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# Proteome analysis provides insight into dormancy and germination of silver fir embryo and megagametophyte

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**Abstract**: Seed structural and physiological properties, together with environmental conditions, control dormancy. Cold stratification is known to alleviate seed physiological dormancy promoting seed germination. The present research aimed to investigate changes in the proteome during seed cold stratification and germination of silver fir (*Abies alba* Mill.), a conifer gymnosperm tree native to the mountains of Europe. Analysis of the identified proteins' functions and associated metabolic pathways would enhance the knowledge of these processes. The proteomes were analysed separately for embryo and megagametophyte using 2D electrophoresis. Forty-nine proteins displaying significant differential abundance during seed dormancy breaking and germiantion were characterised using mass spectrometry. Thirty four proteins were characteristic of the embryo germination and 41 of the megagametophyte. Twenty-six proteins differed between embryos and megagametophytes. Thirteen spots were identified as vicilin-like storage proteins, which were generally abundant in the megagametophyte, slowed down accumulation during cold stratification, and reached minimal abundance during germination. Vicilin-like proteins are the main storage reserves of most angiosperms and gymnosperms. Biological process analysis showed that proteins of both seed tissues were generally associated with protein folding, defence response and seed maturation. Molecular function analysis suggested an involvment of the identified proteins in ATP binding, nutrient reservoir activity and metal ion binding. Proteins were generally predicted to localise in the nucleus, cytosol and plastids. Dormancy breaking and germination of silver fir seed required proteins involved in diverse processes: facilitating plasmodesmata aperture (Gnk2-homologous domain-containing protein), nitrogen remobilisation from protein degradation (glutamine synthetase), abscisic acid signalling (cytochrome P450 protein CYP720PB12 ), transcription (R2R3-Myb14 transcription factor, HDAC) and protein modification (PNGase A).

**Keywords:** dormancy breaking, embryo, gymnosperms, megagametophyte, proteins, proteomics, stratification

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## Introduction

Plants developed seed dormancy to prevent further growth under unfavourable conditions (Sajeev et al., 2024). Dormancy is regulated by the seed's structural and physiological characteristics as well as external environmental factors. Dormancy is initiated as part of the inner program governing seed maturation. It can be induced by the seed covering structures originated from the mother plant or by embryo

itself, either independently or jointly (Sajeev et al., 2024). In most species where dormancy is associated with mature embryo, the underlying mechanisms are linked to reversible metabolic processes, known as physiological dormancy (Bewley et al., 2013). Seeds exhibiting this type of dormancy can be induced to germinate through various treatments, including cold stratification (Yan & Chen, 2020).

Temperature is a critical environmental factor influencing seed dormancy and germination (Xia et al., 2018; Klupczyńska & Pawłowski, 2021; Kurpisz & Pawłowski, 2022). Despite extensive research, the exact mechanism by which temperature promotes germination of dormant seeds remains poorly understood (Carrera-Castaño et al., 2020). Previous research has suggested that dormancy-breaking mechanisms vary based on the depth of dormancy. Nevertheless, similarities have been observed between the dormancy mechanisms in tree seeds and the herbaceous model plant *Arabidopsis thaliana* (Staszak et al., 2019). Proteins associated with energy metabolism, heat shock proteins (HSP), aspartate aminotransferase, elongation factor-2 (EF-2), alfa-tubulin and late embryogenesis abundant (LEA) proteins, have been identified in Norway maple (*Acer platanoides*) and Arabidopsis, playing key roles in controlling germination (Pawłowski, 2009; Ruelland & Zachowski, 2010). Several processes have been proposed as components of the dormancy-breaking and germination pathways: temperature sensing (membrane modifications, cytoskeletal changes, protein conformation), signaling (redox state, ROS production, calcium sequestration, phosphorylation), and cellular responses (alterations in gene expression and metabolism) (Ruelland & Zachowski, 2010). Signal perception and transduction proteins, including abscisic acid (ABA) receptor, are associated with dormancy alleviation and germination of Arabidopsis seeds (Baudouin et al., 2022; Wang et al., 2022). Additionally, calreticulin, a calcium-binding protein, has been linked to dormancy breaking in tree seeds such as Norway maple, sycamore (*Acer pseudoplatanus*) and beech (*Fagus sylvatica*) (Pawłowski, 2007, 2009; Pawłowski & Staszak, 2016). The observed increase in actin levels during dormancy alleviation suggest a shift toward cell elongation and expansion necessary for seed germination (Pawłowski et al., 2020). Transcription regulators also influence the expression of genes involved in dormancy break and germination, alongside translation factors, proteasome activity, and methionine metabolism, which contributes to DNA methylation (Pawłowski, 2010; Zhang et al., 2015; Pawłowski & Staszak, 2016). The final stages of dormancy-breaking involve metabolic activation, including energy production, methionine metabolism, protein degradation, and seed storage reserves mobilisation (Deng et al., 2016; Mei et al., 2017; Wu & Shen, 2021; Ren & Lv, 2024). These processes enable seeds transition from dormancy to germination by triggering cell cycle activation (Pawłowski et al., 2004).

Proteomics has significantly advanced our understanding of seed germination, proving particularly valuable for studying various forest taxa, including *Quercus ilex* (Romero-Rodriguez et al., 2015; Rey et al., 2019; Romero-Rodríguez et al., 2019), *Fagus sylvatica* (Pawłowski, 2007), *Acer* (Pawłowski, 2009; Pawłowski & Staszak, 2016), *Populus* (Zhang et al., 2017) and *Araucaria angustifolia* (Balbuena et al., 2011). However, most research has focused on seeds without dormancy, with limited studies addressing dormancy breaking in tree seeds, especially within the gymnosperms. Similar to other model species with dormant seeds, research on *Taxus chinensis* has revealed that ABA and gibberellic acid (GA) influence protein translation, and the relation between these phytohormones dictates whether seeds remain dormant or germinate (Chen et al., 2023). During germination, energy derived from carbohydrate metabolism (via the tricarboxylic acid cycle (TCA) and glycolysis) supports the pentose phosphate pathway, while lipid-derived energy comes mainly from triacylglycerol lipolysis (Chen et al., 2023).

Silver fir (*Abies alba*) is a coniferous species of significant ecological and economic importance, native to the mountains of Central and Southern Europe (Litkowiec et al., 2016). In Poland, due to the decline of populations caused by air pollution, silver fir is part of a restoration program (Litkowiec et al., 2016). Silver fir seeds exhibit physiological dormancy regardless of their moisture content or age. Their germination requires a period of moistening followed by stratification for approximately three months at 1–5 °C. Proteomics, combined with genome sequencing, offers unprecedented opportunities to identify the full suite of expressed proteins and relate their variations to seed development, maturation, and germination (Staszak & Pawłowski, 2012). In this study, we used a proteomic approach to analyse for the first time dormancy breaking and germination in silver fir seeds, thereby characterising the identity of proteins implicated in the regulation of these seed traits. We expand the current understanding of dormancy and germination, which have been predominantly explored in angiosperms, by focusing on the unique physiological and proteomic changes of a gymnosperm.

## Materials and Methods

### Plant materials and experimental design

Silver fir (*Abies alba* Mill.) seeds were collected from minimum 10 trees, and polled together, in the

Kórnik Arboretum in Poland (N52°14', E17°05') in 2017. The seeds were initially dried at ambient temperature and humidity until they achieved a moisture content of 10% (fresh weight basis). Thereafter, seeds were stored shortly for just a few weeks in plastic boxes at −3 °C. After imbibition, stored seeds underwent cold stratification at 3 °C – a temperature known to break dormancy and induce germination – in closed plastic trays without medium and in the dark. After stratification, seed portions were moved weekly to a constant temperature of 20 °C for germination, starting from week 6 to week 9 of stratification. One portion of stratified seeds was subjected to the germination test at 3 °C. The germination test was conducted following the recommendations of the International Seed Testing Association (ISTA, 2019) and Suszka (Suszka, 2000), using four replicates of 50 seeds for each treatment. Germination was monitored weekly, and seeds were scored as germinated when the radicle protruded through both the testa and pericarp by at least 1 mm. Each week, during both stratification and germination tests, seeds were visually inspected, and water loss was prevented. Analysis of variance (ANOVA) and a Tukey–Kramer HSD were used to assess the influence of treatment on seed germination at $p < 0.05$ (JMP software, SAS Institute, Cary, NC, USA), assumptions to run one-way ANOVA were fulfilled.

## Protein extraction

All analyses were performed using three biological replicates, 50 seeds each. Seed samples were collected weekly, starting with dry dormant seeds, followed by seeds undergoing cold stratification at 3 °C from week 1 to week 6, without any signs of germination, and finally, germinated seeds at 20 °C after 8 weeks of stratification at 3 °C, characterised by a 1 mm radicle protrusion. Seeds were dissected into embryos and megagametophytes, which were then frozen in liquid nitrogen and stored at −80 °C. Protein extracts for electrophoresis were prepared following the protocol of Pawłowski et al. (2019). Samples were homogenised in a 10% [w/v] solution of trichloroacetic acid (TCA) in acetone containing 20 mM dithiothreitol (DTT). After overnight protein precipitation at −20 °C, the homogenate was centrifuged at 16000g for 5 minutes at 4 °C. The resulting pellet was resuspended in acetone with 20 mM DTT and centrifuged again. The supernatant was discarded, and the pellet was vacuum dried for 2 h. Proteins were dissolved in a lysis buffer containing 7 M urea, 2 M thiourea, 2% [w/v] 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1,5% [v/w] DTT, and 0.5% [v/v] Immobiline pH Gradient (IPG) buffer 4–7. After centrifugation at 16000g for 5 minutes at 4 °C, total protein concentration was measured according to the method described by Ramagli & Rodriguez (1985).

## Protein electrophoresis, 2-DE IEF/SDS-PAGE

Proteins (100 mg for silver and 600 µg for colloidal Coomassie Blue staining) were first separated by isoelectric focusing using rehydrated immobiline dry strips (24 cm, linear pH gradient 4–7) in a rehydration buffer containing 6 M urea, 2 M thiourea, 2% [w/v] CHAPS, 20 mM [w/v] DTT, and 0.5% [v/v] IPG buffer (pH 4–7). Separation was performed with an Ettan IPGphor 3 IEF System (GE Healthcare) according to the manufacturer's protocol for 24-cm strips. The focused strips were equilibrated sequentially: first in equilibration solution I (6 M urea, 1.5 M Tris-HCl, pH 8.8, 30% [v/v] glycerol, 2% [w/v] SDS, 1% [w/v] DTT) and then in equilibration solution II (identical to solution I but with 2.5% [w/v] iodoacetamide instead of DTT) for 10 minutes each. For the second dimension (SDS-PAGE), pre-cast Ettan Double-Dimension Analysis using Labcast or Precast Gels (DALT) 12.5% (w/v) polyacrylamide gels (GE Healthcare) were used in an Ettan Dalt Six electrophoresis chamber for 1 h at 80 V followed by 5 h at 500 V. Molecular weight markers (GE Healthcare) were loaded alongside the Immobiline strip for size reference. Three replicate gels were run for each treatment ($n = 3$). Following electrophoresis, gels were stained with silver for densitometric analyses and with colloidal Coomassie Blue for Mass Spectrometry (MS) analyses (Neuhoff et al., 1988).

## Analysis of 2D PAGE gels

Gels were scanned and analysed using 2D Image Master 7 Platinum software (GE Healthcare). After spot detection, gels from three independent biological replicates were aligned and matched, and the normalised spot volumes were calculated. Statistical analyses, including measures of central tendency, dispersion, and overlapping between gel sets/classes, were conducted to identify variation in spot abundance. Assumptions to run one-way ANOVA were fulfilled. Spots with significant variation in abundance were subjected to ANOVA followed by a Tukey–Kramer HSD test (JMP software, SAS Institute, Cary, NC, USA) to identify differences ($p < 0.05$) for two factors: time and seed structure (embryo and megagametophyte). Proteins with significant changes in abundance were subsequently identified using MS.

## Protein identification and quantification

The gel spots were processed using a standard in-gel digestion protocol. Proteins were reduced with 10 mM [w/v] DTT for 30 min at 56 °C, alkylated with 55 mM iodoacetamide for 45 minutes in the dark at room temperature, and digested overnight

with Sequencing Grade Modified Trypsin (Promega V5111) in 25 mM ammonium bicarbonate (25 ng $\mu l^{-1}$ of trypsin). The resulting peptides were eluted from the gel matrix with 0.1% [v/v] trifluoroacetic acid (TFA) in 2% [v/v] acetonitrile (ACN).

Peptide mixtures were analysed using liquid chromatography coupled with a mass spectrometer. Samples were first concentrated and desalted on a RP-C18 pre-column (nanoACQUITY Symmetry® C18, Waters, USA), and further peptide separation was carried out on a nano-Ultra Performance Liquid Chromatography (UPLC) RP-C18 column (Waters, BEH130 C18 column, 75 µm i.d., 250 mm long) using a nanoACQUITY UPLC system (Waters). Separation was achieved with a linear gradient (0–60% [v/v] ACN over 120 min) in the presence of 0.05% [v/v] formic acid at a flow rate of 150 nl/min. The column outlet was directly connected to the Electrospray Ionization (ESI) ion source of the 3'-Orbitrap Velos mass spectrometer (Thermo Scientific, San Jose, USA), operating in a data-dependent MS to MS/MS switch mode. An electrospray voltage of 1.5 kV was applied. A blank run was performed before each analysis to avoid cross-contamination from previous samples.

The Mascot search algorithm (http://www.matrixscience.com) was utilised for protein identification against the NCBInr database (http://www.ncbi.nig.gov). Protein identification was performed using the Mascot probability-based molecular weight search and MOWSE score. The ions score was calculated as $-10 \times \log(P)$, where P represents the probability that the observed match was random. Peptides with a Mascot score exceeding the threshold value, corresponding to < 5% false positive rate, were considered positively identified.

## Statistical and bioinformatic analyses of proteins

EPCLUST hierarchical clustering was chosen to identify the main classes of variations in the data matrix of mean-centred spot percentage volumes. Correlation-measured distances and the UPGMA algorithm were employed for the analysis. Identified proteins were categorised based on biological processes, molecular functions, and subcellular localisation according to gene ontology (GO) annotations, using the UniProt database and Arabidopsis reference genome (https://www.uniprot.org/) (Table S5). The R software was used for statistical analysis and graphical presentation of the GO and heatmap data (R Core Team). The analysis involved ggplot2 (Wickham, 2016), dplyr (Wickham et al., 2023), tidyr (Wickham H, Vaughan D, Girlich M, 2024), stringr (Wickham, 2023) tidyverse (Wickham et al., 2019) and readxl (Wickham H, Bryan J, 2023) libraries. A Venn diagram of protein quantification according to seed stratification and germination conditions was generated using the Bioinformatics and Evolutionary Genomics tool (http://bioinformatics.psb.ugent.be/webtools/Venn/).

# Results

## Germination

The analysis of silver fir seed germination capacity (i.e. the total percentage of seeds that germinated at a certain time) revealed that germination was slowest and reduced when seeds were stratified and kept at 3 °C, reaching only 52% by week 14 (Fig. 1). In contrast, transferring the seeds to 20 °C after 8 weeks of cold stratification resulted in the highest germination capacity, reaching 84% by week 10. Similarly, moving the seeds to 20 °C after 6, 7, and 9 weeks of cold stratification led to the germination capacities of 76, 68, and 50%, respectively, within only two weeks. Thus, exposure to 20 °C, compared to continuous stratification at 3 °C, increased and significantly accelerated germination, reducing the time of reaching maximal germination capacity from 7 to 2 weeks.

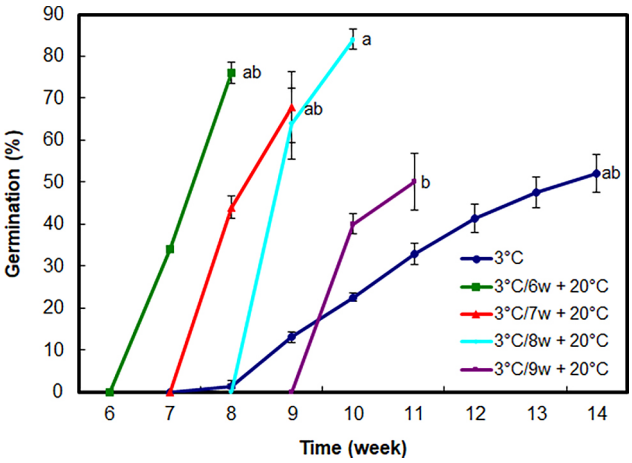


Fig. 1. Germination of silver fir seeds assessed after seed imbibition, stratification at 3 °C, and subsequent transfer to 20 °C, starting from week 6 to week 9 of stratification. Germination of seeds maintained at 3 °C throughout the stratification period was also observed. Error bars represent standard errors (n = 4, 50 seeds each). Data labelled with different letters indicate significant difference ($p < 0.05$), based on ANOVA and Tukey–Kramer HSD test

## Proteome maps and identification results

Changes in protein abundances were analysed by comparison of the two-dimensional gels across

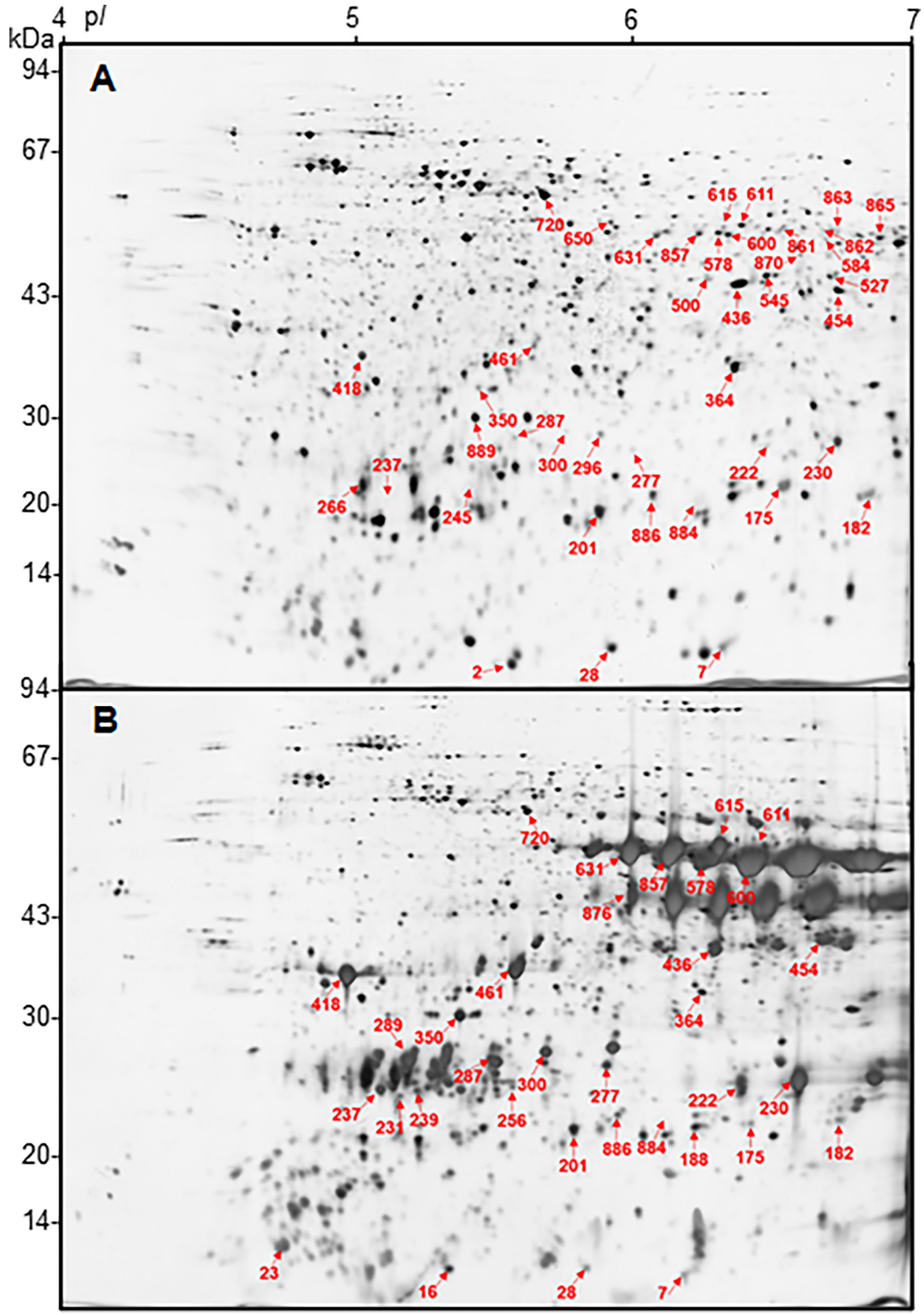


Fig. 2. Positions of the identified protein spots on the silver-stained 2D-PAGE gels of silver fir seeds. Proteome variation during dormancy breaking via stratification and germination in (A) the embryo and (B) the megagametophyte. These numbered positions correspond to the 49 separated and characterised proteins, which combine the results of 1500 spot group analysis, as listed in Table S1 (Supplementary File 1). Specific spots display variations during stratification and between the embryo and megagametophyte

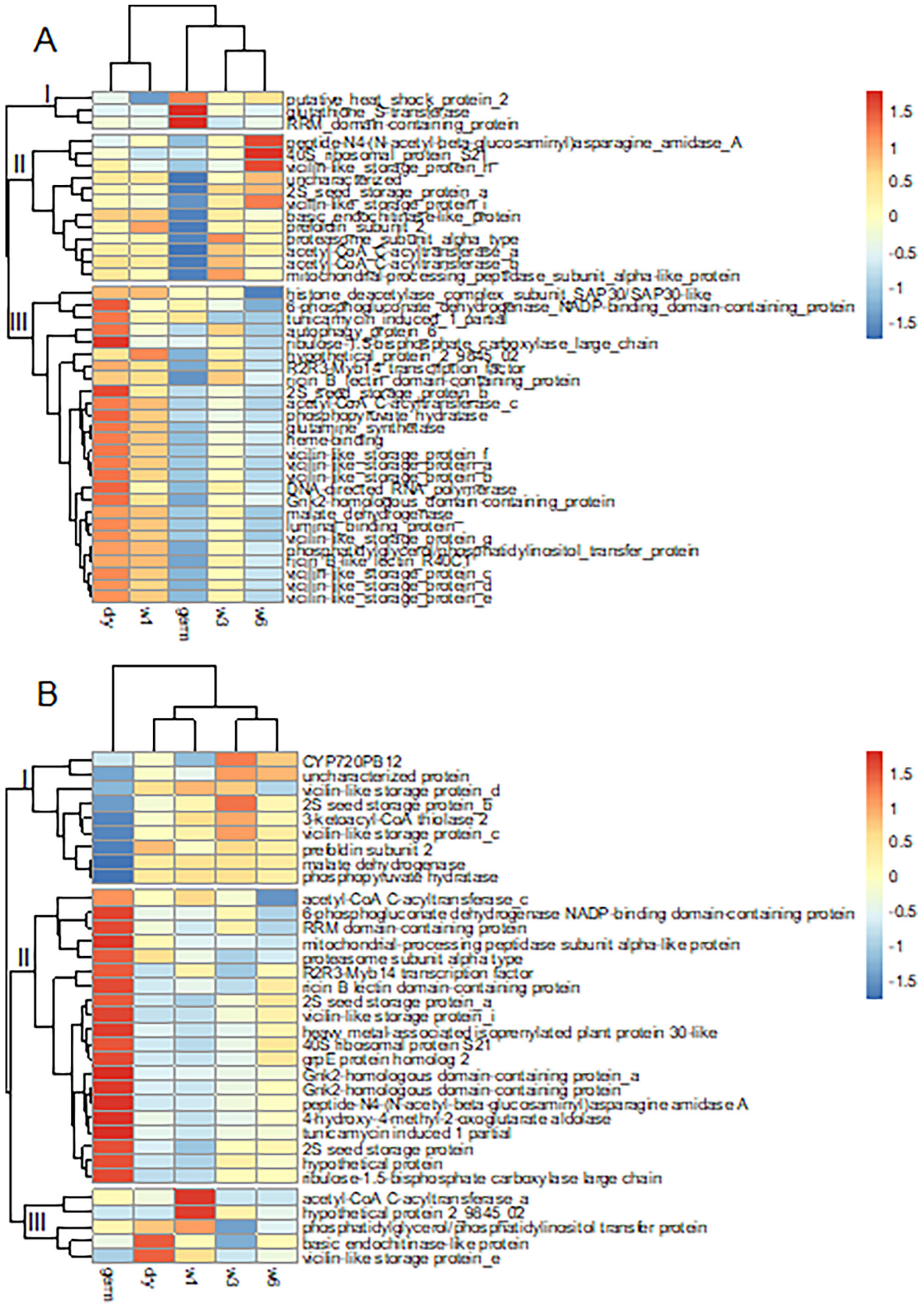


Fig. 3. Heatmap and hierarchical clustering analysis of the 49 identified silver fir seed proteins, which varied during dormancy breaking and germination in the embryo (A) and in megagametophyte (B). The spots indexed in Table S1 were classified based on their volume percentage changes from dry seeds through stratification to germination, using the UPGMA method. Dry – dry dormant seeds; w1, w3, w6 – stratified seeds for weeks 1, 3 and 6; germ – germinated seeds.

successive stages of dormancy breaking, including dry dormant seeds, seeds undergoing cold stratification at 3 °C, and germinated seeds at 20 °C. Significant variations in spot volume between stages were estimated to identify differentially abundant proteins. The effect of stratification was investigated and highligheted key protein variations in the embryo and megagametophyte (Fig. 2). Proteins exhibiting significant up- or down-regulation were identified as key candidates for further characterisation via MS.

Using Image Master 7 Platinum (GE Healthcare), 1500 proteins were found on silver-stained 2D-PAGE gels. Among them, 49 spots showed significant abundance variability (ANOVA), presenting about 3% of the total proteins on the master gels (Supplementary File, Table S1, S2, S3; and Fig. 2). The protein patterns from week three of stratification were chosen to create two master gels for the embryo and megagametophyte (Fig. 2, panels A and B, respectively), incorporating the statistical analysis results of protein volume changes induced by cold stratification.

A total of 34 spots were regulated during seed dormancy breaking in the embryo, and 41 spots in the megagametophyte. Additionally, 26 spots showed variability in both embryos and megagametophytes (Supplementary File, Table S4, Venn diagram). The proteins with modulated expression levels were analysed for amino acid sequences using MS. These amino acid sequence were then matched against protein databases in NCBI using MASCOT, leading to successful identification of all analysed proteins. The MS analysis revealed homology of the identified embryo and megagamethophyte proteins for 46 proteins from the database, with one protein uncharacterised and two labelled as hypothetical (Supplementary File, Table S1).

To summarise the data in Table S1 and classify the proteins with similar expression profiles during seed dormancy breaking, heatmap and hierarchical clustering were applied to the identified spots for both the embryo as well as for the megagametophyte (Fig. 3). Spots were clustered based on the volume percentage variations from the dry seeds to germination, using the unweighted pair group method with arithmetic mean (UPGMA). For the embryo, three main clusters could be distinguished. Cluster I contained 3 protein whose abundance increased during germination (Fig. 3A). Cluster II contained

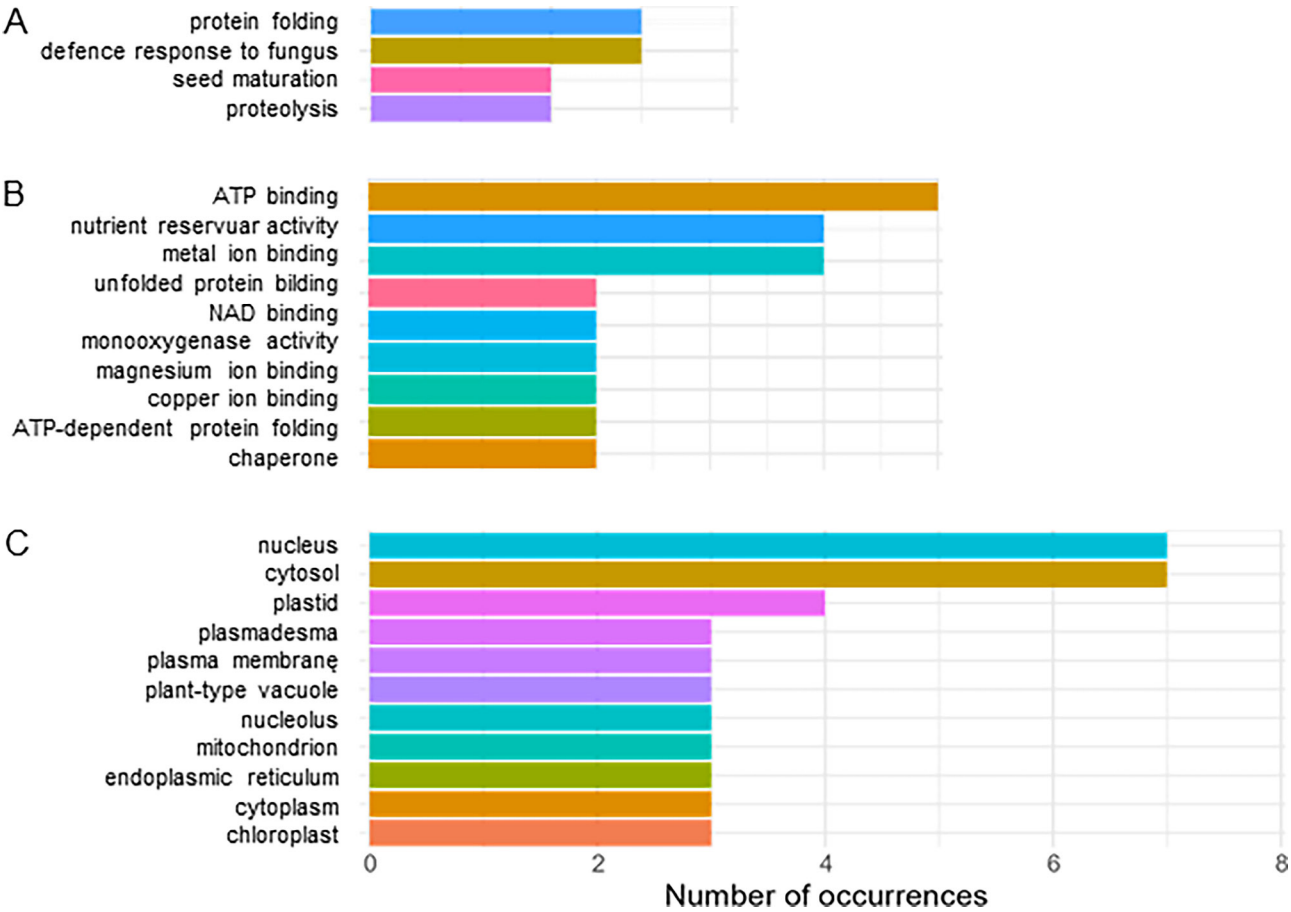


Fig. 4. Gene Ontology (GO) functional annotation of the identified silver fir seed proteins from both embryo and megagametophyte, categorised by biological process (A), molecular function (B), and cellular component (C). UniProt database was used to classify the proteins (Supplementary File, Table S5, S6, S7, S8)

12 proteins, whose abundance decreased during dormancy breaking. Cluster III contained 26 proteins whose abundance decreased during dormancy breaking and germination. For the megagametophyte, three main clusters were also identified. Cluster I included 9 proteins whose abundance decreased during germination, cluster II included 20 proteins whose abundance increased during dormancy breaking and germination, while fewer proteins (5) were grouped in cluster III, whose abundance decreased during dormancy breaking (Fig. 3B).

The spots characterised as the same protein probably denote post-translational modifications (PTMs) of a single protein or various isoforms of the protein.

To gain further information into the biological regulation and functioning of identified proteins in dormancy breaking and germination of silver fir seeds, we performed gene ontology (GO) analysis, based mostly on the Arabidopsis protein homologues (Supplementary File, Table S5). The biological process characterisation revealed that three proteins were associated with protein folding, defence response to fungus, seed maturation, and proteolysis (Fig. 4A; Supplementary File, Table S6). The molecular function analysis indicated that five proteins were linked to ATP binding, four to nutrient reservoir activity, and metal ion binding (Fig. 4B; Supplementary File, Table S7). The cellular component analysis suggested that seven proteins were localised in the nucleus and cytosol, while four were found in the plastid (Fig. 4C; Supplementary File, Table S8).

# Discussion

Seed germination is a paramount topic in plant research, however the mechanisms underlying this process are still not fully elucidated (Pawłowski et al., 2024). Proteomics, as a powerful approach for functional analysis, has been extensively implemented to study seed development, dormancy, and germination (Abril et al., 2011; Galland et al., 2017; Pawłowski et al., 2019). This study utilised proteomic analysis to investigate dormancy breaking and germination of silver fir seeds. The majority of protein accumulated at the end of dormancy breaking and in germinated seeds, marking a clear metabolic resumption as seeds transition from dormancy to germination (Staszak et al., 2017). The roles of the identified proteins and their associated metabolic processes are further discussed.

## Storage reserve

Thirteen spots were identified as vicilin-like storage proteins, which were predominantly abundant in the megagametophyte (Fig. 3B, Table S3). Their accumulation decreased with dormancy breaking and germination. These proteins serve as the main storage reserves in plant species, including both angiosperms and gymnosperms. For example, vicilin-like storage proteins have been isolated during germination from *Araucaria angustifolia*, an endangered Brazilian native conifer (Balbuena et al., 2011). These proteins share identity with 7S globulins found in white spruce (*Picea glauca*) seeds (Newton et al., 1992). Studies on transcript expression suggest that ABA and sucrose directly influence their mRNA synthesis (Newton et al., 1992). In addition to vicilin-like storage proteins, 2S seed storage proteins were also identified in the embryo and megagametophyte of silver fir (Fig. 3, Table S2 and S3). These storage proteins can provide carbon skeletons and energy necessary to support the early growth of silver fir.

## Membrane transport

The Gnk2-homologous domain-containing protein, identified in the embryo (spot 277; Fig. 3A, Table S2) and megagametophyte (spots 239, 277; Fig. 3B, Table S3), has been characterised in gymnosperm as a seed storage protein with antifungal activity (Miyakawa et al., 2014). The GnK2-1 domain of plasmodesmata-located proteins (PDLP7), in association with callose and glucan endo-1,3-β-glucosidase 10 (BG10), has also been implicated in the regulation of plasmodesmata opening and closure, processes essential for the intercellular movement of various molecules (Li et al., 2024; Chen et al., 2024). Additionally, Gnk2 is expressed in the shoot apex and influences the control of Arabidopsis lateral root growth via auxin signalling (Bayer et al., 2008; Sager et al., 2020). Dormancy-breaking conditions altered its abundance: it increased in silver fir megagametophyte and decreased in embryo. The elevated abundance of the Gnk2-homologous domain-containing protein in the megagametophyte (Fig. 3B, Table S3) may be associated with the suppression of plasmodesmal trafficking. In contrast, its reduced abundance in the embryo (Fig. 3A, Table S2) could facilitate plasmodesmata aperture, thereby promoting root growth.

## Metabolism

The abundance of glutamine synthetase (spot 870; Fig. 3A, Table S2) decreased in the embryo of silver fir during dormancy breaking. In Arabidopsis, this enzyme is expressed in root tips, root hairs, and epidermis, where it plays a critical role in maintaining glutamine production homeostasis (Bernard & Habash, 2009). Glutamine synthetase is a central enzyme of nitrogen metabolism and catalyses the assimilation of ammonium into amino

acids. Glutamine synthetase cytosolic isoenzymes assimilate ammonium from primary nitrogen as well as from various internal nitrogen recycling sources. Therefore, cytosolic glutamine synthetase is essential for the remobilisation of nitrogen from protein degradation (Bernard & Habash, 2009). Nitrogen recycling is particularly important during seed germination, dormancy acquisition, active growth resumption, and lignin biosynthesis during wood formation (Balbuena et al., 2011).

Glutathione S-transferase zeta (GSTZ, spot 886; Fig. 3A, Table S2) is a member of the glutathione S-transferase (GST) enzyme family, which plays a crucial role in metabolism, detoxification, and protection against oxidative stress (Öztetik, 2008). GSTZ detoxifies reactive intermediates that can accumulate in cells due to metabolic processes or environmental factors. GSTZ uses glutathione as a cofactor, helping to control oxidative damage and maintain cellular redox homeostasis during seed germination (Frova, 2003; Kalemba & Ratajczak, 2018). In silver fir embryo, GSTZ reached its highest abundance in germinated seeds (Fig. 3A, Table S2). GST activity in *Abies nordmanniana* has been associated with systemic resistance during seed germination and seedling growth (Garcia-Lemos et al., 2020). Similar GSTZ expression patterns were observed during the dormancy breaking of *Acer platanoides* and *A. pseudoplatanus* seeds (Pawłowski, 2009; Pawłowski & Staszak, 2016) suggesting a positive impact on seed germinability through a protective role.

CYP720PB12 (spot 23; Fig. 3B, Table S3) is a cytochrome P450 protein that catalyses various mono-oxygenation and hydroxylation reactions in plant growth and development (Xiang et al., 2023). Cytochrome P450 reductase expression was differentially regulated in the cotyledons, radicle and megagametophyte of Douglas fir seeds (Tranbarger et al., 2000), with its activity increasing during stratification, germination and early seedling development. A similar increase was observed during stratification of silver fir, but only in the megagametophytes (Fig. 3B, Table S3). The CONSTITUTIVE PHOTOMORPHOGENIC DWARF (CPD) gene, encoding a cytochrome P450 monooxygenase is upregulated during Arabidopsis seed germination (Piskurewicz et al., 2024). Xiang et al. (2023) found that cytochrome P450 CYP77A4 directly balanced lipid mobilisation and reactive oxygen species (ROS) synthesis by the epoxidizing lipids during seed germination. Additionally, Arabidopsis CYP707A encodes ABA 8'-hydroxylases, a key enzyme involved in ABA degradation (Kushiro et al., 2004). In summary, cytochrome P450 appears to be involved in seed dormancy breaking and germination of various species, possibly via its role in the hormonal control of these processes (Kushiro et al., 2004; He et al., 2019; Yeon et al., 2022).

Malate dehydrogenase (MDH, spot 454; Fig. 3, Table S2 and S3) is an enzyme of the TCA cycle, while phosphopyruvate hydratase (enolase, spot 436; Fig. 3, Table S2 and S3) is a key enzyme in glycolysis. Both MDH and enolase were isolated from silver fir embryo and megagametophyte, with their abundance decreasing during dormancy breaking (Fig. 3, Table S2 and S3). The release of seed dormancy is associated with ATP accumulation, increased respiration, and metabolic pathways for energy production as glycolysis, electron transport chain and photosynthesis (Romero-Rodriguez et al., 2015; Rey et al., 2019; Romero-Rodríguez et al., 2019). The results of this study align with previous proteomic investigations of *Fagus sylvatica* seed (Pawłowski, 2007).

The 6-phosphogluconate dehydrogenase NADP-binding domain-containing protein (spot 287; Fig. 3, Table S2 and S3) is a homolog of glyoxylate/succinic semialdehyde reductase 1 (GLYR1), which catalyses the NADPH-dependent conversion of glyoxylate to glycolate and succinic semialdehyde (SSA) to gamma-hydroxybutyrate (Zarei et al., 2017). This protein may function in redox homeostasis and contribute to oxidative stress defence by detoxifying toxic aldehydes, such as glyoxylate and SSA, which are generated during glycolate and GABA metabolism under abiotic stress conditions (Zarei et al., 2017). The abundance of GLYR1 in silver fir embryos decreased during dormancy breaking, but increased again in germinated seeds (Fig. 3A, Table S2). In the megagametophyte, GLYR1 reached its highest abundance in the germinated seeds (Fig. 3B, Table S3), suggesting its role in reducing toxic aldehydes in early-growing seed tissues during dormancy breaking by cold stratification.

Ketoacyl-CoA thiolase 2 (PDE1/KAT2, spot 876; Fig. 3B, Table S3) and its homologue acetyl-CoA C-acyltransferase (spots 578, 611, 650; Fig. 3, Table S2 and S3) catalyse a key reaction in fatty acid beta-oxidation, which occurs prior to gluconeogenesis (Germain et al., 2001). The Arabidopsis KAT2 gene is expressed in reproductive tissues during seed germination and throughout seedling emergence (Kato et al., 1996; Germain et al., 2001; Footitt et al., 2007; Wiszniewski et al., 2014). Germain et al. (2001) suggest that although gluconeogenesis using fatty acids is not essential for supporting seedling development, peroxisomal β-oxidation is is necessary to utilse triacylglycerol stored in lipid bodies. In this study, KAT2 abundance increased during stratification in silver fir megagametophyte (Fig. 3B, Table S3), but decreased in germinated seeds in both embryo and megagametophyte (Fig. 3, Table S2 and S3). Therefore, these identified enzymes, likely involved in peroxisomal fatty acid β-oxidation, could mobilise storage lipids during seed dormancy breaking.

Luminal binding protein (spot 584; Fig. 3A, Table S2) is a homologue of Heat shock 70 kDa protein BIP2. In conjunction with other chaperones, Hsp70s play a crucial role in the folding and translocation of new synthesised proteins, and the degradation of damaged proteins under stress conditions. BIP proteins are implicated in endosperm nuclei proliferation (Maruyama et al., 2010, 2020). A luminal binding protein was found exclusively in the embryo axes of silver fir, where its abundance decreased as dormancy was broken and germination progressed (Fig. 3A, Table S2). This protein was also reported during the germination of barley and Arabidopsis seeds, where it was associated with stress defence (Bønsager et al., 2007; Zhao et al., 2021). BIP2 likely involves protective processes during the initiation of seed germination (Pawłowski, 2007; Pawłowski & Staszak, 2016).

The R2R3-Myb14 transcription factor (spot 418; Fig. 3, Table S2 and S3) was identified in both the embryo and megagametophyte of silver fir. R2R3-MYB factors regulate secondary metabolism, cell structure, disease and abiotic stress resistance, and hormone responses (Kranz et al., 1998). R2R3-MYBs specifically control seed dormancy breaking and germination via ABA/GA signalling, influencing the expression of key genes, such as *ABSCISIC ACID INSENSITIVE 5* (*ABI5*), *DELAY OF GERMINATION 1-LIKE 3* (*DOGL3*), and *GA-STIMULATED ARABIDOPSIS 4* (*GASA4*) (Reyes & Chua, 2007; Kim et al., 2015; Zhao et al., 2022; Zhang et al., 2024; Li, Xiang, et al., 2024). The observed increase in R2R3-Myb14 abundance in megagamethophyte during silver fir seed stratification suggests its potential role in enhancing transcriptional activity for dormancy breaking and germination (Fig. 3B, Table S3). The involvement of R2R3-Myb14 in dormancy release, rather than maintenance, has also been reported in other species (Himi et al., 2012; Nagel et al., 2019; Lang et al., 2021; Zhang et al., 2022).

Histone deacetylase complex subunit SAP30/SAP30-like (HDAC, spot 245; Fig. 3A, Table S2) is an enzyme that deacetylates lysin residues of histone and other proteins (Loidl, 2004). This deacetylation leads to chromatin condensation and transcriptional repression (Hollender & Liu, 2008). HDAC plays critical roles in plant defence, adaptation, and development, including seed dormancy breaking and germination (Tai et al., 2005; Wang et al., 2013; Pagano et al., 2019; Zheng et al., 2022). Suppression of HDAC activity using trichostatin A increases global histone acetylation and inhibits seed germination (Zhang et al., 2011). The balance between acetyltransferases (HATs) and HDACs affects the acetylation of the viviparous1 (VP1), with ABA activating its transcription through the aggregation of acetylated histone H3 at the promoter region during seed germination. In Arabidopsis, the acetylases HD2A and HD2C play antagonistic roles in germination, with HD2A restraining and HD2C enhancing the process (Colville et al., 2011). Van Zanten et al. (2014) demonstrated that histone deacetylase HDA9 negatively regulates germination. HDA9 transcripts are abundant in dry seeds, but decrease during germination. Furthermore, HD2A and HD2B deacetylate the *DOG1* locus, repressing its expression, thereby reducing seed dormancy and promoting germination (Han et al., 2023). In silver fir, HDAC abundance was highest in embryo of dormant seeds (Fig. 3A, Table S2), suggesting an inhibitory effect on gene transcription during dormancy.

Peptide-N4-(N-acetyl-beta-glucosaminyl)asparagine amidase A (PNGase A, spot 884; Fig. 3, Table S2 and S3) is enzyme responsible for protein post-translational de-N-glycosylation that regulates protein activity (Berger et al., 1996). During seed germination, numerous temporally regulated morphological and biochemical processes depend on protein regulation. PNGase activity has been studied in a few species, with an increase observed during seed germination (Berger et al., 1996; Chang et al., 2000; Vuylsteker et al., 2000). Additionally, released N-glycans, or free oligosaccharides, have been shown to act as growth-triggering factors (Maeda & Kimura, 2014; Yamamoto et al., 2021). In silver fir, PNGase A abundance increased during dormancy breaking in both the embryo and the megagametophyte, thought it decreased in the embryo of germinated seeds (Fig. 3, Table S2 and S3). Its role is likely associated with the developmental transition from dormancy to germination, potentially through the modification of protein function or the involvement of N-glycans.

Heavy metal-associated isoprenylated plant protein 30-like (HIPP, spot 16; Fig. 3B, Table S3) is metallochaperone that contains a metal binding domain (HMA) and a C-terminal isoprenylation motif, which facilitates post-translational lipid modification of proteins (de Abreu-Neto et al., 2013). HIPPs are implicated in heavy metal homeostasis, detoxification mechanisms, cold and drought responses, and plant-pathogen interactions (de Abreu-Neto et al., 2013). Additionally, HIPPs are potential components of signalling pathways involved in the regulation of plasmodesmata (Barr et al., 2023). Overexpression of HIPP16 from *Prunus avium* has been shown to enhance low-temperature tolerance by increasing germination rate, antioxidant enzyme activity, and concentrations of osmoregulators in transgenic plants (Yu et al., 2024). The increased abundance of HIPP in silver fir megagametophyte during cold stratification (Fig. 3B, Table S3) likely reflects its role in defence against biotic stress accompanying dormancy breaking and germination.

Ricin B-like lectin R40C1 (spots 266 and 600; Fig. 3, Table S2 and S3) showed a decrease in

abundance in silver fir embryo axes and an increase in the megagametophyte during dormancy breaking and germination. Ricin B-like lectins are a family of carbohydrate-binding proteins structurally and functionally associated with the ricin B-chain found in the plant toxin ricin (Damme et al., 1998). These proteins are homologues of toxic N-glycosidase enzymes and ribosome-inactivating proteins, widely distributed in the majority of plant species and in different organs to protect against fungal or viral infections (Liu et al., 2022). The role of ricin B-like lectin R40C1 is likely linked to biotic defence mechanisms during the juvenile stage of plant development, particularly in the organs containing storage materials.

## Conclusions

This study advances knowledge of the seed biology of gymnosperms by identifying proteins, and predicting metabolic pathways and molecular processes involved in the control of seed dormancy breaking and germination. Through a proteome analyses, our study enhances the understanding of seed physiological dormancy, addressing existing gaps in the literature on seed dormancy and germination of gymnosperm species.

Our findings can find practical applications, potentially improving the efficiency of seedling production through a mechanistic understanding of seed physiological dormancy, which is a barrier to germination. Furthermore, this study establishes a foundation for future research by offering a dataset that can be further refined and expanded, for example, through individual gene expression analysis.

Future studies may build upon this work by exploring interactions between environmental factors and gene regulation, including signal perception and transduction, gene expression modulation and metabolite changes. Additionally, integrating proteomics and ecophysiological approaches could provide a more comprehensive understanding of the earliest stages plant development.

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**Supplementary Materials: Supplementary file 1.**

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