Is salinity tolerance of rice lines related to endogenous ABA level or to the cellular ability for ABA synthesis under stress?

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Abstract: As the plant hormone abscisic acid (ABA) is involved in responses to salinity stress. We tested its putative relationship with the degree of tolerance to this abiotic stress. For this purpose we examined the responses of sensitive (IR29) and tolerant (IR651) varieties of indica rice (*Oryza sativa* L.) to a range of salinity (0 (control) and 100 mM NaCl). Shoot and root dry weight was reduced and leaf Na concentration increased in response to salinity for both cultivars with a higher extent in sensitive. Tolerance of IR29 to saline stress was generally improved by ABA treatment and leaves Na content reduced to their respective control treatment. This ABA effect was evident in IR29 with low tolerance, as their ability to recover from stress increased up to seven fold. Independent of the saline treatment, the absolute endogenous leaf ABA content in sensitive variety was significantly more than tolerant one. However, upon stress, the increase in endogenous ABA synthesis was higher in tolerant than in sensitive varieties. These data together with those obtained by using Fluridone, an inhibitor of ABA synthesis, suggested first, there was differential sensitivity to ABA in the tolerant and sensitive leaves cultivars and enhanced concentrations at tolerant levels acted primarily to maintain root and shoot growth salt stress and second, the differences in the level of tolerance to saline stress is related to their different capacity of ABA synthesis under stress conditions.
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1. Introduction

Salinization of arable land is increasing, and could possibly have devastating global effects. Over 6 % of the world's land and 20% of the world's irrigated land are currently affected by salinity (Rhoades et al. 1992; Munns 2005). In such soils, NaCl concentrations typically exceed 40 mM, and much higher values are frequently found (Munns 2005), Enhancing membrane permeability in salt stressed plants has been well documented (Hasegawa et al. 2000), resulting in Na^+ accumulation and increasing Na^+/K^+, conversely reducing K^+ in many plant species i.e. rice (Dionisio-Sese & Tobita, 1998) green bean (Yasar et al. 2006), winter wheat (Zheng et al. 2008), umbu plant (Da silva et al. 2008). From some previous studies, the results showed that rice growth and development are reduced when exposed to the salinity stress (Morsy et al. 2007; Cha-um et al. 2007; Dionisio-Sese and Tobita 1998; Shannon et al. 1998). However, the response of rice to salt stress depends on its salt tolerance abilities. During the last two decades, it has been well established that abscisic acid (ABA) is a vital cellular signal that mediates the expression of a number of salt and water deficit-responsive genes. The direct relation between stress tolerance and increased levels of ABA does not always exist. Thus, in barley and wheat changes in endogenous ABA levels have been reported to be unrelated to freezing tolerance or to cold hardening (Kadlecová et al. 2000; Faltusová-Kadlecová et al. 2002). Among the comparisons between stress-tolerant and stress sensitive cultivars within the same species, some reports have shown higher ABA levels in tolerant cultivars, although the differences appeared in either unstressed, non-acclimated cultivars (Bravo et al. 1998) or following the stress (Chen et al. 2002; Moons et al. 1995; Zheng and Li 2000). In rice, ABA contents increased in a semi salt-tolerant variety but only marginal changes were measured in salt- tolerant and salt sensitive ones (Bohra et al. 1995). Exogenous ABA treatments prior to subjecting the plants or tissues to adverse conditions have been reported to improve tolerance to cold temperatures (Bornman and Jansson 1980; Duncan and Widholm 1987), osmotic stress (Nayyar and Kaushal 2002; Nayyar and Walia 2003), or to salt stress via soluble sugar accumulation in common bean (Khadri et al. 2007) and rice (Asch et al. 1995).In the current study we tested the hypothesis that responses to root-sourced salt stress, are coordinated by elevated ABA concentrations and probably there is tolerance a differential response of tolerant and sensitive cultivars to ABA applications .

2. Materials and methods

Plant material and growth conditions

Two rice cultivars different in tolerance of salt stress during the vegetative stages (Moradi et al.

2003) were selected for this investigation. IR65192-4B-10-3 (abbreviated as IR651) is breeding line tolerant of salt stress at both the seedling and reproductive stages, and IR29 is a cultivar sensitive to salt stress during both stages and is commonly used as a sensitive check in breeding nurseries.

This experiment was conducted in a growth cabinet with light intensity of about 700 µmol m^{-2} s^{-1} and 12 h duration, 70 % relative humidity and 29/22 8C day/night temperature. Pre-germinated seeds of each cultivar were sown in holes made on Styrofoam sheets with a nylon net bottom (Gregorio et al. 1997). Two seeds were sown per hole, with 50 holes per entry. The sheets were first floated on distilled water in 11 L plastic trays for 3 d, after which a nutrient solution (Yoshida et al. 1976) was used until the plants were 21 d old. A control treatment (nutrient solution) and the level of salt stress, 12 dS m^{-1} (100 mM) using NaCl was introduced thereafter. Four hours after imposing the salt treatment, half of the plants of both cultivars were exposed to spraying of Fluridone (50 µM) or ABA (20 µM). The youngest fully expanded leaf (leaves that had completed petioles), harvested at 0 (immediately before starting the salinity), 4, 12, 24, 48, 96 and 168 h after the start of the salt treatments for ABA measurements. Shoot and root dry matter, K^+ and Na^+ concentration of youngest fully expanded leaf measured at the end of this experiment.

The experiment was designed as 2×2 (Cultivars × Salinity) factorials in Completely Randomized Design (CRD) with three replicates. The mean in each treatment was compared by Duncan's New Multiple Range Test (DMRT) at $p<0.01$ and analyzed by SPSS software (SPSS for Windows, SPSS Inc., Chicago, Illinois, USA). The culture solution was renewed once with the pH adjusted daily to 5.5 by adding either NaOH or HCl.

Sodium and potassium measurements

Dried samples were ground to a fine powder. About 0.1 g was transferred to a test tube containing 10 ml 0.2 N acetic acid. Then test tubes heated in a water bath at 80 ^oC for 2 h. The extracted tissue was cooled at room temperature and left overnight and then filtered by using Whitman filter paper number 42. Sodium and potassium concentrations were determined by using an atomic absorption spectrometer (Perkins Elmer, Norwalk, CT,USA).

Leaf gas exchange measurement

Stomatal conductance (g_s) was made on fully expanded youngest leaf of each plant using an open system LCA-4ADS portable infrared gas analyzer (Analytical Development Company, Hoddesdon, England). Measurements were performed with the following specifications/adjustment: molar flow of air per unit leaf area 403.3 mmol m^{-2} s^{-1}, atmospheric pressure 99.9 kPa, water vapor pressure into chamber ranged from 600 Pa to 890 Pa, Par at leaf surface was maximum up to 1711 µmol m^{-2} s^{-1}, temperature of leaf ranged from 28.4 0C to 32.4 0C, ambient temperature ranged from 22.4 to 27.9 0C, and ambient CO2 concentration 352 µmol mol^{-1}.

Measurement of endogenous ABA levels

Endogenous ABA analyses were performed as described in Gómez-Cadenas et al. (2002), Frozen rice leaves of 1.5 g were finely ground in liquid nitrogen and 10 mLof 80% methanol was added together with 0.01 g of ascorbic acid and 0.01 g polyvinylpyrrolidone (PVP) to prevent oxidation reactions during extraction. The homogenate was stirred overnight at 4 °C. After centrifugation (4000×g, 15 min), the supernatant was recovered and adjusted to pH 8.0. The aqueous methanol was evaporated under reduced pressure at 35 °C. The residue was dissolved in 5 mL of water. Then it was frozen and thawed for three cycles. After centrifugation (4000×g, 15 min), the supernatant was recovered and adjusted to pH 2.5 and partitioned against ethyl acetate. Then the solution with the free ABA in ethyl acetate was collected. This process was repeated thrice. After that, the collection was adjusted to pH 8.0 and dried. The resulting dried precipitate was dissolved in 1 mL of 3% methanol containing 0.1 M acetic acid, and was filtered through a 0.45 mm membrane filter. The extract (100 µL) was automatically injected and processed by HPLC (Agilent 1100 Series. USA) equipped with a reverse phase column (4.6×250 mm Diamonsic C18, 5 µm). It was eluted with a linear gradient of methanol (3–97%), containing 0.01% acetic acid, at a flow rate of 4 mL/min. The detection was run at 260 nm with a diode array detector. The retention time of ABA was 36.4 min and shifted 0.1 to 0.5 min. Quantification was obtained by comparing the peak areas with those of known amounts of ABA.

3. Results

Effect of stress on K & Na concentration with impaired ability for ABA synthesis

A different approach was used to study the implication of ABA in the mechanism of stress-tolerance. This experiment was performed with Fluridone treated, an inhibitor of ABA biosynthesis. Irrespective of Fluridone application no statistically significant differences were measured between tolerance and sensitive cultivars in Na concentration in control treatment of both cultivars investigated (Figure.2a&b). Salinity treatment increased 2 fold the

Na level in IR29 but became unchanged in IR651with their respective control (Figure.2b). The Na accumulation in young leaves of both cultivars became more pronounced upon applied Fluridone in salinity treatment (Figure.2a&b) and cause to rapid raise of Na, 7.3 and 2.8 fold in sensitive and tolerant cultivars respectively, contrast to controls treatments). Fluridone had a dramatic effect on their response to stress in both cultivars.

Thus, when plants of both cultivars were sprayed with Fluridone, they showed higher sensitivity to a subsequent salinity stress than stressed plants that had not been sprayed with the inhibitor (Figure.2). Salt stress induced an approximate 30% increase in endogenous leaf ABA content of tolerant cultivar, while spraying with 50 µM Fluridone during the salinity treatment not only suppressed this increase but declined it more than 30% (Figure. 3).

In a further experiment, plants from tolerant and sensitive cultivars were treated with exogenous ABA and subjected to salinity (Figure.2). When plants were treated with ABA (20 µM), leaf tolerant cultivar did not change significantly their Na level response to the subsequent stress. By comparison, in IR29 plants application of exogenous ABA, lead to dramatically reduction the Na level and dropped the value to normal treatment (Figure.2b).

It has to be pointed out that the effect of ABA was totally counteracted with the inhibitor.

Regardless of the salinity, ABA or Fluridone treatments, a very similar changing pattern was observed for K concentration in leaves of both cultivars and no significant differences was observed (Figure.2c&d).

Effect of stress on endogenous ABA levels

Endogenous ABA levels of tolerant and sensitive of both cultivars were determined in order to check whether the different degree of tolerance to stress was correlated with hormone contents. Irrespective of salinity treatment, leaves ABA concentration in IR29 was higher than IR651. Endogenous ABA levels of leaves under non stress conditions varied in the range of 13.4 to 14.10 µg g^{-1}Fwt in IR29. Compare to sensitive cultivar, the tolerant cultivar had a lower ABA concentration, and the values varied in the range of 6 to 9.5 µg g^{-1}Fwt in control treatment. Leaves ABA content of stressed IR651 plants was significantly bigger than that of control plant throughout sampling time and the differences became more pronounced and achieved a maximum value by end of experiment (Figure. 3). Opposite to tolerant cultivar, leaves ABA levels in sensitive cultivar increased slightly but did not reach the level of statistical significance at the end of experiment compare to their respective control treatment (Figure. 3).

Stomatal conductance and biomass accumulation

Stomatal conductance under no stress conditions was two fold in IR651 than IR29, and the differences keep constant throughout of experiment (Table 1). Salinity was significantly lowered the stomatal conductance, the rate of stomatal conductance of IR29 and IR651 plants was reduced by 50% (Table.1). Result regarding plant biomass showed inverse relationship with salinity. A general trend of decrease in dry weight of plant with salinity was noted in both cultivars. However, shoot and root weight reduction was largest in the susceptible variety compare to tolerant variety (Figure.1b&d). Fluridone application lead to drastically decline in root and shoot dry weight of both cultivars (Figure.1a&c). Regardless of salinity, shoot and root dry weight in IR651 was two fold than IR29.

4. Discussion

The plant hormone abscisic acid has been implicated in plant responses to abiotic factors causing water stress. In most studies, elevated ABA levels prior to stress, or as a first step in the response to adverse conditions, are involved in tolerance mechanisms. Under this rationale, it is to be expected that ABA increased in leaves of tolerant variety have a positive role in alleviating stress effects. In the present work differences in ABA contents were observed between sensitive and tolerant of the two varieties studied, and maybe we can ascribe their different response to stress to a different endogenous hormone level under control, non-stress conditions (Figure. 2) when plants were subjected to stress we could detect differences in ABA synthesis in varieties with respect to non-stressed controls (Figure.3). Tolerant variety seems to have a higher ability for ABA synthesis than sensitive. This agrees with reported results comparing sensitive/tolerant cultivars of different species (Chen et al. 2002; Lee et al. 1993; Zheng and Li 2000). Moons et al. (1995) reported that although salt-tolerant rice cultivars showed higher increases of ABA than the sensitive cultivars, there was not a direct correlation between absolute ABA content and degree of tolerance. Therefore, they pointed out the importance of both the rate of ABA increase as well as the absolute ABA levels. The fact that IR29 produce less ABA under stress, could explain their sensitivity response to stress (Figure.3).

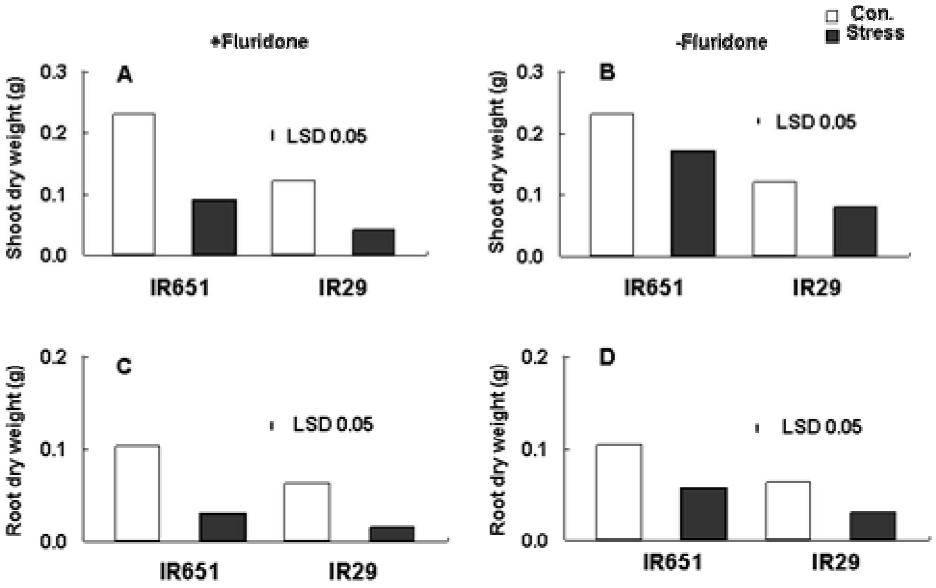

Figure 1. Shoot and rood dry weight of two rice cultivars during the seedling stage under control and salt stress of 12 dsm^{-1}, with (A and C) or without (B and D) spraying Fluridone (50 µM). The data are mean values of three replications with three subsamples per replication, and vertical bars are $LSD_{0.01}$.

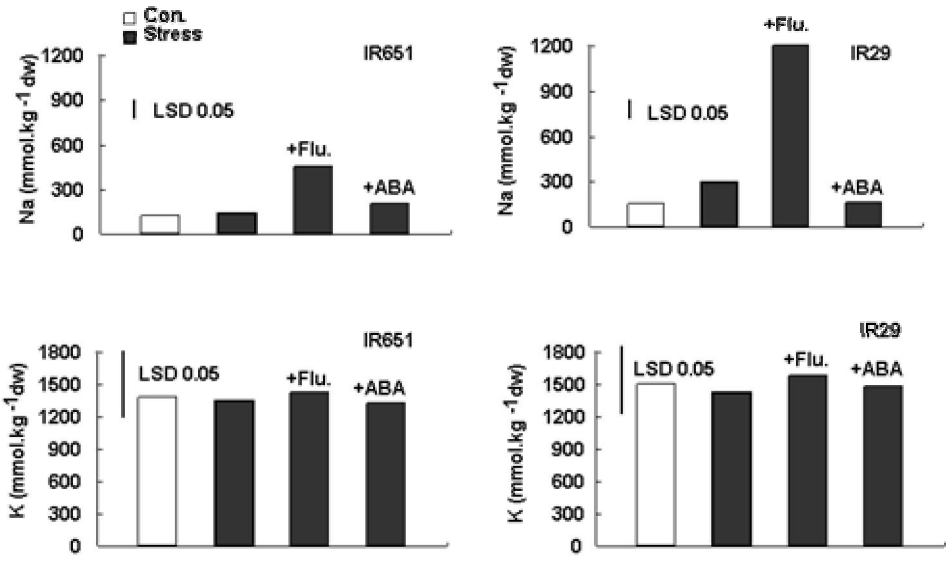

Figure 2. Na and K concentration of youngest full expanded leaf of two rice cultivars during the seedling stage under control and salt stress of 12 dsm^{-1}, with spraying Fluridone (50 µM)or ABA(20 µM). The data are mean values of three replications with three subsamples per replication, and vertical bars are $LSD_{0.01}$.

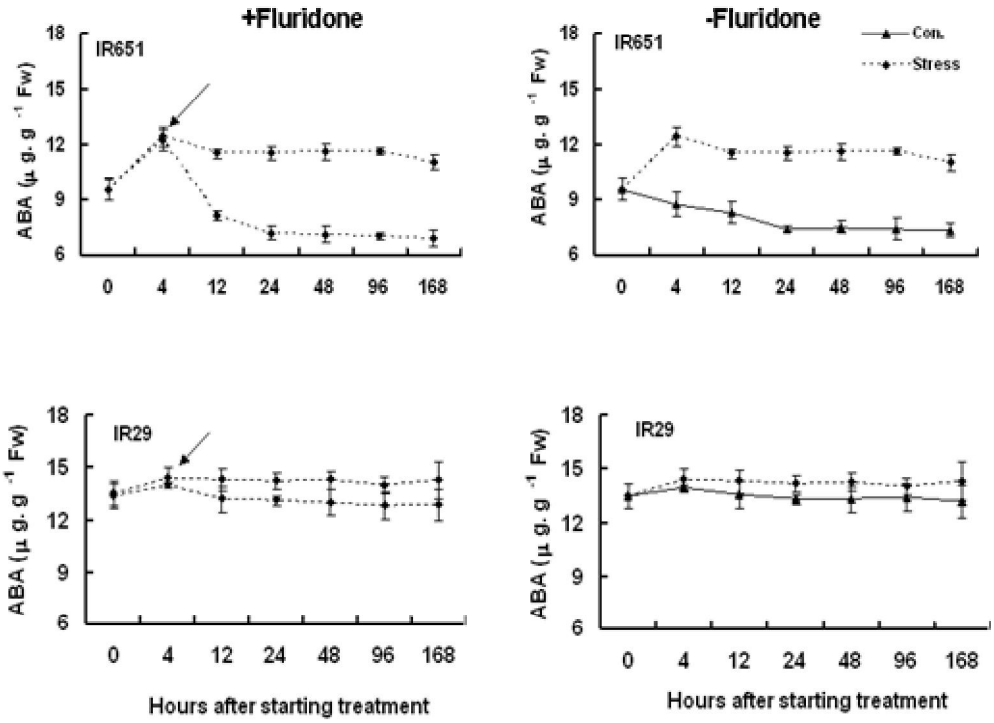

Figure 3. ABA measured on the youngest fully expanded leaf of two rice cultivars during the seedling stage under control and salt stress of 12 dsm^{-1}. The data are mean values of three replications with three measurements per replication, and vertical bars represent ±SE of the mean (n=3) where these exceed the size of the symbol. The values on the x-axis represent of hours after the start of salt stress treatment of 21-day-old seedlings. The *arrows* indicate the spraying of Fluridone.

Table1. Stomatal conductance (μmol m^{-2} s^{-1}) of rice varieties under control (0 Mm NaCl = 1.56 $ds.m^{-1}$) and salinity (100 Mm NaCl = 12 $ds.m^{-1}$) conditions.

		Sampling time					
Variety	Salinity ($ds.m^{-1}$)	12	24	48	72	96	120
IR29	0	0.5±0.01	0.5±0.01	0.5±0.02	0.5±0.05	0.5±0.03	0.6±0.03
	12	0.1±0.02	0.2±0.02	0.2±0.04	0.2±0.02	0.2±0.01	0.2±0.03
IR651	0	1.2±0.03	1.1±0.1	1.4±0.3	1.4±0.2	1.3±0.1	1.4±0.3
	12	0.3±0.03	0.8±0.04	0.7±0.1	0.7±0.1	0.8±0.1	0.7±0.04

Each value is a mean of three replicates ± se.

Further experiments, using Fluridone, confirm the involvement of ABA synthesis in the mechanism of response to stress. Independent of the cultivar used, spraying 50 μM Fluridone, drastically increased leaf Na concentration the tolerance and sensitive varieties when exposed to saline stress (Figure.2). However, the extent of this increment varied as a function of the tolerance level of the

varieties used. Thus, in tolerance variety the increment was of 3 fold, while in sensitive variety it reached 8 fold in their respective to control treatments (Figure.2). Thus, when plants of both cultivars were sprayed with Fluridone, they showed higher sensitivity to a subsequent saline stress than stressed plants that had not been sprayed with the inhibitor. This protective role of ABA is confirmed in the present work. While stress induced dramatically increased endogenous ABA, spraying with 50 µM Fluridone during the salinity treatment suppressed this increase (Figure.3). We observed that an ABA treatment during saline stress nearly to 7 times reduced the leaf Na content of sensitive cultivar to recover from stress in comparison to untreated plants subjected to the same stress conditions (Figure.2). In contrast, tolerance cultivar was not affected by the hormonal treatment. In agreement with this result, Bohra et al. (1995) have also shown that exogenous ABA treatments improved tolerance to salinity in sensitive but not in tolerant rice cultivars. It is interesting to note that non-stressed barley varieties with lower ABA contents were more sensitive to freezing and responded better to exogenous ABA treatments than other varieties with higher hormone levels (Bravo et al. 1998). In fact, growth rate and leaf Na concentration of plants were significantly affected by this inhibitor (Figure.1&2), which interfere with ABA action. Similar results have been obtained by Bianco-Trinchant and Le Page-Degivry (1998) in protoplasts from different plant origin.

Our results demonstrate the importance of ABA synthesis in their tolerance to stress, tolerance variety with a higher capacity of ABA synthesis (Figure.3).

In summary, our study lead to the following conclusions: 1- The degree of stress tolerance of a given variety does not appear to be related to endogenous ABA levels under control conditions, but to the different rate of ABA synthesis under stress. 2- The fact that cells from IR651 produce more ABA when subjected to stress, could explain their better response to salinity condition. 3- The beneficial effect of exogenous ABA on the tolerance to saline stress is mainly restricted to IR29 with low tolerance (sensitive cultivar). 4- There was a different threshold irritability for ABA content, because opposite to the bigger leave ABA level in sensitive variety, more deleterious effect observed in this cultivar. 5- Regardless to present or absent of salinity treatment, IR651 shows a higher growth rate, and low accumulation of Na with a Fluridone application in contrast to IR29, suggesting that improved growth probably alleviating Na^+ toxicity by diluting, this finding consistent with Nooden (1988).

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