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Research article

Comparative analysis of abscisic acid levels and expression of abscisic acid-related genes in Scots pine and Norway spruce seedlings under water deficit

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A B S T R A C T

Abscisic acid (ABA) is one of the main participants in the regulation of plant responses to water deficiency. Knowledge of the ABA signal transduction pathways in gymnosperms is rather limited, especially in comparison with those in angiosperms. Seedlings of Scots pine and Norway spruce are known for their contrasting behaviour strategies under water deficit. To characterize the possible role of ABA in these differences, ABA dynamics were investigated under conditions of water deficit in seedlings of these two species. The content of ABA and its catabolites was followed in the roots and needles of seedlings of *Pinus sylvestris* and *Picea abies* under conditions of polyethylene glycol (PEG)-induced water deficiency (−0.15 and −0.5 MPa) for 10 days. The expression of the main genes for ABA-biosynthetic enzymes was also analysed. ABA showed more pronounced stress-dependent dynamics in pine roots than in spruce roots, whereas in needles, the response was greater for spruce than pine. The ABA increase during drought was mainly due to *de novo* synthesis and the shift in the balance between ABA synthesis and catabolism towards synthesis. The ABA-glucosyl ester did not serve as a reserve for the release of free ABA under water deficiency. The expression levels of the main ABA biosynthetic genes showed a weak or no correlation with changes in ABA content under water stress, i.e., the ABA content in the seedlings of both species was not directly linked to the transcript levels of the main ABA biosynthetic genes. Less-pronounced stress-induced changes in ABA in pine needles than in spruce needles may be related to pine seedlings having a less conservative strategy of growth and maintenance of water balance under water deficit.

1. Introduction

Abscisic acid (ABA) is one of the main phytohormones that acts to control a variety of different plant processes, including growth, sex determination, seed dormancy and stress tolerance. ABA is synthesized in all plants from algae to angiosperms; however, the physiological role of ABA in individual taxa or even species may be different (Sussmilch et al., 2017; Arend et al., 2009; Sreenivasulu et al., 2012).

Undoubtedly, ABA is one of the main hormones orchestrating the performance of angiosperms under water deficit (Walter Lorenz et al., 2011; Vishwakarma et al., 2017; Seiler et al., 2011; Sreenivasulu et al., 2012). ABA plays a vital role in maintaining plant water status under water deficiency, regulating the stomatal aperture and increasing the hydraulic conductivity of the tissues (Tardieu et al., 2010). However, under a prolonged water deficit, an ABA-induced reduction in gas exchange impairs carbon assimilation and nutrient acquisition processes, leading to a decline in plant growth and biomass accumulation (Sreenivasulu et al., 2012; Popko et al., 2010; Claeys and Inzé, 2013). In addition, ABA has non-hydraulic, intrinsic direct effects on plants. For example, ABA stimulates root elongation due to the suppression of ethylene biosynthesis and changes the leaf growth rate by changing the expression of expansins and cell-wall stiffening (Tardieu et al., 2010; Arend et al., 2009; Claeys and Inzé, 2013). As a result of the interaction among all these regulatory pathways, there can be large differences in the overall effects of ABA action in plants under drought stress (Tardieu et al., 2010; Sreenivasulu et al., 2012; Vishwakarma et al., 2017).

Considering the importance and diversity of ABA effects, its homeostasis must be strictly regulated. For angiosperms, the regulation of ABA homeostasis has been well studied (e.g., Prerostova et al., 2018). Regulation of intracellular ABA content largely occurs at the level of its biosynthetic gene expression (Vishwakarma et al., 2017). The main enzymes of ABA biosynthesis include β-carotene hydroxylase (BCH),

Abbreviations: 9OH-ABA, 9-hydroxy-ABA; ABA, abscisic acid; ABA-GE, ABA-glucosyl ester; DPA, dihydrophaseic acid; PA, phaseic acid; PEG, polyethylene glycol

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zeaxanthin epoxidases (ZEP), 9-cis-epoxycarotenoid dioxygenase (NCED), short-chain dehydrogenase/reductase-like enzyme (SDR1) and abscisic aldehyde oxidase (AAO) (Finkelstein, 2013). The NCED-catalysed cleavage reaction is assumed to be a control point of ABA biosynthesis (Popko et al., 2010; Seo and Koshiba, 2002; Sreenivasulu et al., 2012). The expression level of the *NCED* gene, encoding the rate-limiting NCED enzyme, is upregulated in leaves within minutes after an increase in vapour pressure deficit (Sussmilch et al., 2017; Vishwakarma et al., 2017; Daszkowska-Golec, 2016; Ikegami et al., 2009; Thompson et al., 2000). For most other genes involved in ABA biosynthesis, activation of transcription under osmotic stress has also been noted (Xiong and Zhu, 2003; Seiler et al., 2011; Seo and Koshiba, 2002). In addition to biosynthesis, ABA catabolism plays an important role in regulating the level of active ABA. The catabolism of ABA occurs mainly through oxidative and conjugative processes (Kepka et al., 2011). ABA oxidation is irreversible and leads to the formation of phaseic, dihydrophaseic and neophaseic acids (Daszkowska-Golec, 2016; Feurtado et al., 2004; Kepka et al., 2011). In contrast, ABA-glucosyl ester (ABA-GE) can be an important source of active ABA under water deficit (Seiler et al., 2011; Daszkowska-Golec, 2016; Lee et al., 2006; Sauter et al., 2002). Processes of ABA biosynthesis, degradation and conjugation act in a coordinated manner to fine-tune the ABA level in plants (Feurtado et al., 2004; Sussmilch et al., 2017).

The metabolism of ABA and its role in adaptation to water stress have been much less studied in gymnosperms than angiosperms, but substantial differences have been indicated (Sussmilch et al., 2017; McAdam et al., 2011). In contrast to mainly ABA-driven stomatal regulation in angiosperms, the regulation of gas exchange processes in gymnosperms relies on different combinations of active ABA-driven and passive hydraulic stomatal regulation (Brodribb and McAdam, 2013; Brodribb et al., 2014). More isohydric species use permanently high ABA levels to close stomata and maintain leaf water homeostasis during drought, while more anisohydric species rely on leaf desiccation to passively drive stomatal closure (Brodribb et al., 2014). ABA-mediated regulation of gas exchange in such species occurs only during the initial periods of water stress (Brodribb and McAdam, 2013). Gymnosperms contain significant amounts of ABA, often significantly more than angiosperms (McAdam et al., 2011; McAdam and Brodribb, 2015; Brodribb and McAdam, 2013). At the same time, under water deficiency, the rate of ABA content increase and the induction of ABA-mediated physiological responses are significantly lower in conifers than in angiosperms (Sussmilch et al., 2017; McAdam and Brodribb, 2015). The mechanisms of regulating ABA content in gymnosperms have not been studied; however, the homology of the primary structure for most ABA biosynthetic genes in angiosperms and gymnosperms has been found (Feurtado, 2006; Du et al., 2018; Walter Lorenz et al., 2011; Fox et al., 2017; Lu et al., 2018).

Our earlier studies revealed that Scots pine and Norway spruce seedlings implement different strategies to adapt to water deficit conditions. Under drought conditions, pine seedlings maintained growth of their root system and aboveground parts, at least in the initial period of exposure to water deficiency. At the same time, the water content of pine seedlings decreased significantly (Ivanov et al., 2018, 2019). In contrast, spruce seedlings under similar conditions better maintained their water status but sharply reduced their growth (Zlobin et al., 2018). The ABA signalling pathway acts as a hub to coordinate water and carbon interactions (Pantin et al., 2012). Previously, the exogenous ABA treatment of *Picea asperata* plants under drought stress was shown to increase the water use efficiency of the plant but suppress the assimilation and structural growth due to the decrease in gas exchange (Duan et al., 2007). Differences in ABA dynamics under water deficit may result in differences in adaptation strategies even within the same species (Beis and Patakas, 2010; De Diego et al., 2012, 2015; Duan et al., 2007; Corcuera et al., 2012).

The mechanisms of the specific stress responses of pine and spruce seedlings to water deficiency may be based on different ABA balances in the compared species. In this regard, we established two tasks:

- to analyse the dynamics of ABA and its catabolites in Scots pine and Norway spruce seedlings under water deficiency and
- to analyse the relationship between the expression profiles of the main ABA-related genes and the changes in ABA content under water stress.

2. Materials and methods

2.1. Experimental design

Seeds of Scots pine (*P. sylvestris* L.) and Norway spruce (*P. abies* (L.) H. Karst) were provided by the Training and Experimental Forestry Enterprise of the Bryansk State Technological University of Engineering (Bryansk, Russia). The seeds were germinated via hydroculture. After their cotyledons expanded, the seedlings were transferred to culture media containing 2.0 mM NH_4NO_3, 1.5 mM KH_2PO_4, 1.0 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.1 mM Na_2SO_4, 55 μM H_3BO_3, 5 μM $MnSO_4$, 1.26 μM $ZnSO_4$, 0.32 μM $CuSO_4$, 0.1 μM Na_2MoO_4, 0.02 μM $Co(NO_3)_2$, 1.0 μM KI, 9.5 μM $FeSO_4$, and 9.5 μM Na_2-EDTA at a pH of 4.5 (Ivanov et al., 2016; Zlobin et al., 2018). The seedlings were cultivated on 6-L plastic trays (171 seed beds per tray) in a growth chamber that provided a constant air temperature of 24 ± 2 °C and a 16-h photoperiod under fluorescent lighting (L36W/765, JSC OSRAM, Russia, 90 ± 20 μmol m^{-2} s^{-1}) for 6 weeks after mass seed germination. The nutrient solution was constantly aerated and changed twice a week. After 6 weeks, trays were randomly distributed among three experimental variants with 3 trays (approximately 500 seedlings at the beginning of treatment) per variant. Samples at the initial point were collected after 41 days of growth, and PEG 6000 was then progressively added to the nutrient solution. Over the course of 5 days, the water potential decreased daily by 0.03 and 0.1 MPa. Thus, after 5 days, we obtained experimental variants that had the following water potentials: −0.15 (100 g PEG/kg H_2O) and −0.5 MPa (200 g/kg H_2O) (Michel and Kaufmann, 1973). Trays not supplemented with PEG were used as controls. The experiment progressed for 10 days after the above-mentioned values of the culture medium water potential were reached. The volume of the culture medium was controlled and corrected daily. The plants were collected on the 1st (actual seedling age: 47 days) and 10th (56 days) days of full exposure.

2.2. Abscisic acid analysis

Samples of roots and needles were taken before the onset of water stress and on the 1st and 10th day of the experiment. The samples were frozen in liquid nitrogen and stored at −80 °C. They were homogenized using a mortar and pestle and extracted with cold (−20 °C) methanol/water/formic acid (15/4/1, v/v/v) as described previously (Dobrev and Kaminek, 2002; Dobrev and Vankova, 2012). The following isotope-labelled internal standards (10 pmol/sample) were then added: $^2H_6$-ABA, $^2H_3$-PA, $^2H_3$-DPA, $^2H_4$–7OH-ABA, and $^2H_5$-ABA-GE (Olchemim, Czech Republic). ABA and its metabolites were purified with a reverse phase-cation exchange solid phase extraction (SPE) column (Oasis-MCX, Waters) and eluted with methanol. The analysis was performed using high-performance liquid chromatography (HPLC) (Ultimate 3000, Dionex) coupled to a 3200 Q TRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems). Hormone quantification was performed by the isotope dilution method with multilevel calibration curves ($r2 > 0.99$). Data processing was performed with the Analyst 1.5 software package (Applied Biosystems).

2.3. RNA extraction and purification

RNA isolation was performed according to the method of Kolosova et al. (2004), with some modifications. For the *P. abies* and *P. sylvestris*

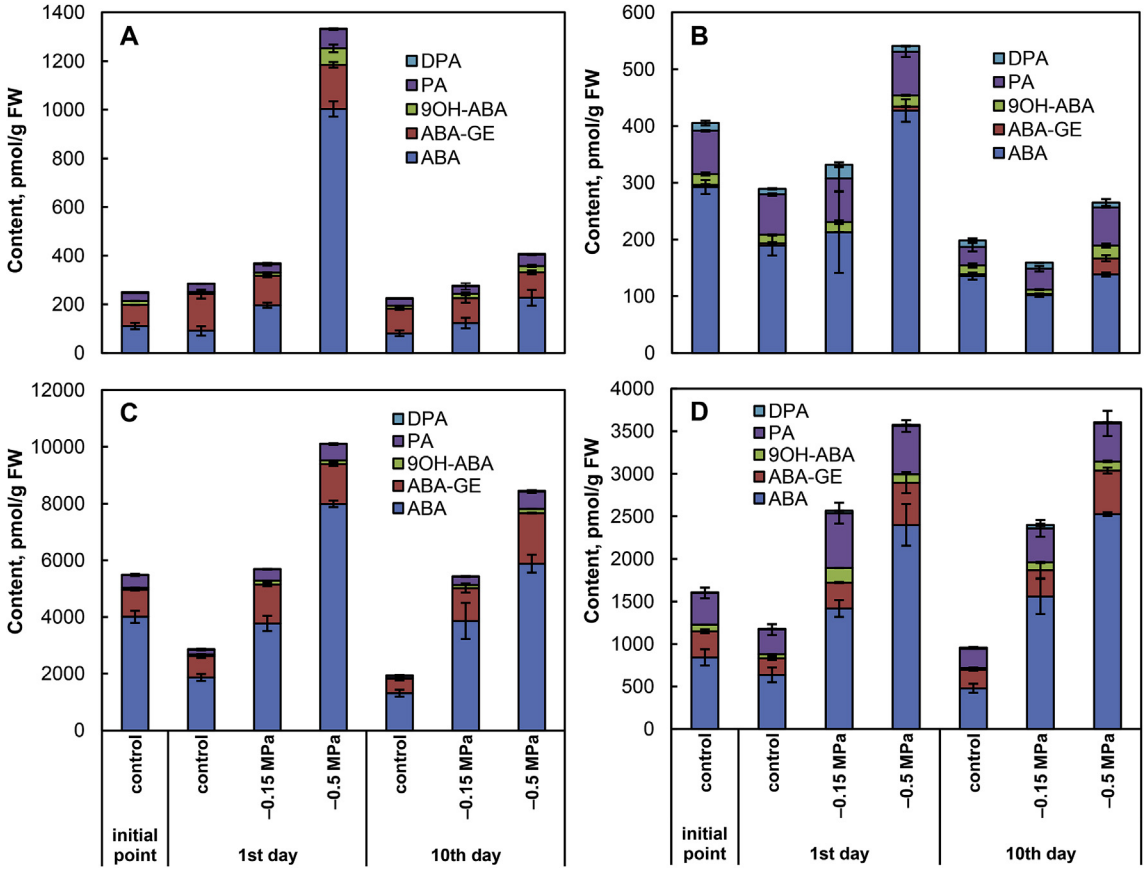

Fig. 1. Contents of ABA and ABA metabolites (pmol/g FW) in Scots pine (A, C) and Norway spruce (B, D) organs under water deficit: roots (A, B) and needles (C, D). ABA, abscisic acid; ABA-GE, ABA-glucosyl ester; 9OH-ABA, 9-hydroxy-ABA; DPA, dihydrophaseic acid; PA, phaseic acid. The graphs show relative mean values ± SE from 3 independent biological replicates.

RNA extractions, the following buffer was used: 200 mM Tris-HCl, pH 8.5, 1.5% lithium dodecylsulfate, 300 mM LiCl, 10 mM Na_2·EDTA, 1% sodium deoxycholate, and 1% Tergitol Nonidet® P-40 (NP40), and just before use, 5 mM thiourea, 1 mM aurintricarboxylic acid, 10 mM dithiothreitol, and 2% polyvinylpolypyrrolidone were added. Plant tissue (200 mg FW) was ground in liquid nitrogen using a mortar and pestle. The powder was transferred to a 2-mL tube, and 1.5 mL of extraction buffer was added and vigorously shaken. The suspension was snap-frozen in liquid nitrogen. After thawing on ice, the extracts were centrifuged at 8000 g for 60 min at 4 °C. Then, 40 μL of 3.3 M sodium acetate (pH 6.1) and 100 μL of 100% ethanol were added, and the mixture was chilled on ice for 10 min to precipitate polysaccharides. Polysaccharides were pelleted by centrifugation at 8000*g* for 30 min at 4 °C. To precipitate nucleic acids, 100 μL of 3.3 M sodium acetate and ice-cold isopropanol were added until a test tube was full, and the solution was left at −20 °C for 24 h. Nucleic acid pellets were collected by centrifugation for 60 min at 3000 g at 4 °C. The supernatant was removed, and the pellet was resuspended in 600 μL of TE (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) and 600 μL of 5 M NaCl and kept on ice for 30 min with periodic vortex mixing. Then, the samples were mixed with 300 μL of 10% cetyltrimethylammonium bromide at room temperature and incubated for 5 min at 65 °C to remove residual polysaccharides. Mixtures were extracted with an equal volume of chloroform/isoamyl alcohol (24:1, v/v), 10 M LiCl was added to each test tube until filled, and test tubes were mixed and kept at 4 °C overnight. RNA was pelleted by centrifugation at 16000*g* for 60 min at 4 °C. The RNA pellet was washed in 100% ethanol, dried by centrifugation and dissolved in 20 μL TE buffer on ice.

2.4. Quantitative RT-PCR

The quantity and quality of the total RNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). cDNA synthesis was performed using the M-MLV Reverse Transcriptase Kit (Fermentas, Canada) and the oligo (dT) 21 primer. The expression patterns of the genes were assessed using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA). Gene-specific primers were selected using nucleotide sequences from the National Center of Bioinformatics (NCBI) database (www.ncbi.nlm.nih.gov, USA) with Vector NTI Suite 9 software (Invitrogen, USA). The list of gene-specific primers is given in Table S1. The transcript levels were normalized to the expression of the *Actin* gene. The experiments were performed in three biological replicates and three analytical replicates. The mRNA levels were expressed as a percentage of the corresponding values for the control plants. The relative gene-expression signal intensity at the initial point was considered 100%.

2.5. Statistical analysis

The number of biological replicates to determine the ABA content was 3, and the gene expression analysis varied from 3 to 4. Statistical analyses of data on ABA content were performed using SigmaPlot 12.3 (Systat Software Inc., USA) with one-way analysis of variance (ANOVA) followed by Duncan's method. Statistical analyses of gene expression data were performed with ANOVA followed by Duncan's method for normally distributed data and with Kruskal-Wallis one-way ANOVA on ranks followed by Student-Newman-Keuls post hoc test for non-normally distributed data and data with unequal variance. Pairwise comparisons of the means were performed using Student's t-test. Different

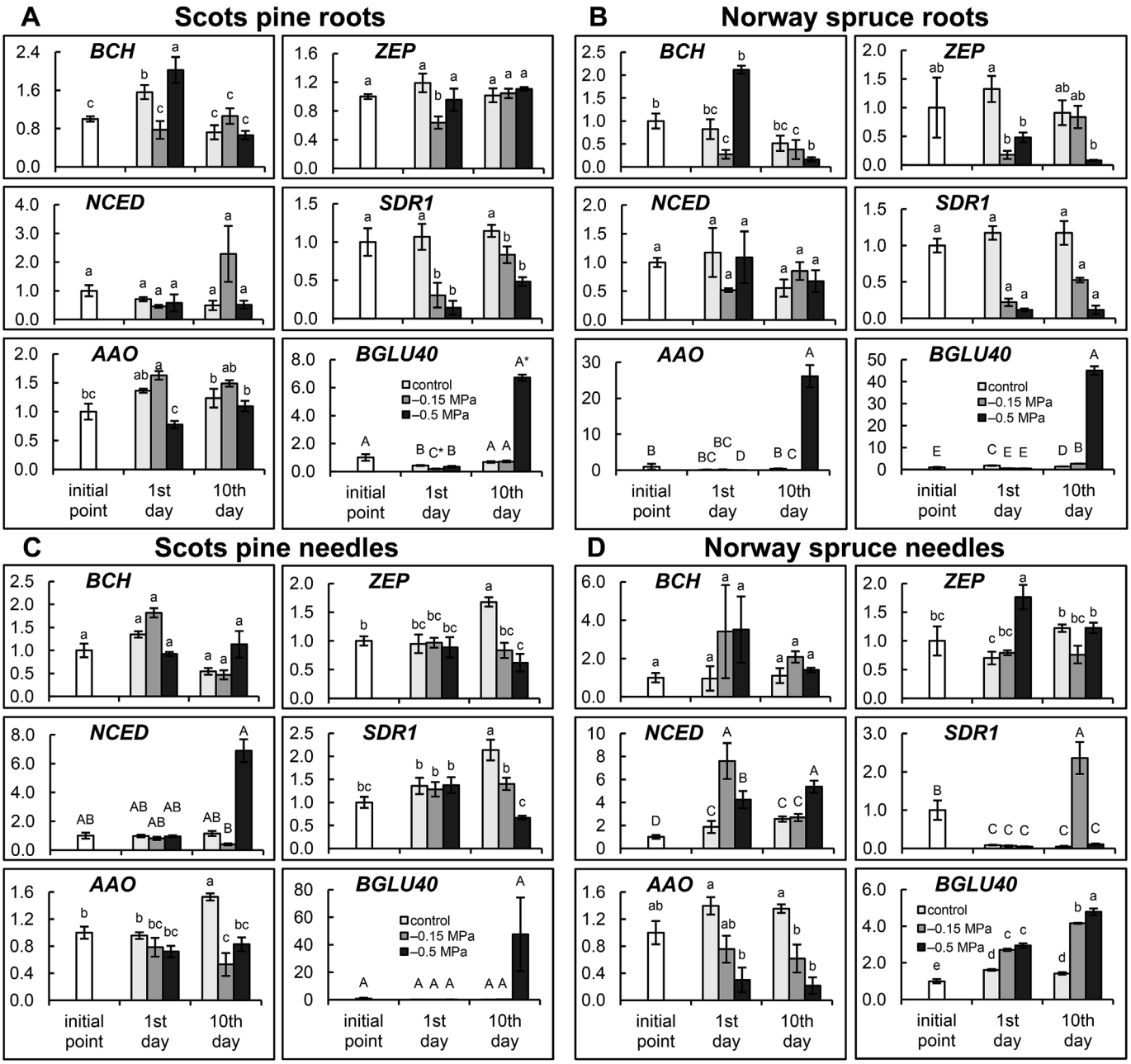

Fig. 2. Differential expression of ABA biosynthetic and deconjugation genes in Scots pine (A, C) and Norway spruce (B, D) seedlings under water deficit analysed by qRT-PCR. The graphs show relative mean values ± SE from 3 to 4 independent biological replicates, with values at the initial point taken as 1.0. Different lower-case letters denote statistically significant differences in the means at $p < 0.05$ (ANOVA followed by Duncan's post hoc test). Different upper-case letters denote statistically significant differences in the means at $p < 0.05$ (ANOVA on ranks followed by Student-Newman-Keuls post hoc test). Asterisks (*) (*t*-test) denote statistically significant differences ($p < 0.05$) in experimental variants with their corresponding controls at the same time points.

letters were used to represent significance at $p < 0.05$. The data presented in the tables and figures are the arithmetic mean values ± standard errors.

3. Results

3.1. Content of ABA and its catabolites in roots and needles of Scots pine and Norway spruce seedlings

In the pine roots, ABA and ABA-GE were the major compounds (Fig. 1A, Table S2). On the 1st day of the experiment, there was a significant increase in the content of ABA, especially pronounced at −0.5 MPa. Absolute amounts of ABA-GE did not decrease under water stress compared to control conditions. However, the ratio of ABA-GE to free ABA decreased in a stress dose-dependent manner. Whereas the ratio could be higher than 100% in the control, it decreased to 18.0% at −0.5 MPa on the 1st day. Similar changes were observed under water deficiency for the phaseic acid. Its absolute amounts under water stress were similar or exceeded those under control levels, but the ratio of phaseic acid to free ABA decreased from more than 30%–7.9% on the 1st day at −0.5 MPa 9OH-ABA and especially DPA were minor compounds, and their dynamics under water stress were less pronounced. However, in general, the proportion of these compounds to free ABA decreased in the initial period of the water deficit (Fig. 1A).

The ABA content was significantly higher in spruce seedling roots than in pine roots. Phaseic acid was clearly the predominant catabolite.

The ABA response was significantly less pronounced in spruce roots than in pine roots, and a noticeable increase in the ABA content was observed only on the 1st day at −0.5 MPa (Fig. 1B). Due to this ABA increase, the relative amounts of ABA catabolites (9-OH-ABA, phaseic acid and DPA) decreased at this point, although their absolute amounts did not decrease. Unlike pine roots, the root system of spruce seedlings did not contain ABA-GE, and the ABA-GE content increased significantly only at −0.5 MPa by the end of the experiment.

The ABA content was many-fold higher in pine needles than in pine roots. Under −0.15 MPa, the content of active ABA in the needles did not change throughout the experiment. At −0.5 MPa, the ABA content doubled on the 1st day compared to the initial point, and on the 10th day, the difference was less pronounced. At the same time, under control conditions, the ABA content decreased several-fold during the experiment compared with the initial ABA content (Fig. 1C). As a result, on the 1st and 10th days, compared to the corresponding control, the experimental variants contained a significantly higher amount of ABA. ABA-GE was the main ABA catabolite in the pine needles. The ABA-GE proportion was approximately 30% of the ABA content in both the control condition and under water stress, and on the 1st day at −0.5 MPa, the ABA-GE proportion decreased to 17.6%. Other ABA catabolites did not show a pronounced response to water deficiency.

Initially, in comparison to the pine needles, the spruce needles contained significantly less ABA. However, in the needles, water stress caused a rapid and sustained increase in the content of active ABA compared with the initial ABA content. Together with a decrease in the ABA content in the control conditions during the experiment, this resulted in several-fold higher ABA content under water stress in comparison with the corresponding controls. Unlike the spruce roots, the spruce needles contained ABA-GE in amounts comparable to the content of another major catabolite, phaseic acid. The ratio of ABA-GE to free ABA decreased 1.5 times under water stress compared with the control, and the ratio of phaseic acid to free ABA decreased twice. 9-OH-ABA and DPA were minor compounds, and their content did not show a clear dependence on water deficiency (Fig. 1D).

Thus, in general, the main metabolites of ABA in pine and spruce plants were phaseic acid and ABA-GE (except spruce roots). 9-OH-ABA and DPA were minor compounds. The contents of two additional ABA metabolites, neophaseic acid and 7-OH-ABA, which were also analysed in the seedlings, were below the detection limit.

3.2. Expression of the major ABA metabolism-related genes in Scots pine and Norway spruce seedlings

The expression profiles of the main genes for the ABA biosynthetic enzymes in pine and spruce seedlings were analysed under conditions of water deficiency. We were unable to detect the expression of the *CYP707* and *UGT* genes, whose protein products are involved in the oxidation of ABA and the formation of ABA-GE in angiosperms, respectively. Therefore, the regulation of the expression of ABA catabolism-related genes remained unexplored.

In the roots of pine seedlings, the expression of the early biosynthetic gene *BCH* was relatively stable during the experiment, significantly increasing only on the 1st day of the experiment under control conditions and −0.5 MPa (Fig. 2A). Similarly, the expression of the *ZEP* and *NCED* genes also did not change substantially during the experiment. For the *NCED* gene, high expression variability was detected on the 10th day under −0.15 MPa, resulting in a nonsignificant observed increase. For the *SDR1* gene, a significant decrease in the expression level was found under water stress, which was especially pronounced on the 1st day of the experiment. The expression level of the *AAO* gene increased during the experiment under control conditions and −0.15 MPa. For the *BGLU40* gene, a very strong (up to 7-fold) increase in the expression level at −0.5 MPa was found on the 10th day of the experiment.

In the roots of spruce seedlings, a trend of decreased expression of the *BCH* gene occurred by the end of the experiment compared to the starting point (Fig. 2B). Under −0.5 MPa, an increase in *BCH* gene expression occurred on the 1st day, and a strong decrease occurred on the 10th day. Despite the high variability in *ZEP* gene expression under control conditions, a decrease in its expression level was found in both experimental variants on the 1st day of the experiment. Under −0.5 MPa, this decrease was maintained on the 10th day. The expression level of the *NCED* gene did not significantly change throughout the experiment. As in pine roots, in spruce roots, water deficiency led to a significant dose-dependent decrease in the expression of the *SDR1* gene. In contrast to pine roots, in spruce roots, the *AAO* gene expression was greatly reduced under −0.15 MPa throughout the experiment and increased sharply on the 10th day at −0.5 MPa. For the *BGLU40* gene, the increase in expression by the end of the experiment was even more pronounced in spruce roots than in pine roots, increasing twice at −0.15 MPa and many times at −0.5 MPa.

In the needles of the pine seedlings, no pronounced stress-dependent expression dynamics of the *BCH* and *ZEP* genes were detected throughout the experiment (Fig. 2C). In contrast, the *NCED* gene exhibited significant multidirectional changes in expression levels after prolonged (10 days) exposure to water deficiency. At −0.15 MPa, there was a decrease in the expression level, whereas at −0.5 MPa, a many-fold increase was observed. For the *SDR1* and *AAO* genes, the expression change over the course of the experiment was less pronounced under experimental conditions than under control conditions. The *BGLU40* gene expression in pine needles was greatly increased under prolonged exposure to −0.5 MPa, but due to the high expression variability between samples, this increase was not statistically significant.

In the needles of the spruce seedlings, *BCH* gene expression tended to increase under water stress; however, due to the high variability of expression levels between samples, the increase was not statistically significant (Fig. 2D). An increase in *ZEP* gene expression was observed only under −0.5 MPa on the 1st day. *NCED* expression increased under water deficit. Under −0.15 MPa, the expression of the *NCED* gene was highest on the 1st day and then decreased, whereas under −0.5 MPa, a steady increase in expression was observed throughout the experiment. The expression of the *SDR1* gene clearly decreased on the 1st day of the experiment in comparison with the starting point, regardless of the experimental conditions. Only on the 10th day under −0.15 MPa, increased expression of this gene was noted. Expression of the *AAO* gene occurred in a dose-dependent manner and was lower under water deficiency than under control conditions. The most consistent increase in expression under water stress in the spruce needles was observed in the *BGLU40* gene. An increase in the expression level was observed in both experimental variants on the 1st day of the experiment and amplified during the experiment.

4. Discussion

4.1. Dynamics of ABA in pine and spruce seedlings under water stress

A plant response to water deficit is a complex process involving both cellular and inter-organ signalling systems. ABA is widely accepted as one of the key signal messengers coordinating the reactions of roots and aboveground plant parts to water deficit (Daszkowska-Golec, 2016; Ikegami et al., 2009; Vishwakarma et al., 2017). For many years, the root apex has been considered the main site for ABA synthesis under water deficiency, and root-derived ABA was believed to be transported to the aboveground plant parts to direct their stress response (Brodribb and McAdam, 2013; Manzi et al., 2015; McAdam et al., 2016; Sussmilch et al., 2017). However, the concept that leaves may be the main place of ABA biosynthesis in plants has been more strongly confirmed, and leaf-derived ABA plays a major role in the long-term adaptation of the aboveground parts to water deficiency (Christmann et al., 2005, 2006; Manzi et al., 2015; Ikegami et al., 2009; McAdam et al., 2016;

Sussmilch et al., 2017). In both species, we observed that the ABA content was several-fold higher in the shoots than in the roots. Taking into account the significantly greater aerial part biomass than root biomass, shoots contain many times more ABA than roots. The stress-induced synthesis of ABA in roots is unlikely to provide the high ABA amounts found in the aerial parts, given that roots contain low levels of carotenoids, which are biosynthetic precursors of ABA (Ikegami et al., 2009; Manzi et al., 2015; Xiong and Zhu, 2003). Moreover, under water deficiency, no relationship between the dynamics of ABA in the roots and in the aerial part was observed. In particular, spruce, which exhibited a rapid and sustained increase in the ABA content in the aerial parts during water deficiency, did not show pronounced stress-dependent dynamics in the ABA content in the root system. Based on the data obtained, we assume that in pine and spruce seedlings, the needles are the main place ABA biosynthesis occurs under water deficiency.

The actual content of physiologically active ABA is the result of its synthesis *de novo*, release from the conjugates, degradation and conjugation (De Diego et al., 2012; Feurtado et al., 2004). We observed that under both normal conditions and water stress, ABA quantitatively prevailed in the roots and needles of both plant species, not only over its catabolites (9-OH-ABA, phaseic and dihydrophaseic acid) but also over ABA-GE (except for pine roots). Water deficiency conditions caused an increase in the ABA content, which was especially pronounced for pine roots and spruce needles, while the content of the main ABA catabolites did not decrease. Consequently, the ABA increase under water stress resulted mainly from *de novo* biosynthesis. However, the proportion of the main ABA catabolites to free ABA decreased in most cases under water stress. Therefore, in addition to *de novo* biosynthesis, a balance between biosynthesis and ABA decay/conjugation shifted towards biosynthesis under water stress, which augmented ABA accumulation under these conditions. ABA-GE can be found in much larger quantities than free ABA in plant tissues (Sauter et al., 2002; Seiler et al., 2011) and can be the dominant source of biologically active ABA under water stress (Daszkowska-Golec, 2016; Lee et al., 2006). However, we did not observe a reciprocal decrease in the ABA-GE content even in cases of the most drastic increase in free ABA content in organs, such as in pine roots under −0.5 MPa. Thus, hydrolysis of ABA-GE does not seem to be a mechanism utilized in either studied species to release free ABA under water deficit.

4.2. What is the relationship between ABA content and the expression of its biosynthesis-related genes?

The differential expression of ABA homeostasis genes is one of the major means of regulating ABA dynamics under water deficits in angiosperms (Sreenivasulu et al., 2012; Vishwakarma et al., 2017). Transcriptomic surveys conducted in recent years on gymnosperms also indicate changes in the expression of ABA-related genes under drought conditions. Thus, the expression of the *NCED* gene, which is often recognized as a limiting step in ABA biosynthesis, was found to increase in conifers under conditions of water deficiency (Du et al., 2018; Walter Lorenz et al., 2011), although not in all cases (Fox et al., 2017). However, the lack of determination of ABA content in these studies did not allow confirmation of the relationship between changes in the expression of *NCED* genes and the accumulation of ABA. In our study, an increase in *NCED* expression was also observed under water stress in pine roots and needles, as well as in spruce needles. However, in the case of pine, there was clearly no relationship between the dynamics of *NCED* expression, which increased by the 10th day of the water deficit only, and changes in the ABA content, which were observed in the initial period of stress exposure (Fig. 1 A, C; Fig. 2 A, C). A relatively consistent relationship between ABA level and *NCED* expression was observed in only spruce needles, where an increase in both factors was detected in the initial period of water stress. For most other genes of the ABA biosynthetic pathway, the expression level did not correlate with the ABA level or the intensity and duration of water stress. For the genes encoding β-glycosidases, which hydrolyse ABA-GE and release free ABA, a pronounced induction of expression under drought was observed (Fig. 2); however, this induction was not accompanied by a decrease in the content of ABA-GEs (Fig. 1). Therefore, ABA biosynthesis in pine and spruce seedlings is apparently regulated not at the transcription level but more likely at the posttranscriptional level.

4.3. Are the differences in the performance of pine and spruce seedlings during drought caused by the differences in ABA dynamics?

In our earlier works, we observed a pronounced difference in the adaptation strategies of pine and spruce seedlings to the conditions of water deficit (Ivanov et al., 2018; Zlobin et al., 2018). Pine seedlings were characterized by more active growth and relatively loose maintenance of water status under drought, while pine seedlings better maintained water balance but sharply slowed growth processes. In this study, we investigated the ABA dynamics in these species under water deficit. The question arises as to whether differences in the dynamics of ABA between the two species explain their different reactions to water stress.

For the root system, a more direct link between the growth processes and the dynamics of ABA could be expected because ABA-mediated root growth enhancement under water deficiency conditions is a well-described phenomenon (Duan et al., 2007; McAdam et al., 2016; Rowe et al., 2016; Sharp et al., 2004; Tardieu et al., 2010). ABA activates main root elongation by inhibiting ethylene biosynthesis and suppresses the development of lateral roots (McAdam et al., 2016; Xiong et al., 2006). In our earlier works, we observed contrasting growth dynamics of pine and spruce root systems under water deficit; pine seedlings significantly increased dry root biomass accumulation even under a strong water deficit (Ivanov et al., 2018), whereas growth of spruce root system growth was rapidly inhibited (Zlobin et al., 2018). The accumulation of ABA showed the same contrasting dynamics, with a pronounced dose-dependent increase in pine roots but not spruce roots in the initial period of water stress. However, the activation of root growth is unlikely to be directly mediated by ABA in pine because we previously observed that the main root elongation in pine was suppressed quickly under water stress (Ivanov et al., 2018). A strong increase in the ABA content in the pine root system may be connected with other ABA effects, such as an increase in the hydraulic conductivity of the root system under water deficiency (Tardieu et al., 2010; Pantin et al., 2012; Sharp et al., 2004).

Due to its role in the regulation of gas exchange under water deficiency, ABA can be one of the most important determinants of the isohydric vs. anisohydric plant strategy under drought (Brodribb et al., 2014). Differences in the growth and water homeostasis of pine (Ivanov et al., 2018) and spruce (Zlobin et al., 2018) seedlings in combination with a significantly less pronounced increase in ABA content in pine needles than in spruce needles under water deficiency (Fig. 1C and D) may indicate that in comparison to spruce seedlings, pine seedlings follow more anisohydric strategies. In general, both Scots pine and Norway spruce are considered typical isohydric species that respond to water stress with a steady increase in ABA levels and a reduction in stomatal gas exchange (Brodribb et al., 2014; De Diego et al., 2015; Sturm et al., 1998). However, species of the *Pinus* genus are characterized by high physiological plasticity, and their water use strategy may vary even within the same genotype, depending on the habitat and other factors (Poyatos et al., 2007). An isohydric strategy ensures plant survival by limiting water loss. However, if the stress is only temporary, then limitation of assimilation and growth can lead to a competitive disadvantage (Claeys and Inzé, 2013). The advantage of a more anisohydric strategy compared to a strictly isohydric strategy is more active assimilation under water availability fluctuations in the soil (Brodribb and McAdam, 2013), which are characteristic of light sandy soils, often inhabited by pine (Tor-ngern et al., 2017). Maintaining assimilation and rapid growth of pine in early age stages can be crucial

because of the competition for light with herbaceous vegetation in open areas pioneered by pine seedlings (Yunusa et al., 2005). However, continued growth can threaten survival when water limitation is long and severe (Claeys and Inzé, 2013), as we observed in pine seedlings under conditions of prolonged water deficiency (Ivanov et al., 2019). Differences in ABA dynamics can likely contribute to differences in the adaptation strategy of Scots pine and Norway spruce seedlings to water deficit.

5. Conclusion

Scots pine and Norway spruce showed pronounced stress-dependent dynamics of ABA under conditions of water deficiency. ABA changes were presumably due to an increase in *de novo* ABA synthesis in the shoots of both species. The dynamics of the ABA content were not determined by the levels of its biosynthetic gene expression. A greater ABA increase in spruce shoots compared with pine could underlie the more isohydric behaviour of spruce seedlings under water deficiency.

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Author statement

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.04.037.

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