



## Different forms of osmotic stress evoke qualitatively different responses in rice



Mohamed Hazman<sup>a,b,\*</sup>, Bettina Hause<sup>c</sup>, Elisabeth Eiche<sup>d</sup>, Michael Riemann<sup>a</sup>, Peter Nick<sup>a</sup>

<sup>a</sup> Botanical Institute, Molecular Cell Biology, Karlsruhe Institute of Technology, Kaiserstr. 2, 76131 Karlsruhe, Germany

<sup>b</sup> Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC), 9, Gamma st, Giza, 12619, Egypt

<sup>c</sup> Cell and Metabolic Biology, Leibniz Institute of Plant Biochemistry (IPB), Weinberg 3, 06120 Halle (Saale), Germany

<sup>d</sup> Institute of Applied Geosciences, Karlsruhe Institute of Technology (KIT), Adenauerring 20b, 76131 Karlsruhe, Germany

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### ABSTRACT

Drought, salinity and alkalinity are distinct forms of osmotic stress with serious impacts on rice productivity. We investigated, for a salt-sensitive rice cultivar, the response to osmotically equivalent doses of these stresses. Drought, experimentally mimicked by mannitol (single factor: osmotic stress), salinity (two factors: osmotic stress and ion toxicity), and alkalinity (three factors: osmotic stress, ion toxicity, and depletion of nutrients and protons) produced different profiles of adaptive and damage responses, both locally (in the root) as well as systemically (in the shoot). The combination of several stress factors was not necessarily additive, and we even observed cases of mitigation, when two (salinity), or three stressors (alkalinity) were compared to the single stressor (drought). The response to combinations of individual stress factors is therefore not a mere addition of the partial stress responses, but rather represents a new quality of response. We interpret this finding in a model, where the output to signaling molecules is not determined by their abundance *per se*, but qualitatively depends on their adequate integration into an adaptive signaling network. This output generates a systemic signal that will determine the quality of the shoot response to local concentrations of ions.

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### 1. Introduction

In the agricultural reality, drought and salinity stress are often accompanied by alkalinity stress as third stressor. Saline soils frequently not only contain high amounts of Na<sup>+</sup>, but also HCO<sub>3</sub><sup>-</sup>. The impact of hydrogen carbonate accentuates the damage imposed by sodium ions, and has therefore attracted a lot of attention. The economic consequences of alkalinity are substantial because relatively large areas of agricultural land suffer from this problem as a consequence of progressive desertification (Liu and Guo, 2011). Globally, about 434 million ha of arable soil are estimated to be impaired by this combination of salinity and alkalinity, which exceeds the 397 million ha of land affected by salinity alone (Wang et al., 2011).

Due to the fact that soil salinization and soil alkalinization frequently co-occur in nature (Patil et al., 2012), plants under alkalinity stress actually face a combination of three stress factors: physiological drought, ion toxicity, and a depletion of protons in the

rhizosphere (Ibraheem et al., 2011). Although present in saline soils, water is not physiologically available to plants because the sodium ions in the soil decrease the water potential  $\psi$ , such that water is withdrawn from the roots. The resulting dehydration will damage membranes, impair enzyme activities, and disrupt cellular metabolism (Horie et al., 2012). Dehydration stress is accentuated by the toxicity of sodium ions that enter the cell through non-selective cations channels (reviewed in Tester and Davenport, 2003). Perturbation of K<sup>+</sup> homeostasis represents a crucial target of sodium toxicity (Szczerba et al., 2008), but also many enzymes are negatively affected because the electrostatic interactions necessary to maintain a functional protein structure are affected leading to metabolic imbalance (Hasegawa et al., 2000). For instance, strong downregulation of the tricarboxylic acid cycle had been found in maize leaves under salt stress (Richter et al., 2015). Alkalinity will result in the precipitation of metal ions (especially calcium and magnesium), and the depletion of phosphate (Lajtha and Schlesinger, 1988). Furthermore, direct impact on root cell structure and physiology has been reported (Yang et al., 2008a).

Adaptation to stress requires specificity in the cellular and physiological responses depending on the context of the stress factor. For instance, in order to reinstall turgidity in the context of

\* Corresponding author at: Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC), 9, Gamma st, Giza 12619, Egypt.  
E-mail address: [yusof.3@hotmail.com](mailto:yusof.3@hotmail.com) (M. Hazman).

water scarcity, production of compatible solutes is a good strategy, whereas in the context of salinity, translocation of sodium into the vacuole is more efficient, since it removes ionic stress and at the same time maintains turgor as driving force for growth (reviewed in Martinoia et al., 2012). The specificity of responses at first sight would imply a specificity of signaling transduced by specific molecular components. However, the number of molecular players that convey stress signals in plants is rather limited, and many of these molecular players are shared between different stresses (reviewed for salinity stress in Ismail et al., 2014). Alternatively, specificity might be generated by particular spatiotemporal patterns (so called signatures) of these overlapping signals and their signaling pathways. For instance, the role of calcium influx in cold, drought and salinity stress differs due to different temporal signatures for this signal resulting in the activation of different downstream events (reviewed in Xiong et al., 2002). Also shifts in the relative timing of primary signals will result in qualitatively different responses of cortical microtubules (reviewed in Nick, 2013). The three aspects of alkalinity stress (water scarcity, ionic stress, proton depletion) must differ in their primary inputs, in order to be discriminated. Water scarcity will affect membrane tension, which can be perceived by mechanosensitive ion channels (reviewed in Kung, 2005). In plant cells, unknown mechanosensitive channels drive an influx of calcium. The recently discovered calcium channel OSCA1 (Reduced Hyperosmolality-induced  $[Ca^{2+}]$  Increase 1) which is gated by hyperosmotic stress (Yuan et al., 2014) might be a molecular candidate for such channels. Calcium-dependent kinases can translate this calcium influx into the activation of membrane-located NADPH oxidases that produce superoxide in the apoplast such that the primary calcium influx is followed (with some delay) by a transient oxidative burst (Dubielia et al., 2013). In case of salinity stress, the osmotically induced  $Ca^{2+}$  influx is accompanied by influx of sodium ions via nonselective cation channels, which activates the adaptive SOS (for salt-overly sensitive) module that not only will extrude sodium from the cytoplasm, but also links cytosolic sodium with the activity of calcium binding signaling proteins (reviewed in Ismail et al., 2014). Alkalinity will affect the apoplastic pH, which is normally kept slightly acidic (at  $\sim 5.5$ ) by proton ATPases localised in the plasma membrane to sustain cell expansion growth (Haruta et al., 2010). Also, alkaline pH will impair the activity of osmotically induced calcium influx, because calcium enters the cell by co-transport with protons. In addition, the superoxide anions generated by the NADPH oxidases will accumulate to higher levels, because they are not dissipated due to the absence of protons as electron acceptors. Thus, the oxidative burst induced by ionic stress is expected to be more persistent under alkalinity.

Based on these differences in the primary inputs among drought, salinity, and alkalinity stress, a stress-signature mechanism should lead to outcomes that are not mere additions of the responses to the individual stress components. Instead, combinations of stressors should result in non-additive interactions and even qualitative differences of the stress response. To address this, we designed a comparative approach where the individual components of alkalinity stress were tested along with their combination. As an experimental system, we used the economically relevant Egyptian rice cultivar Sakha 102, and monitored cellular and organismic responses, while titrating the stress dosage such that each input was equivalent with respect to osmotic challenge.

## 2. Materials and methods

### 2.1. Plant materials, growth and stress conditions

In this study, *Oryza sativa* L. ssp. *japonica* cv. Sakha 102 was used, kindly provided by the Agricultural Research Center (ARC), Giza,

Egypt. The caryopses were dehusked and surface sterilized according to Hazman et al. (2015). The seeds were sown on sterilized 0.5% phytoagar medium (Duchefa, Netherlands) containing 1/10X strength MS medium basal salt (Sigma Aldrich). After one week under continuous light of  $120 \mu\text{mol}/\text{m}^2\text{s}$  at  $25^\circ\text{C}$  the healthy well grown seedlings were transferred to custom made floating racks and moved to a glass container containing 1/20 X MS medium as nutrient solution for extra 5 days. Subsequently, the seedlings were transferred into a glass container containing 2 liters of 1/20X new fresh MS medium solution as control or the same solution containing mannitol ( $\approx 205 \text{ mM}$ ), NaCl ( $\approx 102 \text{ mM}$ ), or  $\text{NaHCO}_3$  ( $\approx 102 \text{ mM}$ ), respectively. Thus, the stress treatment was administered in a step-up manner, and not by gradual increments of osmotic pressure. The treatments were chosen such that the osmotic challenge in all three conditions was  $-0.5 \text{ MPa}$ . However, the treatments with NaCl and  $\text{NaHCO}_3$  complemented this osmotic challenge by additional stressors (ionic stress in case of NaCl; ionic stress in combination with alkalinity stress in case of  $\text{NaHCO}_3$ ). Osmotic potential (OP) level of mannitol, NaCl and  $\text{NaHCO}_3$  were created based on the equation of Van't Hoff (Ben-Gal et al., 2009). The shoots of control and stressed plants were harvested after 24 and 72 h, frozen in liquid nitrogen, and then stored at  $-80^\circ\text{C}$  to be used for subsequent analysis.

### 2.2. Analysis of root elongation

Root elongation was evaluated as the mean of the seminal root length of seedlings raised in darkness ( $25^\circ\text{C}$ , 7 days). The seeds were surface sterilized as described above, and sown on 0.5% phytoagar medium with different osmotic pressure of, NaCl and  $\text{NaHCO}_3$  (0,  $-0.2$ ,  $-0.4$ ,  $-0.6$  and  $-0.8 \text{ MPa}$ ), for drought, salinity and alkalinity, respectively. The levels of osmotic pressure were calculated as described above. The seedlings were scanned and the root length was measured using Image J (<http://imagej.nih.gov/ij/>). The length of the seminal root was measured for  $n = 70$  seedlings from at least 3 independent biological replications.

### 2.3. Carbon isotope discrimination

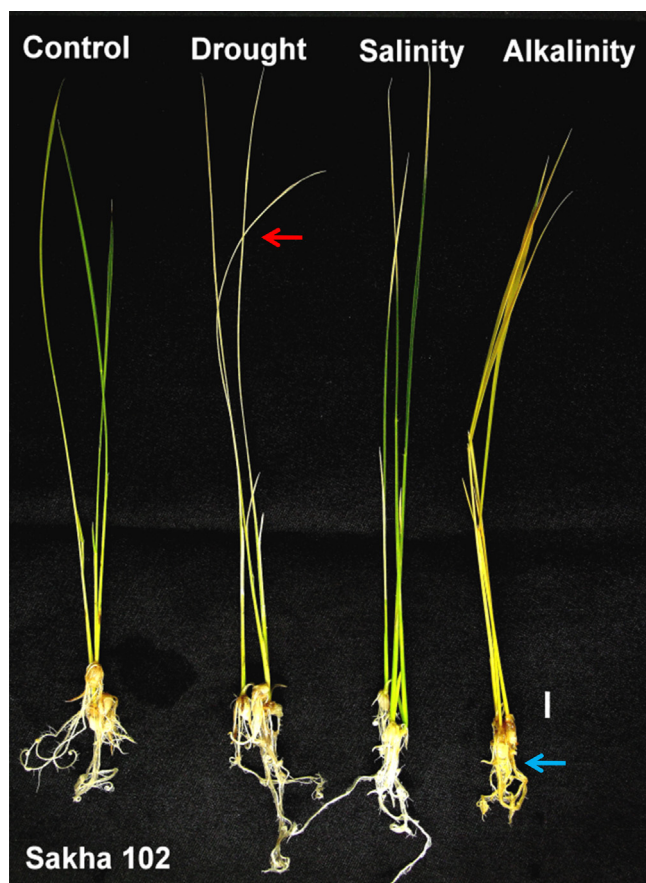
Plant materials (shoots) of five independent repeats were dried in an oven at  $80^\circ\text{C}$  for 3 days. Subsequently they were ground to fine powder. For the analysis,  $100 \mu\text{g}$  of the powder were weighed into a tin cartridge and compressed to free from air. Carbon isotope discrimination was calculated according to Cernusak et al. (2013).

### 2.4. Determination of lipid peroxidation

Lipid peroxidation of shoots (representing 3 independent biological replica) was estimated by the level of MDA (Malondialdehyde) using the thiobarbituric acid (TBA) method as described by Heath and Packer (1968). The value of the non-specific absorption at  $600 \text{ nm}$  was subtracted. The amount of MDA-TBA complex (red pigment) was calculated from the extinction coefficient  $155 \text{ mM}^{-1} \text{ cm}^{-1}$ .

### 2.5. Measurement of ion content

Leaves and roots of control and treated plants were harvested, then washed gently several times with deionized water, and subsequently incubated at  $80^\circ\text{C}$  for 3 days. The dry tissues were homogenized using a mortar and pestle and collected in digestion tubes (Gerhardt, UK), supplemented with 5 ml of concentrated nitric acid ( $\text{HNO}_3$ ) and then incubated for at least 24 h at room temperature while vortexing at 6 and 24 h. The measurements were done at the Institute of Applied Geosciences, Karlsruhe Institute

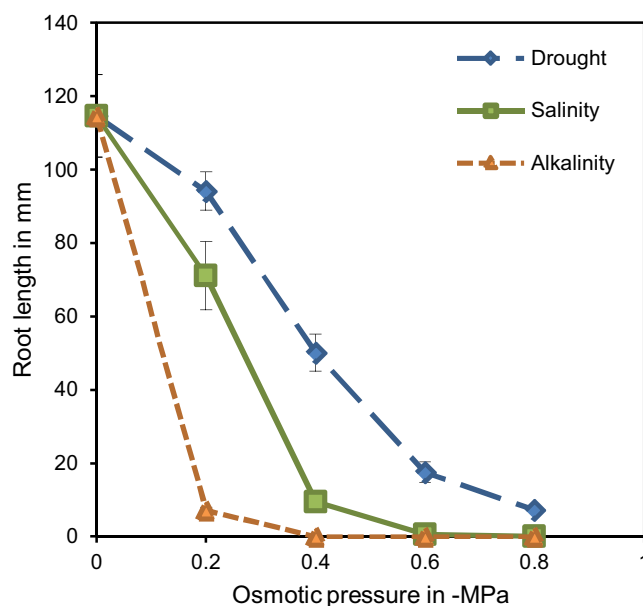


**Fig. 1.** Phenotyping of Sakha 102 plants under drought, salinity and alkalinity stresses comparing to control conditions. 12 days old rice seedlings were subjected for 72 h to the following stresses: Mannitol (to mimic drought), salinity (NaCl), or alkalinity (NaHCO<sub>3</sub>). The solvent was 1/20 MS medium in all treatments, including the control. The osmotic pressure of all stress solutions was adjusted to  $-0.5$  MPa throughout corresponding to 205 mM of mannitol, 102 mM of NaCl and 102 mM of NaHCO<sub>3</sub>, respectively. The red arrow indicates discolored leaves of mannitol-treated plants (the condition leading to maximal damage of the shoots), the blue arrow indicates the stunted roots of alkalinity-treated plants (most condition leading to maximal damage of the roots). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of Technology. The methodologies are described in Hazman et al. (2015).

#### 2.6. Protein extraction and antioxidant enzyme activity measurement

For estimating the activity of catalase (CAT), peroxidase (POD) and glutathione reductase (GR), roots and leaves of control and treated seedlings were homogenized in 1 ml of ice cold extraction buffer according to Venisse et al. (2001). The mixture was centrifuged at 18,000g for 30 min at 0°C; the filtrate was used in total protein estimation according to Bradford (1976). For CAT (EC1.11.1.6), the activity was estimated spectrophotometrically by following the disappearance of hydrogen peroxide at 240 nm (extinction coefficient  $39.4 \text{ mM}^{-1} \text{ cm}^{-1}$ ). CAT activities were calculated according to Abei (1984). One unit of CAT activity was defined as the amount of enzyme required to oxidize  $1 \mu\text{mol}$  of H<sub>2</sub>O<sub>2</sub> per minute (Wedgert and Cullen, 2009). POD (EC 1.11.1.7) activity was measured by following the increasing  $A_{470}$  due to the formation of tetraguaiacol (extinction coefficient of  $26.6 \text{ mM}^{-1} \text{ cm}^{-1}$ ) as described by Chance and Maehly (1955). GR (EC 1.6.4.2) activity was determined by following the oxidation of NADPH at 340 nm



**Fig. 2.** Dose-response curves for root length for mannitol (drought), NaCl (salinity) and NaHCO<sub>3</sub> (alkalinity). Seedlings of the cultivar Sakha 102, pre-cultivated for 7 d in the dark, were grown on solid medium establishing osmotic pressures between 0 to  $-0.8$  MPa by addition of either mannitol (to mimic drought stress), NaCl (salinity stress), or NaHCO<sub>3</sub> (alkalinity stress), respectively. The length of the seminal roots was measured for  $n = 70$ – $100$  seedlings from at least three independent biological replications.

(extinction coefficient of  $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$ ) according to Halliwell and Foyer (1978).

#### 2.7. Total RNA extraction and quantitative real-time PCR

Total RNA was isolated from the shoots of control, drought, salinity and alkalinity stressed plants (24 and 72 h of 3 independent biological repeats) using the InnuPrep plant RNA kit (Analytika Jena RNA kit) according to the manufacturer's instructions. The cDNA synthesis was performed with Dynamo cDNA synthesis kit (Finnzymes, Finland) using total RNA as a template. Real time (qPCR) was performed on the Opticon 2 system (Biorad, USA) as follows:  $95^\circ\text{C}$  for 3 min, and 40 cycles ( $95^\circ\text{C}$  for 15 s, annealing at  $66^\circ\text{C}$  for 30 s and extension at  $72^\circ\text{C}$  for 30 s). Data were exported from the Opticon cyler and imported into the Opticon Monitor (Biorad, USA). The oligonucleotide primer sequences for the genes of interest are listed in Table 1 (see Supplemental Table S1 in the online version at DOI: [10.1016/j.jplph.2016.05.027](https://doi.org/10.1016/j.jplph.2016.05.027)). To compare the transcript levels between different samples, the  $2^{-\Delta\Delta\text{Ct}}$  method was used, the difference in the cycle threshold ( $C_t$ ) values between the endogenous control genes,  $\beta$ -actin and target gene was calculated (Livak and Schmittgen, 2001).

#### 2.8. Estimation of hormones content

Shoots of both control and stressed plants were harvested after 24 h, weighed and frozen in liquid nitrogen for hormonal analysis. Jasmonic acid (JA), JA-isoleucine (JA-Ile), 12-oxophytodienoic acid (OPDA) and abscisic acid (ABA) were quantified simultaneously using a standardised UPLC-MS/MS based method according to Balcke et al. (2012) using  $^2\text{H}_5$ -OPDA,  $^2\text{H}_6$ -JA,  $^2\text{H}_2$ -JA-Ile, and  $^2\text{H}_6$ -ABA as internal standards. The measured samples were collected from 3 independent biological repeats.



## 2.9. Statistical analysis

Testing of statistical significance of the mean values obtained for the different applied abiotic stress forms was performed by Tukey's Honestly Significant Difference (HSD) test, with a significance level of  $P \leq 0.05$ . All analyzed data were collected from experiments conducted in at least three independent biological replications.

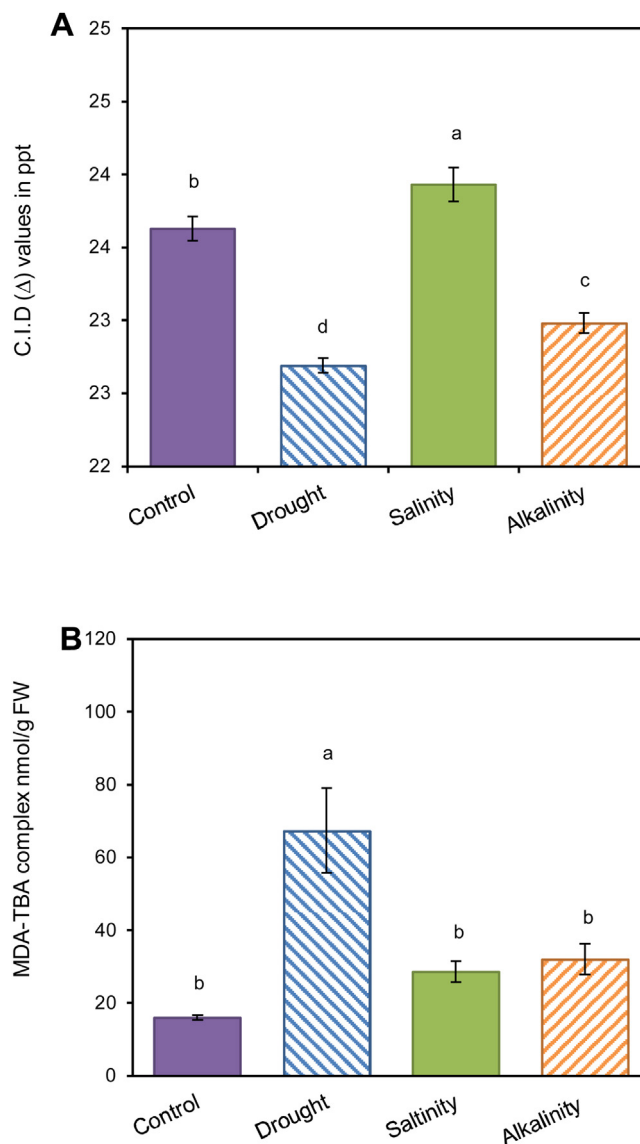
## 3. Results

### 3.1. Morphological responses of rice seedlings

We applied three different types of osmotic stress to rice seedlings (age 12 days). A treatment with mannitol was used to mimic the osmotic stress imposed by drought. Because real drought stress is difficult to standardize under experimental conditions, we used mannitol-induced water scarcity as an experimental approximation of drought, and for simplicity we will use in the following the term "drought" to describe this stress treatment, which actually imposes osmotic stress as single factor and was compared to salinity and alkalinity stress. From the experimental point of view, the osmotic challenge imposed by this "physiological drought" is not distinct from an osmotic challenge caused by water loss in consequence of interrupted water supply. To reach comparable stringencies of the stress inputs, the medium was adjusted to equal values of osmotic pressure. Since the objective of the experiment was to dissect temporal sequences, all stress treatments were given in a step-up mode (i.e. not by a ramp, where stress stringency was gradually increased). This allows to assign a clear starting point ( $t=0$ ), and also avoids modulations of the response by gradual adaptation. Drought stress (experimentally achieved by mannitol) caused significant wilting and discoloration of leaves (Fig. 1), and a dark red compound, probably anthocyanin, was observed in root tips in seedlings of Sakha 102 from about one week after the onset of the treatment (see Supplemental Fig. S1 in the online version at DOI: [10.1016/j.jplph.2016.05.027](https://doi.org/10.1016/j.jplph.2016.05.027)). In response to osmotically equivalent salinity stress, wilting and discoloration were observed as well, but remained confined to the tips of third leaves. Rice seedlings suffering from osmotically equivalent alkalinity stress showed symptoms that differed from those of plants under drought and salinity stress (Fig. 1). Here, the third leaves were not wilted, however completely yellowish (Fig. 1). In addition, the root growth was strongly reduced compared to osmotically equivalent mannitol and salinity stress.

### 3.2. Alkalinity stress inhibits root growth more severely than drought and salinity

To gain insight into the mechanisms responsible for the strong inhibition of root growth by alkalinity stress, dose-response curves over root growth were recorded for osmotically equivalent stress levels imposed by drought (mannitol), salinity, and alkalinity for Sakha 102. The length of seminal roots was measured after 7 days in complete darkness at different osmotic pressures established by the respective stress factor. All three types of treatments led to a reduction of root length (Fig. 2). However, alkaline stress caused the strongest inhibition of root elongation, while osmotic stress alone produced the mildest effect. For alkaline stress, primary roots even failed to germinate at  $-0.4$  MPa, while at the same osmotic pressure seminal roots still developed in the presence of NaCl (9.4 mm), and even just were inhibited to 50% of the control value in case of drought stress (50.1 mm). Generally, the shape of the dose-response curves was similar, but the curve for salinity stress was shifted to lower values of osmotic potential by a factor of around 1.5, and the curve for alkalinity stress even by a factor of

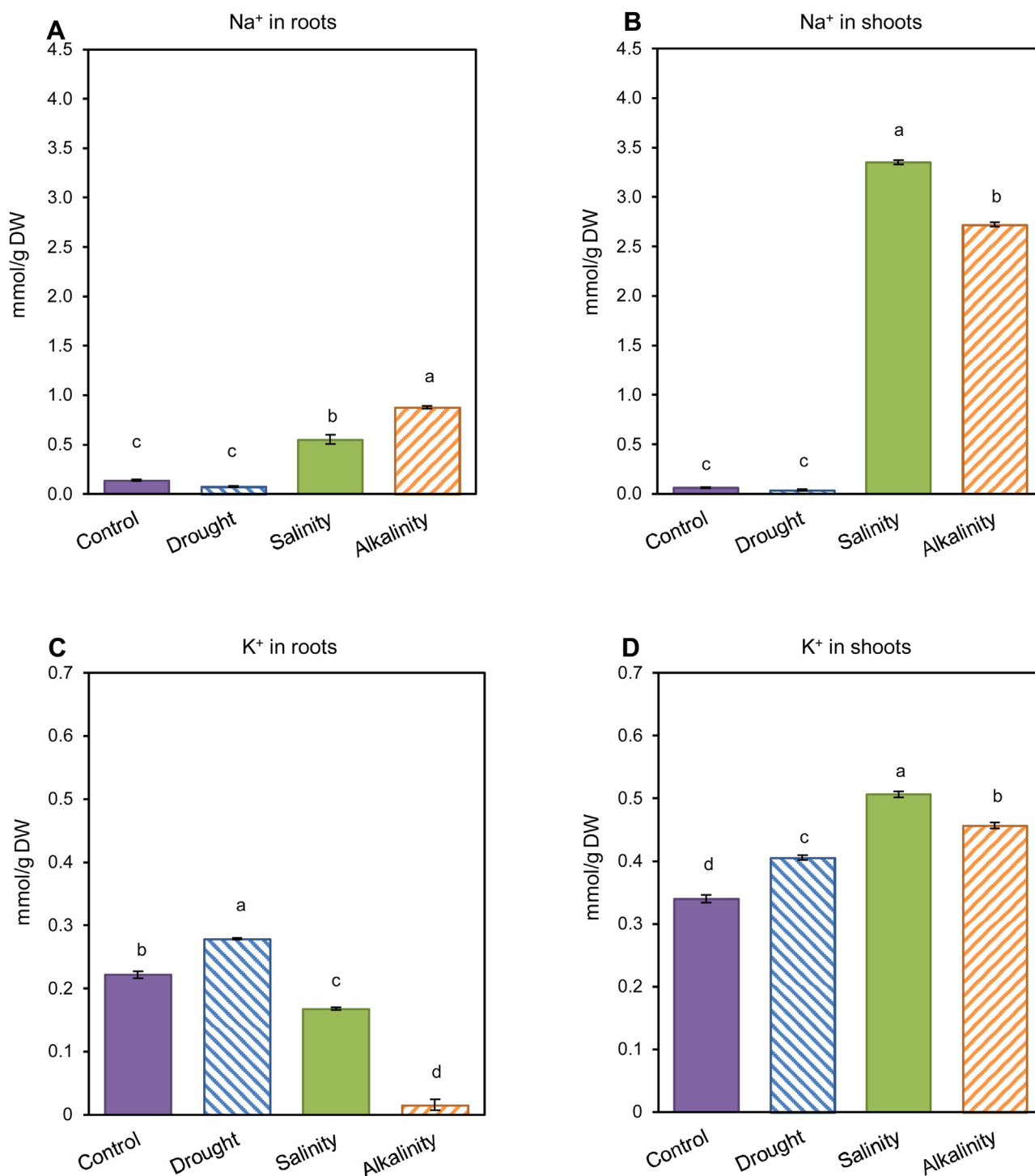


**Fig. 3.** Carbon Isotope Discrimination (C.I.D or  $\Delta$ ) and lipid peroxidation levels in leaves of Sakha 102 under drought, salinity and alkalinity. Plants pre-cultivated in the light for 12 days, were subjected to the respective stress treatment for further 3 days, then shoots were harvested and analyzed for Carbon Isotope Discrimination (C.I.D or  $\Delta$ ) as readout for stomatal aperture (A), and for lipid peroxidation in nmol per g fresh weight (FW) as readout for oxidative damage (B). The osmotic pressure of all stress solutions was adjusted to  $-0.5$  MPa (For details refer to Fig. 1). Values represent the mean of at least three independent experiments  $\pm$ SE. Significant differences amongst different treatments are indicated by different letters, according to Tukey's Honest Significant Difference (HSD) test ( $P < 0.05$ ).

around 4 compared to the curve measured for mannitol-induced drought stress.

### 3.3. Drought stress inhibits stomatal gas exchange and induces lipid peroxidation level in leaves of Sakha 102

To assess how the stress conditions administered to the roots will impair the shoot, the amplitude of carbon isotope discrimination (C.I.D or  $\Delta$ ) as a physiological marker, integrating over several gas exchange parameters, including stomatal closure and internal  $\text{CO}_2$  concentration in the leaves ( $C_i$ ), was determined (Fig. 3). Whereas mannitol reduced  $\Delta$  most severely, closely followed by alkalinity stress, salinity did not lower  $\Delta$ , but even produced a small, but significant increase. Additionally, the level of MDA as



**Fig. 4.** Contents of sodium and potassium ions in both roots and shoots in rice seedlings subjected to mannitol drought, salinity and alkalinity. Plants were pre-cultivated for 12 days in the light and then subjected for additional 3 days to osmotically equivalent ( $P=0.5$  MPa) mannitol stress (to simulate drought stress), salinity, or alkalinity, respectively. For details refer to Fig. 1. The content of sodium, in mmol per g dry weight (DW), in both roots (A) and shoots (B), and of potassium in both roots (C) and in shoots (D) were determined. Values represent the mean of at least three independent experiments  $\pm$ SE. Significant differences amongst different treatments are indicated by different letters, according to Tukey's Honest Significant Difference (HSD) test ( $P < 0.05$ ).

a biomarker for lipid peroxidation was determined to estimate the effect of the treatments on photosynthetic redox homeostasis. Here, drought stress caused a massive accumulation of MDA in shoots, while we did not find a statistically significant difference from the control in the cases of salinity and alkalinity (Fig. 3B).

#### 3.4. Alkalinity stress reduces potassium uptake into the root, but enhances potassium translocation into the shoot

As the different types of osmotic stresses inhibited root growth in a different manner (Fig. 2), and the morphological symptoms appearing on leaves appeared to be specific for each type of stress (Fig. 1), we tested how the uptake of  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{2+}$  ions into rice

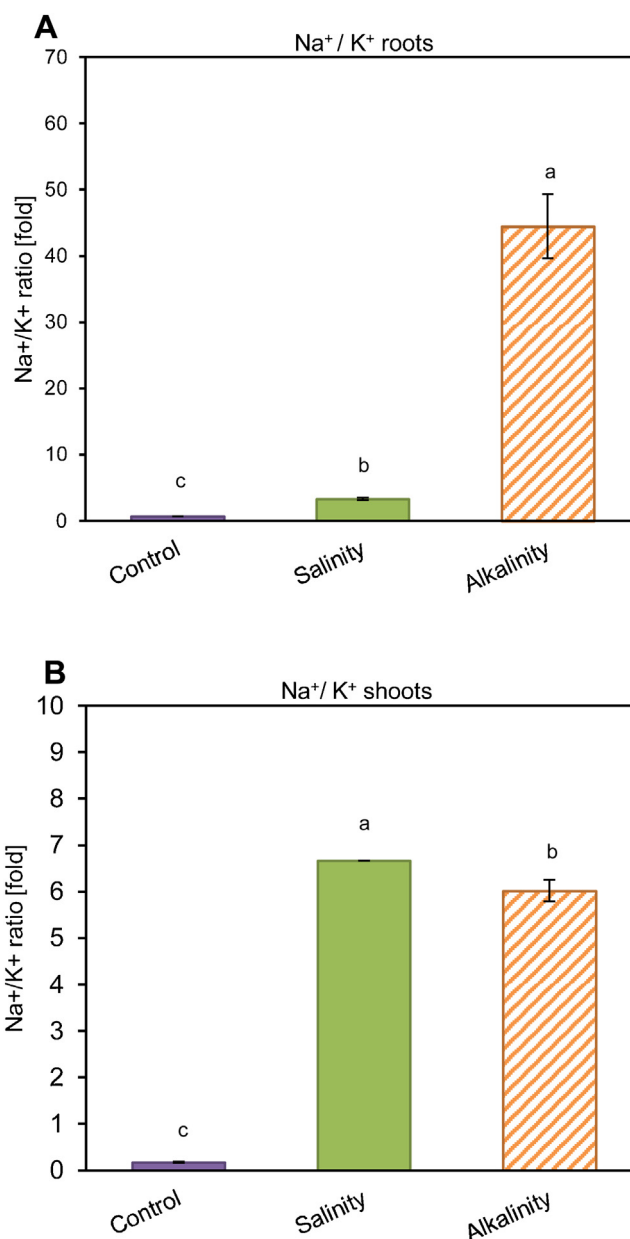
seedlings differed under mannitol-simulated drought, salinity and alkalinity.

#### 3.4.1. Sodium ions are differentially taken up and partitioned under salinity, and alkalinity stress

As shown in Fig. 4A and B, both salinity and alkalinity stress produced a strong accumulation of  $\text{Na}^+$  ions in roots (4–6-fold higher than the control), and this accumulation was further enhanced in the shoots (approximately 50-fold higher than the control). Compared to the sodium content in the root, this accumulation in the shoot was in the range of 3–6-fold higher, whereby salinity and alkalinity differed somewhat: alkalinity stress caused a slightly, but significantly higher increase of  $\text{Na}^+$  ions in roots relative to salt stress (0.88–0.55 mmol/g DW, respectively). However, this higher sodium content was then less efficiently partitioned into the shoots, because under salinity 3.35 mmol/g dry weight were found in the shoots compared to significantly lower amounts (2.72 mmol/g DW) for alkalinity.

#### 3.4.2. Enhanced relocation of potassium and calcium ions into shoots under stress

For the uptake of potassium ions, a qualitatively different result was observed (Fig. 4C and D). Under mannitol stress, rice roots accumulated slightly but significantly more  $\text{K}^+$  ions than under control conditions, and this increase was followed by a similar increase in the shoots. In contrast, salinity and alkalinity stress clearly reduced the uptake of potassium into the root. This was particularly conspicuous for alkalinity stress, where  $\text{K}^+$  content was 15-fold lower than in the control. Interestingly, this reduction of potassium content in the root was fully compensated when potassium contents in the shoot were analyzed. Here, for salinity and alkalinity, significantly higher levels were observed compared to the control and also compared to mannitol stress. In other words, potassium partitioning was strongly channeled to the shoots under sodium stress. Since root growth is severely reduced in response to alkalinity, the reduction of potassium uptake might just reflect the reduction of root growth. However, in the same retarded root, sodium uptake is increased. Thus, the central finding of an active shift of ion preferences remains valid. Similar to potassium, calcium partitioning to the shoot seemed to be enhanced under stress. Whereas potassium partitioning to the shoot was strongly increased for alkalinity over that of salinity, calcium partitioning is similar between salinity and alkalinity (see Supplemental Fig. S2A and B in the online version at DOI: [10.1016/j.jplph.2016.05.027](https://doi.org/10.1016/j.jplph.2016.05.027)). Since alkalinity is often discussed to cause potassium depletion stress, we also calculated the ratio between sodium and potassium ions. Both, salinity and alkalinity caused a clear increase in the ratio of  $\text{Na}^+/\text{K}^+$  in roots and shoots (Fig. 5A and B). For the shoots, both salinity and alkalinity caused a 6-fold molar excess of sodium over potassium which contrasts with the control situation, where sodium was low compared to potassium. In the roots, the two stresses differed qualitatively: whereas under salinity, sodium exceeded potassium by a similar factor as in the shoots, alkalinity produced an excess of sodium over potassium by more than 40-fold. To understand the reason for this difference, we have calculated the ratio of sodium and potassium ion concentrations in shoots relatively to roots (Supplemental Fig. 2C and D). Compared to the control and mannitol stress, the partitioning of sodium ions to the shoots was generally elevated in response to salinity and alkalinity stresses, with higher shoot-to-root ratios for salinity compared to alkalinity stress. On the other hand, the partitioning of potassium ions from roots to shoots was dramatically increased in case of alkalinity over all other conditions (including salinity).

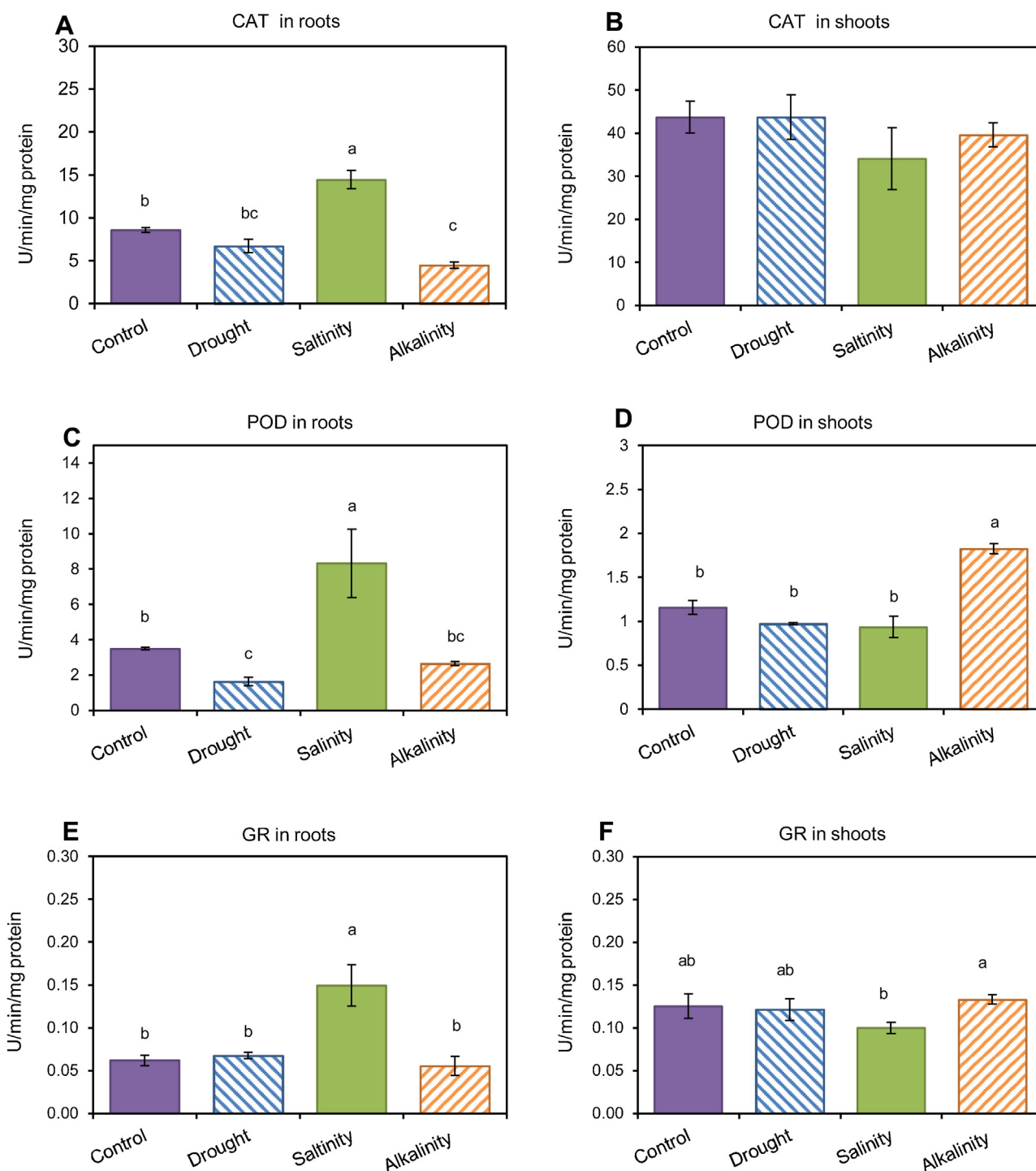


**Fig. 5.** Effect of salinity and alkalinity on the ratio of  $\text{Na}^+/\text{K}^+$  in roots (A) and shoots (B) of stressed rice plants. Values of  $\text{Na}^+/\text{K}^+$  ratios represent the mean of at least three independent experiments  $\pm$  SE. Significant differences amongst different treatments are indicated by different letters, according to Tukey's Honest Significant Difference (HSD) test ( $P < 0.05$ ).

These data show that alkalinity stress strongly reduces the uptake of potassium into the root, but this reduction is compensated by enhanced partitioning of potassium towards the shoot.

#### 3.5. $\text{H}_2\text{O}_2$ detoxifying enzymes are activated in roots under salinity stress

We measured the activities of three different antioxidant enzymes (catalase, peroxidases and glutathione reductase) to address the question of which mechanisms may control ROS levels. As shown in Fig. 6, CAT activity in roots differed depending on the stress treatment: Drought stress did not significantly alter CAT activity compared to control conditions. However, salinity caused a significant stimulation by almost 75%. Alkalinity stress instead caused a reduction in the enzymatic activity of CAT by almost 50%.



**Fig. 6.** The level of activity of antioxidative enzymes CAT (catalase), POD (peroxidases), and GR (glutathione reductase) in both roots and shoots of rice seedling exposed to drought, salinity and alkalinity. Activities of antioxidative enzymes in roots and shoots of Sakha 102 under drought, salinity and alkalinity stress (conditions are as specified in Fig. 1). (A) and (B) catalase (CAT), (C) and (D) peroxidase (POD), (E) and (F) glutathione reductase (GR). Values represent the mean of at least three independent experiments  $\pm$ SE. Significant differences amongst different treatments are indicated by different letters, according to Tukey's Honest Significant Difference (HSD) test ( $P < 0.05$ ).

In spite that CAT activity in shoots was much higher than in roots, its activity in shoots showed no statistically differences between control and treatments.

In the case of peroxidase (POD), we observed that the activity of these enzymes was generally higher in the roots than in the shoots. Salt stress increased POD activity in the root more than twofold, whereas the other two stresses were without effect. For the shoots, only alkalinity triggered specifically an increase of POD activity by around 25%. With respect to glutathione reductase (GR), a specific

and strong increase by a factor of more than 20-fold was seen in roots in response to salinity. In summary, we observed a stimulatory effect of salinity on hydrogen peroxide degrading enzymes, while osmotic and alkalinity stress had less impact.

To gain insight into the physiological effect of these enzymes, we probed steady-state levels of hydrogen peroxide in the root. We observed that the abundance of hydrogen peroxide did not change in response to salinity stress ( $32.9 \mu\text{mol/g}$  fresh weight compared to  $33.0 \mu\text{mol/g}$  fresh weight in unchallenged roots),



and only slightly (27.7  $\mu\text{mol/g}$  fresh weight corresponding to 10%) decreased under alkalinity stress. These findings indicate that the observed changes of enzyme activity are sufficient to compensate potential increases of hydrogen peroxide even under alkalinity stress.

### 3.6. Gene expression in the shoot responds to specific signals from the roots

We measured the expression of six selected genes at two time points (24 h and 72 h) as molecular indicators of the response in the shoot (Fig. 7). The  $\text{H}^+/\text{Na}^+$  vacuolar exchanger tonoplast antiporter protein (*OsNHX1*) can sequester sodium to the vacuole and therefore is an important factor for sodium adaptation. Nitrate reductase (*OsNR*) is not only important for the reduction of the plant nutrient nitrate, but also the major source for the important stress signal nitrogen oxide (NO), oxalate oxidase (*OsOXO4*), can produce large quantities of the important stress signal hydrogen peroxide. The protein Similar to Radical-induced cell death One (*OsSRO-1c*) has been implied with stomatal closure, 1-aminocyclopropane-1-carboxylic acid synthase (*OsACS2*) encodes the rate-limiting step of ethylene synthesis, and the calmodulin-related calcium sensor *OsMCL31* is important to relay primary stress signaling to different transducers (reviewed in Ismail et al., 2014). As shown in Fig. 7, *OsNHX1* expression was elevated in both time points, and in response to all treatments. However, alkalinity stress induced its expression much stronger and more rapidly than drought and salinity. This strong expression had ceased at 72 h, but still was much higher than for drought and salinity, respectively although transcripts had increased for those two conditions in the interval between 24 and 72 h. Whereas *OsNHX1* was most responsive to alkalinity, for *OsNR* it was salinity that produced a strong and rapid response. The other genes *OsOXO4*, *OsACS-2*, *OsSRO-1c*, and *OsMCL31* were all strongly induced by alkalinity stress, but this response was relatively slow and developed mostly during the interval between 24 and 72 h.

### 3.7. Alkaline salinity stress leads to accumulation of jasmonates in shoots

We investigated the impact of drought, salinity and alkalinity stress (24 h at osmotic pressure of  $-0.5$  MPa) on the levels of jasmonic acid (JA), JA-isoleucine (JA-Ile), 12-oxophytodienoic acid (OPDA), and abscisic acid (ABA) in Sakha 102 shoots. Since the morphological and molecular responses in the shoots were specific for the quality of each applied stress (drought, salinity and alkalinity), this led to the question whether these differences would be mirrored in differences of hormonal levels. Whereas the levels of JA, JA-Ile and OPDA (Fig. 8A–C) were significantly elevated in response to alkalinity stress, drought and salinity did not produce significant changes, especially the levels of the bioactive conjugate JA-Ile reached extreme values under alkalinity (Fig. 8A–C). A similar pattern was observed for ABA (Fig. 8D), although the strong increase in response to alkalinity was not consistently observed, such that due to the large variation between experimental repeats this difference was not significant.

## 4. Discussion

### 4.1. Experimental design and comparability

The response of rice to salinity and/or alkalinity stress has been characterized previously (Wang et al., 2011; Yang et al., 2012). Likewise, the differential responses to osmotic and ionic stress have been investigated in several plants including rice (Alam et al., 1999; Mokhberdoran et al., 2009). However, the developmental status,

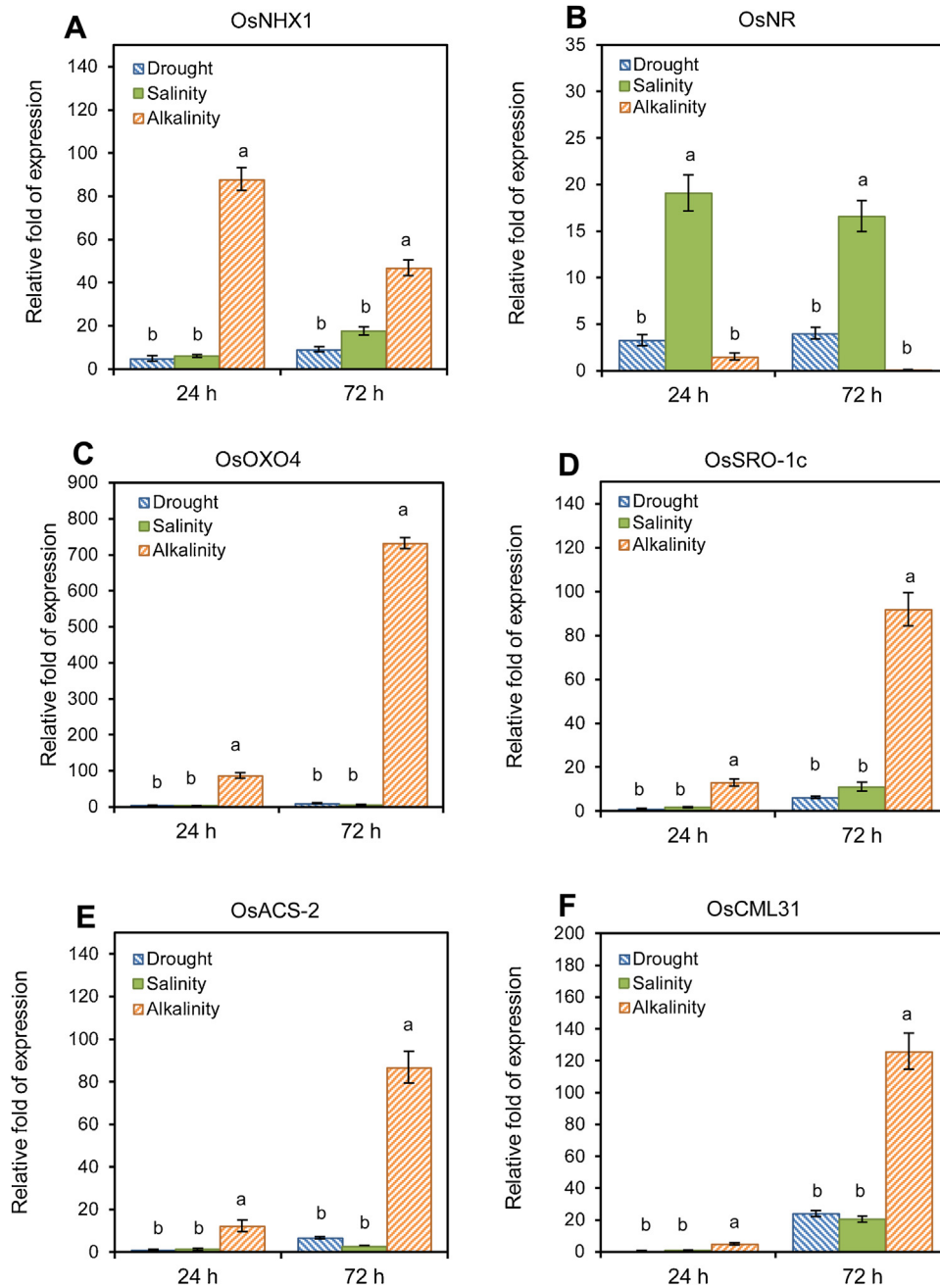
the conditions of the experiments, the genotype of the material, and the stringency of stress differ between different studies, which makes it difficult, or even impossible to derive conclusions upon the underlying signaling. We therefore decided to compare three versions of osmotic stress in the same system, under the same conditions, at the same stringency of osmotic challenge. The aim was to understand, how rice plants respond to combinations of individual stress factors (stressors) as they occur under salinity and alkalinity. In order to create a meaningful comparison, we have designed three different stress treatments that were equivalent with respect to osmotic potential ( $\Psi_p$ ), while differing with respect to additional stressors (ionic stress and/or pH). We therefore adjusted the concentrations of mannitol to 205 mM, that of NaCl and  $\text{NaHCO}_3$  to 102 mM, such that the resulting drought, salinity and alkalinity stresses were equivalent with respect to their osmotic stress component.

### 4.2. Morphological and physiological responses to drought, salinity and alkalinity are not additive, but differ in quality

The primary targets of osmotic stress are the roots, and as expected, we observed a progressive inhibition of root growth with increasing osmotic stress (Fig. 2), whereby alkalinity was more effective than equiosmolar salinity in terms of root length reduction, and where salinity, in turn, was more effective than equiosmolar mannitol-induced drought. The interaction between the individual stress components might be merely additive. The inhibition caused by alkalinity stress would then be summed up from the inhibition caused by mannitol-induced stress, added up by a constant caused by pH and ionic stress. This is not the case: The curve observed for alkalinity stress was similar in shape to that observed for mannitol-induced drought, but instead shifted to around 4-fold lower values of osmotic stress. For salinity, the situation was similar; here it was a factor of around 1.5-fold compared to the curve for mannitol-induced drought. This means that the interaction between the individual stress components is not additive, but multiplicative. Multiplicative interaction indicates that there must be common targets early in signaling, such that the outcome of a combined stress extends beyond the sum of the partial components.

The conclusion of a new quality of response is also supported by a comparison of the morphological responses produced by the three stress treatments. For instance, while drought induced obvious necrotic symptoms such as severe leaf wilting and almost complete discoloration, plants under alkalinity stress, consistent with the published record (Mghase et al., 2011), showed leaves that were still light green, whereas roots were extremely short and stunted. This inhibition of root growth must be seen in the context of the extreme  $\text{Na}^+/\text{K}^+$  ratio (Figs. 4 A and C, and 5 A), which reflects a strongly impaired potassium influx. Depletion of this essential macronutrient under saline-alkaline stress has been proposed as central factor for impaired growth (Very and Sentenac, 2003). In contrast to the damaged roots, leaves were not wilted under alkalinity stress. In contrast, for salt stress, leaf tips bleached and second leaves were stunted as found previously (Hazman et al., 2015). Thus, each stress type evoked a specific and different morphological response. Additionally, we speculated that hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) is a possible candidate. Due to the higher activity of  $\text{H}_2\text{O}_2$  detoxifying antioxidative enzymes (CAT, POD and GR), this signal was expected to be more abundant in roots subjected to salinity stress (Fig. 6A–D). Compared to other ROS species,  $\text{H}_2\text{O}_2$  is thought to be more suited for long distance signaling, because of its relative lower reactivity and higher water solubility (Jammes et al., 2009). However, when we probed for potential modulations, we found the level of hydrogen peroxide under tight control, even for alkalinity



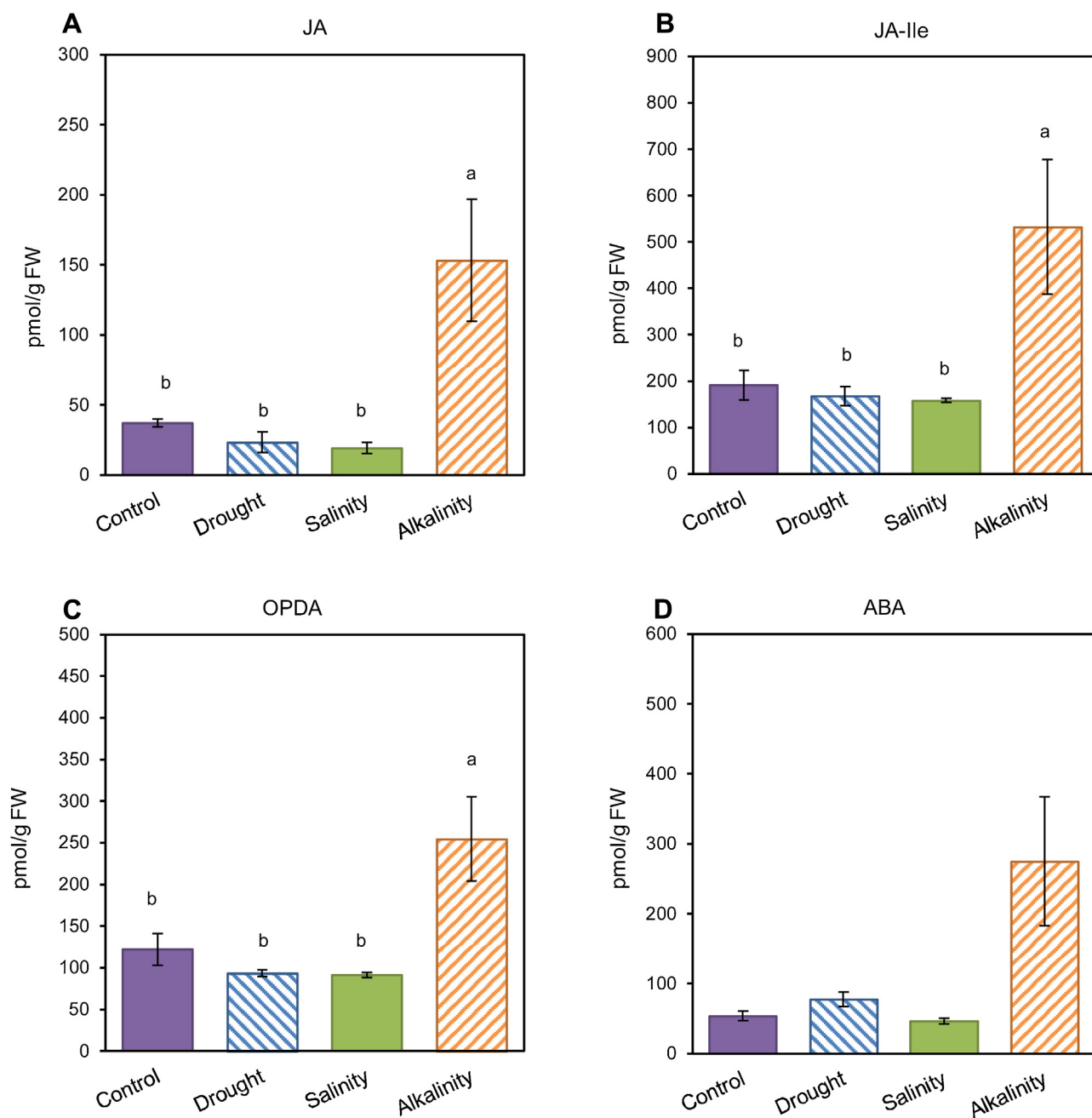


**Fig. 7.** Alterations in transcript accumulations of stress-related genes in the shoots in response to drought, salinity and alkalinity. Plants were pre-cultivated for 12 days in the light and then subjected for additional 24 h or 72 h to osmotically equivalent ( $P=0.5$  MPa) mannitol stress (to simulate drought stress), salinity, or alkalinity, respectively. For details refer to Fig. 1. Steady-state levels of transcripts for A: the vacuolar sodium-proton exchanger (*OsNHX1*), B: the enzyme nitrate reductase (*OsNR*), C the enzyme oxalate oxidase (*OsOXO4*), D: Similar to Radical-induced cell death One (*OsSRO-1c*), E: 1-aminocyclopropane-1-carboxylic acid synthase (*OsACS-2*), F: calmodulin-related calcium sensor proteins (*OsCML31*). Values represent the mean of at least three independent experiments  $\pm$ SE. Significant differences amongst different treatments are indicated by different letters, according to Tukey's Honest Significant Difference (HSD) test ( $P < 0.05$ ).

stress. This indicates that the observed activation of antioxidative enzymes is sufficient to keep control over this signal.

In order to understand the mechanisms causing leaf necrosis in response to drought and salinity, we measured, in parallel, carbon isotope discrimination (Fig. 3A), and lipid peroxidation levels (Fig. 3B) as readout of gas/water exchange parameters and oxidative damage, respectively. Discrimination of RubisCo against the stable isotope  $^{13}\text{C}$  is classically used to integrate stomatal closure over time (Farquhar et al., 1982), and has been used to monitor the leaf response under drought stress (for instance, Brito et al.,

2014). The survival of rice as a  $\text{C}_3$  semi-aquatic crop under applied abiotic stress conditions is mainly related to its ability to sustain stomatal closure as an adaptive response to avoid excessive tissue dehydration and prevent that toxic ions such as  $\text{Na}^+$  and  $\text{Cl}^-$  accumulate in the leaves (Centritto et al., 2009). Drought stress severely reduced isotope discrimination and at the same time elevated lipid peroxidation. This is consistent with a model where the fast and prolonged stomatal closure in response to water scarcity (experimentally induced by mannitol) strongly reduces carbon dioxide levels in the intercellular spaces of the mesophyll, such that the



**Fig. 8.** The level of jasmonates (JA, JA-Ile and OPDA) and ABA in rice shoots under control and 1 day of drought, salinity and alkalinity. Plants were pre-cultivated for 12 days in the light and then subjected for additional 24 h or 72 h to osmotically equivalent ( $P=0.5$  MPa) mannitol stress (to simulate drought stress), salinity, or alkalinity, respectively. A: jasmonic acid (JA), B: JA-isoleucine (JA-Ile), C: 12-oxophytodienoic acid (OPDA), and D: abscisic acid (ABA). Results for the drought, salinity and alkalinity are indicated by gray, dark grey and striped bars, respectively. Values represent the mean of at least three independent experiments  $\pm$ SE. Significant differences amongst different treatments are indicated by different letters, according to Tukey's Honest Significant Difference (HSD) test ( $P < 0.05$ ).

resulting disruption in photosynthetic electron flux will generate overproduction of reactive oxygen species (De Carvallho, 2008). In the case of salinity stress, the same osmotic stress component, accompanied by ionic stress, surprisingly does not result in an aggravated situation, but allows keeping stomata open (which also improves oxidative balance as evident from the lower level of MDA as compared to drought). Again, the response to a combination of stress factors seems to be different in quality and cannot be explained in terms of mere addition of individual stresses.

#### 4.3. Different stress qualities in the root produce different response qualities in the shoot

Roots are the first line of defense against soil-related biotic and abiotic stresses, and root growth therefore reflects the adaptive state of a challenged plant. Moreover, it depends on the adaptive status of the root, whether the leaves will experience the full challenge of the stress situation, or whether this stress will be buffered by the root. For instance, the disruption of potassium homeostasis by excessive sodium has been recognized as one of the severest constraints for plant growth and development (Kronzucker et al.,

2013). Sakha 102 is a salt sensitive Egyptian rice cultivar that accumulates higher  $\text{Na}^+$  amounts in leaves compared to salt tolerant cultivars (Darwish et al., 2009; Mekawy et al., 2015). Interestingly, more sodium was partitioned to the shoot under salinity if compared to alkalinity (Fig. 4B), which correlated with a stronger carbon isotope discrimination (indicative of incomplete stomatal closure). Although roots under alkalinity were strongly depleted in potassium (Fig. 4C), the shoots were found to contain sufficient potassium (Fig. 4D), which might mitigate sodium toxicity and suppress necrosis. However, similarly high potassium content in the shoots of salinity stressed plants did not save leaves from wilting and necrosis. We observed that *OsNHX1* transcripts were induced rapidly and dramatically in response to alkalinity, indicating that these leaves were able to escape necrosis by efficiently sequestering sodium ions in the vacuole (Yamagushi and Blumwald, 2005). In contrast, salinity produced only a comparatively slow and sluggish induction of this transporter, indicative of a poor activation of adaptive responses.

This leads to the conclusion that the qualitatively different responses of the leaves cannot be explained in differences of local ion concentrations in the mesophyll, but must be modulated by signals originating from the root, and that these signals must be different from the sodium or potassium ions translocated in the transpiration stream.

In the search for potential targets for these unknown signals, we probed for the expression of genes known to act in stress signaling. Most of these transcripts were activated late and most strongly under alkalinity, i.e. at the condition that was impairing root growth most severely. It is therefore likely that these transcripts are located in the downstream response to root damage rather than acting in stress signaling or adaptation. Among the tested genes, besides *OsNHX1*, only the gene encoding nitrate reductase (*OsNR*) was activated early (within the first day of stress), and this rapid activation was not only very strong, but it was also specific for salinity stress, since it was not observed for alkalinity stress.

The enzyme nitrate reductase (NR) is a substrate inducible enzyme controlling the degree of nitrogen assimilation in plants (Harper and Paulsen, 1968). Although there is some debate, NR has been widely accepted as major source for the important gaseous and signaling molecule nitric oxide (NO) (reviewed in Meyer et al., 2005; Salgado et al., 2013). Accumulating evidence suggests that NO acts as systemic signal promoting salinity adaptation in several plants, including cereals (Bai et al., 2011), for instance by activating the enzymatic antioxidant system or by promoting stomatal closure (reviewed in Molassiotis et al., 2010). Although NR enzyme activity in response to salt stress is reported to be rapidly enhanced in cucumber roots, it is inhibited in maize leaves (Abd-El Baki et al., 2000; Reda et al., 2001), consistent with our findings in shoots. The proposed specific activation of NR in response to salinity stress (higher *OsNR* expression, Fig. 7B) might be interpreted as event of adaptive signaling due to inorganic nitrate ( $\text{NO}_3^-$ ) partitioned in favour of shoots (unlike ammonium which remains in roots) in response to salinity stress under used N-rich MS medium through xylem transport (Peuke et al., 1996). This is in accordance with the strong decrease in *OsNR* expression in response to alkalinity which is known to trigger nutritional depletion of non-organic ions including  $\text{NO}_3^-$ , therefore lowering NR enzyme activity in rice seedlings (Wang et al., 2011; Singh et al., 2014). Nevertheless, it remains to be elucidated in future work using more extensive examinations, whether in salt tolerant cultivars this event is activated more rapidly or more efficiently compared to Sakha 102, which is salt sensitive.

Under alkalinity, the bioactive jasmonate compound JA-Ile accumulated to extremely high levels (Fig. 8). Interaction of NO and JA signaling has been found repeatedly (for review see Wendehenne et al., 2004) – while JA-biosynthesis genes are activated by NO, JA-

responsive genes are down-regulated. In rice seminal roots, NO was found to activate transcription of *OsAOS1*, a gene encoding the enzyme performing the first committed step in JA biosynthesis (Chen et al., 2015). However, since several JA-biosynthesis genes are activated by JA, the downregulation of JA responsiveness by NO might affect also the JA biosynthesis. The differences in JA-Ile accumulation between salinity and alkalinity stress might therefore be linked with the (inverse) difference in the activation of NR transcripts. When the root is subjected to salinity stress, it not only partitions the sodium to the shoots, but might also transmit a signal that will activate NR, such that the generated NO will counteract the accumulation of JA-Ile that otherwise would result from the presence of sodium ions (reviewed in Ismail et al., 2014). In case of alkalinity, the strongly impaired root fails to generate this signal, such that NR is not activated, NO will not be formed and JA-Ile synthesis will go unrestrained. In case of drought, the root signal might be generated, but since it is acting in the absence of local ionic stress, the resulting response of leaves is different (no induction of NR, no induction of JA-Ile). In future experiments, we will therefore focus on the temporal and spatial dynamics of NO synthesis. In contrast, hydrogen peroxide levels seem to be well balanced even under alkalinity stress and therefore cannot account for the observed differences in physiological adaptation. Furthermore, in order to assign the events described in the current study to either stress damage or stress adaptation, we plan to conduct comparative studies with genotypes that differ with respect to drought, salinity, or alkalinity tolerance.

## 5. Conclusion

We asked the question, whether the combination of several stressors in both salt and alkalinity stress might trigger a stress-signature yielding responses that are qualitatively different from a mere additions of the responses to the individual stress components. Applying three different forms of abiotic stress that are equalized with respect to their osmotic pressure, our results showed that the combination of osmotic stress, ionic stress, and proton-depletion stress leads to a qualitatively different output that cannot be explained by mere addition of single-stressor responses. In other words: drought, salinity, and alkalinity are sensed as different stress qualities.

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