

Chlorophyll fluorescence and lipid peroxidation changes in rice somaclonal lines subjected to salt stress

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Abstract The aim of the present work was to explore physiological changes provoked by somaclonal variation in response to salinity. Two parental cultivars (La Candelaria and Yerua) and their derived somaclones were used as a source for breeding new rice lines with improved salt tolerance. We studied the effect of NaCl salt stress on chlorophyll fluorescence-related parameters, such as the maximum quantum yield of primary PSII photochemistry (F_v/F_m) and the performance index for energy conservation from photon absorbed by PSII antenna (PI_{ABS}). In addition malondialdehyde (MDA) content and leaf temperature (LT) responses were also measured. In somaclonal lines, F_v/F_m , PI_{ABS} , MDA and LT showed coefficients of variation of 13.7, 39.3, 25.5, and 3 %, respectively, for La Candelaria and 1.4, 17.6, 34.4 and 3 % for Yerua. However, the fragrant character did not differ in the aromatic somaclonal lines with respect to their parents. Our results suggest that the F_v/F_m ratio would not be as good marker of PSII vitality as PI_{ABS} for salinized rice somaclones, unless they are highly susceptible to salinity. On other hand, the MDA content showed a strong negative correlation with the PI_{ABS} content in somaclones of both rice cultivars, suggesting that MDA levels could also be used as an oxidative damage index in rice somaclones.

Keywords Callus · Rice · Somaclones · Salinity · F_v/F_m · PI_{ABS} · Malondialdehyde

Introduction

Rice (*Oryza sativa* L.) is considered the most important crop, due to the fact that it is the main nutrient source for one half of the world population (Sasaki 2005), accounting for 20 % of the caloric intake. Varieties that differ from the typical indica or japonica varieties in their quality and characterized by certain special quality features may be defined as speciality rices (Chaudhary 2003). In general, aromatic rice cultivars (another type of speciality rice) present superior flavor and taste than common speciality rice. Recent variations in agricultural commodity prices affected the rice trade market, but aromatic rice prices remained the highest priced sector, turning them into an economically interesting crop alternative for farmers (Chaudhary 2003).

Somaclonal variation is any variability obtained by means of an in vitro tissue culture (Larkin and Scowcroft 1981). This process usually causes a change in few traits of the parental genetic background and, therefore, it becomes particularly relevant, where the preservation of parental organoleptic qualities is crucial (Scowcroft and Larkin 1988).

Changes in single nucleotide polymorphism (snp), insertion and deletions have been identified in regenerated rice plants (Miyao et al. 2012). High frequencies of variations were observed in somaclonal rice traits such as chloroplast, plant height and tillering (Oono 1978; Kucherenko 1979). These variations have been repeatedly used to generate novel rice varieties for breeding purposes (Bertin et al. 1996; Roy and Mandal 2005; Jubair et al. 2008).

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Salinity is one of the most important yield constraints in rice production. Soils with conductivities higher than 4 and 8 dS/m are considered, respectively, moderate and highly saline for rice crops (Gao et al. 2007; Kushwaha et al. 2012). In the southern rice area of Argentina, moderate and high salts contents in the irrigation water were reported (Valenti et al. 2006), representing a potential risk for this crop. However, the somaclonal variation has not been exploited for the genetic improvement of Argentine rices so far. Very limited research has been conducted addressing the influence of this stress on rice speciality quality.

Salinity decreases the photosynthetic efficiency, since the photosynthesis complex II (PSII) is a sensitive target of this stress (Xia et al. 2004; Mehta et al. 2010; Bacarin et al. 2011). The PSII vitality may be measured through the analysis of transient chlorophyll fluorescence rise (OJIP test) after closing all PSII reaction centers with sudden illumination (Strasser et al. 1995).

Data extracted from OJIP analysis can be used to characterize the behavior of PSII. Most used parameters are ratio of variable to maximal chlorophyll *a* fluorescence (F_v/F_m) and the performance index PI_{ABS} (González Moreno et al. 2008). Both F_v/F_m and PI_{ABS} formula have been widely used to screen for salt stress tolerance in several plant species (Percival et al. 2003; Eyidogan and Tufan 2007; Redondo-Gómez et al. 2007; Singh and Sarkar 2014). Other parameters related with PSII, as the density of reaction centers (RC/CS) and the pool size of the electron acceptors of PSII (S_m), have also shown to be affected by salinity in wheat and barley (Mehta et al. 2010; Kalaji et al. 2011).

Malondialdehyde (MDA) is an end-product of fatty acids peroxidation (Møller et al. 2007) and its increment has been related to diminished salt stress tolerance in Durum wheat (Esfandiari et al. 2011).

Infrared measurement can be used to measure leaf temperature, which changes with transpiration rate of the leaves. Therefore, leaf temperature could be used to estimate the water status of the leaves and indirectly its stomatal conductivity (Takai et al. 2010). Sirault et al. (2009) studied salt-stressed barley seedlings, and estimated the physiological status of the plants using leaf temperature registered with infrared measurement.

In Argentina, the most important speciality rice is the double Caroline-type grain, with about 30 % of the market, being Yerua the most commonly used variety. On the other hand, La Candelaria is the outcome of a crossing between a variety of Pakistani origin with excellent aromatic quality. This rice line is cultivated in Argentina, with very good agronomic characteristics. In this work, we hypothesize that somaclones derived from Yerua and La Candelaria rice cultivars vary in their response to salt tolerance (measured as PSII vitality and MDA contents), with respect to the corresponding parental lines.

To the best knowledge of the authors, this is the first study on the effect of somaclonal variation in the parameters of chlorophyll fluorescence analyzed with the OJIP test in rice varieties of special quality. Therefore, the aim of the present work was to explore physiological changes provoked by somaclonal variation on the response to salinity.

Materials and methods

Callus induction and regeneration

Mature dehusked seeds of the rice cultivars La Candelaria (LC) and Yerua (Y) were used in this work. For callus induction, seeds were surface sterilized with 50 % NaOCl in 1 M KH_2PO_4 buffer (pH 5) for 30 min, sown in Petri dishes containing MS basal medium (Murashige and Skoog 1962), 3 % sucrose, 2.5 g/L Phytigel® and 2 mg/L 2,4-Dichlorophenoxyacetic acid (24-D) and cultivated in the dark at 25 °C. After 4 weeks of culture, regeneration was carried out in MS basal medium, 3 % sucrose and 2.5 g/L Phytigel® supplemented with 2 mg/L 6-Benzylaminopurine (BAP), in addition to 0.5 mg/L 1-Naphthaleneacetic acid (NAA). There were 5 calluses per Petri dish, which were incubated at 25 °C with a 16/8-h photoperiod (day/night, 2000 lux light intensity) and subcultured every 2 weeks to fresh, same culture medium thereof. Regenerated shoots were subcultured for rooting, in MS basal medium with 3 % sucrose, then transplanted to pots with soil and further grown in greenhouse until obtaining the R1 seeds.

Assessment of salt stress response

Seeds of LC and Y cultivars, and R1 somaclonal lines were sown on filter paper placed in Petri dishes containing 7 ml of carbendazim solution (25 mg I.A./100 mL) and cultured at 30 °C for germination. Young seedlings were then transplanted to a net covering a 3-L plastic box full of distilled water. After 3 days, the water was replaced by Yoshida nutrient solution (Yoshida et al. 1976) and plantlets were further cultivated at 27–21 °C (day–night) with a 12/12-h photoperiod (day/night, 200 $\mu\text{mol}/\text{m}^2/\text{s}$ of photosynthetic active radiation (PAR) light intensity). 15-day-old plants were salinized with 40 mM NaCl for 24 h and then, salt concentration was adjusted to 80 mM. Addition of 40 mM NaCl was performed, to alleviate the osmotic shock provoked by salt, in agreement to biphasic growth model (Munns and Termaat 1986). NaCl was dissolved in the nutrient solution and pH adjusted as recommended by Gregorio et al. (1997). Nutrient solution without salt addition was used as control treatment.

Final salt concentration was chosen based on previous results of this group (Maiale et al. 2010) where 23 rice lines of 15-day-old plants were subjected to 0, 20, 40 and 80 mM NaCl for 15 days in the same growing conditions as described above. Na/K ratio, fresh and dry weight were measured and the results indicated that 80 mM was the optimal concentration showing differences between rice lines.

In the case of Y and LC cultivars, ten plants were used as replicates and five in the case of R1 somaclonal lines.

Parameters used to evaluate plant response to salt stress were chlorophyll fluorescence fast-transient test (OJIP test), malondialdehyde (MDA) content and leaf temperature. Additionally, the presence of molecular markers for fragrant character in R1 La Candelaria somaclonal lines was also evaluated.

In the experiments performed exclusively with the parental LC and Y lines, F_v/F_m , and PI_{ABS} data were registered 1, 3, 5 and 10 days after the NaCl concentration was adjusted to 80 mM, whereas RC/CS and S_m were registered at the last time point. For R1 somaclonal lines, all measurements were performed after 10 days of salinization.

Chlorophyll fluorescence fast-transient test

Transient fluorescence of PSII (Photosystem II) chlorophyll measurements were made in the newest fully developed leaf with a fluorometer (PocketPEA, Hansatech Instruments®). Data were analyzed by the PEA Plus software (Hansatech Instruments®) and OJIP analysis was performed (Stirbet and Govindjee 2011).

Illuminating a dark-adapted leaf with saturating light pulse results in fast chlorophyll fluorescence rise and in a slower decline known as the Kautsky effect (Kautsky and Hirsch 1931). The fast chlorophyll fluorescence rise recorded with a high-accuracy fluorimeter showed a polyphasic graph when plotted on a logarithmic timescale (Strasser et al. 1995).

This graph shows a basal fluorescence level (F_o), a maximum fluorescence level ($F_p = F_m$) and two intermediate steps namely J (as well as F_j) and I (as well as F_I) (Strasser and Govindjee 1992). The rising phase in fluorescence transient is named as OJIP.

Basal fluorescence level indicates that all reaction centers (RC) are in reduced form (RC open), while F_m indicates that reaction centers are in oxidized form (RC closed).

J step ($F = 2$ ms) is related to reduced Quinone A (QA^-) and I step ($F = 20$ ms) is related to reduction of Quinone B.

The complementary area, calculated as the area above the OJIP curve at the time to reach the F_m , represents pool of electron acceptor. Normalized area ($S_m = \text{area}/F_o - F_m$) indicates the energy needed to close all reaction centers (Stirbet and Govindjee 2011).

Simplified energy flux model is observed in Fig. 1, J_{ABS} is the energy absorbed by chlorophyll pool, J_{TR} is the energy trapped by RC, J_{ET} is the energy in the electron transport chain and JDI is energy dissipated as heat and fluorescence. For another hand, difference between F_o and F_m is called fluorescence variable (F_v) and ratio between F_v and F_m ($F_v/F_m = \phi_{Pho}$) is the maximum quantum yield of primary PSII photochemistry (J_{TR}/J_{ABS}) (Strasser et al. 2000).

Variable fluorescence is normalized with F_v value and different technical fluorescence parameters as V_j ($F_j - F_o/F_v$) or V_i ($F_i - F_o/F_v$) are calculated (Strasser et al. 2000).

Fluorescence signal recorded at 300 μ s is used to calculate the initial slope (M_o) and this indicates the net ratio of RCs closure. M_o is determined as energy trapped for RCs, and energy transfer as electron transport for RCs ($M_o = J_{TR}/RC - J_{ET}/RC$). In DCMU [3-(3',4'-dichlorophenyl)-1,1'-dimethyl urea, inhibitor of reoxidizing QA^-]-treated sample $MoDCMU = J_{TR}/RC = 4$ ms. ($F_{0.3ms} - F_o$)/($F_m - F_o$). However, in the untreated DCMU sample, experimental results indicate that J_{TR}/RC can be obtained as $M_o/V_j = [4 \text{ ms} \times (F_{0.3ms} - F_o)/(F_m - F_o)]/[(F_{2ms} - F_o)/(F_m - F_o)] = 4 \text{ ms} \times (F_{0.3ms} - F_o)/(F_{2ms} - F_o)$ (Stirbet and Govindjee 2011).

As $F_v/F_m = J_{TR}/J_{ABS}$ and $J_{TR}/RC = M_o/V_j$ then, $J_{ABS}/RC = (M_o/V_j)/(F_v/F_m)$ and $RC/J_{ABS} = 1/(J_{ABS}/RC)$.

As M_o indicates net ratio of RCs closure, then $J_{ET}/RC = M_o/V_j - M_o = (M_o/V_j) \times (1 - V_j)$ and then $J_{ET}/J_{TR} = 1 - V_j = \Psi_{ET}$. Phenomenological energy fluxes per excited cross section are an important information derived from the OJIP analysis.

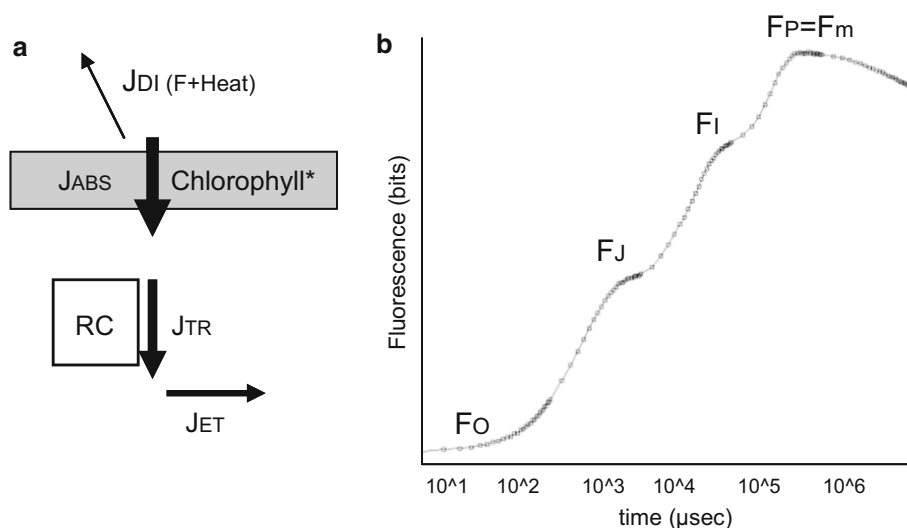
Energy absorbed per cross section (J_{ABS}/CS) is represented by F_o and then RC/CS can be calculated as $RC/CS = (RC/J_{ABS}) \times (J_{ABS}/CS) = RC/J_{ABS} \times F_o$.

Srivastava et al. (1999) introduced a new and a more responsive parameter called performance index (PI) based on absorbed energy (PI_{ABS}). PI_{ABS} can be defined as performance index for energy conservation from photons absorbed by PSII antenna, to the reduction of quinone b. This index is calculated with three components, in which the terms were introduced as ratios and combined properties of PSII. These terms included density of reaction center per energy absorbed basis [$RC/J_{ABS} = \gamma_{rc}/(1 - \gamma_{rc})$], energy trapping efficiency [$\phi_{Pho}/(1 - \phi_{Pho})$] and the electron transport efficiency [$\Psi_{ET}/(1 - \Psi_{ET})$]

$$PI_{ABS} = (\gamma_{rc}/(1 - \gamma_{rc})) \times (\phi_{Pho}/(1 - \phi_{Pho})) \times (\Psi_{ET}/(1 - \Psi_{ET}))$$

In summary, we registered the following parameters: 1—the maximum quantum yield of primary PSII photochemistry (F_v/F_m), 2—performance index for energy conservation from photons absorbed by PSII antenna, to the reduction of quinone b (PI_{ABS}), 3—the active reaction

Fig. 1 Energy flux and OJIP fluorescence curve. Simplified model of energy flux (a); energy absorbed by excited chlorophyll (J_{ABS}), energy trapped by reaction center (J_{TR}), energy dissipated as fluorescence and heat (JDI) and energy in the electron transport chain (J_{ET}). OJIP curve plotted in logarithmic timescale (b); basal fluorescence (F_o), fluorescence a J step (F_J), fluorescence an I step (F_I) and maximum fluorescence ($F_p = F_m$)



centers for cross section (RC/CS) and 4—the energy needed to closed all reaction centers (S_m).

Malondialdehyde measurement

Malondialdehyde (MDA) was indirectly measured in the newest fully developed leaf, 1, 3, 5 and 10 days after NaCl adjustment to 80 mM, through the substances that react with thiobarbituric acid (TBA; Hodges et al. 1999). The tissue was homogenized in a mortar and 40 mg of the homogenate was added to 1 mL of an extraction solution (ethanol:water 80:20 v/v plus 0.01 % butyl-hydroxy toluene). After centrifugation, 0.2 mL of the supernatant was heated with 0.2 mL of 0.65 % TBA in TCA 20 % at 95 °C, 30 min. Then, 0.6 mL of distilled water was added, centrifuged and 0.2 mL of the supernatant was placed in 96-well plates and the absorbance measured at 440, 532 and 600 nm in a micro-plate reader Synergy®. Data were expressed as malondialdehyde equivalents (nmol/g FW) according to Du and Bramlage (1992).

Leaf temperature measurement

Leaf temperature (LT) was measured on whole plant at 1, 5 and 10 days after NaCl adjustment to 80 mM, with a thermographic camera FLIR E-30 (160 × 120 pixels resolution), 0.1 °C thermal sensitivity and 7.5–13 μm spectral range. Measurement was carried out with 0.98 emissivity. Thermal pictures were recorded and analyzed with the ThermoCam Research Pro Software.

Fragrant character evaluation

DNA extraction was carried out according to Murray and Thompson (1980). For PCR the following protocol was used: 2.5 μl of Taq polymerase 10× buffer (PB-L),

0.1 mM dNTPs, 0.25 μL of Taq polymerase (*Taq* Pegasus 5 u/μL, PB-L), and 0.5 μL of 5 μM primer solution and MilliQ water to 25 μL final volume. As template, 1 μL of DNA sample was used. Reaction was performed in a PCR cyclor IVEMA T-18 (Ivema Desarrollos®) as follows: 94 °C 5 min, 94 °C 15 s (30 cycles), 58 °C 15 s, 72 °C 40 s, and a final elongation at 72 °C 5 min. Primers used were those described by Bradbury et al. (2005). Products of PCR were identified by electrophoresis in agarose gel 1.5 % (w/v), and stained with ethidium bromide (ladder 50, PB-L®). Digitalized images of the gel were taken using a FOTO/analyst investigator (CP Photo/Eclipse series 6-1900 Fotodyne).

Statistical data analysis

Statistic analysis was performed with ANOVA ($p < 0.05$) and post test of Tukey was used in parental experiment and Dunnett's in somalconal experiment.

Prism 5® (Graph Pad Prism Inc. CA) software package was used in this work for the statistical analysis and graphics.

Results

Evaluation of LC and Y cultivars' response to saline stress

No significant salt-induced changes were observed in the F_v/F_m values in the LC and Y cultivars, 5 days upon salinization (Fig. 2). In contrast, drastic decreases in the PI_{ABS} were recorded after 10 days of salt treatment in both cultivars (Fig. 2). In parallel, both cultivars showed a sharp and statistically significant salt-induced decrease in RC/CS and S_m at the 10th day after adjustment of salt

Fig. 2 F_v/F_m and PI_{ABS} in rice cultivars subjected to salt stress. F_v/F_m and PI_{ABS} obtained from the OJIP test in La Candelaria (LC) and Yerua (Y) cultivars after 1, 3, 5 and 10 days of treatment with 0 mM NaCl (white bars) and 80 mM NaCl (grey bars). Data were analyzed by ANOVA and Tukey test; asterisk, double asterisk, triple asterisk significant differences between salinized and control plants at each experimental time point, with $p \leq 0.05$, $p \leq 0.001$, $p \leq 0.0001$, respectively ($n = 10$)

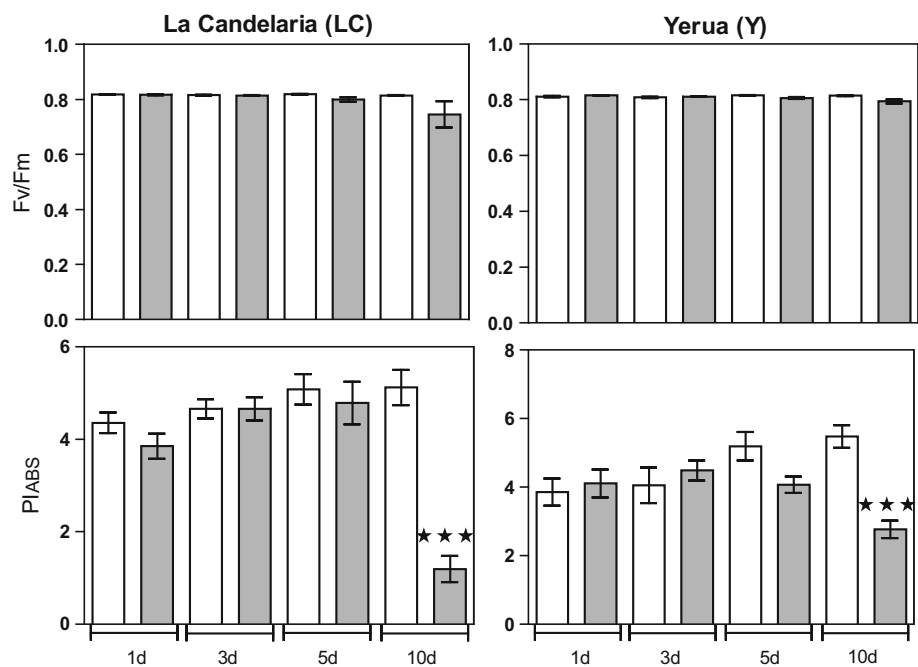
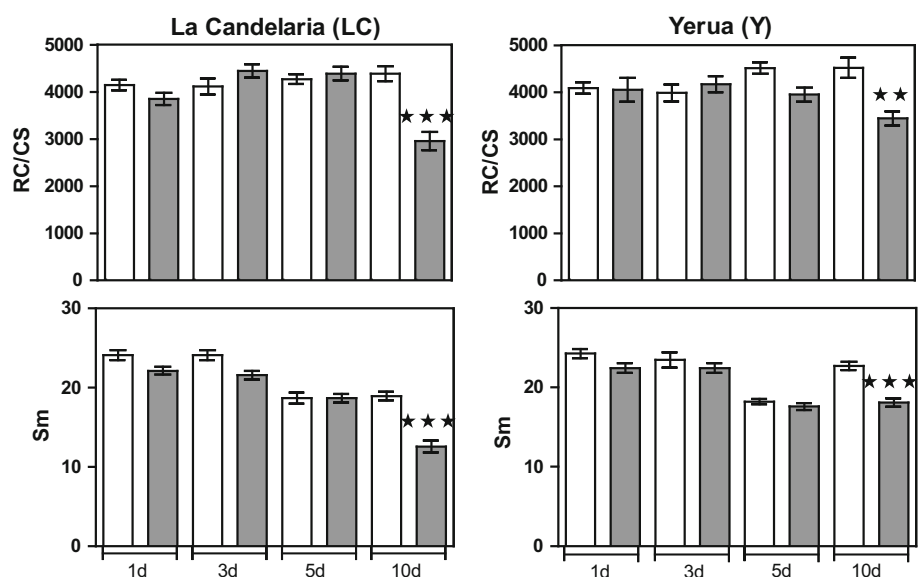


Fig. 3 RC/CS and S_m in rice cultivars subjected to salt stress. RC/CS and S_m obtained from the OJIP test in La Candelaria (LC) and Yerua (Y) after 1, 3, 5 and 10 days of treatment with 0 mM NaCl (white bars) and 80 mM NaCl (grey bars). Data were analyzed by ANOVA and Tukey test; asterisk, double asterisk, triple asterisk significant differences between salinized and control at each experimental time point, with $p \leq 0.05$, $p \leq 0.001$, $p \leq 0.0001$, respectively ($n = 10$)



concentration (Fig. 3), this effect being more pronounced in the LC than in the Y cultivar. On other hand, leaf temperature of salinized plants was about 1 °C higher than that of control plants, in both cultivars for the whole time-lapse (Fig. 4). An exception for the Y cultivar at the 10th day was no difference between salt-treated and control plants was recorded (Fig. 4). Figure 5 shows results from MDA measurements performed at 1, 3, 5, and 10 days upon salinization with 80 mM NaCl. MDA levels sharply increased from the 5th day after the treatment was started in both salinized cultivars, but not in desalinized controls. At the 10th day of salinization, MDA levels were,

respectively, five- and threefold higher in Y and LC, compared with corresponding desalinized controls.

Evaluation of OJIP parameters in salinized R1 somaclonal lines

From former results, it could be concluded that highest response to salt stress can be detected just after 10 days of salt treatment. Therefore, further analysis on somaclonal lines was performed at the 10th day.

The evaluation of R1 somaclonal lines' response to salinization through OJIP parameters showed that LC

somaclones presented a higher variability level in F_v/F_m and PI_{ABS} indexes than Y ones (Figs. 6, 7; Table 1). In fact, coefficients of variation for the F_v/F_m ratio were 13.7 and 1.4 % for LC and Y R1 somaclonal lines, respectively. Coefficients of variation for PI_{ABS} were 39.2 % and 17.6 to LC and Y R1 somaclonal lines, respectively. Also, RC/CS and S_m parameters varied among LC and Y somaclones (Figs. 8, 9), with coefficients of variation within LC and Y somaclonal lines, respectively, being 23.2 and 11.7 % for RC/CS , and 18.3 and 5.9 % for S_m (Table 1).

Although some LC R1 somaclones tended to have higher PI_{ABS} than the parental line under the saline condition, these differences were not statistically significant. In contrast, one somaclone of the Y cultivar presented higher PI_{ABS} than its parental line. Additionally, LT was measured in R1 somaclonal rices at the 10th day after salinity reached 80 mM (Fig. 10). Among LC somaclones, five

lines showed a lower leaf temperature and nine presented a higher leaf temperature, compared with the parental line. In contrast, leaf temperature did not vary in Y somaclonal lines. On other hand, the coefficient of variation was 3 % for both sets of R1 somaclonal lines (Table 1), indicating a low variation level in this parameter.

Despite the few lines with MDA values significantly diverging from those of the parentals (Fig. 11), the coefficients of variation were relatively high in this parameter: 25.5 and 34.4 % for LC and Y, respectively (Table 1). Interestingly, several strong correlations were revealed between MDA and OJIP parameters, in particular PI_{ABS} , with $r = -0.91$ ($p < 0.0001$) for LC, and $r = -0.50$ ($p < 0.0261$) for Y (Table 2).

Finally, changes in the fragrant character of R1 somaclonal LC lines were evaluated with a molecular marker, using the non-aromatic cultivar (Pokkali) as a negative control (Fig. 12). Our marker results showed that all R1 somaclonal lines were homozygous for the aromatic character and similar to the LC parental line.

Discussion

Somaclonal variation as a source of variability for plant improvement has attracted much attention from geneticists and breeders. In this work, we tested the hypothesis that somaclones derived from Yerua and La Candelaria rice cultivars vary in their response to salt tolerance, with respect to the corresponding parental lines. Our results, showing that variation coefficients of the different measured traits related with PSII vitality oscillate from 13.7 to 39.3 % in LC and from 1.4 to 17.6 % in Y R1 somaclones, confirmed the hypothesis. In addition, our results would indicate that R1 somaclones obtained from the LC variety

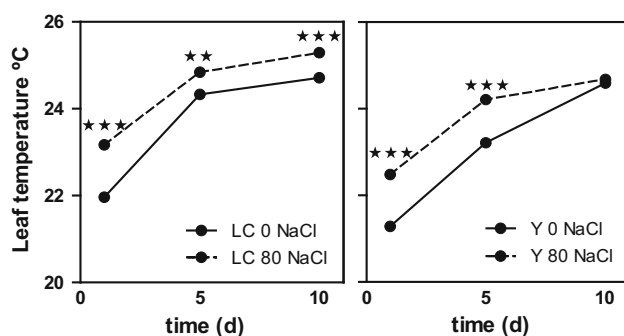


Fig. 4 Leaf temperature in rice cultivars subjected to salt stress. Leaf temperature in La Candelaria (LC) and Yerua (Y) after 1, 5 and 10 days of treatment with 0 mM NaCl (filled line) and 80 mM (dashed line). Data were analyzed by ANOVA and Tukey test; asterisk, double asterisk, triple asterisk significant differences between salinized and control at each experimental time point, with $p \leq 0.05$, $p \leq 0.001$, $p \leq 0.0001$, respectively ($n = 10$)

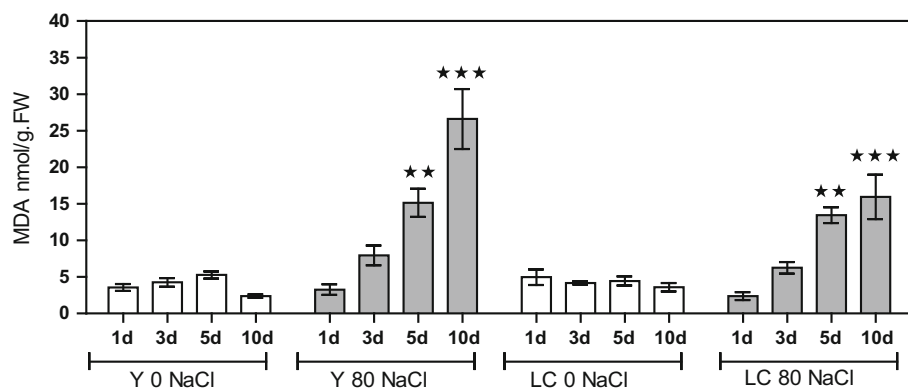


Fig. 5 Lipid peroxidation in rice cultivars subjected to salt stress. Malondialdehyde (MDA) level of plants treated with 0 mM NaCl (white bars) and 80 mM NaCl (grey bars) in cultivars La Candelaria (LC) and Yerua (Y), after 1, 3, 5 and 10 days of treatment with

80 mM NaCl. Data were analyzed by ANOVA and Tukey post test; asterisk, double asterisk, triple asterisk significant differences between salinized and control at each experimental time point, with $p \leq 0.05$, $p \leq 0.001$, $p \leq 0.0001$, respectively ($n = 10$)

Fig. 6 F_v/F_m in somaclonal rice subjected to salt stress. F_v/F_m was obtained from the OJIP test in somaclonal R1 lines from La Candelaria (LC) and Yerua (Y). LCwt and Ywt (grey bars) correspond to parental cultivars. Data were recorded 10 days after treatment reached 80 mM NaCl, and analyzed by ANOVA and Dunnett's post test; *asterisk*, *double asterisk*, *triple asterisk* significant difference from the parental at $p \leq 0.05$, $p \leq 0.001$, $p \leq 0.0001$, respectively, $n = 5$

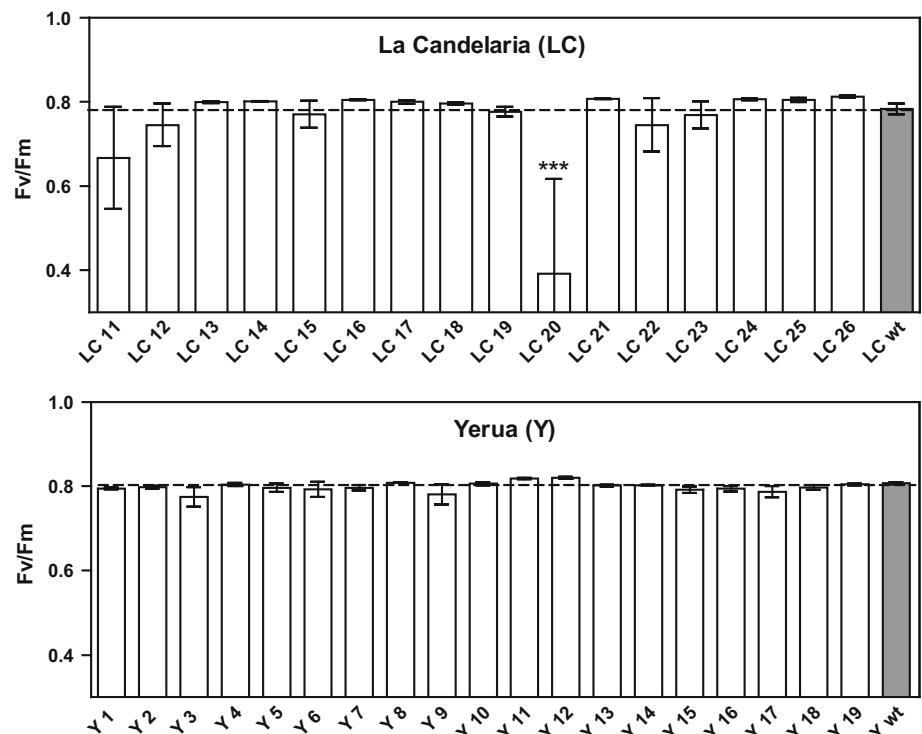
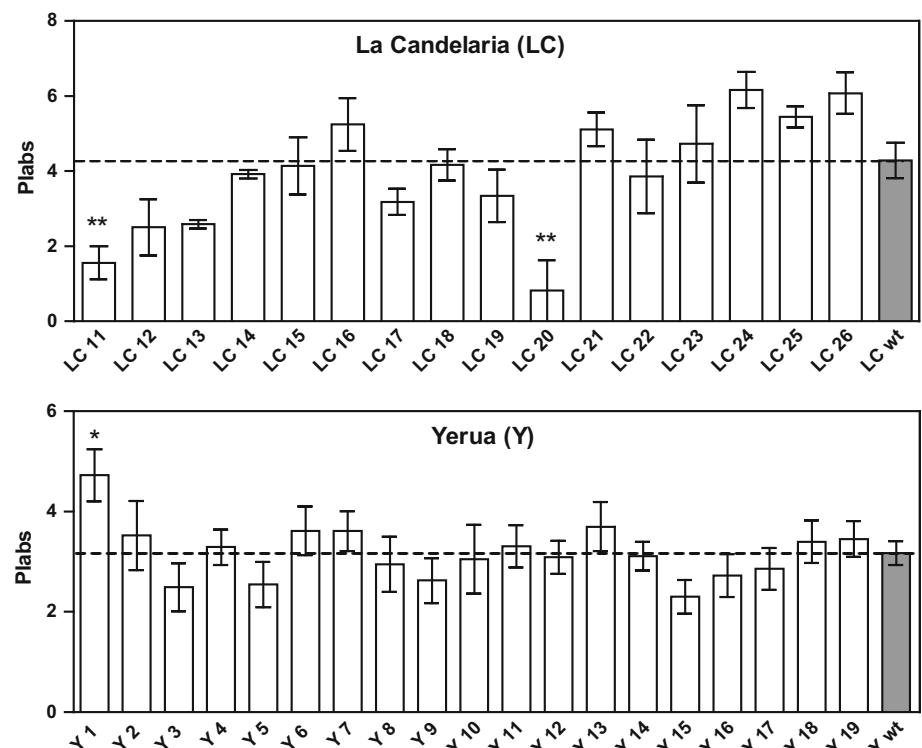


Fig. 7 PI_{ABS} in somaclonal rice subjected to salt stress. PI_{ABS} was obtained from the OJIP test performed on somaclonal R1 lines from La Candelaria (LC) and Yerua (Y). LCwt and Ywt (grey bars) correspond to parental cultivars. Data were recorded 10 days after treatment reached 80 mM NaCl and analyzed by ANOVA and Dunnett's post test; *asterisk*, *double asterisk*, *triple asterisk* significant difference from the parental at $p \leq 0.05$, $p \leq 0.001$, $p \leq 0.0001$, respectively, $n = 5$



experienced a higher degree of variation in their PSII vitality than those of the Y one.

The F_v/F_m ratio reflects the photochemical efficiency of PSII (González Moreno et al. 2008). This ratio presented

the lowest variability among the measured PSII-related parameters. However, F_v/F_m significantly diminished in plants of the LC20 somaclone, which was the only one not surviving to 10 days of 80 mM NaCl. This result is

congruent with the decline in the F_v/F_m values observed in salt stress rice cultivars presenting mortality under an NaCl concentration equivalent to EC 6 dS/m (Singh and Sarkar 2014). Taken together, these results suggest that the F_v/F_m ratio would not be a good marker of PSII vitality as PI_{ABS} for salinized rice somaclones, unless they are highly susceptible to salinity.

The density of the active reaction centers (RC/CS) indicates the number of active RCs in PSII cross section, whereas its decrease reflects a conversion from active to inactive RCs (Mehta et al. 2010). Inactive RCs may trap the energy flux efficiently but fails in reducing the primer electron acceptor (Q_A) in the photosynthetic electron transport chain. Consequently, RCs dissipate all their excitation energy as heat. Our results showing a higher LT

value in salinized plants of the LC and Y cultivars compared with control ones (Fig. 4) could be explained, at least in part, by the reduction in active RCs. However, increased LT could also be due to reduced transpiration, presumably due to stomatal closure, according to the biphasic growth model (Munns and Termaat 1986). The early phase of salinity is characterized by hyperosmotic stress-derived water imbalance.

In chloroplasts, limitation of CO_2 fixation coupled with over-reduction of the electron transport chains is the main cause of ROS production. Whenever the photochemical dissipation and photoprotective mechanisms are insufficient, excited chlorophyll reacts with oxygen to form singlet oxygen (1O_2), and oxygen species especially reactive with polyunsaturated fatty acid (Møller et al. 2007). Malondialdehyde (MDA) is an end-product of peroxidized linoleic acid (Møller et al. 2007), which is a major fatty acid in galactolipids of thylakoid membranes of the plants. It represents about 1 % of fatty acid composition in rice (Lugay and Juliano 1964). Extensive peroxidation of polyunsaturated fatty acids causes oxidative modification in thylakoid proteins of the PSII, such as D1 and D2, affecting the normal PSII function (Okada et al. 1996; Møller et al. 2007; Yamauchi et al. 2008; Yamauchi and Sugimoto 2010). Our results in LC and Y cultivars, showing an increase of MDA over the time of salinization (Fig. 5), are in line with similar observations in salinized plants of rice and other plant species (Li et al. 2010; Wankhade and Sanz 2013). On other hand, MDA levels inversely correlated with PI_{ABS} in LC and Y somaclones, although this

Table 1 Coefficients of variation of OJIP, foliar temperature and malondialdehyde parameters, for R1 somaclonal lines subjected to salt stress

	CV (%)	
	La Candelaria	Yerua
PI_{ABS}	39.3	17.6
F_v/F_m	13.7	1.4
RC/CS	23.2	11.7
S_m	18.3	5.9
LT	3.0	3.0
MDA	25.5	34.4

Coefficient of variation in somaclonal R1 lines of La Candelaria and Yerua at 10 days after treatment with 80 mM NaCl

Fig. 8 RC/CS in La Candelaria and Yerua somaclonal rice subjected to salt stress. RC/CS was obtained from the OJIP test performed on somaclonal R1 lines (white bars) of La Candelaria (LC) and Yerua (Y). LCwt and Ywt correspond to La Candelaria and Yerua parental lines, respectively (grey bars). Data were recorded 10 days after treatment with 80 mM NaCl and analyzed by ANOVA and Dunnett's post test; asterisk, double asterisk, triple asterisk significant difference from the parental at $p \leq 0.05$, $p \leq 0.001$, $p \leq 0.0001$, respectively, $n = 5$

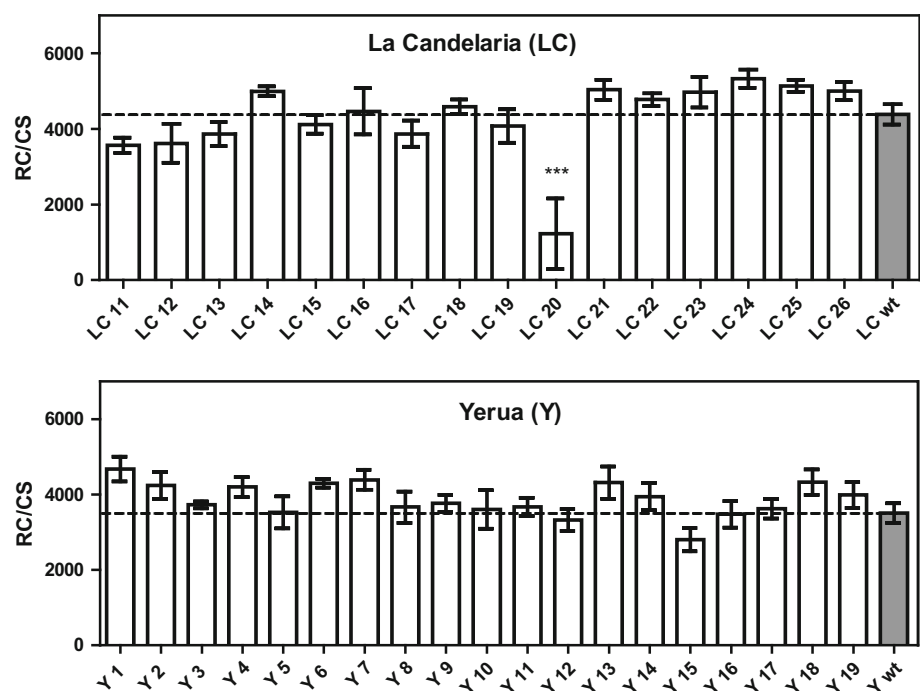


Fig. 9 S_m in La Candelaria and Yerua somaclonal rice subjected to salt stress. S_m data were obtained from the OJIP test performed on somaclonal R1 lines (white bars) of La Candelaria (LC) and Yerua (Y). LCwt and Ywt correspond to La Candelaria and Yerua parental lines, respectively (grey bars). Data were recorded 10 days after treatment with 80 mM NaCl and analyzed by ANOVA and Dunnett's post test; asterisk, double asterisk, triple asterisk significant difference from the parental at $p \leq 0.05$, $p \leq 0.001$, $p \leq 0.0001$, respectively, $n = 5$

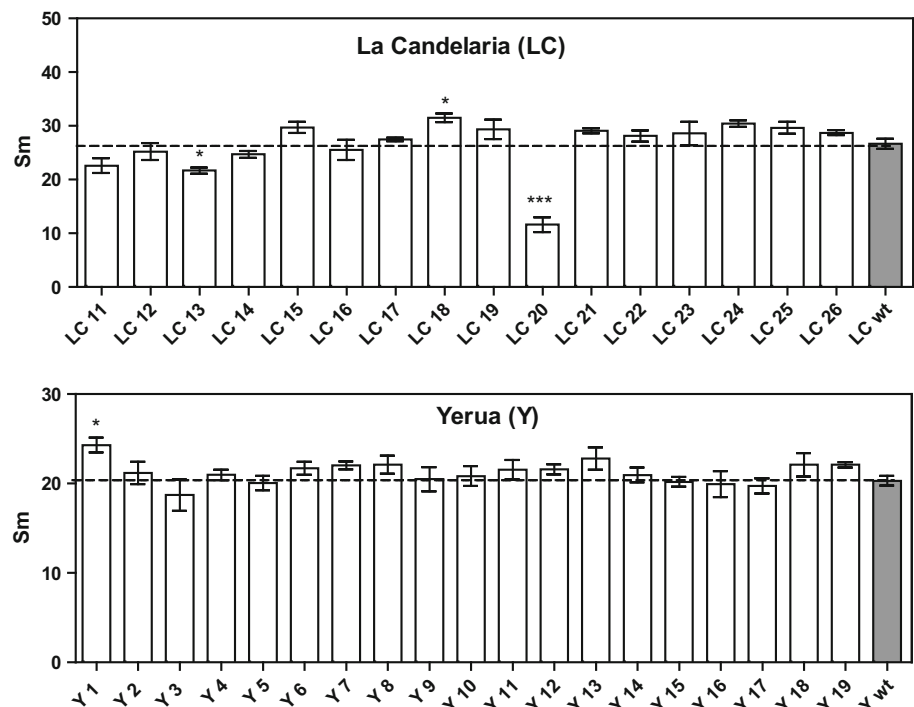
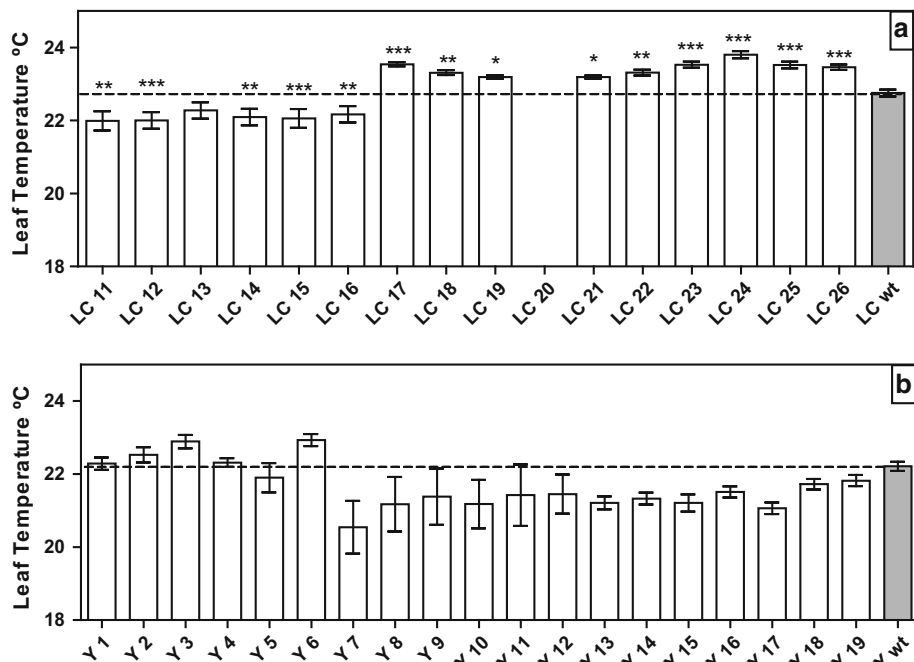


Fig. 10 Leaf temperature in somaclonal R1 rice cultivars subjected to salt stress. Leaf temperature in R1 somaclonal La Candelaria (a) line (white bars) and R1 somaclonal Yerua (b) line (white bars) after 10 days of treatment with 80 mM NaCl. La Candelaria parental line (LCwt) and Yerua parental line (Ywt) are represented with greys bars. Data were analyzed by ANOVA and Dunnett's post test, asterisk, double asterisk, triple asterisk significant differences between salinized and control at each experimental time point, with $p \leq 0.05$, $p \leq 0.001$, $p \leq 0.0001$, respectively ($n = 10$). Data for LC20 were not available on day 10 of salinity treatment due to mortality of all plants



correlation was stronger in LC than in Y-derived somaclones (Table 2). These data suggest not only a differential plant response between LC and Y somaclonal lines when confronted with salinity, but also a higher somaclonal variation in LC, compared with Y. Taken together, these results suggest that MDA levels could also be used as a marker for salinity tolerance in rice somaclones.

The underlying aim of the present work was to explore the use of somaclonal variation as a source for breeding new rice lines, with improved salt tolerance. Our data suggest that somaclonal variation derived from tissue culture may be used in breeding of speciality rice, as it was shown for the Pokkali variety (Mandal et al. 1999). However, although some degree of somaclonal variation was

Fig. 11 Lipid peroxidation in rice somaclonal subjected to salt stress. Malondialdehyde (MDA) levels in somaclonal R1 lines from La Candelaria (**a**) and Yerua (**b**), respectively (white bars). LCwt and Ywt (grey bars) indicate La Candelaria and Yerua parental cultivars. Data were recorded after 10 days of treatment with 80 mM NaCl and analyzed by ANOVA and Dunnett's post test; asterisk, double asterisk, triple asterisk significant difference from the parental at $p \leq 0.05$, $p \leq 0.001$, $p \leq 0.0001$, respectively, $n = 5$

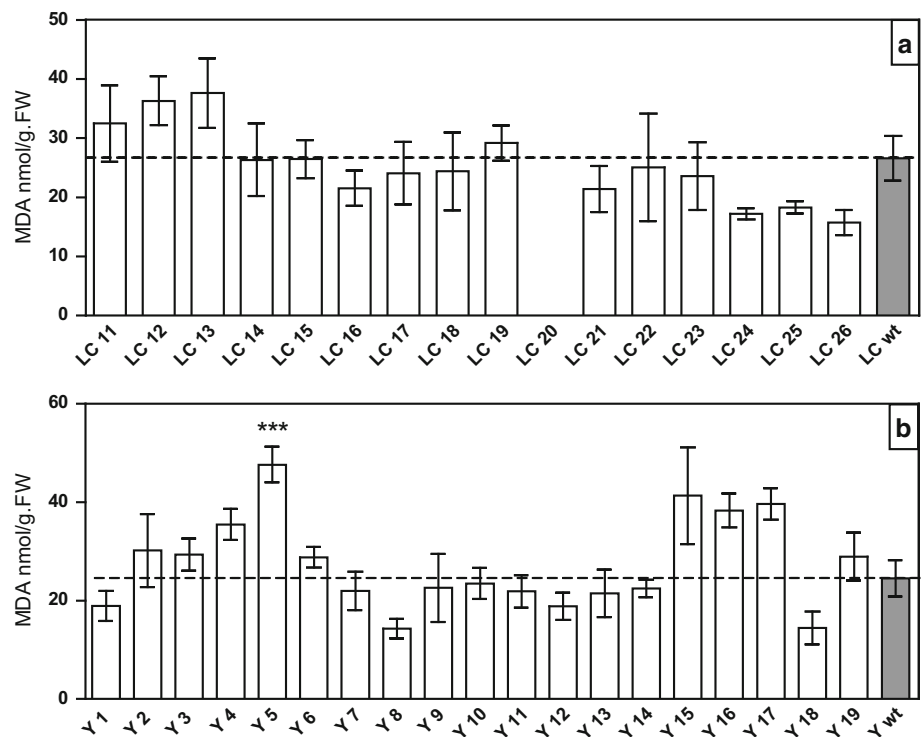


Table 2 Pearson correlations among OJIP, foliar temperature and malondialdehyde parameters, in R1 somaclonal lines subjected to salt stress

LC	PI _{ABS}	F_v/F_m	RC/CS	S_m	LT	MDA
PI _{ABS}	1					
F_v/F_m	0.71**	1				
RC/CS	0.86***	0.88***	1			
S_m	0.75***	0.84***	0.85***	1		
LT	0.58*	0.42 ns	0.60*	0.72**	1	
MDA	-0.91***	-0.54*	-0.82***	-0.69**	-0.68**	1
Y	PI _{ABS}	F_v/F_m	RC/CS	S_m	LT	MDA
PI _{ABS}	1					
F_v/F_m	0.27 ns	1				
RC/CS	0.84***	-0.05 ns	1			
S_m	0.88***	0.46*	0.64**	1		
LT	0.19 ns	-0.29 ns	0.32 ns	-0.06 ns	1	
MDA	-0.50*	-0.36 ns	-0.43 ns	-0.64**	0.17 ns	1

Pearson correlation in somaclonal R1 lines of La Candelaria (LC) and Yerua (Y) after 10 days of treatment with 80 mM NaCl

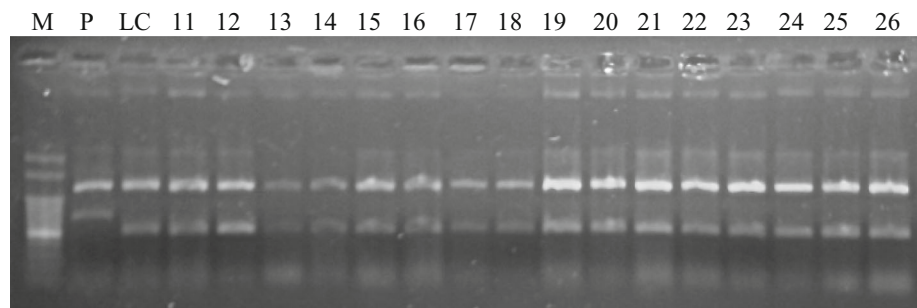
*, **, *** significant difference at $p \leq 0.05$, $p \leq 0.001$, $p \leq 0.0001$, respectively, $n = 5$

detected in this work; somaclones with improved PSII functioning were not obtained. It has been suggested that the induced mutational effect of the hormone 2,4-D contained in the culture media could be a possible source for variations induced by the tissue culture (Sun et al. 1983). Therefore, future research should consider the adjustment of the 2,4-D concentration for the increase of variability.

Interestingly, LC R1 somaclones did not present variations in their fragrant character. The last result encourages further studies in search of somaclones of this aromatic rice variety, with better tolerance to stress.

Author contribution statement Monica Rachoski: experimental work. Ayelen Gazquez: experimental work.

Fig. 12 Image shows the marker for fragrant character (Bradbury et al. 2005) on agarose 1, 5 % (w/v) gel, stained with ethidium bromide *M* marker, *P* Pokkali (homozygous not aromatic), *LC* La Candelaria (homozygous aromatic), *11–26* R1 LC somaclonal lines



Pablo Calzadilla: data analysis. Rodolfo Bezus: seed supplier. Andres Rodriguez: data analysis. Oscar Ruiz: laboratory chief. Ana Menendez: data analysis and manuscript reduction. Santiago Maiale: data analysis and manuscript reduction.

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