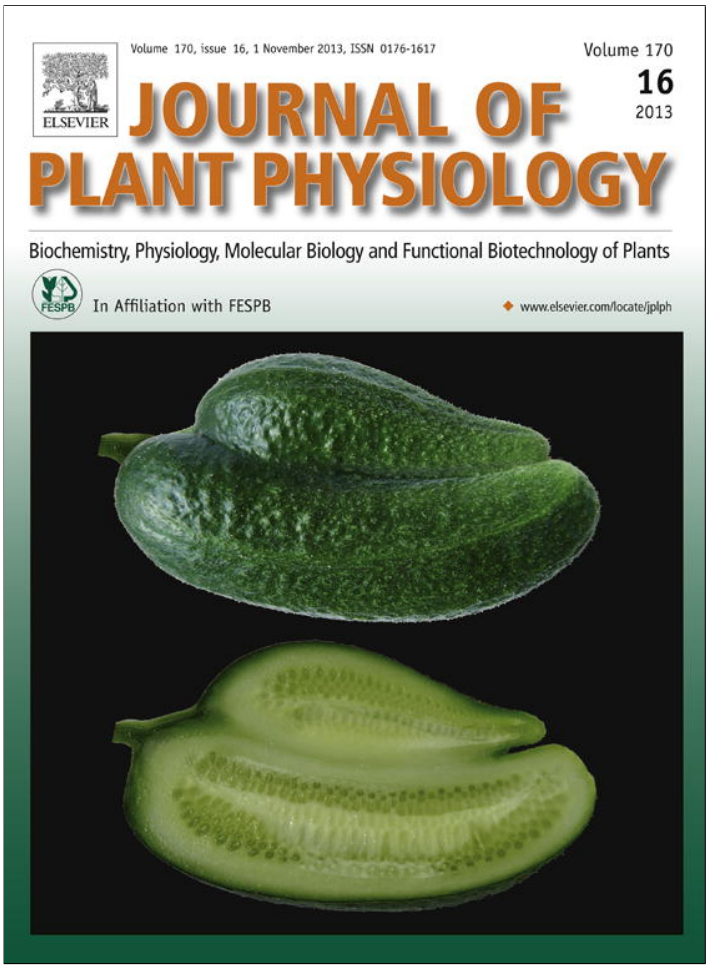

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Physiology

Isohydric and anisohydric strategies of wheat genotypes under osmotic stress: Biosynthesis and function of ABA in stress responses

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ABSTRACT

Changes in water potential (ψ_w), stomatal conductance, abscisic acid (ABA) accumulation, expression of the major genes involved in ABA biosynthesis, activities of abscisic aldehyde oxidase (AO, EC 1.2.3.1) and antioxidant enzymes were studied in two wheat cultivars with contrasting acclimation strategies subjected to medium strength osmotic stress (−0.976 MPa) induced by polyethylene glycol (PEG 6000). Because the biosynthetic pathway of ABA involves multiple gene products, the aim of this study was to unravel how these genes are regulated in isohydric and anisohydric wheat genotypes. In the root tissues of the isohydric cultivar, *Triticum aestivum* cv. Kobomugi, osmotic stress increased the transcript levels of 9-*cis*-epoxycarotenoid dioxygenase (NCED) gene, controlling the rate limiting step of ABA biosynthesis. Moreover, this cultivar exhibited a higher basal activity and a higher induction of aldehyde oxidase isoenzymes (AAO2-AAO3), responsible for converting ABAldehyde to ABA. It was found that the fast activation of the ABA biosynthesis in the roots generated an enhanced ABA pool in the shoot, which brought about a faster closure of the stomata upon increasing osmotic stress and, as a result, the plants could maintain ψ_w in the tissues close to the control level. In contrast, the anisohydric genotype, cv. GK Öthalom, exhibited a moderate induction of ABA biosynthesis in the roots, leading to the maintenance but no increase in the concentration of ABA on the basis of tissue water content in the leaves. Due to the slower response of their stomata to water deficit, the tissues of cv. GK Öthalom have to acclimate to much more negative water potentials during increasing osmotic stress. A decreased activity of superoxide dismutase (SOD) was found in the leaves and roots of both cultivars exposed to osmotic stress, but in the roots elevated activities of catalase (CAT), peroxidase (POX), glutathione reductase (GR) and glutathione transferase (GST) were detected in the isohydric cultivar, suggesting that this genotype was more successful in the elimination of reactive oxygen species caused by the stress conditions.

Introduction

The response of wheat genotypes to drought stress has been investigated extensively because soil drought represents a major constraint for successful crop production. Plants can readily change their metabolic and physiological processes, as well as the morphology of the above-ground parts and the root system in response to water deficit.

Crop plants can cope with drought stress by avoiding tissue dehydration, thus, these isohydric plants are able to keep their tissue water potential almost unchanged by the fast closure of their stomata as well as by alternative water saving mechanisms. Anisohydric plants, on the other hand, tolerate soil drought and respond to the decrease of water availability in the environment by tissue dehydration (Drew, 2006).

The first reaction of plants belonging to the former group is a decrease in their stomatal conductance, in which root-to-shoot chemical or hydraulic signalling events are involved. Abscisic acid (ABA) is a plant hormone involved in many phases of plant development and in the response of plants to various environmental stresses (Wilkinson and Davies, 2002; Blum, 2011; Pantin et al., 2012). Because many of the physiological processes are correlated with endogenous ABA levels, the regulation of ABA biosynthesis has a pivotal role in the elucidation of these physiological characteristics. ABA biosynthesis increases first in the roots, and then the hormone is translocated to the shoot via the xylem and functions as a long-distance chemical signal from the root to the shoot during water stress (Zhang and Davies, 1987; Wilkinson and Davies, 2002, 2010). In addition to this type of chemical signalling,

Abbreviations: ABA, abscisic acid; AO, aldehyde oxidase; CAT, catalase; GR, glutathione reductase; GST, glutathione transferase; MDA, maldondialdehyde; NCED, 9-*cis*-epoxycarotenoid dioxygenase; PEG, polyethylene glycol; POD, peroxidase; SOD, superoxide dismutase; ZEP, zeaxanthin epoxidase.

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ABA can promote stomatal closure by its indirect hydraulic effect through decreasing the water permeability of the leaf vascular tissues (Pantin et al., 2013). Since there are large differences between the apparent sensitivity of leaf conductance to the concentration of ABA in the xylem (Correia and Pereira, 1995), chemical signalling can account for more than 40–70% decrease in the stomatal conductance as opposed to hydraulic events (Zhang and Davies, 1990; Khalil and Grace, 1993).

Identification of genes encoding enzymes involved in the biosynthesis of ABA has revealed details of the main biosynthetic pathways (Seo and Koshiba, 2002). Expression of 9-*cis*-epoxycarotenoid dioxygenase (NCED) in *Arabidopsis*, which represents a rate limiting step in controlling drought stress-induced ABA biosynthesis, is up-regulated significantly during drought stress (Xiong et al., 2002). Five members of the NCED family in *Arabidopsis* are implicated in ABA biosynthesis, among them the product of *NCED3* gene makes the major contribution to increase ABA levels leading to the induction of tolerance mechanisms in vegetative tissues (Urano et al., 2009; Frey et al., 2012). In avocado, another isoform, *PaNCED1* is highly expressed in the leaves and its expression is induced by dehydration (Chernys and Zeevaart, 2000). NCED was thought to be the key regulatory enzyme of ABA biosynthesis, since the transcript amount of this enzyme was directly proportional to the ABA content, and it was induced early after water withdrawal (Chernys and Zeevaart, 2000; Taylor et al., 2000; Thompson et al., 2007a). The initial accumulation of ABA up-regulates the expression of other genes involved in the biosynthesis of ABA, such as zeaxanthin epoxidase (*ZEP*), abscisic aldehyde oxidase (*AAO*) and molybdate cofactor sulfurase (*MCSU*) in *Arabidopsis*. Increased expressions of these genes lead to a fast accumulation of ABA in an autocatalytic process (Xiong et al., 2002).

The tobacco mutants impared in ZEP expression and activity proved to be ABA-deficients and exhibited lower xylem ABA contents during drought stress (Borel et al., 2001). The members of the aldehyde oxidase (AO EC 1.2.3.1) gene family catalyse the oxidation of various aldehydes to carboxylic acids and certain isoenzymes efficiently transform abscisic aldehyde to ABA (Seo et al., 2000a, 2000b). Different isoenzymes exhibited different expression levels in plant organs. *PsAO1* and *PsAO2* were mainly expressed in the leaves of pea plants, while *PsAO3* was expressed in ageing leaves and seeds (Zdunek-Zastocka, 2008). Aldehyde oxidase isoforms, which use abscisic aldehyde as a physiological substrate, were identified in *Arabidopsis* rosette leaves (Seo et al., 2000a), and two isoforms, AO2 and AO3, were also detected in barley roots (Omarov et al., 2003).

Soil drought decreased stomatal conductance (g_s) and ψ_w of wheat genotypes significantly (Guóth et al., 2009). However, other authors did not find such a fast response of g_s to decreased soil water potentials or atmospheric vapour pressure deficit (VPD) (Inoue et al., 1989). This suggests that the responses of g_s to soil water stress or VPD were related to the cultivars (Condon et al., 1992) or to the degree of the drought stress (Rawson et al., 1977). The rate of ABA biosynthesis determines the decrease in stomatal conductance, thereby the diffusion rate of CO_2 into the chloroplast stroma and the carboxylating efficiency of ribulose-1,5-*bis*-phosphate carboxylase/oxygenase. Thus, photosynthesis, which is the most significant process influencing crop production, may also be inhibited by drought stress (Guóth et al., 2009).

Serious water-stress can trigger an increased formation of reactive oxygen species (ROS), such as superoxide radical and hydrogen peroxide, which can directly attack membrane lipids and damage proteins (Navari-Izzo et al., 1994; Bartoli et al., 1999). Detoxification of ROS is accomplished by the antioxidant defence system comprising nonenzymatic components (ascorbate, glutathione, α-tocopherol, carotenoids) and a series of enzymes (Alscher et al., 1997; Foyer and Noctor, 2005; Nikolaeva et al., 2008). One of the most important antioxidant enzymes is superoxide dismutase (SOD), which converts superoxide radicals to the less harmful H_2O_2. Hydrogen peroxide can then be scavenged by catalase (CAT) and peroxidases and also indirectly by glutathione-related enzymes, such as glutathione reductase (GR), which is a component of the ascorbate-glutathione cycle and reduces glutathione disulfide (GSSG) to glutathione (GSH). GSH contributes to the maintenance of cellular redox potential and generates reduced GSH for other enzymatic reactions such as for the detoxification of harmful metabolites by glutathione transferases (GST) (Gallé et al., 2009). These detoxification processes also play important roles in the successful acclimation of plants and can be controlled by ABA.

Certain environmental factors, such as drought stress, induce ABA biosynthesis principally through the transcriptional regulation of ABA biosynthetic genes. However, it may vary not only between different species but also between developmental stages and plant parts.

Here we present a comparative study between an isohydric and anisohydric wheat cultivar carried out in order to elucidate the putative role of tissue dehydration in the regulation of ABA biosynthesis. The patterns of acclimation of an anisohydric (cv. GK Öthalom) and an isohydric wheat genotype (cv. Kobomugi) to PEG 6000-induced osmotic stress were compared with special emphasis on the induction of *ZEP*, *NCED* and *AO*, changes in AO activities and accumulation of ABA both in leaf and root tissues. The effect of osmotic stress on water potential, stomatal conductance, enzymatic antioxidant defence of the two wheat cultivars were also compared to reveal the differences between isohydric and anisohydric strategies during osmotic stress.

Materials and methods

In our experiments, two wheat cultivars, *Triticum aestivum* L. GK Öthalom, a modern cultivar with medium drought tolerance, and Kobomugi, a drought tolerant landrace from Central Asia, were subjected to osmotic stress. The seedlings were grown in plastic dishes containing 10 l of Hoagland solution (5 mM $Ca(NO_3)_2$, 5 mM KNO_3, 1 mM KH_2PO_4, 2 mM $MgSO_4$, 1 μM Fe-EDTA, 0.048 μM H_2BO_3, 14.48 μM $MnCl_2$, 0.815 μM $ZnCl_2$, 0.373 μM $CuCl_2$, 0.001 μM Na_2MoO_4) in Conviron cabinet chambers, at 24/19 °C day/night temperature, 12 h/12 h light period and at 200 μmol m^{-2} s^{-1} light intensity. One hundred plants were grown in one dish and the culture solution was changed twice a week. The roots were aerated with aquarium pump.

Osmotic stress was induced by polyethylene glycol treatments (PEG 6000) (Money, 1989). Increasing amounts of PEG 6000 reaching the final value of 400 mOsm (−0.976 MPa) were applied gradually in the culture media of one-week-old plants. The seedlings were exposed to 100 mOsm PEG on day 7, then the concentration was raised stepwise every second day, on day 9–200 and on day 11–400 mOsm (Csiszár et al., 2012). On the 7th, 9th, 11th days samples were prepared before increasing the osmotic potential of the culture solution. The experiments were performed in three biological replicates.

Water status of the plants

Midday leaf water potentials (ψ_w) were measured using a pressure chamber (PMS Instrument Co., Corvallis, Oregon, USA) on the second fully expanded leaves. Stomatal conductance (g_s) was determined in the middle of the apical leaflets of the second expanded leaves using a steady-state porometer (PMR-2, PP Systems, UK and USA).

Table 1
The primers used for gene expression analyses.

Sequence	Primers	
	Forward	Reverse
18S rRNA	GTGACGGGTGACGGAGAATT	GACACTAATGCGCCCGGTAT
EF 1 α subunit	AACTTCACCTCCCAGGTCAT	GTCACCAGCTCAGCAAACTT
AO2 TC447676	ACGAGGACTAGGCGACGAA	TCAACGTAGGGATCTTGTACGT
NCED TC404702	CCTCGAAGCCCAGCACTAAT	GAGAGCGAGAGGTCCAATGG
ZEP AF384103.2	GGAGTTATGAGAAGGAGAGAAAGC	AAAACGACAAAGGTCCCAGA

Searching for sequences participating in ABA biosynthesis in wheat

Wheat *NCED*, *AAO*, and *ZEP* sequences were identified using an in silico approach. Screening for wheat sequences was initially performed on the DFCI-Gene Index (http://compbio.dfci.harvard.edu/tgi/) wheat database using published plant aldehyde oxidase and 9-*cis*-epoxycarotenoid dioxygenase sequences from DDBJ/EMBL/GenBank sequence database. For Real Time PCR measurement three sequences were used. The chosen aldehyde oxidase is similar to other aldehyde oxidase 2 proteins: blastx searching resulted in aldehyde oxidase 2 proteins from *Oryza sativa* (Acc. No: Q852M1.1, Exp: 3e-88) and *Brachypodium distachylon* (Acc. No: XP_003557918.1, Exp: 1e-92). According to DFCI Gene Index the sequence was annotated to show similarity to aldehyde oxidase 2 from *Zea mays*. The chosen 9-*cis*-epoxycarotenoid dioxygenase (*NCED*) sequence (TC404702) is homologous to *Hordeum vulgare NCED* according to the annotation in DFCI Gene Index, and is highly homologous to *HvNCED1* (AK36199.1, Exp: 0.0) and to *HvNCED2* (AK358040.1, Exp: 2e-144) genes described by Millar et al. (2006) and Seiler et al. (2011), respectively. Furthermore, one wheat zeaxantin expoxidase (AF384103.2) was chosen for the measurements, the homolog of this sequence (AK362500.1, Exp: 0.0) was identified by Seiler et al. (2011) as *ZEP2* in *H. vulgare* (Table 1).

RNA purification and expression analyses with real-time PCR

RNA was extracted from root samples at different developmental stages (9, 11, 22 days) according to Chomczynski and Sacchi (1987) as published earlier (Gallé et al., 2009). DNase digestions were applied (Fermentas). First strand cDNA was synthesized using MMLV reverse transcriptase (Fermentas). Primers were designed using Primer 3 software (Rozen and Skaletsky, 2000) and were synthesized in the Nucleic acid synthesis laboratory, Biological Research Centre (Szeged, Hungary). Primer pairs are shown in Table 1. The expression rate of the ABS biosynthesis sequences was monitored by quantitative real-time PCR (BioRad, MJ Research) using SYBR Green probes (Applied Biosystems; Karsai et al., 2002). Each reaction was repeated at least three times. QRT-PCR was initiated by denaturation at 95 °C for 10 min followed by 41 cycles of denaturation at 95 °C for 15 s and annealing, extension at 60 °C for 1 min. Data analysis was performed using Opticon monitor software. To determine the specificity of the reaction, a melting curve analysis of the product was performed immediately after the final PCR cycle by increasing the temperature from 55 °C to 90 °C (0.2 °C 0.2 s^{-1}). 18 S ribosomal RNA and Elongation factor 1 α subunit were used for high and low controls (Nicot et al., 2005). Data were normalized using the initial control samples (values of the 7-day-old seedlings).

Abscisic aldehyde oxidase (AAO, EC 1.2.3.1.) activity

AAO tissue extraction and native-polyacrylamide gel electrophoresis (PAGE) were carried out as described by Sagi et al. (1998). Root and shoot tissues (1 g) were homogenized using 250 mM Tris–HCl buffer (pH 8.5) containing 1 mM EDTA, 1 mM 1,4-dithio-DL-threitol, 5 mM L-cysteine, 80 μM Na_2MoO_4, 10 μM antipain, 0.1 mM phenazine methosulphate, 10 mM glutathione and 0.03 mM FAD. The samples were centrifuged at 30,000 × *g* for 15 min at 4 °C. The resulting supernatants were used for native PAGE. After the quantitation of the total protein content using the method of Bradford (1976), (1) 5-mm-thick slabs of 7.5% polyacrylamide gel were loaded with 100 and 300 μg of proteins from the root and shoot tissue extracts, respectively. Enzyme activities were determined by incubating the gels in 0.2 M phosphate buffer (pH 8) for 10 min, and then in a reaction mixture containing 0.1 mM phenazine methosulphate, 1 mM 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide in 0.1 M Tris–HCl buffer (pH 8,5) at 25 °C in the presence of, 1 mM indole-3-aldehyde (IAld) substrates. The bands were analysed using a Kodak EDAS-290 Gel Analysis System.

Determination of abscisic acid by ELISA

The quantitative determination of ABA was carried out via an enzyme linked immuno-sorbent assay (ELISA) (Phytodetek-ABA, Sigma–Aldrich, St. Louis, MO). Plant tissues (1.0 g) were extracted with 15 ml of a cold mixture of 100 mM $NaHCO_3$ and methanol (80:20, v/v) containing 1 mg of butylated hydroxytoluene in a volume of 100 ml. The samples were extracted twice at 4 °C for 24 h each, and were then evaporated. The assay utilizes a monoclonal antibody for ABA, and the determination of (+)-*cis*-ABA (Sigma–Aldrich, St. Louis, MO) in the plant extract was based on the competitive binding of ABA and the tracer (alkaline phosphatase-labelled ABA) to the antibody-coated microwell. Tracer and standard solutions were prepared following the manufacturer's instructions. 100 μl of standard ABA or plant extract and then 100 μl of diluted tracer were added to each well. After incubation for 3 h at 4 °C, the wells were washed three times by adding 200 μl of wash solution. The alkaline phosphatase reaction was started by the addition of 200 μl of substrate solution. After 60 min at 37 °C, the reaction was stopped with 50 μl of stop reagent and the absorbance was determined at 405 nm, using a MR 4000 microplate reader (Dynatech) (Guóth et al., 2009). ABA concentration is expressed as nmol ABA in 1 g of tissue water content of the wheat samples.

Activity of antioxidant enzymes

Enzyme activities were determined both in roots and shoots of the PEG-treated and control plants. 0.75 g of plant tissue was homogenized on ice in 3 ml extraction buffer (50 mM phosphate buffer pH 7.0, containing 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, PMSF and 1% polyvinyl-polypyrrolidone (PVPP). The homogenate was filtered through two layers of cheese-cloth and centrifuged for 25 min at 15,000 × *g* at 4 °C. The supernatant was used for enzyme activity assays. The homogenization was repeated two or three times, the mean ± SD were calculated from the data of at least 3 independent measurements.

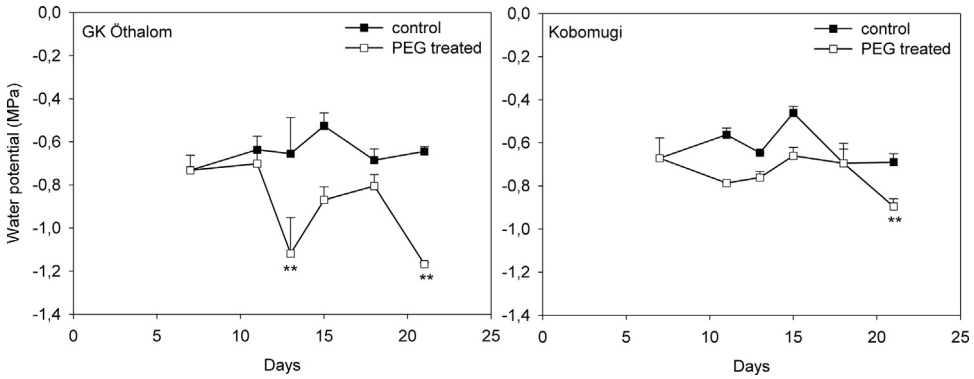

Fig. 1. Water potential changes in the second leaves of wheat cultivars GK Öthalom and Kobomugi in the function of time after exposure to 100 (on day 7), 200 (on day 9) and 400 mOsm (on day 11) PEG 6000 treatment (mean ± SD, $n = 10$). Data labelled with *differed significantly from the untreated controls at *$P \leq 0.05$, **0.01 or ***0.001 level (Student's *t*-test).

Superoxide dismutase (SOD, EC. 1.15.1.1) activity was determined by measuring the ability of the enzyme to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) in the presence of riboflavin in the light (Dhindsa et al., 1981). One unit (U) of SOD was the amount of enzyme that caused a 50% inhibition of NBT reduction and the specific enzyme activity was expressed as U mg^{-1} protein.

Catalase (CAT, EC. 1.11.1.6) activity was determined by the decomposition of H_2O_2 measured spectrophotometrically by following the decrease in absorbance at 240 nm (Upadhyaya et al., 1985). One U equals the amount of H_2O_2 (in μmol) decomposed in 1 min.

Peroxidase (POD, EC 1.11.1.7) activity was determined by monitoring the increase in absorbance at 470 nm during the oxidation of guaiacol (Upadhyaya et al., 1985). The amount of enzyme producing 1 μmol min^{-1} of oxidized guaiacol was defined as 1 U.

Glutathione reductase (GR, EC 1.6.4.2) activity was determined by measuring the absorbance increment at 412 nm when 5,5′-dithio-*bis* (2-nitrobenzoic acid) (DTNB) was reduced by glutathione (GSH), generated from glutathione disulfide (GSSG) (Smith et al., 1988). The specific activity was calculated as the amount of reduced DTNB, in μmol min^{-1} protein mg^{-1}, $\varepsilon_{420} = 13.6\ mM^{-1}\ cm^{-1}$.

Glutathione transferase (GST, EC 2.5.1.18) activity was determined spectrophotometrically by using an artificial substrate, 1-chloro-2,4-dinitrobenzene (CDNB), according to Habig et al. (1994). The reaction was initiated by the addition of CDNB, and the increase in A_{340} was determined. One U is the amount of the enzyme producing 1 μmol conjugated product in 1 min, $\varepsilon_{340} = 9.6\ mM^{-1}cm^{-1}$. The protein contents of the extracts were determined by the method of Bradford (1976).

Malondialdehyde determination

MDA formation was assayed by using the thiobarbituric acid method (Ederli et al., 1997). 100 mg leaf tissue was homogenized with 1 ml 0.1% trichloroacetic acid (TCA); to avoid further lipid peroxidation 100 μl 4% butylhydroxytoluene (BHT) was added to the extract. After centrifugation at 12,000 × *g* for 20 min, 250 μl of supernatant was mixed with 1 ml 0.5% thiobarbituric acid in 20% TCA and the mixture was incubated in boiling water for 30 min. The absorbance was measured at 532 nm and adjusted for nonspecific absorbance at 600 nm. MDA concentration was estimated by using an extinction coefficient of 155 $mM^{-1}\ cm^{-1}$.

Statistical analysis

Significant differences between the control and treated samples prepared at the same time points were determined by Student's *t*-test. Differences were considered significant if $P \leq 0.05$. In some cases, the mean values were compared by Duncan's test and the differences were considered significant if $P \leq 0.05$. Statistical analysis was carried out with SigmaStat 3.1. statistical software. All data presented are means ± SD.

Results

Water relations

Physiological responses to water deficit were measured in two wheat cultivars exposed to 400 mOsm PEG 6000 in hydroponic culture. Water deficit decreased the relative growth rate of the leaves and roots in both cultivars (data not shown). The third leaf developed only after 15 days, therefore water potentials and stomatal conductivities were measured on the days of sampling in all fully expanded leaves. Since similar tendencies were detectable in all cases, only the data of the second leaf are represented. Water potential of the leaves decreased significantly in cv. GK Öthalom during the first week of PEG exposure, but the difference exhibited some fluctuations and at the end of experiment ψ_w declined to −1.18 MPa. In cv. Kobomugi only small and non-significant differences were found in the water potential between the control and PEG-treated leaves from day 11 to day 21 ($\Delta\psi_w = -0.2$ MPa) (Fig. 1). These data suggest that cv. GK Öthalom follows an anisohydric strategy, while the response of cv. Kobomugi was close to isohydric.

Stomatal conductivity

In cv. Kobomugi stomata were closed in all leaves after 2 days of exposure to even the lowest concentration of PEG 6000 (100 mOsm, −0.245 MPa). Stomatal conductivity of the leaves exposed to osmotic stress decreased significantly on day 11 and the values remained under the control level on every sampling day from that time (Fig. 2). The tendency of the changes was similar in the first leaves (data not shown). In cv. GK Öthalom, stomata did not respond to even 200 mOsm PEG and closure of stomata became significant only on day 13, two days after the exposure to 400 mOsm PEG (Fig. 2).

Expression pattern of genes participating in ABA biosynthesis

It was a general response that under drought stress the biosynthesis of ABA was induced first in the roots and the hormone was readily transported in the xylem to the leaves. To follow the changes in ABA biosynthesis in different plant parts, the transcript amounts of *ZEP* (AF384103.2), *NCED* (TC404702) and *AO2* (TC354638) were

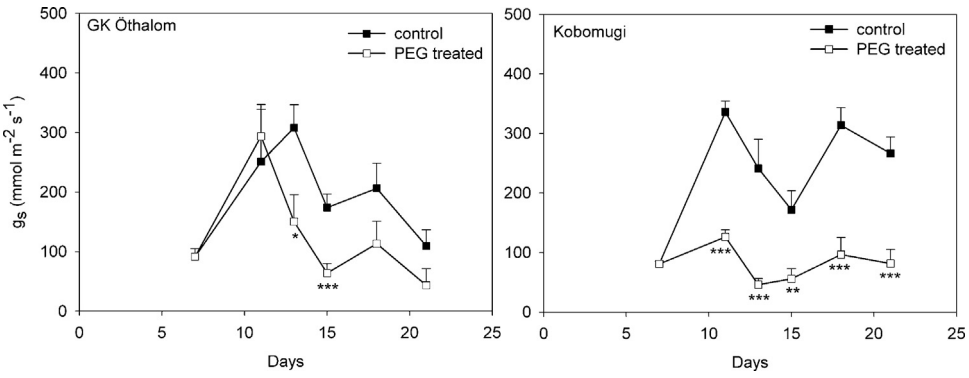

Fig. 2. Changes in total stomatal conductance of the second leaves of wheat cultivars GK Öthalom and Kobomugi in the function of time after exposure to 100 (on day 7), 200 (on day 9) and 400 mOsm (on day 11) PEG 6000 treatment (mean ± SD, $n = 10$). Data labelled with *differed significantly from the untreated controls at $^{*}P \leq 0.05$, **0.01 or ***0.001 level (Student's *t*-test).

measured in the course of the acclimation process. For the selection of the *NCED* and *AO2* sequences, the reported aldehyde oxidase and epoxycarotenoid dioxygenase genes of other Poaceae species were used for searching among the wheat tentative consensus sequences (TC). In the leaves the transcript amount of the three chosen sequences showed a time-dependent decrease in almost every case, both in control and treated plants, suggesting a developmental phase-specific regulation of ABA biosynthesis. The pattern of transcript abundance in roots was different in the two cultivars between control condition and osmotic stress. In Kobomugi, the transcript amounts of the two ABA biosynthetic enzymes studied, *NCED* and *AAO*, were elevated by the osmotic stress while there was no induction in the expression of *ZEP*. The expression of *NCED* was significantly induced in both cultivars due to PEG treatment and the highest transcript levels were detected in cv. Kobomugi on day 11, two days after applying 100 mOsm PEG. The increase observed in the relative transcript level of *NCED* on days 11 and 13 were 18- and 11-fold in Kobomugi and 7- and 8-fold in GK Öthalom (Fig. 3).

Aldehyde oxidase activities

To follow the changes in the subsequent steps of ABA biosynthesis during the experimental period, the activity of the AO isoenzymes was studied in non-denaturating gels. Staining for AO activity using indole-3-aldehyde (IAld) as a substrate resulted in five isoforms in the roots of cv. Kobomugi and six bands in cv. GK Öthalom. In an earlier work it was reported that in the presence of IAld substrate the band of AAO1 isoform could not be detected in barley, so the band with the least mobility in our samples originates from the activity of AAO2 (Omarov et al., 2003). In our experiments, the basic activities of AO2-3 bands were more intense in the control plants and the PEG-induced isoforms exhibited much higher activities in the isohydric genotype cv. Kobomugi than in cv. GK Öthalom (Figs. 4 and 5). Three AO isoforms were detectable in the leaves of both cultivars but there were no significant differences between the control and PEG-treated samples during the study period (data not shown).

ABA content

It is well known that ABA synthesis is induced first in the roots and so the ABA accumulated in the shoot is derived mainly from the root system. Leaves of isohydric cultivars may have a much lower water loss than those of anisohydric plants. Similarly, photosynthetic activity and biomass production can also be differently affected in plants belonging to different water stress acclimation strategies (Guóth et al., 2009). In order to exclude these differences, ABA concentrations were calculated on the basis of tissue water content. Unexpectedly, plants were able to maintain a constant ABA concentration during osmotic stress (Fig. 6). In GK Öthalom plants the highest ABA content was measured in the shoot on the second sampling day and a small, but non-significant increase due to osmotic stress was detected in the leaves on day 13, two days after the nutrient solution reached the final 400 mOsm value. In the roots a significant accumulation of ABA was observed on day 21. PEG treatment caused an enhanced accumulation of ABA in the leaves of cv. Kobomugi on day 13, but in contrast to leaves, the ABA content of the roots decreased significantly on the last sampling day.

Antioxidant enzyme activities

Measurement of the thiobarbiturate reactive compounds (MDA content) on the 21th day revealed that the osmotic stress caused elevated level of lipid peroxidation in both cultivars, but the increase was higher in GK Öthalom than in Kobomugi plants (Fig. 7). The activities of several antioxidant enzymes were also studied on this sampling day. SOD activity decreased in the leaves and roots of both cultivars after two weeks of PEG treatment. However, the basal activity of the enzyme was high in the control samples and remained much higher during osmotic stress in the leaves of cv. Kobomugi. The stress caused a slight decrease in CAT activity in GK Öthalom but increased it in cv. Kobomugi, and in the roots these changes were significant. Guaiacol peroxidase (POD) activity did not show any significant changes in either of the two wheat cultivars after osmotic treatment although it was higher than in the control both in the shoots and roots of Kobomugi. GR and GST activities increased in the roots of both genotypes as an effect of the PEG treatment, but the changes were statistically significant only in the roots of Kobomugi (Fig. 6).

Discussion

In nature, water is usually the most limiting factor for plant growth. If plants do not receive adequate rainfall or irrigation, the resulting drought stress can reduce growth more than all other environmental stresses combined. This may be true for the relatively ancient and well-adapted landraces as well as for modern cultivars, too.

In the presence of osmotic stress, water status parameters are among the firstly affected physiological traits. As seen from the changes in water potential, the two wheat cultivars investigated in our experiments follow different strategies to cope with osmotic stress: GK Öthalom showed tissue dehydration, which it could tolerate during the acclimation, while Kobomugi proved to be isohydric. One reason for the almost uneffected water potential in

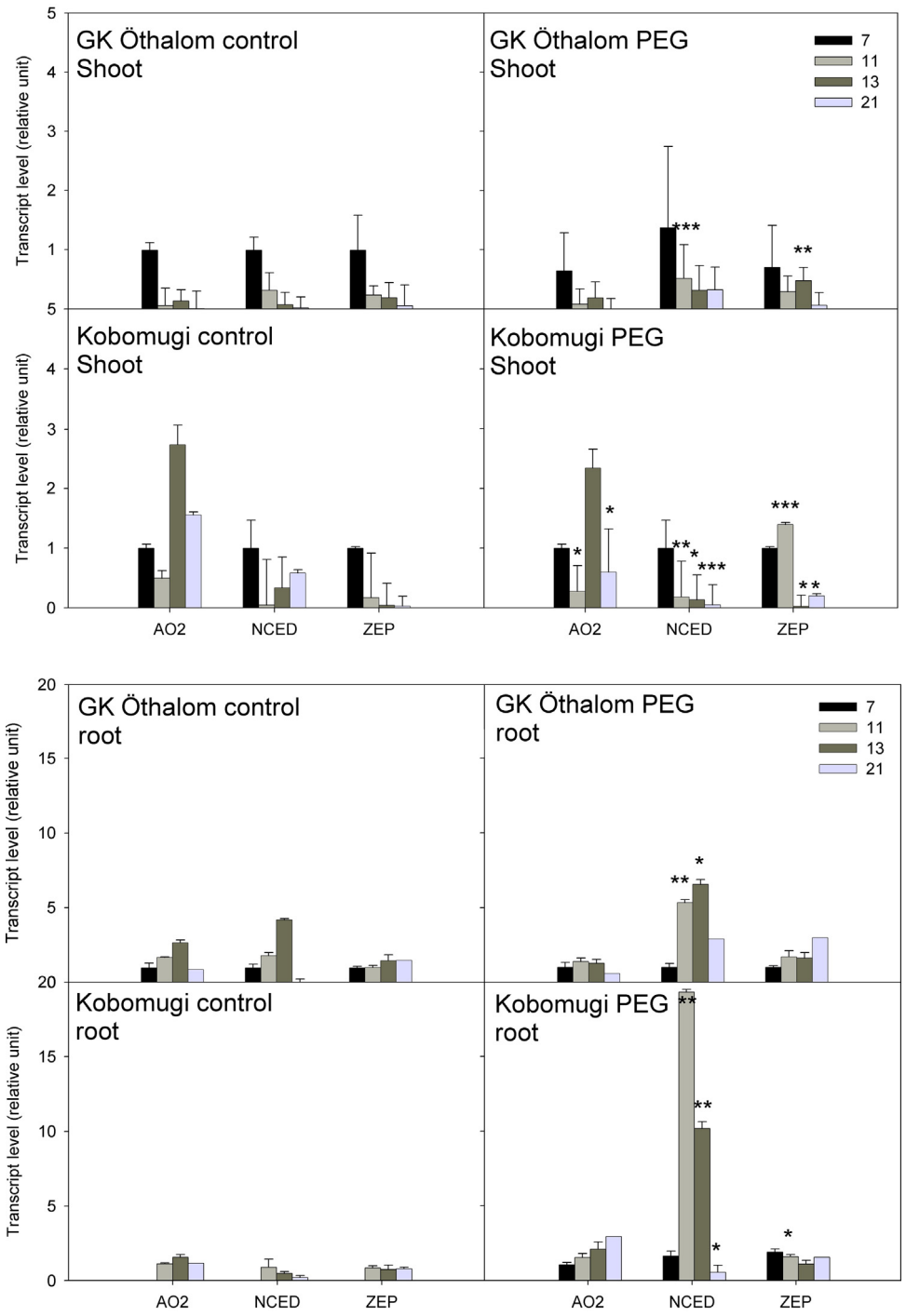

Fig. 3. Transcript levels of *AAO2* (TC354638), *NCED* (TC404702) and *ZEP* (AF384103.2) in the shoots and roots of GK Öthalom and Kobomugi cultivars. The transcript level in the control samples on the first sampling day (initial control) was equalled to one. Data were normalized using the wheat 18S rRNS and elongation factor α subunit (EF-1) as high and low controls, respectively. Statistical differences compared to the controls are indicated by $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

leaves of Kobomugi could be the fast decrease in the stomatal conductance. Stomatal closure caused by osmotic stress in cv. GK Öthalom developed later than in Kobomugi, which is manifested in the decrease in the water potential in this cultivar.

It is well established that stomatal conductance is in correlation with the opening of stomatal pores and is inhibited by water deficit (Quarrie and Jones, 1979; Quick et al., 1992; Tardieu et al., 2006). Closed stomata are an important means to protect the plants from water loss, but this strategy has an unfavourable influence on CO_2 diffusion and, as a consequence, on the photosynthetic rate (Morgan, 1984).

Drought stress is one of the environmental factors that highly activate ABA biosynthesis. Regulation of ABA content can be achieved at transcriptional level especially by the up-regulation of *NCED*. On the other hand, the levels of *AAO* mRNA were increased by water stress in *Arabidopsis* with no change in the amount of the AAO protein (Seo and Koshiba, 2002). *NCED* over-expressing tomato also showed an increased ABA content. This de novo biosynthesis is responsible for enhanced ABA levels in root tissues which contributes to the control of transpiration and leaf expansion in the shoots (Thompson et al., 2007b; Peleg and Blumwald, 2011).

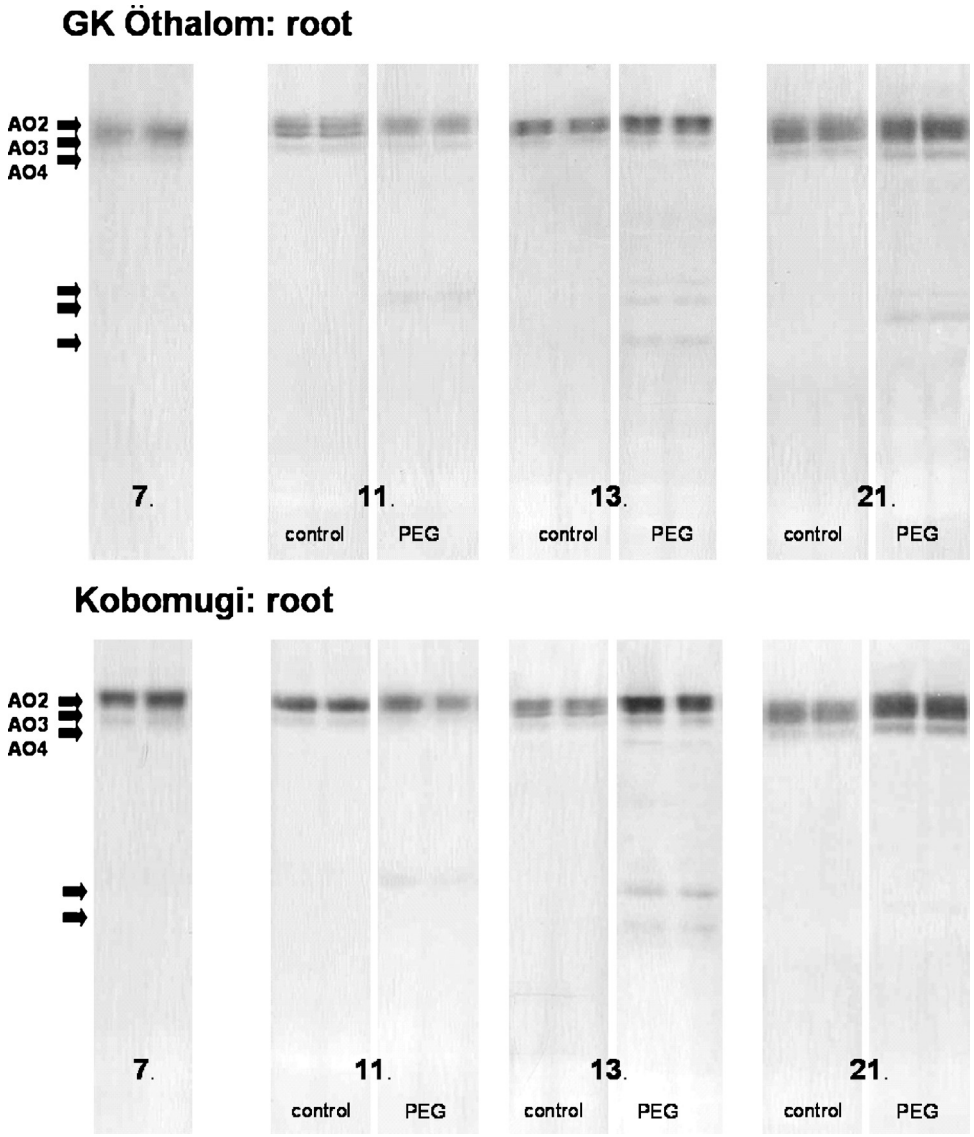

Fig. 4. Changes in aldehyde oxidase (AO) activities in the roots of wheat cultivars GK Öthalom and Kobomugi in the function of time after exposure to 100 (on day 7), 200 (on day 9) and 400 mOsm (on day 11) PEG 6000 treatment. The activity of the enzyme in the gels was determined using 1 mM indole-3-aldehyde as a substrate.

In our experiment, the investigated wheat *ZEP*, *NCED* and *AAO2* genes in the roots showed similar expression patterns to those found in *Arabidopsis* plants (Xiong et al., 2002). The highest induction was detectable in the *NCED* transcript levels in the roots, a lower increase was measured in *AAO2* in cv. Kobomugi and in *ZEP* expression in GK Öthalom cultivar. However, the induction of *NCED* and *AAO*2 occurred earlier and was more pronounced in Kobomugi than in GK Öthalom. At the same time the biosynthesis of ABA was not up-regulated in the leaves.

It has been suggested that the induction of ABA biosynthesis in the roots and ABA transport by the xylem from the roots to shoots is a long-distance signal for shoot tissues, which determines the rate of stomatal closure, thus, it is responsible for the adjustment of leaf water status (Wilkinson and Davies, 2002). It was found that localization of the *AAO3* gene (Koiwai et al., 2004) or NCED3 and AAO3 proteins in *Arabidopsis* plants proved to be tissue specific and the gene was expressed in the vascular parenchyma cells of the roots or shoots (Endo et al., 2008), which permits fast loading of ABA into the xylem sap. In our experiments it seems likely that *AAO* activity can be regulated not only at transcriptional but also at the protein level or by a direct control of the enzyme activity. Although no changes in the gene expression were found, higher AAO2 activities could be detected in the roots of cv. Öthalom exposed to osmotic stress on day 13. Moreover, the activity of AAO2-3 was enhanced very significantly as an effect of the osmotic stress in the roots of cv. Kobomugi.

Water-stressed leaves accumulated large amount of ABA and phaseic acid or dihydrophaseic acid, the oxidative metabolites of the hormone (Seiler et al., 2011). The latters are inactivated forms of ABA and reduce the physiologically active hormone pool (Qin and Zeevaart, 2002). In addition, all the three compounds can be converted to glucosyl ester-conjugate, thus, the steady-state ABA levels are under the control not only of the synthesis but of the rates of catabolism and conjugation. The closure of stomata depends also on the leaf capacity to compartmentalize and metabolize ABA.

If ABA concentration was expressed on tissue water content it was found that the up-regulation of ABA biosynthesis contributed rather to a maintenance than to an increase in ABA concentration. Other calculation methods (ABA content per mg fresh or dry mass) gave very different results. The physiologically relevant concentration of ABA is based on the availability of active hormone molecule for ABA receptors, in other words on the compartmentalization. In

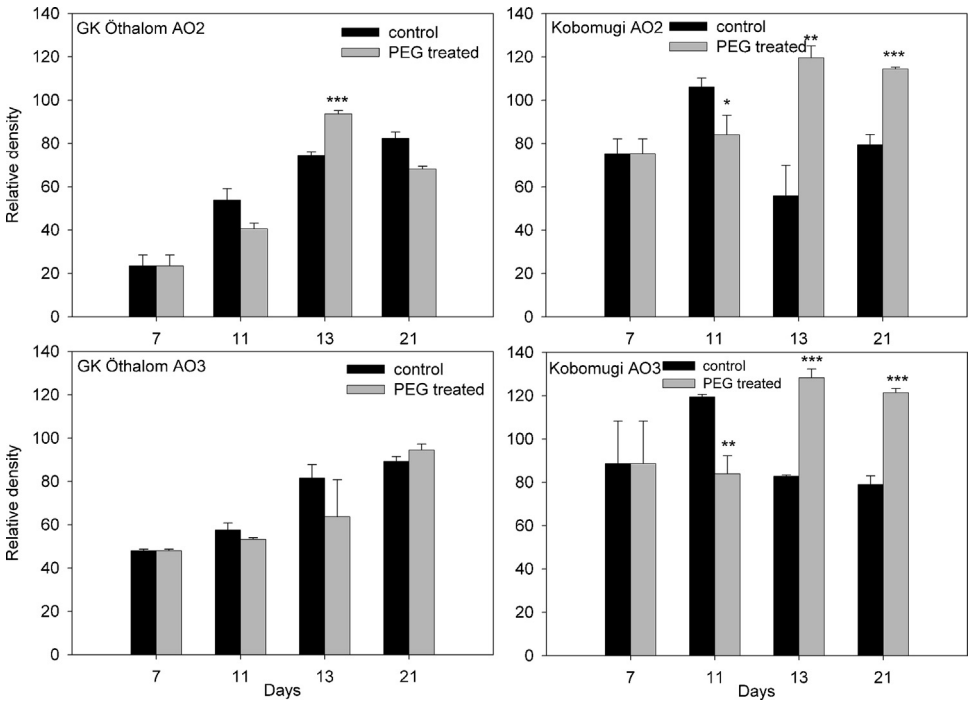

Fig. 5. Changes in the relative densities of aldehyde oxidase (AO) isoenzymes (AO2, 3) in the root tissues of wheat cultivars GK Öthalom and Kobomugi in the function of time after exposure to 100 (on the 7th day), 200 (on the 9th day) and 400 mOsm (on the 11th day) PEG 6000 treatment. (mean ± SD). Data labelled with *differed significantly from the untreated controls at $^{*}P \leq 0.05$, **0.01 or ***0.001 level.

water stressed plants the guard cells respond to small concentration changes in apoplastic ABA transported by the xylem sap from roots to leaf tissues.

Thus, closure of the stomata may be induced by ABA without any change in bulk tissue ABA levels (Cornish and Zeevaart, 1985). Also, the distribution of ABA in sink and source leaves can show differences, source leaves continuously feed young leaves with ABA via the phloem (Cornish and Zeevaart, 1984). In the course of the investigation period, the leaves of cv. Kobomugi showed significantly higher accumulation of ABA related to tissue water content in the stressed plants. Because this strong induction of ABA biosynthesis occurred in the roots, it can result from an effective xylem transport in this cultivar. In cv. Öthalom there was a smaller induction of ABA biosynthesis in the roots, but this enabled the plants to

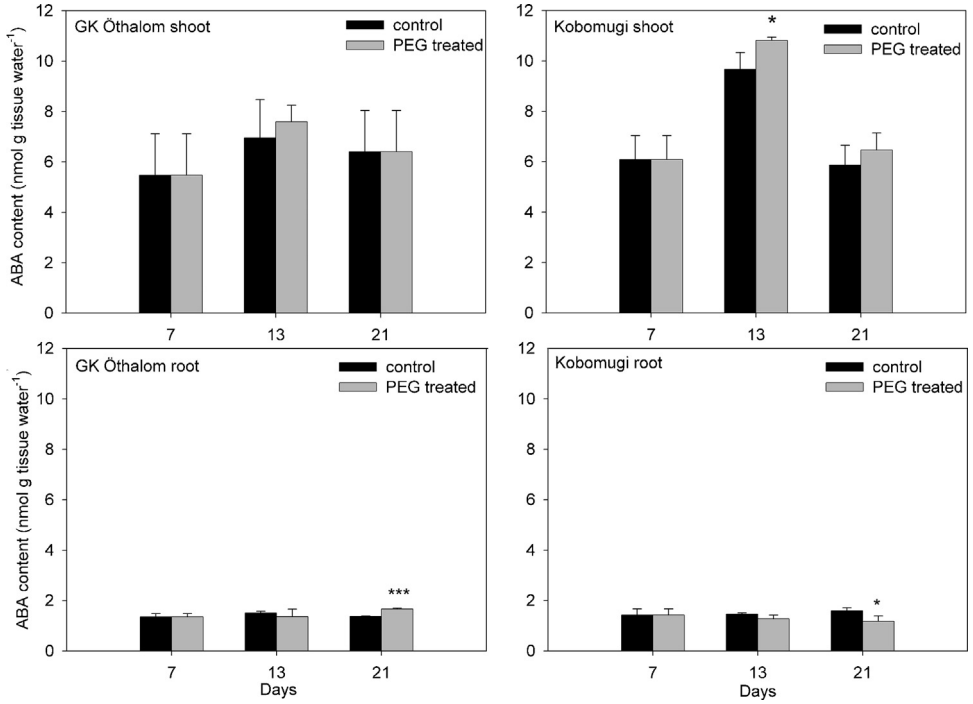

Fig. 6. Changes in the abscisic acid content (ABA) in the leaves and roots of wheat cultivars GK Öthalom and Kobomugi in the function of time, calculated on the basis of tissue water content, after exposure to 100 (on day 7), 200 (on day 9) and 400 mOsm (on day 11) PEG 6000 treatment. (mean ± SD, $n = 3$). Data labelled with *differed significantly from the untreated controls at $^{*}P \leq 0.05$, **0.01 or ***0.001 level.

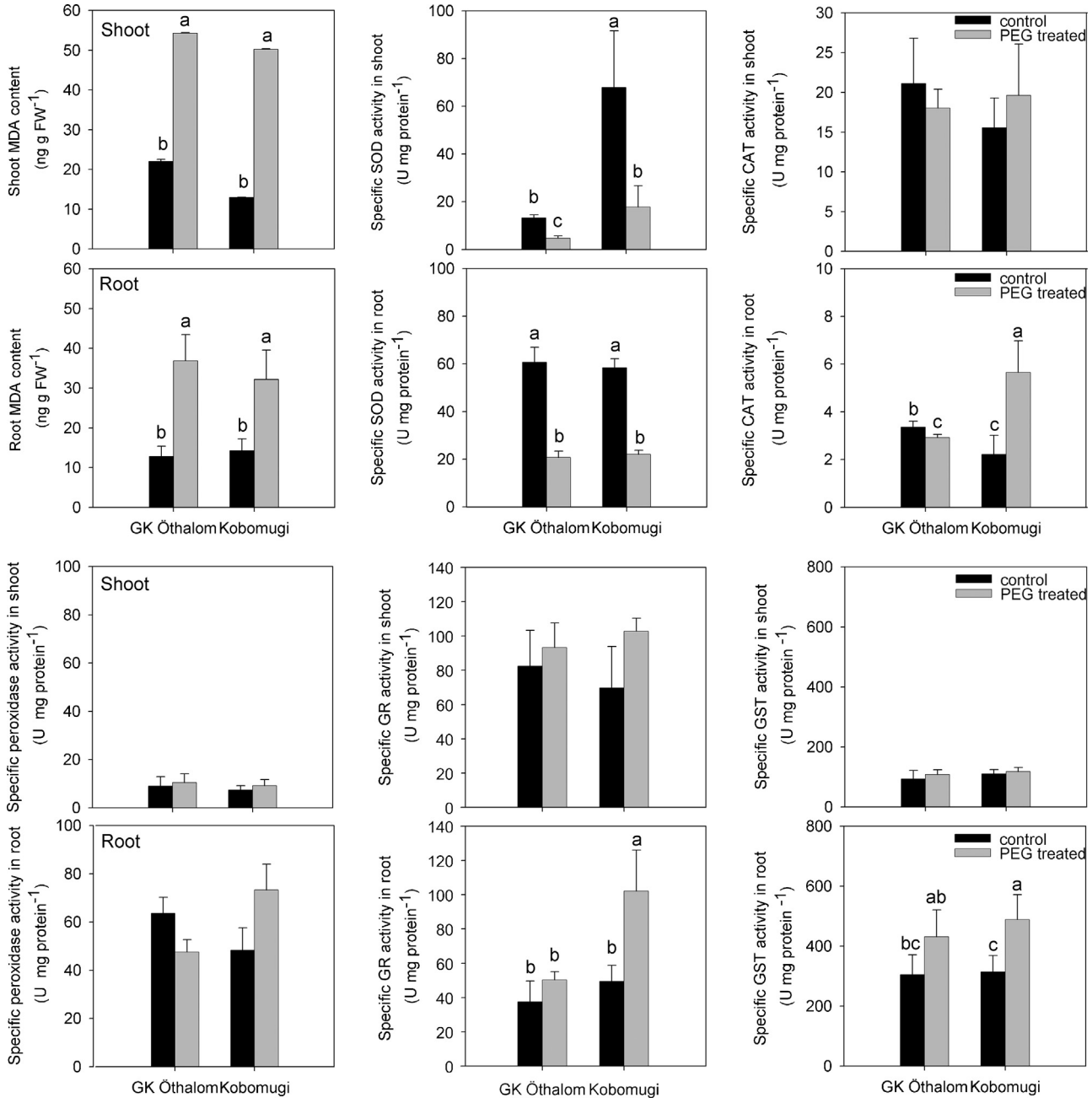

Fig. 7. Changes in MDA content and SOD, CAT, POD, GR, GST activities on day 21 in the leaves and roots of wheat cultivars GK Öthalom and Kobomugi after two weeks of PEG 6000 treatment. (Mean ± SD). Means denoted by different letters indicate a significant difference ($P < 0.05$, Duncan test).

maintain a steady-state hormone concentration in the leaves even during osmotic stress. ABA levels in the last sampling day were higher in the root tissues, providing a good opportunity for the induction of defence mechanisms in the roots of the anisohydric cultivar.

It was reported by Shatil-Cohen et al. (2011) that ABA transported by the xylem decreased the water permeability of the vascular bundle sheath cells and reduced the leaf hydraulic conductance by down-regulating their aquaporins. This is in accordance with the result of Pantin et al. (2013) who demonstrated that ABA promoted stomatal closure in a dual way: via a hydraulic effect and by direct activation of guard cell receptors. Thus, the stomata in cv. Kobomugi leaves may have a higher sensitivity to small changes in ABA levels.

The coordinative control and regulation of the expression and activity of antioxidant enzymes may be important for the survival of plants during drought stress (Bian and Jiang, 2009).

In the present study, almost all antioxidant enzymes investigated were affected by osmotic stress in a different manner in the two wheat cultivars. In the leaves of cv. GK Öthalom, the investigated enzymes worked at a lower level (SOD) or did not change significantly (CAT, POD, GR, GST). In Kobomugi the high basal activity of SOD declined during osmotic stress, but CAT, POD, GR and GST activities were enhanced in the leaves and, more significantly, in the roots after two weeks of osmotic stress. Our results suggest that a higher activity or the induction of these enzymes in the roots of the isohydric genotype can protect against oxidative damage. The isohydric cv. Kobomugi accumulated less

malondialdehyde than cv. Öthalom during osmotic stress. MDA can be regarded as a biomarker for lipid peroxidation, so the decrease in MDA content indicates higher anti-oxidative ability, which can reflect higher resistance to drought (Dhanda et al., 2004). CAT induction has a pivotal role in the defence and adaptation in the presence of excess H_2O_2 (Vranová et al., 2002; Tari et al., 2008), and drought stress increased CAT activity in the leaves of wheat (Luna et al., 2005). Guaiacol peroxidases are involved not only in scavenging H_2O_2 but also in plant growth, development, lignification, suberization, and cross-linking of cell wall compounds. Salt- or drought-tolerant plants often have higher POD activities than the sensitive ones during stress conditions (Wang et al., 2009; Csiszár et al., 2012). Glutathione reductase is a part of the ascorbate-glutathione enzyme system, converting glutathione disulfide (GSSG) to reduced glutathione (GSH). High GST activity is a common characteristic of several cultivated *Triticum* species, and according to the literature, the differences in the GST activities are in a good correlation with their stress tolerance (Bartoli et al., 1999; Edwards et al., 2000; Gallé et al., 2009). The protecting role of GSTs against different stresses has been proved in several plant species (Edwards et al., 2000), and transgenic tobacco plants overproducing a GST gene with GSH-PX activity exhibited significant oxidative stress tolerance (Roxas et al., 2000).

Activation and induction of antioxidant enzymes under drought stress in wheat shows time dependence and depends on the severity of the stress (Bartoli et al., 1999). It was reported earlier that changes in H_2O_2 contents in apical root segments of wheat genotypes exhibited a genotype-specific pattern during osmotic stress (Csiszár et al., 2012). In the present experiments, the role of antioxidant enzyme activities in the stress response was apparent during more severe water stress conditions (on day 21, one week after applying 400 mOsm PEG) in both cultivars. ABA-responsive elements (ABRE) were found in the promoter regions of several enzymes of the antioxidant defence, e.g. POX (Csiszár et al., 2012), CAT (Scandalios, 2005), SOD (Sakamoto et al., 1995), GR (Kaminaka et al., 1998) and in GST (Xu et al., 2002) isoenzymes, but their expression may be controlled by H_2O_2 or by ABA through independent signal transduction pathways.

Beyond the putative control of ROS scavenging systems it was found that increased ABA contents in tomato constitutively expressing *LeNCED1* led to a higher guttation rate (Iuchi et al., 2001). This suggests that ABA may facilitate xylem loading and the enhancement of root pressure under non-transpiring conditions. The leakage of K± and other inorganic ions from the cells was also dependent on ROS production (Demidchik et al., 2010) and on the antioxidant status of the tissues (Tari et al., 2002). Thus, the induction of antioxidant enzymes in the roots of an isohydric wheat genotype may control the accumulation of inorganic osmolytes, which can contribute to the maintenance of water potential.

In conclusion, the two cultivars responded differently to osmotic stress and the successful acclimation can be attributed to various components in both cultivars. The quick stomatal closure and the increased shoot ABA contents in cv. Kobomugi may play a role in the maintenance of the water potential of the plants at control level. The stress-inducible ABA biosynthesis and antioxidant defence in the root system of Kobomugi could arise from the adaptation of this landrace to semidesert environmental conditions. The closure of stomata in parallel with the ABA biosynthesis in GK Öthalom was a response to a higher osmotic stress and the antioxidant defence was less pronounced in the roots, which led to a higher damage to the membrane structure in the root cells.

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