Physiologia Plantarum
An International Journal for Plant Biology

Physiologia Plantarum 155: 43–54. 2015

Comprehensive phenotypic analysis of rice (*Oryza sativa*) response to salinity stress

Inês S. Pires[a,b], Sónia Negrão[c], M. Margarida Oliveira[a] and Michael D. Purugganan[b,*]

[a]Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, and IBET, 2781-901 Oeiras, Portugal
[b]Department of Biology and Center for Genomics and Systems Biology, New York University, New York, NY, USA
[c]Center for Desert Agriculture, Division of Biological and Environmental Sciences and Engineering, King Abdullah University of Science and Technology, Thuwal 23955-6900, Kingdom of Saudi Arabia

Correspondence
*Corresponding author,
E-mail: mp132@nyu.edu

Received 24 November 2014;
revised 25 May 2015

doi:10.1111/ppl.12356

Increase in soil salinity levels is becoming a major cause of crop yield losses worldwide. Rice (*Oryza sativa*) is the most salt-sensitive cereal crop, and many studies have focused on rice salinity tolerance, but a global understanding of this crop's response to salinity is still lacking. We systematically analyzed phenotypic data previously collected for 56 rice genotypes to assess the extent to which rice uses three known salinity tolerance mechanisms: shoot-ion independent tolerance (or osmotic tolerance), ion exclusion, and tissue tolerance. In general, our analyses of different phenotypic traits agree with results of previous rice salinity tolerance studies. However, we also established that the three salinity tolerance mechanisms mentioned earlier appear among rice genotypes and that none of them is predominant. Against the pervasive view in the literature that the K^+/Na^+ ratio is the most important trait in salinity tolerance, we found that the K^+ concentration was not significantly affected by salt stress in rice, which puts in question the importance of K^+/Na^+ when analyzing rice salt stress response. Not only do our results contribute to improve our global understanding of salt stress response in an important crop, but we also use our results together with an extensive literature research to highlight some issues commonly observed in salinity stress tolerance studies and to propose solutions for future experiments.

Introduction

Almost 20 years ago, Ghassemi et al. (1995) verified that more than 20% of irrigated land was affected by salinity levels due to unsustainable farming practices. Since then, the amount of salt-affected land has increased, and will continue to increase, due to continued unsustainable cultivation practices and climate change (Wassmann et al. 2009, Qadir et al. 2014). A recent estimate claims that the annual cost of salt-induced land degradation in irrigated areas due to loss of crop production could be as high as US$ 27 billion (Qadir et al. 2014). Hence, it has become increasingly important to find ways to understand tolerance mechanisms and to develop more tolerant crop varieties.

Munns and Tester (2008) categorized salinity tolerance mechanisms, which were later detailed in Roy

Abbreviations – 3leafK, potassium content in third leaf; 3leafNa, sodium content in third leaf; ChlA and ChlB, chlorophyll *a* and *b* content, respectively; OP, osmotic potential; RDW, root dry weight; RFW, root fresh weight; RL, root length; rootK, potassium content in roots; rootNa, sodium content in roots; SDW, shoot dry weight; SeedW, average weight of 10 seeds; SES, Standard Evaluation System; SFW, shoot fresh weight; SL, shoot length; ST, Salinity Tolerance index; TDW, total dry weight; tRDW, transformed root dry weight; tRFW, transformed root fresh weight; tRL, transformed root length.

et al. (2014), and which we also employ here. They described three salinity response mechanisms used by plants. In this paper we call trait a specific characteristic in the plant, such as sodium concentration or shoot biomass, and we call mechanism a group of traits that characterize a type of response to the salinity stress. A trait might be associated with one or more salinity tolerance mechanisms, and one tolerance mechanism might involve several different traits. For example, one mechanism is the shoot-ion independent tolerance mechanism, which may involve traits such as signaling cascades and biomass/growth reduction. However, biomass/growth reduction as a trait contributing to salinity tolerance can be affected by both shoot-ion independent response and the accumulation of salt in the shoot.

The early response mechanism activated immediately after salt stress exposure is shoot-ion independent tolerance, also known as osmotic tolerance. This fast response, which is independent from the accumulation of sodium in shoots, is related to Na^+ sensing and signaling in the root, and ends up in shoot growth reduction and stomata closure under saline conditions (Munns and Tester 2008, Roy et al. 2014). Shoot-ion independent tolerance is active with exposure to salinity stress and overlaps with two other response mechanisms, ion exclusion and tissue tolerance, which are both ion-dependent and related to the build-up of Na^+ in the shoot.

In our opinion, shoot-ion independent tolerance is a more accurate name than osmotic tolerance. The reason is that the designation 'osmotic tolerance mechanism' could lead readers to think that the plant response is triggered by differences in osmotic pressure, due to the presence of salt (NaCl) around the root. However, thirty years ago Termaat et al. (1985) showed, by applying pressure to the pots where plants were growing, that the presence of NaCl on its own is enough to promote growth reduction. Plants under the same osmotic pressure caused by the presence of Na^+ and Cl^- ions, but not in the presence of either ion, did not exhibit growth reduction as plants that where in the presence of NaCl, which lead the authors to hypothesize the existence of a signaling cascade from the root to the shoot being activated when NaCl is present around the roots. Much more recently, Choi et al. (2014) showed in *Arabidopsis thaliana* that NaCl around the roots elicits a calcium (Ca^{2+}) wave signal that propagates throughout the plant that might be responsible for the initial and fast plant responses to salinity. Choi et al. (2014) also showed that exposing Arabidopsis root tips to sorbitol, an osmotic control for NaCl, did not elicit the same type of long-distance Ca^{2+} signaling as NaCl did. This agrees with the hypothesis proposed earlier by Termaat et al. (1985) and explains why we preferred to use the term 'shoot-ion independent tolerance' rather than 'osmotic tolerance'. On the other hand, Yeo et al. (1991) observed rice growth reduction after the application of NaCl, KCl or mannitol to the growth medium and concluded that the initial growth reduction was due to a limitation in water supply caused by a variation in osmotic pressure. However, in light of the results obtained by Termaat et al. (1985) and, especially by Choi et al. (2014), we hypothesize that the growth reduction seems to be a specific signaling and sensing response to NaCl stress which is independent of osmotic pressure and also independent from other agents such as KCl or mannitol.

The second mechanism is ion exclusion, which is important in reducing shoot Na content, and is probably the most studied mechanism due to the simplicity in its phenotyping (Roy et al. 2014). The third mechanism, tissue tolerance, is achieved by Na^+ compartmentalization in vacuoles, or in different tissues/organs of the plant, and by the accumulation of compatible solutes such as proline, sucrose or glycine betaine. Most salinity tolerance studies focus on the second tolerance mechanism (Hauser and Horie 2010, Munns et al. 2012, Platten et al. 2013) or in a general response to salinity that does not discriminate between the three tolerance mechanisms (Bhowmik et al. 2007, Theerakulpisut et al. 2011). For this reason, it is still unclear if a specific mechanism is preferred by some species or if a plant can shift tolerance strategies depending on salt concentrations (Roy et al. 2014).

Rice (*Oryza sativa*) feeds the human population more than any other crop (Wassmann et al. 2009), but it is a moderately salt sensitive crop in risk of greater exposure to brackish water due to the elevation of sea level, especially in the delta regions where rice is mainly produced (Wassmann et al. 2009). Efforts have been made to use naturally tolerant genotypes, such as NonaBokra and Pokkali, to introduce salinity tolerance quantitative trait loci (QTLs) into high yielding rice genotypes (Thomson et al. 2010). However, the main QTLs identified and used by breeders, *Saltol* (Gregorio 1997) and *SNC-7* (Lin et al. 2004, Ren et al. 2005), have been related to ion transport, specifically ion exclusion from the plant or K^+ homeostasis maintenance.

Rice salinity stress tolerance has been extensively studied (Kanawapee et al. 2012, Coskun et al. 2013, Negrão et al. 2013, Platten et al. 2013, Ueda et al. 2013, Ali et al. 2014, Hairmansis et al. 2014), but there is still a need to assess if rice uses all three salinity tolerance mechanisms previously described by Munns and Tester (2008), and if there is a predominant salinity mechanism used by the majority of rice cultivars.

To address these issues, we analyzed phenotypic data available for 56 rice genotypes selected from a target

gene study conducted on 392 accessions representative of rice genetic diversity (5 rice-variety groups) (Negrão et al. 2013). Our aim with the present work was to assess if rice exhibits the three salinity tolerance mechanisms and if one mechanism is preferentially used. Finally, in light of our results and previous reports we identify some issues commonly observed in salinity stress tolerance studies and propose ways of addressing those issues for future experiments.

Materials and methods

Plant material and salinity experiment

For this paper, we analyzed phenotypic data collected by Negrão et al. (2013), in which 59 phenotypes were chosen from among 392 rice accessions as representative of the different haplotype groups found by EcoTILLING in 5 genes relevant for salinity tolerance, Os*NHX*1, Os*HKT*1;5, Os*CPK*17, Os*RMC* and Os*Sal*T. Os*NHX*1 and Os*HKT*1;5 are Na^+ and K^+ transporters, Os*CPK*17 and Os*RMC* seem to be involved in salt stress signal perception and transduction pathways, and Os*Sal*T is possibly associated with the production of compatible solutes (Negrão et al. 2013). Only 56 genotypes, out of the 59 phenotyped by Negrão et al. (2013), were included in our analyses. This was because we eliminated two genotypes (TOS7564 – IRGC47017, and Issamo – IRGC63494) that we believe are from another species, the African rice *Oryza glaberrima* (Pires et al., unpublished data). We also excluded IR52724 – IRTP 22005, as no viable seeds were available at the time of this study.

The phenotyping experiment was conducted in a greenhouse maintained approximately at 29°C/22°C day/night with 70% relative humidity, and was designed as a split plot experiment with 11 genotypes per tray and 16 plants per genotype. Ten days after sowing, control plants were kept at 0 $dS\,m^{-1}$ of electrical conductivity (EC) and salt stressed plants had 6 $g\,L^{-1}$ of NaCl (EC = 12 $dS\,m^{-1}$) added to the Yoshida medium (Yoshida et al. 1976) in which they were growing. Ten days after salinization of salt stressed plants' trays, different traits were measured for both control and salt stressed plants (see *Phenotypic traits evaluated* below for a list of traits analyzed and see Negrão et al. (2013) for a complete description of how the different traits were measured).

While in the paper by Negrão et al. (2013) we used phenotypic traits for candidate gene association, here we intend to have a broader view of rice's phenotypic response to salinity by using statistics to base our conclusions (see section Statistical analysis below) and by trying to follow a coherent conceptualization of salinity tolerance mechanisms as explained in the section Introduction.

Phenotypic traits evaluated

The traits analyzed here were Na and K content in roots (rootNa and rootK); Na and K content in third fully expanded leaf (3leafNa and 3leafK); leaf osmotic potential (OP); content of chlorophyll *a* (ChlA) and *b* (ChlB) in the fourth fully expanded leaf; shoot length (SL), fresh weight (SFW) and dry weight (SDW); and root length (RL), fresh weight (RFW) and dry weight (RDW) (see Negrão et al. (2013) for details on how the traits were measured). Also from Negrão et al. (2013) we obtained the visual scoring of response to salt stress, which was adapted from Gregorio et al. (1997) and it is named Standard Evaluation System (SES). SES varies from 1 to 9 with one corresponding to the most salt tolerant genotypes. To the previous 14 traits we added average weight of 10 seeds for each genotype (SeedW) and determined Salinity Tolerance index (ST) using total dry weight (TDW) according to $TDW_{salt}/TDW_{control}$.

All 13 continuous traits obtained by Negrão et al. (2013) were used in our analyses as averages of replicates per genotype (see the list of 56 genotypes in Appendix S1, Supporting Information). There were three replicates per genotype except for biomass traits, which had five replicates.

Statistical analysis

We used fBasic R package (http://CRAN.R-project.org/package=fBasics) to determine the mean, standard deviation, skewness and kurtosis of each trait (Appendix S2). We also used the built-in shapiro.test R function, which performs the Shapiro-Wilk normality test (Royston 1982a, 1982b), to determine the probability of each trait not exhibiting a normal distribution both in control and in salt stress conditions (Appendix S2). When needed, the traits were transformed following the indications in Fink (2009) (see Appendix S3 for details on the performed transformations). Both transformed and the original averaged traits were used for all analyses since models with original traits are easier to interpret, but models with normalized traits are more reliable statistically. The results obtained using transformed and original traits were evaluated for discrepancy and are presented and discussed when relevant.

We generated box plots for each trait in control and salt stress conditions and determined if the mean of the trait distribution was significantly different between the two conditions using R's t-test function. Correlations between traits were determined using Kendall's *tau*

method (Hollander and Wolfe 1973) available through the built-in cor.test function in R. We chose Kendall's *tau* to look for correlations between traits because it is a non-parametric test that does not assume a particular distribution of the original data and already corrects for the existence of tied data, as opposed to Spearman's *rho* [see Newson (2002) for an argument on the superiority of Kendall's *tau* over Spearman's *rho*].

Finally, we used nlme R package (http://CRAN.R-project.org/package=nlme) to fit linear mixed models to the traits and check for significance of the effect of condition (control vs salt stress) and genotype. The following mixed linear models were fitted to each trait:

$$\text{model a) } trait \sim condition$$
$$\text{model b) } trait \sim condition + (1|genotype)^{*}$$
$$\text{model c) } trait \sim condition\ (1 + condition|genotype)^{\#}$$

*random intercepts per genotype; #random intercepts and slopes per genotype

Fixed effect was the same in all three models, which allowed us to use restricted maximum likelihood (REML) to estimate the models and still use an analysis of variance (ANOVA) to select the model that best fitted the data. Residual plots and normality of residuals were evaluated to confirm if the models were reliable for explaining the data.

Fitness modeling

We modeled fitness using the measured traits and the lm function available in R. Since in the experiment of Negrão et al. (2013) rice genotypes were only studied during seedling stage, we used total dry weight in salt as a proxy for fitness. And, because we observed a tight correlation between plant size in control and plant size in salt, we used models with and without total dry weight in control as a predictor. The models used were:

$$\text{model d) } TDW_{salt} = rootNa_{salt} + rootK_{salt} + 3leafNa_{salt} + 3leafK_{salt} + OP_{salt} + ChlA_{salt} + SeedW + TDW_{control}$$
$$\text{model e) } TDW_{salt} = rootNa_{salt} + rootK_{salt} + 3leafNa_{salt} + 3leafK_{salt} + OP_{salt} + ChlA_{salt} + SeedW$$

Only chlorophyll *a* content was included in the models because we previously saw that chlorophyll *a* and *b* content are not independent variables, being actually highly correlated both in salt and control conditions.

To narrow down to the most important predictors in the two models above, we used an R script provided by Anne Plessis (NYU, Purugganan Lab, unpublished data), where every combination of 8 or less predictors was tested and the best and simplest model was selected based on Bayesian Information Criterion (Schwarz 1978).

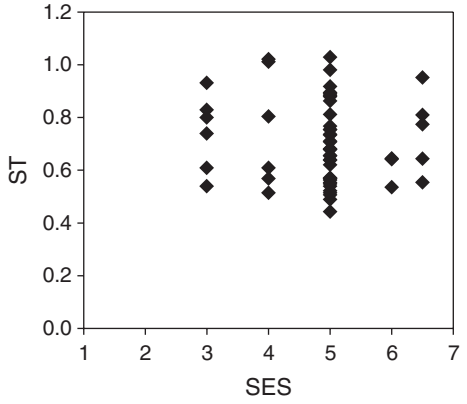

Fig. 1. Salinity Tolerance index (ST) calculated on the basis of total dry weights ($TDW_{salt}/TDW_{control}$) does not correlate with the Standard Evaluation System (SES) of salinity tolerance.

Results

SES and ST comparison

Rice plants were exposed to 12 dS m^{-1} of NaCl for 10 days (approximately 110 mM of NaCl). After this period, visual scores from the SES varied between 3 and 6.5, with 11% and 14% of genotypes falling in the tolerant ($SES \leq 3$) and sensitive ($SES \geq 6$) categories, respectively. ST varied between 0.4 and 1 with an average of 0.7, which is compatible with a growth reduction of 30% of the same genotype in saline conditions. We also observed that there was no correlation between ST and SES measures of tolerance (Fig. 1).

Trait variation with salinity and genotype

Fig. 2 depicts the distribution of each trait values in control and salt stress conditions (see also Appendix S2). Na content in roots and leaves and biomass measurements changed substantially with salt stress (Fig. 2), with Na content in roots and the 3rd leaf increasing and biomass traits (especially shoot biomass) decreasing under saline conditions. The means of trait values in each condition were compared using a two sided t-test and all traits, except for K content in roots (P-value = 0.1) and 3rd leaves (P-value = 1.0), showed a significant difference (P-value < 0.002) between the mean of the trait under control conditions and the mean of the trait under salt stress conditions. These observations clearly show that 10 days after salinization differences in Na content, chlorophyll content and biomass between control plants and salt stressed plants are detectable and significant.

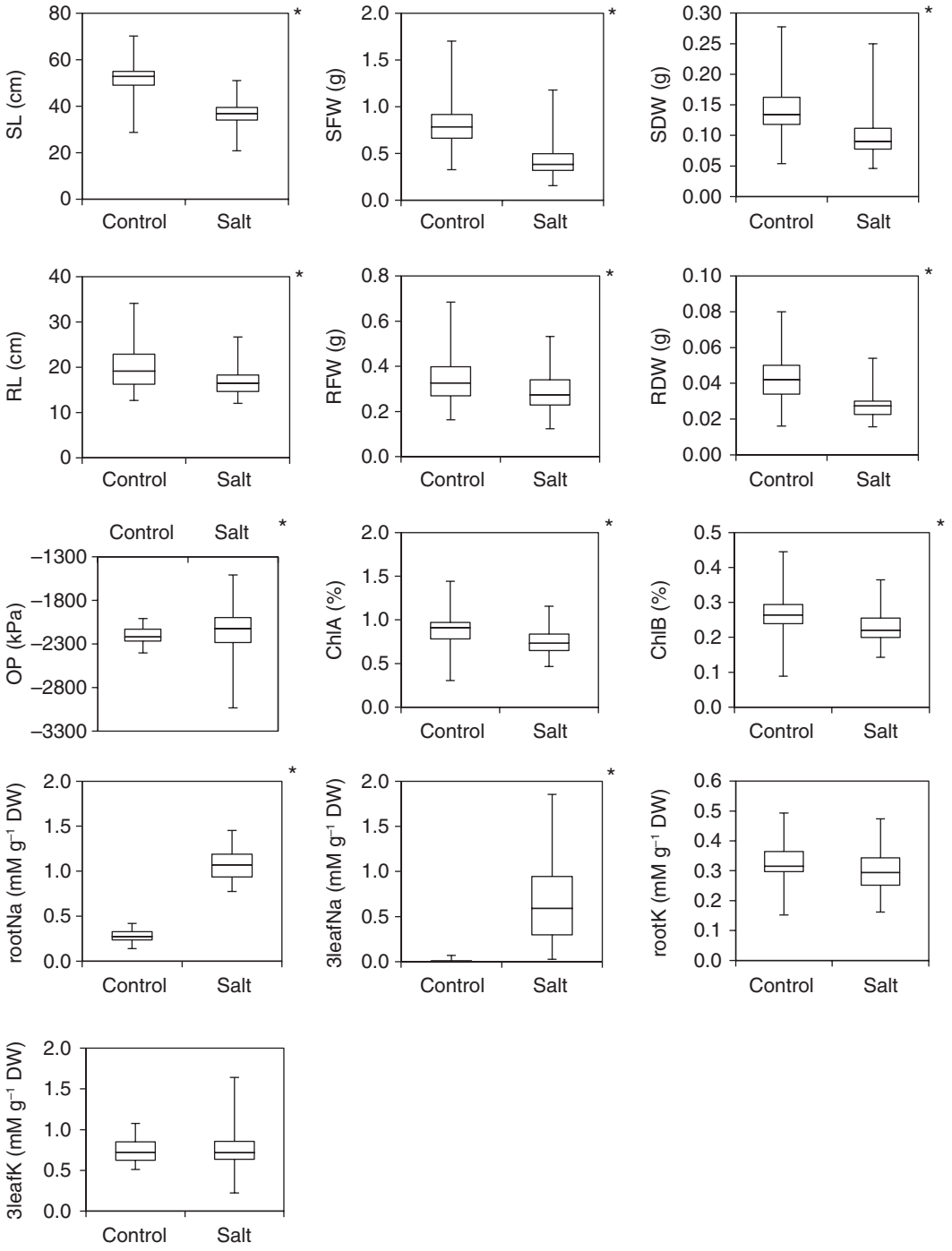

Fig. 2. Box plots for each trait in control and salinity stress conditions. An asterisk (*) in the upper right corner of the trait square means that the trait mean in control is significantly different (*P*-value < 0.002) from the trait mean in salt stress conditions according to a two sided t-test. Among all traits analyzed, only potassium content in roots and 3rd leaves did not change significantly with the presence of salt stress.

Na content in leaves and roots were below detection levels for most plants in control conditions. Moreover, Na content exhibited a large change under salt conditions (Fig. 2), which made this variable appear almost binary and also made it harder to model the contribution of condition and genotype to Na content, even with transformed variables. Despite these limitations, we could observe that condition (salinity) had a strong effect on

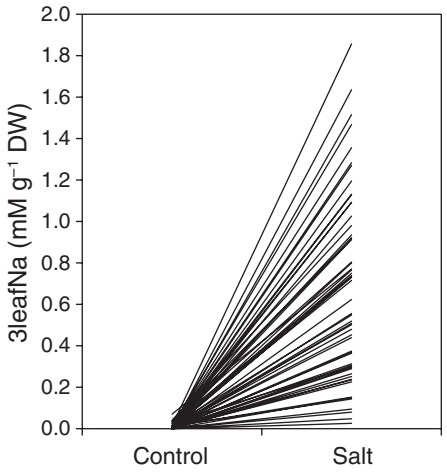

Fig. 3. The reaction norms of each of the 56 genotypes for Na content in 3rd leaf (3leafNa) clearly show that Na content in 3rd leaf increases with exposure to salt stress, but different genotypes exhibit different levels of increase (different slopes of the reaction norms).

Na content (Fig. 2). Additionally, although salinity always increased Na content in the 3rd leaf, there was great variation in how much Na^+ the different genotypes could accumulate, as seen in differences in the slopes in Fig. 3.

For all shoot biomass traits [length (SL), fresh weight (SFW) and dry weight (SDW)] both the condition (as a fixed effect) and the genotype (as a random effect) were significant, as well as the interaction between condition and genotype (Table 1). However, root biomass traits [length (RL), fresh weight (RFW) and dry weight (RDW)] did not follow a normal distribution and had to be transformed (see Appendix S3 for details on transformation). Our analyses suggest that condition and genotype are also very important in modeling root biomass traits (Table 1).

Interestingly, K content in root and the 3rd leaf did not change significantly between control and salt stressed plants (Fig. 2 and Appendix S2). In contrast, chlorophyll *a* (ChlA) and *b* (ChlB) content decreased slightly with the presence of salt stress (Fig. 2 and Appendix S2) and these changes were dependent on genotype (Table 1).

Correlations between traits

Biomass traits exhibit a significant and strong correlation between different conditions (see diagonal in Table 2). The same is true for chlorophyll *a* and *b* content and OP, but with weaker correlation coefficients (diagonal in Table 2). Finally, Na and K content in control conditions are not at all correlated with themselves under salt stress (diagonal in Table 2).

As for correlations between different traits in both conditions, it can be seen in Table 2 that, as expected, biomass traits tend to have significant and strong positive correlations with each other, both under control and salt stress conditions. The exception is root length, which tends to be less correlated with other biomass traits. This may be due to the hydroponic conditions in which the rice roots grow, where growth of rice roots is accomplished by increasing the number of lateral roots and not by growing deeper (with the number of lateral roots being not directly quantified but indirectly accounted for in root weight measures). Another explanation might be that pots in growth chambers are not sufficiently high for root length to be a reliable trait similar to field conditions.

Chlorophyll *a* and *b* content are highly and significantly correlated with each other, both in control and salt stress conditions (Table 2). More importantly, chlorophyll *a* and *b* levels exhibit significant correlations with biomass traits only in control conditions (Table 2).

Na content in 3rd leaf was only significantly and positively correlated with Na content in roots when under salt stress conditions (Table 2). OP did not seem to have strong correlations with Na and K content (Table 2). This may be due to OP being the result of the combination

Table 1. Each trait was modeled using condition as fixed effect and genotype as random effect (models a) *trait ~ condition*, b) *trait ~ condition + (1|genotype)* and c) *trait ~ condition (1 + condition|genotype)*). For the traits that could be reliably explained by this type of model we determined the model that best fitted the trait using an ANOVA and present here its statistics. SL, shoot length; SFW, shoot fresh weight; SDW, shoot dry weight; tRL, transformed root length; tRFW, transformed root fresh weight; tRDW, transformed root dry weight respectively; ChlA, chlorophyll *a* content; ChlB, chlorophyll *b* content.

Trait	Best model	χ^2 (***df***)	*P-value*	*F-value* of condition effect on trait ($\boldsymbol{df}_1, \boldsymbol{df}_2$)	*P-value* of condition effect on trait
SL	c)	18.4 (2)	0.0001	558.2 (1, 55)	<0.0001
SFW	c)	29.8 (2)	< 0.0001	268.6 (1, 55)	< 0.0001
SDW	c)	13.1 (2)	0.002	108.9 (1, 55)	< 0.0001
tRL	b)	18.9 (1)	< 0.0001	32.2 (1, 55)	< 0.0001
tRFW	b)	21.4 (1)	< 0.0001	23.6 (1, 55)	< 0.0001
tRDW	b)	20.6 (1)	< 0.0001	128.3 (1, 55)	< 0.0001
ChlA	b)	13.9 (1)	0.0002	39.7 (1, 55)	< 0.0001
ChlB	b)	14.3 (1)	0.0002	128.3 (1, 55)	< 0.0001

Table 2. Kendall's *tau* correlation matrix. Upper part (white background) presents correlation coefficients for traits under control conditions. Lower part (grey background) presents correlation coefficients for traits under salt stress conditions. And the diagonal (in bold) presents correlation coefficients between control and salt stress conditions for the same trait. **P*-value ≤ 0.05; ***P*-value <0.01

	Rootna	Rootk	3leafNa	3leafK	OP	ChlA	ChlB	SL	SFW	SDW	RL	RFW	RDW	SeedW
rootNa	**0.1**	−0.1	−0.04	0.1	0.04	0.2*	0.2*	−0.04	−0.1	−0.2	−0.1	−0.1	−0.2*	−0.2*
rootK	0.003	**0.1**	0.2	−0.2*	0.1	−0.1	−0.1	0.1	−0.1	0.0	−0.03	0.1	−0.02	−0.03
3leafNa	0.3**	−0.2*	**0.1**	−0.3**	0.2*	−0.2*	−0.2	−0.01	−0.1	−0.2	0.1	−0.01	−0.1	−0.1
3leafK	0.03	−0.03	−0.1	**0.2**	−0.1	0.2**	0.3**	−0.1	−0.04	−0.005	−0.1	−0.2	−0.1	−0.05
OP	−0.04	0.1	−0.2	−0.2*	**−0.2***	−0.2	−0.1	0.01	0.02	−0.03	0.1	0.05	0.1	−0.01
ChlA	−0.1	0.2	−0.1	0.03	0.2*	**0.3****	0.9**	−0.2*	−0.2**	−0.2	−0.1	−0.3**	−0.3*	−0.2*
ChlB	−0.03	0.2	−0.1	−0.01	0.2*	0.9**	**0.3****	−0.2*	−0.2*	−0.2*	−0.1	−0.3**	−0.3**	−0.3**
SL	−0.04	0.1	−0.4**	0.1	−0.02	−0.1	−0.1	**0.6****	0.5**	0.5**	0.2	0.3**	0.4**	0.5**
SFW	0.1	0.1	−0.2*	0.1	0.1	−0.1	−0.1	0.5**	**0.6****	0.8**	0.2*	0.6**	0.6**	0.8**
SDW	0.05	0.1	−0.2*	0.1	0.1	0.0	−0.04	0.5**	0.9**	**0.5****	0.2	0.5**	0.6**	0.9**
RL	−0.2	0.1	−0.001	−0.1	−0.1	−0.1	−0.1	0.1	0.1	0.1	**0.4****	0.3**	0.4**	0.2*
RFW	0.05	0.2*	−0.1	0.1	−0.001	0.1	0.04	0.4**	0.6**	0.6**	0.2*	**0.4****	0.6**	0.6**
RDW	−0.03	0.2	−0.1	0.05	0.1	−0.03	−0.1	0.4**	0.6**	0.6**	0.2*	0.7**	**0.4****	0.7**
SeedW	0.05	0.1	−0.2*	0.1	0.1	−0.03	−0.04	0.5**	0.8**	1.0**	0.1	0.6**	0.7**	-

of multiple ions and solutes in the plant. Chlorophyll *a* and *b* levels had some significant correlations with ion concentrations in control conditions and with OP in salt stress conditions (Table 2).

Most importantly, in salt stress conditions Na content in the 3rd leaf was significantly and negatively correlated with shoot biomass traits (Table 2). Plants that accumulate more Na^+ in leaves tend to arrest shoot growth more strongly than plants with lower levels of Na^+ in leaves.

We also weighed 10 seeds from each genotype and used this value as seed weight (SeedW). Seed weight is highly and strongly correlated with biomass traits both in control and salt stress conditions (Table 2). Heavier seeds tend to generate bigger plants independently of the conditions in which the plants are growing (a linear correlation between SeedW and TDW is shown in Fig. 4). Seed weight is also significantly, but negatively, correlated with Na content in the 3rd leaf under salt stress (Table 2). This indicates that plants with more biomass tend to accumulate less Na^+ in the shoot.

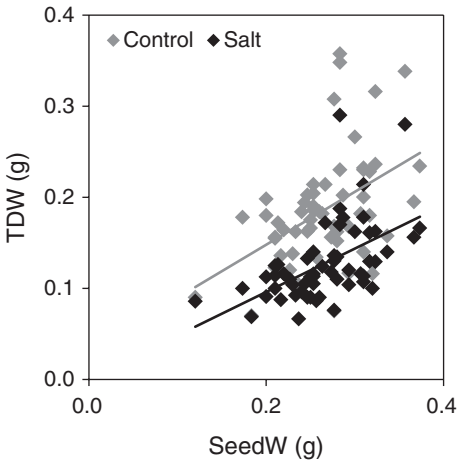

Fig. 4. Total dry weight (TDW) increases with increased average weight of ten seeds (SeedW) both in control (grey regression line: $TDW = 0.58 \times SeedW + 0.03$, $R^2 = 0.21$) and in salt stress (black regression line: $TDW = 0.48 \times SeedW + 0.00$, $R^2 = 0.29$) conditions.

Fitness modeling

The best and simplest model describing a key fitness component (total dry weight, TDW) in salt was obtained with and without considering total dry weight in control as a predictor. We decided to test both cases, because our previous analyses indicated that plant size even under control conditions may be an important factor in determining plant size in stress conditions. However, because $TDW_{control}$ and TDW_{salt} are highly correlated variables (Table 2), the inclusion of $TDW_{control}$ in the model could bias the results. Hence, we decided to model fitness with and without $TDW_{control}$ as a predictor.

As expected, when including $TDW_{control}$ as a predictor the best and simplest model for TDW_{salt} corresponded to $TDW_{salt} = 0.02 + 2^{-5}\ OP_{salt} + 0.20\ SeedW + 0.48\ TDW_{control}$ ($F_{(3,52)} = 41.2$, P-value $= 9 \times 10^{-14}$), which reinforces the effect of general plant size, independent from the stress, in determining overall plant size under salinity stress conditions.

The best and simplest model, without including $TDW_{control}$ as a predictor, corresponded to $TDW_{salt} = -0.06 + 0.08\ rootNa_{salt} - 0.03\ 3leafNa_{salt} + 0.46\ SeedW$ ($F_{(3,52)} = 12.0$, P-value $= 4 \times 10^{-6}$). This result again shows the importance of Na content in roots and shoots, in addition to plant size, in determining the biomass of the plant under salinity stress conditions. Plants that

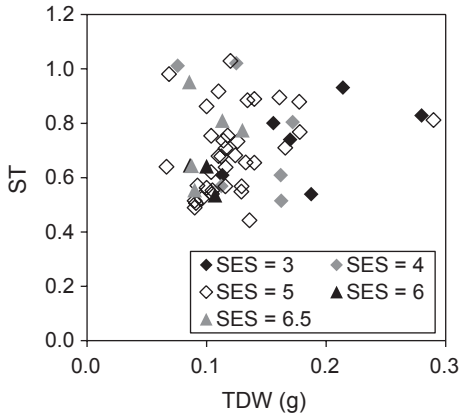

Fig. 5. Plants that differ in total dry weight (TDW) can present the same level of growth reduction (measured as ST = $TDW_{salt}/TDW_{control}$), but present different scores in Standard Evaluation System (SES).

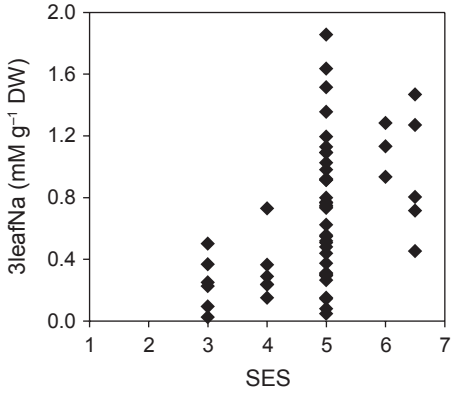

Fig. 6. Na content in 3rd leaf (3leafNa) tends to increase with an increase in Standard Evaluation System (SES) scoring, which corresponds to plants which are performing worse under elevated salinity conditions. However, it is still possible for two genotypes to accumulate the same level of Na^+ in 3rd leaves and exhibit very different tolerance levels.

accumulate Na^+ in leaves arrest growth more strongly than plants that are able to exclude this deleterious ion from their aerial parts.

Combined analysis of traits and salinity tolerance indices

We verified that plants arrest growth when under salt stress (Fig. 2), but the level of growth reduction is not directly associated with salinity tolerance as measured by the SES score (Fig. 5). Growth reduction can be the same in plants with differing biomass, but the plant with more biomass will, in general, be able to tolerate salt much better (Fig. 5).

We also observed that higher Na content in leaves has deleterious effects in the plants (e.g. by promoting a stronger growth reduction, Table 2). Although in general the higher the Na^+ levels in the 3rd leaf, the higher the SES score, genotypes with similar levels of Na^+ accumulated in leaves can exhibit differences in their physiologic response to salt stress (Fig. 6).

Discussion

Indices of salinity tolerance, chlorophyll content and K content in rice salinity

Salt tolerance indices, such as the visual score of damage given by the SES and the ST given as a proxy for biomass reduction under salt stress, are often used to assess plant salinity tolerance. However, these indices are not strongly correlated with each other (Fig. 1). This was not unexpected since ST at seedling stage only accounts for the effect of salt stress on biomass, while SES considers survival/death of plants, level of leaf chlorosis and curving, and visual difference in biomass between plants in control and salt stress conditions (Gregorio et al. 1997).

We observed a slight decrease in chlorophyll *a* (ChlA) and *b* (ChlB) content after salt stress imposition (Fig. 2). Kanawapee et al. (2012) also observed, in 106 rice genotypes grown hydroponically, a significant change in chlorophyll *a* and *b* content under salinity, but while chlorophyll *a* increased with salt stress, chlorophyll *b* showed a decrease. It is possible that a consistent and strong change in chlorophyll content is only observed with extended exposure to salt stress, which causes extensive leaf chlorosis.

Also relevant is the correlation between chlorophyll content and biomass in control conditions (Table 2). This is not unexpected since it is known that the growth of heterotrophic tissues depends on photosynthetic tissues and that disrupting chlorophyll biosynthesis results in impaired growth of the plant, especially the root (Ferrández et al. 2012, Kirchsteiger et al. 2012). When exposed to salt stress, rice plants seem to lose the correlation between chlorophyll content and biomass. Possibly, biomass in salt stress conditions is the result of more complex interactions.

In a previous study (Kanawapee et al. 2012) observed a decrease in K content combined with an increase in Na content with growth in saline conditions. Hence, the authors suggested that the K^+/Na^+ ratio is the most important mechanism controlling salinity tolerance in rice. In our conditions, however, we observed that K content was not significantly affected by salt stress (Fig. 2). In fact, almost 20 years ago Garcia et al. (1997) concluded that in rice K^+/Na^+ is less relevant as a trait than the individual content of Na^+ and K^+, contrary to what

might happen in wheat. Their conclusions where based on the fact that in rice Na^+ uptake is mechanistically different from K^+ uptake (Garcia et al. 1997). This observation is particularly important because several studies assume that a low K^+/Na^+ ratio is the most important goal in terms of ion concentrations in rice salinity tolerance and emphasize this value (Theerakulpisut et al. 2011, Kanawapee et al. 2012). Also, the content of Na^+ and K^+, independently assessed, is less affected by measurement errors, since a ratio always combines the errors associated with the measurement of the variable in the denominator and with the measurement of the variable in the numerator.

Na content and biomass are important but not sufficient traits to assess rice salinity tolerance

An increase of Na content in leaves was correlated with decreased plant biomass under salinity (Figs 2 and 3, Table 2). In fact, an increase in Na content in the plant accompanied by growth reduction has been recurrently observed [see Parida and Das (2005) for a review], and it is known that growth reduction occurs both as consequence of the deleterious effect of Na^+ in the cells and as a general stress response [see Roy et al. (2014) for a review]. There is also a negative correlation between seed weight and Na content in the 3rd leaf (Table 2). This correlation suggests that plants with more biomass tend to accumulate less Na^+ in leaves, eventually due to a dilution effect. These two factors probably contribute to the observation that rice plants with heavier seeds generate plants with more biomass both in control and in salt stress conditions (Table 2, Fig. 4). These conclusions indicate that plants with more biomass have an inherent vigor that allows them to tolerate salinity better than smaller plants, an observation previously noted in rice (Yeo and Flowers 1986), as well as in other plant species, such as *Medicago truncatula* (Veatch et al. 2004). It is important to note that plant size and seed weight have been largely ignored in studies that examine rice salt tolerance, and this may be relevant especially when comparing two or three genotypes with very different plant sizes.

Additionally, the Na content that negatively affects one genotype may be different from that affecting another genotype. In fact, Yeo and Flowers (1983) showed that the Na content that resulted in a chlorophyll content loss of 50% differed between nine rice genotypes, and that leaf chlorosis occurred at different levels of Na content in leaves. We also observed a high variability of physiologic response to salt stress between genotypes with similar levels of Na^+ accumulated in leaves (Fig. 6), and this is probably caused by the existence of other mechanisms for salt tolerance (Munns and Tester 2008, Wang et al. 2012).

In conclusion, plant biomass as well as Na content in roots and shoots, are key traits when studying salinity response in plants. However, neither of these traits is sufficient to define the level of tolerance of the plant.

Rice genotypes do not present a predominant mechanism of salinity tolerance

Taken together, the results of this study confirm that rice exhibits all three mechanisms of response to salinity stress previously described by Munns and Tester (2008), and no one mechanism is preferentially used. This suggests that different genotypes may be needed to study each of the different mechanisms of plant salinity tolerance. Our study also demonstrates that in order to have a complete understanding of salinity tolerance in plants we should focus on studying each tolerance mechanism independently by selecting an appropriate species or specific genotype that best exemplifies a specific mechanism.

Common issues when studying salinity tolerance in general

In light of the conclusions reached in our study regarding rice salinity stress response, although our aim is not to perform an exhaustive evaluation [as Flowers (2004) did over 10 years ago] of the most common issues showing up in published reports (Xu et al. 2013, Zhang et al. 2013), we may point out some of those issues.

One very important aspect to consider in the assessment of salinity tolerance is the appropriate experimental and analytical design. This is true when comparing genotypes, or assessing the impact of a particular gene in the stress response. For this we propose five aspects to consider in future experiments, namely:

First, proper controls have to be analyzed, which also means evaluating the biomass of plants both in control and salt stress conditions, and controlling for the biomass in control conditions when necessary. It is common to use rice cultivar Pokkali as a salt-tolerant reference in salinity stress experiments (Bhowmik et al. 2007, Jiang et al. 2013, Jain et al. 2014). However Pokkali was one of the biggest plants, among the 56 different rice genotypes we analyzed under control conditions.

Second, when there is *a priori* information on the function of a certain gene under study, experiments should be designed to look into the specific salinity tolerance mechanism in which the modified function might be relevant. If the gene under study appears to be involved in the production of a compatible solute, then

the plants being compared should have similar biomass under control conditions and similar Na^+ uptake rates and accumulation levels, or at least these traits should be accounted for when analyzing the data.

Third, quantitative data on the trait of interest should be presented. When proposing that a certain gene is involved in salinity stress tolerance, photographs of a transgenic plant next to a control plant (Kalamaki et al. 2009, Zhang et al. 2013) and germination tests are not enough. Germination in salinity can be uncorrelated with salinity tolerance later in the life cycle of rice and tomato plants (Flowers 2004).

Fourth, tolerance scores measure a combination of several tolerance mechanisms each with an unknown percentage of contribution to the final phenotype of the plant/genotype under study. Nevertheless, salinity tolerance scores are informative as long as they are treated with the proper statistical tools. It is common to see tolerance scores being treated as quantitative variables (Kanawapee et al. 2012, Ali et al. 2014, Ul Haq et al. 2014) when in fact, they are ordinal interval variables. The impact of this treatment might be negligible, but until it is properly tested it should not be ignored and interpretations from such analyses should be considered carefully.

Finally, we would like to point out that genome-wide analysis (GWA) where the trait being associated is a tolerance score (Bandillo et al. 2013) are hard to interpret. Because a tolerance score is the end result of a combination of salinity stress tolerance mechanisms, genes implicated in each of those mechanisms can associate with phenotype, which makes it hard to identify the candidate gene(s) for each peak and its (their) function. Furthermore, if two genes that are close to each other in the genome (are in high linkage disequilibrium) but act in opposite directions in terms of affecting salinity tolerance through different tolerance mechanisms (e.g. one increasing inherent vigor of the plant and the other decreasing tissue tolerance), each time a GWA is performed with a different population a different result may be achieved.

Author contributions

I. S. P. participated in the design of the study, carried out the statistical analysis, and drafted the manuscript. S. N., M. M. O., and M. D. P. conceived the study, participated in its design and coordination, and contributed for the manuscript. All authors read and approved the final manuscript.

Acknowledgements – I. S. P. is thankful for receiving, in 2013, the cross-disciplinary visiting and training grant from the microMORPH Research Coordination Network sponsored by the National Science Foundation (NSF), US, which allowed her to visit Professor Johanna Schmitt, and The Schmitt Lab (University of California, Davis), who contributed critically for the statistical analyses performed in this study. We also thank funding by Fundação para a Ciência e a Tecnologia (FCT) – Portugal through National Funds (# UID/Multi/04551/2013), and in part by a grant from the NSF Plant Genome Research Program to M. D. P. I. S. P. has a PhD fellowship grant (SFRH/BD/68835/2010) from FCT – Portugal. S. N. gratefully acknowledges KAUST for financial support.

References

Ali MN, Yeasmin L, Gantait S, Goswami R, Chakraborty S (2014) Screening of rice landraces for salinity tolerance at seedling stage through morphological and molecular markers. Physiol Mol Biol Plants 20: 411–423

Bandillo N, Raghavan C, Muyco PA, Sevilla MA, Lobina IT, Dilla-Ermita CJ, Tung CW, McCouch S, Thomson M, Mauleon R, Singh RK, Gregorio G, Redona E, Leung H (2013) Multi-parent advanced generation inter-cross (MAGIC) populations in rice: progress and potential for genetics research and breeding. Rice 6: 11

Bhowmik SK, Islam MM, Emon RM, Bequm SN, Siddika A, Sultana S (2007) Identification of salt tolerant rice cultivars via phenotypic and marker-assisted procedures. Pak J Biol Sci 10: 4449–4454

Choi WG, Toyota M, Kim SH, Hilleary R, Gilroy S (2014) Salt stress-induced Ca^{2+} waves are associated with rapid, long-distance root-to-shoot signaling in plants. Proc Natl Acad Sci USA 111: 6497–6502

Coskun D, Britto DT, Jean YK, Kabir I, Tolay I, Torun AA, Kronzucker HJ (2013) K^+ efflux and retention in response to NaCl stress do not predict salt tolerance in contrasting genotypes of rice (*Oryza sativa* L.). PLoS One 8: e57767

Ferrández J, González M, Cejudo F (2012) Chloroplast redox homeostasis is essential for lateral root formation in Arabidopsis. Plant Signal Behav 7: 1177–1179

Fink EL (2009) The FAQs on data transformation. Commun Monogr 76: 379–397

Flowers TJ (2004) Improving crop salt tolerance. J Exp Bot 55: 307–319

Garcia A, Rizzo CA, Ud-Din J, Bartos SL, Senadhira D, Flowers TJ, Yeo AR (1997) Sodium and potassium transport to the xylem are inherited independently in rice, and the mechanism of sodium:potassium selectivity differs between rice and wheat. Plant Cell Environ 20: 1167–1174

Ghassemi F, Jakeman AJ, Nix HA (1995) Salinisation of land and water resources: Human causes, extent, management and case studies. CAB International, Wallingford

Gregorio GB (1997) Tagging salinity tolerance genes in rice using amplified fragment length polymorphism (AFLP). DPhil Thesis. University of the Philippines, Los Baños, Laguna
Gregorio GB, Senadhira D, Mendoza RD (1997) Screening rice for salinity tolerance. IRRI discussion papers. International Rice Research Institute, Manila, Philippines
Hairmansis A, Berger B, Tester M, Roy S (2014) Image-based phenotyping for non-destructive screening of different salinity tolerance traits in rice. Rice 7: 16
Hauser F, Horie T (2010) A conserved primary salt tolerance mechanism mediated by HKT transporters: a mechanism for sodium exclusion and maintenance of high K^+/Na^+ ratio in leaves during salinity stress. Plant Cell Environ 33: 552–565
Hollander M, Wolfe DA (1973) Kendall and Spearman tests. In: Nonparametric Statistical Methods. John Wiley & Sons, New York, pp 185–194
Jain M, Moharana KC, Shankar R, Kumari R, Garg R (2014) Genomewide discovery of DNA polymorphisms in rice cultivars with contrasting drought and salinity stress response and their functional relevance. Plant Biotechnol J 12: 253–264
Jiang SY, Ma A, Ramamoorthy R, Ramachandran S (2013) Genome-wide survey on genomic variation, expression divergence, and evolution in two contrasting rice genotypes under high salinity stress. Genome Biol Evol 5: 2032–2050
Kalamaki MS, Alexandrou D, Lazari D, Merkouropoulos G, Fotopoulos V, Pateraki I, Aggelis A, Carrillo-Lopez A, Rubio-Cabetas MJ, Kanellis AK (2009) Over-expression of a tomato N-acetyl-L-glutamate synthase gene (SlNAGS1) in *Arabidopsis thaliana* results in high ornithine levels and increased tolerance in salt and drought stresses. J Exp Bot 60: 1859–1871
Kanawapee N, Sanitchon J, Lontom W, Threerakulpisut P (2012) Evaluation of salt tolerance at the seedling stage in rice genotypes by growth performance, ion accumulation, proline and chlorophyll content. Plant Soil 358: 235–249
Kirchsteiger K, Ferrandez J, Pascual MB, Gonzalez M, Cejudo FJ (2012) NADPH thioredoxin reductase C is localized in plastids of photosynthetic and nonphotosynthetic tissues and is involved in lateral root formation in Arabidopsis. Plant Cell 24: 1534–1548
Lin HX, Zhu MZ, Yano M, Gao JP, Liang ZW, Su WA, Hu XH, Ren ZH, Chao DY (2004) QTLs for Na^+ and K^+ uptake of the shoots and roots controlling rice salt tolerance. Theor Appl Genet 108: 253–260
Munns R, Tester M (2008) Mechanisms of Salinity Tolerance. Annu Rev Plant Biol 59: 651–681
Munns R, James RA, Xu B, Athman A, Conn SJ, Jordans C, Byrt CS, Hare RA, Tyerman SD, Tester M, Plett D, Gilliham M (2012) Wheat grain yield on saline soils is improved by an ancestral Na^+ transporter gene. Nat Biotechnol 30: 360–364
Negrão S, Almadanim MC, Pires IS, Abreu IA, Maroco J, Courtois B, Gregorio GB, McNally KL, Oliveira MM (2013) New allelic variants found in key rice salt-tolerance genes: an association study. Plant Biotechnol J 11: 87–100
Newson R (2002) Parameters behind "nonparametric" statistics: Kendall's tau, Somers' D and median differences. Stata J 2: 45–64
Parida AK, Das AB (2005) Salt tolerance and salinity effects on plants: a review. Ecotoxicol Environ Saf 60: 324–349
Platten JD, Egdane J, Ismail A (2013) Salinity tolerance, Na^+ exclusion and allele mining of HKT1;5 in *Oryza sativa* and *O. glaberrima*: many sources, many genes, one mechanism? BMC Plant Biol 13: 32
Qadir M, Quillérou E, Nangia V, Murtaza G, Singh M, Thomas RJ, Drechsel P, Noble AD (2014) Economics of salt-induced land degradation and restoration. Nat Resour Forum 38: 282–295
Ren ZH, Gao JP, Li LG, Cai XL, Huang W, Chao DY, Zhu MZ, Wang ZY, Luan S, Lin HX (2005) A rice quantitative trait locus for salt tolerance encodes a sodium transporter. Nat Genet 37: 1141–1146
Roy SJ, Negrão S, Tester M (2014) Salt resistant crop plants. Curr Opin Biotechnol 26: 115–124
Royston P (1982a) Algorithm AS 181: the W test for normality. Appl Stat 31: 176–180
Royston P (1982b) An extension of Shapiro and Wilk's W test for normality to large samples. Appl Stat 31: 115–124
Schwarz G (1978) Estimating the dimension of a model. Ann Stat 6: 461–464
Termaat A, Passioura JB, Munns R (1985) Shoot turgor does not limit shoot growth of NaCl-affected wheat and barley. Plant Physiol 77: 869–872
Theerakulpisut P, Kanawapee N, Sanitchon J, Srihaban P (2011) Genetic diversity analysis of rice cultivars (*Oryza sativa* L.) differing in salinity tolerance based on RAPD and SSR markers. Electron J Biotechnol: 14
Thomson MJ, Ocampo M, Egdane J, Rahman MA, Sajise AG, Adorada DL, Tumimbang-Raiz E, Blumwald E, Seraj ZI, Singh RK, Gregorio GB, Ismail AM (2010) Characterizing the *Saltol* quantitative trait locus for salinity tolerance in rice. Rice 3: 148–160
Ueda A, Yahagi H, Fujikawa Y, Nagaoka T, Esaka M, Calcaño M, González M, Hernández Martich JD, Saneoka H (2013) Comparative physiological analysis of salinity tolerance in rice. Soil Sci Plant Nutr 59: 896–903
Ul Haq T, Akhtar J, Steele KA, Munns R, Gorham J (2014) Reliability of ion accumulation and growth components for selecting salt tolerant lines in large populations of rice. Funct Plant Biol 41: 379

Veatch ME, Smith SE, Vandemark G (2004) Shoot Biomass Production among Accessions of Medicago truncatula Exposed to NaCl. Crop Sci 44:1008–1013

Wang H, Zhang M, Guo R, Shi D, Liu B, Lin X, Yang C (2012) Effects of salt stress on ion balance and nitrogen metabolism of old and young leaves in rice (*Oryza sativa* L.). BMC Plant Biol 12: 194

Wassmann R, Jagadish SVK, Sumfleth K, Pathak H, Howell G, Ismail A, Serraj R, Redona E, Singh RK, Heuer S (2009) Chapter 3 Regional vulnerability of climate change impacts on Asian rice production and scope for adaptation. Adv Agron 102: 91–133

Xu C, Wang M, Zhou L, Quan T, Xia G (2013) Heterologous expression of the wheat aquaporin gene *TaTIP2;2* compromises the abiotic stress tolerance of *Arabidopsis thaliana*. PLoS One 8: e79618

Yeo AR, Flowers TJ (1983) Varietal differences in the toxicity of sodium ions in rice leaves. Physiol Plant 59: 189–195

Yeo A, Flowers T (1986) Salinity Resistance in Rice (*Oryza sativa* L.) and a Pyramiding Approach to Breeding Varieties for Saline Soils. Australian Journal of Plant Physiology 13:161–173

Yeo AR, Lee λ-S, Izard P, Boursier PJ, Flowers TJ (1991) Short- and long-term effects of salinity on leaf growth in rice (*Oryza sativa* L.). J Exp Bot 42: 881–889

Yoshida S, Forno D, Cock J, Gomez K (1976) Laboratory Manual for Physiological Studies of Rice. International Rice Research Institute, Manila, Philippines

Zhang Z, Zhang Q, Wu J, Zheng X, Zheng S, Sun X, Qiu Q, Lu T (2013) Gene knockout study reveals that cytosolic ascorbate peroxidase 2 (OsAPX2) plays a critical role in growth and reproduction in rice under drought, salt and cold stresses. PLoS One 8: e57472

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. List of rice genotypes phenotyped in Negrão et al. (2013) and analyzed in this study.

Appendix S2. Table with number of replicates per genotype (N), mean, standard deviation (SD), skewness, kurtosis and *P*-value of Shapiro-Wilk normality test for each trait under control and salt stress conditions.

Appendix S3. Table with trait transformations performed for statistical analysis.

Edited by S. Pelaz