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Effects of temperature on dehydrin expression in mature somatic embryos of Norway spruce (*Picea abies*)

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Forord

Denne masteroppgaven representerer avslutningen på mitt masterstudie i bioteknologi ved NMBU. Det har vært en fin og lærerik reise, avbrutt av en lengre studiepermisjon. Jeg vil takke alle som har hjulpet meg. Først og fremst mine veiledere, professor Trine Hvoslef-Eide ved fakultet for biovitenskap, institutt for plantevitenskap, og Dr. Carl-Gunnar Fossdal, forskningssjef / Avdelingsleder, NIBIO, som begge har fulgt meg på veien og gitt gode faglige innspill og råd. Jeg skylder også Torfinn Torp, seniorrådgiver i matematisk statistikk, NIBIO, mange takk for å ha tatt seg tid til å hjelpe meg i gang med statistikken. Takk til Dr. Elena Carneros for veiledning og gode råd på lab, og til Dr. Igor Yakovlev, seniorforsker, NIBIO, for å ha delt av sin kunnskap. Jeg er glad for å ha fått mulighet til å gjennomføre eksperimentene til denne masteroppgaven ved Skog og Landskap (nå NIBIO). Tusen takk!

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Abstract: This master thesis deals with genetic and epigenetic effects in two genotypes (denoted A2K and B10) of *in vitro* derived somatic embryos (SEs) of Norway spruce (*Picea abies* (L.) Karst at different temperature conditions. The differential expression profile of selected dehydrins (DHNs) were examined using qRT-PCR. The **aim** was to determine whether the DHNs expression were up – or down-regulated as a result of the temperature conditions the SEs had been developing under. The **results** showed a significant effect for temperature for DHN11.1. Genotype had a significant effect for DHN1, DHN9, DHN11.1, DHN24 and DHN41. Overall, the DHNs transcript levels were higher in B10 than A2K. The main **conclusion** is that genotype is more important than epitype for gene expression of the DHNs observed in mature SE of Norway spruce. In epitypes it is known that DHNs are differentially expressed in buds (Carneros et al. 2017) but mature embryos are a very different tissue in some state of dormancy and thus in retrospect it may not be surprising that transcription of these gene products is not epigenetically impacted at this time. The possibility remains that there are differences at the protein level but this remains to be examined. Earlier stages of embryogenesis are likely better suited for studies of epigenetic and genetic effects of temperature in SEs.

Sammendrag: I denne masteroppgaven undersøkes genetiske og epigenetiske effekter av temperatur på to genotyper (A2K og B10) av somatiske embryoer (SEs) av norsk gran (*Picea abies* (L.) Karst. Transkripsjonsmønsteret til et utvalg dehydrin (DHN) kodende gener ble undersøkt med qRT-PCR for å avgjøre om genuttrykket var opp- eller ned-regulert som følge av temperaturforholdene SEs var utviklet i. Resultatene viser signifikant effekt av genotype for DHN1, DHN9, DHN11,1, DHN24 og DHN41. Transkripsjonsnivåene var høyere i B10 enn A2K. Effekt av temperatur var signifikant for DHN11.1. Hovedkonklusjonen er at genotype er viktigere enn epitype for uttrykket av de utvalgte DHNs i modne somatiske embryoer av norsk gran. Modne embryoer befinner seg i eller på vei inn i en hviletilstand, dermed er det kanskje ikke overraskende at transkripsjonen av DHNs i dem ikke i større grad ble epigenetisk påvirket. Det kan likevel ikke utelukkes at større forskjeller ville vist seg på proteinnivå, men dette er enda ikke undersøkt. SEs i tidligere stadier av embryogenese er kanskje et mer hensiktsmessig valg enn modne somatiske embryo for å studere epigenetiske og genetiske effekter av temperatur på SEs.

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1.0 Introduction

The environments in which plants grow and reproduce includes of a multitude of non-living (abiotic) factors varying in time and geographic location. The abiotic factors can influence one another and they include temperature, light intensity and -quality, water availability and nutrient concentrations, among others. Fluctuations outside of the factors normal range will usually be inducing stresses in the plants. The stresses may have negative physiological and biochemical consequences like destabilizations of membranes and proteins, or changed gene expression and disrupted cellular processes (Taiz & Zeiger, 2010).

In order to cope with the environmental factors, the plants have to adapt (Taiz & Zeiger, 2010). Raven et al (2005) point out three definitions of the term adaption in biology. Firstly, every living being can be thought of as adapted: it is a *state* of being adapted to the environments they thrive in. Secondly, adaption refers to particular characteristics that help the organism *adjust* to its environments. Lastly, the term adaption can refer to the *evolutionary process* that occur over generations, which lead to organisms better suited to changing environments.

According to Mendelian genetics, this third meaning of adaption would refer to changes in the base sequences, the genetic code, over generations. We know now, that this is not the whole story; Phenotypic plasticity phenomena such as epigenetic modifications also, to a lesser degree, have an impact on the phenotype (Watson, Baker et al., 2014; Yakovlev et al, 2012). The word "epi" is Greek, and it means "over" or "on top of" (Taiz & Zeiger, 2010). Epigenetics is referring to stable changes in gene expression that occur *without changes* in the DNA sequence (Taiz & Zeiger, 2010), which may even be established in the organism as an epigenetic memory (Alberts et al, 2014).

By altering the regulation of gene expression, epigenetic modifications may modulate the development, morphology and physiology of an organism, without altering the genetic code (Bräutigam et al. 2013; Pikaard & Mittelsen Scheid 2014). Therefore, epigenetic mechanisms play a role in plasticity responses to the environment and contribute to stress memory and adaption in plants (Baulcombe & Dean, 2014; Crisp et al. 2016). Because of the epigenetic memory, the organism's previous experiences can affect its future responsiveness over longer periods, even over generations (Baulcombe & Dean, 2014). It should be noted that the epigenetic machinery is encoded by genes, so epigenetics is firmly within the research field of genetics.

1.2 Aims and hypothesis

This master thesis deals with genetic and epigenetic effects from temperature in *in vitro* derived somatic embryos from two genotypes of Norway Spruce (*Picea abies* (L.) Karst. The aim is to determine whether the expression of selected dehydrins (DHNs) are up – or down-regulated as a result of the temperature conditions in which the somatic embryos have been developing.

Because temperature is an abiotic factor that can lead to changes in plants gene expression, and because both genotype and epigenetic factors may influence the way the genes are expressed, the somatic embryos different temperature conditions may show differences in transcription levels. Therefore, the hypothesis for this master thesis are:

1) Transcription patterns of one or more DHN will differ between the epitypes of the mature somatic embryos.
2) Transcription patterns of one or more DHN will differ between the genotypes of the mature somatic embryos.

2.0. Theoretical background

2.1 Gymnosperms were the first seed plants to evolve during evolution

The development of the seed was one of the most dramatic innovations during evolution of the vascular plants (Raven et al, 2007); The seed provides the developing (and mature) embryo both protection and nutrition: an advantage non-seed bearing plants do not have.

The gymnosperms, the first seed plants to evolve, arouse more than 365 million years ago (Raven et al. 2007). There are four phyla of living gymnosperm: Cychadophyta, Ginkophyta, Gnetophyta and the Coniferophyta. Within these, there are only about 840 species living in today's flora. The evolutionary much younger angiosperms, which comprises of at least 300 000 species, is occupying a greater variety of habitats than gymnosperms, yet in some areas in nature individual gymnosperm species are dominant (Raven et al, 2007).

2.1.1 The conifers are the most widespread of the living gymnosperms

The most numerous, ecological important and most widespread of the living gymnosperms are the Coniferophyta (the conifers). This phylum extends back in time at least 300 million years ago. It comprises around 70 genera with circa 630 species (Raven et al. 2007). They are distinguished by their needle-like leaves (Yakovlev et al, 2012).

2.1.2 Norway spruce is a conifer species of ecological and economical importance

Norway spruce (*Picea abies* (L.) Karst is a conifer species that covers three major regions, as the result of post-glacial re-colonization: the Nordic-Baltic-Russian, the Hercyno-Carpathian and Alpine regions (Yakovlev et al. 2008). The species has also been *planted* outside these areas, in particular in Central Europe and in Scandinavia, since the middle of the 19th century (Skrøppa, 2003). Because Norway spruce is a key species in northern regions, it is a tree of high ecological importance (Skrøppa, 2007). Also, it is an *economically* important conifer, showing good yield and quality performance on very different site conditions (Skrøppa, 2003).

2.1.3 Norways spruces developmental processes are influenced by abiotic factors

The juvenile period of Norway spruce are rather long (more than 20 years), during which they will not reproduce and set seeds (Skrøppa, 2003). Floral initiation, development of buds, and development and maturation of seeds in Norway spruce are influenced by temperature and other factors (Skrøppa, 2007). An epigenetic memory regulates bud phenology and cold acclimation, as shown in studies of Norway spruces resulting from zygotic embryogenesis in warm versus cold conditions (Bjørnstad, 1981; Johnsen 1989a and b; Skrøppa et al. 2007 and 2010; Yakovlev et al. 2012 and 2014). The fact that Norway spruces have effectively reinvaded large areas following the recent ice ages, and are well adapted to the local environments, despite their long generation times, leads us to postulate that they are masters of adaptation (Yakovlev et al. 2008). This may be caused by their epigenetic capacity (Yakovlev et al. 2014).

2.2 During embryogenesis, a single cell transforms into a multicellular entity

The term embryogenesis describes the process where a single cell transforms into a multicellular entity having a characteristic, but typically rudimentary, organization (Taiz and Zeiger, 2010). In most seed plants, embryogenesis takes place within the confines of the ovule, by fusion of gametes, resulting in a zygotic embryo. The overall sequence of the development is a highly predictable process, considered strictly under genetic control (Wolpert et al. 2011) in which the basic architecture of the plant is set. This includes the establishment of polarity, differentiation of cells to produce various tissue, the elaboration of forms, and the formation of organized structures (Taiz and Zeiger, 2010).

2.2.1 Somatic embryogenesis bypasses the fusion of gametes

Somatic embryogenesis (SE) is a result of somatic cells (as opposed to gametic cells) being stimulated to undergo embryogenesis (Hvoslef-Eide and Corke, 1997). This is a common process in nature, both during embryonic and post-embryonic plant development (Raghavan, 1976). *Cultivation* of explants from microspores, ovules, seedlings or embryos on a medium added the appropriate balance of plant growth regulators may induce formation of embryos directly from the explanted tissue or by proliferation of the embryo-forming callus (Smertenko and Bozhkov, 2014). During the first steps of the process, the cell(s) gene transcription alters, causing de-differentiation of the cell(s) and initiation of the SE.

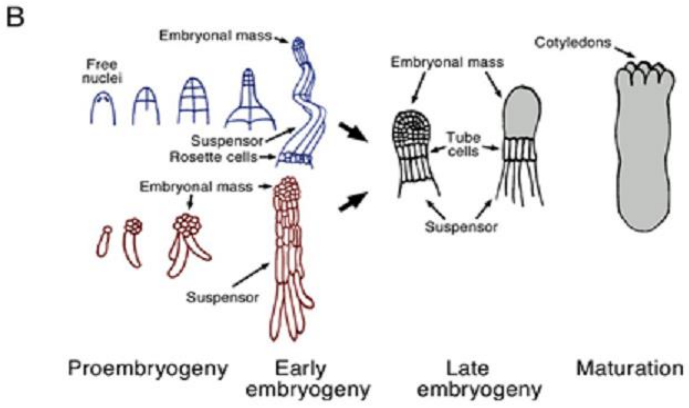


Illustration 1. Comparisons between zygotic and somatic embryogenesis pathways for P. abies (Smertenko and Bozhkov, 2014; © Springer-Verlag Berlin Heidelberg). ZE drawn in blue. ES drawn in red.

2.2.3 Somatic embryos are good models for studying epigenetic prosesses

Somatic embryogenesis has many similarities to the gametic embryogenesis in plant seeds, and the mature SE resembles mature zygotic embryos (ZE) (Smertenko and Bozhkov, 2014; Winkelmann, 2016). This makes it possible to mimic the natural zygotic embryogenic process during natural seed formation (Yakovlev et al. 2014). Because somatic embryos inducted from the same explant is genetically identical, their phenotypic differences is a result of epigenetic processes. Thus, the use somatic embryos a good model for studying epigenetic processes (Yakovlev et al. 2014).

2.3 DNA, genes and gene expression

2.3.1 Eukaryotic DNA is associated with proteins and organized into chromosomes

An organism's biological information is contained in the DNA, where it is encoded in the base sequence, and organized in a number of genes (Watson et al. 2014). Eukaryotic DNA is usually composed of two polynucleotide chains twisted around each other in the shape of a double helix oriented in an antiparallel manner (Watson and Crick, 1953). The fundamental building blocks of the strands are nucleotides, which consist of a phosphate joined to a sugar to which a base is attached. The total amount of DNA in the cell nucleus is referred to as the nuclear genome (Slater et al. 2008). Plants also contain separate DNA in chloroplasts and mitochondria (Slater et al. 2008). This will not be further discussed in this thesis.

Eukaryotic DNA and its associated proteins, called histones and non-histone proteins, are organized into chromosomes to fit the entire DNA into the nucleus of the cells, protecting the bases in the genetic code from damage (Klug et al. 2010). The size and numbers of chromosomes varies between organisms. In diploid organisms, like Norway spruce is (Yakovlev et al. 2012), the chromosomes exists in homologous pairs and each member of such a chromosome pair have identical size and gene sites (Klug et al. 2010).

The chromosomal areas have a condensed appearance as in heterochromatin, or a more open structure as in euchromatin, reflecting their differences in gene expression; Heterochromatin makes up a barrier to gene expression because the DNA here is less available for DNA

recognizing proteins and DNA binding proteins needed for transcription (Reece et al., 2011).

2.3.4 Protein coding genes are transcribed into RNA which are translated into proteins

Eukaryotic genes can be referred to as discrete units of heredity information that consist of specific nucleotide sequences in DNA (Reece et al. 2011), which are responsible for making molecules that do have a function (Slater et al. 2008). Most of the genes code for proteins and the term gene expression then refers to the presence of gene products in the form of mRNAs and proteins in a cell or tissue (Fletcher and Hickey, 2007). Non-protein-coding genes code for functional RNA molecules, of which many can regulate gene expression (Slater et al. 2008).

During gene expression of protein coding genes in eukaryotic cells, genetic information is transcribed into RNA, cleaved into a messenger RNA (mRNA), added adenine residues to its 3`end before travelling out of the nucleus (Watson et al. 2011), and translated into an polypeptide chain, which "folds into protein shape" (Reece et al. 2011). Misfolded proteins are less functional or not functional at all (Alberts et al. 2014).

2.3.2 Gene expression and phenotype is influenced by genetic and epigenetic factors

The two members in a gene pair are influencing the same trait(s), but they are not necessarily identical (Klug et al. 2010). Instead, they are gene alleles; variants of the same gene. Their differences might not only be in the base sequence (=of genetic character), but also in the chromatin structure (=epigenetic character) (Yakovlev et al. 2014). In a population, many different alleles may exist, contributing to genetic variation between individuals (Wolpert et al. 2011).

An organism's genetic composition is referred to as its genotype, and the physical structure of the organism is its phenotype (Watson et al. 2014). Because most gene products function inside the cell, and cells interact with each other, and the organism are influenced by cues in the environment, a combination of genotype and epigenetic factors is likely to modify the gene expression in the cells and the resulting phenotype (Klug et al. 2010). Thus, the phenotypic differences between two genotypes can be resulting both from their DNAs genetic and epigenetic differences, and/or from a combination of them.

The gene expression may also vary between two genetically identical individuals, resulting in phenotypic variants of the same genotype, referred to as epitypes (Yakovlev et al. 2014). Being genetically identical, their phenotypic differences are caused by epigenetic effects. The formation of epitypes may be an effective way to successfully cope with environmental factors that are rapidly changing, and this may be an important mechanism for species with long generation time, like Norway spruce, to adapt to its environments (Yakovlev et al. 2014).

2.3.3 Transcriptional changes are hallmarks of epigenetic changes

Regulation of transcription can result in different sets of genes being transcribed in different cells, or in the same cell at different times or at different amounts (Wolpert et al. 2011). Because such regulations can be induced by the epigenetic machinery, transcriptional changes are hallmarks of epigenetic changes. In epitypes of somatic embryos of Norway spruce, it is shown that temperature treatments during the embryogenesis are related to transcriptomic changes for several genes related to the epigenetic machinery (Yakovlev et al. 2016). The majority of them code for regulators that may be influencing DNA and histone methylation, signaling genes and sRNA pathways (Yakovlev et al. 2016).

2.3.4 Modifications of chromatin might alter gene expression for long time scales

Transient changes of chromatin structure is necessary for transcription, but the chromatin may also undergo dynamic structural changes that are more stable over time (Taiz and Zeiger, 2010). Incorporation of histone variants and adding of chemical groups onto histone tails are examples of epigenetic modifications that might alter chromatin structure. The enzymes that are establishing, reading and erasing these kind of epigenetic marks are called “writers”, “readers” and “erasers” (Watson et al. 2014), and their actions may alter or reset gene expression (Dawson and Kouzarides, 2012). Long and short non-coding RNAs is participating by guiding the writers, readers and erasers to specific regions of the genome (Van Oosten et al. 2014). The epigenetic modifications may be persisting through the process of cell division and thereby continue into the following cell generations, causing the alteration of gene expression to persist. The epigenetic modifications are in that way established as an epigenetic memory, causing the alteration of gene expression to persist even if the factor causing the establishments of the epigenetic marks, may be gone (D`urso and Brickner, 2014).

Epitypes of genetically identical Norway spruces, resulting from different temperatures during embryogenesis, display a life-long shift in events like bud phenomenology and cold acclimation (followed for 20 years and counting); a fact that suggest the involvement of an epigenetic memory (Yakovlev et al, 2016). It is suggested that the mechanisms leading the establishment of the epigenetic memory happens exclusively during the initial stages of embryogenesis (Kvaalen and Johnson, 2008).

2.4 Dehydrins is a multifamily of proteins in plants

Dehydrins (DHNs) are hydrophilic members of the protein family Late Embryogenesis Abundant proteins (LEAs). They are highly abundant during the later stages of seed development, and they are important for seed survival, as emphasized in studies on transgenic seeds showing that reduced levels of DHNs reduces the seeds longevity (Hundermark et al. 2011). DHNs are also important in plants in response to abiotic stress that causes dehydration of the cells (Galau et al. 1986; Taiz and Zeiger, 2010).

2.4.1 Dehydrins contain conserved sequences

Based on the arrangement of the DHNs conserved K-, Y- and S- segments, the DHN architecture generally categorizes in the following categories: K_n, SK_n, K_nS, Y_nSK and Y_nK_n (Close, 1996). The K- segment may be involved in membrane binding (Strimbeck et al. 2015). DHNs are highly disordered proteins *in vitro,* but they often gain structure when bound to a target. This suggests that they may be structured *in vivo* when bound to ligands (Graether and Boddington, 2014).

2.4.2 Dehydrins are providing protection against stress from various abiotic factors

For trees, high levels of DHNs have been associated with tolerance to freezing temperatures, winter dormancy and protection against water stress (Basset et al. 2006; Yakovlev et al. 2008; Perdiguero et al. 2012; Eldhuset et al. 2013; Kjellsen et al. 2013; Strimbeck et al. 2015). Because damage to the cell membrane is a significant consequence of plant stress (Steponkus, 1984), the plants might alter their composition of membrane bound DHNs to adapt to the stress (Takahashi et al. 2013). DHNs may protect the cells by stabilizing nuclear or cytoplasmic macromolecules and membranes under conditions of low water availability (Campbell and Close, 1997; Koag et

al. 2003). The DHNs may be able to local pools of water molecules that sustain the metabolic processes during stress and re-growth (Rinne et al. 1999), DHNs may also to decrease damages during freezing temperatures (Wisniewsky et al. 1999), by promoting intermembrane vitrification via molecular entanglement by interacting with sugar molecules (Strimbeck et al. 2015). In the absence of vitrification, the unstructured regions of the DHNs might be acting sterically as "molecular spacers" preventing membrane-membrane interactions to stop or counteract water loss (Strimbeck et al. 2015).

2.4.3 The expression of DHNs are influenced by temperature and other abiotic factors

Danyluk et al (1998) performed a study on DHNs accumulation in wheat during cold acclimation, by comparing seedlings of a winter cultivar to seedlings of a spring cultivar. They found that DHNs were rapidly upregulated, in both cultivars, after the seedlings were transferred to cold acclimating conditions. The spring cultivar showed inability to maintain DHNs in an upregulated state over time, and the authors suggest that this may explain its poor frost tolerance. Also, the relative abundance of the DHNs in different tissues were measured. Slightly higher levels where detected in leaves than in the crown and the roots, which is suggesting tissue specificity. Accumulation of DHNs were found in the vicinity of the plasma membrane of cold acclimated cells, but not in non-acclimated cells. The authors state that the results prove evidence that the accumulation of the DHNs correlates with the wheat cultivars different capacities to develop frost tolerance.

Yang et al (2012) performed identification of DHN coding genes from grapevine species, and analyzed their responsiveness to various forms of abiotic and biotic stresses. They identified four genes: DNH1, DHN2, DHN3 and DHN4. *Distinct expression patterns* in tissues and organs was indicated: DHN1 was mainly expressed in seeds under normal conditions. DHN2 was detected in all tissues tested (roots, stems, leaves, seeds and fruit peels). Both the expression of DHN1 and DHN2 were varying during seed development. DHN3 was undetectable in roots, stems, leaves and fruit peels, and was only expressed, at very low levels, during seed development during all stages of embryogenesis. DHN4 was expressed only in seeds, specifically during late embryogenesis. The stress-responsiveness of all four genes were tested using leaves subjected to stress conditions, including low temperatures. Neither DHN3 nor DHN4 showed detectable levels

of expression under any of the conditions, but the two other did: DHN1 exhibited a sudden up-regulation, while DHN2 increased more gradually, indicating that DHN1 are more important in stress-response than DHN2. The authors state that the DHNs expression patterns throughout development and stress conditions imply the gene family's functional diversification.

Yakovlev et al (2008) performed a sequence analysis and expression profiling of DHNs genes related to timing of bud burst in Norway spruce. They identified eight groups which all showed significant similarities to DHN from various plant species, and which they classified into the three main types K_2, K_3 and SK_n. The results from measurements of transcript levels indicated an influence of temperature: K_n type DHNs showed decreasing levels toward bud burst, with significant differences between "early" and "late" flushing families, reflecting differences in sensitivity to cues in the environments. The transcript levels were high during late winter and early spring, followed by decreasing levels to the time of bud burst. Exposure to longer days and higher temperatures generally decreased the transcription of all DHNs. The authors hypothesize that DHNs is playing an important role in protecting plants during winter.

Carneros et al (2017) found that the epigenetic memory of temperature during embryogenesis modifies the expression of bud-burst related genes in Norway spruce epitypes. Samples of buds and "last years needles" from two epitypes (variants) of eight years old trees, of the genotype A2K was subject to this study. The epitypes were originating from "cold" embryogenesis (CE) environment at 18°C and "warm" embryogenesis (WE) environment at 28°C. The results of Carneros et al (2017) confirm the existence of an epigenetic memory mechanism operating during embryo development. The mechanism adjust the subsequent timing of bud burst according to the "remembered" temperature conditions (i.e temperature sum) the plant was subjected to during its embryogenesis. This was manifested by the earlier timing of bud burst in epitype originating from CE compared to WE. Also different patterns of DHN gene expression between the two epitypes were detected: transcript levels of 9 of the 12 DHNs tested was significantly higher in WE-buds compared to CE-buds in the spring prior to bud burst. The authors state that this is most likely a result of chromatin modifications that were established during embryogenesis. In other words, expression of DHN genes are affected by an epigenetic memory. An opposite expression pattern was detected between buds and needles, independent of epitype: DHNs expression in buds significantly decreased approaching bud burst, while the levels in

needles increased during the same period. The authors state that it seems like the DHNs needed for frost protection have to be down-regulated before start of cell division and growth in Norway spruce.

Amongst the dehydrins tested for in Carneros et al (20017), was DHN1, DHN6, DHN9, DHN24 and DHN41, which was also used for this master thesis.

3.0 Materials and methods

The experimental system used for this master thesis is developed by Kvaalen and Johnsen (2008). It is considered an optimal experimental system for molecular studies of the mechanisms underlying the establishment and maintenance of epigenetic memory in spruce, and it is based on the induction of epitypes during somatic embryogenesis (SE) at different temperatures (Kvaalen and Johnsen, 2008). The temperatures 18˚C, 23˚C and 28˚C are found to be epitype inducing (Kvaalen and Johnsen, 2008; Yakovlev et al. 2014).

3.1 Plant material and sample collection

The plant material had already been produced before the lab work for this thesis started. Two zygotic seeds (genotypes) were used as starting material ("explants") to induce the embryogenic samples used for this thesis. The seeds were originating from a controlled cross of a defined female (♀#2650) and male (♂#2707) of Norway spruce parents, with those crosses being performed either in outdoors conditions; a cold originated genotype, or in greenhouse conditions; as a warm originated genotype. The two zygotic seeds are denoted A2K and B10.

Each of the two zygotic seeds had been placed on separate AL medium supplemented with inositol (10%, w/v) and vitamin mixture. 2,4-dichlorophenoxyacetic acid (10 µM), sucrose (1%, w/v) had also been added to the medium before it was solidified using Phytagel (P-8169; Sigma, 0,3% w/v). During this step, embryogenetic callus were forming on the zygotic seeds. The genetic material in the embryogenic callus started from the zygotic seed A2K do share the genetic identical material as the A2K zygotic seed itself. The embryogenic callus started from the zygotic seed B10 do share the genetic identical material as the B10 zygotic seed itself.

After separation from the two explants (zygotic seed A2K and B10), the embryogenic tissue had been divided into three cultures (three cultures per genotype) and subjected to different temperature treatments; 18°C, 23°C and 28°C. The temperature conditions will induce epigenetic changes in the embryogenetic cultures, resulting in three different epitypes of each genotype (three epitypes of the genotype A2K, and three epitypes of the genotype B10). The developing somatic embryos were transferred to fresh medium every other week until maturation.

The work for this master thesis started by attending the transferring of somatic embryos to fresh medium during the period of maturation, and later collecting samples of mature somatic embryos.

The somatic embryos were sampled into Eppendorf vials pre-frozen in liquid nitrogen before immediately frozen in liquid nitrogen. All samples were stored at -80°C until time of use.

3.2 Gene searching

The DHNs to be used for this master thesis had already been selected by the research group, from a screened and annotated set of expressed sequence tags (ESTs) in a Norway spruce database from suppressive substraction hybridization cDNA libraries. An EST is a short sub-sequence of a cDNA sequence which may be used to identify gene transcripts (Slater et al, 2008).

3.3 RNA extraction

The samples stored at -80°C were used for this step. For each of the three temperature treatments for the two genotypes, nine somatic embryos were added to Eppendorf tubes. Three somatic embryos per tube. The somatic embryos were crushed to fine powder using a tissue lyser (RETCH MM300) bead mill, set at 1 minute at highest speed. After this step, the plant cells and membranes were lysed and RNA was extracted using a MasterPure™ Plant Purification Kit: 300 µl Plant Tissue and Cell Lysis solution was added each tube. This solution degrades polysaccharides. 1.5 µl 100 mM DTT and 0.5 µl Proteinase K was added to each tube to increase the efficiency of lysis. The proteinase degrades proteins. The solution was mixed by vortexing for 1 minute before being incubated at 56°C for 15 minutes to help the efficiency of the Proteinase K. Every 5 minutes the solution was mixed by vortexing for 15 seconds to improve the yield of nucleic acids in the solution. The debris, containing lysed cells, sugars and other macromolecules,

was pelleted by centrifugation for 5 minutes at 12000 rpm at room temperature. The clarified supernatant was transferred to a new tube before being placed on ice for 5 minutes.

During the next steps in the protocol the nucleic acids was drawn out from the supernatant into solid form: 175 µl of MPC Protein Precipitation Reagent was added to each sample and mixed by vortexing vigorously for 10 seconds. The debris was pelleted by centrifugation in a micro centrifuge for 10 minutes at 12000 rpm at 4°C. The supernatant, containing the nucleic acids in solution, was transferred to a new tube. 500 µl of isopropanol was added to the supernatant and the tube was inverted 40 times to make sure all sides of the tube was washed in the alcohol. The nucleic acids were pelleted by centrifugation in a micro centrifuge for 10 minutes at 12000 rpm at 4°C. The isopropanol was poured off without disturbing the pellet. All of the residual isopropanol was removed with a pipette.

There might be DNA contaminating RNA in the pellet. This had to be removed from the samples: 200 µl of DNase I solution for each sample was prepared; 173 µl RNase-Free water, 20 µl 10x DNase Buffer, 5 µl RNase-Free DNase I and 2 µl RiboGuard RNase inhibitor. To degrade the DNA, the pellet was completely resuspended in 200 µl of the prepared DNase I solution and incubated for 30 minutes. Then 200 µl of 2XT and Lysis Solution was added. This was mixed by vortexing 5 seconds before 200 µl of MPC Protein Precipitation Reagent was added and the sample was mixed again by vortexing 10 seconds and placed on ice for 5 minutes. The debris, containing the contaminations, was pelleted by centrifugation in a micro centrifuge for 10 minutes at 12000 rpm at 4°C. The supernatant, now containing the isolated RNA, was transferred into a new tube and added 500 µl of isopropanol. The tube was inverted 40 times to make sure all sides of the tube were washed in the alcohol. The purified RNA was pelleted by centrifugation in a micro centrifuge for 10 minutes at 12000 rpm at 4 °C. The isopropanol was then poured off without disturbing the pellet. The pellet was washed twice with 70% ethanol and centrifuged briefly. All residual ethanol was removed with a pipette. The pellet was resuspended in 30 µl of RNase-Free water. The quantity of RNA was assessed by a NanoDrop 2000 Spectrophotometer (Thermo Scientific).

3.4 cDNA synthesis

RNA from each of the individual samples extracted during the previous steps was employed for cDNA synthesis and subsequent RT-qPCR analysis. First-strand cDNA was synthesized from 300 ng of total RNA in 50 µl reaction volume using Taq-Man® Reverse Transcription Reagents (Applied Biosystems, Carlsbad, CA, USA, #N8080234); In each Eppendorf vial, the following were added: 16,25 µl RNase-free water, 5 µl 10X TaqMan® RT buffer, 11 µl 25 mM $MgCl_2$, 10 µl of the 2.5 mM deoxyNTP Mixture. Before adding the rest of the components, the vials were vortexed briefly. Now, 2.5 µl Oligo d $(T)_{16}$, 1 µl RNase inhibitor, 1.25 µl Multiscribe Reverese transcriptase and 3 µl of the RNA in solution (≈200 ng RNA) was added. The vials were capped and inverted to mix the components. To remove air bubbles and collect liquid that might be on the walls and in the cap, the capped vials were briefly centrifuged. The vials were placed in a thermal cycler set on following parameters:

Table 1. Thermal parameters

Step	**Incubation**	**RT**	**Reverse Transcription Inactivation**
	HOLD	HOLD	HOLD
Time	10 minutes	30 minutes	5 minutes
Temperature	25 ^{O}C	48 ^{O}C	95 ^{O}C

3.5 RT-qPCR amplification and gene expression analyses

RT-qPCR amplification was performed in a 10 µl reaction volume included 2 µl of cDNA solution as template, 5 µl of 2X Fast®SYBR Green Master Mix and 200 nM of each primer. Gene expression analyses were performed using the ViiA 7 Real-time PCR System (Applied Biosystems) with standard cycling parameters. All reactions were done in triplicates. For data analysis, the arithmetic mean of two biological replicates was calculated, and a no-template control was run for each primer pair. Target gene expression was normalized to the average of transcript levels of the Norway spruce ACTIN (PaACTIN), TRANSLATION INITIATION FACTOR-5-ALPHA (PaelF5a) and α-TUBULIN (Paa-TUB). Quantification was performed using the ViiA 7 Real-time PCR System (Applied Biosystems).

Table 2. Primer sequences used for RT-qPCR analyses of the seven dehydrins and three reference genes. Sequences are listed in 5`- 3`direction.

Gene IDs	Accession no.[a]	Primers forward / reverse	Product lenght (bp)
PaDNH1	MA_95995g0010	GCGGCCTATGCGGCAAGAA / TCGACGCCCCGCCTTCTG	95
PaDHN6	MA_757559g0010	TCCCGGAGGCCGGAACAAGT / CGAAAGCGACATGGAGAGGTAGCC	102
PaDHN9	MA_2408574G0010	TCACGGTCAGCAGGGGCAAG / AACCGGAGCCGGAGCCATGT	101
PaDHN11.1	MA_13855g0010	TCACCGCACGCACAGTTCCA / CCTTGGTCTTGTCCTTGGAGCCTTTC	110
PaDHN24	MA_12179G0010	CCCGGCTGTCTGGAATGCTC/ CCGCCAAAACCCCTAGCAGAACA	90
PaDHN39	MA_86965G0010	CGAGGAGGATAAGGGCGGGAAT / TGCGTGGGTTGTAGCAGGTG	115
PaDHN41	MA_10434136G0010	CCGCGAGAAGCCCGTCCATAC / CACCAGCAAGAACACCGGCTGA	98
PaACTIN	AY961918/ MA_10427661g0030	TGAGCTCCCTGATGGGCAGGTGA / TGGATACCAGCAGCTTCCATCCCAAT	105
Pa aTub	X57980/ MA_93486G0010	GGCATACCGGCAGCTCTTC / AAGTTGTTGGCGGCGTCTT	66
PaelF5a	AY_961932/ MA_103714G0010	GCCGATGCGGGAGCTTCCAA / TGCAGGGCCTGGCCTTAATGACG	88

[a] Accession no. based on Norway spruce genome sequence v.1 (http://congenie.org/)

3.6 Statistical analysis

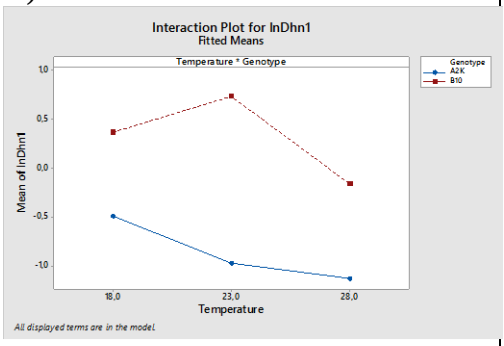
The raw data was sorted in MS excel before the statistical analysis was performed using the statistical software Minitab 18. The residual plots were checked for each dehydrin. For the dehydrins showing deviations from the assumption of normality, a Box-Cox plot was performed to find the best transformation formula for the data to make a better fit. The data was transformed using the formula suggested, before checking the residual plots again, and persuade to the next step of the analysis. Because the presence of an interaction effect between the two factors (temperature and genotype) in the fitted model used for the analysis would make it complicated to persuade further to look for main effects, the interaction plots between the two factors were checked. After concluding that no interaction was present, the main effect plots was checked, and a two-way ANOVA was performed. Tuckey`s test was performed for the fitted values for dehydrin 11.1 regarding temperature.

4.0 Results

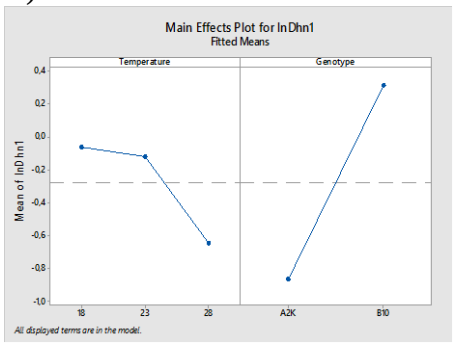
DHN1

As shown in Figure 1, panel A, no interaction was detected between genotypes and temperature for DHN1. The main effects plot and the fitted means are shown in panel B and C, respectively. They show a temperature effect by a decrease in transcript levels by rising temperature. Also, they show that genotype influences the levels of transcripts with higher levels in the B10 genotype than in the A2K. The ANOVA (panel D) shows that the p-value for temperature is higher than 0, 05, for genotype it is significant and for the lack of interaction between the two factors the p-value is high. The normal probability plot (panel E, left hand side) indicates that the residuals are normal distributed. The points in the residual versus fit plot (panel E, right hand side) falls randomly on both sides of 0, without recognizable patterns, indicating that the residuals are randomly distributed and have a constant variance.

A)



B)



C)

Means

Term	Fitted Mean	SE Mean
Temperature		
18	-0,062	0,211
23	-0,120	0,211
28	-0,645	0,211
Genotype		
A2K	-0,864	0,172
B10	0,313	0,172
Temperature*Genotype		
18 A2K	-0,491	0,298
18 B10	0,366	0,298
23 A2K	-0,974	0,298
23 B10	0,734	0,298
28 A2K	-1,128	0,298
28 B10	-0,162	0,298

D)

Analysis of Variance for lnDhn1

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature	2	1,2372	0,6186	2,32	0,141
Genotype	1	6,2314	6,2314	23,33	0,000
Temperature*Genotype	2	0,6420	0,3210	1,20	0,334
Error	12	3,2050	0,2671		
Total	17	11,3155			

E)

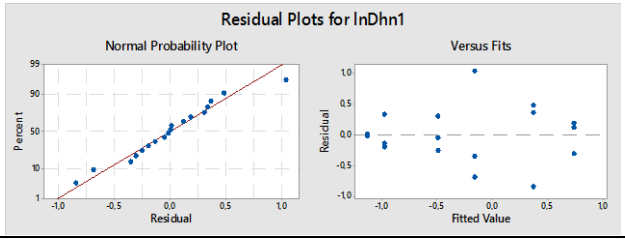
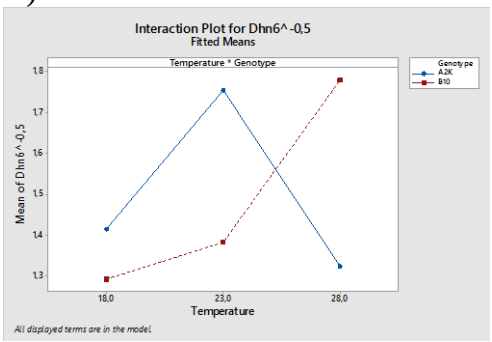


Figure 1. The effect of EpI-temperature on the expression of DHN1 in mature somatic embryos of two genotypes of Norway spruce. The results are shown as Interaction plot (A), main effect plot (B), means (C), analysis of variance (D) and residual plots (E) for the In-transformed observations for DHN1.

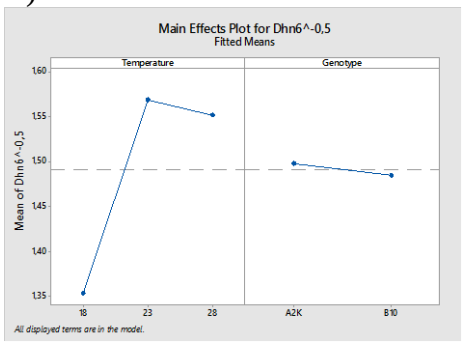
DHN6

As shown in figure 2, panel D, the p-values for effect of temperature, genotype and interaction between the two factors are far too high to take the results for this DHN into account.

A)



B)



C)

Means

Term	Fitted Mean	SE Mean
Temperature		
18	1,354	0,331
23	1,569	0,331
28	1,552	0,331
Genotype		
A2K	1,498	0,271
B10	1,485	0,271
Temperature*Genotype		
18 A2K	1,415	0,469
18 B10	1,292	0,469
23 A2K	1,754	0,469
23 B10	1,383	0,469
28 A2K	1,324	0,469
28 B10	1,779	0,469

D)

Analysis of Variance Dhn6 ^(-0,5)

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature	2	0,17126	0,085631	0,13	0,879
Genotype	1	0,00077	0,000768	0,00	0,973
Temperature*Genotype	2	0,54023	0,270116	0,41	0,673
Error	12	7,91118	0,659265		
Total	17	8,62344			

E)

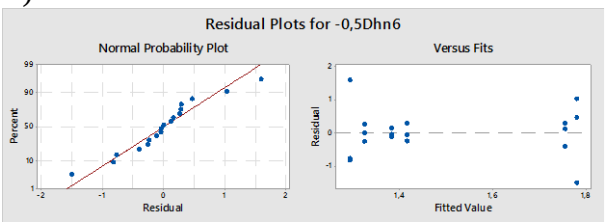
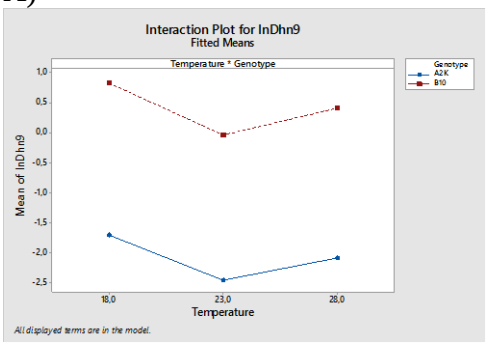


Figure 2. The effect of EpI-temperature on the expression of DHN6 in mature somatic embryos of two genotypes of Norway spruce. The results are shown as Interaction plot (A), main effect plot (B), means (C), analysis of variance (D) and residual plots (E) for the ^(-0,5) transformed observations for DHN6.

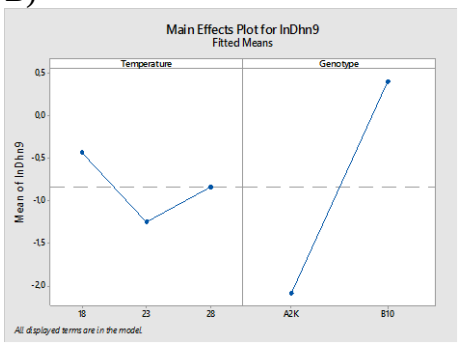
DHN9

As shown in figure 3, panel A, no interaction was detected between the factors genotype and temperature for DHN9, but the p-value (showed in the ANOVA, panel D) for this is high. The main effects plot (panel B) and the fitted mean (panel C) shows an effect of temperature by increased transcript levels from the cold temperature to the middle temperature, before the levels decreases, but the ANOVA (panel D) shows that the p-value is high for the temperature effect. Also, the main effects plot (panel B) and the fitted mean (C) shows that genotype has effect on the levels of transcripts manifested as higher levels in the B10 genotype than in A2K. The ANOVA (panel D) shows a significant p-value for the genotypic effect. The residual plots (panel E) do not show crucial deviations, indicating that the residuals are normally distributed.

A)



B)



C)

Means

Term	Fitted Mean	SE Mean
Temperature		
18	-0,435	0,266
23	-1,247	0,266
28	-0,837	0,266
Genotype		
A2K	-2,081	0,217
B10	0,401	0,217
Temperature*Genotype		
18 A2K	-1,701	0,376
18 B10	0,830	0,376
23 A2K	-2,457	0,376
23 B10	-0,037	0,376
28 A2K	-2,085	0,376
28 B10	0,411	0,376

D)

Analysis of Variance for lnDhn9

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature	2	1,9759	0,9880	2,33	0,140
Genotype	1	27,7338	27,7338	65,42	0,000
Temperature*Genotype	2	0,0097	0,0048	0,01	0,989
Error	12	5,0872	0,4239		
Total	17	34,8066			

E)

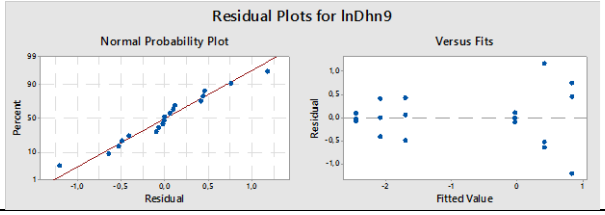


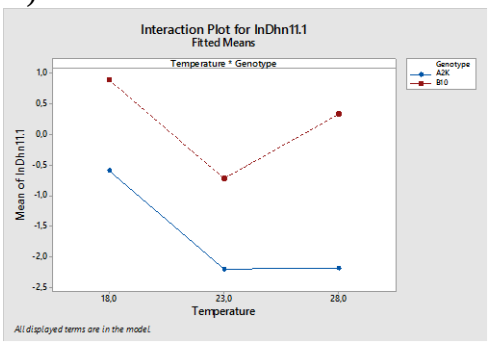
Figure 3. The effect of EpI-temperature on the expression of DHN9 in mature somatic embryos of two genotypes of Norway spruce. The results are shown as Interaction plot (A), main effect plot (B), means (C), analysis of variance (D) and residual plots (E) for the In-transformed observations for DHN9.

DHN11.1

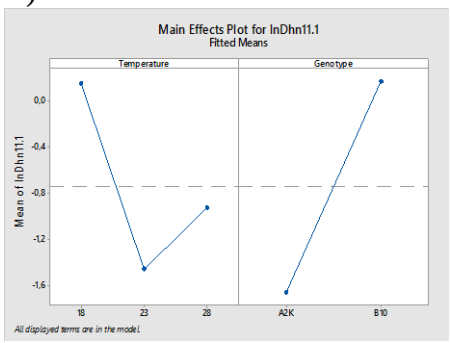
As shown in figure 4, panel A, no interaction was detected between the factors genotype and temperature for DHN11.1. The main effects plot and the fitted means are shown in panel B and C respectively. They show a temperature effect by decreasing transcript levels from the cold temperature to the middle temperature, before increasing levels between middle temperature and warm temperature. Also, they show that genotype has an effect on the levels of transcripts manifested as higher levels in the B10 genotype than in A2K. According to the ANOVA (panel D), the p-value for both temperature and genotype are significant, and the p-value for the lack of interaction between the two factors is high. The residual plots (panel E) do not show crucial deviations.

The post hoc test (figure 5) shows that the fitted means from temperature 18˚C is significantly different from the two other temperatures.

A)



B)



C)

Means

Term	Fitted Mean	SE Mean
Temperature		
18	0,152	0,267
23	-1,458	0,267
28	-0,925	0,267
Genotype		
A2K	-1,657	0,218
B10	0,171	0,218
Temperature*Genotype		
18 A2K	-0,590	0,378
18 B10	0,894	0,378
23 A2K	-2,199	0,378
23 B10	-0,716	0,378
28 A2K	-2,184	0,378
28 B10	0,334	0,378

D)

Analysis of Variance for InDhn11.1

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature	2	8,070	4,0352	9,43	0,003
Genotype	1	15,036	15,0357	35,13	0,000
Temperature*Genotype	2	1,071	0,5354	1,25	0,321
Error	12	5,136	0,4280		
Total	17	29,313			

E)

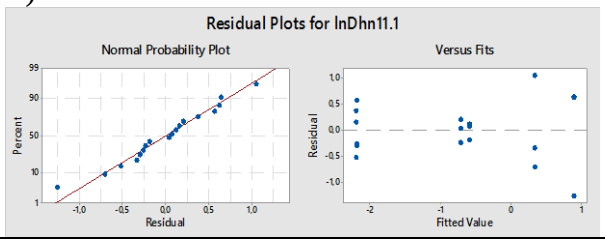


Figure 4. The effect of EpI-temperature on the expression of DHN11.1 in mature somatic embryos of two genotypes of Norway spruce. The results are shown as Interaction plot (A), main effect plot (B), means (C), analysis of variance (D) and residual plots (E) for the In-transformed observations for DHN11.1.

1)

Tukey Pairwise Comparisons: Temperature Grouping Information Using the Tukey Method and 95% Confidence

Temperature	N	Mean	Grouping	
18	6	0,15221	A	
28	6	-0,92469		B
23	6	-1,45759		B

Means that do not share a letter are significantly different.

2)

Tukey Simultaneous Tests for Differences of Means

Difference of Temperature Levels	Difference of Means	SE of Difference	Simultaneous 95% CI	T-Value	Adjusted P-Value
23 - 18	-1,610	0,378	(-2,617; -0,603)	-4,26	0,003
28 - 18	-1,077	0,378	(-2,084; -0,070)	-2,85	0,036
28 - 23	0,533	0,378	(-0,474; 1,540)	1,41	0,366

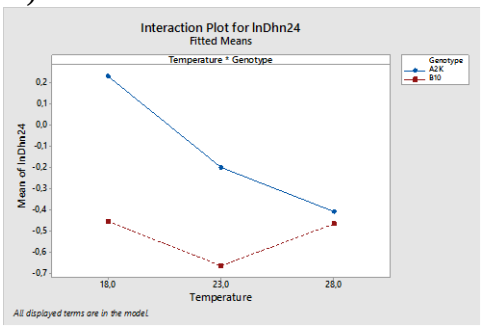
Individual confidence level = 97,94%

Figure 5. Post hoc test for the In-transformed observations of DHN11.1. The results are shown as Tuckey pairwise comparision (panel 1) and Tuckey simultaneous tests for differences of means (panel 2).

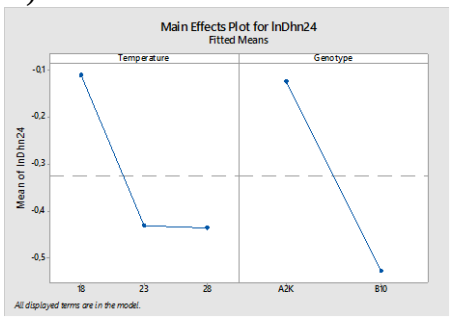
DHN24

As shown in figure 6, panel A, no interaction was detected between the factors genotype and temperature for DHN24. The ANOVA (panel D) shows a high p-value for this. The main effects plot (panel B) and the fitted means (panel C) shows an effect of temperature by decreasing transcript levels from the cold temperature to the middle temperature, but the ANOVA (panel D) shows a high p-value for this finding. Also, the main effect plot (panel B) and the fitted means (panel C) shows an effect from genotype has an effect on the levels of transcripts manifested as lower levels of transcripts in the B10 genotype than in A2K. According to the ANOVA (panel D), the p-value for this finding is significant. The residual plots (panel E) do not show crucial deviations.

A)



B)



C)

Means

Term	Fitted Mean	SE Mean
Temperature		
18	-0,110	0,159
23	-0,430	0,159
28	-0,435	0,159
Genotype		
A2K	-0,124	0,130
B10	-0,527	0,130
Temperature*Genotype		
18 A2K	0,232	0,225
18 B10	-0,453	0,225
23 A2K	-0,198	0,225
23 B10	-0,663	0,225
28 A2K	-0,407	0,225
28 B10	-0,464	0,225

D)

Analysis of Variance for lnDhn24

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature	2	0,4162	0,2081	1,37	0,292
Genotype	1	0,7280	0,7280	4,78	0,049
Temperature*Genotype	2	0,3035	0,1518	1,00	0,398
Error	12	1,8291	0,1524		
Total	17	3,2769			

E)

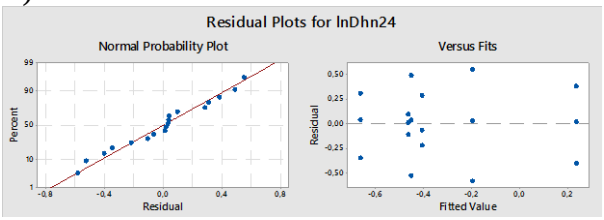
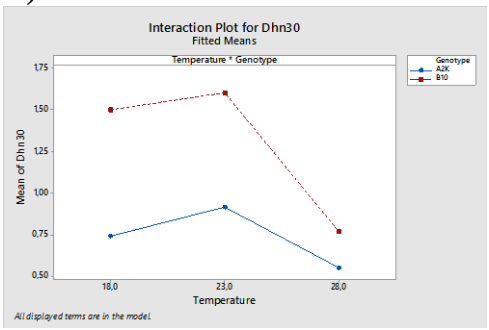


Figure 6. The effect of Epi-temperature on the expression of DHN24 in mature somatic embryos of two genotypes of Norway spruce. The results are shown as Interaction plot (A), main effect plot (B), means (C), analysis of variance (D) and residual plots (E) for the In-transformed observations for DHN24.

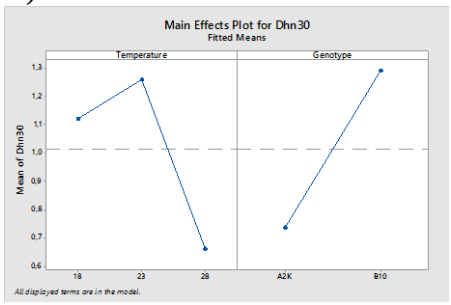
DHN30

As shown in figure 7, panel A, no interaction was detected between the factors genotype and temperature for DHN30. The ANOVA (panel D) shows a high p-value for this. The main effects plot (panel B) and the fitted mean (panel C) shows an effect of temperature by increasing transcript levels from the cold temperature to the middle temperature before a steep decrease in levels between middle temperature and warm temperature. The ANOVA (panel D) shows that the p-value for the temperature effect is high. For genotype, the main effect plot (panel B) and the fitted mean (panel C) shows an effect on the levels of transcripts manifested as higher levels in the B10 genotype than in A2K. According to the ANOVA (panel D), the p-value for the genotypic effect is over, but not far from 0.05. The residual plots (panel E) do not show crucial deviations.

A)



B)



C)

Means

Term	Fitted Mean	SE Mean
Temperature		
18	-0,342	0,615
23	-0,397	0,615
28	-1,282	0,615
Genotype		
A2K	-0,770	0,502
B10	-0,577	0,502
Temperature*Genotype		
18 A2K	-0,605	0,870
18 B10	-0,079	0,870
23 A2K	-0,577	0,870
23 B10	-0,216	0,870
28 A2K	-1,127	0,870
28 B10	-1,436	0,870

D)

Analysis of Variance for Dhn30

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature	2	1,1772	0,5886	1,81	0,206
Genotype	1	1,3817	1,3817	4,25	0,062
Temperature*Genotype	2	0,2570	0,1285	0,39	0,682
Error	12	3,9045	0,3254		
Total	17	6,7203			

E)

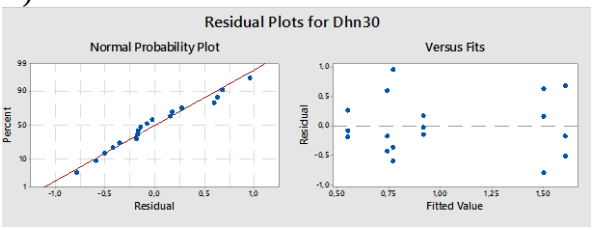
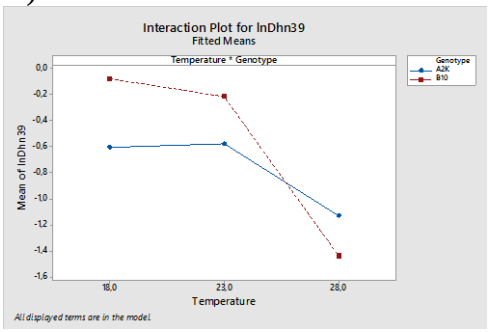


Figure 7. The effect of EpI-temperature on the expression of DHN30 in mature somatic embryos of two genotypes of Norway spruce. The results are shown as Interaction plot (A), main effect plot (B), means (C), analysis of variance (D) and residual plots (E) for the observations for DHN30.

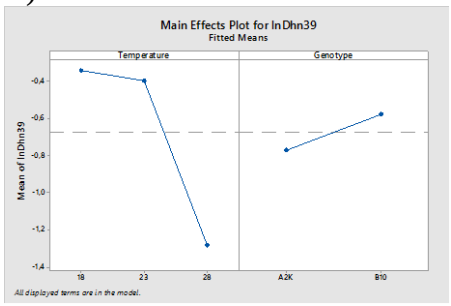
DHN39

As shown in figure 8, panel D, the P-values for effect of temperature, genotype and interaction between them are far too high to take the results for this DHN into account.

A)



B)



C)

Means

Term	Fitted Mean	SE Mean
Temperature		
18	1,121	0,233
23	1,259	0,233
28	0,661	0,233
Genotype		
A2K	0,737	0,190
B10	1,291	0,190
Temperature*Genotype		
18 A2K	0,743	0,329
18 B10	1,500	0,329
23 A2K	0,916	0,329
23 B10	1,603	0,329
28 A2K	0,552	0,329
28 B10	0,770	0,329

D)

Analysis of Variance for the lnDhn39

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature	2	3,3387	1,6693	0,73	0,500
Genotype	1	0,1675	0,1675	0,07	0,791
Temperature*Genotype	2	0,5867	0,2934	0,13	0,880
Error	12	27,2605	2,2717		
Total	17	31,3535			

E)

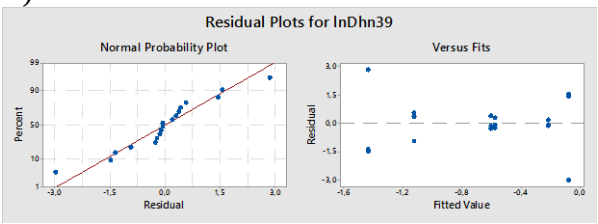
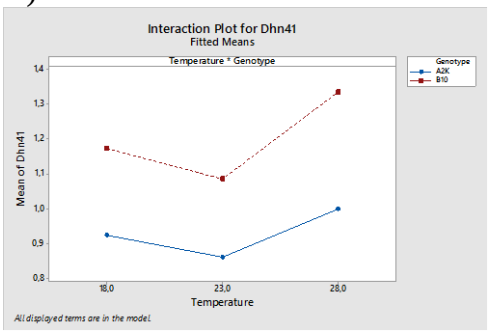


Figure 8. The effect of EpI-temperature on the expression of DHN39 in mature somatic embryos of two genotypes of Norway spruce. The results are shown as Interaction plot (A), main effect plot (B), means (C), analysis of variance (D) and residual plots (E) for the In-transformed observations for DHN39.

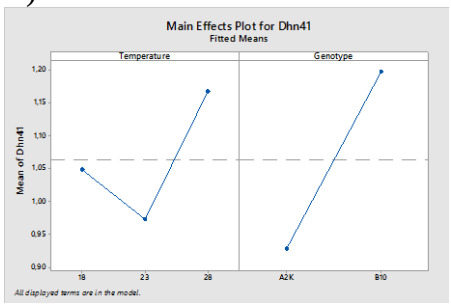
DHN41

As shown in figure 9, panel A, there was no interaction between the factors genotype and temperature for DHN41, but according to the ANOVA (panel D) the p-value for this is high. For temperature, the main effects plot (panel B) and fitted means (panel C) shows an effect by decreasing transcript levels from the cold temperature to the middle temperature before an increase in levels between middle temperature and warm temperature. The ANOVA (panel D) shows a high p-value for this finding. For genotype, the main effects plot (panel B) and fitted means (panel C) an effect manifested as higher levels of transcripts in the B10 genotype than in A2K. According to the ANOVA (panel D), this finding is significant. The residual plots (E) do not show crucial deviations.

A)



B)



C)

Means

Term	Fitted Mean	SE Mean
Temperature		
18	1,0489	0,0714
23	0,9734	0,0714
28	1,1676	0,0714
Genotype		
A2K	0,9285	0,0583
B10	1,1981	0,0583
Temperature*Genotype		
18 A2K	0,925	0,101
18 B10	1,173	0,101
23 A2K	0,861	0,101
23 B10	1,086	0,101
28 A2K	1,000	0,101
28 B10	1,335	0,101

D)

Analysis of Variance for Dhn41

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature	2	0,11495	0,057475	1,88	0,195
Genotype	1	0,32701	0,327008	10,69	0,007
Temperature*Genotype	2	0,01017	0,005083	0,17	0,849
Error	12	0,36694	0,030579		
Total	17	0,81907			

E)

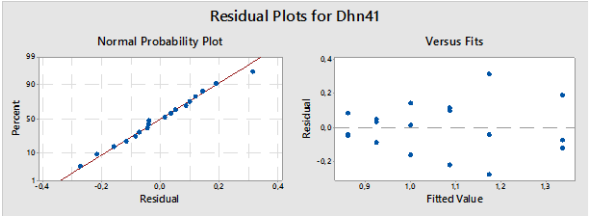


Figure 9. The effect of EpI-temperature on the expression of DHN41 in mature somatic embryos of two genotypes of Norway. The results are shown as Interaction plot (A), main effect plot (B), means (C), analysis of variance (D) and residual plots (E) for DHN41.

Summary of all results

Genotype had a significant effect and was more important than temperature for DHN1, DHN9, DHN11.1, DHN24 and DHN41. For DHN30, the p-value for the effect of genotype was close to significant. Overall, the level of DHN transcripts were higher in B10 than A2K, except for DHN24 showing an opposite trend. Temperature had a significant effect only for the expression of DHN11.1. The expression of the other DHNs were not affected by temperature.

5.0 Discussion

Temperature is an abiotic factor that can lead to changes in plants gene expression, and because both genotype and epigenetic factors may influence the way the genes are expressed, it was expected that the somatic embryos from the different temperature conditions could show differences in transcription levels of one or more DHNs. The results in this master thesis suggests a higher level of expression of DHNs in embryos from the colder conditions versus the warmest one. However, the differences were minor in genotype A2K and more distinct in B10. Also, only one DHN demonstrated the postulated epigenetic effect significantly. This leads us to conclude that the differences in epitype inducing temperature have much less impact on transcription of the selected DHNs in mature embryos, than the differences in genotypes do have.

5.1 Genotype effects on DHNs

The distinct and statistically significant differences in transcription levels of DHNs between the genotypes showed in the results of this master thesis, was to be expected, due to the fact that different genotypes differ in their gene sequence in some of their alleles and regulatory regions in the genome. The genotypic differences between A2K and B10 makes them express their genes differently, leading to different phenotypical traits like sensitivity for temperature (Klug et al. 2010).

5.1.1 B10 might be more robust than A2K

Differences in transcription patterns of DHNs between genotypes are previously described, e.g in Danyluk et al (1998) where differences in DHNs expression levels correlates with the genotypes

different capacities in tolerance. Hence, this might also be the case for A2K and B10 in this present master thesis. Abiotic factors like drough and frost, in addition to desiccation of the embryo, are factors that all leads to dehydration of plant cells, which plants must protect themselves from by the expression of DHNs. Because DHNs functions may overlap (Yang et al. 2012), the general higher transcript levels in B10 versus A2K might be because B10s genotype makes it better protected against dehydrative stress during embryogenesis and seed dormancy, or makes it better suited for stress responsiveness to protect itself from abiotic stress, or a combination of both. Either way, as B10 showed generally higher levels of the DHNs, and the DHNs do in some way or another protect the embryo against dehydration related damages, B10 can be hypothesized as being more robust than A2K. Thus, B10 would maybe be the better choice for breeding and planting purposes. Further testing should be performed in future studies before making a conclusion on this matter.

5.1.2 Epigenetic modifications set in the zygotic seed may persist in the somatic embryos

Most gene products function inside the cell, and cells interact with each other, and the organism are influenced by environmental factors (Klug et al. 2010). Therefore, a combination of genotype and epigenetic factors is likely to modify the gene expression in the cells and the resulting phenotype (Klug et al. 2010). Thus, phenotypic differences between two genotypes of the same species can be resulting both from their DNAs genetic and epigenetic differences, and/or from a combination of them. Therefore, the phenotypic differences between the two zygotic seeds (A2K and B10) used for induction of the somatic embryos for this master thesis, maybe not be resulting from differences in their DNAs base sequence alone, but also in differences in their chromatin structure. A2K were produced during a controlled cross performed in outdoors-conditions, and B10 was produced during a controlled cross in warmer environments; in a greenhouse. These environmental factors might have lead to epigenetic mechanisms in some way altering the chromatin structure. Such a phenomena could contribute to a greater differences between zygotic seed A2K and zygotic seed B10, than their base sequence alone. If such modifications were established during the early steps of their embryogenesis, they might cause an epigenetic memory establishment (Kvaalen and Johnsen, 2008). If so, the modifications may have been passed on to the somatic embryos inducted from the zygotic seed. The somatic embryos may then “remember”

the temperature conditions from the embryogenesis of their respectively explants (=the zygotic seeds), like previously shown for buds, by Carneros et al (2017). This might be contributing to greater variation of gene expression in the two genotypes of somatic embryos than the differences in the base sequence alone.

5.2. Temperature effects on DHNs

Norway spruce is a species that have effectively reinvaded large areas following the recent ice ages, and are well adapted to the local environments, despite their long generation times (Yakovlev et al. 2008), and some are hypothesizing this to be caused by their epigenetic capacity (Yakovlev et al. 2014). Epigenetic mechanisms do exist in this species, and an epigenetic memory regulates bud phenology and cold acclimation, as shown in studies of Norway spruces resulting from zygotic embryogenesis in warm versus cold conditions (Bjørnstad, 1981; Johnsen 1989a and b; Skrøppa et al. 2007 and 2010; Yakovlev et al 2012 and 2014). Also the expression of DHNs have been shown to be under the influence of temperature in Norway spruce (Yakovlev et al, 2008; Kjellsen et al, 2013; Carneros et al. 2017). Despite all this, the only DHN showing statistically significant effect from temperature in this master thesis was DHN11.1. This DHN was not tested by Carneros et al (2017) or in any of the other studies summarized in chapter 2.4.6 of this master thesis (Danyluk et al. 1998; Yakovlev et al, 2008; Yang et al. 2012). This is a novel finding. According to the post hoc test, the expression is significantly different in embryos developed at the lowest temperature (18°C). Even though this temperature is far from freezing and actually the conditions closest to field conditions in Norway, it might still be low enough to cause more accumulation of DHNs to protect the cells. On the other hand, it might also be possible that the higher epitype inducing temperatures influence transcription factors to cause a reduction of DHNs related to frost tolerance. In order to gain knowledge about whether it is the levels of mRNA in the embryos developed in the colder temperature that are the abnormal ones or if actually the samples from the two warmer conditions are, additional testing must be done, including testing of transcription factors in embryos from the two warmer temperatures, to check for the mentioned possibility.

5.2.1 Why would epitypes of the same genotype express different levels of DHNs?

It is well known that epigenetic mechanisms can lead to structural changes of chromatin that are more stable over time than the transient chromatin changes needed for transcription, and that this may lead to epigenetic effects on gene expression (Taiz and Zeiger, 2010; Reece et al. 2011; Wolpert et al. 2011). The differential transcription levels of DHN11.1 between genetically identical somatic embryos from epitype inducing temperatures in this present master thesis indicates that transcription is influenced by the epitype inducing temperature during embryogenesis, as described by others (e.g Kvaalen and Johnsen, 2008; Yakovlev et al. 2016). Because epitypes are variants of the same genotype, the differential transcription patterns is resulting from epigenenic modifications that alters chromatin structure, rather than differences in the base sequence of DNA (Yakovlev et al. 2014). The induction of the callus did not involve different temperature treatments, one can assume that all developing somatic embryos in the two cultures during that period were still of the same genotype and epitype as its explants (zygotic seed B10, zygotic seed A2K, respectively). Thus, it is likely that the epigenetic modifications were induced after dividing of the embryos of the same genotype into three different treatments. Now, they are still the same genotype, but they are no longer expressing the same genes at the same rates, at least for the expression of DHN11.1, which is now different from the lowest temperature epitypes and the two other temperatures.

5.2.2 May the somatic embryos be removing old marks or setting new ones?

It is known that external cues can lead to adding or removal of specific epigenetic marks in the chromatin, leading to transcriptional changes (Watson et al, 2014), and for Norway spruce, temperature both during somatic and zygotic embryogenesis may lead to epigenetic changes (Kvaalen and Johnsen 2008). Therefore, the epigenetic modifications leading to the differential transcript patterns observed for DHN11.1 in this master thesis may have been induced during the embryogenesis of the somatic embryos in epitype inducing temperatures, or during the first stages of development of the *zygotic seeds* the somatic embryos are derived from. In the case of the latter, the epigenetic modifications induced in the zygotic seed(s) must have passed on from the mother cells (in the zygotic seed(s)) to the daughter cells (in the cells dividing in the callus). In other words, they are imprinted as an epigenetic memory (Alberts et al. 2014) in the cells of the developing somatic embryos. Thus, an interesting possibility may be raised; may the

epigenetic changes induced during the *somatic* embryogenesis in the lowest epitype inducing temperature in this master thesis, be a consequence of the *removing* epigenetic marks that were set and imprinted in the *zygotic* seed, or is it rather a consequence of completely *new marks* being set in the developing *somatic embryos*. Or is it maybe a combination of both.

Thus, in order to gain understanding of the modifications behind the differential transcription pattern for DHN11.1, testing for various epigenetic regulators could be performed. After all they do regulate transcription; one of the hallmarks of epigenetics. Epigenetic modifications proven to make an impact on gene expression include incorporation of histone variants (Watson et al, 2014), histone methylation (Yakovlev et al. 2016) and the action of long and short non-coding RNA (Van Oosten et al. 2014; Yakovlev et al. 2016)).

The crucial genes underlying the phenomenon of epigenetic memory are coding for regulators such as DNA methylases (=”a writer”) and DNA demetyltransferase (“=an eraser”) (Yakovlev et al. 2016). Their transcriptional indications would be better to study in earlier stages of development as the modifications leading to an epigenetic memory is believed to be established exclusively during the initial stages of the embryogenesis (Kvaalen and Johnsen, 2008).

5.2.3 Epitype inducing temperatures may be remembered by the tree long after planting

Epitype inducing temperature conditions from zygotic embryogenesis is remembered by the tree long after planting (Skrøppa, 2007; Csrneros et al. 2017). Thus, chances are that the epitypes from this master thesis experiments would show differences in their DHNs transcription levels, if they were germinated and the seedlings planted. In that case, based on the main effect plot from this study, it can be hypothesized that the somatic embryos from the lowest epitype inducing temperature will be more protected from damages related to dehydration and desiccation. This seems to be true at least for DHN 11.1. It should be noted that the moderate temperature effect for the other DHNs makes the assumption an uncertain one as a general rule for all DHNs. Further studies is needed before making any firm conclusions, because the differences in DHNs levels observed here in mature embryos may not be a good measure for the epigenetic effect in Norway spruce but rather mainly temperature and genetic effects.

5.3 The DHNs chosen were maybe not the right ones to choose

One might ask what reasons could explain that there's only one DHN showing significant temperature effect. One reason might be that the DHNs chosen were not the right ones in order to look for epigenetic effects from temperature in embryos. For trees, high levels of DHNs have been associated with tolerance to freezing temperatures, winter dormancy and protection against water stress (Basset et al. 2006; Yakovlev et al. 2008; Perdiguero et al. 2012; Eldhuset et al. 2013; Kjellsen et al. 2013; Strimbeck et al. 2015). Downregulated levels are associated with bud burst (Yakovlev et al. 2008; Carneros et al. 2017). As we already know, DHNs have shown different transcription patterns between vegetative tissue and embryogenic tissue in other species (Yang et al, 2012). Therefore, it is not given that the same DHNs showing transcriptional effect from temperature in vegetative tissue like buds would show the same kind of effect in embryos. This, in turn, might be related to the specific DHNs functions (Graether and Boddington, 2014). The DHNs possible ability to create local pools of water molecules that sustain the metabolic processes during stress and re-growth (Rinne et al. 1999), and to decrease damages during freezing temperatures (Wisniewsky et al. 1999) by possibly preventing membrane-membrane interactions to stop or counteract water loss (Strimbeck et al. 2015) are likely more important in vegetative tissue, than in mature embryos. After all, the mature embryos naturally should *loose* water in order to survive until germination. Therefore, protection against desiccation are a more likely main function for DHNs in mature embryos than frost tolerance and temperature stress is.

DHNs from this thesis are maybe more likely parts of the normal protection of the mature embryo, then part of its "first line stress response DHNs", but some DHNs may also have overlapping functions. This is indicated in Yang et al (2012), where DHNs is found during "normal conditions" in plant tissue, and also is accumulated in tissue induced to abiotic stress. DHN11.1 might very well have such an overlapping function by possibly playing a natural protective role in mature embryos, while also being one of the DHNs that play a role in a stress response against abiotic factors.

5.4 Mature somatic embryos might not be the optimal stage

Maybe the minor epigenetic effect of temperature and the more distinct effect of genotype in somatic embryos shown in this master thesis is not peculiar at all – even in Norway spruce, a

species which is believed to have a great epigenetic capacity. After all, embryogenesis is a process considered strictly underlying genetic control (Wolpert et al. 2011). Successful development and maturation of embryos are in nature crucial for the survival of the species. If the embryo fail to develop or mature properly, the chances for its survival and later germination into a new plant are low. Should embryogenesis easily be disturbed by abiotic factors, the embryos might would have to start downregulating its DHNs needed in natural development and maturation, in order to upregulate DHNs needed for protection against the abiotic factors. Not only could this disturb the embryogenesis in earlier stages, but for mature embryos it would mean that they would have to become metabolically active, in order to upregulate DHNs. We know that the mature embryos loose water content and become metabolically inactive in order to protect its cell membranes and cell constituents (Galau et al. 1986; Taiz and Zeiger, 2010). Becoming metabolically active before the time is right, could potentially harm the embryo, jeopardizing its chances of survival until germination. Hence, mature somatic embryos may continue expressing the DHNs needed as part of natural maturation, instead of upregulating the expression at this time, even in the presence of temperature conditions, which in seedlings and grown plants would lead to epigenetic transcriptional changes.

5.5. Statistical and experimental bias

The sample size in this master thesis was quite small. A greater sample sizes could have made a statistical better foundation for the conclusions made in this thesis.

The though the effects in this master thesis appear to be mainly genetic, with minor effect from temperature on transcription of DHNs, it cannot be completely ruled out that some of those apparent genotypic differences are epigenetic and that these were not established during somatic embryogenesis.

It should also be mentioned that even though *in vitro* somatic embryogenesis mimics how the process of zygotic embryogenesis works *in vivo,* somatic embryogenesis is still a “staged” system. Thus, the transcription patterns of the selected DHNs is not a completely accurate picture of their transcription pattern *in vivo*. Also, it is not to be forgotten that in the cells it is not the mRNA that performs the protective function, rather it is the protein itself. The protein levels may

be different from transcript levels as posttranscriptional regulations may affect the amount of mRNA that in the cells are translated into proteins.

6.0 Conclusions

The main conclusion from this study is that genotype is more important than epitype for gene expression of DHNs in mature SE of Norway spruce. The observed differences between the genotypes may also include an epigenetic memory established in the two zygotic seeds and passed on to the SEs during somatic embryogenesis. It is possible that the observed differences on DHNs transcription between epitypes in this study may be a consequence of the SEs removing epigenetic marks "inherited" from zygotic seed, and/or the setting of new marks in the SEs themselves. It would be better to study the transcriptional indications of epigenetic memory in earlier stages of development as these are likely more crucial in forming the epigenetic marks (Kvaalen and Johnsen, 2008) underlying the differences later seen in Norway spruce epitype trees.

In epitypes it is known that DHNs are differentially expressed in buds (Carneros et al. 2017) but mature embryos are a very different tissue in some state of dormancy and thus it is likely that they will need to express DHNs as part of natural maturation, instead of prioritizing upregulating DHNs at this stage. Therefore, in retrospect, it may not be surprising that transcription of DHNs is not easily epigenetically impacted at this time. The possibility remains that there are differences at the protein level but this remains to be examined.

7.0 References

Alberts B, Johnson A, Lewis J, Morgan D, Raff M, Roberts K, Walters P (2014) Molecular Biology of the Cell (sixth edition) New York: Garland Publishing Inc

Baulcombe DC, Dean C (2014) Epigenetic regulation in plant responses to the environments. Cold Spring Harbor Perspectives in Biology 6(9), retrieved from http://cshperspectives.cshlp.org/content/6/9/a019471.full.pdf+html

Basset CL, Wiesniewsky ME, Timothy SA, Norelli L, Renaut J, Farrell RE (2006) Global analysis of genes regulated by low temperature and photoperiod in peach bark. Journal of the American Society for Horticultural Science 131 (4):551-563

Bjørnstad Å (1981) Photpperiodical after effect of parent plant environment in Norway spruce (Picea abies (L.) Karst.) seedlings. Meddelelser fra Norsk Institutt for Skogforskning

Bräutigam K, Vining KJ, Lafon-Placette C, Fossdal CG, Mirouze M, Marcos JG, Fluch S, Fraga MF, Guevara MÀ, Abarca D, Johnsen Ø, Maury S, Strauss SH, Campbell MM, Rohde A, Diaz-Sala C, Cervera M-T (2013). Epigenetic regulation of adaptive responses of forest tree species to the environment. Ecology and Evolution 3(2): 399-415

Campbell SA, Close TJ (1997) Dehydrins: genes, proteins and associations with phenotypic traits. New Phytologist 137:61-74

Carneros E, Yakovlev IA, Viejo M, Olsen JE, Fossdal CG (2017) The epigenetic memory of temperature during embryogenesis modifies the expression of bud burst-related genes in Norway spruce epitypes. Planta 246:553-566

Chrisp PA, Ganguly D, Eictern SR, Borevitz JO, Pogson BJ (2016) Reconsidering plant memory: intersections between stress recovery. RNA turnover and epigenetics. Science Advances 2(2):el501340

Danyluk J, Perron A, Houde M, Limin A, Fowler B, Benhamou N, Sarhan F (1998). Accumulation of an acidic dehydrin in vicinity of the plasma membrane during cold acclimation of wheat. The Plant Cell 10:623-638

Dawson MA, Kouzarides T (2012) Cancer epigenetics: From Mechanisms to Therapy. Cell 150 (1):12-27

D`Urso A, Brickner JH (2014) Mechanisms of epigenetic memory. Trends in Genetics 30(6):230-236

Eldhuset TD, Nagy NE, Volarik D, Børja I, Gebauer R, Yakovlev IA, Krokene P. (2013) Drought affects tracheid structure, dehydrin expression, and above and belowground growth in 5-year old Norway spruce. Plant Soil 388:305-320

Graether, SP, Boddington KF (2014) Disorder and function: a review of the dehydrin protein family. Frontiers in Plant sciences 5 (576):1-12

Heard E, Martienssen RA (2014) Transgenerational epigenetic inheritance: myths and mechanisms. Cell 157:95-109

Herman JJ, Sultan SE (2011) Adaptive transgenerational plasticity in plants: case studies, machanisms, and implications for natural populations. Frontiers in Plant Sciences 2:1-10

Hvoslef-Eide AK, Corke FMK (1997) Embryogenesis specific changes in birch suspension cultures. Plant Cell, Tissue and Organ Culture 51(1):35-41

Hundtermark M, Buitnik J, Leprince O, Hicha DK (2011) The reduction of seed specific dehydrins reduces seed longevity in *Arabidopsis thaliana.* Seed Science Research 21:165:173

Johnsen Ø (1989a) Phenotypic changes in progenies of northern clones of Picea abies (L.) Karst. Grown in a southern seed orchard. I. Frost hardiness in phytotron experiment. Scandinavian Journal of Forest Research 4;331-341

Johnsen Ø (1989b) Phenotypic changes in progenies of northern clones of Picea abies (L.) Karst. Grown in a southern seed orchard. II. Seasonal growth rhythm and height in field trials. Scandinavian Journal for Forest Research 4:317-330

Kjellesen TD, Yakovlev IA, Fossdal CG, Strimbeck R (2013) Dehydrin accumulation and extreme low temperature tolerance in Siberaian spruce (Picea obovate) Tree Physiology 33 (12):1354-1366

Klug WS, Cummings MR, Spencer CA, Palladino MA (2010) Essentials of genetics (seventh edition). San Fransisco: Pearson/Benjamin Cummings

Koag MC, Fenton RD, Wilkens S, Close TJ (2003) The binding of maize DHN1 to lipid vesicles. Gain of structure and lipid specificity. Plant physiology 131:309-316

Kvaalen H, Johnsen O (2008= Timing of bud set in Picea abies is regulated by a memory of temperature during somatic and zygotic embryogenesis. New Phytologist 177:309-316

Perdiguero P, Barbero MC, Cervera MT, Soto A, Collada C (2012) Novel conserved segments are associated with differential expression patterns for Pinaceae dehydrins. Planta 236:1863-1874

Pikaard CS, Mittelsen Scheid O (2014) Epigenetic regulation in plants. Cold spring Harbour Perspectives in Biology 6(12):a019315

Raghavan V (1976) Adventive embryogenesis: induction of diploid embryos. In: Experimental embryogenesis in vanscular plants. London: Academic Press 349-381

Reece JB, Urry LA, Cain M, Wesserman SA, Minorsky PV, Jackson RB (2011) Campbell Biology (ninth edition) San Fransisco: Pearson/ Benjamin Cummings

Raven PH, Evert RF, Eichorn SE (2008) Biology of plants (seventh edition) New York: W.H Freeman and Company Publishers

Rinne PL, Kaikuranta PL, van der Plas LH, van der Schoot C (1999) Dehydrins in cold acclimated aspices of birch (*Betula pubescens* Ehrh): production, localization and potential role in rescuing enzyme function during dehydration. Planta 209:377-388

Skrøppa T (2003) Picea abies – Norway spruce; Technical guidelines for genetic conservation and use. PDF retrieved from: http://www.euforgen.org/fileadmin/templates/euforgen.org/upload/Publications/Technical_guidelines/856_Technical_guidelines_for_genetic_conservation_and_use_for_Norway_spruce__Picea_abies_.pdf

Skrøppa T, Kohmann K, Johnsen Ø, Steffenrem A, Edvardsen ØM (2007) Field performance and early test resuts of offspring from two Norway spruce seed orchards containing clones

transferred to warmer climates. Canadian Journal of Research 37:515-522

Skrøppa T, Tollefsrud MM, Sperisen C, Johnsen Ø (2010) Rapid change in adaptive performance from one generation to the next in *Picea abies* – Central European trees in Nordic environments. Tree Genetics & Genomes 6:93-99

Slater A, Scott NW, Fowler MR (2008) Plant Biotechnology the genetic manipulation of plants (second edition) New York: Oxford University Press

Smertenko A, Bozhkov P (2014) Somatic embryogenesis: life and death processes during apical-basal patterning. Journal of experimental Botany 65(5):1343-1360

Steponkus PL (1984) Role of the plasma membrane in freezing injury and cold acclimation. Annual Reviev of Plant Physiology 35:543-584

Strimbeck GR, Schaberg PG, Fossdal CG, Schöder WP, KJellsen TD (2015) Extreme low temperature tolerance in woody plants. Frontiers in Plant Science

Takahashi D, Li B, Nakayama T, Kawamura Y, Uemura M (2013) Plant plasma membrane proteomics for improving cold tolerance. Frontiers in plant sciences 4 (Article 90) 1-5

Taiz L, Seiger E (2010) Plant Physiology (fifth edition) Massachussets: Sinauer Associates Inc

Van Osten MJ, Bressan RA, Zhu J-K, Bohnert HJ, Chinnusamy V (2014) The role of the epigenome in gene expression and control and the epimark changes in response to the environment. Chritical Reviews in Plant Sciences 33(1):64-84

Von Arnold S, Sabala I, Bozhkov P, Dyachock J, Filonova L (2002) Developmental pathways of somatic embryogenesis. Plant Cell, Tissue and Organ Culture 69:233-249

Watson JD, Baker TA, Bell SP, Gann A, Levine M, Losic R (2014) Moelcular Biology of the gene (seventh edition). New York: Cold Spring Harbor Laboratory Press

Watson JD, Crick FHCA (1953) Structure for deoxyribose nuclei acid. Nature 171: 737-738

Wiesniewsky ME, Webb R, Balsamo R, Close TJ, Yu-Xiau M, Griffith M (1999) Purification, immunolocalization, cryoprotective and antifreeze activity of PCA60: a dehydrin from peach (*Prunus persica*) Plant Physiology 105:600-608

Winkelmann T (2016) Somatic versus Zygotic Embryogenesis: Learning from Seeds. Methods in Molecular Biology 1359:25-46

Wolpert L, Tickle C, Jessel T, Lawrence P, Meyerowitz E, Robertson E, Smith J (2011) Principles of development (fourth edition) New York: Oxford University Press

Yakovlev IA, Asante DKA, Fossdal CG, Partanen J, Juntila O, Johnsen Ø (2008) Dehydrins expression related to timing of bud burst in Norway spruce. Planta 228:459-472

Yakovlev IA, Fossdal CG, Johnsen Ø (2010) MicroRNAs, the epigenetic memory and climatic adaption in Norway spruce. New Phytologist 187:1154-1169

Yakovlev IA, Fossdal CG, Skrøppa T, Olsen JE, Jahren AH, Johnsen Ø (2012) An adaptive epigenetic memory in conifers with important implications for seed production. Seed Science Research 22(2)63:76

Yakovlev IA, Lee YY, Rotter B, Olsen JE, Skrøppa T, Johnsen Ø, Fossdal CG (2014) Temperature-dependent differential transcriptomes during formation of an epigenetic memory in Norway spruce embryogenesis. Tree Genetics & Genomes 10:355-366

Yang Y, Mingyang H, Zhu Z, Li S, Xu Y, Zhang C, Singer SD, Wang Y (2012) Identification of the dehydrin gene family from grapevine species and analysis of their responsiveness to various forms of abiotic and biotic stress. BMC Plant Biology 12:140

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