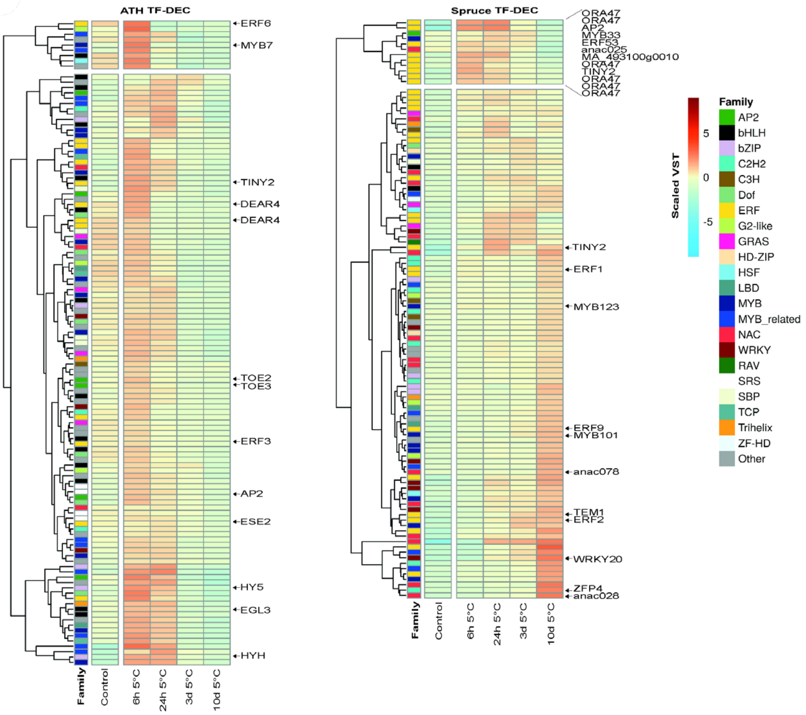

Figure 2 **Transcription factor analysis**. TF differentially expressed by cold (TF-DEC) were analysed in both Arabidopsis leaves and Norway spruce needles. TF with positive changes relative to control are shown (corrected p ≤ 0.01 and fold change ≥2). VST data were scaled by row means.

In summary, our results indicate that Norway spruce needles and Arabidopsis leaves share a partially conserved early cold regulation hub. However, differences in timing, species-specific induction of distinct cold-related processes, and the apparent absence of *CBF*-like TFs in Norway spruce indicate a fundamental divergence in cold-regulatory networks between these species, likely reflecting deep evolutionary separation and contrasting leaf morphologies. The enrichment in light-stress related responses in spruce highlights its adaptation to high-light winter-conditions. Notably, spruce needles exhibit a stronger indication of a trade-off between sustaining growth and activating stress responses, reflecting both the high metabolic cost of stress activation and the necessity to maximize the short boreal growth season. The delayed transcriptional activation of stress pathways may therefore represent an adaptive strategy; whereby growth-related processes are maintained until prolonged cold stress necessitates a full shift towards stress regulation.

4.1.2 Above and below-ground cold responses in Norway spruce and Arabidopsis

I. Common responses

A central question in plant stress biology is whether the same abiotic stimulus triggers similar biological responses across different tissues, and to what extent these responses are coordinated by shared versus tissue-specific genes. While above- and below-ground comparison of low temperature stress have been studied in Arabidopsis and aspen *(Populus trichocarpa*) (Kreps *et al.* 2002; Tao *et al.* 2022), comparison on coniferous species is lacking. **Papers I** and **II** address this gap by comparing leaf and root low temperature in Norway spruce, with the model herbaceous species Arabidopsis Col-0 included for comparison.

In **Paper I and II**, we demonstrate that while both Norway spruce and Arabidopsis exhibit distinct transcriptional responses to progressive cold and freezing stress in roots versus needles/leaves, a small group of overlapping responses were found. In Arabidopsis, enriched categories among shared upregulated genes included translational and transcriptional regulation, as well as cold and water stress responses (**Paper II**, Figure S5). In spruce, overlapping upregulated GO categories included responses to transport, abscisic acid (ABA), general stress and salt stress (**Paper I**, Figure 4c & **Paper II**, Figure S8). Further DEG analysis in spruce grouped up-regulated genes into three shared clusters, enriched with terms such as oxidation-reduction, biotic stress, transport and plasma membrane processes (**Paper I**, Table S8). Cold acclimation is a dynamic process that requires continuous regulation of gene expression and cellular transport, which likely explains the prominence of these broad stress related processes. The adaptive significance of shared stress responses is consistent with recent findings by Whiting *et al.* (2024), showing that genes with greater tissue pleiotropy, genes whose effects extend across multiple tissues, play an important role in local adaptation of climate-related traits in Arabidopsis. However, the analysis of transcription factors shared between the two tissues (37%) revealed that most TF-DECs exhibited differences either in their temporal expression or in the strength of their induction (**Paper I**, Figure 6, Table S10). Taken together, these results suggest that genes central to temperature adaptation are generally expressed across tissues, with stress-related genes particularly showing substantial overlap between below- and above-ground responses. Nevertheless, these tissues show highly different transcriptional control during cold stress.

II. Tissue-specific responses

Across both species, approximately 20% of cold-induced DEGs were shared between the tissues. In Arabidopsis, only 15% of up-regulated DEGs overlapped between both tissues, compared with 25% in Norway spruce (**Paper II**, Figure 1,2). This discrepancy in gene expression may be explained by the slower transcriptional response in spruce; the 10-day cold treatment may not have been long enough to fully capture late-responding genes (**Paper II**, Figure S2). In our experimental setup, soil temperatures declined in a broadly similar pattern for both species, though soil volume differed (Appendix 1,2). The delayed cold induction in spruce roots may be related to an evolutionary adaptation to buffered soil environment, where insulation dampens abrupt temperature fluctuations and consequently delays the initiation of stress signalling. Similar pattern was found in the roots of other tree species (**Paper II**, Figure S9). Interestingly, the majority of DEGs were downregulated in response to cold in roots (**Paper I**, Figure 4b, **Paper II**, Figure 1&2), a pattern that has not been observed in other abiotic stress studies as with drought (Haas *et al.* 2021), indicating this repression may be cold-specific. Finally, roots generally showed a larger number of cold-induced DEGs, whereas needle and leaf GO analysis exhibited a more diverse and functionally enriched response (**Paper I**, Figure 4C; **Paper**

II, S4&S7). Similar trends have been observed in maize (*Zea mays* L.) and Norway spruce, where roots displayed a greater number of DEG in response to abiotic stress compared to above-ground tissues (Haas *et al.* 2021; Zhao *et al.* 2021). Leaf-tissues are exposed to everchanging temperature and light conditions, requiring constant active regulation between photosynthesis and stress regulation, explaining more diverse stress responses.

In Norway spruce, tissue-specific differences in cold responses were more pronounced than in *Arabidopsis* (**Paper I**, Figure 4; **Paper II**, S3&S4, S6&S7). Needles upregulated pathways related to light signalling and oxidative stress, while roots showed enhanced expression of genes involved in metabolic regulation, solute transport and sugar metabolism, while downregulating photosynthesis and plasma membrane processes. In Norway spruce, root-specific enriched GO terms included “response to copper ion”, “galactose metabolic process” and response to salt stress and nitrate starvation, highlighting a strong emphasis on nutrient uptake and ion homeostasis under cold conditions (Pregitzer & King 2005). Additionally, the enrichment of terms such as “lateral root development” and “D-glucose import” indicates continued root growth and glucose-mediated signalling during stress, consistent with known roles of glucose in abiotic stress responses (Ehness *et al.* 1997; Rolland *et al.* 2006). Lastly, we analysed the cold regulatory network to identify key TF hubs in Norway spruce regulating cold responsive genes (**Paper I**, Figure 7). The most connected hub (MA_448849g0010) was identified as a *ICE1*-like *bHLH* TF, highly connected to *COR* genes in both tissues. Interestingly, the third-most connected TF hub MA_68586g0010 (BHLH101-like TF) was nearly exclusively regulating root-specific subset of cold-responsive genes. In Arabidopsis, *bHLH101* is associated with iron homeostasis and photo-oxidative stress (Sivitz *et al.* 2012; Noshi *et al.* 2018). Its established role in oxidative stress regulation, together with our observation of root H_2O_2 accumulation under cold conditions (**Paper I**, Figure S11), supports its involvement in root-specific cold responses.

Together, these results highlight roots as highly responsive tissue under cold stress, displaying both strong transcriptional repression and distinct functional enrichments. These finding raise questions how root-specific responses are orchestrated across boreal tree species and whether common regulatory modules are responsible their adaptation to cold. In the following section, we assess in more detail the root responses and regulatory networks in boreal trees to uncover the mechanisms shaping their cold resilience.

4.2 Core transcriptional cold response mechanisms in roots

Above-ground plant organs, such as flowers, fruits, stems and leaves have long been the focus of plant scientists. In contrast, below-ground tissues, primarily roots, have received less attention, overshadowed by their sun-exposed counterparts. The primary challenge in root research lies in the inaccessibility of root systems, complicating specifically molecular studies hindering the progress in understanding the importance of roots from the species level to global ecological scales. To address the limited molecular knowledge of root cold responses, **Papers I** and **II** investigate tissue-specific transcriptional regulation under low temperature in both the herbaceous Arabidopsis Col-0 and the coniferous Norway spruce (*Picea abies),* concluding highly tissue-specific responses and regulatory patterns. In **paper I**, we observed ~80% root-specific cold-responsive genes in both species, emphasizing the distinct nature of root transcriptional responses. We also identified several root-specific transcription factor clusters and a highly connected regulatory network in Norway spruce, in which a *bHLH101*-like TF (MA_68586g0010) emerged as a central hub in low temperature regulation, indicating that roots employ a unique transcriptional pathway network to tolerate low temperature stress.

In **paper II**, we extended this investigation to unravel the mysteries of roots core responses under cold, focusing on the dominant boreal tree species. We conducted a comparative study on cold responses across coniferous and broadleaf species native in Fennoscandia, Norway spruce (*Picea abies*), Scots pine (*Pinus sylvestris*), silver birch (*Betula pendula*) and aspen (*Populus tremula*), all of which are ecologically, societally and economically significant in the region (Kullman 2008; Jäppinen & Heliölä 2015; Nilsson *et al.* 2021; Hämäläinen *et al.* 2023; Vainio *et al.* 2024). As in **paper I**, Arabidopsis was included as a model herbaceous species to complement the limited transcriptional data on root cold responses in woody species. As previous studies suggest ecotypic variation in cold response within Arabidopsis ecotypes (Hannah *et al.* 2006; Park *et al.* 2018), we included both the widely used ecotype Col-0, originating from Germany (Passardi *et al.* 2007) and a northern ecotype Ost-0 (N 60°15’, E 18° 18’), to evaluate possible ecotypic differentiation under cold, and compare them with the responses of northern-adapted tree species.

I. Roots show low conservation genes regulated by cold across species

Transcriptional analyses enable the identification of causal genes and regulatory mechanism underlying stress responses (Martin *et al.* 2013; Montenegro 2022). In **paper II**, our aim was to capture both direct and indirect root responses to cold. Direct responses, identified through differential expression analysis, reflect immediate changes in gene or transcription factor expression levels in reaction to environmental shifts (Guo *et al.* 2009; Peng *et al.* 2014). Indirect responses, in contrast, arise from broader network-level interactions among stress-responsive genes (Weston *et al.* 2008; Ohama *et al.* 2017; Lv *et al.* 2020). To identify conserved direct responses, we constructed cold-response profiles of transcriptional factors (TF-DECs) by their temporal expression patterns (Figure 3). To understand the biological processes associated with these superclusters, we identified the corresponding DEGs and TF-DEGs and performed a "signature" Gene Ontology (GO) analysis to highlight functional categories consistently characterized in each supercluster (Figure 3c). Indirect responses were investigated using co-expression network analysis, which groups genes into modules based on their shared expression dynamics across conditions (Wolfe *et al.* 2005). Closely connected gene clusters tend to have similar expression patterns and have uniformed regulation (Heyndrickx & Vandepoele 2012). Genes are represented as nodes in these networks, with edges indicating correlated expression patterns. Gene centrality, the degree of connectivity and influence of a gene within a network, is often conserved across species (Stuart *et al.* 2003; Netotea *et al.* 2014) and this helps to identify the key regulatory hubs.

The comparative co-expression analysis in **Paper II** followed three steps using the root transcriptomics data under cold: 1. **Identification of co-expressologs**: Orthologous gene pairs between two species were compared based on the similarity of their co-expression neighbourhoods, identifying those with similar expression patterns. 2. **Detection of conserved co-expressologs:** co-expressologs (orthologous gene pairs with similar expression) showing similar expression patterns across all the species were detected. 3. **Clique identification**: Cliques were identified as groups of orthologous genes in which each member is significantly co-expressed with every other member across all species networks examined. A clique thus represents a fully interconnected, evolutionary conserved co-expression subnetwork.

The comparative analysis across species throughout **paper II**, requires the use of gene orthologs, that are essential to identify functional information using high-quality annotations of A. thaliana to other species with more recent or less-quality annotation (Lee *et al.* 2015), as in our case with trees. Ortholog-analysis can determine core functional regulatory networks and distinguish evolutionary conservation and divergence in regulatory and response processes between species (Fang *et al.* 2010).

All the boreal tree species share both the climatic growth conditions and the insulation properties of soil that is surrounding their root system. While the soil properties can affect the rate of cooling (Thiffault 2019), conserved cold responses were expected due to the similar thermal environment that roots are sharing. In addition, similar absence of physiological acclimation was found across tree species (Appendix 3). This conserved nature of the regulation patterns in cold-responsive genes in roots was investigated with both co-expression networks and temporal DEG supercluster analysis. First, across all pairwise co-expression network comparisons the number of unique orthogroups overlapping with other species were detected (**Paper II**, Figure S12). The largest sets of common orthogroups were within Arabidopsis ecotypes, followed by conifer- and broadleaf species (1579, 612 and 293, respectively). The grouping of orthogroups based on their phylogeny was expected, while in contrast, the small number (181) of orthogroups conserved across all species was a surprise.

In addition to this indirect method to study expression similarities during cold, a more direct analysis by clustering temporal DEG expression patterns were performed (Figure 3). Four regulatory superclusters representing conserved temporal cold-expression patterns across the species were determined. However, the conservation of orthogroups across species within these superclusters were low, aligning with the results of low conservation in cold regulation and responses in co-expression analysis. These results indicate that roots regulate and response to cold in highly species- and lineage specific manner.

I. Core conserved transcriptional regulation and response in roots

Co-expression analysis identified 181 highly conserved orthogroups with shared expression patterns across all studied species. Of these, 116 orthogroups formed fully interconnected subnetworks that were conserved across all species, meeting the definition of cliques. Gene expression within these cliques clustered into three distinct groups (**Paper II**, Figure 5). Two clusters (1 and 2) were enriched for GO terms related to developmental and growth processes, while the third (3) was enriched for stress-response processes. The largest cluster, comprising 50 orthogroups, with enrichments for cell division processes was corresponding to the main supercluster SC1 (Figure 3). SC1 showed developmental and root-specific growth responses with progressive upregulation throughout the 10-day cold treatment. Growth reduction is a primary plant response to cold stress (Qari *et al.* 2022) and boreal tree roots can decrease growth rates at soil temperature as high as 9°C (Kilpeläinen *et al.* 2019). Nevertheless, growth maintenance and cell wall remodelling are among the most critical processes during low temperatures (Nagelmüller *et al.* 2016). Together, these findings suggests that the predominant root response across species is to sustain growth-related processes despite cold stress at 5°C.

The second clique cluster was enriched for methylation and metabolic processes and displayed a distinct temporal pattern across species; Arabidopsis ecotypes exhibiting fastest upregulation, followed by broadleaved trees, while conifers reached their highest expression levels only at 10-days. This indicates a strong reprogramming of gene expression and broad physiological adjustments in roots during cold, which follow also similar temporal patterns across lineages while proceeding at different speeds. In parallel, supercluster SC3 and clique cluster 2 both showed temporally structured regulation (Figure 3; **Paper II**, Figure 5), indicating that all species induce epigenetic and post-transcriptional regulation together with wide-ranging protein metabolic processes as an early and sustained strategy to improve root cold tolerance (Shan *et al.* 2013; Liu & He 2020).

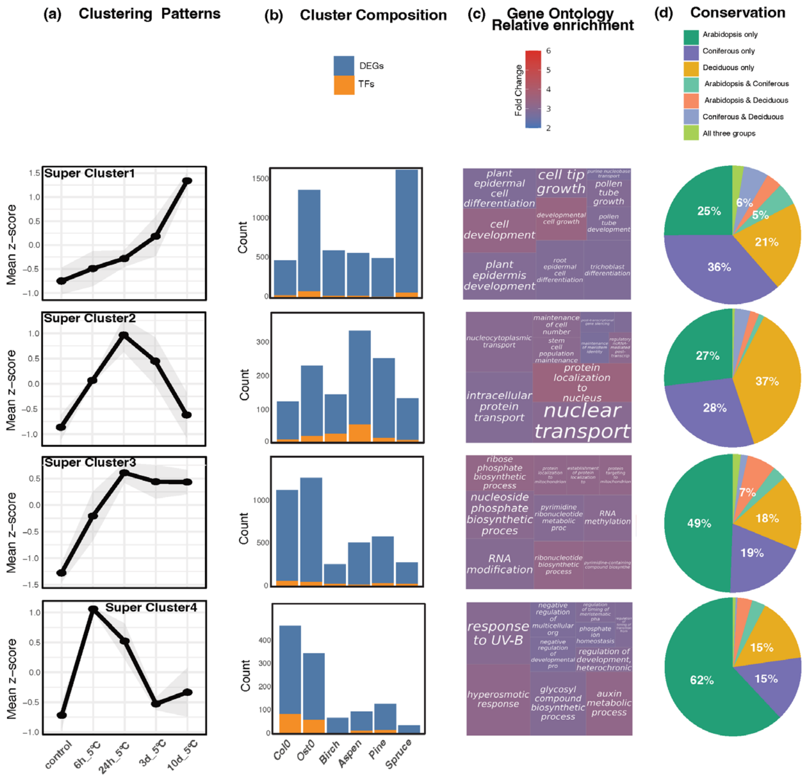

Figure 3 Supercluster characterization. (a) SC TF-DEC expression trends. (b) Number of cold-responsive DEGs and TFs across species. (c) Treemaps of functionally characterizing superclusters through GO analysis. Comparative analysis between superclusters identified the most differentially enriched GO terms relative to other superclusters, with fold change measurements quantifying functional divergence regarding the other super cluster. (d) Display of orthologous relationships among supercluster members across species.

Two additional superclusters, SC2 and SC6, showed early expression peaks that were not reflected in the clique clusters. SC3 peaked at 24h and was subsequently downregulated, with signature GO terms including nuclear transport, protein localization and cell number maintenance. In **paper I,** Norway spruce roots exhibited similar pattern of transport and localization responses (**Paper I**, Figure 4).

SC6 represented the earliest transcriptional response among superclusters, with a sharp increase in expression immediately after cold exposure. Significant GO terms included osmotic stress, UV-B processes and auxin metabolism. Hong *et al.* (2017) has proposed auxin as an important mediator in root growth under cold stress and orthogroup overlap across the lineages (**Paper II**, Figure 4) suggests a strong hormonal response in roots under cold. The third clique cluster consisted of 20 orthogroups, enriched for signalling and stress-related processes.

Taken together, the low level of orthogroup conservation within superclusters indicates that temporal cold-regulatory patterns are lineage-specific. In contrast, co-expression networks are less dependent on temporal dynamics and therefore capture more deeply conserved responses. However, even in co-expression analysis, the number of shared orthogroups was low, suggesting that most cold-regulatory and response pathways are diverged through speciation. Thus, although studied tree species are inhabiting similar environments, they respond to cold stress largely via lineage-specific orthogroups, showing more enriched groups for stress related terms compared to conserved orthogroups across species (**Paper II,** Figure 4).

II. Cold-related transcription factors in roots

A highly interconnected set of cold-related transcription factors was identified in roots across species through co-expression analysis. Eight highly conserved orthogroups, representing seven TF-families, *LBD, bZIP, MYB, NAC, WRKY, HSF* and *G2*-like, were identified (**Paper II**, Figure 6).

Several of these TF-families have well-established roles in cold stress regulation. *NACs* are key regulators of *CBFs* and *COR*-genes, with roles in cold responses in tree species such as silver birch (Puranik *et al.* 2012; Gong *et al.* 2018; Chen *et al.* 2019; Diao *et al.* 2020), but also in lateral root formation (Yarra & Wei 2021). *WRKYs* are likewise responsive to cold stress in trees (Wang *et al.* 2014; Aguayo *et al.* 2019), with *WRKY42* specifically inducing root hair expansion under cold conditions (Moison *et al.* 2021). In this study both *NAC (ANAC018/ANAC002)* and *WRKY* (WRKY48/WRKY23) TFs were up-regulated in aspen and silver birch after 3 days of cold exposure, whereas in Norway spruce and Scots pine NAC expression peaked at 24 h and WRKYs showed even earlier induction in them compared to angiosperms. WRKY48 has shown cold response in grape (*Vitis vinifera) (Wang et al. 2014),* while *WRKY23* is involved in auxin signalling in Arabidopsis roots (Prát *et al.* 2018) and regulating defence responses in aspen (Levée *et al.* 2009).

The bZIP family is regulating cold signalling in radish (*Rhaphanus sativus*) roots, and part of the cold stress response in angiosperm trees (Ito *et al.* 2006; Ma *et al.* 2021; Dong *et al.* 2023), while heat shock factors (HSFs) are regulating abiotic stress responses in woody species (Li *et al.* 2019; Tian *et al.* 2021). LBD-family transcription factors primarily regulate root development and participate in several abiotic stress pathways, though their direct role in low temperature stress has not been confirmed (Rong *et al.* 2024). *MYBs* are known to regulate cold responses in trees, in addition to their roles in several abiotic stresses in roots (Dubos *et al.* 2010; Gong *et al.* 2018; Chen *et al.* 2022). However, their expression profiles in this study remained relatively flat across species. The *G2*-like TFs (*GOLDEN2*-LIKE) from the *GARP* family regulate the expression of several genes involved in cold acclimation and freezing tolerance (Hernández-Verdeja & Lundgren 2024). *G2, bZIP* and *LBD*-family TFs were all downregulated in aspen and birch.

Notably, the *AP2/ERF* and *bHLH* TF families, which were identified as central hubs regulating COR genes in Norway spruce roots (**Paper I,** Figure S5) were absent from the conserved cliques. This absence is slightly unexpected, given their well-established roles as core cold regulators across plant species. *bHLH* TFs accumulate during cold stress to improve physiological cold tolerance in Arabidopsis and several angiosperm tree species (Xie *et al.* 2012; Guo *et al.* 2021), while several *bHLH* subfamilies are involved in regulating the trade-off between cold responses and plant growth (Kidokoro *et al.* 2009; Lee & Thomashow 2012). Similarly, the *AP2/ERF* family is known for its importance in cold regulation (Illgen *et al.* 2020; Ritonga *et al.*

2021), specifically through the *CBF* regulon; the central transcriptional module for cold responses.

4.2.1 CBF-dependent cold regulation

I. CBFs in roots

The *CBF/DREB1* (CRT-binding factor/DRE-binding protein) transcription factor family is well-established as a central regulator of cold responses in many plant species. However, in coniferous species, the expression of homologous *CBFs* genes appear not to be induced by cold stress (Holliday *et al.* 2008; Chang *et al.* 2021). Aligning with these observations, *CBF*-orthologs were entirely absent in the spruce needle cold expression profile in **paper I,** as well as in needles in both coniferous species in **paper II**. Despite the lack of *CBF* induction, several members of the APETALA2/ethylene-responsive factor (*AP2/ERF*) transcription superfamily, of which *CBFs* are a subgroup, were upregulated in spruce needles during both early and late cold responses (**Paper I**, Figure 3b). A similar result was reported in Sitka spruce, where only *AP2/ERF* transcription factors, excluding *CBFs*, were significantly upregulated in response to cold (Holliday et al. 2008). *AP2/ERF* is considered one of the main cold-stress related TF-families and they are important enhancers of cold tolerance in a number of plant species (Ritonga et al. 2021). **In paper I**, orthologs (homologous genes diverged due to speciation) of *CBF1* and *CBF3* showed low-level expression during early cold exposure in Norway spruce roots but did not meet the statistical threshold for differential expression.

Indication of Norway spruce *CBF*-like TFs in cold treated roots led us to investigate their role in roots of other boreal tree species. Potential *CBF1-3* orthologs in silver birch, aspen, Scots pine and Norway spruce were investigated by looking at significant expressologs of Arabidopsis *CBFs* (**Paper II**, Figure 7). The *CBF*-ortholog patterns in birch and aspen were almost identical with high expression peaks at 3 days into cold treatment. This indicates that *CBFs* have a role in cold regulation in roots of these angiosperm trees, as they have in above-ground tissues (Benedict *et al.* 2006; Welling & Palva 2008). These results also align with findings of several *CBF* orthologs upregulated in roots under cold stress of other angiosperm trees, such as maple (*Acer* spp.) and *Liriodendron chinense* (Guan *et al.* 2021; Zhao *et al.* 2023a). In Norway spruce, a *CBF*-ortholog search found two hits, however, neither showed clear upregulation. Combined with the complete lack of needle-specific CBFs, our results align with previous observations that Norway spruce regulates cold responses in a *CBF*-independent manner, and other *AP2/ERF* family regulators might play more crucial role in its cold responses. Interestingly and slightly unexpectedly, Scots pine showed an early peak of one *CBF3*-ortholog in roots (PS_chr06_G023928) at 6 hours of cold treatment, exhibiting a similar pattern as *CBF3* in Arabidopsis.

We also found a clear difference in the expression patterns of *CBFs* between the two Arabidopsis ecotypes (**Paper II**, Figure 7). In Ost-0, *CBF1* and other *CBF*-family members exhibited downregulation until 3 days and were then upregulated. However, *CBF3* showed similar, while slightly delayed, early upregulation as shown in Col-0. In Arabidopsis Col-0 leaves*, CBF3* expression pattern follows a fast induction at first 6 hours, increasing already after 15 min of cold exposure (Gilmour *et al.* 1998a). Interestingly, only *CBF3* expressed similar temporal pattern in roots and leaves (**Paper II**, Figure 7; **Paper I**, Figure 3a). This aligns with previous study by Perez-Garcia *et al.* (2023) showing that *CBF3* has a central role between growth and cold response in Arabidopsis Col-0 (Serrano-Ron *et al.* 2021). The clear up-regulation of *CBF3* compared to other *CBFs* reflects its role as key cold regulator in *Arabidopsis* leaves, since it has shown to account for 40-60% of cold acclimation in both Col-0 and a northern ecotype (SW, Rödåsen-47) (Zhao *et al.* 2016; Park *et al.* 2018). Park *et al.* (2018) showed distinct cold transcriptional responses between northern and southern Arabidopsis ecotypes, revealing a strong difference in their *CBF*-regulation and expression levels, specifically with *CBF2*. In our results we can confirm the distinct ecotype *CBF*-regulation in Arabidopsis root, although *CBF3* appears to act in similar way between ecotypes as well as across tissues.

The (almost) conserved *CBF3 expression* and strong development and growth responses in roots during cold stress across species may be linked to unique root specific mechanism. Cold induces DNA damage in root stem cells (Koukalová *et al.* 1997; Ning *et al.* 2002). Hong *et al.* (2017) have proposed a unique "sacrifice-for-survival" mechanism where cold causes newly formed stem cells to die to maintain functional stem cell niche activity via auxin regulation and slowing down the rate of further stem cell division. More recently, Perez-Garcia *et al.* (2023) has proposed a role of *CBF3* as a mediator between cold and root development by regulating stem cell activity. The clear induction of *CBF3* across species as well as indications of root apical meristem activity in SC2 could supports their link to these newer theories on complex cold and developmental interconnection in roots.

The low overlap of supercluster TF orthogroups (Figure 3d) and the highly conserved co-expressed orthogroups across species (**Paper II**, S12) suggests that both the genes underlying cold responses and the TFs orchestrating them are often species-specific. This highlights the need of detailed transcriptional and molecular studies to improve applied transgenic and gene editing technologies in coniferous species, which are already widely used with several commercial species (Lu & Hu 2011; Ricroch *et al.* 2022). However, despite recent progress in sequencing tree genomes and ongoing molecular research, reliable and reproducible gene-editing methods for coniferous species remain scarce. Current somatic embryogenesis (SE) methods using *Agrobacterium* are not only slow but also inconsistent in introducing foreign genetic material (Yin *et al.* 2021). This difficulty of generating transgenic conifers (Zhao *et al.* 2024), may, at least partly, be reflected in the high divergence of regulators behind stress responses observed across species in **paper II.** Although **paper II** focused specifically on root responses to cold, when these findings are considered alongside the absence of *CBFs* in Norway spruce and Scots pine needles under cold and freezing temperatures (**paper I** and **II),** it becomes evident that the core regulators od stress in conifers differ substantially from those in angiosperm species. Together, these results highlight the need of lineage-specific model plants with advanced molecular toolkits and further suggest that Arabidopsis may not be an appropriate model for studying stress responses in species separated from it by large evolutionary distances, at least until molecular methods in conifers have advanced sufficiently.

4.3 Utilization of basal spring frost tolerance in Norway spruce breeding

Papers I and **II** examined the transcriptional responses of Norway spruce to low temperatures during its non-acclimated stages, highlighting the extensive diversity of transcriptional changes following exposure to cold and freezing conditions. These non-acclimated stages represent the greatest vulnerability to sudden low-temperature events, underscoring the importance to understand the tolerance and avoidance mechanisms that operate at these vulnerable developmental phases. Overexpressing of core cold-responsive genes such as *CBFs* has been shown to increase cold tolerance in non-acclimated plants across several plant species (Jaglo-Ottosen *et al.* 1998; Walworth *et al.* 2012). However, no equivalent core cold regulators have been identified in Norway spruce or Scots pine, and the technical challenges associated with transgenic methods in conifers further limit the application of such strategies. Moreover, low temperature tolerance, as most abiotic and biotic stress responses, is a polygenic, quantitative trait regulated by hundreds of genes (Zobel & Talbert 1984). This complexity complicates transgenic approaches, as targeted modifications may inadvertently alter other growth and developmental traits. While molecular tools for genetic improvement of trees continue to advance, practical applications in forestry remain limited.

Consequently, we change our focus to a long-established approach that utilizes natural genetic variation to improve growth, wood quality and stress resilience, and which is well-suited for polygenic traits.

In **paper III**, we extended our insight into the genetic variation, focusing on spring frost tolerance in Norway spruce during its most vulnerable developmental stage and assessed whether genomic selection could be applied to predict this trait. Using ion-leakage assay to quantify frost damage (FD) in a multi-year field trial, we combined frost damage scores with bud burst and height in multivariate genomic models to evaluate selection efficiency. Moreover, we identified a novel relationship between bud burst and frost tolerance of non-acclimation needles, an important genetic correlation to consider in the Norway spruce breeding programs in future.

II. Genomic prediction models and spring frost tolerance

Genomic selection (GS, or genomic prediction) is a breeding approach that uses genome-wide molecular marker data to predict phenotypic performance of individuals. Prediction models are trained on a reference population for which both genotypic (e.g. SNP) and phenotypic data are available, and then applied to selected candidates based solely on their genotypes (Meuwissen *et al.* 2001). This approach reduces phenotyping costs and facilitates the inclusion of novel or difficult-to-measure traits in breeding programs (Meuwissen *et al.* 2001; Wong & Bernardo 2008). In **paper III**, we estimated a narrow-sense heritability (h^2) of approximately 0.21 for frost damage (FD) (**Paper III**, Table 2) and detected a strong correlation between bud burst (BB) and FD in the northern Swedish Norway spruce population (Figure 4). For clarification: we used FD in all our analyses, which is the opposite to tolerance. Stress-related traits in forest trees frequently exhibit lower h^2 values, e.g. due to the high environmental impact on the traits, reflecting limited additive genetic variance that parents are passing through their offsprings (Skrøppa *et al.* 2015; Steffenrem *et al.* 2016), a pattern consistent with our results (**Paper III**, Table 2). Such low estimates may also be influenced by sampling error or phenotypic inconsistencies. In comparison, Chen *et al.* (2023) reported even lower h^2 values (~0.07) for FD in their prediction models, which might be reflected by their choice of assessment method for FD.

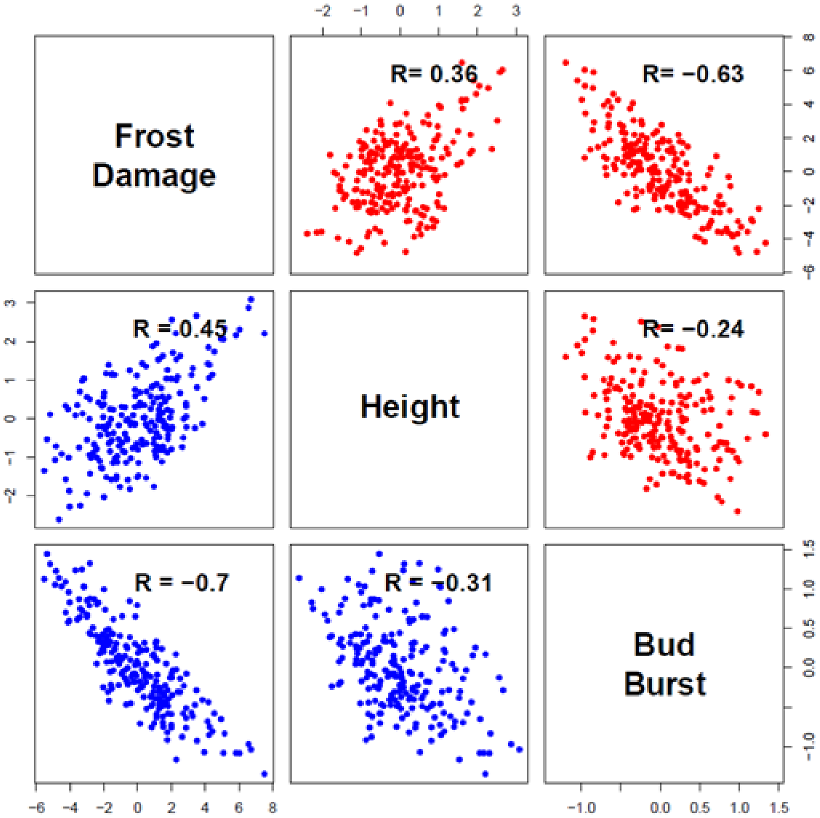

Figure 4 Genetic correlations among frost damage, height and bud burst. The lower left panel represents the breeding values estimated using the pedigree-based multivariate model (MVPBLUP) for these three traits, while the upper right panel shows the breeding values estimated from the genomic-based multivariate model (MVPBLUP) for the same traits. The x- and y-axes in each panel represent breeding values of the respective traits (in their original measurement units: frost damage in % electrolyte leakage, bud burst in score units 0 - 8, height in dm). The R values in each figure indicate the genetic correlation between each pair of traits under these two different model estimations.

The genetic variance in FD found in **Paper III** was utilized in both univariate and multivariate genomic selection models to evaluate the genomic prediction accuracy (PACC) of frost damage. In univariate analyses, the prediction accuracy and prediction ability for FD did not improve using genomic best linear unbiased prediction (GBLUP) models compared with pedigree-based BLUP (PBLUP) (**Paper III**, Table 2). The relatively low GBLUP performance may be due to the limited number of families and genotypes in the training population (**Paper III**, Table S2), shown to affect the GBLUP in previous studies (Papin *et al.* 2024). Nevertheless, the Akaike information criteria (AIC) values for GBLUP were the lowest among all tested models (**Paper III**, Table 2), suggesting that, from a statistical standpoint, GBLUP outperformed PBLUP (Akaike 2011). Although genomic models did not surpass pedigree-based models in predictive accuracy in our study, GBLUP offers advantages by capturing Mendelian segregation and partitioning additive and non-additive genetic effects (Muñoz *et al.* 2014; Calleja-Rodriguez *et al.* 2021). Furthermore, genomic models can help mitigate errors in pedigree records or incomplete family data (Suontama *et al.* 2019).

Given the lack of substantial improvement in univariate PACC, but the availability of field measurements and high genomic correlation for bud burst and height (Figure 4), this encouraged us to shift our focus to multivariate genomic prediction (MVBLUP and MVPLUB), to explore their potential accuracy gains. In contrast to univariate models, MV prediction models incorporate phenotypic information from one or more correlated traits to improve predictions of the target trait (Henderson & Quaas 1976; Jia & Jannink 2012; Lee & van der Werf 2016). The magnitude of accuracy on MV models depends largely on the genetic correlation between the assisting and main trait (Schaeffer 1984; Thompson & Meyer 1986). In our study, the strong genetic correlation between spring frost damage and bud burst explains the observed improvement in prediction accuracy for MV-models relative to univariate models (Figure 5). Previous research in forest tree species, including *Eucalyptus dunnii, E. nitens*, radiata pine (*Pinus radiata)*, lodgepole pine (*Pinus contorta)* and white spruce (*Picea glauca)*, have reported higher predictive accuracy from MV-models, especially for growth, wood properties (Klápště *et al.* 2020; Jurcic *et al.* 2021; Cappa *et al.* 2022) and stress resistance traits (Lenz *et al.* 2020; Laverdière *et al.* 2022). Given the typically low heritability estimates of abiotic and biotic traits in forest trees, MV-models present a promising approach for improving prediction accuracy (Cappa *et al.* 2018). In addition, incorporating BB as an assisting trait enhanced prediction accuracy particularly when TS and VS were distantly related, enabling the inclusion of more genetically diverse individuals without loss of accuracy (Figure 5). This is particularly relevant for Swedish breeding programs, where distant relatedness is common due to crossing strategies in which each parent is used only one to three times (Jia & Jannink 2012). However, accuracy gains were also evident in closely related populations, indicating that multivariate approaches can be beneficial without broadening the genetic base, thereby supporting more targeted breeding strategies.

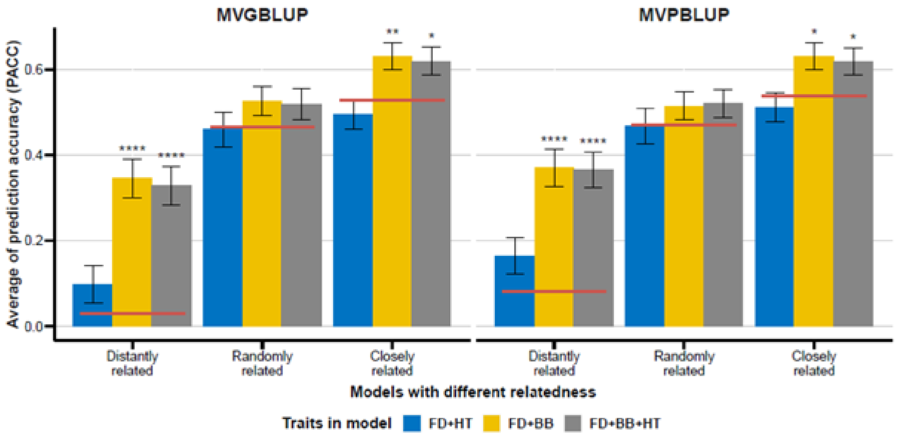

Figure 5 Average prediction accuracy (PACC) of frost damage in multivariate models with different trait combinations across levels of relatedness. Each coloured bar plot shows the average PACC for frost damage prediction in multivariate models that include combinations of traits: frost damage and height (FD+HT), frost damage and bud burst (FD+BB), and all three traits (FD+BB+HT). The x-axis represents three levels of relatedness between training set and validation set. Each scenario was conducted using two types of models: pedigree-based multivariate model (MVPBLUP) and genomic-based multivariate model (MVGBLUP). The red lines represent the average PACC values for frost damage from a univariate model across the same relatedness levels. Error bars denote the standard error calculated from 100 repetitions. Asterisks (*) indicate the level of significance compared to the univariate model based on a Wilcoxon test, *: p <= 0.05; **: p<=0.01; ****: p<=0.0001.

Another key advantage of genomic selection is its potential to shorten breeding cycles (Crossa *et al.* 2017). Accelerating genetic gain is major focus on forest tree breeding programs, that is heavily reduced by long-breeding cycles of coniferous trees (Falconer 1996; Grattapaglia *et al.* 2018). Advancing the breeding cycle can be achieved through early selection of young seedlings or embryos, thereby eliminating the need for progeny testing (Wu 1999). In Norway spruce, however, acceleration has instead been achieved by skipping conventional seedling progeny test and relying on clonally replicated field trials (Rosvall 2019). We therefore investigated whether MV-models could outperform univariate models without incorporating BB phenotyping in VS - that is, whether FD could be predicted using genotypic data alone, to avoid phenotyping completely. Unfortunately, prediction estimates did not exceed those of the univariate models (**Paper III**, Figure 5). Although the inability to predict FD solely with SNP data may seem discouraging for breeders, BB is a simple and inexpensive trait to phenotype at an early age. It also exhibits a strong genetic correlation between juvenile and mature stages, making it a great assisting trait for improving FD genomic prediction (Ekberg et al. 1994; Hannerz et al. 1999; Danusevičius et al. 2024).

III. Applied methods to utilize spring frost tolerance

Norway spruce is highly susceptible to early spring frost events, particularly in southern and central regions of Sweden, where frequent temperature fluctuations occur during the spring (Perttu 1981; Hannerz 1999). Despite the regular occurrence of frost damage, the genetic basis of basal spring frost tolerance (FT) in spruce remains insufficiently characterized. In **paper III** we investigated this trait by collecting samples during the shoot elongation phase – recognized as the most frost-sensitive developmental stage in Norway spruce, occurring shortly after bud burst initiation (Christersson 1971; Repo 1992; Langvall et al. 2001).

Given the temporal co-occurrence of the most vulnerable developmental stage and the occurrence of spring frost, we hypothesized that genetic variation in spring FT would be detectable. Traditionally, frost risk in Norway spruce has been assessed by scoring tissue damage following frost events (Chen *et al.* 2023). However, this approach measures frost tolerance only indirectly, as the extent of damage depends largely on the development stage at which each genotype experiences frost. Genotypes that have already reached the most susceptible bud burst stage are disproportionally affected. As a result, the genetic correlation between frost damage and bud burst is expected to be close to 1, as demonstrated by Chen *et al.* (2021). Therefore, both bud burst stage and the timing of frost events must be considered when drawing conclusions about the frost tolerance of different genotypes. In addition, variation in the intensity and duration of frost events can affect the observed damage under field conditions.

To acquire phenotypic and genotypic variation of basal frost tolerance we decided to collect samples in three consecutive springs when trees were at the bud developmental stage 2 according to Krutzsch-scale (Krutzsch 1973). Ion leakage method was followed as described in **paper III** to treat all the samples with -3 °C. Consistent with our hypothesis, we estimated a moderate narrow-sense heritability of ~0.21 for FD indicating frost shaping the genetic landscape of the studied northern Norway spruce population (**Paper**

III, Table 2). This *h*^{2} in our study was higher than in previous study (Chen *et al.* 2023), perhaps due to more precise sampling method and multi-year sampling. In addition to FD, height and bud burst were also phenotyped, allowing us to estimate genetic correlation among these traits. A strong negative correlation was observed between FD and BB (Figure 4), indicating that individuals with earlier growth initiation exhibited lower FD, whereas trees with later bud burst were more susceptible to FD. Similar results have been found from broadleaves, where early-leafing species are more frost resistant than later leafing (Wang *et al.* 2025). Evolutionary theories suggest that plants with early growth initiation experience stronger selective pressures from frost events compared to the ones that avoid this pressure by delaying their growth (Fineblum & Rausher 1995; Agrawal *et al.* 2004). And since temperature is the main factor restricting the distribution of plants in northern hemisphere, phenological timing of growth is a plausible target for adaptational pressure. As a results, it is possible that the narrow-sense heritability of FT and its genetic correlation to growth in southern Swedish populations may be higher, reflecting possible local adaptation to higher frost events in the southern latitude (Jönsson *et al.* 2004). In our study this correlation was moderate (Figure 4), that might reflect the lower number of frost event in northern Sweden or high growth recovery ability after frost damage.

Spring frost is also a strong evolutionary pressure for trees with broad distribution range to locally adapt in a certain climate. Since species have intraspecific variation in freezing tolerance, this allows the selection of suitable frost tolerant genotypes in frost prone location. This is essential in Nordic countries with limited number of native tree species adapted to the harsh climate, and limited amount of research and general reluctance to introduce non-native tree species in the area (Kjær *et al.* 2014).

Currently, forestry practices in Fennoscandinavia predominantly rely on a frost-avoidance strategy, which involves relocating genotypes with delayed bud burst to frost-prone areas (Rostad 1988; Hannerz 1996; Hannerz & Langvall 2000). However, this approach does not account for basal frost tolerance, potentially leading to the unintended selection of individuals with inherently low basal FT. Also, the current model for assisted migration of Norway spruce in changing climate conditions in Fennoscandinavia does not accounting for frost due to its stochastic nature (Liziniewicz et al. 2023), although models in Northern America highlights the risk of frost with climate change and assisted migration (Montwé *et al.* 2018). The observed correlation between increased height and low FD in **paper III** may encourage the exclusive selection of trees with later bud burst as a strategy to avoid frost damage (Figure 4). However, this approach can introduce several issues. First, selecting solely for late BB could diminish the genetic diversity of Norway spruce, potentially eliminating genotypes with inherent basal frost tolerance identified in this study. Second, the anticipated advancement of spring temperatures due to climate change may outpace the delayed BB phenotype, as BB timing is primarily temperature dependent (Worrall & Mergen 1967). Third, a delayed bud burst is often connected to later bud set in autumn, potentially increasing the risk of frost damage before winter acclimation (Skrøppa & Dietrichson 1986; Hannerz 1994).

Altogether, the results in **Paper III** present a novel phenotyping method for assessing spring frost tolerance in Norway spruce, as well as a noteworthy correlation between basal frost tolerance and bud burst. Together, these findings enabled the development of multivariate prediction models that estimated frost tolerance with good accuracy, supporting the potential inclusion of abiotic stress traits in genomic selection.

5. Conclusions

The climate is warming rapidly, changing growth conditions and challenging the adaptive capacity of plant species. However, anthropogenic climate change is not only a challenge for ecosystems and species – it also disrupts human lives, forcing us to adapt alongside the very changes we have set in motion. To sustain our societies, we are highly dependent on plants with high productivity under environmental conditions that are becoming more extreme. In this thesis, I have focused on one small fraction of climate change, narrowing the scope to boreal forests and a single abiotic stress: cold. Even this one factor alone can reshape tree growth and wood production in profound ways. Throughout this thesis I have assessed the potential impacts of climate warming to above- and below ground boreal tree tissues and the low temperature effects on their transcriptional responses.

First, in section 3.1, I describe transcriptomics analysis in **(**presented in **paper I**) comparing the responses of Norway spruce needles and Arabidopsis Col-0 leaves exposed to low-temperature stress and show a strong divergence between them in the cold-response, reflecting their deep evolutionary separation. While Norway spruce needles are highly specialized for cold environments, the pronounced interspecific differences in root responses described in **paper II** suggest that evolutionary distance, rather than tissue-specific characteristics alone, may be the primary drivers of the observed variation. In addition, comparing needles and roots of Norway spruce and Arabidopsis revealed distinct tissue-specific responses to low temperature. Although the gene set shared between the different tissues was small, they represent the fundamental stress responses that are regulated in a highly divergent manner across tissues. This highlights the complexity of gene regulation between tissues, and how deeper knowledge on tissue-specific regulators and their timing are required for possible transgenic approaches and advanced molecular breeding methods.

Second, with advanced transcriptional analyses in **paper II**, the divergence of cold regulation and responses in the roots of boreal trees species was demonstrated – showing that knowledge gained from model plants may not be enough and that species most likely respond to climate change in unique ways. In section 3.2 comparative transcriptional analysis of root cold-regulation and responses were examined across boreal tree species and Arabidopsis ecotypes by combining differential gene analysis and co-expression network analysis. Together, the results demonstrate a diverse growth and developmental responses during exposure to cold, indicating that instead of strong activation of stress responses, roots try to maintain growth and development at 5°C, this is in accordance with the lack of physiological cold acclimation in tree roots. *CBF3* activity in roots across species (except Norway spruce and perhaps Scots pine) support the importance of the *CBF* regulation in roots under cold stress.

Finally in section 3.3, I use multivariate genomic selection models to assess the potential of predicting spring frost tolerance in Norway spruce population. Furthermore, we discovered a higher basal spring tolerance in individuals starting the growing season early, revealing an interesting evolutionary mechanism in frost tolerance and avoidance mechanisms during bud burst. Introducing spring frost damage into multivariate GBLUB models with highly correlated bud burst trait, demonstrated an increased prediction accuracy when predicting spring frost compared to univariate models. These results complement the findings of **Papers I** and **II** by demonstrating that the large-scale transcriptional diversity observed in response to cold is reflected at the population level in significant genetic variation for basal frost tolerance. Consequently, the variation in basal frost tolerance found could be applicable for improved climate resilience in Norway spruce and worth to consider in breeding programs, specifically if the main selection decisions are focused on selecting trees with later bud burst for frost avoidance. To conclude, precise phenotyping methods for training GS models and empowering genome-wide association studies are essential for plant improvement (Cobb *et al.* 2013), while molecular stress response information is also required for implementing molecular breeding approaches.

This thesis, with the combination of **papers I – III**, expands current knowledge of transcriptional regulation and low temperature stress responses in boreal tree species, while also addressing future challenges and potential solutions related to increasing cold and freezing stress. Taken together, the transcriptomic and genomic perspectives presented here highlight both the molecular basis and the heritable variation underlying cold tolerance in non-acclimated plant tissues – insights that are particularly relevant in the context of ongoing climate change. Although this work provides new understanding on low-temperature stress in boreal trees, uncertainty regarding the future impacts of climate change complicates predictions about abiotic stress responses in boreal forests. The limited number of stress-related responses detected in fine roots may indicate a restricted adaptive capacity to changing climate conditions. Combined with the limited physiological acclimation capacity potential of roots, this suggests that fine root damage could increase as soil become colder and experience deeper freezing in the future. Furthermore, the absence of conserved gene groups associated with cold responses across species underscores the importance of species-specific research, specifically on both transcriptional and physiological adaptational capacity of roots in the main boreal trees.

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Popular science summary

Plants in northern regions are well adapted to the long, cold winters. However, climate change is disrupting the timing and conditions of the seasonal transitions, increasing low temperature risks for plants tissues. Late spring frosts and periods of cold soil due to the altered snowpack are expected to occur more frequently, threatening sensitive plant tissues while altering tree growth and development.

This thesis investigates how boreal tree species respond to cold in the molecular level. The findings reveal that plant tissues and species respond to low temperature stress in unique ways. From more practical perspective, the thesis also evaluates the potential of genetic tools to enhance frost tolerance in Norway spruce. By integrating genomic prediction models with information of bud burst, the study demonstrates that resistance to damaging spring frosts can be improved. Together, these findings offer new insights into how boreal tree species can cope with low temperature and highlight strategies that may help forests adapt to the challenges of a changing climate.

Populärvetenskaplig sammanfattning

Växter i norliga områden är väl anpassade till de långa, kalla vintrarna. Klimatförändringarna stör dock tidpunkten och förutsättningarna för årstidernas växlingar, vilket ökar risken för köldskador i växtvävnader. Sena vårfroster och kalla perioder i marken till följd av ett förändrat snötäcke förväntas bli allt vanligare. Detta hotar känsliga vävnader och påverkar samtidigt trädens tillväxt och utveckling.

I denna avhandling undersöks hur boreala trädslag reagerar på kyla på molekylär nivå. Resultaten visar att olika vävnader och arter svarar på köldstress på unika sätt. Från ett mer praktiskt perspektiv utvärderas även möjligheterna att använda genetiska verktyg för att förbättra frosttoleransen hos gran. Genom att kombinera genomiska prediktionsmodeller med information om knoppsprickning visar studien att motståndskraften mot skadliga vårfroster kan stärkas.

Tillsammans ger dessa resultat nya insikter i hur boreala trädslag kan hantera kyla och pekar på strategier som kan hjälpa skogarna att anpassa sig till utmaningarna i ett förändrat klimat.

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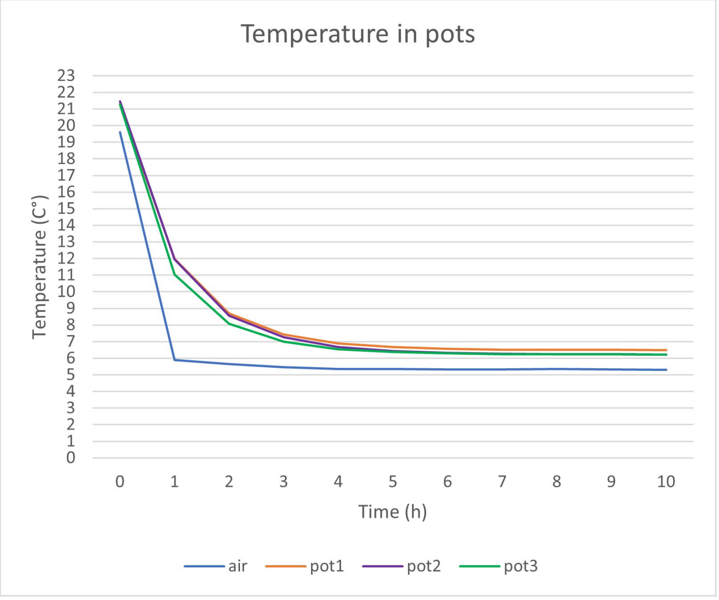
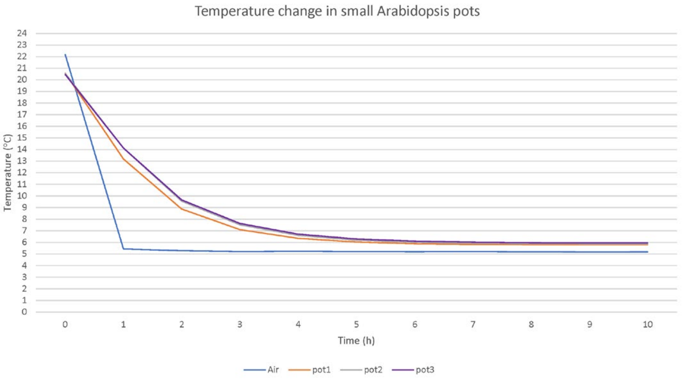
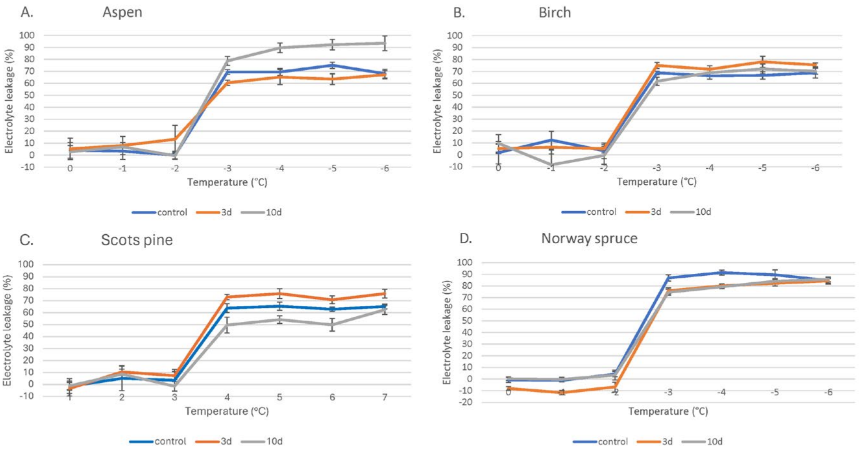
Appendix



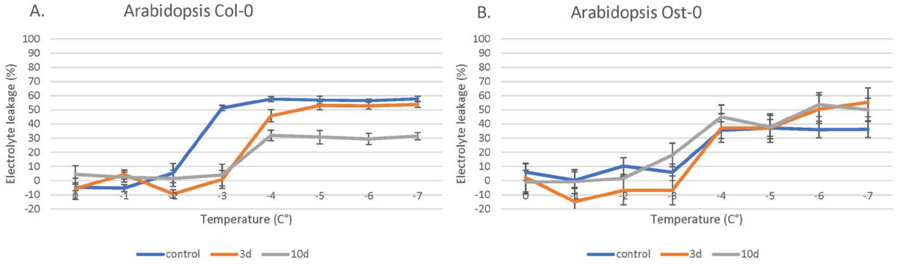
Appendix 1 Temperature change in growth pots (1l) used in **Paper II.** Air temperature and three pots were measured after the temperature was decreased from 22°C to 5°C.



Appendix 2 Temperature change in growth pots (small used with Arabidopsis) used in **Paper II**. Air temperature and three pots were measured after the temperature was decreased from 22°C to 5°C.



Appendix 3 Electrolyte leakage in roots of A. aspen, B. birch, C. Scots pine and D. Norway spruce after subjected to 5°C for 0h (control), 3 days (3d) and 10 days (10d). Methodology of the electrolyte leakage assay is described with detail in "Methods" section.



Appendix 4 Electrolyte leakage in roots of A. Arabidopsis Col-0 and B. Arabidopsis Ost-0 after subjected to 5°C for 0h (control), 3 days (3d) and 10 days (10d). Methodology of the electrolyte leakage assay is described with detail in “Methods” section.

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ORIGINAL ARTICLE

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Norway spruce deploys tissue-specific responses during acclimation to cold

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Abstract

Climate change in the conifer-dominated boreal forest is expected to lead to warmer but more dynamic winter air temperatures, reducing the depth and duration of snow cover and lowering winter soil temperatures. To gain insight into the mechanisms that have enabled conifers to dominate extreme cold environments, we performed genome-wide RNA-Seq analysis from needles and roots of non-dormant two-year Norway spruce (*Picea abies* (L.) H. Karst), and contrasted these response to herbaceous model Arabidopsis We show that the main transcriptional response of Norway spruce needles exposed to cold was delayed relative to Arabidopsis, and this delay was associated with slower development of freezing tolerance. Despite this difference in timing, Norway spruce principally utilizes early response transcription factors (TFs) belonging to the same gene families as Arabidopsis, indicating broad evolutionary conservation of cold response networks. In keeping with their different metabolic and developmental states, needles and root of Norway spruce showed contrasting results. Regulatory network analysis identified both conserved TFs with known roles in cold acclimation (e.g. homologs of *ICE1*, *AKS3*, and of the NAC and AP2/ERF superfamilies), but also a root-specific *bHLH101* homolog, providing functional insights into cold stress response strategies in Norway spruce.

KEYWORDS
cold, Norway spruce, transcriptome

1 | INTRODUCTION

Plants vary in their capacity to tolerate cold stress and, globally, low temperatures strongly limit species distribution and plant productivity (Levitt, 1980). Temperate herbaceous crop and model plant species, such as *Arabidopsis thaliana* L. (Arabidopsis), acclimate to cold temperatures after exposure to chilling, nonfreezing temperatures (0–15°C) and increase the freezing tolerance after exposure to temperatures below 0°C (Miura & Furumoto, 2013). Studies on these species have provided insight into the complex molecular mechanisms involved and changes in global gene expression have been shown to support a multitude of metabolic and physiological modifications such as the accumulation of cryoprotective molecules (amino acids, amines, proteins and carbohydrates) and antioxidants,

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Alexander Vergara and Julia C. Haas contributed equally to this study.

as well as adaptations in membrane fluidity in response to cold (Cook et al., 2004; Hoermiller et al., 2016; Hurry et al., 1995; Janska et al., 2010; Murata & Los, 1997; Renaut et al., 2006; Strand et al., 1999, 2003). The best-studied of the cold response pathways is that regulated by the CBF/DREB1 (CRT-binding factor/DRE-binding protein) family of transcription factors (Cook et al., 2004; Fowler & Thomashow, 2002; Shinozaki & Yamaguchi-Shinozaki, 1996), which bind to a CRT/DRE (C-repeat/dehydration responsive element) element in the promoter region of target genes (Stockinger et al., 1997). Investigations directed at improving frost tolerance in agricultural plants often target the CBF pathway because of its wide conservation in plants (Thomashow, 2010). However, in recent genome-wide transcriptomic analyses, other early cold-induced transcription factors and their role in regulating cold-responsive (COR) genes have received attention, highlighting the need for whole-genome transcriptional analyses to unravel the complexity of the low-temperature gene regulatory network (Park et al., 2015).

In woody perennials, overwintering is initiated after sensing the change of season (Welling et al., 2004), with the shortening of the photoperiod in late summer/early autumn inducing growth cessation, the development of dormancy and cold hardening (Bigras et al., 2001; Chang et al., 2015; Cooke et al., 2012; Guy, 1990; Li et al., 2004; Rossi et al., 2008; Welling et al., 2004). However, maximal frost tolerance is only acquired after exposure to temperatures below 0°C (Beck et al., 2004; Bigras et al., 2001; Greer & Warrington, 1982; Sakai, 1966; Sogaard et al., 2009; Weiser, 1970). The establishment of frost tolerance to extremely low temperatures (<−60°C) in perennial or overwintering tissues makes it possible for above-ground parts to survive above the snow cover and may be a result of greater cellular dehydration than found in herbaceous species (Lang et al., 1994; Rinne et al., 1998; Welling et al., 1997, 2004), coupled to more intensive accumulation of cryoprotective compounds (Coleman et al., 1991; Kuroda & Sagisaka, 1993; Rinne et al., 1994; Sauter & Wellenkamp, 1998). High concentrations of solutes also increase intracellular viscosity, stabilizing cells when stressed and leading to the formation of aqueous glasses in woody plants (Wisniewski et al., 2003). Nevertheless, woody perennials and herbaceous plant species also share mechanisms of cold regulation and cold-regulated target genes. For example, CBFs have been found in deciduous (poplar, birch) (Benedict et al., 2006; Nanjo et al., 2004; Welling & Palva, 2008) and evergreen angiosperm tree species (eucalyptus) (El Kayal et al., 2006; Navarro et al., 2009) and are involved in the activation of the cold responses of both leaves and winter dormant tissue (Benedict et al., 2006; Welling & Palva, 2008), where specialization after perennial-driven evolution might explain differences in the transcriptomes.

Boreal forests cover about 11% of the earth's surface (Bonan & Shugart, 1989) and are dominated by evergreen conifers of the genera *Abies*, *Picea* and *Pinus*, which can survive extended periods of temperatures below −40°C when fully cold-acclimated (Sakai & Weiser, 1973; Sakai, 1966; Strimbeck et al., 2007, 2008). In contrast to deciduous trees of temperate and boreal regions, evergreen conifers such as Norway spruce (*Picea abies* (L.) H. Karst) maintain their photosynthetic tissues for several years. To keep the evergreen foliage alive throughout the winter, the needles have to acquire extreme low-temperature tolerance (Strimbeck et al., 2007, 2008). Similar to angiosperms, changes in carbohydrates (Strimbeck et al., 2008), accumulation of low-molecular-weight cryoprotectant metabolites (Chang et al., 2015; Crosatti et al., 2013) and cryoprotective proteins such as dehydrins (Kjellsen et al., 2013), contribute to acquired freezing tolerance in conifer needles. Frost injuries can also occur in belowground tissues of plants, with soil frost causing fine root dieback, reducing nutrient and water uptake by trees (Groffman et al., 2001). Normally, snow cover insulates the soil from the cold air temperatures and reduces freeze-thaw events in the soil (Campbell et al., 2005), protecting roots from extreme temperature changes. Although over-wintering conifer roots have been shown to cold acclimate (Burr et al., 2001; Flint et al., 1967) the mechanisms used by roots to develop cold tolerance have received little attention. Arabidopsis has been reported to show as little as 14% overlap in cold-induced leaf and root transcriptomes, based on 8 K microarray chips (Kreps et al., 2002). Furthermore, the limited data available suggests that conifer roots remain metabolically active longer than above-ground tissues and, while they do cold acclimate, they do not develop the same deep frost tolerance as the above-ground tissues (Bigras et al., 2001), suggesting that the molecular responses of roots to changing seasonal temperatures warrant closer investigation.

Climate change models predict that temperatures will increase and become more variable at higher latitudes, particularly during the winter months (Christensen et al., 2007). These changes in seasonal temperatures will not only increase the length of the growing season (Barichivich et al., 2013) but possibly also delay the onset of cold acclimation, impair the development of freezing tolerance in the autumn and lead to early deacclimation during the late winter (Chang et al., 2016; Frechette et al., 2016; Guak et al., 1998; Repo et al., 1996; Stinziano et al., 2015; Wang, 1996). Furthermore, current evidence also suggests that the risk of belowground frost injury is increasing (Campbell et al., 2005), with warmer winter air temperatures reducing the depth and duration of the snow cover, leading to colder soil temperatures (Decker et al., 2003; Groffman et al., 2001). Conifers have evolved separately from angiosperms for more than 300 million years (Bowe et al., 2000) and large-scale whole-genome transcriptional profiling experiments are needed to understand whether the same gene regulatory networks are involved in cold acclimation in conifers. The assembly of a draft genome of Norway spruce (Nystedt et al., 2013) has made this species an ideal coniferous model. To gain insight into the mechanisms that have enabled conifers to dominate the boreal forest under current climatic conditions, we performed genome-wide RNA-Seq analysis from needles and roots of nondormant 2-year-old Norway spruce seedlings exposed to cold (5°C) and freezing (−5°C) temperatures. This experimental design allowed us to focus only on the temperature responses, without influence from growth cessation and dormancy-related mechanisms. For direct comparison with the standard herbaceous model, Arabidopsis leaves exposed to cold (5°C) were sampled at equivalent time points.

2 | MATERIALS AND METHODS

2.1 | Experimental design and sampling

Two-year-old Norway spruce (*Picea abies* (L.) Karst.) seedlings of the seed provenance Lilla Istad (56°30′N) were planted in peat in 3 L pots and grown at 18°C/15°C light/dark in a 16-h light period. Plants had been potted up from 1 L pots 10 weeks before the start of the experiment so that the roots had explored the soil volume. At the beginning of the experiment, 2 h into the light period, 40 seedlings were shifted to controlled environment rooms with constant 5°C and the 16-h light period was maintained to ensure that only temperature was changed. Needles and roots were collected immediately before transfer and then 6 h, 24 h, 3 days and 10 days after the start of the cold treatment. After this 10-day cold acclimation period at 5°C, the seedlings were transferred to subzero freezing temperature at −5°C (16-h light period) and samples were again collected after 6 h, 24 h, 3 days and 10 days. Samples were always taken from previously unsampled plants. Needles were sampled from the most recently expanded mature shoots. Actively growing fine roots (>1 mm in diameter) were sampled, rinsed with distilled water, and blotted dry. Every sampling time point was replicated by needles from five seedlings, three of which were also sampled for roots. Samples were collected onto dry ice and then stored at −80°C until further processing.

Seeds of *Arabidopsis thaliana* (Col-0) were sown into soil and kept in an 8 h photoperiod (150 μmol photon $m^{-2} s^{-1}$) at 23°C. After 14 days seedlings were transplanted into individual pots. After a further 30 days plants were shifted to 5°C, 2 h into the photoperiod and the most recently expanded mature leaves of these plants were sampled after 0 h, 6 h, 24 h, 3 days and 10 days at 5°C. Single leaves were harvested from 5 to 10 randomly chosen plants and were pooled. Three pools were collected per time point.

2.2 | RNA preparation and sequencing

Norway spruce samples were prepared by the CTAB method (Chang et al., 1993) with the following modifications: Addition of warm extraction buffer, including polyvinyl pyrrolidinone (PVP) 40, and vortex mixing of the ground sample material was followed by an incubation step for 5 min at 65°C. Precipitation with ¼ volume 10 M LiCl took place at −20°C for 2 h and RNA was then harvested by centrifugation at 14 000 rpm for 20 min and 4°C. The RNA was further purified using the RNeasy mini kit (Qiagen). A DNase Digestion with the RNase-free DNase set (Qiagen) was included in the procedure. High-quality total RNA with an RIN ≥ 7.5, OD 260/280 ratio of ≥2.0 and concentrations ≥50 ng/μl were sequenced by SciLifeLab for paired-end (2 × 125 bps). The sequencing library preparation included enrichment for poly-adenylated messenger RNAs and all samples yielded >9.4 million read pairs.

Arabidopsis thaliana (Col-0) leaf samples were isolated using the Plant RNA Mini kit (E.Z.N.A.) according to the manufacturer's instructions. DNase treatment was performed after RNA extraction using DNA-free™ DNA Removal Kit (Ambion, Life Technologies). Samples were sequenced by BGI (Beijing Genomics Institute) and all yielded >23 million paired-end reads.

All RNA samples' integrity was analysed by Agilent RNA 6000 Nano kit (Agilent Technologies) on a Bioanalyzer 2100 (Agilent Technologies) and purity was measured with a NanoDrop 2000 spectrophotometer (Nanodrop Technologies). All samples that passed quality controls were sequenced by Illumina HiSeq. 2000 platform.

2.3 | Freezing tolerance, cold index injury and H_2O_2 measurements

To analyse the freezing tolerance of Arabidopsis and Norway spruce tissues we followed the protocol described by Strand et al. (2003). Briefly, for Arabidopsis, two leaf discs (1 cm^2) from the most recently fully expanded leaves of 6-week-old plants (non-acclimated as well as 3- and 10-days 5°C cold-acclimated) were put into individual glass tubes containing 200 μl high-performance liquid chromatography (HPLC)-grade double deionized water. Two-year-old Norway spruce plants growing at 18°C were cold acclimated at 5°C for 3 and 10 days and needles and roots samples were collected. Additionally, seedlings that had been cold acclimated for 10 days at 5°C were subsequently exposed to freezing at −5°C for 3 days after which needles and roots were sampled. For the freezing tolerance assay, five needles from twigs of four seedlings were collected and cut into 10 5-mm pieces and placed in glass vials with 1 ml of HPLC-grade double deionized water (4°C). Additionally, fine roots from four seedlings were collected and three sections (10 mm) were placed in glass vials with 500 μl of HPLC-grade double deionized water. All the root samples were rinsed with 4°C deionized water before being placed in the vials. All samples were kept on ice before moving to the freezing bath. For both species, the samples were incubated in a programmable bath (Julabo FP45) first at −2°C for 1 h, at which point ice formation was initiated using a metal probe cooled by liquid nitrogen. The samples were then subjected to decreasing temperatures at a cooling rate of −2°C/h. At designated intervals, the samples were removed from the freezing bath and kept on ice at 4°C overnight. On the following day, 2 and 1.3 ml of double deionized water were added to Norway spruce and Arabidopsis sample vials, respectively, and placed on a shaker at room temperature (RT) overnight. Electrolyte leakage was measured, followed by flash freezing the samples in liquid Nitrogen and shaking again overnight at RT. Final conductivity was then determined. For both species, ion leakage measurements were made using a conductivity metre (CDM210; Radiometer).

For the comparison of different Norway spruce tissues, freezing injury was expressed as an index of injury. Index of injury accounts for the ion leakage present in the tissues aside from that caused by freezing treatment by converting the percentage release of electrolytes to a scale from 0 (unfrozen samples) to 100 (damaged samples). Roots contain higher amounts of residual electrolytes than needles due to the soil around them and the usage of the index of injury to express the freezing damage addresses this issue (Burr et al., 2001; Flint et al., 1967).

Freezing injury of Norway spruce tissues was expressed as index of injury and calculated using the following equation (Driessche, 1976):

$$I_t = 100 \times \frac{(L_t - B_o)}{(1 - B_o)},$$

$$L_t = \frac{L_i}{L_f} \text{ and } B_o = \frac{B_i}{B_f},$$

where L_i is the initial conductivity of a sample, L_f is the final conductivity of a sample, B_i is the Initial conductivity of a blank and B_f is the final conductivity of a blank.

For H_2O_2 measurements, 2-year-old Norway spruce seedlings grown and subjected to the same treatments used for the RNA extraction were sampled and analysed using Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes). Tissues were harvested and ground by using liquid Nitrogen. For each tissue four biological replicates were analysed.

2.4 | Data processing

Sequence read preprocessing and quality assessment of the raw data was performed as in Delhomme et al. (2014) following the standard guidelines using FastQC-0.10.1 https://www.bioinformatics.babraham.ac.uk/projects/fastqc/for quality control and SortmeRNA-2.0 to filter RNA contaminants (Kopylova et al., 2012). Trimmomatic-0.022 (Bolger et al., 2014) was used for trimming and adapter removal and STAR-2.4.0f1 for read alignments (Dobin et al., 2013). To summarize read counts per transcript and obtain count data we used HTSeq-0.6.1 (Anders et al., 2015) and to identify candidate genes that were differentially expressed and to obtain variance stabilizing transformationnormalized expression values we used the DESeq. 2-1.16.1 package (Love et al., 2014). Hierarchical clustering was performed using Pvclust R package (Suzuki & Shimodaira, 2006).

2.5 | Functional analysis

The predicted TF genes and their associated families in Norway spruce were obtained from PlantTFDB 3.0 (Jin et al., 2014). Norway spruce sequences and GO annotations were obtained from Conifer Genome Integrative Explorer (ConGenIE; http://congenie.org) (Nystedt et al., 2013). Gene Ontology and GOSlim enrichment analyses were performed with a standalone version of GeneMerge (Castillo-Davis & Hartl, 2003) using as background population a transcriptome of 43 398 genes expressed in the current data set. To compare globally the functions against *Arabidopsis thaliana* the GOSlim tags were assigned to Norway spruce genes using the plant slims subset available from the Gene Ontology Consortium website (Ashburner et al., 2000; Beike et al., 2015) using the Map2Slim function from Owltools https://github.com/owlcollab/owltools/wiki/Map2Slim. To identify previously characterized COR genes in Norway Spruce, we performed a bidirectional BlastP (Altschul et al., 1990) using *Arabidopsis thaliana* (TAIR 10) (Garcia-Hernandez et al., 2002; Rhee et al., 2003) and *Picea abies* v1.0 references proteomes (Nystedt et al., 2013), which are available in PlantGenIE (Sundell et al., 2015). COR genes were obtained from Park et al. (2015). The best BLAST matches were used to assign gene aliases. Peptide sequences of 10 or fewer amino acids were removed. A total amount of 58 587 amino acid sequences were analysed in Norway spruce after excluding low-confidence sequences. Orthology analysis was performed using the PLAZA resource (Van Bel et al., 2018).

2.6 | Regulatory network analysis to find pivotal TF of cold stress response

The upstream regions of Norway spruce gene models containing start and stop codons were obtained from the FTP website available in ConGenIE and dropped all of them at the same lengths of 1 Kb using a Perl script. Genes with upstream regions shorter than 1 Kb were excluded from the analysis. In total 37 621 upstream regions were analysed. Counts of the different analysed motifs in the promoters regions (Table 1) were obtained with the stand-alone version of PatMatch (Yan et al., 2005). A motif was considered over-represented in a target gene promoter if was present in an upstream region more than the respective confidence interval (CI) upper bound of the abundance of the different analysed motifs (Chawade et al., 2007). The analysed motifs were consensus target sequences for ERF, NAC, MYB, bZIP, bHLH, AP2, DRE, LBD and WRKY families. The upper limit of the (CIs was below one, with an exception for bHLH where the upper limit was over four. In these cases, just a single occurrence was considered as a case of over-representation because these sequences are not sporadically distributed on the promoter regions. For bHLH five or more times was considered as over-representation of the motif in a promoter. We got the coexpression values between all the identified COR genes and identified TF from Norway spruce using Spearman correlations values, performing a significance test of the correlation coefficients and FDR-corrected p values were obtained for them. A threshold of Spearman correlation ≥0.8 and adjusted $p \leq 0.01$ was used to filter the correlations. A regulatory interaction in our network was obtained by the combination of a co-expression data and over-representation of binding motifs. So, if a pair TF–target gene overcome a correlation threshold and the target gene has an over-representation of the motif, which recognize the TF in the promoter, then these TF–target gene pair make up a regulatory interaction in our regulatory network. Pajek software was used to obtain network parameters and visualizations (de Nooy et al., 2005).

TABLE 1 Analysed transcription factors (TF) and consensus binding site motifs

TF family	Binding motif	Means	SD	Confidence interval (95%) Lower bound	Upper bound
AP2/ERF Superfamily	CCGAC	0.253	0.558	0.247	0.259
AP2/ERF Superfamily	RCCGAC	0.133	0.399	0.1291	0.137
AP2/ERF Superfamily	AGCCGCC	0.013	0.121	0.0120	0.014
AP2/ERF Superfamily	GCCGCC	0.048	0.241	0.0461	0.051
bHLH	CANNTG	4.270	2.379	4.247	4.295
bZIP	ATGACGTCAT	0.001	0.033	0.001	0.001
bZIP	TGACGTGG	0.010	0.105	0.0085	0.016
bZIP	CACGTGG	0.040	0.241	0.0374	0.042
LBD	GCGGCG	0.029	0.185	0.0275	0.031
MYB	AAMAATCT	0.119	0.366	0.1157	0.123
NAC	CATGTG	0.337	0.637	0.3301	0.344
WRKY	TTTGACY	0.215	0.500	0.2099	0.220

Note: TF selected for regulatory network analysis are presented here. Binding motif were analysed according to IUPAC nomenclature (Cornish-Bowden, 1985). 'M' = A or C, 'N' = any nucleotide A or T or C or G, Y = C or T.

3 | RESULTS

3.1 | Comparing the cold acclimation transcriptional response of Norway spruce needles and Arabidopsis leaves

RNA-Seq analysis of needles and leaves exposed to cold (5°C) over a time course of 10 days revealed that the bulk of the transcriptional response of Norway spruce needles was slower than for Arabidopsis leaves (Figure 1a,b). Overall, both species differentially expressed approximately 15% of all transcribed genes in response to cold, with approximately equal numbers up- and down-regulated (Figure 1b). Comparing the homology relationship of the differentially expressed genes (DEGs) between species using orthology information from the Gymno PLAZA resource (Van Bel et al., 2018) (Figure 1c) it was found that of the 1949 Arabidopsis cold-induced DEGs, 340 had an identified ortholog that also responded to cold in Norway spruce (validated orthologs). A further 47 had a potential ortholog, representing homologous genes identified by best match BLAST (Pearson, 2013), which were also differentially regulated by cold in Norway spruce but not classified as orthologs by PLAZA (Figure 1c and Table S1). 1395 (72%) of the Arabidopsis DEGs had identified homologs in Norway spruce that were not cold-responsive in Norway spruce. An additional 167 cold-induced Arabidopsis DEGs had no identifiable homolog in Norway spruce (Arabidopsis singletons). Similarly, of the 3251 cold-induced DEGs in Norway spruce, 401 had orthologs in Arabidopsis and an additional 74 had potential Arabidopsis orthologs. There were 2068 (64%) Norway spruce cold-induced DEGs with homologs that were not cold-responsive in Arabidopsis and another 708 that had no identifiable homolog in Arabidopsis (Norway spruce singletons). These data revealed a core cold-induced regulon of 387 orthologous genes in Arabidopsis and 475 orthologous COR genes in Norway spruce, which represented 20% and 15% of the cold-induced DEGs, respectively. The orthologous genes were associated with the Gene Ontology (GO) categories 'stress response', 'ion binding' and 'nucleic acid binding transcription factor activity', consistent with the known importance of these processes in cold acclimation and demonstrating conservation of the transcriptional response between these two divergent species (Tables S2 and S3).

To compare the functional response of the two species in more detail, we performed DEG-enrichment analysis using GOSlim categories. We found that the number of enriched categories increased over time in both species, indicating an extensive and progressive remodulation of the transcriptome with the progression of cold exposure (Figure 2 and Tables S4 and S5). Both species showed significant enrichment of genes involved in 'response to stress', 'nucleic acid binding transcription factor activity', 'plasma membrane' and 'carbohydrate metabolic processes'. A distinct response in Arabidopsis resulted in significant enrichment of up-regulated genes in the 'lipid metabolic process', 'secondary metabolic process', 'cell wall organization or biogenesis' and 'catabolic process' that were not significantly enriched in Norway spruce. On the contrary, only Norway spruce showed significant enrichment for 'vacuole', 'transport', 'transmembrane transporter activity', 'ion binding', 'cellular protein modification process', 'signal transduction' and 'Golgi apparatus' and these processes became significant only after 10 days. DEGs within these categories were also induced in Arabidopsis but did not become significantly enriched at any time (Figure 2 and Tables S4 and S5). Among down-regulated genes, Arabidopsis showed strong

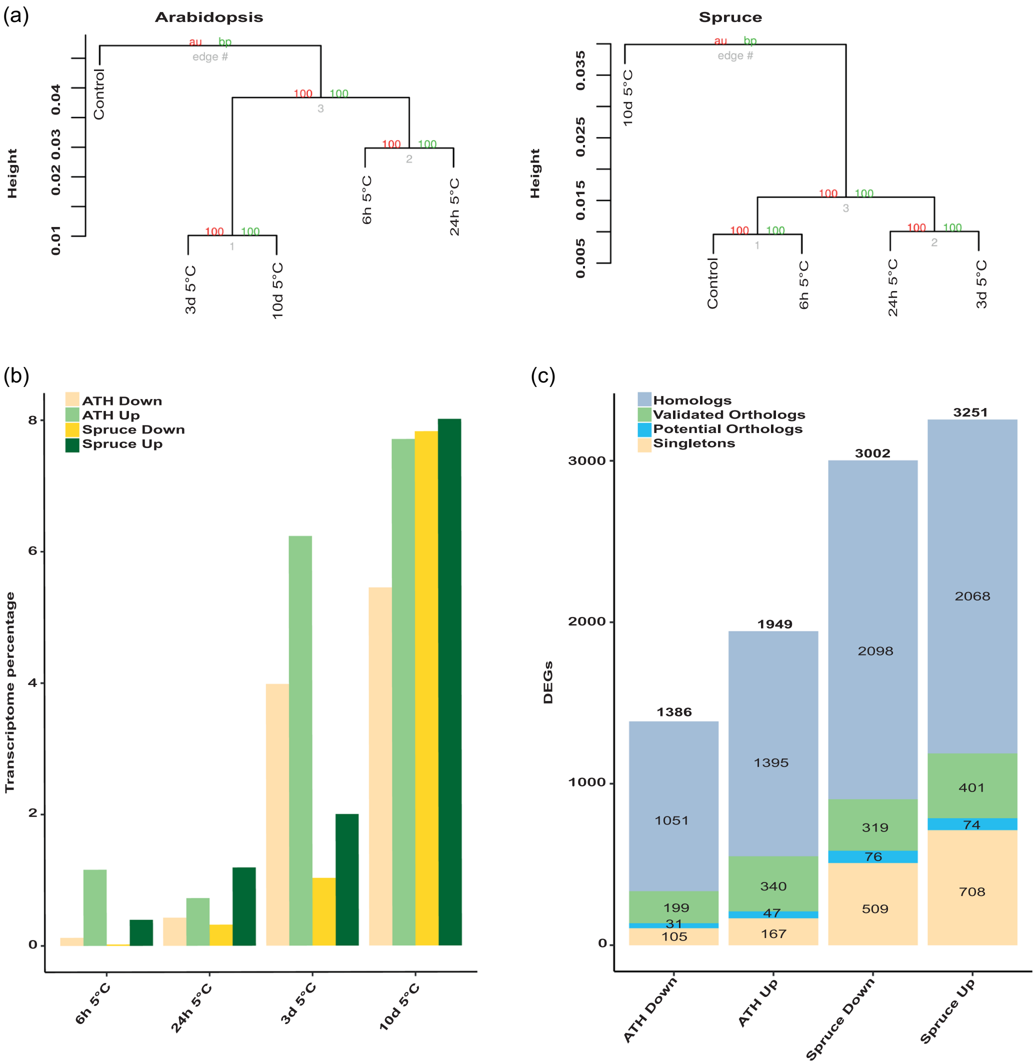

FIGURE 1 Comparing the response of *Arabidopsis thaliana* leaf and *Picea abies* (Norway spruce) needle transcriptomes exposed to 5°C. (a) Hierarchical clustering using normalized data (see methods). The red numbers correspond to approximately unbiased (AU) values and the green ones to bootstrap probability (BP) values. (b) Analysis of transcriptome progression in response to cold. Differentially expressed gene lists (DEGs) were obtained at each point in the time series, compared against the control, and then represented as a percentage of the transcriptome. DEGs significantly induced in Arabidopsis (green) and Spruce (dark green) and significantly repressed DEGs in Arabidopsis (yellow) and Spruce (dark yellow) were obtained by filtering the data by corrected $p \leq 0.01$ and fold change ≥2. (c) Orthologs, homologs and species-specific DEGs for both species (down and up-regulated). Validated orthologs correspond to orthologous genes that are differentially regulated by cold in both species. Gene lists for each group and functional information are available in Table S1

enrichment for genes related to 'structural constituent of ribosome', 'translation' and 'RNA binding' that were all largely absent in the Norway spruce response, suggesting a strong reorganization of the translational machinery in the herbaceous leaf that was not present in the Norway spruce needle. Similarly, Arabidopsis strongly and rapidly down-regulated 'plastid' and 'photosynthesis' genes but Norway spruce did so much less and much later (Figure 2).

To identify the transcription factors (TFs) that drove the transcriptional responses in both species, the composition and expression profiles of TFs Differentially Expressed in response to Cold (TF-DEC)

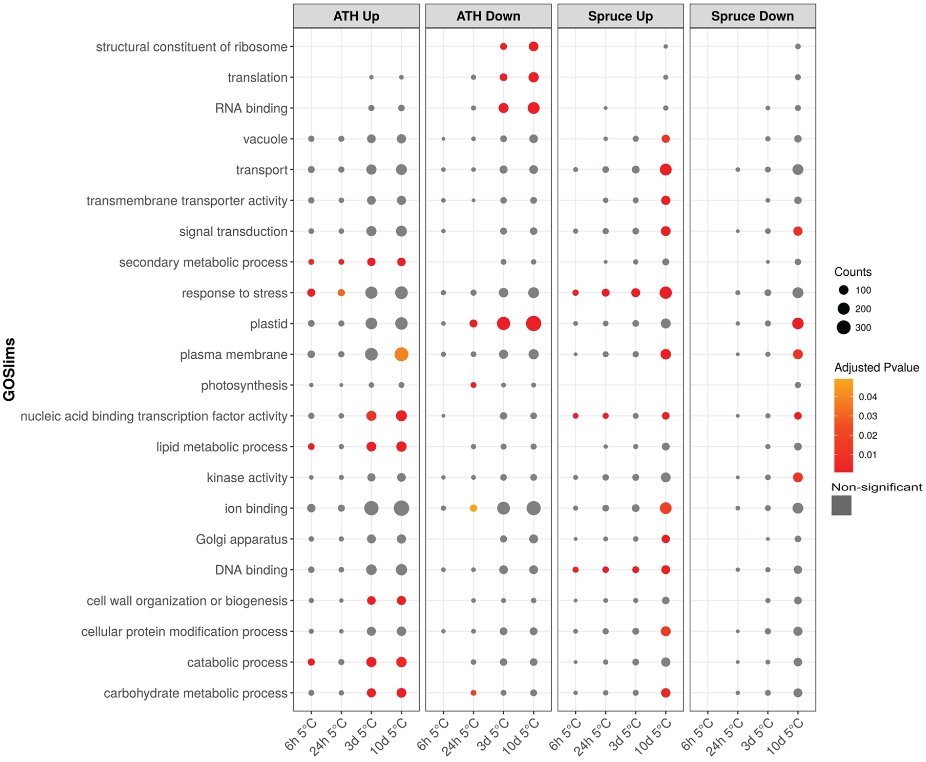

FIGURE 2 GOSlim functional analysis of species comparison. Aerial tissue transcriptomic responses of *Arabidopsis thaliana* (ATH) and *Picea abies* (Spruce) to chilling (5°C) were compared functionally by GOSlim analysis. Differentially expressed gene (DEGs) lists were analysed along the time series treatments with different GOSlim tags assigned in the up and down-regulated DEGs. The number of genes in each category is represented by bubble sizes (counts). GOSlim enrichments with Bonferroni corrected $p \leq 0.05$ are represented with a red colour scale. Nonsignificant p values (corrected $p > 0.05$) are in grey. Here only a selected group of GOSlim categories are represented. A full list including all the categories and p values is included in Table S4 and a description of the genes and their homologs in Table S5 [Color figure can be viewed at wileyonlinelibrary.com]

were analysed (Figure 3). First, transcriptional regulators known to respond to cold in Arabidopsis were analysed and showed an induction at 6 h with a fast decline observed by 24 h (Figure 3a), as shown previously (Chinnusamy et al., 2003; Kim et al., 2013; Kim et al., 2017; Shen et al., 2015; Vogel et al., 2005). Overall, all of the 120 up-regulated TF-DECs in Arabidopsis responded to cold between 6 and 24 h at 5°C (Figure 3b, S1, and S2) and most belong to ERF, bHLH and MYB families, which were previously reported as families containing members involved in plant cold and dehydration responses (Chinnusamy et al., 2003; Fowler & Thomashow, 2002; Vogel et al., 2005; Yamaguchi-Shinozaki & Shinozaki, 2006). These Arabidopsis TF-DEC fell into two main clusters; one, including *ERF6* and *MYB7*, with a transient expression peak at 6 h (the top cluster in Figure 3b); a second larger cluster with a broader expression peak in which expression was induced by 6 h and remained high until 24 h after exposure to cold (bottom cluster, Figure 3b). The second cluster included several AP2/ERF superfamily members, such as *TINY2*, *DEAR2*, *DEAR4*, *ERF3* and *AP2* (Figure 3b). This contrasted sharply with what was observed for the 107 up-regulated TF-DEC from Norway spruce, where only a few TFs were induced early (at 6 or 24 h) and these were six *ORA47* homologs, one *AP2*, one *MYB33*, one *ERF53*, one *NAC025* and one *TINY2*-like homolog (Figure 3b). The main response of TFs in Norway spruce was delayed relative to Arabidopsis and formed two subclusters, one showing induction between 24 h and 3 days and a second subcluster with an even later expression maximum at 10 days that was completely absent in the Arabidopsis response (Figure 3b). This delayed response in Norway spruce was associated with TFs that belong to AP2/ERF superfamily such as *ERF1*, *ERF2*, *ERF9* and *TEM1* homologs, along with other TFs such as *ZFP4*; *MYB123* and *MYB101*; *anac028* and *anac078* and *WRKY20*-like genes whose role in the cold stress response has not been directly reported in Arabidopsis (Figure 3b). This delayed response in cold-induced gene expression was also reflected in the slower acquisition of frost tolerance by Norway spruce needles

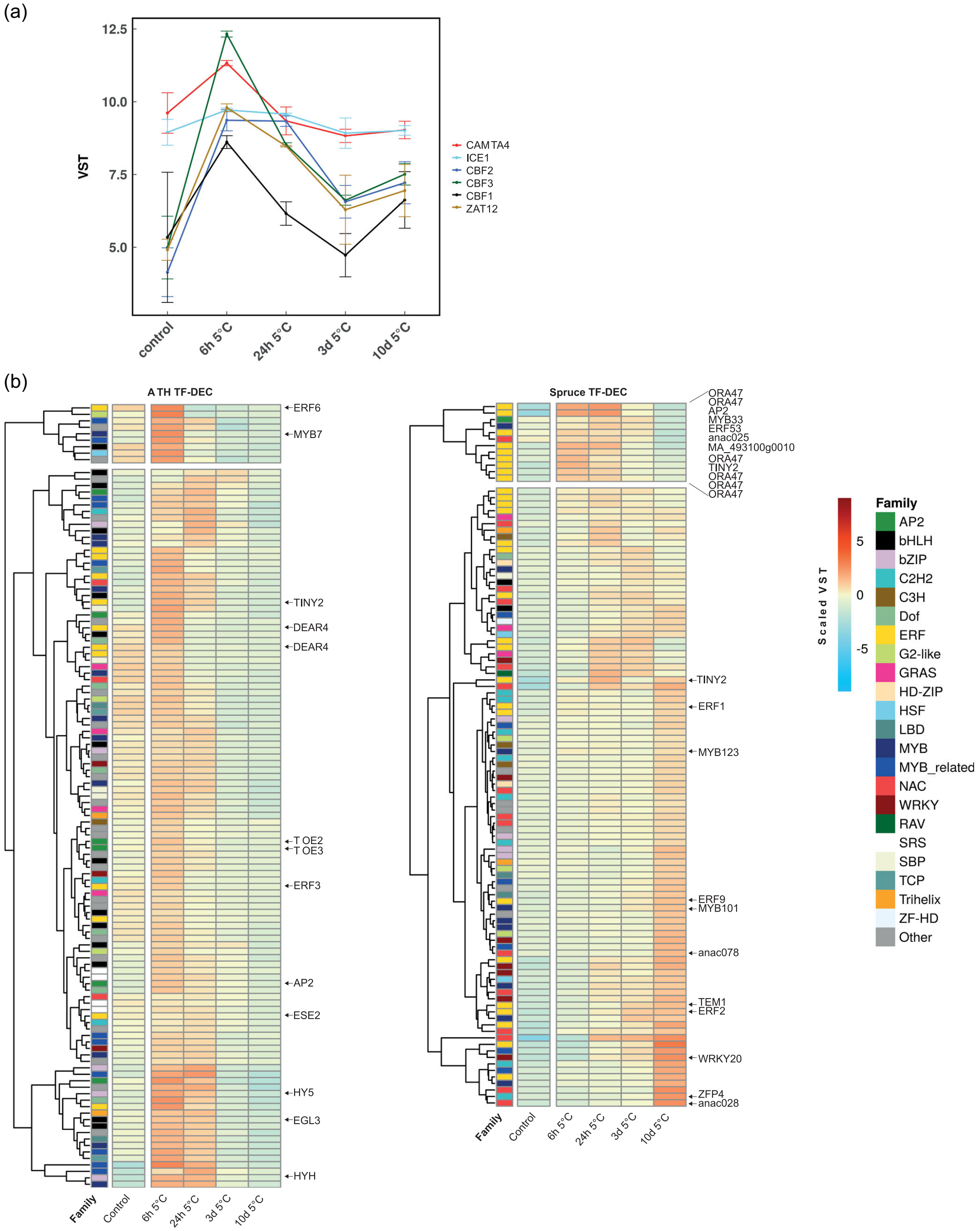

FIGURE 3 (See caption on next page)

relative to Arabidopsis leaves (Figure S12). Thus, both the freezing tolerance and related gene expression data indicated that while Norway spruce shared an early transcriptional response with Arabidopsis at 6 to 24 h, the more extremophile conifer mounted a later transcriptional response following 3 and 10 days of exposure to cold.

3.2 | Characterizing Norway spruce responses in needles and roots to cold and freezing

The effect of a progressive exposure to cold (5°C) on the transcriptomes of developmentally and metabolically distinct needles and roots of Norway spruce revealed prominent transcriptional changes in both tissues after 3–10 days at 5°C (Figure 4). Although the root response lagged behind at 6 h, presumably due to the effect of soil acting as a temperature buffer, after 24 h the number of DEGs was largely similar between the two tissues. Subsequent exposure of both tissues to freezing at −5°C had no noticeable effect on the total number of DEGs in needles but did result in a further increase in root DEGs after 3 and 10 days at −5°C, giving a total of 4324 and 4407 up-regulated DEGs in needles and roots respectively (Figure 4b). This progressive increase in DEGs was reflected in increased freezing tolerance in both tissues at 3 and 10 days at 5°C. However, following subsequent exposure to −5°C for 3 days, both tissues showed evidence of freeze injury indicative of only partial cold acclimation, where needles showed higher injury than roots (Figure S13).

GOSlim enrichment analysis showed that while both needles and roots deployed similar numbers of up-regulated DEGs and most GOSlim categories were common between the tissues, in needles, more categories were significantly enriched (Figure 4c). For example, genes in the ‘transport’ category were enriched in up-regulated DEGs common to both tissues and also in needle-specific genes, showing that modulating the transport of solutes and ions across membranes is an important response to cold in both tissues, but the response by needles is stronger and more diverse. This stronger response by needles to exposure to cold is further demonstrated by the finding that ‘plasma membrane’, ‘transmembrane transporter activity’ and ‘ion binding’ categories were only enriched in the up-regulated needle-specific DEGs.

Unsurprisingly, in the down-regulated DEGs photosynthesis and plastid categories were the main gene classes significantly enriched in the needle-specific list. In addition, down-regulation of ‘nucleic acid binding transcription factor activity’ genes at 6 and 24 h under freezing, reflected deactivation of bHLH, bZIP, MYB, NAC and AP2/ERF superfamily TFs in needles following exposure to freezing temperatures (Figure 4c and Table S6 and S7). On the contrary, root-specific and common down-regulated DEGs were enriched in ‘plasma membrane’, ‘kinase activity’ and ‘signal transduction’ categories, indicating that the silencing of some components of these categories occurred in both tissues but that this suppression response was more pronounced in roots exposed to subzero temperatures.

3.3 | Analysis of common and tissue-specific responses to cold

Despite being developmentally and metabolically distinct, needles and roots shared 2002 up-regulated DEGs, with a further 2322 being needle-specific and 2405 root-specific (Figure 5a). To identify coordinated positive responses to cold and contrast the tissue-specific responses, we performed hierarchical cluster analysis on the up-regulated DEGs. The identified clusters were designated as N prefix clusters for Needle-specific (seven clusters), C prefix clusters for those Common to both tissues (11 clusters) and R prefix clusters indicating those that were Root-specific (eight clusters) (Figure 5b). Correlation and cluster analysis was then performed with all identified clusters and seven main responses were found, termed Super Clusters (SC) (Figure 5c and Table S9). SC-6 identified a needle-specific behaviour enriched in the GO term ‘response to chitin’, characterized by early induction that peaked at 6 h, followed by expression falling below control levels by 10 days of cold. SC-7 also showed early response behaviour but was composed of DEGs from both tissues, and reached an expression peak after 24 h of cold. This cluster contained GO enrichments in ‘response to biotic stimulus’, ‘response to wounding’, ‘protein serine/threonine phosphatase activity’ and in terms related to regulation of transcription (Table S9). On the contrary, expression of SC-1 showed a progressive increase under cold starting at 6 h, and showed a more pronounced increase in expression following exposure to freezing. Similarly, SC-2 and SC-3 showed a progressive increase in gene expression until 10 days at 5°C, but displayed a flat profile under freezing. These three SCs (SC-1, SC-2 and SC-3), represent gene expression profiles from both tissues and included GO enrichments related to oxidation–reduction process, response to biotic stress, transport and plasma membrane (Table S9). SC-4 was identified as a root-specific response that showed a progressive increase in gene expression until 10 days at 5°C, and then a continuous drop during freezing. This cluster showed GO enrichments in ‘cysteine-type peptidase activity’, ‘response to nonionic osmotic stress’, ‘response to lipid hydroperoxide’, ‘apoplast’, ‘pyridoxine biosynthetic process’ (vitamin B6) and unexpectedly in

FIGURE 3 Transcription factor analysis. (a) Expression profiles of previously characterized transcription factors (TF) involved in the cold response in *Arabidopsis thaliana: CAMTA4* (AT1G67310), *ICE1* (AT3G26744), *CBF1* (AT4G25490), *CBF2* (AT4G25470), *CBF3* (AT4G25480) and *ZAT12* (AT5G59820) are shown from our experiment ($n = 3$) using variance stabilizing transformation (VST) gene expression values. Errors bars represent SD. (b) TF differentially expressed by cold (TF-DEC) were analysed in both Arabidopsis and Norway spruce. TF with positive changes relative to control are shown (corrected $p \le 0.01$ and fold change ≥2). VST data were scaled by row means. For each heatmap, zoom versions including all the identifiers are available in Figures S1 and S2 [Color figure can be viewed at wileyonlinelibrary.com]

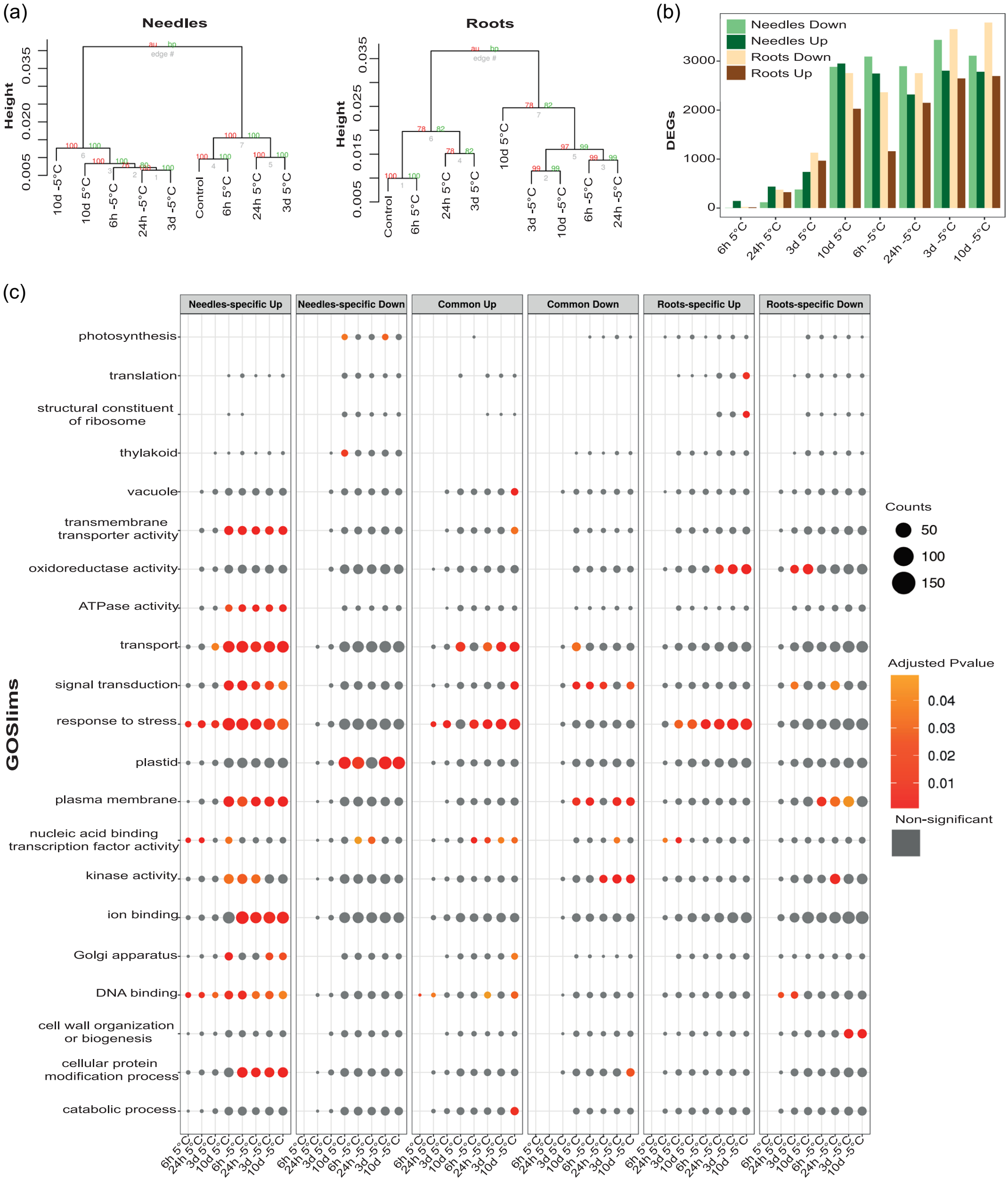

FIGURE 4 The response of needle and root transcriptomes from Norway spruce to exposure to cold (5°C) and freezing (−5°C). (a) Hierarchical clustering of gene expression data from needle and root samples (see Section 2). Red numbers correspond to approximately unbiased (AU) values and green ones to Bootstrap Probability (BP) values. (b) Analysis of transcriptome progression in response to cold (5°C) and freezing (−5°C). Differentially expressed gene lists (DEGs) were obtained at each point in the time series, compared against the control. DEGs in needles (dark green represents induced genes and light green represents repressed genes) and roots (brown represents induced genes and tan represents repressed genes) were obtained by filtering the data by corrected $p \leq 0.01$ and fold change ≥2. (c) The number of DEGs (counts) with different GOSlim tags assigned are represented by bubble size. GOSlim enrichments with Bonferroni corrected $p \leq 0.05$ are represented with a red colour scale. Nonsignificant p values (corrected $p > 0.05$) are in grey. A full list of GOSlim enrichments is included in Table S6 and a complete description of each GOSlim category and the gene functions in Table S7 [Color figure can be viewed at wileyonlinelibrary.com]

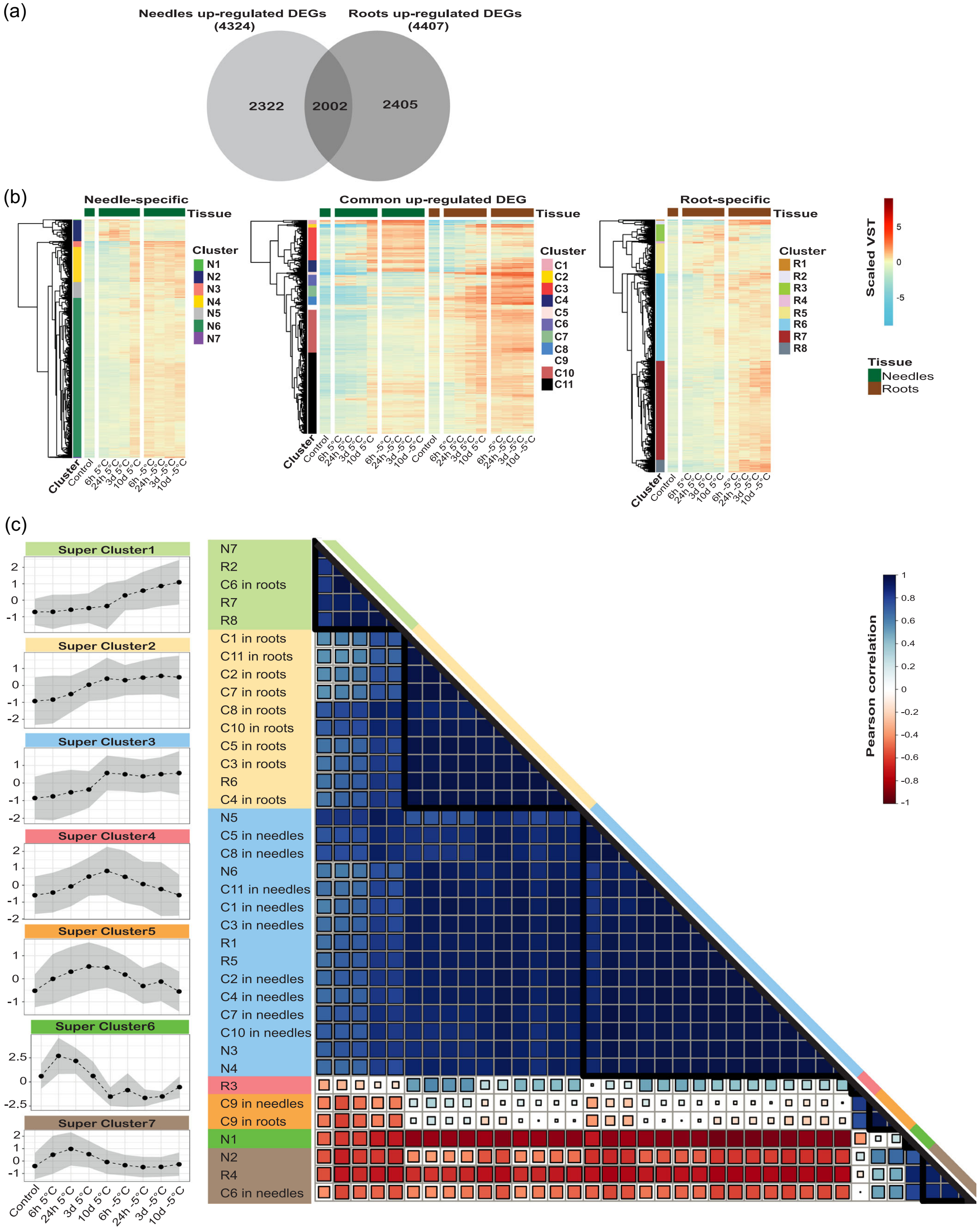

FIGURE 5 (See caption on next page)

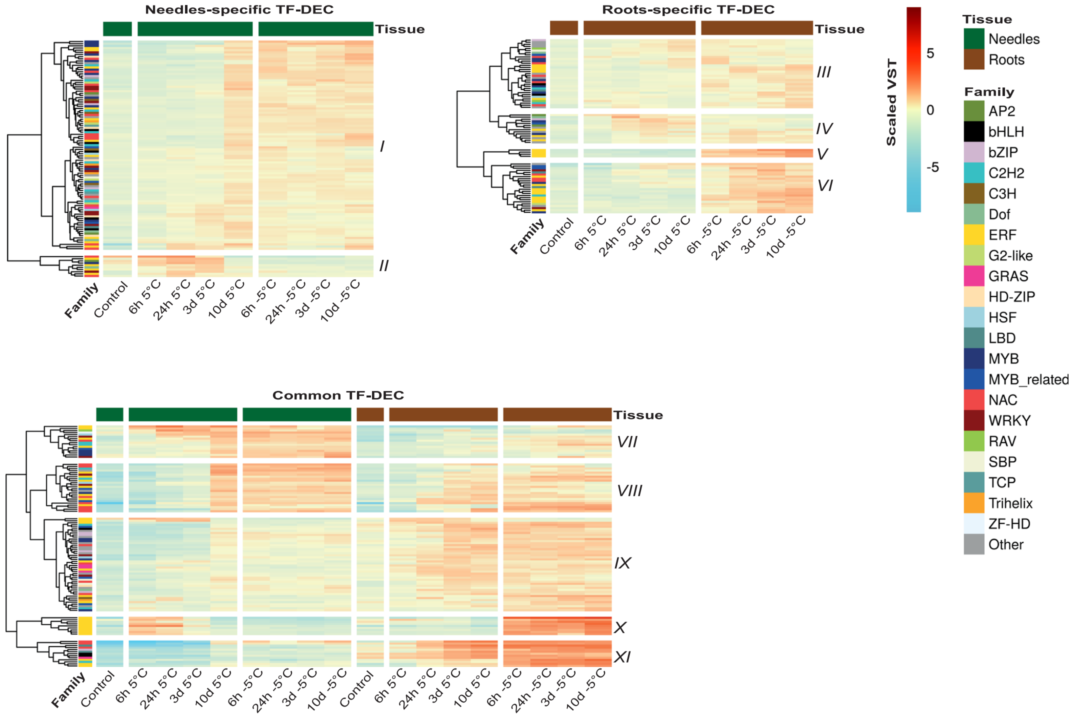

FIGURE 6 Transcription factors differentially regulated by cold (TF-DEC). TF-DEC were analysed by heat maps and hierarchical clustering (corrected $p \leq 0.01$ and fold change ≥2) crossing normalized needle and root gene expression data. Family members were obtained from the Plant Transcription Factor Database (Jin et al., 2014) and normalized VST expressions were scaled by row means. A file including gene descriptions for each cluster is available in Table S10 [Color figure can be viewed at wileyonlinelibrary.com]

'chlorophyll metabolic process' (Table S9). Thus, early and late response clusters were identified in both tissues, but with maximum induction levels at different time points. In addition to this different timing, while the needle-specific responses identified were composed of genes with biotic and abiotic stress annotations, the root-specific responses were enriched for genes related to protein degradation, vitamin B6 and redox metabolism, possibly reflecting the different metabolism of these contrasting tissues and demonstrating the need to independently investigate the stress responses of these different tissues.

3.4 | Identification and tissue specificity of transcription factors significantly induced by cold

The transcription factors associated with these different cold acclimation responses in Norway spruce needles and roots were examined and 215 TFs significantly induced in response to cold (TF-DEC) were identified from needles and 183 from roots, of which 107 (37%) were needle-specific, 75 (26%) root-specific and 108 (37%) common to both tissues (Figure 6 and Table S10). In addition, 20% (57 genes) of these Norway spruce genes lacked orthologs in Arabidopsis (Figure S4).

FIGURE 5 Identification of coordinated responses to cold. (a) Venn diagram representing needle-specific, root-specific and common up-regulated differentially expressed genes (DEGs). A Gene Ontology (GO) enrichment analysis of these gene lists is available in Table S8. (b) Heat maps and main clusters of tissue-specific and common up-regulated DEGs. Common DEGs were analysed from both tissues and each identified cluster (C) was separated according to cluster trends in needles (N) or in roots (R). (c) Super Clusters (SC) were defined by comparing clusters from (b) using Pearson correlation analysis. Common clusters (C) were separated by tissues to compare against tissue-specific clusters. Thus, a cluster trend for a common cluster number '*i*' was called 'C_i in needles' or 'C_i in roots' according to the tissue analysed. For each SC, scaled gene expression mean values are represented with dotted lines. Grey areas represent variability by two standard deviations. An analysis of SC distribution in the network is available in Figure S3. A GO enrichment analysis for these SC is available in Table S9 [Color figure can be viewed at wileyonlinelibrary.com]

Needle-specific TF-DEC formed two main clusters (Figure 6); cluster *II* TFs were induced between 6 h and 3 days after exposure to cold and returned to pre-cold exposure expression levels or lower by Day 10, cluster *I* was comprised of TFs induced from 3 to 10 days after exposure to cold. No freezing-specific induced TFs were found in the needle-specific group. Of the root-specific group, cluster *IV* was comprised of TFs induced early by cold while cluster *III* was comprised of TFs induced after 10 days of cold and reaching induction peaks under freezing. Unique to roots, clusters *V* and *VI* were strongly induced only following exposure to freezing at −5°C (Figure 6 and Table S10). Within the group of significant induced TFs common to both tissues, cluster *X* was comprised of a group of ERF TFs that were induced sharply in needles at 6–24 h in the cold and then returned to control levels. However, these same genes were markedly induced in roots only after freezing. Cluster *VIII* included TFs that reached an induction peak in needles at 10 days in the cold and thereafter expression levels remained high during freezing. However, in roots members of this cluster were induced much earlier, from 24 h exposure to cold, and stayed at high levels under freezing. Cluster *IX* and *XI* showed stronger inductions in roots than in needles. Thus, even though there were a large number of common TFs significantly induced in both tissues by exposure to cold and freezing, the majority of these showed differential regulation between the two tissues, either in timing or the strength of their induction and therefore when this is added to the evidence of clusters of tissue-specific TFs, it demonstrates that these dissimilar tissues display very different transcriptional control during acclimation to cold.

3.5 | Cold response regulatory network of Norway spruce

To analyse in detail how COR genes were regulated and to identify TF 'hubs' playing a key role in COR gene regulation, we performed a regulatory network analysis of TF-DEC and COR genes combining coexpression and promoter motif analysis of all COR genes. A network model of inferred transcriptional regulations of COR genes was built using an approach previously used to identify putative plant cold acclimation pathways and key TFs in Arabidopsis and rice (Chawade et al., 2007; Lindlof et al., 2009). The analysed motifs were consensus target sequences for ERF, NAC, MYB, bZIP, bHLH, AP2, DRE, LBD and WRKY families, which have previously been associated with plant cold stress (Chawade et al., 2007; Peng et al., 2015). For each motif, (CI were obtained (Table 1) and used as a criterion of over-representation. Thus, regulatory interaction between two nodes (genes) was the result of a combination between TF-target co-expression and the over-representation of the recognized motif for the respective TF in the target COR gene promoter. Using a strict correlation threshold, the resulting regulatory network included 2135 links of TF and target genes and 910 nodes (genes). 784 were COR target genes and 126 were TFs, with mainly bHLH, MYB, NAC, and ERF family members (Figure 7a). Analysis of the connectivity of the nodes (degree) in the network showed that the network followed a scale-free behaviour (Figure 7b), with only a few genes highly connected. These node degree measurements were then used as a gene prioritization criterion (Moreau & Tranchevent, 2012) and the top 10 most connected nodes (hubs) were analysed, all of which were TFs (Figure 7c). The most connected hub corresponded to the gene MA_448849g0010; a bHLH TF (*ICE1*-like) that putatively regulated (on the basis of TF-COR co-expression and TF binding motif presence) 159 COR genes. Interestingly, the third most-connected hub (MA_68586g0010) was a *bHLH101*-like that was only differentially regulated in roots with 88% of the target genes having root-specific expression (Figure 7c and Table S13). These results demonstrated that the cold regulatory network in Norway spruce is highly interconnected, that most of the cold response circuits were common to both tissues and that these include homologs of TFs previously reported as regulators of stress response genes in other species such as Arabidopsis (Figure S5 and Table S13). A notable exception to this was the root hub *bHLH101* homolog (MA_68586g0010), which suggests that tissue-specific responses are present in this species, although the function of this hub and its downstream genes remain to be elucidated.

4 | DISCUSSION

A qualitative comparative analysis of Norway spruce needles with leaves of the temperate herbaceous model plant Arabidopsis showed that, despite their long evolutionary separation, both species share a core of orthologous genes that respond to cold (Figure 1), suggesting that this core response predated the split of the lineages. This is in common with the presence of a conserved, core drought response (Haas et al., 2021). The orthologous responses include the upregulation of several TFs of the ERF3 family, including TCP2 and HB13, that are known to play a role in plant stress responses (Cabello et al., 2012; Chawade et al., 2007; Liu et al., 1998; Nakano et al., 2006; Stockinger et al., 1997) (Table S3). In addition, construction of a gene regulatory network demonstrated that an ICE1-like TF was, like its counterpart in Arabidopsis, a central player in mediating the cold response in needles and roots of Norway spruce. However, Norway spruce demonstrated different transcriptional dynamics relative to Arabidopsis, and this contrasted delayed response was associated with the induction of a large cohort of TFs, including *ERF1*, *ERF3*, *anac028* and *anac078* homologs, that have not previously been described to have a function in cold acclimation. In addition, a *bHLH101-like* TF was shown to be co-expressed with a root-specific subset of genes in the gene-regulatory network. No central regulators have previously been identified as root-specific and this finding indicates that tissue-specific responses are important in the cold hardiness response, at least in perennial species such as conifers (Figures 4–6 and S10). This is in agreement with similar findings for drought response in Norway spruce (Haas et al., 2021).

Evergreen plants such as Norway spruce maintain their needles above the snowpack during winter and thus require mechanisms to protect the needles from extreme low temperatures and the associated desiccation. On the contrary, roots face less extreme

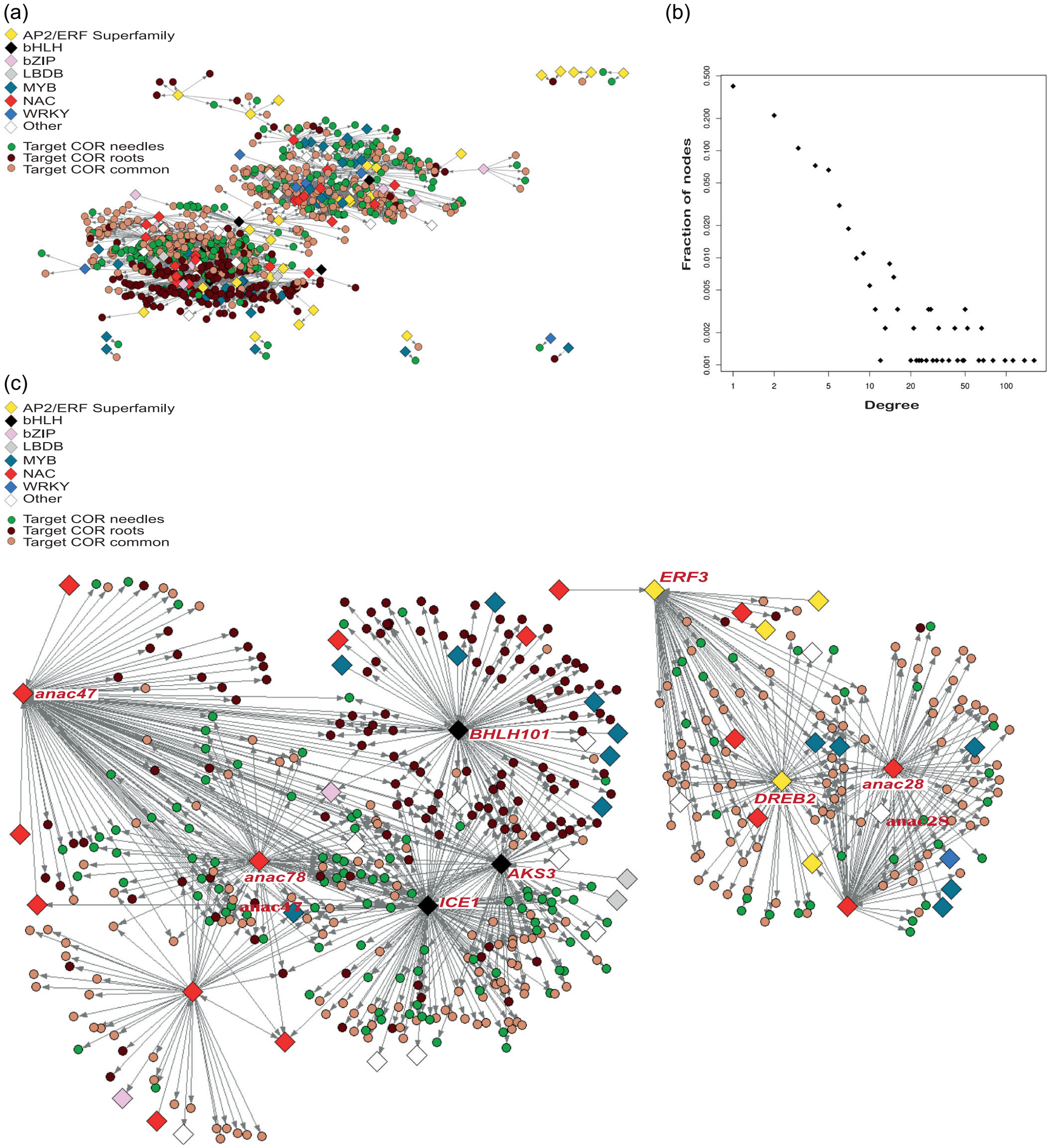

FIGURE 7 Regulatory network analysis. (a) Network representation of predicted regulatory interactions between transcription factors (TF) and cold-responsive (COR) genes. TFs are represented by diamonds and their family by colours. COR genes are represented by circles and coloured according to the tissue in which they are differentially regulated. (b) Network Degree distribution in Log10/Log10 scale. (c) Subnetwork of the 10 genes with the highest centrality. Gene Ontology (GO) enrichments in the hub neighbourhoods are available in Table S12 and topology information and gene aliases are available in Table S13 [Color figure can be viewed at wileyonlinelibrary.com]

temperatures (Figure S6) but they must also be protected from freezing damage and from anoxia stemming from ice encasement. To date, metabolomic and proteomic analyses have shown that, as for herbaceous leaves, carbohydrate and lipid metabolism and the accumulation of dehydrins play key roles in survival at extremely low temperatures in perennial evergreen needles (Angelcheva et al., 2014; Kjellsen et al., 2013; Strimbeck et al., 2015) and that both needles and roots increased cold acclimation in response to exposure to low temperatures, as measured by electrolyte leakage (Burr et al., 2001; Flint et al., 1967). In our analysis, we found that both tissues mounted a progressive transcriptional response to cold and that following subsequent freezing, most common cold acclimation responses were maintained in both tissues. GO enrichment analysis performed on all DEGs that were induced in Norway spruce revealed that regardless of the tissue, common genes induced in response to cold were over-represented for genes within the core stress categories of 'regulation of transcription', 'transport' and 'response to wounding' (Figure 5b and Table S8). However, the responses of the two tissues did vary (Figure 4c) and GO enrichment analysis showed that needle-specific induced genes were enriched for 'plasma membrane', 'ATP-binding', 'DNA binding', 'integral component of membrane' and 'Golgi apparatus'; while root-specific induced DEGs were enriched for the categories 'peroxidase activity', 'oxidation–reduction process', 'metal ion binding' and 'structural constituent of ribosome' and 'translation' (Table S8). These contrasting responses indicated that while needles are exposed to extreme cold and desiccation, demanding membrane reorganization and protection; roots, which remain more metabolically active during winter (Law & Anthoni, 1999; Martz et al., 2016), may be under higher oxidative stress. Consistent with this conclusion, the Arabidopsis ortholog of the novel *bHLH101-like* TF identified in Norway spruce roots has previously been reported to be involved in iron homeostasis (H. Y. Wang et al., 2007) and photo-oxidative stress responses (Noshi et al., 2018). While we cannot yet assign any specific function for this TF in roots, the root-specific transient accumulation of H_2O_2 (Figure S11) coupled with our evidence that the first-degree neighbourhood of this TF is enriched in oxidoreductase activity genes (Table S12), suggests it may play a central role in the oxidative stress response of roots to cold stress. However, further studies will be required to establish the role of this TF and its root-associated regulon.

The best studies of the cold response pathways in herbaceous species is that regulated by the CBF/DREB1 (CRT-binding factor/DRE-binding protein) family of TFs, which belong to the ERF family (Cook et al., 2004; Fowler & Thomashow, 2002; Jin et al., 2014; Shinozaki & Yamaguchi-Shinozaki, 1996). In Arabidopsis, the CBFs show a transient early induction by cold in roots and leaves (Hruz et al., 2008; Kilian et al., 2007). A single ortholog of CBF1 and CBF3 was found in Norway spruce (MA_20987g0010), which was induced between 6 and 24 h at 5°C in roots, although it did not pass the statistical filters to be classified as an up-regulated DEG or COR gene and it was not responsive to cold in needles (Figure S7). Cluster *X* of the common TF-DEC (Figure 6) contained nine ERF TFs showing early induction in needles, indicating that other genes of the same TF family have a role in cold stress regulation in aerial tissues of Norway spruce and that this stress response may be supported by an expansion of the ERF family of TFs in Norway spruce (Figures S8 and S9). On the basis of co-expression and *cis*-element over-representation analysis, we built a regulatory network to identify potential key conserved TFs mediating cold acclimation in Norway spruce (Figure 7). We found extensive crosstalk between COR genes and TFs and a hierarchical structure in their connections, being COR genes regulated by upstream TFs and also a module with a root-specific bias. Interestingly, the most connected TF in the network was an *ICE1*-like gene (MA_448849g0010), orthologous to the Arabidopsis bHLH transcription factor INDUCER OF CBF EXPRESSION1 (ICE1). Although recently a direct role of ICE1 in the regulation of CBFs has been questioned by data showing that an ice1-1 mutation does not impair cold induction of the CBF genes (Kidokoro et al., 2020), other studies provide evidence that ICE1 does play a role in cold response transcriptional regulation by binding to promoters of CBFs following activation by the kinase OST1 (Chinnusamy et al., 2003; Ding et al., 2015; Kim et al., 2015; Tang et al., 2020). These data demonstrate that early response TFs and COR genes can be induced by ICE1 homologs under cold in several plant species such as *Zea mays* (Lu et al., 2017), rice (Zhang et al., 2017), tomato (Feng et al., 2013), *Vitis amurensis* (Xu et al., 2014), *Pyrus ussuriensis* (Huang, Li, et al., 2015) and *Poncirus trifoliata* (Huang, Zhang, et al., 2015), in line with our finding that in Norway spruce this ICE1-like protein is an important potential regulator of many COR genes, where 150 of these genes appear as targets of ICE1 in our regulatory network (Table S13), and whose progressive late induction suggests that its role may become even stronger following freezing (Figure S10). The recent finding that an ICE2 homolog has been associated with local adaptions to shade and cold at northern latitudes in Norway spruce (Ranade & García-Gil, 2021) further supports a key role for ICE-like proteins in cold tolerance in Norway spruce.

In conclusion, our study provides a comprehensive overview of the transcriptome of Norway spruce involved in cold stress and reveals potential regulators of COR. The TF-DECs and the hubs identified from our regulatory network can be used to identify the most suitable candidate genes for targeted genetic modifications or for directed breeding to generate high-yielding cold stress-tolerant trees to aid the forest industry as it confronts new environmental challenges resulting from climate change.

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DATA AVAILABILITY STATEMENT

Sequencing data are available at the European Nucleotide Archive (ENA) as accession PRJEB26934. Code for reproducibility is available at the Github page https://github.com/avergro/Spruce-cold-stress.

The expression profiles of Norway Spruce and Arabidopsis genes can be viewed interactively through a web service at https://hurrylab.shinyapps.io/spruce-cold-stress.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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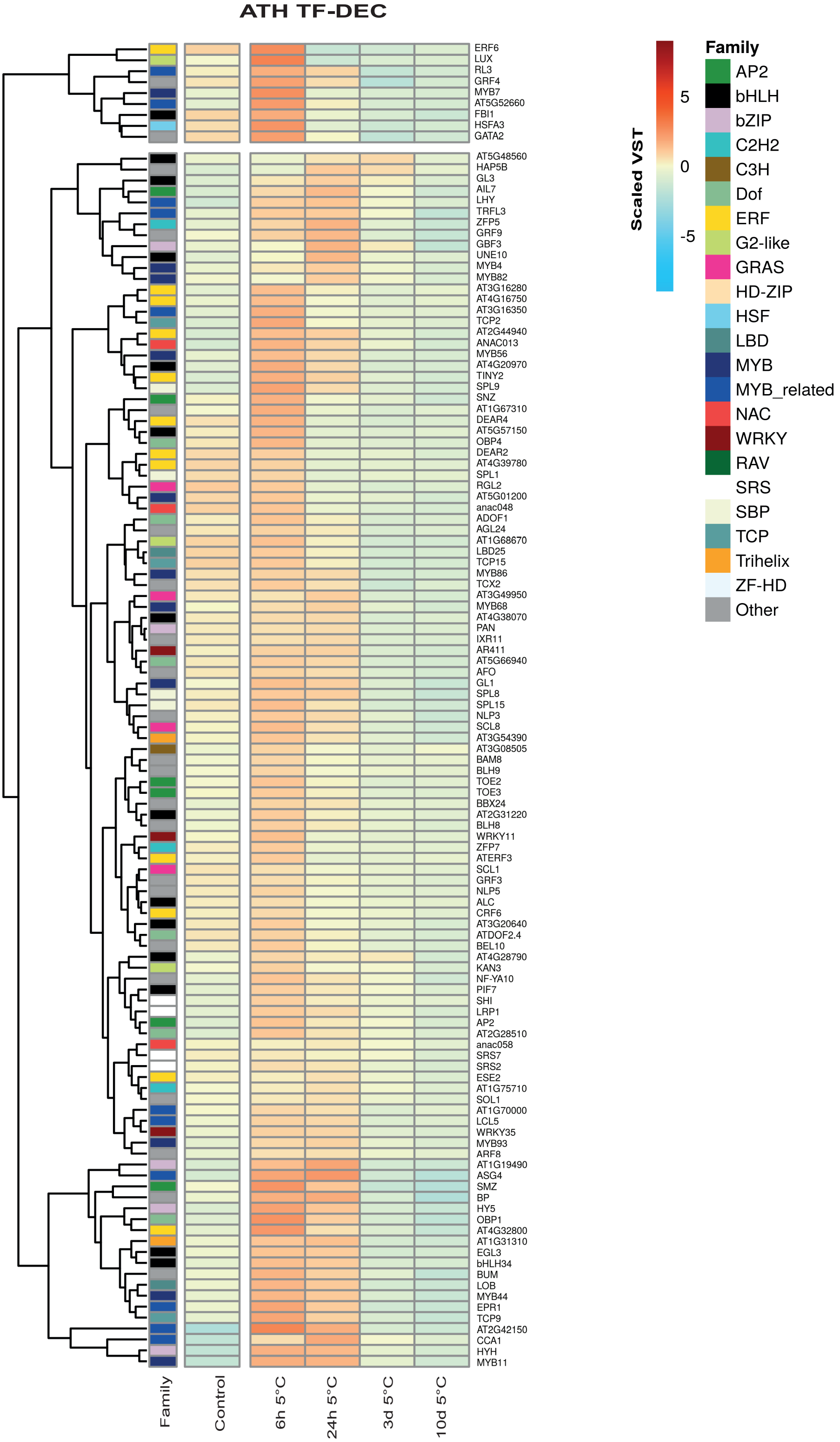

Figure S1. **Arabidopsis TF-DEC.** Heatmap of transcription factors differently regulated by cold (TF-DEC) in *Arabidopsis thaliana*. TF with positive changes relative to control are shown (corrected P-value ≤0.01 and Fold Change ≥2). VST data were scaled by row means.

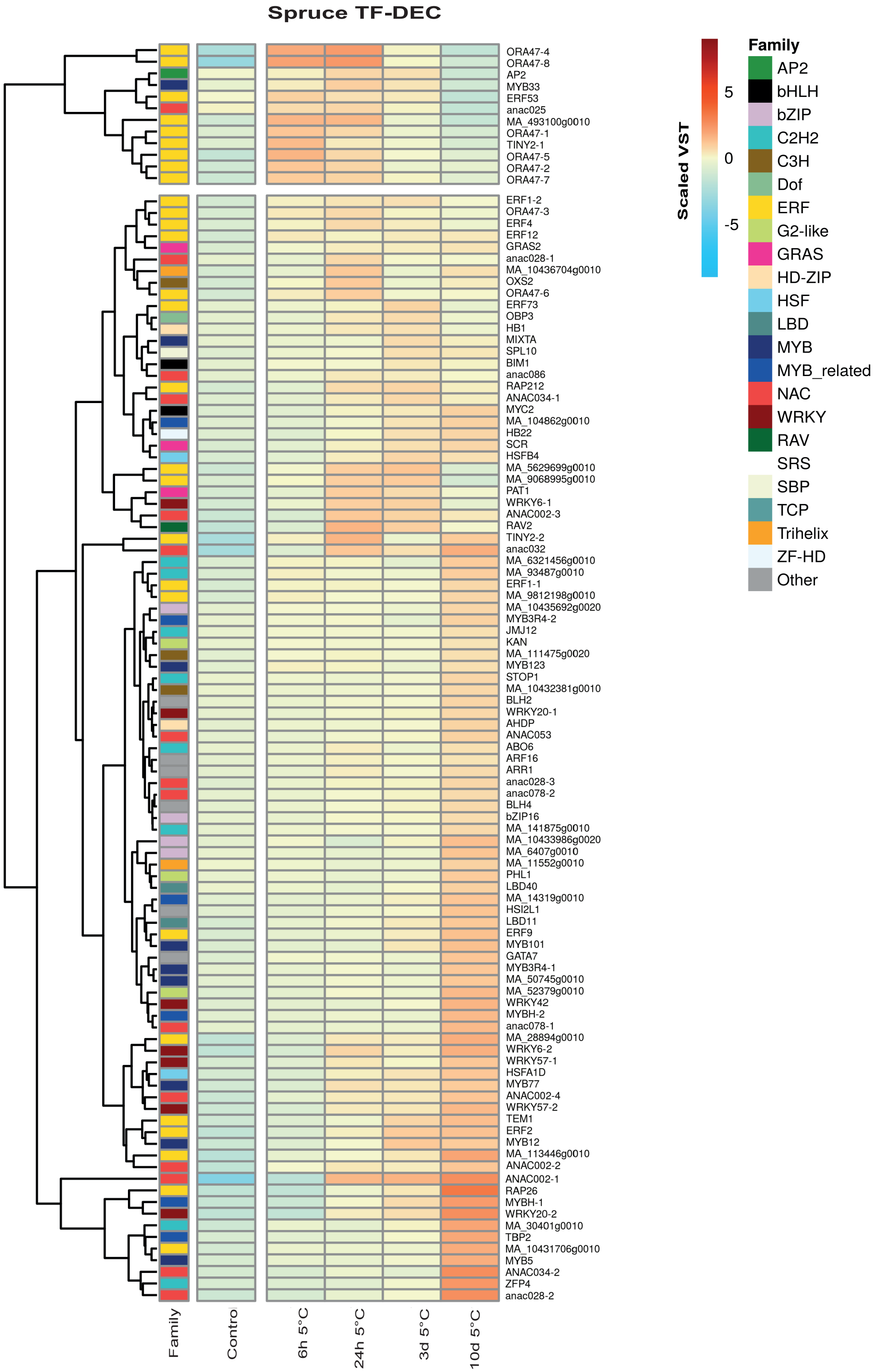

Figure S2. Spruce TF-DEC. Heatmap of transcription factors differently regulated by cold (TF-DEC) in *Picea abies* (Norway spruce). TF-DEC with positive changes regarding control were represented (corrected P-value ≤0.01 and Fold Change ≥2). VST data were scaled by row means. Families members were obtained from Plant Transcription Factor Database (Jin, Zhang et al. 2014) and represented by different colours.

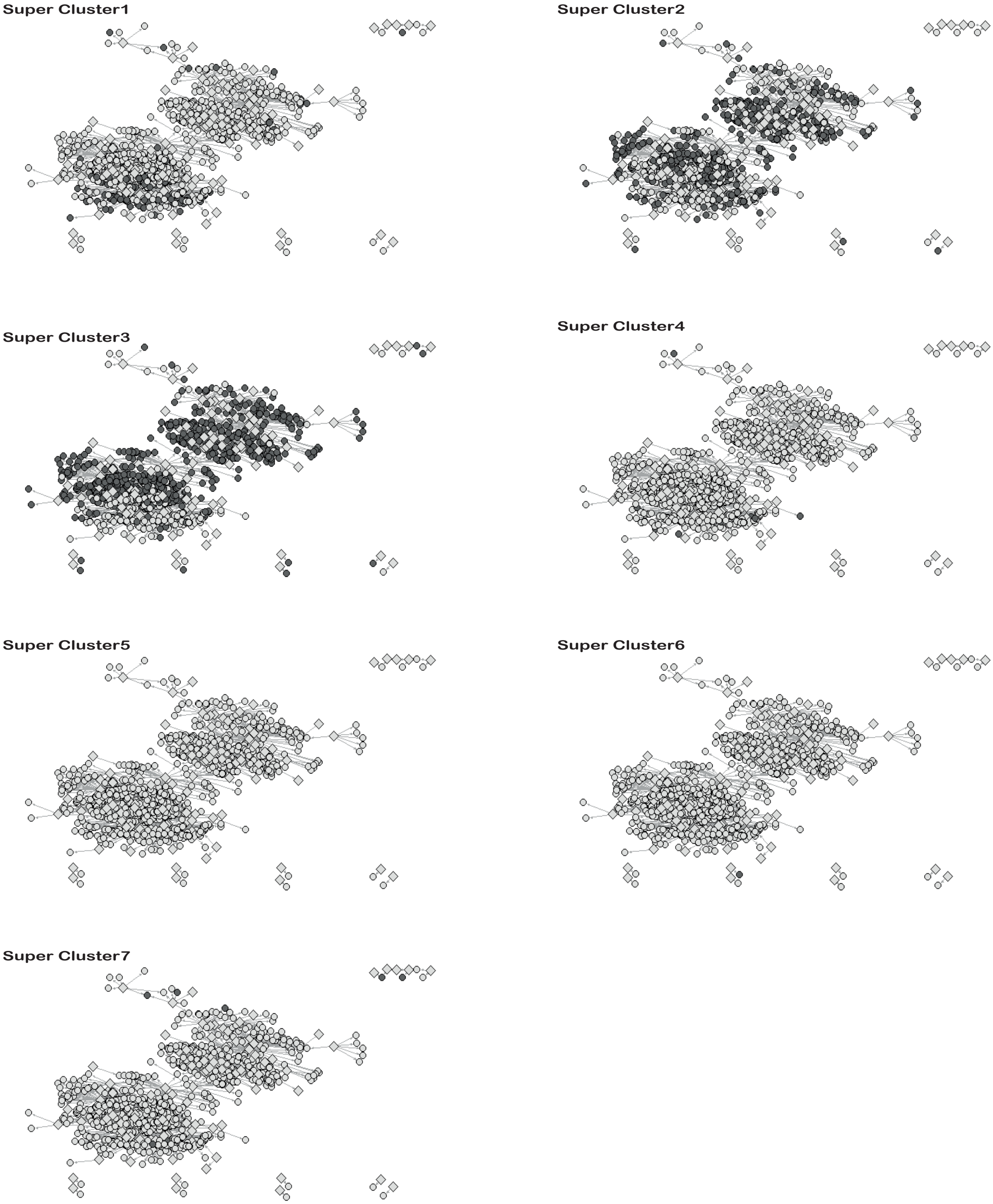

Figure S3. Super clusters (SC) member distribution in the predicted regulatory network. Regulatory networks representation highlighting SC members in the network. Each SC is highlighted coloring their members in gray.

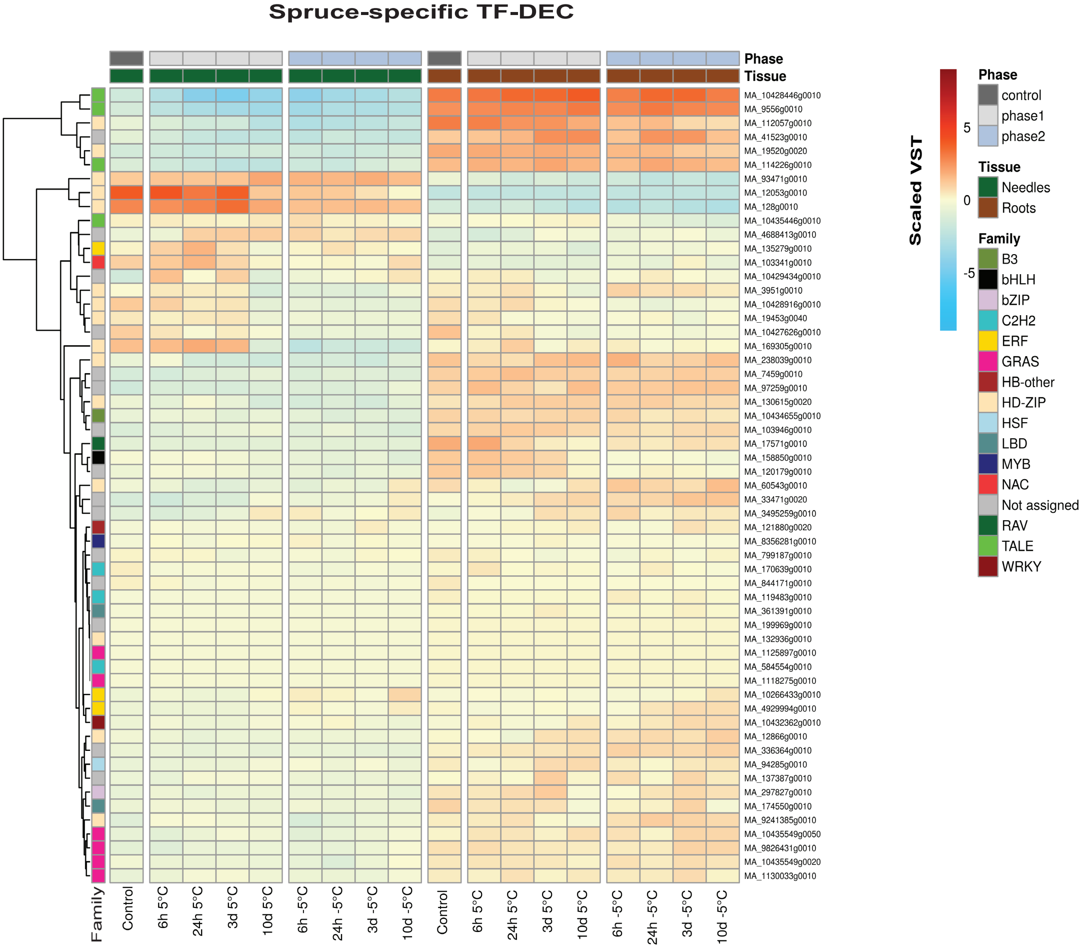

Figure S4. Heatmap of Norway spruce-specific transcription factors differently regulated by cold (TF-DEC). These are TF-DEC with positive changes relative to control (corrected P-value ≤0.01 and Fold Change ≥2) that did not have orthologs predicted in Gymno-PLAZA. VST data were scaled by row means. Families members were obtained from the Plant Transcription Factor Database (Jin, Zhang et al. 2014) and represented by different colors.

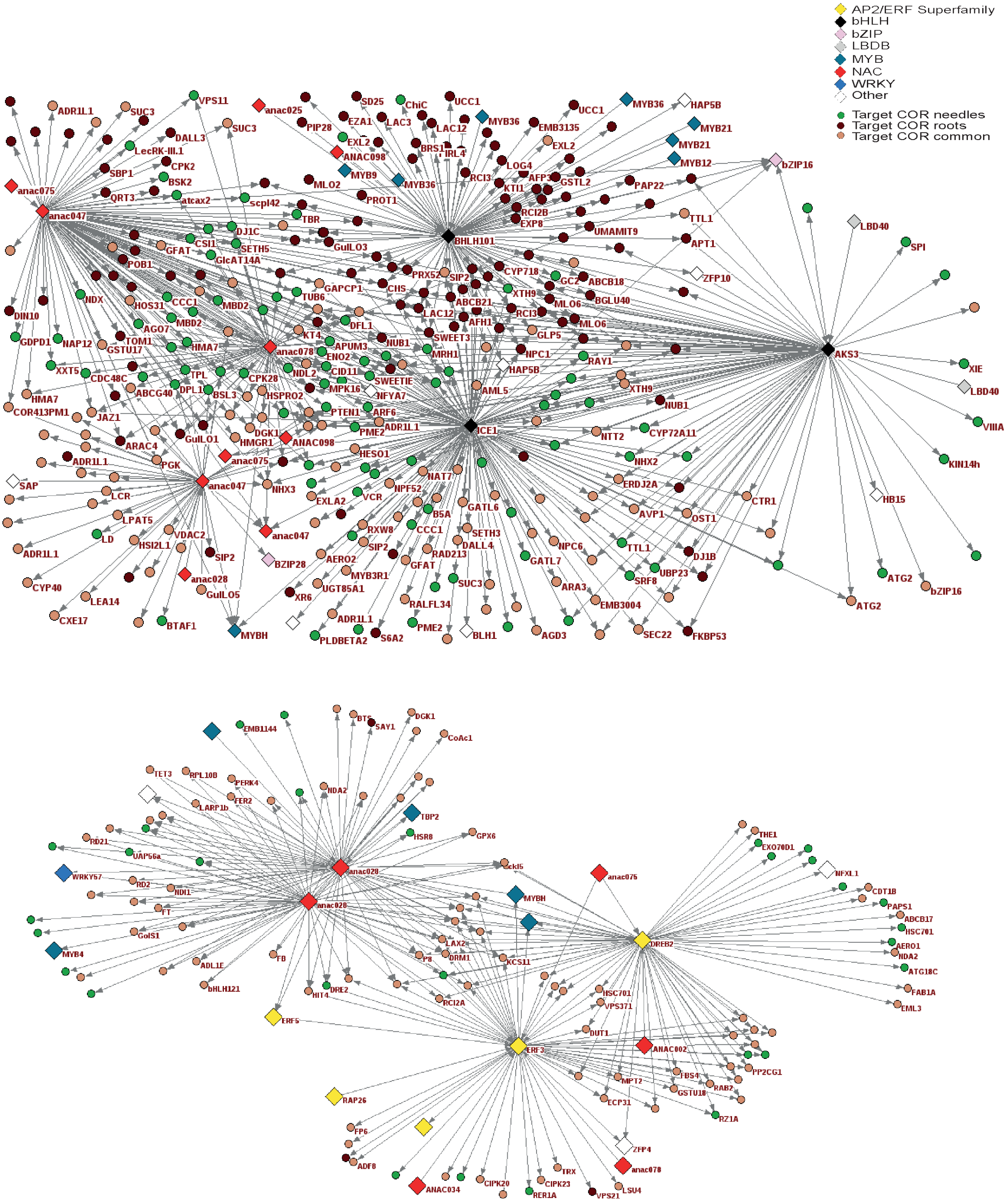

Figure S5. Aliases of the first neighborhood genes of the 10 hubs with the highest centrality in the predicted regulatory network. Gene aliases were obtained using aliases assigned to best matches in *Arabidopsis thaliana.*

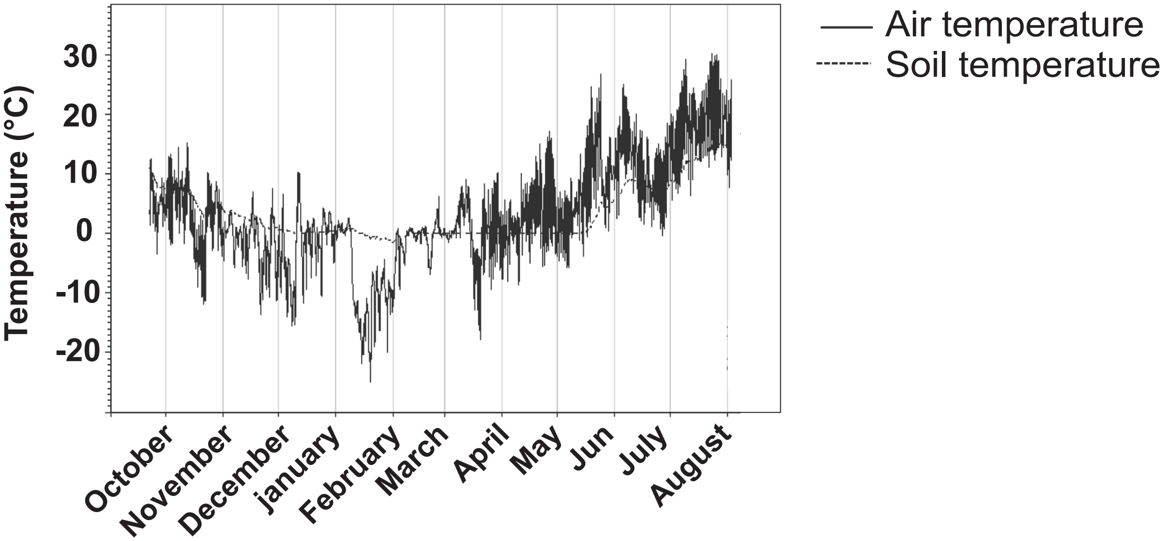

Figure S6. Norway Spruce temperature measurements on soil and air. Air and soil (15 cm deep) temperatures under a mature Norway spruce forest in northern Sweden (64° 00′ 21.24”N, 19° 54′ 00.24“E) throughout the year.

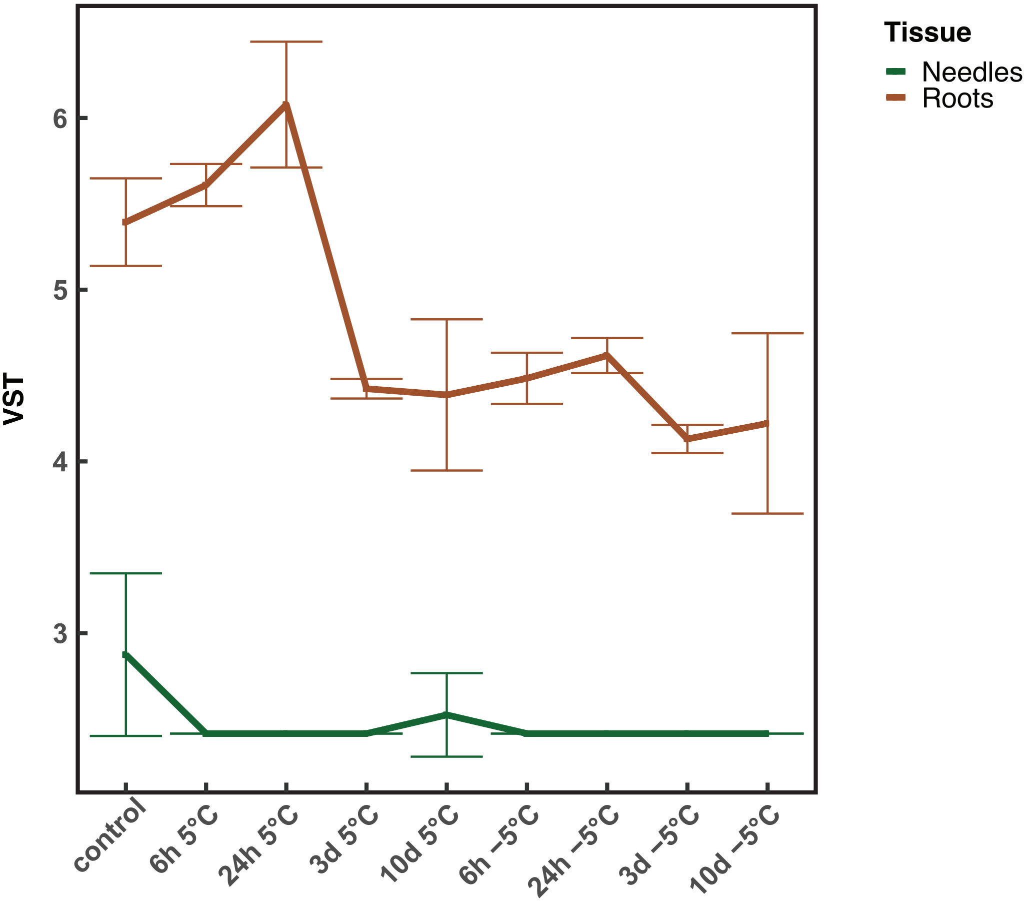

Figure S7. Expression profile of CBF ortholog in Norway spruce. Expression profiles of MA_20987g0010 (PLAZA id: PAB00032324) gene in needles and roots. Normalized expression values by Variance-stabilized data transformation (VST) are represented. Error bars correspond to SD values obtained from the replicates.

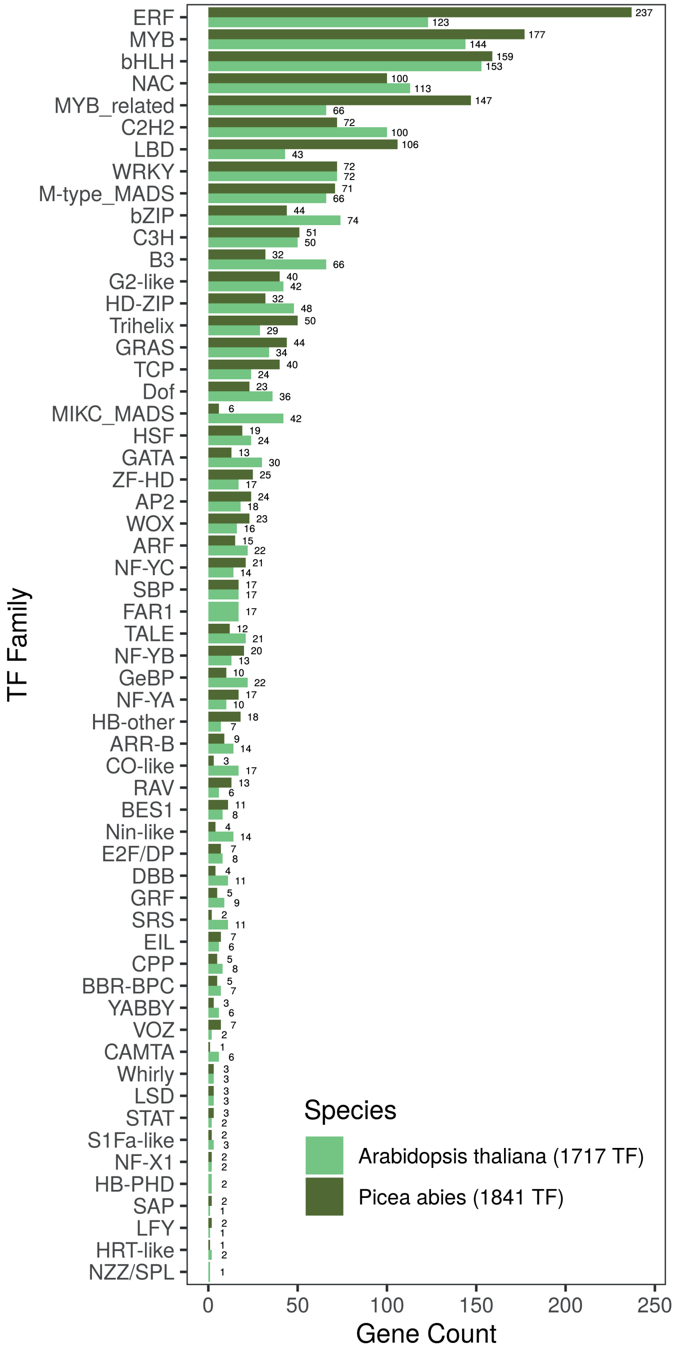

Figure S8. TF family distribution. Bar plot representation of transcription factor (TF) family distribution in Arabidopsis thaliana and Picea abies (Norway spruce).

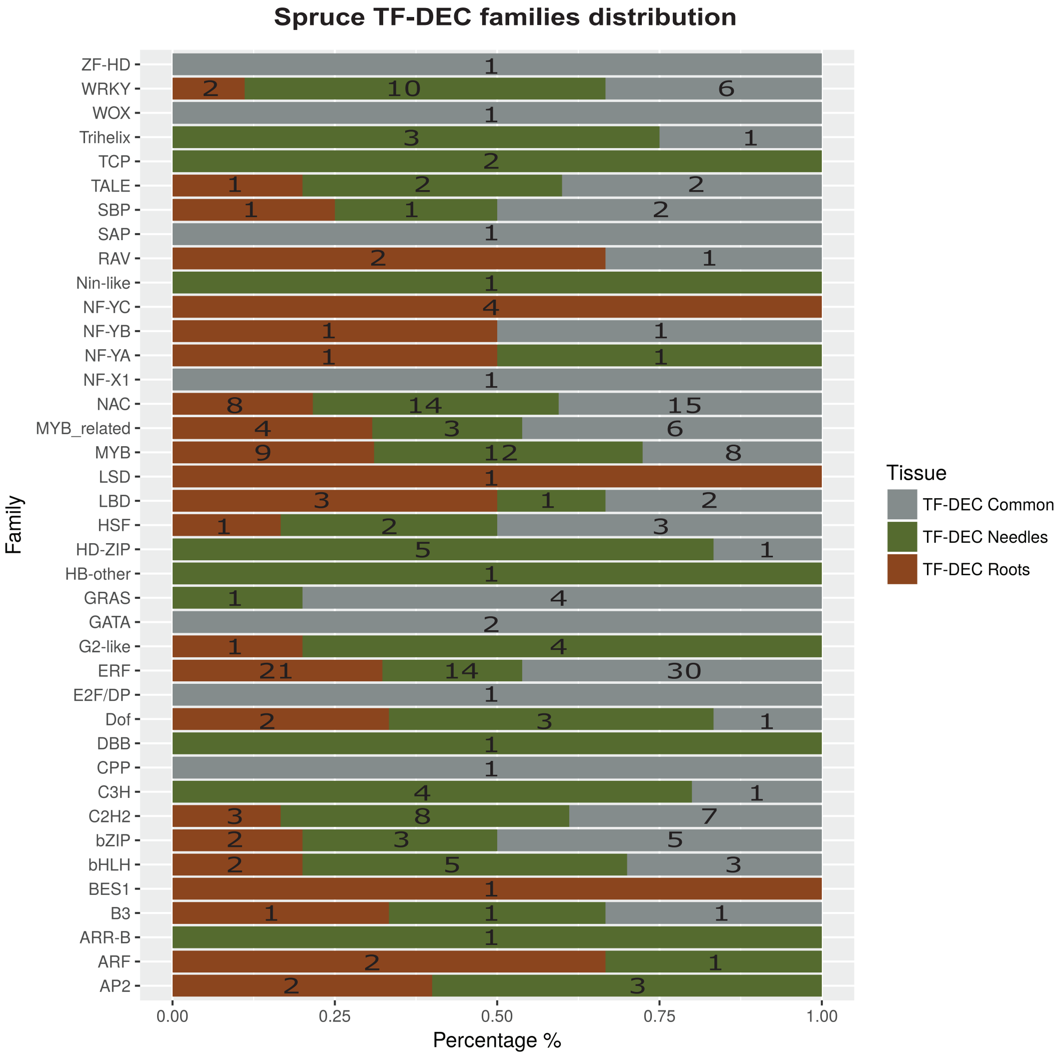

Figure S9. Distribution of Norway spruce TF-DEC families. Bar plot representation of percentages of transcription factors differently regulated by cold (TF-DEC) in Norway spruce in common and tissue-specific groups. The numbers inside each bar represent the counts of each TF group.

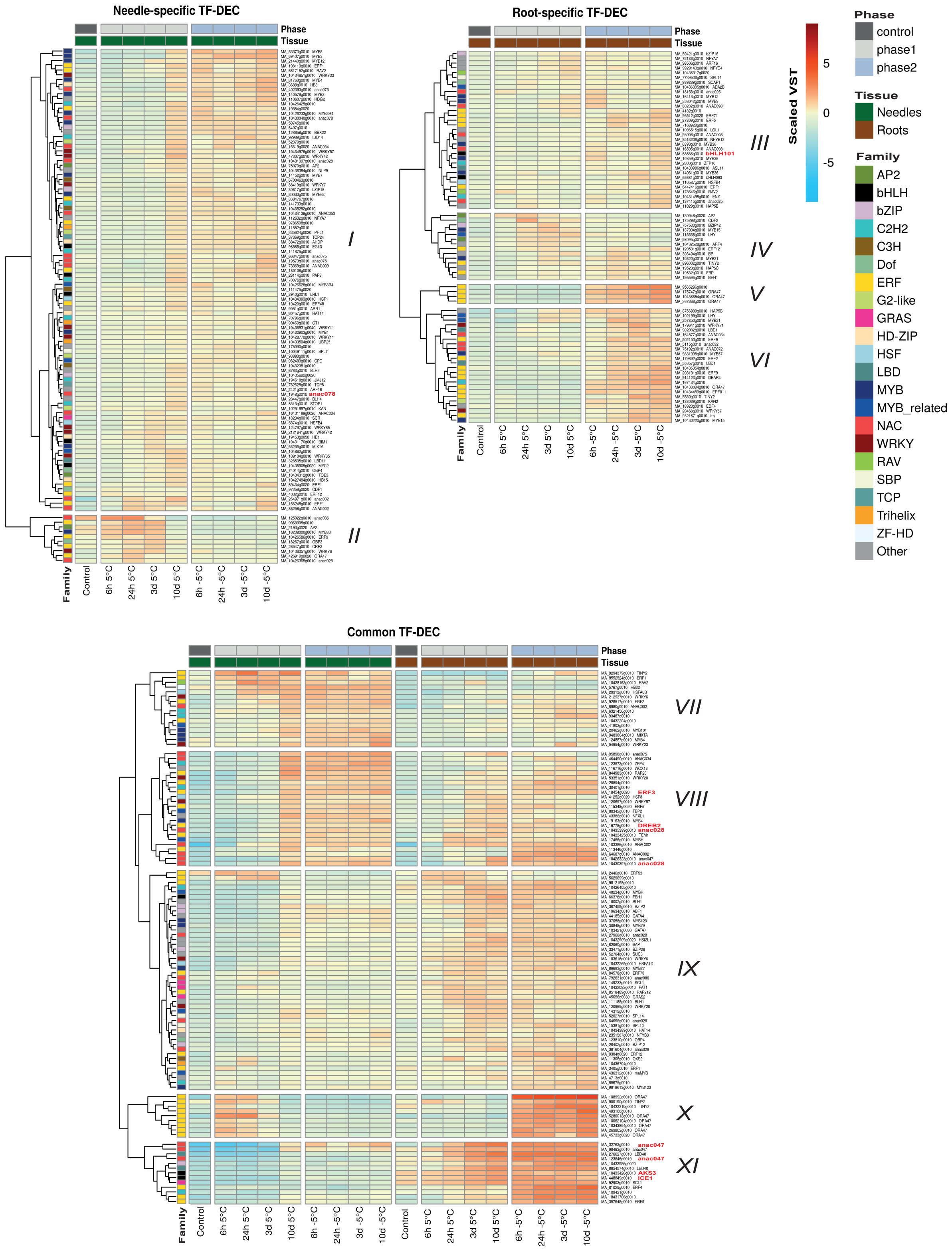

Figure S10. Heatmaps of expression levels of transcription factors differently regulated by cold (TF-DEC). This is an extended version of Figure 6 that includes gene ids and aliases. **A)** Needle-specific TF-DEC. **B)** Root-specific TF-DEC. **C)** Common TF-DEC. Hub genes identified in the predicted regulatory network are highlighted in red.

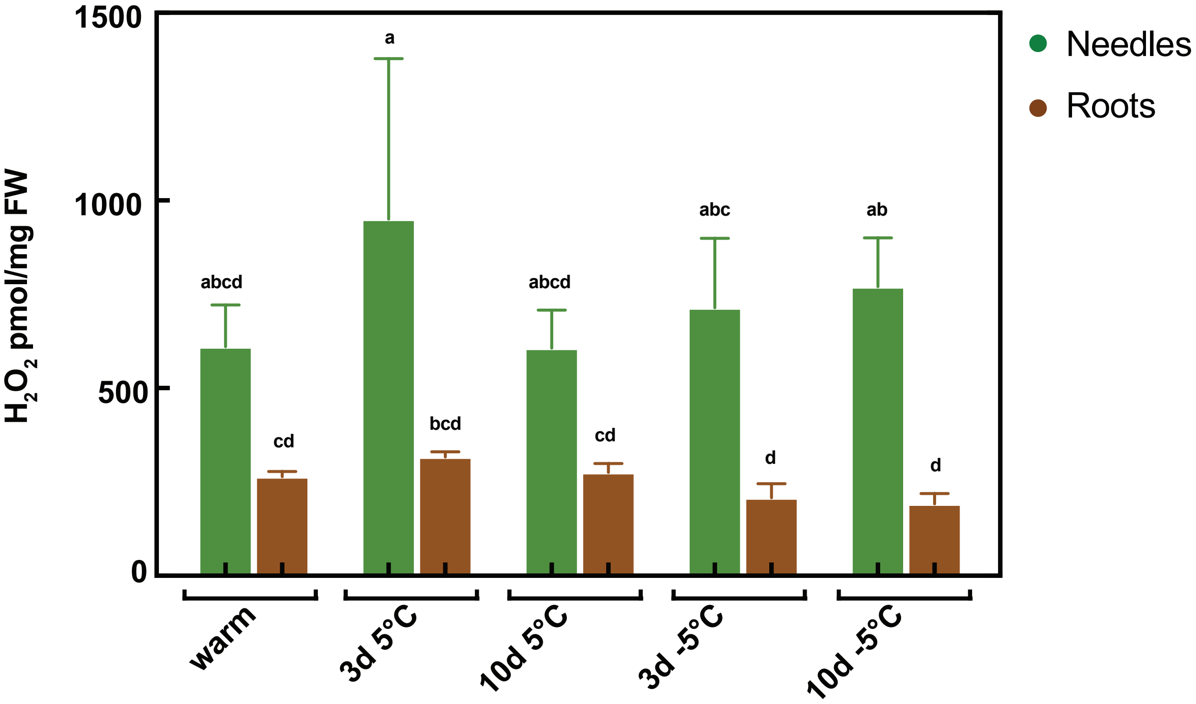

Figure S11. H_2O_2 measurements in needles and roots of Norway spruce. Error bars correspond to SD values obtained from four biological replicates. Letter represent statistically significant differences ($p < 0.05$; Tukey's HSD) between time points. Means and SD of four replicates are indicated.

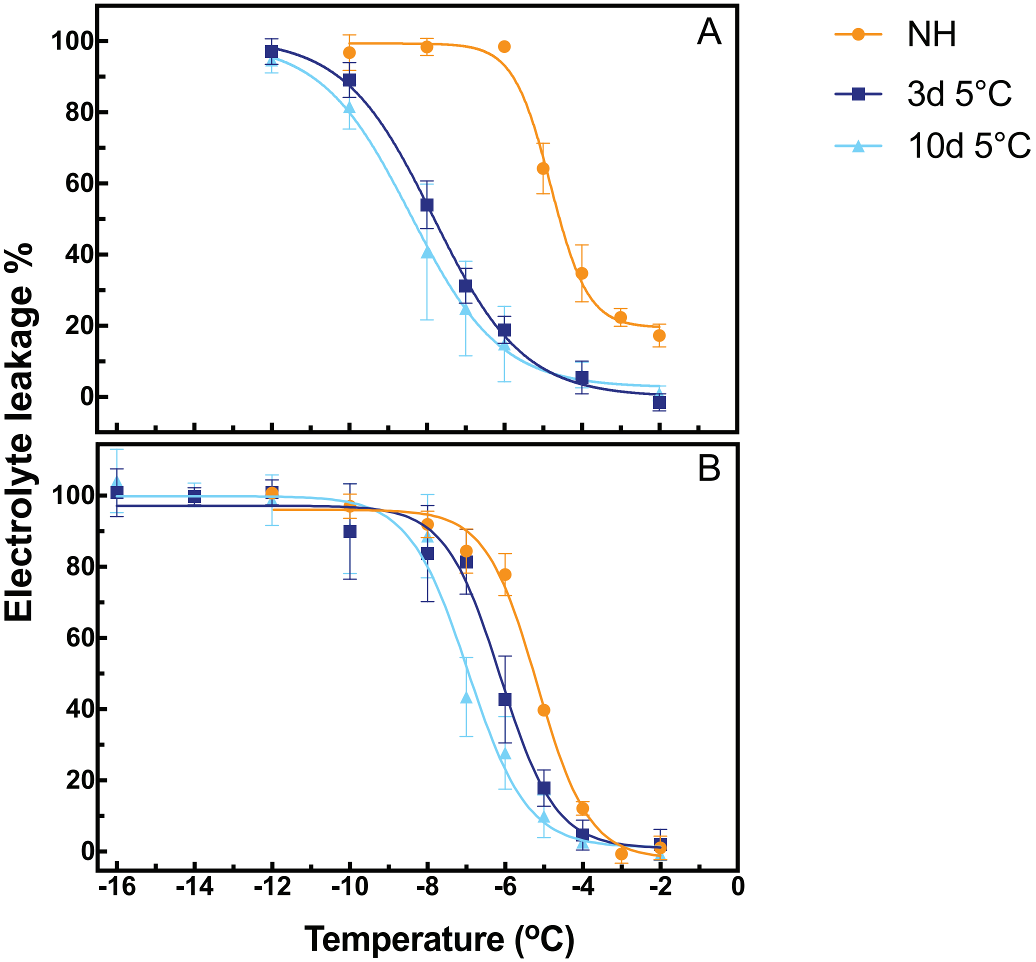

Figure S12. Freezing tolerance estimated by electrolyte leakage A) *Arabidopsis thaliana* leaves and **B)** Norway spruce *(Picea abies)* needles. NH=non-hardened. Error bars correspond to SD values obtained from four replicates.

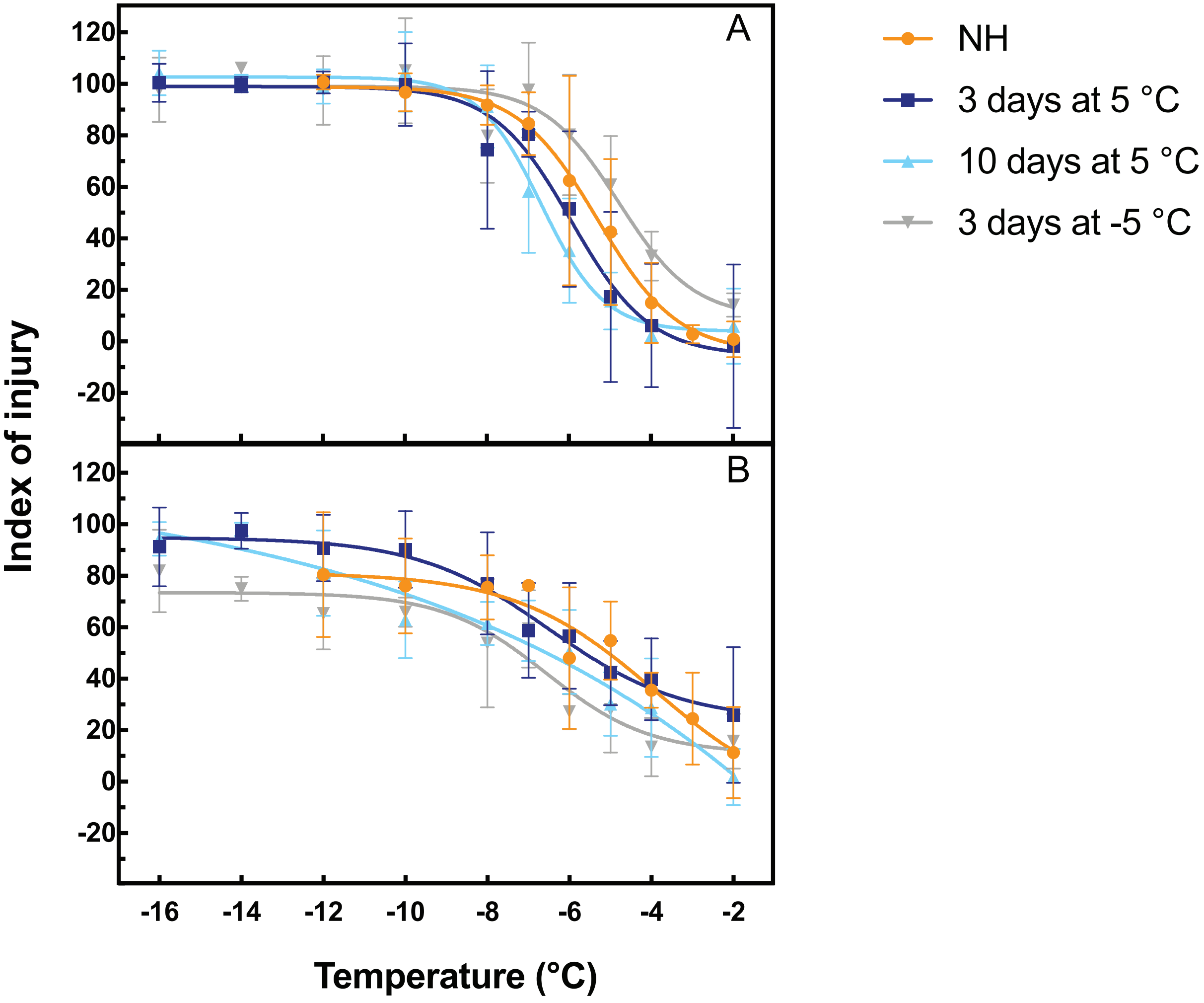

Figure S13. Norway spruce freezing tolerance estimated by electrolyte leakage and expressed as index of injury. **A)** *Picea abies* needles. **B)** *Picea abies* roots. NH= non-hardened. Error bars correspond to SD values obtained from four replicates. One replicate from needles at -5°C was removed because it was consider as outlier.

Supplementary tables of the publication are found in the publishers website: https://doi.org/10.1111/pce.14241