Riina Muilu-Mäkelä

POLYAMINE METABOLISM OF SCOTS PINE UNDER ABIOTIC STRESS
RIINA MUILU-MÄKELÄ

POLYAMINE METABOLISM OF
SCOTS PINE UNDER ABIOTIC
STRESS

Academic dissertation to be presented with the assent of
the Doctoral Training Committee of Health and
Biosciences of the University of Oulu for public defence in
Kaljusensali (KTK112), Linnanmaa, on 11 December 2015,
at 13 p.m.
Muilu-Mäkelä, Riina, Polyamine metabolism of Scots pine under abiotic stress.
University of Oulu Graduate School; University of Oulu, Faculty of Science; Natural Resources Institute Finland (Luke), Parkano Research Unit
University of Oulu, P.O. Box 8000, FI-90014 University of Oulu, Finland

**Abstract**

Changes in climate will impose abiotic stress on plant species, and eventually affect their distributional range. This is a particular challenge especially to tree species with long generation times and slow rates of evolution. In the boreal zone, most of these species also hold high economic value. The aim of the study was to enhance understanding of the abiotic stress coping strategies of Scots pine (*Pinus sylvestris* L.) in its vulnerable early growth phase. In particular, the study evaluates the role of polyamine (PA) metabolism of Scots pine under abiotic stress. Polyamines (PAs) are small, ubiquitous nitrogenous compounds involved in fundamental biological processes of plants such as growth, organogenesis, embryogenesis and abiotic and biotic stress defence.

The effects of different water availabilities and spring frost temperatures on shoots and roots of young seedlings were investigated in two growth chamber experiments. Proembryogenic liquid cell culture system was established and used as a controllable platform. Effects of polyethylene glycol (PEG) induced osmotic stress was investigated at the cellular level in liquid cultures. Responses to treatments were evaluated by quantifying changes in the expression of PA metabolism, stress, cell death and cell division-related genes, metabolite concentrations, as well as stress-induced physiology and morphology.

The results revealed that PA metabolism is tissue specific and strictly regulated. Drought stress induced the accumulation of putrescine (Put) in photosynthesizing tissues, whereas the prime response to spring frost was a reorganization of root growth. Generally, drought and osmotic stress decreased the expression of PA catabolizing genes, leading to consistent PA levels in tissues even under severe stress. To conclude, PAs have a protective role in maintaining the growth and development of pine tissues subjected to abiotic stress.

**Keywords:** abiotic stress, development, embryogenic cells, gene expression, liquid cell culture, phenylpropanoids, polyamines, Scots pine
Muilu-Mäkelä, Riina, Polyamiiniaineenvaihdunnan rooli metsämännyn abioottisissa stressivasteissa.

Oulun yliopiston tutkijakoulu; Oulun yliopisto, Luonnontieteellinen tiedekunta; Luonnontaidon keskus (Luke), Parkanon toimipaikka

Oulun yliopisto, PL 8000, 90014 Oulun yliopisto

Tiivistelmä

Ilmastonmuutos vaikuttaa eri kasvilajien levineissyyteen. Nopeasti muuttuva ilmasto on haaste erityisesti metsäpuulajeille, jotka pitkän sukupolvenvälin takia kehittyvät evolutiivisesti hitaasti. Erilaisten ympäristötökköiden aiheuttamien puolustusaineenvaihdunnan muutosten tunteminen metsäpuilla on tulevaisuuden kannalta myös taloudellisesti tärkeää. Työn tavoitteena oli selvittää millainen merkitys polyamiineilla on metsämännyn (Pinus sylvestris L.) abioottisessa stressivasteessa. Polyamiinit (PAt) ovat pienimolekyylisä typpiyhdisteitä, joiden tiedetään olevan mukana solujen perusaineenvaihdunnassa, kasvien kasvussa ja kehityksessä ja stressivasteissa.


Asiasanat: abioottinen stressi, erilaistumaton somaattinen alkiosolukko, fenyylipropanoidit, geenien ilmentyminen, nesteviljely, metsämänty, polyamiinit
Acknowledgements

First of all I want to thank my supervisors Docent Tytti Sarjala and Prof. Hely Häggman for offering me this interesting research topic which has developed during my thesis work. The atmosphere has always been helpful and understanding, which has encouraged me in my work. I thank you for your expert guidance and for the opportunity to be involved in such high quality science. Working in two institutions, at the University and at the Research Institute, has expanded my perspective on Finnish academic life. I am grateful to Dr. Jaana Vuosku for her valuable advises and for being my unofficial supervisor during the journey. I wish to thank Prof. Esa Läärä for teaching and inspiration in statistical issues.

I am grateful to Dr. Sergio Ochatt and Prof. C. Peter Constable for efficiently reviewing this thesis. I warmly thank all my co-authors, especially Dr. Markku Saarinen, Dr. Leena Hamberg, Dr. Juha Heiskanen and Harri Latva-Mäenpää for your contribution to this study. I want to express my thanks to Dr. Suvi Sutela for being my co-author but also peer support, ever since we started to work in a same lab over twelve years ago.

I wish to thank all my collaborators and personnel at the Parkano unit of the Natural Resource Institute Finland (Luke). I thank Dr. Meeri Pearson for peer support and for revising the language of two of the original papers. I thank Prof. Jori Uusitalo, Eveliina Oinas, Tomi Kaakkurivaara, Harri Lindeman and Maarit Haavisto for the long but rewarding drives to work between Tampere and Parkano. I think that after those kilometers we have learned to know each other pretty well. I am grateful to Hanna Leppälämmi, Anneli Käänmäki and Eeva Pihlajaviita for their skillful help in developing the methods in a lab and producing the data. I thank Tuire Kilponen for the graphic layout of the publications. I wish to thank the great people who have shared the office in Hervanta with me. You have made the working days enjoyable. Especially, I am grateful to Dr. Otso Huitu, who revised the language of this thesis and one original paper, gave last moment valuable advice and in that way “pushed the man to the top of the hill”.

I wish to give my loving thanks to my parents Pirkko and Matti Muilu who have loved and encouraged me and trusted in me at every turn of my life. Warmest thanks to my children Ilona and Konsta for being the most precious things of my life. They had great patience during the last summer, when in spite of their vacation, mother just worked. I thank my husband Pekka Mäkelä, for all his support during the process. Especially, I thank him for the encouraging words during the moments
when I totally lost faith. He is the love of my life. Finally, I extend my warmest thanks to all my friends and relatives. Thank you for being here for me.

This research was conducted at the Natural Resource Institute Finland (Luke), Parkano unit, and at the Genetics and Physiology Department of the University of Oulu. I acknowledge financial support by the Graduate School of Forest Sciences, the Academy of Finland and the Thule institute of Oulu.

Tampere, October 2015

Riina Muilu-Mäkelä
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABA</td>
<td>abscissic acid</td>
</tr>
<tr>
<td>ABRE</td>
<td>ABA responsive cis-regulatory element</td>
</tr>
<tr>
<td>ACC</td>
<td>1-amino-cyclopropane-1-carboxylic acid</td>
</tr>
<tr>
<td>ACCS</td>
<td>1-amino-cyclopropane-1-carboxylic acid synthase</td>
</tr>
<tr>
<td>ACL5</td>
<td>thermospermine synthase</td>
</tr>
<tr>
<td>ACO</td>
<td>1-amino-cyclopropane-1-carboxylic acid oxidase</td>
</tr>
<tr>
<td>ADC</td>
<td>arginine decarboxylase</td>
</tr>
<tr>
<td>ADH</td>
<td>alcohol dehydrogenase</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>calcium ion</td>
</tr>
<tr>
<td>CAT</td>
<td>catalase</td>
</tr>
<tr>
<td>CBF</td>
<td>C-repeat binding factor</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>CO_{2}</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>Cp</td>
<td>crossing point</td>
</tr>
<tr>
<td>CPA</td>
<td>N-carbamoyl putrescine amidohydrolase</td>
</tr>
<tr>
<td>DAO</td>
<td>diamine oxidase</td>
</tr>
<tr>
<td>DCR</td>
<td>Douglas-fir cotyledon revised medium</td>
</tr>
<tr>
<td>DREB</td>
<td>dehydration-responsive element</td>
</tr>
<tr>
<td>DW</td>
<td>dry weight</td>
</tr>
<tr>
<td>eIF5A</td>
<td>eukaryotic translation initiation factor 5A</td>
</tr>
<tr>
<td>E2F</td>
<td>cell cycle related transcription factor</td>
</tr>
<tr>
<td>EST</td>
<td>expressed sequence tag</td>
</tr>
<tr>
<td>FW</td>
<td>fresh weight</td>
</tr>
<tr>
<td>F_{0}</td>
<td>minimum fluorescence</td>
</tr>
<tr>
<td>F_{v}</td>
<td>variable fluorescence</td>
</tr>
<tr>
<td>F_{m}</td>
<td>maximum fluorescence</td>
</tr>
<tr>
<td>GS</td>
<td>glutamate synthetase</td>
</tr>
<tr>
<td>GCL</td>
<td>glutamyl cysteine ligase</td>
</tr>
<tr>
<td>HCAA</td>
<td>hydroxycinnamic acid amide</td>
</tr>
<tr>
<td>HClO_{4}</td>
<td>perchloric acid</td>
</tr>
<tr>
<td>H_{2}O_{2}</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>ICE1</td>
<td>freezing stress related transcription factor</td>
</tr>
<tr>
<td>LEA</td>
<td>late-embryogenesis-abundant protein</td>
</tr>
<tr>
<td>MCA</td>
<td>metacaspase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>MD</td>
<td>mean difference</td>
</tr>
<tr>
<td>NH₃</td>
<td>ammonia</td>
</tr>
<tr>
<td>ODC</td>
<td>ornithine decarboxylase</td>
</tr>
<tr>
<td>PA</td>
<td>polyamine</td>
</tr>
<tr>
<td>PAL</td>
<td>phenylalanine ammonia lyase</td>
</tr>
<tr>
<td>PAO</td>
<td>polyamine oxidase</td>
</tr>
<tr>
<td>P5CS</td>
<td>1-pyrroline-5-carboxylate synthase</td>
</tr>
<tr>
<td>PCD</td>
<td>programmed cell death</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDC</td>
<td>pyruvate decarboxylase</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PMT</td>
<td>pinosylvin monomethyl transferase</td>
</tr>
<tr>
<td>PSII</td>
<td>photosystem II</td>
</tr>
<tr>
<td>Put</td>
<td>putrescine</td>
</tr>
<tr>
<td>R2R3-MYB</td>
<td>transcription factor family related to phenylpropanoid synthesis</td>
</tr>
<tr>
<td>RBR</td>
<td>retinoblastoma-related protein</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosyl methionine</td>
</tr>
<tr>
<td>SAMDC</td>
<td>S-adenosyl methionine decarboxylase</td>
</tr>
<tr>
<td>Spd</td>
<td>spermidine</td>
</tr>
<tr>
<td>SPDS</td>
<td>spermidine synthase</td>
</tr>
<tr>
<td>Spm</td>
<td>spermine</td>
</tr>
<tr>
<td>SPMS</td>
<td>spermine synthase</td>
</tr>
<tr>
<td>psSTS</td>
<td>pinosylvin synthase</td>
</tr>
<tr>
<td>TAT-D</td>
<td>cell death related nuclease</td>
</tr>
<tr>
<td>Tspm</td>
<td>thermospermine</td>
</tr>
<tr>
<td>TTC</td>
<td>2,3,5-triphenyl tetrazolium chloride</td>
</tr>
<tr>
<td>TUBA</td>
<td>alfa-tubulin</td>
</tr>
<tr>
<td>UBQ</td>
<td>ubiquitini</td>
</tr>
<tr>
<td>4CL</td>
<td>4-coumarate:CoA ligase</td>
</tr>
</tbody>
</table>
List of original publications

This thesis is based on the following publications, which are referred to throughout the text by their Roman numerals:


Author contributions

<table>
<thead>
<tr>
<th>Paper</th>
<th>Original idea</th>
<th>Study design</th>
<th>Data collection</th>
<th>Data analysis</th>
<th>Statistical analysis</th>
<th>Manuscript preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>HH, TS, JV</td>
<td>TS, RMM, JH,</td>
<td>RMM</td>
<td>EL</td>
<td>RMM, JH/TS, HH</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MS</td>
<td>RMM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>TS, HH</td>
<td>RMM, TS</td>
<td>RMM, HLM</td>
<td>LH, RMM</td>
<td>RMM, JH/TS, HH</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RMM, JH/TS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>HH</td>
<td>RMM, TS, MS</td>
<td>RMM, TS</td>
<td>LH, RMM</td>
<td>RMM, HH/TS, HH</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RMM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>HH, JV, PN</td>
<td>JV, MaS, MR,</td>
<td>JH/TS</td>
<td>JH/TS</td>
<td>JH/TS</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>JV, MaS, JS</td>
<td>RMM</td>
<td>RMM</td>
<td>RMM, JH/TS, HH</td>
<td></td>
</tr>
</tbody>
</table>

Riina Muilu-Mäkelä (RMM), Leena Hamberg (LH), Juha Heiskanen (JH), Hely Häggman (HH), Riitta Julkunen-Tiitto (RJT), Harri Latva-Mäenpää (HLM), Esa Läärä (EL), Peter Neubauer (PN), Maria Ruottinen (MR), Markku Saarinen (MS), Tytti Sarjala (TS), Marja Suorsa (MaS), Suvi Sutela (SS), Jaana Vuosku (JV)
## Contents

**Abstract**  
**Tiivistelmä**  
**Acknowledgements**  
**Abbreviations**  
**List of original publications**  
**Contents**  

### 1 Introduction

1.1 Climate change and peatland forest regeneration as challenges to early growth of Scots pine ................................................................. 15

1.2 Abiotic stress physiology ........................................................................ 16

1.3 Polyamines related to the stress and development of plants ................... 18

1.4 PAs and phenylpropanoids ...................................................................... 21

1.5 Aim of the research ................................................................................. 23

### 2 Material and Methods

2.1 Plant material and abiotic stress (I, II, III, V) .......................................... 25

2.1.1 Drought stress and water excess (I)...................................................... 25

2.1.2 Spring frost (III) ........................................................................... 25

2.1.3 Polyethyleneglycol (PEG)-induced osmotic stress in liquid cell cultures (II,IV) ............................................................. 26

2.2 Morphological and physiological parameters ......................................... 27

2.2.1 Chlorophyll fluorescence and leaf yellowing (I).......................... 27

2.2.2 Root morphology and mycorrhizal root tips (I)............................ 28

2.2.3 Cell viability assay (II, IV)........................................................... 28

2.2.4 Cell mass growth (II,IV) ............................................................... 28

2.3 Nutrient, pH and conductivity measurements (II, IV)............................. 28

2.4 Polyamine analysis (I, II, III, IV)............................................................ 29

2.5 Phenol analysis (II, III) ........................................................................... 29

2.5.1 Total phenol content (II,III) .......................................................... 29

2.5.2 HPLC analysis for singular phenols (II, III) ................................... 29

2.6 PCR primer design and sequencing (I, II, III)........................................ 30

2.7 RNA extraction and reverse transcription (I, II, III, IV).......................... 31

2.8 Quantitative real-time RT-PCR analyses (I, II, III, IV).......................... 31

2.9 Statistical methods .................................................................................. 32

### 3 Results

3.1 Scots pine sequence information............................................................... 33
3.2 Put accumulated in needles under drought stress (I) .............................. 33
3.3 Spring frost induced expression of DAO and PAL in roots (III)........... 34
3.4 Put accumulates during proliferation growth of proembryogenic
cells (II, IV) ............................................................................................. 35
  3.4.1 Transition to exponential growth (IV) ......................................... 36
  3.4.2 Osmotic stress affects PA homeostasis (II) .................................. 36
  3.4.3 Phenylpropanoid synthesis was up-regulated during
      proliferative growth of proembryogenic cells (II) ......................... 37
3.5 PA catabolizing genes were down-regulated under water deficit........ 38

4 Discussion 39
  4.1 Seedling physiology under water stress in controlled experiments
      vs. in the field ...................................................................................... 39
  4.2 Put accumulated in proliferating cells and photosynthesizing
      organs of Scots pine ............................................................................ 40
  4.3 Are consistent PA levels advantageous to Scots pine under
      abiotic stress? .................................................................................... 42
  4.4 PA metabolism related to phenylpropanoid synthesis under
      abiotic stresses (II,III) ........................................................................ 45

5 Conclusion 47
References 49
Appendices 57
Original publications 65
1 Introduction

1.1 Climate change and peatland forest regeneration as challenges to early growth of Scots pine

Climate change is expected to increase regional variation in precipitation (NRC report 2011) and alter the composition and biogeography of forests (Allen et al. 2011). Warmer temperatures and increasing atmospheric carbon dioxide (CO₂) content, on the other hand, are expected to enhance forest growth and wood production in Northern and Western Europe (Lindner et al. 2010). Scots pine (Pinus sylvestris L.) is one of the most widespread coniferous species in the boreal zone and as such important also to Fennoscandian forestry. Long and warm summer periods and reduced water supply have been implicated in a recent decline of Scots pine forests in many dry inner-Alpine valleys (Rigling et al. 2013) and in Mediterranean regions (Poyatos et al. 2008). Although pines, also Scots pine, are one of the most drought-tolerant tree species (Jarvis and Jarvis 1963) the limited plasticity of their xylem and needle properties may promote their vulnerability to drought, as recently experienced throughout most of its range (Martinez-Vilalta et al. 2009). Paradoxically, in the boreal zones a warming climate has been predicted to increase frost damage to trees. Higher winter and spring temperatures will promote earlier dehardening and growth onset, extending the risk period of frost damage (Beck et al. 2004, Hänninen et al. 2006). Scots pine inhabits a large variety of soil types, and is adapted to a diverse array of water availabilities and temperatures. However, pines are most vulnerable to abiotic stress factors during their early growth as seedlings. Tree organs which develop in the early stages of phenology are suggested to be more susceptible to frost damage than organs from the previous years (Linkosalo et al. 2000).

In Finland, over 700 000 ha of peatland forests are approaching renewal age (70–80 years) (Saarinen 2013). This is due to the passing of the first Finnish forest improvement act in 1928, which led to extensive drainage of nearly 5.5 million ha of mires to improve wood production capacity (Päivänen and Hånell 2012). Drained peatland habitats pose challenges to successful forest regeneration. The water retention capacity of peat is vastly different from that of mineral soil. Undecomposed peat particles have a large pore space and thus a great capacity to retain water, which may lead to adversely wet growing conditions. Conversely, dry peat is hydrophobic, which poses drought-related challenges to artificially or
naturally regenerated conifer seedlings. After clearcutting, seedling stands experience large temperature fluctuations. This may be accentuated by different soil preparation methods, which form diverse microhabitats, leading to exposure of seeds and seedlings to subzero early summer temperatures as well as to variable moisture conditions. Scots pine is predicted to become the most common tree species in peatland forests.

1.2 Abiotic stress physiology

Plants are sessile organisms that have complicated metabolic responses to variation in environmental conditions. Most types of environmental stressors induce general physiological responses, such as reduction in growth and photosynthesis, oxidative damage, hormonal changes and accumulation of stress-related proteins (Aroca et al. 2012). Tissue dehydration occurs when there is an imbalance between root water uptake and leaf transpiration, i.e., under drought, low temperature, heat, saline, or flooding conditions (Aroca et al. 2012). The primary site for sensing abiotic stress are membranes, from which a cascade of cellular processes is initiated (Theocharis et al. 2012). Sugars, amino acids, organic acids, polyamines (PA) and lipids belong to stress responsive metabolites in plant cells (Krasensky and Jonak 2012).

The ability to alleviate hydration-related stress is dependent on the capacity of a plant to minimize water loss and maximize water uptake. Many forest tree species promote water uptake by extensive and deep roots (Hamanishi and Campbell 2011). A common early symptom of drought stress in leaves is usually a partial or complete closure of stomata, which decreases the assimilation of carbon dioxide and reduces photosynthetic rate (Akinci and Lösel 2012). Naturally, reductions in photosynthetic activity and increases in leaf senescence reduce crop growth. Plant cells have three main responses to abiotic stress. Firstly, cells try to restore their osmotic and ionic equilibrium to maintain cellular homeostasis. Secondly, cells control and repair damage due to stress by detoxification. Thirdly, cell division is coordinated as to meet the requirements of the plant under stress (Tuteja 2009).

Many hormonal signaling pathways, including ethylene, auxin, abscissic acid (ABA) and jasmonic acid, regulate growth, development and defence responses of plants, which implies the existence of complicated signaling networks. ABA is considered as the most important plant hormone regarding the regulation of stress responses (Yoshida et al. 2014). ABA binds to membrane receptors, thus launching an intercellular cascade which leads to calcium (Ca$^{2+}$) accumulation. Numerous physiological processes, including stomatal movements, are regulated by
oscillations in calcium levels. In plants, dehydration leads to an increase in ABA levels, which in turn induces the expression of multiple genes involved in defence against abiotic stress (Yoshida et al. 2014). Abiotic stress response pathways can be divided into two categories: ABA dependent and ABA independent pathways. The ABA independent pathway induces dehydration-responsive element binding (DREB)/C-repeat binding (CBF) transcription factors that bind to dehydration-responsive element/C-repeat binding elements of the target gene promoter. Instead, ABA inducible genes contain ABA responsive cis-regulatory elements named ABRE in their promoters. Transcription factors that bind to ABRE elements are known as ABRE binding proteins/factors (Yoshida et al. 2014). Transcription factors that bind to DREB/CFB or ABRE elements belongs to different gene families e.g. DREBs, MYBs, MYCs or NACs (Tuteja 2009, Yoshida et al. 2014). Many drought-responsive genes identified from Pinus species belong to proteins with known functions in photosynthesis, carbohydrate metabolism, cell wall synthesis and plant defence (Doubos et al. 2003, Lorenz et al. 2005).

Abiotic stress leads to oxidative stress, which is often rapidly manifested as an accumulation of reactive oxygen species (ROS), such as hydrogen peroxide (H$_2$O$_2$). ROS are important signaling molecules, but toxic at high concentrations. ROS affect many cellular functions by damaging nucleic acids, oxidizing proteins and causing lipid peroxidation (Gill and Tuteja 2010). Stress-induced ROS accumulation is counteracted by enzymatic antioxidant systems, including a variety of scavengers. Catalase (CAT) is a peroxisomal enzyme and protects the cells from the toxic effects of H$_2$O$_2$ by converting it into water and oxygen (Mhamdi et al. 2012). The expression of genes coding for CAT reflects the H$_2$O$_2$ content of the plant cells (Luna et al. 2004). Another central component of maintaining redox state in plant cells under oxidative stress is the balance between reduced and oxidized forms of glutathione (Galant et al. 2011). Glutathione is produced in a two-step reaction by glutamyl cysteine ligase (GCL) and glutamate synthetase (GS).

In addition to enzymatic scavengers, oxidative stress is diminished by non-enzymatic low molecular metabolites such as proline, phenolics and amines. As a consequence of drought and freezing stress, higher plants, including pines (Lorenz et al. 2011, Velasco-Conde et al. 2012), are reported to accumulate late embryogenesis abundant (LEA) proteins and other dehydrins (Yang et al. 2012). LEA proteins are well-known osmoprotectors (Battaglia et al. 2008), protecting other proteins from degradation.

Abiotic stress often accelerates senescence, which is a type of programmed cell death (PCD) in plants (Quirino et al. 2000). Senescence involves the recycling of
resources accumulated during prior development for use in other growing parts of the plant. In plants under abiotic stress, a common form of PCD is necrosis which is characterized by an early rupture of the plasma membrane and shrinkage of the protoplast (Del Duca et al. 2014). Ethylene is a gaseous plant hormone which regulates developmental PCD and senescence and also plays a role in cell cycle arrest under abiotic stress (Skirycz et al. 2011). The expression of the 1-amino-cyclopropane-1-carboxylic acid synthase (ACCS) gene is the limiting step of ethylene biosynthesis in plant tissues (Shen et al. 2014). Ethylene has been shown to regulate some ABA-independent pathway genes under stress (Kazan 2015).

Abiotic stress retards cell growth and division. The expression of CAT and retinoblastoma-related protein (RBR) genes has recently been associated with cell death processes in the zygotic embryogenesis of Scots pine (Vuosku et al. 2015). The phosphorylation of RBR proteins regulates the cell cycle by preventing transition from the G1 phase to the DNA-replicating S phase (Desvoyes et al. 2014). RBR proteins bind to transcription factor E2F, which leads to an at least partial arrest of the cell cycle in plant cells (Desvoyes et al. 2014). Osmotic stress seems to increase the expression of RBR, specifically in nucellar layers of Scots pine seeds (Vuosku et al. 2015).

1.3 Polyamines related to the stress and development of plants

Stress tolerance in plants has been improved by exogenously and transgenetically elevated levels of PAs (Shi and Chan 2014). PAs, putrescine (Put), spermidine (Spd), spermine (Spm) and thermospermine (Tspm) are small, positively charged nitrogenous compounds that have an important and intricate role in the stress and developmental pathways of plants (Tiburcio et al. 2014). PA levels are strictly regulated by biosynthesis, degradation, conjugation, back-conversion and transport and by interactions with other pathways associated with stress (Tiburcio et al. 2014). In Scots pine, Put is mainly synthesized from arginine via intermediate steps by arginine decarboxylase (ADC), agmatine iminohydrolase (AIH) and N-carbamoylputrescine amidohydrolase (CPA) (Vuosku et al. 2006) (Fig. 1). Aminopropyl groups are detached from S-adenosylmethionine (SAM) by S-adenosylmethionine decarboxylase (SAMDC) and added to Put and Spd by the aminopropyltransferases spermidine synthase (SPDS) and spermine synthase (SPMS), forming Spd and Spm, respectively (Kusano et al. 2008, Kakehi et al. 2010). Tspm is a structural isomer of Spm and their synthesis resembles each other (Knott et al. 2007). Scots pine has a putative thermospermine synthase encoding ACL5 gene (Vuosku 2011), whereas
the SPMS gene has not been found in gymnosperms (Minguet et al. 2008). PAs are catabolized by diamine oxidase (DAO) and polyamine oxidase (PAO) in deamination reactions, producing H$_2$O$_2$ and ammonia (NH$_3$) (Cona et al. 2006). Biosynthesis of the plant hormone ethylene and the amino acid proline, both also involved in regulating plant tolerance to abiotic stress, are associated with PA metabolism (Moschou et al. 2012).

Fig. 1. Polyamine metabolic pathway in Scots pine. ACCS=ACC synthase, ACO=ACC oxidase, ADC=arginine decarboxylase, ACL5=thermospermine synthase, AIH = agmatine iminohydrolase, CPA = N-carbamoyl putrescine amidohydrolase, DAO=diamine oxidase, PAO = polyamine oxidase, P5CS = delta 1-pyrroline-5-carboxylate synthase, SPDS = spermidine synthase.

**PAs and development**

PAs are implicated in pathways regulating cell division, PCD (Moschou and Roubelakis-Angelakis 2014), organogenesis (Vera-Sirera et al. 2010), embryogenesis (Minocha et al. 1999; Vuosku et al. 2006) as well as development
and stress tolerance of many plant species (Alcázar et al. 2011) including pines (Tang et al. 2007). PAs are considered to be essential for life, as inhibition of PA synthesis terminates cell growth. In particular, Spd and Put are essential for growth, as demonstrated by adc/spds/samdc knockout mutants, which have been shown to be lethal (Urano et al. 2005, Ge et al. 2006). One mechanism how Spd affects the viability of eukaryotic cells is a specific post-translational hypusination of the translational initiation factor 5A (eIF5A) (Park et al. 2010). Spd is a precursor for the rare amino acid hypusine, which is a specific residue of the eIF5A (Park et al. 2010). The cell cycle is controlled by eIF5A, which makes Put and Spd essential for the cellular viability. Conversely, acl5/spms Arabidopsis mutants lacking Spm or Tspm grow normally and are able to produce fertile seeds (Imai et al. 2004). Therefore, Spm and Tspm are not essential for cell survival, but they do seem to have an essential role in organ function. For example, Tspm is an important PA for the translational control of transcripts involved in stem elongation (Vera-Sirera et al. 2010). Moreover, Arabidopsis acl5/spms double mutant plants, unable to produce spm, were hypersensitive to salt and drought stress (Yamaguchi et al. 2007).

PAs and stress defence

Plants under stressful conditions often activate PA synthesis. PAs have a dual role against ROS accumulation under stressful conditions. PAs have been considered to be osmoprotectors and ROS scavengers. On the other hand, catabolism of PAs releases H₂O₂. Positively charged PAs can bind to negatively charged groups in cell membranes and prevent injury caused by stress factors (Pottosin et al. 2014). PAs have been shown to protect photosystem II (PSII) in isolated thylakoid membranes of spinach (Spinacia oleracea L.) (Hamdani et al. 2011). PSII is the first protein complex involved in the light-dependent reactions of oxygenic photosynthesis in thylakoid membranes. Exogenous PAs prevent senescence in leaves. ABA induces PA synthesis in cells and PA homeostasis is involved in ABA regulated stomatal movements (Alcázar et al. 2010a). Binding of PAs to macromolecules can cause conformational changes in DNA (Iacomino et al. 2012), affect the activity of several antioxidative enzymes or alter the function of ion channels (Kusano et al. 2008, Pottosin et al. 2014). In addition, there exists a link between PAs, Ca²⁺ homeostasis and stress responses (Kusano et al. 2008). Stress induced PAs regulate K⁺/Na⁺ homeostasis, leading to modulation of the activity of a several sets of ion channels to adapt ionic fluxes in response to environmental changes. Moreover, PA
synthesis is followed by a stimulation of PA oxidation, in which H$_2$O$_2$ and 4-Aminobutyrate (GABA) are released (Cona et al. 2006, Moschou et al. 2012). Depending on the severity of stress, H$_2$O$_2$ released in PA catabolism may act as a signalling molecule leading to the expression of defence genes or to a hypersensitive response and PCD (Moschou et al. 2008). High apoplastic amino oxidase activity induces PCD under abiotic stress (Moschou et al. 2008), affecting the ability of plants to react to environmental conditions.

**PA genes belong to gene families**

PA enzymes are regulated on transcriptional, translational and post-translational levels. Most plant PA genes are considered to belong to gene families (Tiburcio et al. 2014). Gene isoforms are mRNAs that are produced from the same locus but which differ in their transcription beginning sites, protein coding DNA sequences and/or untranslated regions, potentially altering the function of the genes. For instance the model plant *Arabidopsis* has two different ADC genes that are differentially expressed (Alcázar et al. 2010a). ADC2 is induced by abiotic stress, whereas ADC1 is more related to developmental processes (Alcázar et al. 2010b). Similarly, the other PA metabolism related genes, SPDS, SAMDC, DAO and PAO, belong to gene families recently reviewed by Tiburcio and co-authors (2014). Different isoforms of PA genes have variable responses to abiotic stress and ABA treatment, indicating their different role in stress defence. Both PA biosynthesis and catabolism seem to play a role in stress tolerance and the development of plants (Tiburcio et al. 2014). Not much is known of the PA gene isoforms of coniferous species in general. So far, only one member of the PA enzyme genes of Scots pine has been identified (Vuosku 2011).

### 1.4 PAs and phenylpropanoids

H$_2$O$_2$ released in PA oxidation has been suggested to affect cell wall maturation and lignification during development as well as wound-healing and cell wall reinforcement during pathogen invasion (Cona et al. 2006). Complex phenolic compounds related to defence and/or cell wall structures e.g., lignin, flavonoids and stilbenes, are produced via the phenylpropanoid pathway. They have been shown to accumulate under abiotic and biotic stress also in pine tissues (Boudet 2007, Häggman et al. 2009). The phenylpropanoids are a diverse family of organic
compounds that are synthesized by plants from the amino acid phenylalanine. The first step in phenylpropanoid synthesis is the conversion of phenylalanine to cinnamic acid by phenylalanine ammonia lyase (PAL) (Fig. 2). Several simple phenylpropanoids (with the basic C6-C3 carbon skeleton of phenylalanine), i.e., phenolic acids, are produced from cinnamic acid, including p-coumaric, caffeic, ferulic, and sinapic acids and simple coumarins. The phenolamides, also called hydroxycinnamic acid amides (HCAA), are a group of phenolic derivatives formed by the conjugation of PAs with hydroxycinnamic acids (caffeic, chlorogenic, feluric and p-coumaric acids) (Gaquerel et al. 2014), thus forming a link between PA and phenolic metabolism. HCAAs are produced by N-acylation of PAs with coenzyme A (CoA)-activated thioesters (Fuell et al. 2010). The conjugation of PAs with phenolics reduces their polarity and hydrophility (Bassard et al. 2010). The transcription factor proteins belonging to The R2R3-MYB gene family are important regulators of primary and secondary metabolism in plants (Doubos et al. 2010, Bomal et al. 2014). For example, R2R3-MYB8 up-regulates phenylpropanoid synthesis in Pinus species (Craven-Bartle et al. 2013, Bomal et al. 2014).
The phenylpropanoid biosynthesis pathway is linked with polyamines via conjugation (4CL = 4-coumarate:CoA ligase, PAL = phenylalanine ammonia lyase, HCAA = Hydroxycinnamic acid amide).

1.5 Aim of the research

The study of abiotic stress physiology and coping strategies of Scots pine seedlings is topical, due to both the projected effects of climate change on forest ecosystems and the foreseeable large-scale forest regeneration on peatland soils in Finland. The aim of the study was to enhance knowledge of the coping strategies of Scots pine during its vulnerable seedling phase and elucidate the role of PA metabolism in the process. PA metabolism in the plant kingdom has been investigated for 50 years. It is well known that PAs have a fundamental role in cellular functioning. However, the exact role of PAs remains indistinct, as their relative contents and composition vary widely between plant species, plant organs and physiological stages. We hypothesized that PA metabolism is an important component of stress metabolism in Scots pine. Stress-related responses were expected to manifest as systematic changes in PA metabolism-related gene expression or in metabolite levels. To form
a more comprehensive picture of abiotic stress-related metabolism of pines, PA metabolism was compared with associated changes in physiology, growth and expression of stress indicator genes. This study enhances the understanding of environmental effects on PA metabolism and of the general stress physiology of Scots pine.
2 Material and Methods

2.1 Plant material and abiotic stress (I, II, III, V)

2.1.1 Drought stress and water excess (I)

The Scots pine (*Pinus sylvestris* L.) material exposed to drought stress and water excess (I) was reared from seed orchard seeds (Hiirola, Finland, 61°49'N, 27°15'E). About 80 seeds were sown in flowerpots filled with fertilized (Kekkilä Oy, Vantaa, Finland) and non-fertilized (Biolan Oy, Kau tuua, Finland) horticultural peat, at a ratio of 1:9, respectively. The water content of the peat in all pots was maintained at ca. 75% during the growth of seedlings. Photoperiod in the growth chamber during the experiment was 6 h darkness and 18 h daylight with a light intensity of ca. 200 µmol m⁻² s⁻¹, and temperature was maintained at 25 °C.

In this experiment (I) the watering treatments (wet, optimal and dry) were designed in accordance with the water retention characteristics of peat (Heiskanen 1993). In the wet treatment, the volumetric water content of the peat was kept as high as possible without reaching the minimum air-filled porosity level (20 vol.%) (Wall and Heiskanen 2003). In the dry treatment, the water content was kept above wilting point (Hillel 1971), which here was 9.7 vol.%. The optimal water availability was targeted to be around field capacity (-10 kPa matric potential) (Hillel 1971).

2.1.2 Spring frost (III)

The spring frost experiment (III) employed seeds from three controlled Scots pine crossings, with northern mother grafts and pollen clones originating from Kolari (67°17'N, 23°46'E), but pollinations made in Punkaharju, South Finland (61°45’20”N, 29°23’20”E).

The freezing treatments were based on climate data from Punkaharju. In the experiment, nine-week old seedlings were subjected to freezing stress in growth chambers (Conviron CMP 3246, Saskatoon, Canada; light intensity in chambers ca. 150 µmol/m²/s and humidity 80%). The time points of the experiment were chosen to mimic the duration of the freezing periods under natural conditions. During the first night, temperature was lowered to +5 °C for two hours and subsequently raised to +20 °C for the day, with a temperature change rate of 2 °C/h. During the second
night temperature was lowered to +5 °C in the control treatment and to -3 °C in the freezing stress treatment. Seedlings were exposed to -3 °C for two hours before sampling. After the first sampling, temperature was allowed to increase back to +5 °C. Samples representing the recovery state were collected one hour after this.

2.1.3 Polyethyleneglycol (PEG)-induced osmotic stress in liquid cell cultures (II, IV)

Embryogenic cell line (II, IV)

One-year-old immature seed cones were collected from an open-pollinated elite Scots pine clone (K884) in Punkaharju, Finland (61° 48’ N; 29° 17’ E) during one growing season as described in detail by Vuosku et al. (2009). Embryogenic cultures were established using immature zygotic embryos with suspensor and megagametophyte tissues. The inoculation, induction and proliferation of the embryogenic cell masses were carried out as described in Sarjala et al. (1997) on a solid DCR medium (Gupta and Durzan 1985).

Transition to proliferation growth (II, IV)

Prombryogenic cells were cultivated on solid DCR medium before transfer into liquid DCR medium (II, IV). The pH value of the culture medium was adjusted to 5.8 before autoclaving. In the beginning, two to three grams of proembryogenic cell mass was mixed with 50 ml of DCR medium and transferred into Erlenmeyer bottles (250 ml) that were sealed with silicone sponge closures (Sigma-Aldrich Co., St. Louis, USA). The DCR media contained the plant hormones cytokinin (BAP) (0.5 mg/l) and auxin (2,4-D) (0.5 mg/l) to maintain proliferation growth. Prior to the experiments, the pH electrodes (autoclavable EGA 186-L Meinsberger Elektroden, Germany) were calibrated, autoclaved (121 °C, 20 min) and placed in Erlenmeyer-type shake flasks with three side necks for sensors and sampling (Glasgerätebau Ochs GmbH, Bovden, Germany). The SENBIT® receiver unit was set to monitor the cultures every 90 seconds. Data was managed with SENBIT® Control software. The proembryogenic cell suspensions were cultured on shaking tables at room temperature in the dark.
Osmotic stress induced by PEG (II)

The proembryogenic cells were adapted for 13 days to the liquid environment before osmotic stress. This was induced by replacing 20 ml of the medium in the bottles with a combination of DCR and polyethylene glycol (PEG) (with molecular weight 6000) solution to reach 5% (w/v) or 10% (w/v) PEG (6000) content. Only liquid DCR medium without PEG (6000) was used in the control treatment. The PEG content of the medium was increased gradually during three days to allow cell masses to adapt to the osmotic environment. The final PEG level was 5% in 12 bottles, 10% in 12 bottles, and 11 bottles were used as controls without PEG (i.e., PEG 0% treatment).

The effect of different PEG contents on cell mass growth was investigated in preliminary tests, in which 5% and 10% PEG contents turned out to be most appropriate for purposes of the experiment. The osmotic potential of the PEG solutions was not measured during the experiment, but according to Michael and Kaufmann (1973), 5% and 10% PEG 6000 solutions have an osmotic potential of -0.5 and -1.5 bar, respectively.

2.2 Morphological and physiological parameters

2.2.1 Chlorophyll fluorescence and leaf yellowing (I)

Chlorophyll fluorescence is considered a tool for interpreting the stress tolerance of plants (Peeva and Cornic 2009). As a non-destructive method, chlorophyll fluorescence, especially the ratio of variable (Fv) to maximum fluorescence (Fm), has been widely used for assessing plant physiological status and the state of Photosystem II (PSII) (Krause and Weis 1991). In this study, chlorophyll fluorescence emission was measured from green needles by means of a Plant Efficiency Analyzer (Hansatech Instruments Ltd., Norfolk, England). The ratio of Fv to Fm was measured from three seedlings per pot. Needles were dark-adapted at room temperature in leaf clips supplied with the analyser for at least 30 min before measurement. The minimum fluorescence (F0), Fm and Fv (Fv=Fm-F0) as well as the ratio Fv/Fm were recorded at 15 s intervals at a 100% intensity level of photon flux density (4000 µmol m⁻²s⁻¹). Moreover, the needles of four seedlings per pot were categorized and counted according to their colour: green, greenish brown, brown.
2.2.2 Root morphology and mycorrhizal root tips (I)

The fresh weight (FW) and dry weight (DW) (samples dried overnight at +105 °C) of the roots and shoots (= stem with needles) of four seedlings from each pot (n=12) were measured. The total number of the root tips was calculated under a stereomicroscope, also from four seedlings per pot.

2.2.3 Cell viability assay (II, IV)

The viability of proembryogenic cells was determined by biochemical marker 2,3,5-triphenyl tetrazolium chloride (TTC) staining, which is a commonly used technique based on the reduction of tetrazolium salts to red-colored end products in viable cells (Towill and Mazur 1975). Two 0.2 g cell samples were collected, washed and stained with 1.5 ml of TTC solution (0.6% TTC in Tris buffer, pH 7.5). Cells were incubated for 24 h at 30 °C in the dark, after which red formazan was released from cells with 80% ethanol and absorbance measured with a spectrophotometer at 485 nm wavelength (Jenway Genova MK2 Life Science Analyser, Dunmow, Essex, UK).

2.2.4 Cell mass growth (II, IV)

The relative growth rate of embryogenic cell masses per day (RG, % dry weight (DW) increase per day) in the 4-week cultivation was calculated with the formula

\[ RG (\%) = 100 \times \left( \frac{\ln W_2 - \ln W_1}{t} \right) \]

where W1 = initial biomass at harvest time t1 (DW), W2 = biomass at the harvest time t2 (DW) and t = t2 – t1, time of the growth (days) (Ingestad and Lund 1986).

2.3 Nutrient, pH and conductivity measurements (II, IV)

After harvesting, the pH values and the conductivity of the cell culture medium were determined from the filtrates by using a pH meter (Philips PW9422) and YSI 3200 conductivity instrument (YSI, 1700/1725 Brannum Lane, Yellow Springs OH45387 USA), respectively. Also, the concentrations of total nitrogen, ammonium (NH₄⁺) and nitrate (NO₃⁻) were analyzed from the filtrated medium by flow injection analysis (FIA Star 5020, Tecator, Hillerød, Denmark).
2.4 Polyamine analysis (I, II, III, IV)

For the PA analyses, 100 mg (FW) of the somatic proembryogenic cultures (II, IV) or needle, stem or root tissues (I, III) were extracted in 5% (w/v) perchloric acid (HClO₄). PA dansyl derivatives were analyzed with high-performance liquid chromatography (HPLC) (Merck-Hitachi, Darmstadt, Germany) as described in Fornalé et al. (1999). Spm and thermospermine (Tspm) are isomers and in the present study they were included in the same fraction.

2.5 Phenol analysis (II, III)

For the analysis of secondary metabolites, one gram of filtrated proembryogenic cell mass (II) or needle or root tissue (III) was ground in 6 ml of 100% methanol. The supernatant was filtered and divided into Eppendorf tubes, after which samples were dried and stored in -80 °C.

2.5.1 Total phenol content (II,III)

Total phenol contents were analyzed by the Folin-Ciocalteu procedure (Singleton et al. 1999), modified for use on 96-well microplates (BD Labware, Franklin Lakes, NJ. USA), as described in Viitala et al. 2011. The Folin-Ciocalteu assay is also described as an antioxidant capacity analysis method due to its ability to react with phenols but also with any reducing substances, such as some vitamins, thiols, glyceraldehyde, inorganic ions and nitrogen-containing molecules (Everette et al. 2010). Thus, the Folin-Ciocalteu reagent gives a rough estimation of both the phenolic content and the reducing capacity of the samples.

2.5.2 HPLC analysis for singular phenols (II, III)

For HPLC analysis, the samples were dissolved in 500 µl of 50% methanol. HPLC analysis was performed using a Shimadzu Prominence Liquid Chromatograph system including LC-20AP pumps, UFLC Prominence Communication Bus Module CBM-20A and Prominence Photo diode array SPD-M20A Detector (Shimadzu USA Manufacturing Inc.) and a Waters XBridge C18 5 µm 4.6*150 mm column (Waters Corporation USA). The wavelengths of the detector were set at 270 and 320 nm. The stepwise HPLC mobile phase conditions were as follows: initial methanol content 2% increased to 20% at 10 min, to 30% at 20 min, to 45%
at 33 min, to 95% at 35 min, where after it was held at 95% for 10 min; the injection volume was 25 µl. Phenylalanine (Fluka P1150000) (II), tryptophan (Fluka PHR1176) (II, III), cinnamic acid (Fluka 97013) (II, III) and pinosylvin (Arbonova) (II, III) were used as external standards to quantify the concentrations of compounds in pine tissues.

2.6 PCR primer design and sequencing (I, II, III)

The PCR primers for the amplification of the cDNA fragments of the Scots pine genes were based on the Scots pine mRNA gene sequences for phenyl alanine ammonia lyase (PAL) [GenBank: AF353986] (II, III), ACC-synthase (ACCS) [GenBank: AY366206] (II), alfa-tubulin (TUBA) [GenBank: FN546172] (I, III) and late embryogenesis abundant protein (LEA) [GenBank: FJ201571] genes (I, II, III); on the mRNA gene sequences of other Pinus species for ACC oxidase (ACO) [GenBank: FN824808] (II), glutamyl cysteine ligase (GCL) [GenBank: AJ132540.1] (I) and transcription factor related to phenylpropanoid synthesis (R2R3-MYB8) [GenBank: DQ399057]. (IV); and on Pinus expressed sequence tags (EST) sequences for prolin synthesis related delta 1-pyrroline-5-carboxylate synthase (P5CS) [GenBank: CF394415.1] (II), flooding stress related pyruvate decarboxylase (PDC) [GenBank: CO161777.1] (I) and alcohol dehydrogenase (ADH) [GenBank: U48367.1] (I), freezing stress related transcription factor (ICE1) [GenBank: DR384895.1] (IV) and glutamate synthase (GS) [GenBank: CF478539.1] (I) genes. The EST sequences were selected on the basis of their similarity to known plant genes at the nucleotide or amino acid level. Circa 600 base pairs long cDNA fragments were amplified by standard PCR using cDNA from the needles, stems or roots of the seedlings or of the proembryogenic cells as a template. Fragments with a length of six to seven hundred base pairs were amplified by standard PCR using needle, root or proembryogenic cDNA as a template and DNA Polymerase (5U/µl) (Biotools B&M Labs, S.A. Madrid Spain). The fragments with an appropriate length were gel-purified using a purification kit (Wizard SV Gel and PCR Clean-Up System Promega Corporation Madison USA) and sequenced with the Applied Biosystems 3730xl DNA Analyzer in Macrogen Europe (Meibergdreef 31, 1105 AZ Amsterdam Zuid-oost, Netherlands). The partial gene sequences were aligned with the protein sequences of other plant species by using a translated nucleotide query.
2.7 RNA extraction and reverse transcription (I, II, III, IV)

Total RNA was extracted for the gene expression studies from the proembryogenic cell masses (100 mg) using the total RNA purification PureLinkTM Plant RNA Reagent (Invitrogen Corporation, California, USA). The RNA samples were treated with rDNase set (Magherey-Nagel, Duren, Germany) to eliminate contaminating genomic DNA. RNA samples were purified with the NucleoSpin® RNA Clean-Up kit (Macherey-Nagel, Duren, Germany). The RNA yields were measured three times with an OD260 analysis (Jenway Genova MK2 Life Science Analyser, Dunmow, Essex, UK), and 1 μg of each RNA sample was used for the gene expression analyses. cDNA was reverse-transcribed using a SuperScript VILOTM cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA).

2.8 Quantitative real-time RT-PCR analyses (I, II, III, IV)

Gene expression was studied by quantitative real-time RT-PCR analysis. Ubiquitin (UBQ), glyceraldehyde-tri-phosphate (GAPDH) and alfa-tubulin (TUBA) are commonly used reference genes in RT-PCR investigations with plants (Jarosova and Kundu 2010). The RT-PCR results of the present studies (I, II, III and IV) were verified by using three different reference genes UBQ [AF461687], GAPDH [L07501], and TUBA. The accumulation of proteins, polysaccharides, polyphenols etc. might decrease the quality of RNA in samples collected under stressful conditions. Therefore, a relative quantification strategy was used in experiments with stress treatments (I, II, III). However, absolute quantification was used, and described in detail in article IV. The PCR primers for the Scots pine genes (P5CS, ACO, ACCS, GCL, GS, ADH, LEA, ICE1, R2R3-MYB8 and the reference gene TUBA) were designed on the basis of the sequence data discovered in this study. The PCR primers for the expression studies of the PA biosynthesis genes ADC [GenBank: HM236823], ODC [GenBank: HM236831], SPDS [GenBank: HM236827], SAMDC [GenBank: HM236826], ACL5 [GenBank: HM236828], DAO [GenBank: HM236829] and PAO [GenBank: HM236830] gene were based on Scots pine PA gene sequences. Similarly, CAT [GenBank: EU513163], PAL [GenBank: AF353986], psSTS [GenBank: S50350], TAT-D [GenBank: EU513167] as well as S-adenosylmethionine synthase (SAMS) [GeneBank: JQ970126] primers were based on the known gene sequences of Scots pine. Primers for the cell division regulator genes RBR and E2F were based on the sequences submitted to genebank explained in detail in the study (IV).
2.9 Statistical methods

The effects of water stress (I), PEG induced osmotic stress (II), time, and their interactions on PA concentrations and gene expression were analysed using linear mixed models (Fitzmaurice et al. 2004). Individual models were constructed for the free and soluble conjugated fractions of Put, Spd and Spm and the relative expressions of the examined genes (see above). Gene expression data were log-transformed when necessary to meet assumptions of normality. Linear mixed models were used also in the spring frost experiment (III), using PAs, phenols (pinosylvin, PSM and total phenol contents) as well as gene expressions as response variables. Treatments were included in the models as fixed explanatory variables, and the origin of seedlings (three families, S1, S2 and S3) as a random factor. The estimated regression coefficients and their 95% confidence intervals (CI) were used to assess statistical significance. Analyses were carried out using function lme in the package nlme of the program R (Pinheiro et al. 2014, R Core Team 2014) (I, II, III)
3 Results

3.1 Scots pine sequence information

The alignments of putative Scots pine genes; flooding stress \textit{PDC}, \textit{ADH}, oxidative stress \textit{GCL}, \textit{GS}, freezing stress \textit{ICE1}, phenylpropanoid synthesis \textit{R2R3-MYB8}, prolin synthesis \textit{P5CS} and ethylene synthesis \textit{ACO} related genes with appropriate protein sequences of other species are shown in appendices 1–7.

3.2 Put accumulated in needles under drought stress (I)

\textit{Stress induced physiological and morphological changes (I)}

The water content of pine needles remained relatively stable in the different water level treatments, whereas root water content was positively associated with water availability in peat (Fig. 3). The number of root tips decreased under drought stress. Conversely, mycorrhizal root tips became withered under both wet and dry treatments. Water excess and drought decreased the proportion of green needles, as compared to optimal growth conditions. However, chlorophyll fluorescence (F\textsubscript{v}/F\textsubscript{m} value) remained high in green needles under all treatments up until severe drought stress, when PSII efficiency collapsed. Variation in F\textsubscript{v}/F\textsubscript{m} values increased under the wet treatment.

\textit{Expression of stress related genes (I)}

Patterns of expression in \textit{LEA} and \textit{CAT} indicate that protective processes were launched in seedlings under severe drought stress. \textit{LEA} was up-regulated in all parts of the Scots pine seedlings following severe drought. Expression of \textit{CAT} was slightly elevated in roots under the dry treatment protocol.

The excess water treatment did not enhance the expression of flooding stress related genes. While the expression of \textit{GCL} was too low to be investigated (low Cp values), \textit{GS} expression levels remained stable in all plant organs throughout the experiment. This suggests that the glutathione synthesis pathway was not transcriptionally up-regulated in stressed seedlings. The \textit{PDC} gene, involved in the regulation of alcohol fermentation under hypoxia, was not up-regulated in the roots under the wet treatment. The expression of \textit{ADH} was too low to be detected.
**PA metabolism was organ specifically regulated (I)**

Free and soluble conjugated Put accumulated in needles under drought stress, suggesting that Put plays a role in the function of photosynthesizing organs. Otherwise, PA profiles remained relatively unchanged under wet and dry growth conditions. The transcription profiles of the genes involved in PA biosynthesis and catabolism suggested that, under drought stress, PA levels in the needles and stems were maintained rather by the inhibition of PA oxidation than by the induction of PA synthesis. Water deficit decreased the expression of PAO in needles and stems and DAO in stems. Furthermore, free Spd levels were higher in roots with a reduced amount of mychorrizaal root tips, as was prevalent under the wet and dry treatments. This was accompanied with lower expression of ACL5 in stems and roots under both water excess and drought stress.

<table>
<thead>
<tr>
<th>Wet treatment, peat water content 75%</th>
<th>Optimal treatment (control), peat water content 39%</th>
<th>Dry treatment, peat water content 15%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groen needles (42%)</td>
<td>Groen needles (63%)</td>
<td>Groen needles (32%)</td>
</tr>
<tr>
<td>Free Spd</td>
<td>Free and soluble conjugated Put</td>
<td>Free and soluble conjugated Put</td>
</tr>
<tr>
<td>Water content (82%)</td>
<td>Water content (69%)</td>
<td>Water content (46%)</td>
</tr>
<tr>
<td>Mycorrhizaal root tips (MD=13%)</td>
<td>Mycorrhizaal root tips (MD=10%)</td>
<td>Mycorrhizaal root tips (MD&lt;3.3)</td>
</tr>
<tr>
<td>Free Spd</td>
<td>Free Spd</td>
<td>Free Spd</td>
</tr>
<tr>
<td>PA genes</td>
<td>PAO</td>
<td>PAO</td>
</tr>
<tr>
<td>ACL5</td>
<td>LEA</td>
<td>LEA</td>
</tr>
<tr>
<td>ADC</td>
<td>Stress genes</td>
<td>Stress genes</td>
</tr>
<tr>
<td>Spd</td>
<td>PAO</td>
<td>PAO</td>
</tr>
<tr>
<td>Spd</td>
<td>LEA</td>
<td>LEA</td>
</tr>
<tr>
<td>Spd</td>
<td>ACL5</td>
<td>ACL5</td>
</tr>
</tbody>
</table>

Fig. 3. Effect of watering treatments on morphological and physiological parameters and polyamine metabolism in different parts of Scots pine seedlings.

3.3 **Spring frost induced expression of DAO and PAL in roots (III)**

Gene expression of needles and roots varied also under short freezing stress (III). The temperature change from +5 to -3 °C decreased the expression of the PA synthesizing genes ADC and SPDS in needles. The expression of SPDS remained low also after recovery. Conversely, the expression of ADC increased in the roots after recovery. Frost also increased expression of the PA catabolizing gene DAO in roots, whereas the expression of PAO was down-regulated both in needles and in...
roots after recovery. Although the treatments had specific effect on the expression of PA metabolism related genes, the treatments did not affect levels of the free or soluble conjugated PA in the seedlings.

Furthermore, the only stress-related genes affected by the frost were LEA and ICE1, whose expression decreased in needles. The phenylpropanoid synthesis regulating putative transcription factor gene R2R3-MYB8 was expressed in the roots but not in the needles of Scots pine seedlings. The R2R3-MYB8 gene has been shown to up-regulate carbohydrate, shikimate and monolignol pathways in white spruce (Picea glauca (Moench.) Voss) (Bomal et al. 2014) and the phenylalanine pathway in maritime pine (Pinus pinaster L.) (Craven-Bartle et al. 2013). Treatments in the present study did not affect the expression of R2R3-MYB8 in roots. Instead, PAL gene was up-regulated in the roots under frost (-3 °C) and recovery. The stilbenes, i.e. pinosylvin and pinosylvin o-methyl ether (PSM) as well as the stilbene-producing gene psSTS remained constant during the experiment.

3.4 Put accumulates during proliferation growth of proembryogenic cells (II, IV)

Cell mass proliferation in the liquid cultures correlated negatively with pH and conductivity (IV). Continuously measured pH and conductivity of the growth media was shown to be a good indicator of the transition of proliferation to the exponential growth phase (Fig. 4).

![Tetrazolium (TTC) stained Scots pine proembryogenic cells.](image)
3.4.1 Transition to exponential growth (IV)

The transition phase of cell mass to exponential growth was characterised by stress reactions. All oxidative stress, cell cycle and PCD indicator genes such as RBR/E2F, TAT-D, metacaspase (MCA) and CAT displayed up-regulation before the acceleration of cell mass growth. While free Spd was the main PA in the beginning of the suspension cultures, the transition towards exponential growth was characterized by an increase in Put levels, implicating its role in proliferation growth. All PA biosynthesizing genes, ADC, AIH, CPA, SPDS and ACL5, had similar expression profiles with an expression peak after six days of cultivation. Conversely, the PA catabolizing gene DAO was up-regulated and PAO remained stable during the transition to exponential growth.

3.4.2 Osmotic stress affects PA homeostasis (II)

In the osmotic stress experiment, cell mass growth and viability increased in the control and under the 5% PEG treatment but decreased under the 10% PEG treatment (Fig 5). Expression of the cell cycle-related genes RBR and E2F and the PCD related gene TAT-D indicated inhibition of cell cycle intensity and PCD in the 10% PEG treated samples. Free PA levels remained mostly relatively constant, whereas free Spm/Tspm and methyl-Put contents of cells increased under osmotic stress. The expressions of SAMDC, ACL5 and PAO decreased in the 10% PEG treatment group, in which cell mass growth and viability were inhibited (Table 2).

![Fig. 5. Growth and viability of proembryogenic cell masses of Scots pine in liquid culture. The bars indicate arithmetic mean values of cell mass growth or viability under](image-url)
control (dark grey bars), 5% PEG (light grey bars) and 10% PEG (open bars) treatment and their 95% confidence intervals at incubation time (1, 4 and 7 days).

Table 2. Temporal changes (stable, zero, up or down) in the content of PAs and in the expression of PA related genes ADC, SPDS, ACL5, DAO, PAO and SAMDC in Scots pine proembryogenic cell masses exposed to 5 or 10% PEG treatment for seven days.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>5% PEG</th>
<th>10% PEG</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA contents</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free Spm/Tspm</td>
<td>stable</td>
<td>stable</td>
<td>stable</td>
</tr>
<tr>
<td>Methyl-Put</td>
<td>zero</td>
<td>up</td>
<td>up</td>
</tr>
<tr>
<td>Expression of PA genes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADC</td>
<td>down</td>
<td>down</td>
<td>down</td>
</tr>
<tr>
<td>SPDS</td>
<td>stable</td>
<td>stable</td>
<td>stable</td>
</tr>
<tr>
<td>ACL5</td>
<td>stable</td>
<td>stable</td>
<td>down</td>
</tr>
<tr>
<td>DAO</td>
<td>stable</td>
<td>stable</td>
<td>stable</td>
</tr>
<tr>
<td>PAO</td>
<td>stable</td>
<td>stable</td>
<td>down</td>
</tr>
<tr>
<td>SAMDC</td>
<td>stable</td>
<td>stable</td>
<td>down</td>
</tr>
</tbody>
</table>

3.4.3 Phenylpropanoid synthesis was up-regulated during proliferative growth of proembryogenic cells (II)

Expression of the PAL gene tended to be elevated in the control group and down-regulated in the 5% PEG treatment between days one and seven, while remaining stable in the 10% PEG treatment. Furthermore, cinnamic acid content increased in control and 5% PEG treated cells during the experiment. This suggests phenylpropanoid synthesis in proliferating cells (Table 3).

Table 3. Temporal changes (stable, up or down) in the content of phenolic compounds and in the expression of PAL and psSTS genes in Scots pine proembryogenic cell masses exposed to 5 or 10% PEG treatment for seven days.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>5% PEG</th>
<th>10% PEG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total phenol content</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>stable</td>
<td>down</td>
<td>down</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>up</td>
<td>up</td>
<td>stable</td>
</tr>
<tr>
<td>Gene expressions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAL</td>
<td>up</td>
<td>down</td>
<td>stable</td>
</tr>
<tr>
<td>psSTS</td>
<td>down</td>
<td>down</td>
<td>stable</td>
</tr>
</tbody>
</table>
3.5 PA catabolizing genes were down-regulated under water deficit

The drought stress experiment with Scots pine seedlings and the osmotic stress experiment with proembryogenic liquid cell cultures had common features regarding the regulation of genes involved in PA metabolism (Table 4). Expression of PA biosynthesizing and catabolizing genes remained constant or were down-regulated under stress treatments in both the seedlings (I) and in the proembryogenic cells (II). More specifically, the expression of ADC and SPDS remained at a constant level even under severe drought (I) or osmotic stresses (II), but ACL5 decreased in both experiments. Expression of DAO decreased in stems and PAO in stems, needles (I) and embryogenic cell cultures (II) under water deficiency. The transcription level results from different experiments indicate that, under water deficiency, PA levels were maintained via down-regulation of PA catabolism rather than by activation of synthesis. It seems to be advantageous for the different tissues to maintain relatively steady PA levels despite severe drought (I) or osmotic stress (II). Conversely, expression of DAO was up-regulated under spring frost in roots (III).

Table 4. Simplified summary of the changes in PA metabolism under water deficit (I) and spring frost (III) conditions in needles, stems and roots of Scots pine seedlings and osmotic stress in proembryogenic cells (II). Arrows indicate the direction of stress-induced changes in PA contents or gene expression compared to control conditions.

<table>
<thead>
<tr>
<th>Expression of PA genes</th>
<th>PA contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADC  SPDS  ACL5  DAO  PAO</td>
<td>Put  Spd  Spm/Tspm</td>
</tr>
<tr>
<td>Drought stress</td>
<td></td>
</tr>
<tr>
<td>Needle</td>
<td></td>
</tr>
<tr>
<td>Stem</td>
<td></td>
</tr>
<tr>
<td>Root</td>
<td></td>
</tr>
<tr>
<td>Osmotic stress</td>
<td></td>
</tr>
<tr>
<td>Proembryogenic cells</td>
<td></td>
</tr>
<tr>
<td>Spring frost</td>
<td></td>
</tr>
<tr>
<td>Needles</td>
<td></td>
</tr>
<tr>
<td>Roots</td>
<td></td>
</tr>
</tbody>
</table>
4 Discussion

4.1 Seedling physiology under water stress in controlled experiments vs. in the field

When subjected to environmental stress, plants actively reduce their vegetative growth as a way to conserve and redistribute resources and increase chances of survival (Quirino et al. 2000). Under abiotic stress, plants close their stomata, repress cell growth and photosynthesis, activate respiration, reduce leaf expansion, and start scarifying older leaves to reduce transpiration area (Akinci and Lösel 2012). Many tree species invest in relative root growth, thus enhancing the capacity of the root system to extract more water from deeper soil layers (Hamanishi et al. 2011). Therefore, the ratio of roots over shoots is a commonly used measure indicative of stress responses (Joslin et al. 2000). In the present study we show that during early growth Scots pine seedlings maintain water in their shoots while water levels in the soil substrate decline. Likewise, the PSII efficiency of needles was maintained up until the most extreme drought, at which point it collapsed. Similar responses by Scots pine seedlings to severe water deficit have also been demonstrated under field conditions (Pearson et al. 2013). We also discovered that while photosynthesis was actively maintained and protected, Scots pine roots became atrophied under drought stress.

In general, vulnerability of trees to xylem embolism (occlusion of a vascular tissue by an air bubble) is related to their vulnerability to water deficit. At dry sites, tolerance is increased if the plant is able to decrease its minimum shoot water content (Martinez-Vilalta et al. 2009). In different Scots pine populations investigated around the Europe the needle water potentials remained constant despite large variation in water availabilities (Martinez-Vilalta et al. 2009). Therefore, although Scots pine is a xerophyte and adapted to the large variety of soil moisture levels, the limited plasticity of its xylem and leaf properties may make the species vulnerable to the predicted decline in water availability across most of its distribution areas in future (Martinez-Vilalta et al. 2009).

Mycorrhizal fungi have been considered to be symbiotic with trees, specifically by facilitation the expansion of the root system, and hence water and nutrient uptake (Lehto and Zwiazek 2011). However, the role of ectomycorrhizas in water uptake is not clear cut. Mycorrhizas can facilitate water uptake, but there are also reports where the symbiosis has had no, or even negative effects on the water
content of the trees (Lehto and Zwiazek 2011). In the present (I) study, water content affected mycorrhiza formation in the seedling roots, indicating that microbial symbiosis might not be central for seedling surviving during early growth phases. Limited growth of roots and fungi has been suggested to be a consequence of the reduced availability of photosynthates when stomata start closing (Lehto and Zwiazek 2011).

4.2 Put accumulated in proliferating cells and photosynthesizing organs of Scots pine

*PAs are essential factors for cell growth*

PAs are essential factors for cell growth and differentiation (Moschou and Roubelakis-Angelakis 2014). In the present study (IV) free Put content increased in the proembryogenic liquid cell cultures during their transition to the exponential growth phase, which suggested a role for Put in proliferating cells. However, Spd and Spm levels decreased during the same phase, which suggests that these compounds, especially Spd, accumulate when cell growth is hindered. Similarly, free Spm accumulated in proembryogenic cells under 10% PEG (II) and free Spd in roots of seedlings under severe drought stress, where growth was reverted (I). Similar results have been found in Virginia pine (*Pinus virginiana* Mill.) plantlets, where Put was required for root growth, while Spd and Spm behaved as growth inhibitors (Tang *et al.* 2007). Likewise, exogenous Spm reduced cellular growth in and affected the physiology of Brazilian pine (*Araucaria angustifolia* (Bertol.) Kuntze) embryogenic cultures (Dutra *et al.* 2013). The mechanism by which Spm affected the maturation process of Brazilian pine was potentially associated with a reduction in the activity of proton pumps (Dutra *et al.* 2013). Furthermore, transgenic tobacco (*Nicotiana tabacum* L.) with decreased PAO activity increased Spd and Spm content and enhanced tolerance to salinity (Tisi *et al.* 2011a). In the present study, PEG-induced osmotic stress decreased the expression of *PAO*, leading to an accumulation of free Spm/Tspm, which may be a consequence of the demoted cell division intensity.

Ethylene is the main hormone regulating senescence in plant cells. Proliferating *Arabidopsis* leaves were found to exhibit rapid up-regulation of ethylene biosynthesis and signalling genes when exposed to osmotic stress, leading to accumulation of ACC (Skirycz *et al.* 2011). As soon as *Arabidopsis* leaf cells
became osmotically stressed, cell cycle progression rapidly ceased, but cells were remained in a latent state, allowing quick recovery (Skirycz et al. 2011). In the present study with proembryogenic cells of Scots pine (II), expression of the ethylene synthesis related ACCS or ACO was not up-regulated under osmotic stress, although other indicators, such as cell mass growth, viability and expression of the PCD related gene TAT-D and cell division regulator protein RBR, indicated inhibition in cell division rate. The gene expression results of ACCS and ACO indicate that there was no tangible role for ethylene during the stress treatments. However, ethylene can post-transcriptionally arrest cell proliferation, allowing a fast but reversible mechanism by which to react to changes in environmental conditions (Skirycz et al. 2011). When stress persists, the cells are irreversibly pushed into differentiation.

Free and soluble conjugated Put accumulated in needles

Yellowing leaves in plants are a visible cue of senescence and PCD (Del Duca et al. 2014). In yellowing the coordinated degradation of chlorophyll and other macromolecules leads to their subsequent mobilization to other plant parts. In the present study (I), water stress enhanced leaf yellowing. Despite severe stress, stable PSII efficiency and the simultaneous accumulation of free and soluble conjugated Put suggest a role for Put in photosynthetizing organs in Scots pine. Transglutaminases (TGases) are a family of enzymes that cross-link intracellular and extracellular proteins and stabilize dying cells (Del Duca et al. 2014). TGases are involved in all types of plant PCD and their activity maintains cellular and tissue integrity and remodeling (Griffin and Verderio 2000, Del Duca et al. 2014). Chloroplast senescence is regulated by TGases, which catalyze the formation of covalent bonds between PAs and proteins (Sobieszczuk-Nowicka et al. 2009). Thus, conjugation of PAs may be the mechanism through which PAs are involved in the protection of cells under senescence (Del Duca et al. 2014). The positive charges of PAs provide stability to the conformation of thylakoid proteins against various modes of stress, helping maintain photosynthetic activity (Shu et al. 2012). In chloroplasts, the light harvesting complex (LHClI) and PSII membranes, especially PSII core and the reaction centre of PSII, are enriched with PAs (Navakoudis et al. 2007). However, the precise role of PAs in the regulation of photosynthesis is far from being fully understood (Shu et al. 2012).

As mentioned, pines are characterized by vulnerability to xylem embolism under water shortage, which is compensated by strong stomatal control (Martinez-
Vilalta et al. 2009). Under drought stress, PAs may modulate the activity of certain ion channels on the plasma membrane and stimulate stomatal closure, which in turn would enhance photosynthetic efficiency (Shu et al. 2012). Particularly the accumulation of Put has been associated with stomatal closure (Alcázar et al. 2010a).

In plant cells, the non-selective vacuolar cation channels are blocked by a high affinity of PAs (Pottosin and Shabala 2012). Stomatal movements are regulated by neighbouring guard cells which have potassium (K⁺)-selective channels in their tonoplast (Hamamoto et al. 2008). The channels mediate vacuolar K⁺ release, leading to stomatal movements. The K⁺ channels of tonoplast are weakly sensitive to Spd and Spm but insensitive to Put (Hamamoto et al. 2008). Thus, accumulation of PAs can inhibit the activity of non-selective vacuolar ion channels, increasing K⁺/Na⁺ ratio in the cells under conditions where Na⁺ normally dominates, e.g., under salt stress (Elmaghrabi et al. 2013). Furthermore, nutrient uptake can be restricted under severe drought stress (Akinci and Lössel 2012). Finnish peatland soil is often poor in nutrients, especially potassium (Kaunisto 1997). Put has been shown to accumulate in the needles of Scots pine grown on peatland soil due to potassium deficiency (Sarjala and Kaunisto 1993) as well as in proembryogenic cells in liquid cultures (our unpublished data). Osmotic stress itself did not induce Put accumulation in proembryogenic cells of the present study (II). Therefore, the accumulation of Put in photosynthesizing organs (I) may be related to the role of Put in regulation of vacuolar ion channels. Ion homeostasis in guard cells regulates stomatal closure and reduces transpiration in seedlings. In a liquid environment (II), nutrient uptake was not limited as it may have been in seedlings grown on a peat substrate, where nutrients are distributed by the water (I). Therefore, it is possible that in the seedlings exposed to severe drought, nutrient uptake was restricted and Put accumulation was a response to an unbalance in K⁺/Na⁺-ratio (I).

### 4.3 Are consistent PA levels advantageous to Scots pine under abiotic stress?

PAs have direct roles in cell death i.e. through their regulatory effect on vacuolar ion channels. In plants, stress-induced PCD is associated with cell shrinkage, which requires the activity of plasma membrane and vacuolar ion channels (Pottosin et al. 2014). An indirect PCD regulatory mechanism of PAs is via PA oxidation. Tisi et al. (2011b) proposed that PAs affect plant development and stress responses in diverse ways, depending on the expression levels of PA oxidases, which in turn
may lead to different plant responses by altering PA/H$_2$O$_2$ balance. Under abiotic stress, Spd is secreted into the apoplast, where it is oxidized by apoplastic PAO, producing PA catabolic products—this suggests that cell fate depends on the rate of Spd oxidation by PAO (Moschou et al. 2008). If apoplastic PAO is at a moderate level and oxidation of Spd is low, amounts of produced H$_2$O$_2$ remain moderate, thus inducing the ROS-dependent protective pathway which leads to activation of tolerance responses. If PAO is at high levels and H$_2$O$_2$ levels increase, a specific PCD pathway is employed (Moschou et al. 2008). Furthermore, in plants, apoplastic PA oxidation alone cannot launch PCD; rather, a combination of a low rate of PA synthesis and an increased rate of PA catabolism regulates PCD (Moschou and Roubelakis-Angelakis 2014). However, constitutive PA oxidation leads to chronic oxidative stress. In our present studies (I, II), a common feature in experiments was the stable or downward trend in expression of PA biosynthetizing genes, followed by a down-regulation of PA catabolizing genes, suggesting that the ratio between PA and H$_2$O$_2$ ratio was in favor of the former. Stress did not decrease PA contents in any of the experimental treatments (I, II, III). We also investigated the expression of CAT to denote over-production of H$_2$O$_2$ in the cells (I, II, III, IV). We found that the expression of CAT increased in root tissue under severe drought stress (I). However, results on PA metabolism rather indicated an accumulation of PAs (free Spd in the roots) as well as down-regulation of PA catabolism. Given that the roots in this study were strongly atrophied, our results suggest that PA metabolism has a protective role in the seedlings and is important in the maintenance of cellular functioning. However, information on the cellular location of the different components of PA metabolism (genes, enzymes and PAs), would be needed to assess their exact role in different tissues.

Moreover, investigating gene expression will reveal only part of the picture. Many of the analysed genes in the present study are reported to be in gene families (Tiburcio et al. 2014). For example, multiple PAO genes have been identified in Arabidopsis (Takahashi et al. 2010). The PAO genes can be differentially regulated depending on the tissue, developmental stage or environmental factors. It is likely that other PA genes are similarly diverse and in gene families also in Scots pine. In the present studies, conserved domains of the coding sequences were amplified. From the data presented it is impossible to discern how many additional members/isoforms of each of the PA enzymes are expressed, or if the most relevant/highly expressed members have been assayed. There may also be tissue specific isoforms, which can cause perceived differences between the needles, roots and stems of the seedlings. Investigation of the whole PA gene families of pines
would be needed to elucidate the role of different PA genes under abiotic stress or development. Moreover, the fluxes and levels of PAs are likely influenced by post-transcriptional regulatory mechanisms, which might be an explanation for the poor correlation of PA gene expressions and PA contents.

Nevertheless, the gene expression results highlight the role of \textit{ACL5} in vasculature tissue development (I). \textit{ACL5} was down-regulated in the stems and in atrophied roots of Scots pine seedlings under drought stress. Biosynthesis of Tspm by \textit{ACL5} and catabolism by intracellular PAO adjust Tspm levels and affect vasculature development (Moschou and Roubelakis-Angelakis 2014). In transgenic \textit{Arabidopsis} plants with an acl5 knockout mutation the cell death of the xylem vessel elements proceeded too fast and the time required for xylem cell maturation, including the extensive deposition of cell wall material was inadequate (Mûniz \textit{et al.} 2008). Inhibition of the \textit{ACL5} gene and the consequent decrease in Tspm levels led to an \textit{Arabidopsis} phenotype which lacks cell wall thickening structures in xylem elements. This supports the notion of Tspm having a protective role in the xylem. Tspm is needed to maintain the survival of differentiating xylem elements until the differentiation process has been completed (Vera-Sirera \textit{et al.} 2010). Cell enlargement is strongly dependent on the extensibility of the cell wall, and also on the turgor pressure inside the cell (Donelly \textit{et al.} 1999). \textit{ACL5} might also control the expression of genes that influence the mechanical properties of cell walls (Vera-Sirera \textit{et al.} 2010), e.g., through enzymes which participate in cell wall loosening activities necessary for cell expansion (Fry \textit{et al.} 1992) or negative regulators of cell death related genes (Vera-Sirera \textit{et al.} 2010). The results of the present study (I) suggest that drought stress blocked the developmental processes in the roots of Scots pine seedlings by decreasing expression of \textit{ACL5} and leading to a consequent transcriptional down-regulation of \textit{PAO}. In fact, PA catabolism was up-regulated at the transcription level in proliferating tissue. This was found in both proembryogenic cell cultures (II, IV) and in seedlings under optimal growth conditions (I). Also in this study, the results are merely a part of a larger picture; we have no knowledge of how the different putative gene isoforms are expressed and regulated and how the post-transcriptional and translational regulation affects the cellular PA contents.
4.4 PA metabolism related to phenylpropanoid synthesis under abiotic stresses (II,III)

Plant cells are surrounded by cell walls. Primary cell walls are formed by growing cells and contain cellulose microfibrils and a polysaccharide matrix (Passardi et al. 2004). Usually primary cell walls are thin and have a simpler structure than secondary walls. Secondary cell walls are formed between the primary cell wall and cell membrane, after cell enlargement ceases. Cell wall structure varies among different plant tissues, depending on tissue function (Zhong and Ye 2007). Cells of water-conducting xylem tissue, such as tracheids and vessels, produce thick cell walls strengthened by lignin. Lignin is a complex phenolic polymer built of phenylpropanoid subunits called monolignols (Vanholme et al. 2010). The first step in phenylpropanoid synthesis is a conversion of phenylalanine to cinnamic acid. PAs conjugate with hydroxycinnamic acids, proteins and cell wall components and are shown to stabilize membranes under abiotic stress (Bassard et al. 2010). Exogenous Spd application inhibits the elongation of root cells, promotes the deposition of phenolics in cell walls and lowers cell cycle intensity in maize (Zea mays L.) (Tisi et al. 2011a). In the present study, phenolic compounds synthesized via phenylpropanoid pathway and PA metabolism were investigated in proembryogenic liquid cell cultures (II) and in seedlings exposed to spring frost (III).

The phenylpropanoid synthesis-related genes in maritime pine (Pinus pinaster Aiton) were induced by R2R3-MYB8, which was highly expressed in lignifying tissues (Craven-Bartle et al. 2013). In spruce, the R2R3-MYB8 gene was expressed in differentiating root and stem xylem, having a very similar expression profile than that of 4CL (Bomal et al. 2014). In the present study, the putative R2R3-MYB8 gene of Scots pine was mainly expressed in the roots, but only to a mild degree in needles (III), perhaps indicating a specific role in root tissues. Expression of the PAL gene increased in roots under freezing stress, and remained high during the recovery phase, indicating activation of the phenylpropanoid pathway (III). Furthermore, expression of DAO also increased in Scots pine roots under spring frost (III). Transcriptional up-regulation of both DAO and PAL are potentially associated with root growth inhibition and/or cell wall stiffening processes in Scots pine seedlings (III). Up-regulation of DAO expression in roots may indicate the launching of a signaling pathway, in which PA catabolism in up-regulated and H2O2 produced, leading to alterations in root development. Biotic or abiotic stress has also been shown to increase cell wall amine oxidase levels in the roots of tobacco (Tisi et al.)
H$_2$O$_2$ derived from PA catabolism has been found to induce early xylem differentiation by triggering cell wall stiffening and developmental PCD in xylem precursors (Tisi et al. 2011b). H$_2$O$_2$ has been reported to promote cell wall stiffening through formation of covalent cross-links between cell wall components (Pottosin et al. 2014). Thus, the results of the present study (III) are in line with previous reports (Tisi et al. 2011b; Pottosin et al. 2014), emphasizing the role of H$_2$O$_2$ in xylem differentiation by a triggering of cell wall stiffening.

Osmotic stress increased the content of total phenolic compounds in cells (II), which might indicate induction of antioxidant capacity. In the cell cultures of grape vine (Vitis vinifera L.) PA oxidation was associated with an up-regulation of stress-induced phenylpropanoid metabolism (Hatmi et al. 2014). Proembryogenic cells of Scots pine are highly undifferentiated and they are characterized by a low content of phenolic compounds (Häggman et al. 2009). However, increased expression of PAL and accumulation of phenylalanine and cinnamic acid indicated up-regulation of the phenylpropanoid pathway during proliferation growth in the control group (II). Similarly, the PA-catabolizing gene DAO was up-regulated in cells during their transition to exponential growth (IV).
5 Conclusion

PA metabolism is well-known to be part of the fundamental cellular functions in plants during growth, development and stress defences. However, the exact role of PAs has remained elusive, as various factors, e.g., plant species, developmental stage and environmental factors, all affect PA metabolism and its associations with other metabolic processes in a complex manner. The present study increases understanding of the developmental processes of Scots pine seedlings under different abiotic stress conditions, and how these manifest at organism, tissue and molecular levels. The role of PAs in the strategies of Scots pine for coping with abiotic stress was investigated in four different experiments. In two of these, the above and below ground parts of the seedlings were investigated separately. Cellular-level metabolism was investigated in experiments employing a coniferous controllable liquid cell culture system, in which proembryogenic cells were exposed to osmotic stress.

The results from the different experiments revealed that PA metabolism was strictly and tissue-specifically regulated. Water deficit and osmotic stress decreased expression of genes related to PA catabolism in seedlings and proembryogenic cells, respectively, which might be a consequence of the down-regulation of PA biosynthesis. The PA anabolism / PA catabolism -ratio has also previously been shown to be the mechanism by which PAs affect cell fate in PCD (Moschou et al. 2008). Stress-induced PA oxidation and H2O2 accumulation lead to a hypersensitive response and cell death. However, in the present study, PA levels were maintained at relatively constant levels despite severe stress, suggesting that PAs have a protective role in Scots pine tissues. Accumulation of Put in photosynthesizing organs may indicate the ability of PAs to protect thylakoid membranes, or the specificity of Put in regulation of vacuolar ion channels in guard cells. In the present study, ACL5 was clearly a stress-repressible gene. ACL5 expression and Tspm are connected with root cell expansion processes during xylem differentiation in Arabidopsis (Vera-Sirera et al. 2010). In the present study, the expression of ACL5 was down-regulated in roots, stems and proembryogenic cells under stress, i.e., when growth was repressed. Thus, the transcriptional regulation of ACL5 in Scots pine is possibly related to cell expansion. Furthermore, DAO was up-regulated simultaneously with the phenylpropanoid synthesis-related gene PAL in roots under the frost treatment, suggesting a contribution of PA catabolism and phenylpropanoids to cell wall stiffening (III).
Several stress-inducible genes were investigated in the present study. Expression of the R2R3-MYB8 gene is related to lignification in pine species. The putative R2R3-MYB8 gene was shown to be expressed in roots but not in the needles of Scots pine, suggesting its specific role in root tissues. The LEA gene was up-regulated under drought stress in all seedling parts, thus acting as a good indicator of drought stress.

All in all, Scots pine seedlings invest more in their shoots than in their roots during abiotically stressful conditions during early growth. Water deficiency induced similar changes to PA metabolism in seedlings and proembryogenic cells, suggesting that PA catabolism is down-regulated to maintain PA levels at a consistent level in tissues. The results also suggest that PA metabolism reacts more rapidly to stress than some stress-related genes investigated in the present study. The controllable cultivation environment for Scots pine proembryogenic cell cultures developed in this study can be applied and utilized also in future research, e.g., on the role of PAs in potassium deficiency and ion channel regulation. So far, only one member from the PA gene families of Scots pine has been sequenced. In the future, investigation on all putative gene members would be needed to deepen the understanding of PA metabolism in specific cellular functions.

Research onto epigenetic regulation might provide insight to processes enabling long-generation tree species to respond to changing environmental conditions of the future. The environment experienced by the mother tree can be transferred to the next generation. Thus, the present study can be used as a background for further studies on the relative roles of environmental and intrinsic effects on gene regulation and stress responses of Scots pine.
References


R Core Team (2014) R: A language and environment for statistical computing. Wien, R

factors of a vegetation shift from Scots pine to pubescent oak in dry Alpine forests.

Saarinen M (2013) Artificial and natural seeding of Scots pine in old drainage areas – Unique
features of forest regeneration on peat lands. Dissertationes Forestales 164, University
of Helsinki.

Sarjala T, Häggman H, Aronen T (1997) Effect of exogenous polyamines and inhibitors of
polyamine biosynthesis on growth and free polyamine contents of embryogenic Scots

Sarjala T, Kaunisto S (1993) Needle polyamine concentrations and potassium nutrition in

profiling revealed an important role of cell wall remodeling and ethylene signaling

Shi H, Chan Z (2014) Improvement of plant abiotic stress tolerance through modulation of

at: URI: http://www.intechopen.com/books/advances-in-photosynthesis-fundamental-
aspects. Cited 2015/11/17

oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods

stress on cell proliferation during early leaf development in Arabidopsis and a role for

Sobieszczuk-Nowicka E, Wieczorek P, Legocka J (2009) Kinetin affects the level of
chloroplast polyamines and transglutaminase activity during senescence of barley

Characterization of five polyamine oxidase isoforms in Arabidopsis thaliana. Plant Cell

expressing the pepper CaPF1 gene is associated with the polyamine biosynthesis. Plant

Theocharis A, Clément C, Barka EA (2012) Physiological and molecular changes in plants


Appendices

<table>
<thead>
<tr>
<th>1. P. sylvestris</th>
<th>2. PDC P. euphratica</th>
<th>3. PDC M. domestica</th>
<th>4. PDC P. serrulata</th>
<th>5. PDC S. lycopersicum</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDXKGSLOTCRPSNIMNFTNVAAIQLSSVPTVINSSEATLGHKARLVLVQIGVNDV</td>
<td>FSVGDFHNLDDLHAEPEFLNLNCCLNAGYAADGVARSKGAGCVVTVFGGSLSLN</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E</td>
<td>D</td>
<td>MN</td>
<td>A</td>
<td>-</td>
</tr>
<tr>
<td>MKDFLAARKKVKHNPTSRLEHRIFVPDGHPKSAPEPLRVNFLKFHGMLTBLDTAVI</td>
<td>MKDFLAARKKVKHNPTSRLEHRIFVPDGHPKSAPEPLRVNFLKFHGMLTBLDTAVI</td>
<td>MKDFLAARKKVKHNPTSRLEHRIFVPDGHPKSAPEPLRVNFLKFHGMLTBLDTAVI</td>
<td>MKDFLAARKKVKHNPTSRLEHRIFVPDGHPKSAPEPLRVNFLKFHGMLTBLDTAVI</td>
<td>MKDFLAARKKVKHNPTSRLEHRIFVPDGHPKSAPEPLRVNFLKFHGMLTBLDTAVI</td>
</tr>
</tbody>
</table>

Appendix 1. Alignment of the predicted amino acid sequence of the Pinus sylvestris putative pyruvate decarboxylase (PDC) with Populus euphratica (XP_011046856.1), Malus domestica (XP_008356242.1) and Prunus serrulata (AGO61957.1), Solanum lycopersicum (XP_004246778.1). Identical and similar amino acids are highlighted in black and grey, respectively.
Appendix 2. Alignment of the predicted amino acid sequence of the *Pinus sylvestris* putative alcohol dehydrogenase (ADH) with *Pinus banksiana* (AAC49545.1), *Pinus pinaster* (CBL95265.1) and *Glycine max* (AAC97495.1). Identical and similar amino acids are highlighted in black and grey, respectively.
Appendix 3. Alignment of the predicted amino acid sequence of the *Pinus sylvestris* putative glutamate cysteine ligase (GCL) with *Picea abies* (CAC27145.1), *Citrus cinnensis* (XP_006476577.1), *Arabidopsis thaliana* (CAAB2626.1), *Solanum lycopersicum* (NP_001234010.1), *Elaeis guineensis* (XP_010920822.1). Identical and similar amino acids are highlighted in black and grey, respectively.
Theobroma cacao putative glutamate synthetase (GS) with respect to (XP_010921965.1). Identical and similar amino acids are highlighted in black and grey, respectively.

Appendix 4. Alignment of the predicted amino acid sequence of the *Pinus sylvestris* putative glutamate synthetase (GS) with *Malus domestica* (XP_008370519.1), *Theobroma cacao* (XP_007044086.1), *Glycine max* (XP_003554669.2), *Elaeis guineensis* (XP_010921965.1). Identical and similar amino acids are highlighted in black and grey, respectively.

<table>
<thead>
<tr>
<th>1</th>
<th>P. sylvestris</th>
<th>2</th>
<th>G. max</th>
<th>3</th>
<th>T. cacao</th>
<th>4</th>
<th>M. domestica</th>
<th>5</th>
<th>E. guineensis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FVPQKG</td>
<td>P</td>
<td>KS</td>
<td>I</td>
<td>A</td>
<td>E</td>
<td>FV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>ETVL</td>
<td>G</td>
<td>L</td>
<td>G</td>
<td>S</td>
<td>D</td>
<td>ETVL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>QPSQ</td>
<td>D</td>
<td>R</td>
<td>L</td>
<td>D</td>
<td>G</td>
<td>QPSQ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>GLPQ</td>
<td>R</td>
<td>T</td>
<td>S</td>
<td>L</td>
<td>L</td>
<td>GLPQ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>GFL</td>
<td>L</td>
<td>L</td>
<td>Q</td>
<td>I</td>
<td>N</td>
<td>GFL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

60
Appendix 5. Alignment of the predicted amino acid sequence of the *Pinus sylvestris* putative CBF (C-repeat binding factor) 1 inducer protein (ICE1) with *Citrus sinensis*
(XP_006485105.1), Arabidopsis lyrata (NP_189309.2), Amborella trichopoda (XP_006844542.1), Theobroma cacao (XP_007038116.1) and Phalaenopsis aphrodite (AIE56161.1). Identical and similar amino acids are highlighted in black and grey, respectively.

Appendix 6. Alignment of the predicted amino acid sequence of the *Pinus sylvestris* putative phenylpropanoid synthesis related transcription factor (R2R3-MYB8) with *Pinus taeda* (ABD60280.1), *Pinus pinaster* (CBM40481.1) and *Picea glauca* (ABQ51224.1). Identical and similar amino acids are highlighted in black and grey, respectively.
Appendix 7. Alignment of the predicted amino acid sequence of the *Pinus sylvestris* putative 1-pyrroline-5-carboxylate synthase (P5CS) with *Theobroma cacao* (XP_007009199.1), *Suaeda salsa* (AAM28630.1), *Tamarix hispida* (AIL24121.1), *Arabidopsis thaliana* (BAD94287.1). Identical and similar amino acids are highlighted in black and grey, respectively.
Appendix 8. Alignment of the predicted amino acid sequence of the *Pinus sylvestris* putative 1-amino-cyclopropane-1-carboxylic acid oxidase (ACO) with *Pinus taeda* (ADD65760.1), *Picea sitchensis* (ABF20552.1), *Pinus pinaster* (CBL95267.1), *Elaeis guineensis* (XP_010930700.1). Identical and similar amino acids are highlighted in black and grey, respectively.

<table>
<thead>
<tr>
<th>1. P. sylvestris</th>
<th>2. ACO P. taeda</th>
<th>3. ACO P. sitchensis</th>
<th>4. ACO P. pinaster</th>
<th>5. ACO E. guineensis</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLYFSRXK</td>
<td>VPEAVPI</td>
<td>VPEAVPI</td>
<td>VPEAVPI</td>
<td>VPEAVPI</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LLYFSRXK</td>
<td>VPEAVPI</td>
<td>VPEAVPI</td>
<td>VPEAVPI</td>
</tr>
</tbody>
</table>

1. P. sylvestris
2. ACO P. taeda
3. ACO P. sitchensis
4. ACO P. pinaster
5. ACO E. guineensis
Original publications


Reprinted with permission from Elsevier Masson (I), Springer (II) and Oxford University Press (IV).

Original publications are not included in the electronic version of the dissertation.
647. Cherevatova, Maria (2014) Electrical conductivity structure of the lithosphere in western Fennoscandia from three-dimensional magnetotelluric data


650. Shao, Xiuyan (2015) Understanding information systems (IS) security investments in organizations


652. Tolkkinen, Misko (2015) Biodiversity and ecosystem functioning in boreal streams: the effects of anthropogenic disturbances and naturally stressful environments


655. Li, Ying (2015) Users’ information systems (IS) security behavior in different contexts


657. Lappalainen, Katja (2015) Modification of native and waste starch by depolymerization and cationization: utilization of modified starch in binding of heavy metal ions from an aqueous solution


Book orders:
Granum: Virtual book store
http://granum.uta.fi/granum/
Riina Muilu-Mäkelä

POLYAMINE METABOLISM OF SCOTs PINE UNDER ABIOTIC STRESS