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Drought and salinity adaptive mechanisms in japonica and indica/japonica rice varieties: a bilateral comparative study

Mohamed Hazman[1,2], Longbiao Guo[3], Hanhua Tong[3], Bettina Hause[4], Elisabeth Eiche[5], Michael Riemann[6], Samer Fawzy[7], Peter Nick[8]

[1]*Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Centre (ARC), Giza, Egypt*
[2]*School of Biotechnology and Bioinformatics, Nile University, Giza, Egypt*
[3]*State Key Laboratory for Rice Biology and Breeding, China National Rice Research Institute, 311401 Hangzhou, China*
[4]*Cell and Metabolic Biology, Leibniz Institute of Plant Biochemistry (IPB), Halle, Weinberg 3, 06120 Halle (Saale), Germany*
[5]*Institute of Applied Geosciences, Karlsruhe Institute of Technology (KIT), Karlsruhe. Adenauerring 20b, 76131 Karlsruhe, Germany*
[6]*Department of Crop Sciences, FiBL Switzerland, Ackerstrasse 113, CH-5070 Frick*
[7]*School of Chemistry and Chemical Engineering, Queen's University Belfast, Belfast BT9 5AG, Northern Ireland, UK*
[8]*Molecular Cell Biology, Joseph Kölreuter Institute for Plant Sciences, Karlsruhe Institute of Technology, Fritz-Haber-Weg 4, D-76131 Karlsruhe, Germany*

This work investigated physiological, biochemical, and molecular adaptive responses of two Egyptian rice cultivars representing two different subspecies, Giza 177 (japonica) and Giza 178 (indica/japonica), to osmo-equivalent concentrations of mannitol and NaCl to mimic drought and salinity stress, respectively. Phenotypically, Giza 178 was less sensitive to drought than Giza 177; however, salt stress generated comparable damage symptoms and sodium content in either variety. In Giza 178, more K+ were accumulated in shoots under drought compared to Giza 177 with a 1.48-fold increase, while Ca++ content was significantly higher in shoots under drought and salt treatments (2 and 1.7-fold increase, respectively). Carbon isotope discrimination (CID) implied a prolonged stomatal closure in Giza 177 under drought. Additionally, the oxidative damage marker malondialdehyde (MDA) was more severely accumulated in Giza 177 shoots with 1.67-fold increase. The antioxidative enzymatic activity of Glutathione Reductase (GR) increased in Giza to 178 shoots under drought, indicating better antioxidative power. Interestingly, OsNHX1 gene expression (Na+/H+ antiporter) was significantly induced in Giza 178 shoots under drought stress, indicating better osmotic turgor pressure and less degree of nutrition deficiency. Furthermore, both phytohormone abscisic acid (ABA) and jasmonate iso-leucine (JA-Ile) were significantly elevated in Giza 177 shoots compared to Giza 178 under drought and salt stress. Generally, it is suggested that Giza 177 shoots displayed greater sensitivity to drought, mainly due to extensive oxidative damage caused by a prolonged period of stomatal closure.

Keywords: Rice; Mannitol; NaCl; ROS; Antioxidants enzymes; Gene expression

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CORRESPONDENCE TO
Mohamed Hazman,
Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Centre (ARC), Giza, Egypt
Email: yousof.hazman@outlook.com
Longbiao Guo,
State Key Laboratory for Rice Biology and Breeding, China National Rice Research Institute, 311401 Hangzhou, China
Email: guolongbiao@caas.cn

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INTRODUCTION

Expanding agricultural systems in low-fertile sandy agroecological zones is an urgent solution needed with high priority to address global food security challenges, especially in developing countries with rapidly growing populations and limited agricultural resources under climate change (Shin et al., 2022). Nevertheless, these efforts to expand are seriously challenged with several obstacles, wherein water scarcity and soil salinity come at the top. Indeed, fresh irrigation surface water scarcity, saline groundwater, and salt-affected soil are the most destructive edaphic stress types in arid and semi-arid regions as newly reclaimed desert land in Egypt (Hazman et al., 2023).

Cereals are a fundamental source of carbohydrates and daily energy uptake for humanity, with rice being one of the most important crops globally that provides food for more than half of the world's population (Zhang et al., 2022). Developing rice lines with efficient adaptive response under harsh edaphic conditions including drought and salinity is an essential strategy to fill the gap between growing world population and current food production under climatic changes along with region-specific extreme environmental conditions (Hossen et al., 2022).

Revealing the molecular basis of adaptive mechanisms in morphologically distinctive rice subspecies (japonica, indica, or the cross between them: indica/japonica) is believed to consolidate the breeding and engineering of new resilient rice lines (Civáň et al., 2015). Indica and japonica, the two main rice subspecies, are apparently different in many aspects including phenology, habitats, geographical distribution, and cultivation practices (Wang et al., 2023). Although genome sequences of both indica and japonica rice subspecies were publicly presented several years ago, the adaptive distinctive molecular mechanisms for each subspecies under abiotic stress are still not fully understood (Kong et al., 2021). Indica rice species are more adaptive to tropical and subtropical ecosystems (low latitude of 1200 m), while japonica cultivars are more productive in low temperature agroecosystems (high latitude regions of 1600 m), (Xiong et al., 2010). Although it is widely recognized that indica rice genotypes exhibit greater tolerance to abiotic stresses such as drought and salinity, recent perspectives suggest that differences in how indica and japonica rice respond to these stresses should not be viewed as a consistent pattern (Kong et al., 2021; Hossen et al., 2022). This is probably due to drought and/or salinity tolerance could be driven under the auspice of complicated quantitative trait loci (QTL), i.e., not only controlled by multiple genes, but also one trait might be regulated by multiple mechanisms (Palmgren and Shabala 2024). Therefore, crossing between indica and japonica rice varieties is a successful strategy for selecting tolerant and high yield rice lines, including inbreds and hybrids cultivars (Zhang et al., 2022). Nevertheless, generally indica/japonica hybrids/ inbreds cultivars exhibited poor cooking and eating qualities, in addition, demand extra costs for nitrogen fertilizers high input relative to conventional rice, i.e., japonica or indica varieties (Zhang and Zhang 2022). Nitrogen (N) is a crucial macronutrient for plant growth and development; it is basically involved in synthesizing essential and vital macromolecules as proteins, genetic materials (DNA and RNA), chlorophyll, in addition to great number of important secondary metabolites (Hazman et al., 2024). Drought and salinity stress significantly disrupt cell ability to uptake, assimilate thus transmit nitrogen, the condition which eventually lead to plant death (Ding et al., 2018). Therefore, a well-balanced nitrogen fertilization is essential for plants to cope drought and/or salinity stress by enhancing adaptive resilience through strengthening cell wall, osmotic adjustment, and synthesizing antioxidative enzymes. On the other hand, applying overdoses of nitrogen fertilizers can harm plants root and shoot systems and even make plants more vulnerable to environmental stresses (Sun et al., 2020).

Drought and salinity stress are two distinctive abiotic stress types, individually or combined, can cause destructive catastrophic effects on crop production specifically with rice as a semi-aquatic cereal monocot crop (Lv et al., 2022; Vanani et al., 2024). Both types of stress induce physiological drought due to reduced water potential in the rhizosphere, the limited hydration level is associated with general reduction of nutrients uptake. Salt stress triggers two additional types of direct negative consequences on plant growth and development. The first is specific ions toxicity, mainly sodium, and the second is nutrition deficiency towing to the competition between sodium and other nutritional ions specifically potassium (Mal and Panchal 2024).

Reactive oxygen species (ROS) are natural by-products for regular respiration and photosynthesis rates in plants. These harmful active species could be contained by the antioxidative system, enzymatically and non-enzymatically, for avoiding possible oxidative damage of macromolecules (DNA, RNA, and proteins), in addition to cell plasma membrane (Riemann et al., 2015). As indirect yet extremely serious consequence, drought and/or salinity trigger the overproduction of ROS such as superoxide radicals ($O_2\cdot$), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH.) (Budran et al., 2024; Wang et al., 2024; Mohammed et al., 2025). Excessive ROS production might be due to a disruption in electron transport and energy sinking in photosynthesis upon extended unbalanced CO_2/O_2 exchange that accompany stomatal adaptive extended closure. Moreover, ROS could be enzymatically generated by ROS-producing enzymes, ex: membrane NADPH oxidase (respiratory burst oxidase homolog: RBOH) and cell-wall oxalic acid oxidase (Hazman et al. 2023).

Due to their sessile life nature, plants could develop several adaptive responses to survive undesirable environmental conditions through different types of homeostatic mechanisms including molecular alternations based on fine tuning gene expression regulation and hormonal crosstalk (Hazman et al., 2016; Alzahrani 2024). Molecular players (ex: signaling molecules) in plants are limited, several of these molecules perform a dual role- one as adaptive tolerant response and, if stress effect could not be mitigated, damage response is adopted (Ismail et al., 2014). For example, under either drought or salt stress, calcium ions could be absorbed by cells through the mechanosensitive calcium channel OSCA1 which is over-expressed in response to osmotic stress (Yuan et al., 2014). High cytosolic calcium ions could trigger the production of H_2O_2 via the apoplastic ROS-producing enzyme NADPH oxidase which eventually led to an oxidative burst (Dubiella et al., 2013). On the other hand, under salinity stress, cytosolic calcium could be utilized to activate salt overlay sensitive (SOS) module for excluding Na^+ extracellularly (Ismail et al., 2014).

Rice genotypes or subspecies can perceive and react to drought and salinity stresses in distinct, individualized ways. It is reported that rice plant could differentially respond to equivalent doses of osmotic and salinity stress (Hazman et al., 2016; Hazman et al., 2019). In this study, several phenotypical, physiological and molecular aspects were examined to determine whether distinct rice subspecies (specifically japonica and indica japonica) exhibit unique responses to drought and/or salinity stresses.

The findings from this research could support ongoing efforts in precision breeding and genome engineering to create rice subspecies with enhanced resilience to specific soil-related challenges, such as drought and salinity.

MATERIALS AND METHODS

Plant materials, growth, and stress treatments

Rice seeds of Egyptian rice varieties Giza 177 (subspecies japonica) and Giza 178 (subspecies indica/japonica) were kindly provided by Rice Research and Training Center (RRTC), Field Crop Research Institute (FCRI), Agricultural Research Center (ARC), Giza, Egypt. The dehusked kernels were surface disinfected by incubation in Ethanol (70% v/v) for one min, and in Chlorox® (2.5% NaOCl), with few drops of Tween™ 20, for 30 min with gentle shaking at room temperature, finally rinsed 5 times with sterilized distilled water. The seeds were sown on sterilized 0.7% phytoagar medium (Duchefa, Netherlands) containing 1/20X strength MS medium basal salt (Sigma Aldrich, Germany). After 12 days under continuous light of 120 µmol/m^{2}s at 25 ℃, healthy well-grown seedlings were transferred to custom made floating racks installed in a glass container containing 1/20 X MS medium as nutrient solution for extra 2 days to adapt before applying stress treatments. Subsequently, the seedlings were transferred into a glass reservoir containing 2 liters of 1/20X new fresh MS medium solution as control or the same solution containing mannitol (≈205 mM) and NaCl (≈102 mM). Osmotic pressure (ψ) was adjusted to be -0.5 MPa (Ben-Gal et al., 2009). The stress exposure extended over 4 days, at the end of which stress symptoms clearly emerged on the second and third leaves. Root and/or shoot tissues were collected individually then either dried in oven for ions content analysis or frozen in liquid nitrogen and then kept in -80℃ for subsequent biochemical, physiological, and molecular examinations. To evaluate the hypothesis that the indica/japonica cultivar Giza 178 may require more nitrogen fertilizers than the Japonica variety Giza 177 during the seedling stage, seeds were germinated in double-distilled water for 10 days, after which the seedlings were moved to a custom-modified 96-well plastic rack for imaging. The shoots were examined in terms of exogenous nitrogen-deficiency symptoms such as chlorosis and yellowing in leaves. Plant responses to *real* drought and saline irrigation stress were assessed. Rice seeds were sown in soil mixture (70% commercial sand, 20% topsoil, and 10% compost) and allowed to grow till the age of 3 weeks when subjected to drought by preventing irrigation for 10 days. For salt stress treatment, the soil was fully saturated and irrigated with a 100 mM NaCl solution twice over the course of 10 days. The control plants were grown by sowing the seeds in the same soil mixture with tap water irrigation.

Root length assay

Root elongation was measured as the mean value of seminal roots length of seedlings raised in darkness (25 ℃, 7 days). Seeds were dehusked, surface disinfected, then germinated on phyto-agar medium (0.7%) with different osmotic pressure of mannitol and NaCl (0, −0.3, −0.6, and −1.2 MPa) for mimicking drought and salinity, respectively, according to Hazman et al (2016). After one week, seedlings were gently removed from agar medium and then scanned using regular scanner. Roots length was measured using Image J (https://imagej.net/ij/) for n=40 seedlings from at least 3 independent biological replications).

Sodium, potassium and calcium ions contents

Shoots of both control and stressed plants were harvested, gently washed three times using deionized water, and dried at 80℃ oven for 3 days. The dried tissues were manually grinded using a mortar and pestle and collected in digestion tubes (Gerhardt, UK) containing 5 ml of concentrated nitric acid (HNO3) for 4 h. Contents of sodium, potassium, and calcium ions were measured by flame atomic absorption spectrometry (AAnalyst200, Perkin Elmer) in an air acetylene flame. Blank samples were prepared by adding 5ml of concentrated nitric acid to an empty digestion vessel and processed as described above.

Carbon isotope discrimination (C.I.D)

Shoots of rice plants (control and treated) were incubated in an oven at 80°C for three days. Subsequently, they were ground into fine powder. 100 µg of the powder was weighed into a tin cartridge and compressed to remove air. Carbon isotope discrimination was calculated according to Cernusak et al (2013).

Determination of lipid peroxidation

Thiobarbituric acid (TBA) was used to evaluate the lipid peroxidation of shoots based on the level of MDA (malondialdehyde). The absorbance of the supernatant was recorded at 532 nm and 600nm. The value of the non-specific absorption at 600 nm was subtracted. The amount of MDA–TBA complex (red pigment) was calculated from the extinction coefficient 155 mM^{-1}cm^{-1} (Heath and Packer (1968).

Measurement of leaves relative water content (RWC)

For all treated samples, shoots were collected and used to calculate RWC according to Barrs and Weatherley (1962). Fresh mass (FM) was first calculated by submerging the leaves in distilled water in a 50 mL Falcon® Polypropylene tube. Secondary, the turgid mass (TM) was measured. For doing so, any remaining water was removed from the leaf surface by gently wiping the leaf samples with tissue paper after 12 hours. Thirdly, dry mass (DM) was determined after samples were dried for three days at 80 °C in a drying oven till constant weight. The formula used to calculate RWC was (RWC in %) = [(FM - DM) / (TM - DM)] * 100.

Antioxidant enzymes activities

The activity of the antioxidant enzymes catalase (CAT), peroxidase (POD), glutathione reductase (GR), and superoxide dismutase (SOD) were estimated in both roots and shoots of control and treated seedlings. 1 ml of ice-cold extraction buffer, prepared according to Venisse et al (2001), was used to homogenize plants tissues. The mixture was centrifuged at 18,000g for 30 min under cooling; the filtrate was used in total protein estimation according to Bradford (1976). For CAT (EC1.11.1.6), the activity was spectrophotometrically measured by following the disappearance of hydrogen peroxide at 240 nm (extinction coefficient 39.4 $mM^{-1}cm^{-1}$), the activities were calculated according to Aebi (1984). CAT activity unit was defined as the amount of enzyme required to oxidize 1 mol of H_2O_2 per minute (Weydert and Cullen 2010). POD (EC 1.11.1.7) activity was estimated by following the increasing A_{470} due to the formation of tetraguaiacol (extinction coefficient of 26.6 $mM^{-1}cm^{-1}$) according to Chance and Maehly (1955). The activity of GR (EC 1.6.4.2) was estimated by following NADPH oxidation at 340 nm. The activity of SOD (EC 1.5.1.1) was determined by tracking the photochemical reduction inhibition of nitroblue tetrazolium at 560 nm (Beauchamp and Fridovich, 1971).

Reverse-transcription Quantitative PCR

Total RNA was isolated from the shoots of control and stressed plants 4 days after treatment according to InnuPrep plant RNA kit (Analytika Jena RNA kit). 1000 ng of previously isolated RNA were used for the cDNA synthesis with Dynamo cDNA synthesis kit (Finnzymes, Finland). Real time (qPCR) was performed by the Opticon 2 system (Biorad, USA) based on the following program: 95°C for 3 min, and 40 cycles (95°C for 15 s, 66°C for 30 s and 72 °C for 30s). The oligonucleotide primer sequences for the reference gene and target genes of interest are illustrated in Table S1 according to (Hazman et al., 2015; Hazman et al., 2023). $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) was used for quantifying the relative gene expression based on the difference in the cycle threshold (Ct) values between the endogenous control gene, β-actin, and each target gene.

Hormones content

Jasmonic acid (JA), jasmonoyl-isoleucine (JA-Ile), 12-oxophytodienoic acid (OPDA) and abscisic acid (ABA) contents were quantified in fresh shoots and roots of control and stressed rice plants. The measurements were achieved simultaneously using a standardized UPLC-MS/MS based method according to (Balcke et al., 2012) using [2H5]-OPDA, [2H6]-JA, [2H2]-JA-Ile, and [2H6]-ABA as internal standards.

Statistical analysis

The experiment plots design was complete randomized design (CRD) and SPSS 20 software (IBM Statistics, USA) was used for statistical analysis including mean separations by Tukey's Honestly Significant Difference (HSD) test ($P \le 0.05$). Experimental data collected and analyzed in this work were from at least three independent biological replications.

RESULTS

Phenotyping of Giza 177 and Giza 178 under control and stress conditions

Seeds of Giza 177 and Giza 178 were sowed using custom-made racks floating on tap water under control light conditions for 7 days. Roots of Giza 177 showed distinctive severe coiled phenotype, where the tips of seminal roots are coiled several times around themselves, on the other hand, seminal roots of Giza 178 were totally down-straighten (Figure 1a). Roots of Giza 177 could not touch the bottom of the jar, while roots of Giza 178 were totally straight and touched the bottom of the jar. Due to the reduction of root length under mannitol and NaCl stress, root length was diminished, and root coiling trend was reduced accordingly (data not shown). Interestingly, the root coiling phenotype of Giza 177 was totally absent or partially attenuated in control dark conditions (Figure S1). Additionally, when the seeds were germinated in double distilled water for 10 days, Giza 178 shoots showed nitrogen deficiency symptoms, i.e., leaf light necrosis, yellowing and chlorosis. In contrary, Giza 177 leaves exhibited more greenish and healthier phenotype without any visible necrosis or chlorosis symptomes (Figure 1b).

Indica/japonica Giza 178 was less sensitive to drought than Japonica Giza 177

We have challenged Giza 177 and Giza 178 with osmotically equivalent doses of mannitol (mimicking drought stress) and NaCl (mimicking salt stress). Under osmotic stress, the second leaves and third leaves of Giza 177 were entirely dehydrated, wilted and discolored, while Giza 178 leaves remained discolored only at the tip (Figure 1c,d). In case of salt stress (NaCl), the two genotypes seemed to be comparable with respect to wilting and necrosis, although Giza 178 second leaf looks partially pale while Giza 177 leaves were entirely stunted and discolored. Furthermore, the drought stress which is triggered by preventing irrigation for one week showed that Giza 177 was severely sensitive where third leaves were severely stunted and rolled compared to Giza 178 (Figure 1e, Figure S2). On the other hand, the applied salt stress triggered by irrigating rice seedlings with 100 mM NaCl along 10 days produced typical parallel salt stress symptoms with both cultivars in terms of leaf tips discoloration and general stunting of shoot system (Figure 1f, Figure S2). Generally, after 2-3 weeks on optimum growth conditions, the indica/japonica cultivar Giza 178 was much shorter than Giza 177 in total plant height, nevertheless, the second leaf of Giza 178 was much longer than in Giza 177.

Osmotic stress generated milder effect on root length in Giza 177

A dose-response curves over root growth were recorded for osmotically equivalent stress levels imposed by drought (mannitol) and salinity (NaCl) for Giza 177 and Giza 178. Both applied osmotic and salt stress resulted in a reduction in root length in either examined rice genotype (Figure 1g,h). Giza 177 showed longer root length than Giza 178 under osmotic stress with a reduction ratio of 43.78% and 73.34% in relative control. Under salinity stress, both genotypes showed comparable root length reduction ratios. At -1.2MPa of salinity stress, both genotype seeds failed to germinate while the germination occurred, yet with small root length, in case of equal mannitol-derived osmotic pressure. Generally, the pattern of dose response curve shows that the effect of osmotic stress was milder than salt stress, and this effect was more announced with Giza 177 (japonica) compared to Giza 178 (indica/japonica).

Giza 178 accumulated more K^+ and Ca^{++} than Giza 177 under drought and salinity

As previously illustrated in Figure 1, the morphological symptoms noted in shoots have appeared to be linked to stress quality and rice subspecies. We have estimated the content of sodium, potassium and calcium ions in leaves under mannitol-triggered drought stress and NaCl-triggered salt stress, in relative to control conditions (Figure 2). Both Giza 177 and Giza 178 leaves accumulated comparable amounts of sodium ions under salinity stress with mean value of 78.6-fold increase (Figure 2a), furthermore, Na^+/K^+ ratios were also comparable in either rice genotype (Figure 2c). On the other hand, Giza 178 absorbed more potassium ions than Giza 177 under both control and mannitol stress by 1.27- and 1.48-fold increase, respectively (Figure 2b). Applied salt stress enhanced K^+ accumulation in shoots of Giza 177 and Giza 178, yet with parallel levels (mean value of 1.31-fold increase). Interestingly, Giza 178 seemed to be a highly calcium extractable genotype as leaves accumulated higher significant levels of Ca^{++} under control, osmotic, and salinity with fold increase ratios of nearly 2.4, 2.0, and 1.7, respectively, in comparison to Giza 177 (Figure 2d). It is worth noting that neither mannitol nor NaCl could alter calcium ions uptake in Giza 177 or Giza 178 compared to control conditions.

Drought stress instigated more oxidative damage in leaves of Giza 177

To assess how applied stress conditions can develop oxidative damage, both genotypes' leaves were analyzed in terms of stomatal aperture movement and consequent cell membrane degradation by overproduced ROS. The amplitude of carbon isotope discrimination (C.I.D or Δ), a physiological marker to stomatal closure and internal CO_2 concentration in the leaves (C_i), was determined (Figure 3a). The most severe reduction in Δ was in case of Giza 177 with ratios of 16% and 8% under mannitol and NaCl stresses, respectively, in relative to control plants. Furthermore, the level of MDA was increased in both genotypes leaves in response to drought and salinity, with mean fold increase values of 2.1 and 3.38, respectively, compared to control plants (Figure 3b). Nevertheless, Giza 177 accumulated 1.67 times more MDA than Giza 178 under drought stress, while salinity-induced MDA level was comparable in both genotypes. Relative water content (RWC) in leaves of all tested genotypes (Giza 177 and Giza 178) showed no significant alterations in response to either stress type (Figure S2).

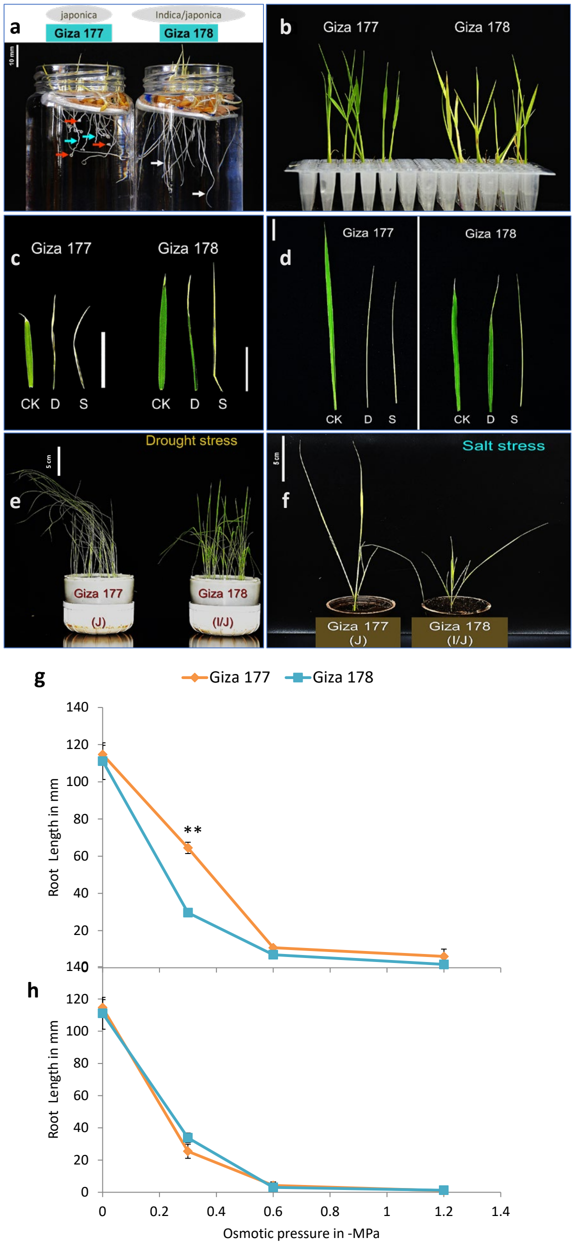

Figure 1. Phenotypic response of Giza 177 and Giza 178 to control (CK), drought (D), and salt stress (S). a root coiling phenotype in Giza 177 compared to straightened roots of Giza 178 (indica/japonica) under control conditions in light, red arrows point at totally coiled roots, blue arrows point at curly roots (both in Giza 177), and white arrows for straightened roots in Giza 178. b phenotyping of nitrogen deficiency symptoms appeared with Giza 178 (leaves yellowing and chlorosis) compared to Giza 177 with more greenish and healthy leaves. Phenotyping of the second leaves (c) and the third leaves (d) of Giza 177 and Giza 178 with mannitol-simulated drought and NaCl-simulated salt stress (scale bar equals to 1 cm). e phenotyping of Giza 177 and Giza 178 in response to drought triggered by holding irrigation for 5 days in sandy soil containers. f phenotyping of Giza 177 and Giza 178 in response to saline irrigation of 100 mM NaCl. Root length assay of Giza 177 and Giza 178 under different levels of osmotic potentials (0, -0.3, -0.6, and -1.2MPa) of mannitol (g) and NaCl (h). **significant difference at P<0.01 in a student's t-test.

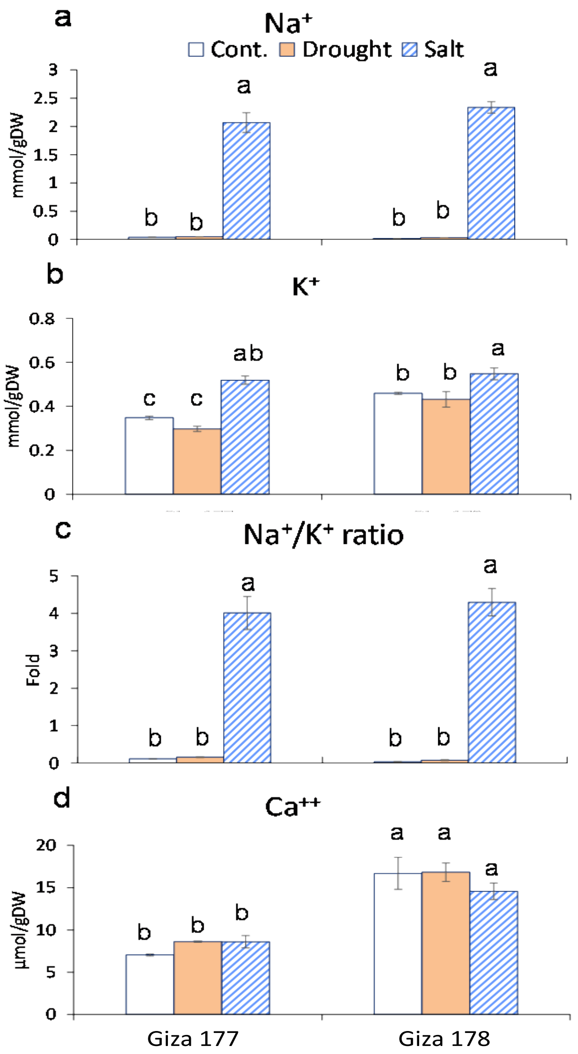

Figure 2. Sodium (a), potassium (b), sodium/potassium ratio (c) and calcium (d) ions contents in shoots in rice seedlings subjected to drought (mannitol) and salinity (NaCl). Values represent the mean of at least three independent experiments ±SE. Significant differences amongst different treatments are indicated by different letters, according to Tukey's Honest Significant Difference (HSD) test ($P < 0.05$).

Antioxidative enzymatic activity varied in response to drought and salinity

The antioxidative enzymatic machinery was estimated to shed more light on mechanisms responsible for detoxifying generated ROS under applied stress types. The activity of four different antioxidant enzymes (CAT, POD, GR and POD) were measured in roots and shoots under control and stress conditions (Figure 4). Figure 4a shows that CAT activity was more induced under drought and less induced with salinity stress, compared to control conditions with mean fold increase values of 11.4 and 3.9, respectively, in both genotypes.

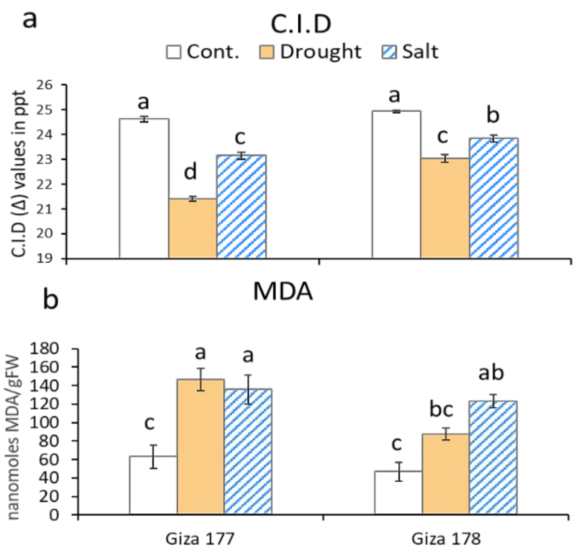

Figure 3. Carbon Isotope Discrimination (C.I.D or Δ) as readout for stomatal aperture (**a**), and lipid peroxidation as readout for oxidative damage (**b**). Values represent the mean of at least three independent experiments ±SE. Significant differences amongst different treatments are indicated by different letters, according to Tukey's Honest Significant Difference (HSD) test ($P < 0.05$).

Furthermore, catalase activity was much higher in Giza 177 roots than Giza 178 in either drought or salinity. In shoots, CAT activity was much higher than roots with a mean value of 17.11-fold increase, nevertheless, there were no significant differences between all treatments (Figure 4b). POD (peroxidases) activity pattern was different in roots for all treatments wherein only salinity stress could raise its activity by 1.86 and 2.43-fold in case of Giza 177 and Giza 178, respectively (Figure 4c). In shoots, unlike catalase, the activity of POD was generally much lower than roots by a mean reduction ratio of ≈72%. Figure 4d illustrates that POD activity in shoots was significantly induced in response to salinity in Giza 177 and Giza 178 with fold increase ratios of 1.37 and 2.3, respectively. There were no significant differences between POD activities under salinity and/or drought stress in Giza 177 compared to Giza 178.

GR (Glutathione Reductase) activity was also estimated in roots; whereas drought slightly enhanced GR activity in Giza 177 (1.17-fold), the same stress reduced GR activity by a reduction ratio of 58.8% in case of Giza 178 (Figure 4e). Under salinity stress, GR activity was reduced in Giza 177 by a reduction ratio of 20.4%, nevertheless, in Giza 178, GR activity showed insignificant differences compared to control conditions. In shoots, GR activity generally increased by 2.23-fold compared to the mean activity in roots. It is noted that both Giza 177 and Giza 178 showed higher GR activity in both drought and salinity in relative to control conditions (Figure 4f). Nevertheless, Giza 178 showed higher GR activity compared to Giza 177 with 1.35 and 1.57-fold increase under drought and salt stress, respectively.

For superoxide dismutase activity (SOD) in roots, drought and salinity could significantly enhance activity compared to control conditions in both genotypes. Figure 4g shows that SOD activities in Giza 178 under drought and salinity stress were significantly higher than those in Giza 177 with 1.28 and 1.46-fold increase. On the other hand, SOD mean activity in shoots were more induced by 5.5-fold compared to roots. Mannitol-induced drought stress triggers the activity of SOD by 1.55 and 1.7-fold increase, in Giza 177 and Giza 178, respectively (Figure 4h). In summary, antioxidative enzyme machinery in Giza 178 was altered under stress conditions, compared with Giza 177, whereby the activities of POD and SOD in roots and GR activity in shoots in response to drought and salinity stress were upregulated.

Gene expression profile implied an adaptive osmoregulation response in Giza 178

We quantified the expression profile of five stress key genes in shoots under control and stress conditions (Figure 5). Under salt stress, as expected, the transcripts of OsNHX1 (encoding tonoplast Na^+/H^+ vacuolar antiporter) were highly accumulated in shoots of both Giza 177 and Giza 178 with comparable levels. Interestingly, the OsNHX1 expression was significantly regulated in Giza 178 under drought stress (4.23-fold) than Giza 177 (Figure 5a). OsNR, encoding nitrate reductase enzyme, was also comparably upregulated in response to salt stress in either tested genotype with a mean value of 15-fold increase compared to drought stress (Figure 5b). It is noted that OsNR expression was strongly upregulated in response to salinity stress with a mean value of 31-fold increase in relative to drought stress. Additionally, we have quantified the expression of OsCML3 which encode calmodulin-related like protein (calcium signaling). Figure 5c shows that the mRNA level of OsCML31 was dramatically reduced in Giza 178 under both of drought and salinity by a mean reduction ratio of 58.3%, compared to Giza 177. It is noted that Giza 177 and Giza 178 showed no significant variation in terms of OsCML31 gene expression under either drought or salinity stress.

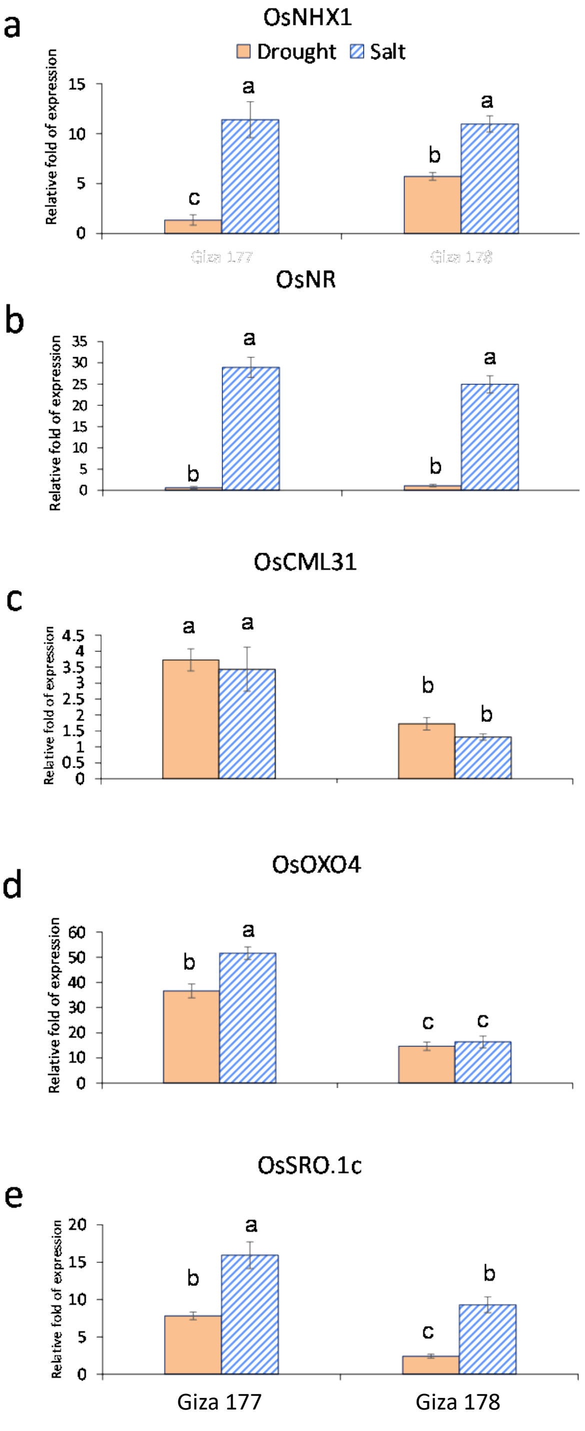

Figure 5. Alterations in transcripts accumulations of stress-related genes in the shoots in response to drought and salinity. a: the vacuolar sodium-proton exchanger (OsNHX1), b: the enzyme nitrate reductase (OsNR), c calmodulin-related calcium sensor proteins (OsCML31), d: the enzyme oxalate oxidase (OsOXO.4), e: Like Radical-induced cell death One (OsSRO-1c). Values represent the mean of at least three independent experiments ±SE. Significant differences amongst different treatments are indicated by different letters, according to Tukey's Honest Significant Difference (HSD) test ($P < 0.05$).

Similarly, the same trend was observed in case of OsOXO4 (encoding H_2O_2 production). Figure 5d illustrates that both mannitol-simulated drought and NaCl-simulated salinity could clearly induce OsOXO4 expression in Giza 177 shoots more than Giza 178 with a mean value of 2.8-fold increase. Like OsCML31, neither Giza 177 nor Giza 178 could show significant difference between drought and salinity stress in terms of OsOXO4 expression. OsSRO.1c produces the protein Similar to Radical-induced cell death One (associated with stomatal closure).

It is found that salinity stress in both Giza 177 and Giza 178 upregulated the expression of OsSRO.1c more strongly than drought with a mean fold increase value of ≈2.5 (Figure 5e). Importantly, Giza 177 shows an elevated higher level of OsSRO.1c in response to all applied stress types, compared to Giza 178. Under drought stress, Giza 177 accumulated OsSRO.1c transcripts 2 times more than Giza 178. Under salinity stress, OsSRO.1c transcripts were 1.7 times higher in Giza 177 leaves in relative to Giza 178.

ABA and JA-Ile were accumulated in Giza 178 shots under both stresses

Levels of JA, JA-Ile, OPDA, and ABA were estimated in either rice genotype (Giza 177 and Giza 178) under stress and control conditions, in both roots and shoot tissues (Figure 6). 12-oxophytodenoic acid (OPDA) level was not significantly altered in Giza 177 roots, while Giza 178 showed a significant reduction ratio of 57.3% and 54% in relative to control conditions, under drought and salinity, respectively (Figure 6a). In shoots, OPDA level trend was much higher compared to roots with a mean value of 14-fold increase (Figure 6b). Unlike in roots, OPDA level was much more responsive whereby its level was much higher in Giza 177 leaves under either stress type with a mean value of 11-fold increase, in relative to control conditions. It is worth noting that OPDA in Giza 178 was strongly regulated in response to drought, but not salt, by an amplitude level of 30 compared to control conditions.

Jasmonic acid (JA) was significantly elevated in Giza 177 roots in response to salt stress (1.74-fold), while drought stress did not lead to a significant alternation (Figure 6c). Although Giza 178 roots did not significantly produce too much of JA in response to drought and salt stress, the level of JA in Giza 177 under salt stress was 3-times higher than in Giza 178. Additionally, Giza 177 roots accumulated more JA under control conditions compared to Giza 178. In shoots, JA level under both stress conditions was generally stronger than its level in roots (16-fold).

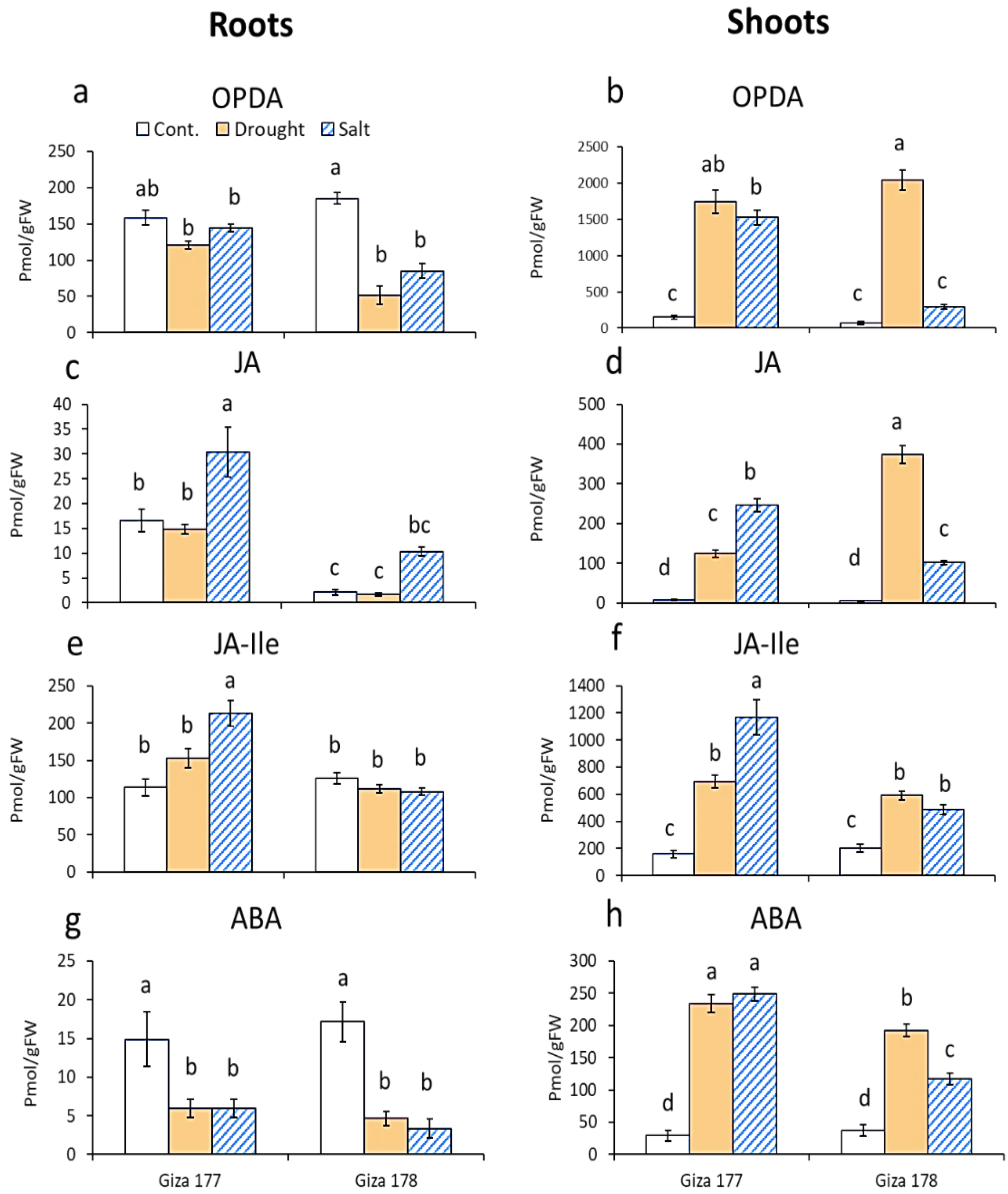

Figure 6. The level of jasmonates (OPDA, JA, and JA-Ile) and ABA under control and 1 day of drought and salinity in both roots and shoots of rice seedling exposed to drought and salinity. a and b 12-oxyphytodenoic acid (OPDA), c and d: Jasmonic acid (JA), e and f JA-isoleucine (JA-Ile), g and h: abscisic acid (ABA). Values represent the mean of at least three independent experiments ±SE. Significant differences amongst different treatments are indicated by different letters, according to Tukey's Honest Significant Difference (HSD) test ($P < 0.05$).

As shown in Figure 6d, while drought stress strongly enhanced the accumulation of JA in Giza 178 shoots (74.6-fold compared to control), salt stress in Giza 177 showed the second most fold increase value of 35, in relative to control conditions. Jasmonate isoleucine (JA-Ile) was induced in response to salt stress in Giza 177 roots, while in Giza 178, both stress types did not alter JA-Ile levels compared to control conditions (Figure 6e). In shoots, JA-Ile level was greatly elevated in relative to roots under stress conditions with a mean increase value of 5-fold.

In accordance with JA-Ile pattern in roots, Giza 177 also accumulated more JA-Ile in shoots under drought more than Giza 178 with 2.4-fold increase (Figure 6f). ABA (abscisic acid) level was also estimated accordingly. Its pattern in roots was consistent to be clearly reduced in response to both stress types with a reduction ratio of 60 and 76.5% for Giza 177 and Giza 178, respectively (Figure 6g). In shoots, the mean value of ABA level under stress conditions was approximately 40 times compared to the same conditions in roots. Interestingly, the responsive trend of ABA was inverted under either stress type in both genotypes compared to roots (Figure 6h). It is observed that ABA was significantly elevated in shoots under both stress types with a mean value of 8.3 and 4-fold increase in Giza 177 and Giza 178, respectively, compared with control conditions.

DISCUSSION

Experimental design and comparability

Plants responses to drought and salt stress are complicated and widely vary as they are associated to several factors including species and genotype, plant age and size, the rate of progression as well as the intensity and duration of the stresses. Thus, it is very hard to conclude the exact differential impact for each stress factor (stressor) within applied stress in relation to another (Hazman et al., 2016). Therefore, in this work, we have designed two different stress treatments that are equivalent with respect to osmotic potential (ψ_p) to be -0.5MPa, while differing only with respect to stress quality.

Giza 178 is less sensitive to drought, but not salinity, compared to Giza 177

Drought and salinity stress revealed distinctive differences between japonica and indica rice varieties where indica variety was more tolerant to drought and salinity (Hossen et al., 2022). Giza 178 (indica/japonica) showed better adaptive behavior under drought stress, compared to the japonica variety Giza 177 (Figure 1c, d and e). Additionally, root length assay showed that japonica cultivar (Giza 177) has longer seminal root than indica/japonica Giza 178 under moderate osmotic potential of -0.3MPa. Japonica rice genotypes, with few exceptions, are generally characterized with deeper root system for a better soil exploration, nevertheless, this advantage might penalties vegetative and yield under low input environment due to extra metabolic cost required to generate and maintain newly produced root tissues (Hazman et al., 2018; Hazman and Kabil, 2022).

Potassium and calcium partitioning in shoots lead to better adaptive response to drought rather than salinity stress in indica/japonica Giza 178

Sodium accumulation in shoots is a direct expected consequence with practicing saline agriculture where high sodium ions concentration poses severe toxicity at the cellular level thus disrupting sensitive metabolic processes as photosynthesis and respiration (Hazman et al., 2022; Rodríguez Coca et al., 2023). Giza 177 and Giza 178 accumulated comparable amounts of sodium ions in shoots, reflecting the similar degree of damage symptoms on second leaves in either genotype (Mekawy et al., 2024). Nutrients imbalance/deficiency is a major detrimental impact of both drought and salt stress (Dos Santos et al., 2022). Salinity comparably induced potassium ions translocation into shoots in both tested rice subspecies, yet Giza 178 accumulated more potassium than Giza 177 under drought (Figure 2b). Similarly, accumulated calcium ions were significantly higher in Giza178 shoots under all treatments. Together, this could imply that Giza 178 is characterized with better nutritional status under drought thus better adjusted turgor pressure and balanced water status (Wu et al., 2018; Gupta et al., 2023; Bhardwaj et al., 2024). It is worth mentioning that Giza 178 showed exogenous nitrogen deficiency symptoms under nutrient-deprived solutions, i.e., double distilled water. Genotypes are varied in terms of their response to nutrients deficiency and their utilization, nitrogen (N) in particular (Hazman et al., 2025). This is in accordance with the findings of Zhang and Zhang (2021) who reported that most of indica/japonica rice varieties might show sensitivity to nitrogen deficiency at different developmental stages where grain filling comes at the top. We could observe that indica and indica/japonica species might be more sensitive to nitrogen-deficiency than japonica rice lines (data not shown). Nitrogen-deficiency traits and their interaction to drought and salinity tolerance need more investigations with strategic crops like rice, maize, and wheat.

Drought attenuated stomatal gas exchange rate, and intensified oxidative damage in Giza 177 leaves

The extensive exposure to drought and/or salinity stress instigates the overproduction of reactive oxygen species (ROS) particularly in leaves. This could happen mainly due to a prolonged stomatal closure for shifting plants to a water-saving strategy, i.e., reduction of transpiration rate. Nevertheless, this is accompanied with limited CO_2 entrance (assimilation) and thus unbalanced energy sinking in the form of higher electron leakage to molecular O_2 under the auspice of Mehler reaction (Cruz de Carvalho 2008; Samanta et al., 2023; Guadarrama et al., 2024). As a read out of gas/water exchange parameters and induced oxidative damage, C.I.D or Δ (carbon isotope discrimination) and MDA (malondialdehyde), respectively, were estimated in leaves. CID or Δ measures the discrimination of Rubisco against ^{13}C, a stable carbon isotope, which is well acknowledged parameters associated with the degree of stomatal closure (Guadarrama-Escobar et al., 2024). C3 semi-aquatic monocot crops, ideally rice, depend on a carful sustaining of stomatal closure as a survival strategy under drought and/or saline stress by avoiding water loss through transpiration and Na^+ translocation into leaves (Centritto et al., 2009). Stomatal closure in Giza 177 was more severely provoked than Giza 178 under mannitol-simulated drought. The greater stomatal closure (less Δ values), the higher accumulated MDA in leaves, supporting the assumption that Giza 177 sensitivity to drought stress is associated to excessive generated ROS caused by a prolonged stomatal closure (Hazman et al., 2016). The antioxidative machinery in roots and shoots of tested rice genotypes were examined.

Although not experimentally estimated, it is speculated that japonica rice Giza 177 roots produced higher amounts of hydrogen peroxide (H_2O_2) under drought stress than Giza 178. H_2O_2 levels were assumed to be significantly higher than levels triggered by salinity, this is due to the higher activity of the CAT as a hydrogen peroxide scavenging first-order-reaction enzyme (Senthilkumar et al., 2021). H_2O_2 is believed to act as stress signal transducer thus triggering stress damage or adaptation response (Anjum et al., 2022). Root seemed to sense stress signal of mannitol-simulated drought more efficiently than salt stress in either examined rice genotype. In leaves, like roots, the pattern of examined antioxidant enzymes activities varies among genotypes and stress type where glutathione reductase (GR) activity was significantly higher in shoots with indica/japonica rice Giza 178 under both stresses compared to japonica rice Giza 177. This might be associated with better remediated stress symptoms in Giza 178 under drought stress (Verma and Singh 2021). Generally, what has occurred under stress (biotic and abiotic) is the over-production of ROS, however, antioxidant enzymes responses widely vary between stress types as drought and salinity based on stress duration and ROS amounts (Mishra et al., 2023).

Gene expression profile implied higher vacuolar potassium content, mild stomatal closure, and lower levels of ROS

Sodium ions compartmentation in vacuoles is a basic salt-stress tolerance mechanism mainly achieved by the tonoplast Na^+/H^+ antiporter for scavenging sodium ions away from cytoplasm (Wu et al., 2018). Interestingly, under drought stress, the sodium-specific antiporter (Na^+/H^+ antiporter) can sequestrate K^+ in the vacuoles, the strategy which aid the cell to retain its turgor pressure under drought (Barragán et al., 2012). Indeed, OsNHX1 gee was upregulated in either examined rice genotype under mannitol-simulated drought stress whereby Giza 178 was significantly higher than Giza 177. This could be associated with the elevated potassium level in Giza 178 leaves under drought thus better adaptive response. The expression of the stomatal closure marker gene OsSRO.1c (encoding Similar to Radical-induced cell death One) was significantly upregulated in Giza 177 more than Giza 178 under either stress type, aligning with less restricted stomatal gas exchange in Giza 178 relative to Giza 177. For being able to regulate stomatal closure, SRO.1c protein in plants has to be empowered by the presence of accumulated amount of hydrogen peroxide in rice leaves guard cell which is strongly believed to be produced by plasma membrane NADPH oxidase, i.e., the healthy type of H_2O_2 needed to trigger antioxidative response without a significant damage in cellular structures as plasma membranes (You et al., 2013; Shi et al., 2020).

On the other hand, apoplastic H_2O_2 was thought to be produced by the cell-wall oxalic acid oxidase (OXO) through decomposing the organic acid oxalate into CO_2 and H_2O_2 (Verma and Kaur, 2021). Since it is a cell wall embedded enzyme, produce H_2O_2 is thought to be utilized by two ways: i) parthenogenetic action on invading bacteria and fungi, and ii) cross-linking mono-lignols in lignin biosynthesis as defensive or developmental response. Nevertheless, uncontrolled over-expression of OXO under abiotic stress might severely cause an oxidative damage thus cell death (Delisle et al., 2001; Bao et al., 2024). Higher level of OsOXO.4 transcripts was significantly enhanced in Giza 177 more severely than Giza 178 under either stress type which reinforces the assumption that Giza 177 accumulates higher level of OXO-produced- H_2O_2 which can lead to a severer drought-sensitive phenotype.

OsNR (nitrate reductase) was exclusively induced in response to salt stress, but not drought, as a result to ions toxicity (Hazman et al., 2016; Hazman et al; 2023). This could be hypothetically associated with higher synthesized nitric oxide (NO) hormones as a distinctive physiological biochemical response to salt stress, which might promote stomatal closure for minimizing accumulated Na^+ ions in leaves, this might explain parallel amounts of sodium ions in both examined rice subspecies leaves under NaCl stress (Figure 2a) (Raddatz et al., 2020). OsCML31 (or OsMRS2) encodes calmoduline-like protein that is deeply involved in calcium signaling and found to be linked to drought and salinity stress tolerance (Xu et al., 2011). Nevertheless, its expression was unexpectedly lower in Giza 178 compared to Giza 177 under both stresses. This could be attributed to the high level of calcium content in Giza 178 which might trigger feedback mechanism for avoiding cytosolic calcium cytotoxicity (Virdi et al., 2015).

Both stress types triggered ABA and JA-Ile accumulation in shoots

ABA was diminished in response to drought and salinity in roots of both rice subspecies in relative to control conditions. This could imply that root-synthesized-ABA might be translocated into shoots, and the translocated ABA could be utilized to regulate stomatal closure accordingly. Indeed, ABA levels of shoots in Giza 177 and Giza 178 were greatly elevated in response to drought and salt stress, with Giza 177 levels higher than Giza 178. This implies that Giza 178 might maintain a moderate stomatal closure under both stress types. ABA is one of the most chemical signals that connect roots to shoot under regular and challenging conditions (Schachtman and Goodger 2008; Kuromori etal., 2018; Tang et al., 2020). However, ABA transport regulation from biosynthesis to action site is not unidirectional but rather shaping complicated intrinsic networks. Yang et al (2024) highlighted that it is not confirmed whether translocated root-ABA contributes in stomatal closure under drought stress due to the fact that there are many action sites for ABA than guard cells. Jasmonic acid metabolites (OPDA, JA and JA-Ile) were also varied among tissues, stress types, and studied genotypes. Although it is well evidenced that jasmonates are involved in enhancing plant resistance to fungal and insect attacks, its role in abiotic stress tolerance is still not clear (Riemann et al., 2015). OPDA (JA precursor possessing distinctive biological activity) and JA-Ile (the biologically active form of jasmonic acid) could contribute to stomatal closure thus enhance drought and salinity stress tolerance in some species (Wang et al., 2021). Indeed, JA-Ile was significantly higher in Giza 177 shoots, thus might be contributed, along with ABA, in a more prolonged stomatal closure. Interestingly, it is observed that JA was significantly higher than in Giza 177 roots under control compared to Giza 178. Giza 177 seminal roots showed sever root-coiling phenotype (when roots were grown in light transparent container). This could be supported by the findings of Jiang et al (2007) where root coiling was mediated by jasmonic acid signaling in rice. Further studies are needed to reveal how root tips coiling might be involved in adaptation to edaphic stress conditions.

CONCLUSION

Cultivated Asian rice subspecies (japonica, indica, and indica/japonica) displayed significant variations in response to drought and salinity, and that each stress quality is perceived differentially. Giza 178 (indica/japonica) appeared to be less sensitive to drought stress rather than Giza 177 (japonica), while salinity impacted either genotype comparably. Giza 177 showed severe reduction in stomatal gas exchange under the hormonal effect of ABA and JA-Ile, which eventually can impose severer oxidative damage causing leaves wilting and discoloration. Additionally, japonica/indica rice line sequestered more potassium in vacuoles under drought stress, thus better retained cellular turgor pressure and nutritional status. We propose using OsNHX1 and OsSRO.1c gene expression for early screening rice lines at seedling stage against osmotic stress in rice breeding programs. We interpret these findings in a model for revealing different rice molecular responses to non-ion osmotic stress in Giza 177 versus Giza 178 (Figure 7). Finally, we emphasize that further studies are needed to decipher more fine details considering examining larger number of samples, rice cultivars representing japonica, indica, and indica/japonica subspecies.

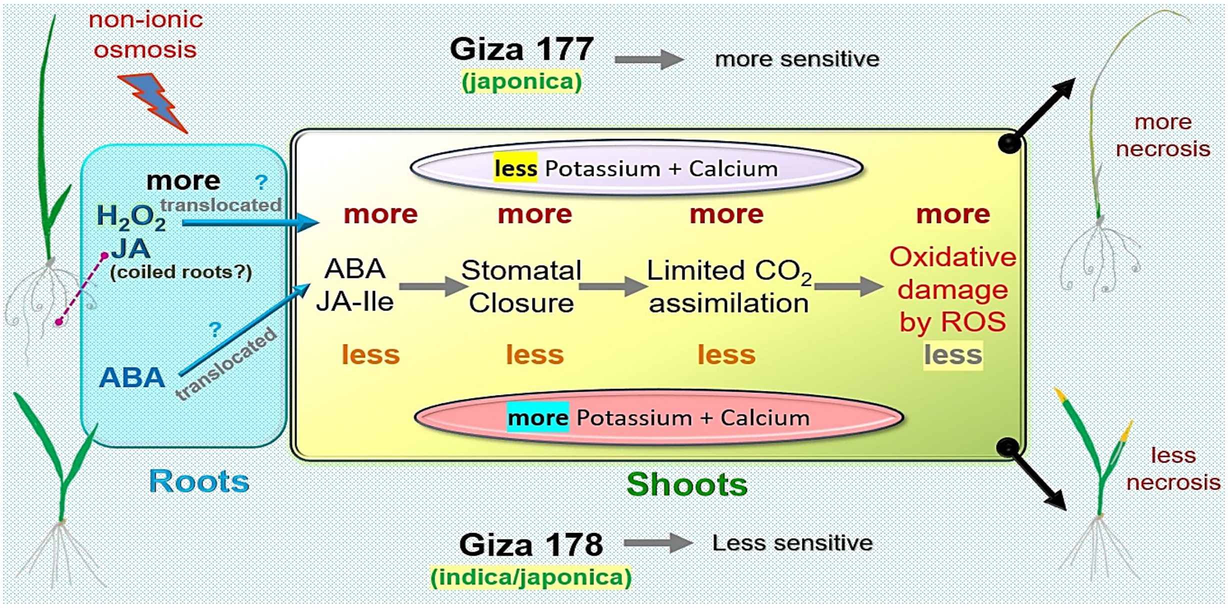

Figure 7. A proposed molecular framework illustrates the differences in how Giza 177 and Giza 178 respond to drought stress. The observed phenotype of drought stress sensitivity in the japonica cultivar Giza 177 could be linked to the high overproduction of ROS thus intensified lipid peroxidation. The highly accumulated ROS is believed to be due to a severe reduction of assimilated CO_2 after prolonged unregulated stomatal closure in response to drought stress (non-ionic osmotic) in Giza 177. We assume that Giza 178 leaves accumulate more potassium (maybe in vacuoles) and cytosolic calcium which support drought resilience by providing better turgor pressure and stress signaling, respectively. Giza 178 leaves accumulated more ABA and JA-Ile in response to drought stress. It is noted that Giza 178 produced less hydrogen peroxide probably due to attenuated activity of cell-wall oxalate oxidase enzyme.

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AUTHORS CONTRIBUTIONS

MH and LG contributed equally to this work. MH, LG, and PN: conceptualization, supervising experimental work, and manuscript preparation. LG and PN: infrastructure. MH and LG: funding acquisition. HT and MR: methodology and data validation. BH and EE: experimental work and data curation. MH and SF: writing the manuscript and data analysis. All authors read and approved the manuscript.

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