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Comparative analysis of drought stress effects on photosynthesis of Eurasian and North African genotypes of wild barley

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Abstract

The impact of drought stress (DS) on eight Eurasian and North African genotypes of wild barley (*Hordeum spontaneum*) was evaluated by analysis of chlorophyll (Chl) *a* fluorescence fast induction curves using the JIP-test. Three-week-old, pot-grown plants were exposed to a DS treatment by withholding water for nine days. The genotype-specific impairment of the functionality of the photosynthetic electron transport chain was quantified using the relative decline of the performance indices (PI_{abs} and PI_{tot}), two key parameters of the JIP-test. The genotypes showing the highest (HOR10164) and lowest (HOR10710) relative PIs under DS were subjected to additional experiments, including measurements of leaf gas exchange, water status, pigment content, key enzyme activity, and protein abundance. The genotypes showed a specific profile of DS-mediated inhibition of photosynthesis, associated with higher relative leaf water contents in HOR10164 at the end of the treatment. Whereas decreased photosynthetic rate in HOR10164 was mainly caused by stomatal closure, nonstomatal limitations (decreased Rubisco content and activity) were detected in HOR10710. Additional genotype specific features were the upregulation of the NADP-malate dehydrogenase in HOR10164 and a decreased fraction of Q_A-reducing reaction centers in HOR10710.

Additional key words: Calvin cycle; chlorophyll fluorescence; JIP-test; performance index; photosynthetic CO_2 assimilation.

Introduction

The accessibility of sufficient amounts of water is a basic prerequisite for plant growth and development. Consequently, drought periods are a major factor limiting crop yield and biomass production. Climate change scenarios (*e.g.* IPCC 2013) predict an increase of regional and seasonal weather extremes, such as drought, heat waves, and heavy rainfall for Central Europe and the Mediterranean basin. Hence, for agricultural purposes it is essential to study the impact of DS on plant functions and, in addition, to search for plants and genotypes adapted to dry conditions.

Having its center of distribution located in the area of the "Fertile Crescent", ranging throughout the Middle East from Israel to Iraq, *H. spontaneum*, the wild ancestor of cultivated barley (*H. vulgare*), is also present in North Africa, Greece, Azerbaijan, and Central Asia (Harlan and Zohary 1966). Since *H. spontaneum* is distributed over a high number of different habitats, including mountainous and desert regions (Volis *et al.* 2002), it promises a broad spectrum of genetic adaptations to abiotic and biotic stresses, beyond the potential of cultivated varieties. The use of these rich resources in breeding programs aiming

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Abbrevations: C_i – intercellular CO_2 concentration; Car – carotenoids; Chl – chlorophyll; cpHSP70 – plastidic heat shock protein 70; Dhn1 – dehydrin 1; DM – dry mass; DS – drought stress; FM – fresh mass; g_s – stomatal conductance; LA – leaf area; LSU – large subunit; NADP-MDH – NADP-malate dehydrogenase; P_N – net photosynthesis rate; PI – performance index; PsbO – psbO – manganese stabilising protein of the oxygen evolving complex of PSII; RA – Rubisco activase; RC – reaction center; RC/ABS – fraction of active (Q_A-reducing) PSII reaction centers; ROS – reactive oxygen species; RWC – relative water content (of leaves); sFBPase – stromal fructose-1,6-bisphosphatase; SWC – soil water content; TM – turgid mass; V_I – relative variable fluorescence at the I-step (30 ms); V_J – relative variable fluorescence at the J-step (2 ms); δ_{Ro} – efficiency of electron transport from the intersystem electron carriers to the PSI acceptor side; ϕ_{Po} – maximum photochemical efficiency of PSII; Ψ_{soil} – soil water potential.
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for an improvement of cultivated crops can provide an enhanced performance under stressful conditions, ensuring high crop yield and quality (Ellis *et al.* 2000, Feuillet *et al.* 2008).

The analysis of Chl *a* fluorescence fast induction curves using the JIP-test method – introduced by Strasser *et al.* (2000, 2004) – is a tool frequently used for the quantification of photosynthetic limitations caused by abiotic stresses (*e.g.* Tóth *et al.* 2005, van Heerden *et al.* 2007). To achieve a quick and systematic analysis, the JIP-test accepts a number of assumptions simplifying the underlying events reflected in the OJIP induction curve (Stirbet and Govindjee 2011). Despite the actual complexity of Chl *a* fluorescence induction (*cf. e.g.* Lazar 2006, Stirbet and Govindjee 2012), the test is useful for monitoring changes of the photosynthetic capability in plants under unfavourable environmental conditions. Recently, the JIP-test has been applied in comparative studies evaluating the differential drought resistance of genotypes (Oukarroum *et al.* 2007, Jedmowski *et al.* 2013). Therein, the assessment was done by evaluation of a key parameter derived from the fast induction curves, the so-called performance index (PI_{abs}). The PI_{abs} is a multifactorial parameter giving an overall measure of PSII electron transport. Later it was shown that the amplitude of the PSI related I-P phase of the OJIP transient is also sensitive to DS (Oukarroum *et al.* 2009). An extension of the PI_{abs} including an I-P phase related parameter, the so-called total PI (PI_{tot}), was introduced by Strasser *et al.* (2010) to include electron transport to the PSI end acceptors. However, photosynthetic performance does not only rely on the functionality of the electron transport chain, but also on the availability of CO_2 and the efficiency of CO_2 fixation, which both may decrease dramatically under DS (*cf.* reviews on stomatal and nonstomatal limitation of photosynthesis, *e.g.* Lawlor and Tezara 2009). None of the cited screening studies included measurements of leaf gas exchange or of the activity of Calvin cycle enzymes, which would provide information about the relations between the functionality of the electron transport chain and the processes of photosynthetic carbon fixation.

The aims of this study were (*1*) to quantify the drought tolerance of wild barley genotypes covering a broad geographical spectrum *via* JIP-test analysis, (*2*) to implement the Chl fluorescence data into a more comprehensive overview of the physiological DS response, and (*3*) to identify genotype specific limitations of photosynthesis due to DS as well as strategies to cope with low water availability.

Materials and methods

Plant material: Seeds of *Hordeum spontaneum* KOCH accessions (Table 1), provided by Leibniz Institute of Plant Genetics and Crop Plant Research (IPK, Gatersleben, Germany) were germinated in darkness on vermiculite for 48 h. Seedlings were transferred to pots filled with 4 l of a commercial peat soil (type *C700, Stender Erden*, Schermbeck, Germany), each pot containing three individuals. Plants were grown in climate chambers at 25°C, 70% RH, 14/10 h of day/night rhythm under 400–500 μmol(quantum) m^{-2} s^{-1} with daily watering.

Imposition of drought stress: DS was applied on 21-d-old plants by preventing further watering for 9 d under the growing conditions mentioned above. On day 1 of the experiment, the soil was adjusted to the maximum water holding capacity. Volumetric soil water content (SWC) was measured using a *Theta Probe ML2x* (*Delta T Devices*, Cambridge, UK). For determination of the corresponding soil water potential (Ψ_{soil}), a soil-specific calibration curve of SWC *vs*. Ψ_{soil} was calculated using a *PSYPRO* (*Wescor*, Logan, USA) psychrometer equipped with *PST-55* soil sensors. SWC decreased from 35% on day 1 to below 5% on day 9 of the experiment, corresponding to Ψ_{soil} below –1 MPa.

Table 1. Accession numbers and origins of *Hordeum spontaneum* genotypes used in this study.

Accession number	Origin
HOR2686	Ahvaz, Iran
HOR10164	Tacnis, Libya
HOR10478	Mosul, Iraq
HOR10710	Baku, Azerbaijan
HOR10977	Khujand, Tajikistan
HOR11017	Chania, Greece
HOR11183	Tal Afar, Iraq
HOR12818	Sede Boker, Israel

Screening *via* JIP-test analysis: Fast induction kinetics of Chl *a* fluorescence was detected utilizing a *Pocket PEA* fluorimeter (*Hansatech,* King's Lynn, GB). All measurements were conducted after an overnight dark adaption of the plants. A weak lamp emitting green light was used for orientation during the measurements. The collected data were analysed with the *PEA-Plus* (version *1.02*) and the *Biolyzer* (version *3.0*, 1999–2001, by R.M. Rodriguez, Bioenergetics Laboratory, Geneva, Switzerland) software according to the JIP-test (Strasser *et al.* 2000, 2004), including I-P phase related parameters (Strasser *et al.* 2010) by self-made extensions of the data files using *Excel* (*Microsoft*, Redmond, USA). The relative decrease of PI_{abs} and PI_{tot} of the tested genotypes, evaluated as $PI_{(rel)} = PI_{day9}/PI_{day1}$, was used as a specific measure for DS-induced inhibitions.

Sampling scheme for HOR10164 and HOR10710: The genotypes showing the highest (HOR10164) and lowest (HOR10710) relative PIs under DS were chosen for further

experiments to achieve a more comprehensive look into the impact of DS on the named genotypes. Measurements using noninvasive methods (Chl fluorescence, gas exchange) were carried out on day 1 (control, well watered conditions) and on days 6–9 of the experiment. Invasive measurements (*e.g.* determination of the leaf water status) were only conducted on days 1 and 9 to avoid an artificial reduction of the overall leaf area (LA). Leaf material of the youngest, fully developed leaves was used for all measurements. Chl *a* fluorescence and gas exchange were measured on the middle section of the leaves. For determination of the water status, leaves were cut and the middle segment was used for calculation of the relative water content (RWC), whereas the remaining parts were utilised for the measurement of the corresponding osmotic potential. Immediately after gas-exchange measurements the leaf was cut, divided, frozen under liquid nitrogen, and stored at –80°C. The collected leaf sections were used for protein extraction, measurements of enzyme activities, and determinations of the pigment content.

Plant water status and total LA: For determination of the RWC, leaf segments were harvested predawn and weighed immediately after harvesting (fresh mass, FM), after rehydration for 5 h at 4°C (turgid mass, TM) and after a subsequent drying at 70°C for 24 h (dry mass, DM). RWC was calculated according to Barrs and Weatherley (1968) as RWC = 100 (FM – DM)/(TM – DM). The corresponding osmotic potential of leaf cell sap extracts was measured with an *Osmomat 030* (*Gonotec,* Berlin, Germany). For the measurement of the total LA, all vital leaves were harvested and the blades were fixed on scaled sheets. After scanning, LA was calculated using the *ImageJ* software (Schneider *et al*. 2012).

Chl *a* fluorescence: Fluorescence measurements were conducted as described above. Additionally average normalised induction curves were calculated to display changes of the relative variable fluorescence over time. Therefore original transients were double normalised (F_0 = 0, F_m = 1) and differential curves of the relative variable fluorescence (ΔV_{OP}-curves) were assessed using the formula:

$$\Delta V_{OP}(t) = [(F_t - F_0)_{DS}/(F_m - F_0)_{DS}] - [(F_t - F_0)_{day\ 1}/(F_m - F_0)_{day\ 1}].$$

Gas exchange: Net photosynthetic rate (P_N), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) were measured using a *GFS-3000* device (*Walz*, Effeltrich, Germany) at 25°C and 70% RH under saturating light conditions [1,000 μmol(quantum) m^{-2} s^{-1}]. The applied CO_2 concentration was set to 400 mg kg^{-1} or 1,400 mg kg^{-1}. To achieve steady-state conditions, samples were adapted to the specific conditions for a period of 10 min before the measurements were started.

Pigment content: Frozen leaf material was ground under liquid nitrogen and the fine powder was extracted using ice-cold, absolute acetone containing 0.05% (w/v) $CaCO_3$ [1 ml mg^{-1}(FM)]. Samples were agitated on ice in the dark for 60 min until the leaf material was completely decolorized. During the extraction procedure samples were kept on ice in the dark. For spectroscopic measurements the acetone was diluted to 80% by addition of specific amounts of distilled water. The spectroscopic determination of the Chl and carotenoid (Car) contents of the leaf samples was conducted using equations given by Lichtenthaler (1987):

$$\text{Chl } a\ [\mu g\ ml^{-1}] = 12.25\,(A_{663} - A_{750}) - 2.79\,(A_{647} - A_{750})$$
$$\text{Chl } b\ [\mu g\ ml^{-1}] = 21.50\,(A_{647} - A_{750}) - 5.10\,(A_{663} - A_{750})$$
$$\text{Car}\ [\mu g\ ml^{-1}] = (1{,}000\,A_{470} - 1.90\,\text{Chl } a - 85.02\,\text{Chl } b)/198$$

Protein extraction, SDS-PAGE, and Western blotting: Extraction of leaf proteins was conducted according to Ashoub *et al.* (2011) using absolute ethanol containing 10% dithiothreitol for protein precipitation. Resuspension solution consisted of 7 M urea, 2 M thiourea, and 2% Nonidet P-40 (v/v). Protein concentrations of the extracts were determined by Bradford (1976) assay using various BSA concentrations in resuspension solution as standards.

For SDS-PAGE, extracts were treated with four-fold concentrated sample buffer [240 mM Tris-HCl, pH 6.8, 8% SDS (w/v), 20% 2-mercaptoethanol (v/v), 40% glycerol (v/v), and 0.2% bromphenol blue (w/v)] and incubated at 95°C for 5 min. Portions of the samples equivalent to 5 μg protein were applied to electrophoresis using the discontinuous method (4% stacking gel and 12.5% separating gel) according to Laemmli (1970). Gels were run at 100 V for approximately 2.5 h.

Western blot transfer on PVDF membranes (*Immobilon-P Transfer Membrane*, *Merck Millipore,* Billerica, USA) was carried out using a semidry blotting apparatus (*Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell*, *Biorad,* Hercules, USA) with a single buffer [48 mM Tris; 39 mM glycine; 20% methanol (v/v); 1 mM SDS]. For immunodetection, membranes were blocked for 1 h with TBST-buffer [20 mM Tris-HCl, pH 7.5; 0.15 M NaCl; 0.05% Tween 20 (v/v)] containing 5% skimmed milk powder. Primary antibodies of the target proteins [Rubisco (135-IV-II anti-Rubisco holoenzyme from rye), T. Berberich, University of Frankfurt)], Rubisco activase (RA, AS10 700, *Agrisera*, Vännäs, Sweden), manganese stabilising protein of the oxygen evolving complex of PSII (psbO, AS06 142-33, *Agrisera*), dehydrin 1 (Dhn1, AS07 206, *Agrisera*), cpHsp70 (AS08 348, *Agrisera*)] were added to the TBST-milk powder solution (dilution 1:5,000). The membranes were incubated for 1 h and washed subsequently with TBST-buffer (3 × 10 min). The secondary antibody (horseradish peroxidase, HRP, *Thermo Scientific,* Rockford, USA, 1:10,000) was applied for 1 h, again followed by three washing steps with TBST. For chemiluminescence detection, membranes were left in *Pierce ECL Western Blotting Substrate* (*Thermo*

Scientific) according to the manufacturer's instructions and the signal was documented on X-ray films. The films were scanned and quantified using the *ImageJ* software.

Enzyme activities: Samples for enzyme assays were collected immediately after gas-exchange measurements to ensure full activation of all light-regulated enzymes. Further, all measurements were conducted under fully activating *in vitro* conditions. The extractions for the measurements of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, E.C. 4.1.1.39), stromal fructose-1,6-bisphosphatase (sFBPase, E.C. 3.1.3.11), and NADP-malate dehydrogenase (NADP-MDH, E.C. 1.1.1.82) were conducted according to Dias and Brüggemann (2007). Activities of Rubisco and NADP-MDH were measured as described in Du *et al.* (1996). Rubisco samples were incubated on ice for 10 min in presence of 10 mM $NaHCO_3$ and 20 mM $MgCl_2$ (Lilley and Walker 1974) before measuring. sFBPase activity was determined according to Brüggemann *et al.* (1994).

Statistical analysis was performed with the *GraphPad Prism 5.0* software (*GraphPad Software Inc.*, LaJolla, USA). In figures and tables, significant changes by treatment (Fig. 3, Table 2) or differences between accessions (all other figures) were marked with one ($p<0.05$), two ($p<0.01$) or three ($p<0.001$) asterisks. All values shown in the figures, tables or in the text are displayed as mean ± SD. Data sets (only if $n \geq 7$) were tested for Gaussian distribution using the *Shapiro-Wilk* normality test (as recommended for Chl *a* fluorescence measurements in Lazar and Naus 1998). Data sets following Gaussian distribution (and if $n < 7$) were tested for statistical significance using the unpaired *t*-test. If Gaussian distribution was rejected, the nonparametric *Mann-Whitney U*-test was applied.

Results

JIP-test analysis: An effect of DS on the photosynthetic electron transport was detected for all tested genotypes, as displayed by changes of the analysed JIP-test parameters. However, the relative decreases of PI_{abs} and PI_{tot} were variable for the tested genotypes (Fig. 1*A,B*). HOR10164 showed no reduction of PI_{abs}, while PI_{tot} was reduced by 12% by drought. In contrast, a decline of PI_{abs} (38%) and PI_{tot} (64%) was revealed in HOR10710. For the remaining genotypes, the magnitudes of changes were located between these two extremes. Using the PIs as a measure for drought susceptibility, genotypes HOR10164 and HOR10710 were used for further experiments.

Comparative analysis of HOR10164 and HOR10710

Growth habit and water status: While HOR10164 showed an upright growth habit and an average LA of 165.2 ± 58.1 cm^2 per plant (n = 18) at day 1 of the experiment, HOR10710 exhibited a rosette-like growth habit and a significantly larger total LA (206.9 ± 56.9 cm^2, n = 20). During the first days of DS, the SWC of HOR10710 declined with a higher rate compared to HOR10164 (Fig. 2). Despite the genotypes showed relatively similar SWC (HOR10164: 4.5%, HOR10710: 3.7%) at day 9, HOR10710 was exposed to higher soil water deficits throughout the experiment. The drought-induced

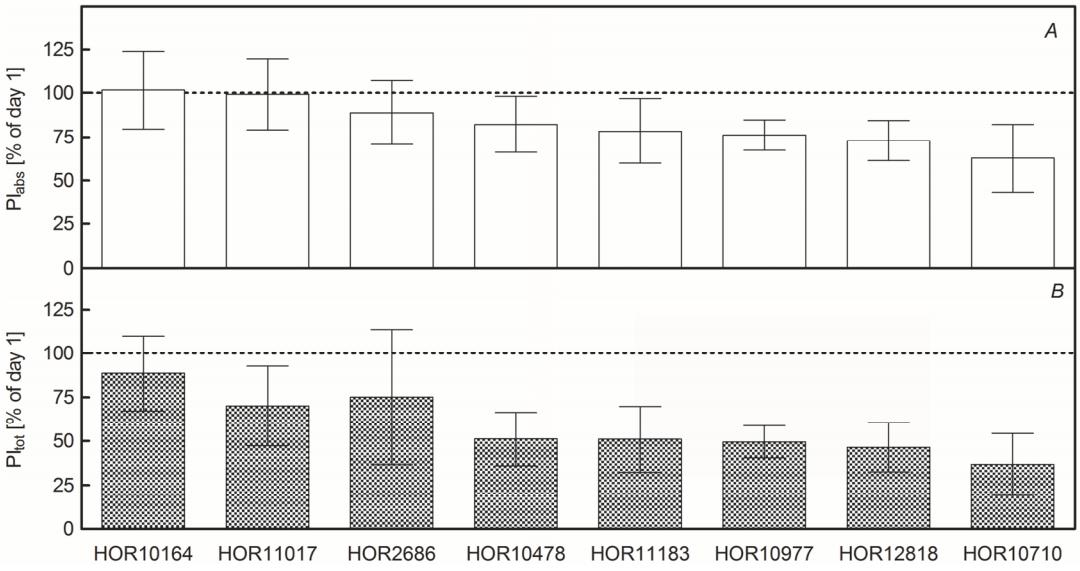

Fig. 1. Relative changes of the JIP-test parameters, performance indices PI_{abs} and PI_{tot}, of the tested genotypes of *Hordeum spontaneum* at the end of drought treatment (mean ± SD; n = 9–14).

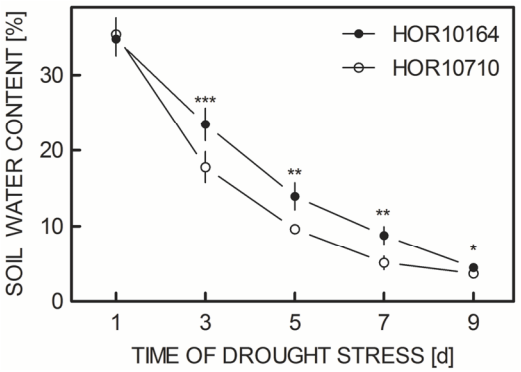

Fig. 2. Changes of the volumetric soil water content of HOR10164 and HOR10710 during the experiment (mean ± SD; n = 6). * – p<0.05, ** – p<0.01, *** – p<0.001.

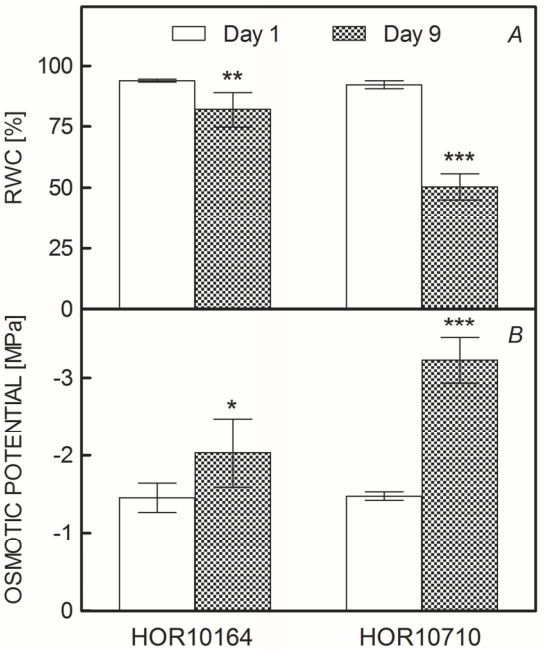

Fig. 3. Plant water status (RWC, *A*) and osmotic potential, (*B*) of HOR10164 and HOR10710 under control and drought conditions (mean ± SD; n = 5–10). * – p<0.05, ** – p<0.01, *** – p<0.001.

changes of plant water status, as determined by leaf RWC, differed significantly between the genotypes (Fig. 3*A*). The changes were much stronger for HOR10710 (control: 92.2%, DS: 50.3%) than for HOR10164 (control: 93.9%, DS: 81.7%). The osmotic potential of the leaves decreased from –1.45 to –2.03 MPa (HOR10164) and from –1.47 to –3.23 MPa (HOR10710), respectively, in consequence of the DS treatment (Fig. 3*B*).

Gas exchange and Chl fluorescence: At ambient CO_2 concentration (400 mg kg^{-1}), P_N of HOR10710 decreased gradually from 21.9 (day 1) to 3.5 μmol(CO_2) m^{-2} s^{-1} at the end of the experiment (Fig. 4*A*). g_s was strongly reduced to 87.3 mmol(H_2O) m^{-2} s^{-1} after 6 d of DS [control: 366.8 mmol(H_2O) m^{-2} s^{-1}] (Fig. 4*C*). Under conditions of elevated CO_2 (1,400 mg kg^{-1}), P_N of unstressed plants was slightly higher compared to ambient CO_2 (Fig. 4*B*). At the end of the drought period, P_N at 1,400 mg kg^{-1} was reduced to 10.0 μmol(CO_2) m^{-2} s^{-1}. C_i decreased from 295 to 152 mg kg^{-1} on day 9 of the experiment (Fig. 4*D*).

For HOR10164, an initial increase of P_N, followed by a slow decrease to 16.8 μmol(CO_2) m^{-2} s^{-1} [control: 21.4 μmol(CO_2) m^{-2} s^{-1}] was observed at 400 mg kg^{-1}(CO_2). g_s declined constantly from 451.9 to 147.7 mmol(H_2O) m^{-2} s^{-1}. Measurements at 1,400 mg kg^{-1}(CO_2) revealed no significant changes of P_N comparing unstressed [24.3 μmol(CO_2) m^{-2} s^{-1}] and stressed [24.4 μmol(CO_2) m^{-2} s^{-1}] plants. C_i was reduced from 305 to 197 mg kg^{-1}.

While ϕ_{Po} (= F_v/F_m, a parameter often used for assessing stress effects), remained unchanged in both genotypes (not shown), the relative PI_{tot} values of HOR10164 slightly decreased on day 9 of the experiment (Fig. 4*E*). PI_{tot} of HOR10710 dropped continuously from day 6 on. Additionally, linear relationship between the daily means of the PIs and P_N at 400 mg kg^{-1} was observed (R^2 = 0.93/0.80) (Fig. 5).

The average ΔV_{OP}-curves shown in Fig. 6*A,B* display the development of the relative variable fluorescence during the days 6–9 of DS relative to day 1. The signal of HOR10164 fluctuated close to the baseline on days 6–8 and finally exhibited a small peak around the I-step of the OJIP-transient (30 ms). Measurements of HOR10710 showed an equivalent peak, though the signal was much stronger. Here, the I-peak appeared already on day 6, increased again on day 7 and lasted stable until day 9. After day 6, the signal also started to increase in the early phase of the transient. The relative fluorescence at the J-step (2 ms) increased gradually until day 9 of DS.

To evaluate JIP-test parameters not as a function of the duration of DS treatment, but of the plant water status, the DS period was prolonged up to 14 d with continuous measurements of RWC and Chl *a* fluorescence. The reaction of PI_{tot} during leaf desiccation was quite similar for both genotypes (Fig. 7*A*). After an initial decrease, PI_{tot} reached a steady state which was stable down to RWCs below 50% in the case of HOR10710. For severely stressed plants (RWC below 70%), no significant difference between the genotypes could be observed. The fraction of active (Q_A-reducing) reaction centers (RCs), expressed by the JIP-test parameter RC/ABS (Fig. 7*B*), was reduced during desiccation. HOR10710 showed a pronounced decrease until RWCs drop below 70%, followed by a slight increase under severe DS. For HOR10164, the decline of RC/ABS was less pronounced with increasing water deficit.

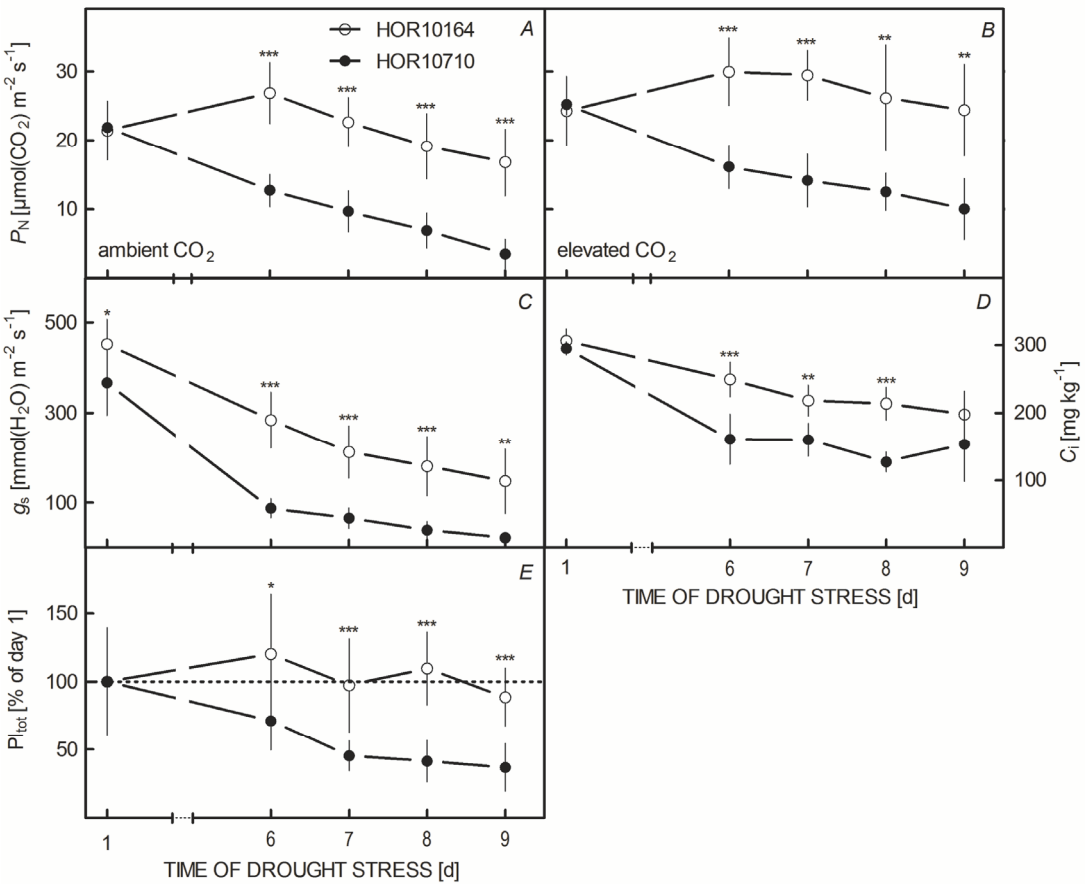

Fig. 4. Changes of net photosynthetic rate (P_N) at ambient (400 mg kg^{-1}, *A*) and elevated (1,400 mg kg^{-1}, *B*) CO_2 concentrations, stomatal conductance (g_s) (*C*), and intercellular CO_2 concentration (C_i) (*D*) at 400 mg(CO_2) kg^{-1} and performance index (PI_{tot}) (*E*) of HOR10164 and HOR10710 during drought treatment (mean ± SD; *A–D*: n = 6, *E*: n = 10–14). * – p<0.05, ** – p<0.01, *** – p<0.001.

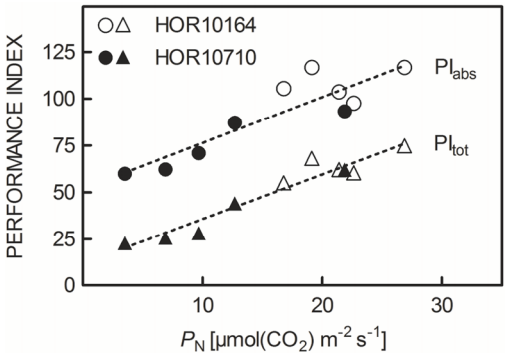

Fig. 5. Correlation of the performance indices PI_{abs} and PI_{tot} with net photosynthetic rate (P_N) at 400 mg(CO_2) kg^{-1} of HOR10164 and HOR10710. Daily mean values from Fig. 4 are plotted in this diagram [R^2 = 0.93 (PI_{tot}), 0.80 (PI_{abs})].

Protein content: The relative amounts of Rubisco large unit (LSU), RA, and psbO did not change significantly in samples of HOR10164 (Table 2). In contrast, a decline of Rubisco (18.5%) and RA (35%) content was detected in HOR10710, psbO was slightly reduced (10.1%) under DS. Both genotypes showed a minor increase of cpHSP70. As Dhn1 was undetectable under control conditions, the relative amount was calculated by defining the value of HOR10710 under DS as 100% (Fig. 3*C*). Here, HOR10710 showed a Dhn1 concentration 6.5-fold higher than HOR10164.

Pigment content: DS had only a minor effect on the overall Chl content of both genotypes (Table 2). Under control and DS, HOR10164 showed higher Chl contents on fresh mass base than that of HOR10710. The Chl *a*/*b* ratios of HOR10164 and HOR10710 – approximately 3:1 – were completely unaffected by DS. In contrast, the concentration of Car slightly decreased in HOR10164 and increased significantly in HOR10710.

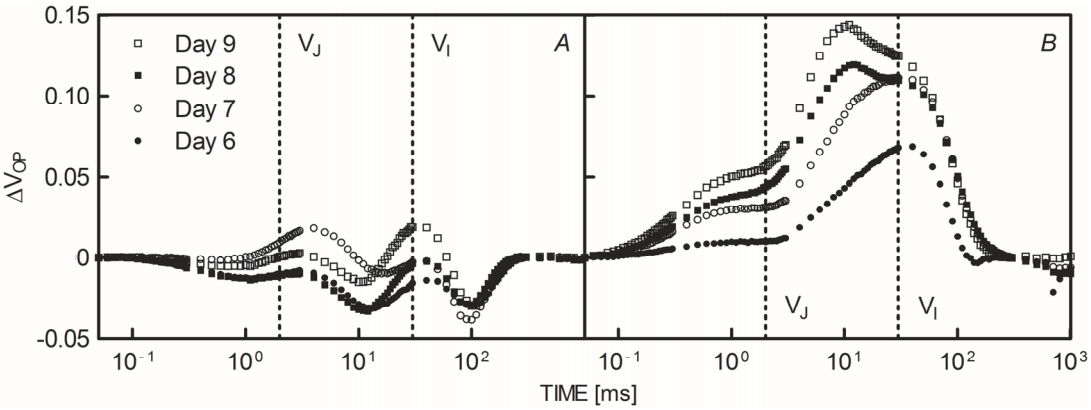

Fig. 6. Changes of the relative variable fluorescence (ΔV_{OP}) of HOR10164 (*A*) and HOR10710 (*B*) during drought treatment (mean ± SD; n = 10–14).

Enzyme activities: Under DS, Rubisco activities of both genotypes decreased (Table 2), but HOR10164 (–16.8%) showed only about half of the decline of HOR10710 (–34.8%). In contrast, the activities of another key enzyme of the Calvin cycle, sFBPase, remained unchanged comparing control and stressed plants. Significant changes were observed in the total activities of NADP-MDH. It almost doubled under DS in case of HOR10164, whereas HOR10710 showed a decline to 70.2% as compared with well watered conditions.

Discussion

Screening *via* JIP-test analysis: As a systematic tool for the analysis of Chl *a* fluorescence fast induction curves, the JIP-test has been used for the detection of inhibitions of the photosynthetic electron transport induced by low water availability (*e.g.* van Heerden *et al.* 2007, Živčák *et al.* 2008). One possible use of the test is the application in comparative screening studies aiming for the detection of drought-resistant genotypes or cultivars. Herein, the changes of the multifactorial and drought-sensitive parameter PI_{abs} (Jedmowski *et al.* 2013) – or the derived drought factor index (DFI, Oukarroum *et al.* 2007) – have been used for the quantification of DS-mediated limitations. In agreement with the findings of the named studies, the relative PI_{abs} of the genotypes used in this study was reduced under DS conditions, with a genotype-depending degree of impairment (Fig. 1*A*). Analysis of the extended PI (PI_{tot}) led to an almost identical ranking of the genotypes, with HOR10164 showing the lowest and HOR10710 showing the highest reduction (Fig. 1*B*). However, PI_{tot} turned out to be much more sensitive than PI_{abs}. Oukarroum *et al.* (2009) observed a similar behavior of the PIs probing varieties of cultivated barley after a two-week drought treatment.

Comparative analysis of HOR10164 and HOR10710: To incorporate the results of the JIP-test into a more comprehensive overview, additional measurements were conducted using the genotypes showing the highest/lowest reaction, as determined by the screening procedure (*i.e.* HOR10164 and HOR10710). For a correct assessment of the measured effect of DS on the genotypes in relation to each other, it is essential to quantify the actual DS intensity the plants were suffering (Lawlor and Tezara 2009). In this study, quantification was achieved by determination of the plant water status under control and DS conditions (Fig. 3*A*).

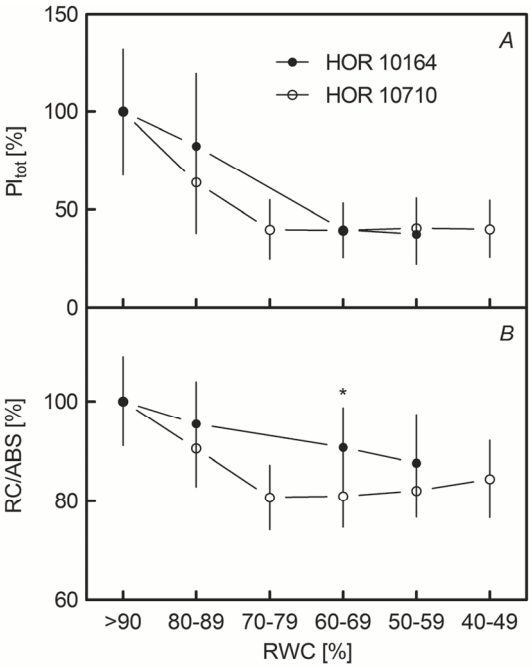

Fig. 7. Relative values of the JIP-test parameters, performance index (PI_{tot}) and fraction of active PSII reaction centers (RC/ABS) of HOR10164 and HOR10710 as a function of leaf water status (mean ± SD; n = 5–26). RWC – relative water content. * – p<0.05, ** – p<0.01, *** – p<0.001.

Table 2. Effect of drought stress on pigment contents, specific protein contents, and enzyme activities of HOR10164 and HOR10710 (mean ± SD; n = 3–6). Chl – chlorophyll; LSU – large subunit; RA – Rubisco activase; psbO – manganese stabilising protein of the oxygen evolving complex of PSII; cpHSP70 – plastidic heat shock protein 70; sFBPase – stromal fructose-1,6-bisphosphatase.

		HOR10164		HOR10710	
		Day 1	Day 9	Day 1	Day 9
Pigment content	Total Chl	2.11 ± 0.46	2.07 ± 0.74	1.44 ± 0.16	1.66 ± 0.34
[μg mg^{-1}(FM)]	Total carotenoids	0.38 ± 0.09	0.31 ± 0.07	0.22 ± 0.02	0.31 ± 0.06**
	Chl *a*/*b*	2.98 ± 0.23	2.90 ± 0.06	3.02 ± 0.07	2.98 ± 0.13
Protein content	Rubisco LSU	100.0 ± 13.9	104.7 ± 12.7	100.0 ± 1.2	81.5 ± 5.1**
[on protein basis, as	RA	100.0 ± 5.3	110.1 ± 3.5	100.0 ± 5.1	65.0 ± 13.4*
% day 1 (dehydrin 1:	psbO	100.0 ± 18.0	95.8 ± 7.4	100.0 ± 20.7	89.9 ± 15.6
HOR10164 as	cpHSP70	100.0 ± 13.4	107.3 ± 10.2	100.0 ± 6.2	111.0 ± 6.7
% of HOR10710)]	dehydrin 1	undetectable	14.1 ± 14.8	undetectable	100.0 ± 4.9
Enzyme activity	Rubisco	100.0 ± 23.8	83.2 ± 48.9	100.0 ± 10.0	65.2 ± 29.6*
[fully activated,	sFBPase	100.0 ± 20.8	104.5 ± 16.6	100.0 ± 15.3	89.1 ± 13.2
% of day 1]	NADP-MDH	100.0 ± 17.5	194.6 ± 42.6***	100.0 ± 16.1	70.2 ± 18.4*

Starting from a similar level of RWC (>90%), HOR10164 and HOR10710 showed considerable differences in leaf desiccation at the end of drought treatment. Hence, a direct comparison of the genotypes in terms of interpretation as drought tolerance (or susceptibility) should be avoided under the conditions of equal drought duration. The higher RWC of HOR10164 was caused by a smaller daily loss of soil water, as displayed by the integral below the curves shown in Fig. 2. Most likely, this effect was due to the observed differences in the genotype growth habit combined with a smaller total LA per plant at day 1 of the experiment. In addition, residual transpiration with closed stomata may vary under DS more than twofold between different *H. spontaneum* genotypes (Suprunova *et al.* 2004). The smaller LA can be interpreted as an adaption leading to drought avoidance, and concomitantly to the higher performance indices of HOR10164 after a given time of DS. The relative contents of Dhn1, as determined for the genotypes under DS (Table 2), are in conformity with the observed differences in the plant water status at the end of the drought period. Suprunova *et al.* (2004) showed that early *Dhn1* gene expression occurred in a sensitive accession, while resistant lines (showing high cuticular resistance) revealed a strong *Dhn1* expression only after severe wilting.

The osmotic potential of the leaves (Fig. 3*B*) increased mainly due to leaf desiccation and consequent rise of the concentration of osmotically active particles. The total number of dissolved molecules in the leaves on total leaf water basis [*i.e.* n = (FM – DM) × osmotic potential] only changed insignificantly between control and DS plants of both genotypes (data not shown).

A comparison of drought tolerance of the PI_{tot} was done exemplary by the assessment of Chl fluorescence as a function of leaf water status by allowing also plants of HOR10164 to dry to below 70% RWC (Fig. 7*A*,*B*). Under these severe DS conditions, no difference between the relative values of PI_{tot} of HOR10164 and HOR10710 was detected (Fig. 7*A*). Under mild DS (*i.e.* 80–89% RWC), HOR10164 showed slightly higher values than HOR10710, though the difference was not statistically significant. In conclusion, there is only a tendency to an increased desiccation tolerance of HOR10164 under mild DS, which disappears under increasing leaf water deficits.

P_N of HOR10164 at day 9 of the DS treatment was only slightly reduced under ambient CO_2 conditions, whereas under elevated CO_2 no decline was observed at all (Fig. 4*A*,*B*). These observations point towards a small stomatal inhibition of photosynthesis in the case of HOR10164 – as indicated by the reduced g_s – which the plants can bypass under an artificially increased CO_2 availability. HOR10710 showed a strong reduction of g_s from day 6 on (Fig. 4*C*), accompanied by reduced P_N and C_i (Fig. 4*A*,*D*). CO_2 elevation led to an increased P_N (Fig. 4*B*), however, the values declined gradually below the P_N under well watered conditions, which is a hint for a nonstomatal limitation of photosynthesis that sums up with the limitation caused by the decreased g_s.

One possible nonstomatal limitation might result from functional or structural changes of the photosynthetic electron transport chain, which can be detected by analysis of the OJIP induction curves. The development of the changes of the relative variable fluorescence is displayed in Fig. 6. Here, it is obvious that in the early stages of DS (day 6, Fig. 6*B*) its effect on the induction curve was exclusively in the region around V_I, whereas V_J remained unchanged. On the consecutive days, V_J values also increased, though to a smaller extent than V_I. PI_{tot}, in contrast to PI_{abs}, includes the relative fluorescence intensity at the I-step of the OJIP transient by the supplemental parameter δ_{Ro}. δ_{Ro} is defined as the efficiency of electron transport from the intersystem electron carriers to the PSI acceptor side (Strasser *et al.* 2010), based on the results of Schansker *et al.* (2005) showing that the IP-Phase is related to the reduction of the PSI end acceptors. Ceppi *et al.* (2012) showed a correlation

of decreased PSI electron transport and the loss of psaD subunits using pot-grown *H. vulgare* plants suffering from DS. Oukarroum *et al.* (2009) suggested PSI to be a source of oxygen radicals under DS, decreasing PSI content and consequently lowering the PSI/PSII ratio. In our study, δ_{Ro} decreased already under mild DS, in HOR10710 stronger than in HOR10164, confirming the finding of Oukarroum *et al.* (2009) in cultivated barley (direct data not shown, but δ_{Ro} was deduced from the intensity of V_I, cf. Fig. 6*B*). However, the results of the pigment analysis showed only a marginal decrease of the Chl *a/b* ratio in HOR10710 (Table 2). The Chl *a*/*b* ratio can be used as an indicator for a changed PSI/PSII ratio or decrease of antenna size (Haldrup *et al.* 1999). PSII structure seemed to be unaffected by DS in both lines, since no change of primary photochemistry (ϕ_{Po}) was observed (data not shown) and the oxygen-evolving complex was not damaged, as indicated by the only insignificant decrease of the psbO protein content. Additionally, analysis of the O-J phase of the OJIP transient did not reveal a prominent K-step (data not shown).

The reduced content and total activity (Table 2) of Rubisco observed for genotype HOR10710, accompanied by a reduced abundance of RA, indicate an impairment of CO_2 fixation during DS. However, DS had no effect on the total activity of sFBPase, another major regulatory enzyme of the Calvin cycle (Kreim and Giersch 2007), which decreased, *e.g.* in bean under DS conditions (Dias and Brüggemann 2010). The strong linear correlation between P_N and the PIs (Figs. 4*A*,*E*, 5), confirming and extending the results of van Heerden *et al.* (2007), suggests an empirical (not a causal) link between the functionality of the dark-adapted photosynthetic electron transport chain and the Calvin cycle activity under DS, allowing the application of the JIP-test as a proxy for the more tedious and expensive measurement of gas-exchange rates in screening experiments.

In light, the NADPH production has to be synchronized with the consumption by Calvin cycle to avoid an overexcitation of the electron transport chain leading to structural damage of the PSs by accumulation of reactive oxygen species (ROS). As the CO_2 fixation under DS is reduced, the consumption of NADPH is impaired. This means that plants need strategies to keep the redox balance in chloroplasts. One possibility for regeneration of NADP is the reduction of oxaloacetate (OAA) by the light-activated NADP-MDH. The occurring malate is exported to the cytosol *via* the malate-OAA shuttle. This so-called "malate valve" results in a shift of (excessive) "redox power" from chloroplast stroma to the cytosol (Scheibe 2004). In HOR10164, a strong increase of NADP-MDH was detected, which was missing in HOR10710, suggesting an importance of the malate valve already under moderate DS.

The JIP-test parameter, RC/ABS – indicating the proportion of active (Q_A-reducing) PSII RCs – decreased already at higher RWC in HOR10710 than in HOR10164 (Fig. 7*B*). Non-Q_A-reducing RCs, so-called heat sinks (Strasser *et al*. 2004), are thought to dissipate exclusively the absorbed light energy, exhibiting a protective function for PSII by reducing the probability of ROS production at the PSII acceptor site.

The plastid Hsp70 seems not to be involved in the reaction of wild barley chloroplasts towards moderate or severe DS, despite the documented protective function of the nuclear-localized NtHSP70-1 in transgenic tobacco overexpressing this chaperone (Cho and Hong 2006, Cho and Choi 2009).

In conclusion, it was shown that the JIP-test is useful for the monitoring of genotype specific limitations of the photosynthetic electron transport due to DS, and genotypes exhibiting better or worse JIP-test parameters also revealed higher/lower P_N. The limitations of JIP-test parameters were mainly PSI-related, especially during moderate DS. We suggest that a major cause for the differential reaction of HOR10164 and HOR10710 was the difference in the leaf water status, leading to a specific profile of photosynthetic limitation. Avoidance of oxidative stress by increasing malate valve activity is an additional protective feature of HOR10164. Photosynthesis in the moderately stressed genotype HOR10164 was mainly inhibited by stomata closure, and its PI_{abs} and PI_{tot} were only slightly affected, whereas the severely stressed HOR10710, with much stronger affected PI_{tot}, additionally showed nonstomatal limitations associated with reduced Rubisco content and activity and reduced RC/ABS values.

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