

USE OF ABA TREATMENT FOR THE ACTIVATION OF DROUGHT PROTECTIVE MECHANISMS IN BARLEY UNDER NON-STRESS CONDITIONS

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Abstract

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The present study evaluated the sensitivity of three different barley genotypes to stress simulated by the application of exogenous abscisic acid ($20 \mu\text{mol.l}^{-1}$) at the early stage of the plant development. We used RIA method, instantaneous water use efficiency (WUE), the discrimination of ^{13}C ($\Delta^{13}\text{C}$) and the expression levels of *Dhn4* gene. The increase of ABA concentration in leaves after exogenous ABA application was detected in all tested genotypes; however, the lowest amount was found in the most tolerant genotype. Increased level of the instantaneous WUE after ABA treatment was found in all genotypes. The $\Delta^{13}\text{C}$ in ABA treated plants decreased, however, relatively drought-tolerant genotype Tadmor showed lower discrimination even in control variant. The genotype-dependent differences were observed in the expression levels of *Dhn4* gene in the leaves. High expression level of this gene was observed in Tadmor. Based on the exogenous ABA level, it was possible to distinguish two types of response of plants to exogenous ABA. Tadmor represented one of them as manifesting high sensitivity to exogenous ABA, leading to fast induction of *Dhn4* gene relative expression. Conversely, spring genotypes of Jersey and Malz manifested slower response to exogenous ABA as well as lower WUE values and relative expression of *Dhn4*. The results supported the idea that ABA application may activate similar stress reactions in plants as drought conditions and additionally the intensity of this reaction is genotype dependent.

Keywords: Abscisic acid, drought stress, Dhn, gene expression, *Hordeum vulgare* L., WUE

INTRODUCTION

The abiotic stressors such as deficiency or excess of light, minerals, temperature and the availability of water affect yield of agriculturally important plants. Water availability is one of the most important factors. Plant drought tolerance is genetically controlled (Teulat *et al.*, 2002), but the assessment of tolerance traits is not simple regarding its complex character and should be done under well defined stress conditions. Relative water content (RWC) and osmotic potential (Teulat

et al., 1997b), evapotranspiration and water use efficiency (WUE) (Xu *et al.*, 2007) or size of the root system (Chloupek *et al.*, 2006) represent frequently evaluated parameters. These parameters can be influenced not only by water deficit, but also by other environmental factors, the growth stage and fitness of the plant.

WUE is an important factor connected with the productivity of plants under drought. Instantaneous WUE at the leaf level is the ratio of photosynthesis rate to transpiration (A/E). In an

agronomic sense, WUE represents the ratio of water use to produced biomass. The indirect method of WUE estimation created by Farquhar and Richards (1984) is based on the analysis of the ratio of carbon isotope ^{13}C to ^{12}C ($\delta^{13}\text{C}$) in plant tissues. The relative ratio of the $^{13}\text{C}/^{12}\text{C}$ in plant biomass adjusted to the $^{13}\text{C}/^{12}\text{C}$ ratio in atmospheric CO_2 is expressed as $\Delta^{13}\text{C}$ (carbon isotope discrimination). During photosynthesis, C_3 plants discriminate CO_2 with ^{13}C to the benefit of CO_2 with ^{12}C (Farquhar *et al.*, 1982; Morison *et al.*, 2008). It is suggested that plants with higher WUE assimilate more carbon per unit of used water. Therefore lower $\Delta^{13}\text{C}$ may indicate plants with higher WUE and higher drought tolerance (Condon *et al.*, 2006).

One of the important aspects of drought stress tolerance is plant response mediated by abscisic acid (ABA). ABA content is markedly increased as a result of water shortage (Schwartz and Zeevaart, 2004). ABA participates significantly in photosynthesis through regulation of the transpiration and stomatal conductance and thus plays a key role for water maintenance in plants (Acharya and Assmann, 2009). During drought stress, ABA is bound to the plasmalemma surface and resulting ion's flux changes lead to the activation of stomatal closure (Procházka *et al.*, 1998). Furthermore, ABA acts as a signal molecule in the activation of processes resulting in the accumulation of proteins with protective functions against cell dehydration (Zhang *et al.*, 2004). The application of exogenous ABA triggers various stress protective mechanisms in plants, e.g. in maize (Correa De Souza *et al.*, 2013).

Survival of plants under stress conditions depends on a rapid regulation of the expression of various genes. Members of *Cor/Lea* gene family (*Cold regulated/Late embryogenesis abundant*) are also often studied in association with protective functions at the cell level. Their expression is induced both during embryo maturation and in response to water shortage (Close, 1997; Tommasini *et al.*, 2008). The corresponding proteins are known to have several protective functions, mainly against dehydration. They occur in small quantities in plants grown under optimal conditions, but their quantity significantly increases as a response to dehydration (Kosová *et al.*, 2010). Generally, more tolerant genotypes respond more sensitively to stress at the level of the *LEA2* group gene expression (Zhang *et al.*, 2004; Park *et al.*, 2006). The *Dhn4* gene is a member of the *LEA2* group in barley and its expression increases upon stress conditions, e.g. under drought. Choi *et al.* (1999) showed that *Dhn4* gene expression can be also modulated by ABA treatment.

Some authors in their studies ascertained that more tolerant genotypes are usually characterised by high ABA concentration and high level of protective gene activation under drought stress (e.g. Suprunova *et al.*, 2004; Park *et al.*, 2006). However, our previous study (Mikulková *et al.*, 2009) has shown that a barley cultivar with high drought tolerance showed slower

activation of *dhn* genes expression and lower ABA concentration in comparison with barley cultivars more sensitive to drought. The aim of this work was to compare the response of barley genotypes with different drought tolerance to ABA treatment both at the physiological level (ABA content, WUE, $\Delta^{13}\text{C}$) and at the molecular level (*Dhn4* gene expression).

MATERIALS AND METHODS

Three barley (*Hordeum vulgare* L.) genotypes were chosen according to their different sensitivity to drought stress in field conditions. Tadmor, a two-row variety selected out of a Syrian landrace and well adapted to dry conditions (Teulat *et al.*, 1997a), together with a drought-sensitive (Jersey) and a moderately tolerant (Malz) cultivars of spring barley were used for the experiments. Plants were grown in hydroponic culture (substrate Perlite), in the nutrient solution of MS salts (Murashige and Skoog 1962) in Sanyo growth cabinet (day/night temperature 22/15 °C, PPFD 350–400 $\mu\text{mol m}^{-2}\text{s}^{-1}$, day/night 14/10 hours). After 14 days of cultivation, ABA solution 20 $\mu\text{mol.l}^{-1}$ (Shen *et al.*, 2001) was added to the hydroponic solution to simulate drought stress. Control plants were cultivated simultaneously without ABA application. The youngest fully developed leaves were used for analyses and measurements. Quantification of ABA in leaves was carried out using radioimmunological method (RIA) according to Quarrie *et al.* (1988). Leaf tissues (youngest fully developed leaves) for ABA analyses were sampled at 12 h, 24 h and 3 days after ABA application, in two replications.

Photosynthesis rate (A) and transpiration (E) were measured using the open gas-exchange system LI-6400 (LI-COR Inc, Lincoln, NE USA) equipped with 2×3 cm broadleaf chamber with an integrated light source (LI-6400-02B) at 400 $\mu\text{mol}(\text{CO}_2)\text{mol}^{-1}$ (air) in the reference chamber. Temperature of the leaf was set to 22 °C, flow rate was 500 $\mu\text{mol s}^{-1}$. Measurements (of the light curves) were carried out since 3rd till 5th day after ABA treatment. The middle sections of two leaves (youngest fully developed leaves) from different plants were used for each measurement to cover the area of the broadleaf chamber. The instantaneous WUE was calculated as A/E ratio. The mean values were estimated from nine different measurements for every genotype and treatment (ABA and control). WUE was calculated from values measured under 350, 500 and 1000 $\mu\text{mol m}^{-2}\text{s}^{-2}$ light curves.

The carbon isotope relative abundance ($\delta^{13}\text{C}$) in leaf biomass was estimated by IRMS (DeltaPlusXL, ThermoFinnigan, Bremen, Germany) interfaced with an elemental analyser Vario Micro Cube (Elementar, Germany) and calculated as

$$\delta^{13}\text{C} = (\text{R}_p - \text{R}_s) / \text{R}_s,$$

where R is the carbon isotope ratio $\text{R} = [^{13}\text{C}]/[^{12}\text{C}]$ in plant sample (index p) and standard (index s),

respectively. Leaves were sampled at 3rd and 7th day after ABA application, in three replications. Plant material was dried to constant weight at 80°C and ground to a fine powder in a ball mill. Tin capsules containing 0.3–0.4 mg of samples were combusted at ca. 1600 °C. Isotopic composition was expressed as per mil (‰) against VPDB (Vienna Pee Dee Belemnite) standard. The precision of $\delta^{13}\text{C}$ estimation was ± 0.05 ‰. The results are means of three biological samples for each variety and treatment. Plant discrimination against CO_2 in air, determined as Δ , is related to ^{13}C abundances in plant (δ_p) and in air (δ_a) as

$$\Delta = (\delta_a - \delta_p) / [(\delta_p / 1000) + 1],$$

where both δ and Δ are in per mill. We used -8.0 ‰ as δ_a of CO_2 for calculation of $\Delta^{13}\text{C}$.

The relative expression (RE) of ABA regulated gene *Dhn4* was estimated by qPCR according to Pfaffl (2001). RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). cDNA was prepared using the Reverse Transcription Kit (Qiagen, Hilden, Germany) and genes activity was analyzed by the QuantiTect™ SYBR Green PCR Kit. (Qiagen, Hilden, Germany). Primers for the *Dhn4* gene were synthesized according to Mikulková *et al.* (2009). Values of the relative expression of the target gene were normalized to the values of the relative expression of the reference gene (α -tubulin) (Suprunova *et al.*, 2004). Youngest fully developed leaves sampled 6 h, 12 h, 24 h and 3 days after ABA application were used for *Dhn4* expression analyses.

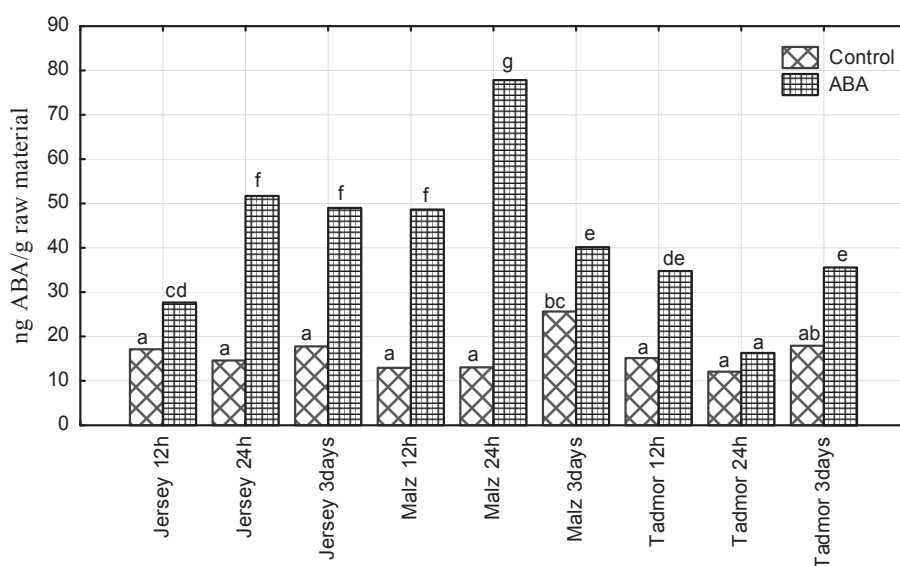
Given the type of experiment, one-way analysis of variance for ABA content, instantaneous WUE and $\Delta^{13}\text{C}$ was used. For comparisons of the means Tukey's HSD test at the significance level of 0.05 was

applied (Statistica 9.0, StatSoft Inc.). Mean values from three independent samples together with standard deviations were calculated for the relative expression of *Dhn4* gene.

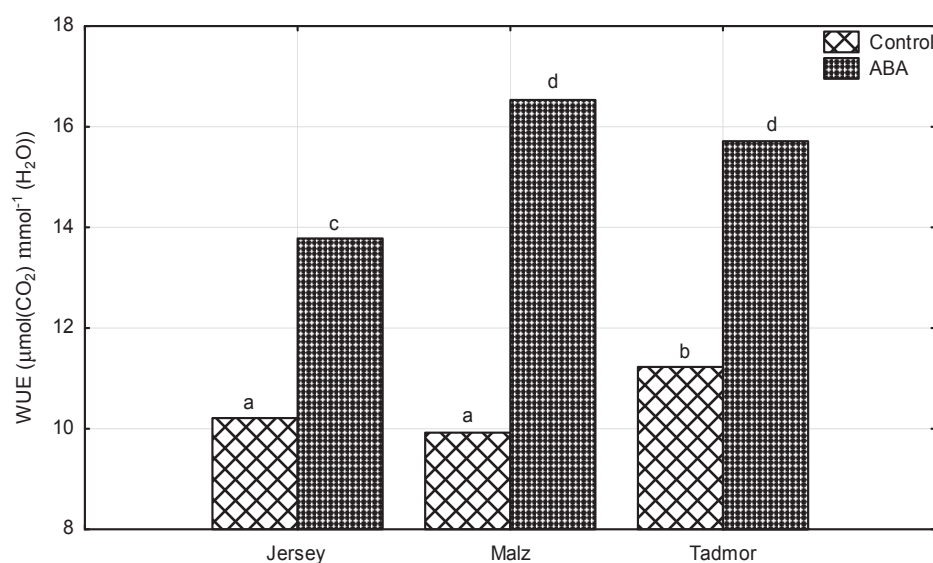
RESULTS AND DISCUSSION

Thomas *et al.* (1997) demonstrated that the application of exogenous ABA can induce drought-like effects in plants. The concentration of ABA in leaf tissues in ABA-treated and control plants are shown in Fig. 1. The effects of treatment and genotype were statistically significant, Tadmor and Malz with ABA levels higher in Malz, can be statistically distinguished (Fig. 1). The ABA concentration increased already after 12 h of ABA treatment in all genotypes. After 24 h, the differences between treated and control plants of Jersey and Malz even increased. Surprisingly, the drought tolerant genotype Tadmor showed a decrease of ABA concentration to the level of control variant after 24 h. Some differences in ABA accumulation have been described by Bandurska and Stroinski (2003), who found an earlier increase of ABA content in roots and a higher level of accumulated ABA in leaves of wild barley *Hordeum spontaneum* compared to the modern variety Maresi (*Hordeum vulgare*). In our experiment plants of Tadmor variety showed distinct reaction to exogenous ABA treatment resulting in lower ABA accumulation in leaves than plants of other tested varieties.

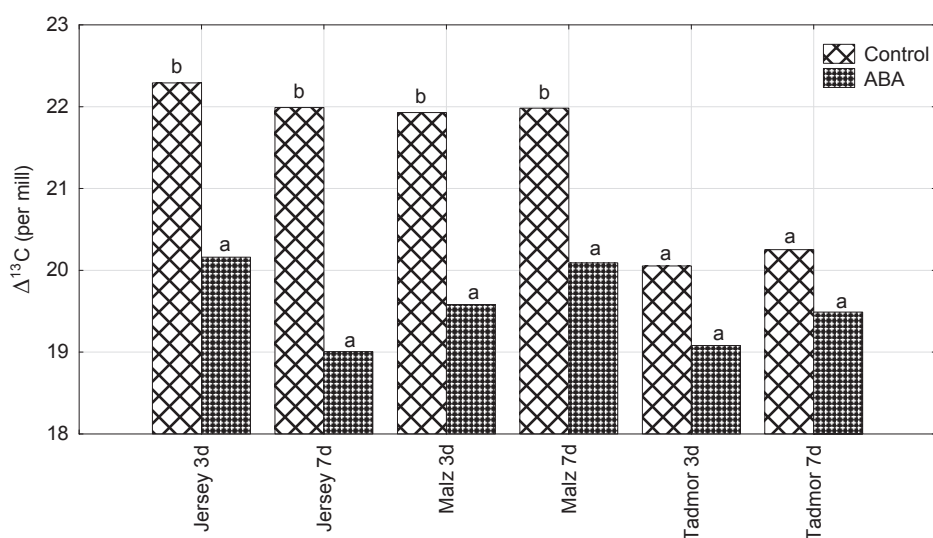
Analysis of variance for instantaneous WUE calculated as a ratio of A/E from gas exchange measurements for control and ABA treated plants is given in Fig. 2. Statistically significant influence of treatment and variety emerged here too (Fig. 2). WUE showed a higher value in non-treated plants of the Syrian drought-tolerant genotype Tadmor.



1: The content of endogenous abscisic acid (ABA) in leaves of three barley varieties at 12 h, 24 h and 3rd day of ABA treatment (control = plants not treated with exogenous ABA). The letters denote statistically significant differences at $p \leq 0.05$, $n = 2$. $F(df 6; 29) = 6.76$, $p = 0.000$.



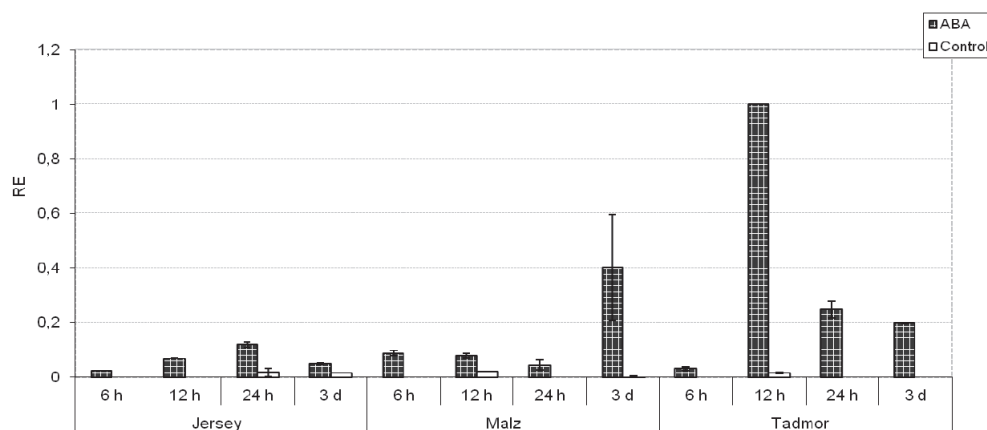
2: The instantaneous water use efficiency (WUE) in leaves of the control and ABA-treated plants of three barley varieties. The letters denote statistically significant differences at $p \leq 0.05$, $n = 9$. $F(df\ 5; 86) = 65.08$; $p = 0.000$.



3: The discrimination of the carbon isotope ($\Delta^{13}\text{C}$) in leaves of the three barley varieties at 3rd and 7th day of ABA treatment. The letters denote statistically significant differences at $p \leq 0.05$, $n = 3$. $F(df\ 17; 33) = 5.20$; $p = 0.000$.

An increase in WUE due to ABA treatment was observed in all three genotypes and there was a statistically significant difference between drought-sensitive Jersey and more tolerant Malz and Tadmor. Estimation of WUE based on gas-exchange measurement is time-consuming and may be variable due to changing environmental conditions during measurement. The indirect method of WUE estimation proposed by Farquhar and Richards (1984) is based on the analysis of the carbon isotope ^{13}C discrimination ($\Delta^{13}\text{C}$) which is negatively correlated with WUE. This can overcome disadvantages of direct WUE determination. Plant biomass samples integrate isotope signal over whole time of biomass accumulation, which make this method more robust. The analysis of variance for $\Delta^{13}\text{C}$ is given in

Fig. 3. In all three genotypes, $\Delta^{13}\text{C}$ in leaf biomass decreased after ABA treatment, probably as a result of stomatal closure which corresponds with water saving and limited CO_2 supply according to the theory of Farquhar *et al.* (1982). Lower $\Delta^{13}\text{C}$ was found in drought-adapted genotype Tadmor, even in control plants. Statistically significant difference between Tadmor ($\Delta^{13}\text{C} = 19.72$ per mill) and Malz and Jersey ($\Delta^{13}\text{C} = 20.90$; 20.86 per mill) was ascertained on 0.05 level (Fig. 3). This corresponds with the results of Chen *et al.* (2011) who concluded that higher $\delta^{13}\text{C}$ (which means lower $\Delta^{13}\text{C}$), especially estimated at sufficient supply of water, can represent a good selection criterion for breeding for drought resistance and maintaining the yield stability of barley genotypes. However, a superior yield of grain and biomass under optimum growth



4: The relative expression (RE) of *Dhn4* gene in leaves of three barley varieties at 6 h, 12 h, 24 h and 3rd day of ABA treatment. Columns represent mean values ($n = 3$) \pm SD.

conditions is usually connected with a relatively high level of ^{13}C discrimination (lower $\delta^{13}\text{C}$) and low WUE values (e.g. Condon *et al.*, 2002; Araus *et al.*, 2003). The relative expression of the *Dhn4* gene is displayed in Fig. 4. The results showed different sensitivity of tested varieties to initial concentrations of exogenously applied ABA ($20 \mu\text{mol.l}^{-1}$). The more sensitive protective reaction was found in drought-tolerant variety Tadmor in contrast to Malz and Jersey. The highest *Dhn4* gene expression level was detected in drought-tolerant Tadmor already 12 h after ABA treatment. The moderately drought-sensitive cultivar Malz showed a noticeable increase of *Dhn4* gene expression up to 3 days of ABA treatment and the drought-sensitive Jersey showed the lowest relative expression level of this gene. In our previous studies a low activity of protective *Dhn* genes was observed in the Jersey cultivar under various stress conditions (Mikulková *et al.*, 2009; Holková *et al.*, 2010). Park *et al.* (2006) also described a faster activation and a higher final expression level of the *Dhn4* gene in drought-tolerant genotypes

of barley. Intensity of *Dhn* gene expression can be affected by the level of ABA sensitivity as well. High level of *Dhn* genes expression in stress condition was found in ABA-sensitive wheat mutants (Kobayashi *et al.*, 2008).

Tadmor represents genotypes that are well-adapted to dry conditions. This was proven by its high osmotic adjustment (Teulat *et al.*, 1997b), good water use efficiency (Teulat *et al.*, 2001; Mikulková *et al.*, 2009) and a high relative expression of some *Dhn* genes (Mikulková *et al.*, 2009; Holková *et al.*, 2010). The results of our experiments suggest that the adaptation of Tadmor may be based on its higher WUE even in optimal conditions and relatively low endogenous ABA concentration together with its enhanced sensitivity to ABA. The high sensitivity of Tadmor to ABA was manifested by more intense stress reaction both at physiological and molecular level in comparison with the other genotypes studied; even though the native content of ABA in leaves was lower than in more drought-sensitive genotypes.

CONCLUSION

In this paper, we studied the responses of three barley genotypes on the main stress signal induced by exogenous ABA application. Results showed that Tadmor demonstrated an unambiguous reaction to ABA in comparison with cultivars less resistant to drought (Jersey and Malz). After ABA treatment, the level of ABA in leaf tissues of Tadmor fluctuated, but did not reach the levels in Malz and Jersey. Instantaneous WUE of Tadmor was comparable with the level of Malz. Levels of WUE estimated according to ^{13}C discrimination increased comparably in all treated variants. Tadmor, however, showed a high relative level of WUE (low $\Delta^{13}\text{C}$) even in control plants with the low level of ABA in leaves. Increased expression of dehydrins was detected in Tadmor already 12 hours after ABA application. These findings supported the theory of high sensitivity of Tadmor to ABA. High sensitivity to ABA is connected with drought tolerance, therefore Tadmor-like genotypes can be utilised as a source of this trait in breeding. Combination of dehydrin expression and isotope discrimination may represent useful criteria for selection of drought-resistant barley genotypes.

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