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A rice mutant defective in antioxidant-defense system and sodium homeostasis possesses increased sensitivity to salt stress

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Abstract

Screening salt-sensitive mutants is a powerful method to identify genes associated with salt tolerance. We used forward genetic screening with sodium azide-mutated rice (*Oryza sativa* L. cv. Tainung 67) to identify mutants showing hypersensitivity to salt stress. A new mutant line, named *salt hypersensitive 1* (*shs1*) and exhibiting a severe salt-sensitivity when grown under a high NaCl concentration, was identified; the salt hypersensitivity was caused by duplicate recessive epistasis with mutations likely in two different loci. The *shs1* salt sensitive phenotypes included a decreased seed germination rate, reduced shoot height and root length, severe and quick wilting, and overaccumulation of sodium ions in shoots as compared with wild-type plants. In addition, *shs1* showed a decreased photosynthetic efficiency and enhanced hydrogen peroxide (H_2O_2) production under the salt stress. An increased superoxide dismutase activity and decreased catalase activity were responsible for the hyperaccumulation of H_2O_2 in *shs1*. The hypersensitivity of *shs1* to the salt stress might be caused by an impaired antioxidant machinery and cellular Na^+ homeostasis.

Additional key words: catalase, chlorophyll fluorescence, hydrogen peroxide, *Oryza sativa*, oxidative stress, salt hypersensitive mutant, superoxide dismutase.

Introduction

Approximately 20 % of the Earth's land and nearly a half of the irrigated land are affected by salinity (Prasad *et al.* 2000, Mahajan and Tuteja 2005, Chikelu *et al.* 2007) and rice is the most salt-sensitive crop among cereals (Munns and Tester 2008). Rice may experience salt stress at germination and seedling or reproductive growth stages and is considered most susceptible to salt stress at the 2- to 3-leaf stage (Zheng *et al.* 2001). Excessive Na^+ accumulation in plants grown under a high salinity greatly reduces the photosynthetic capacity of plant leaves (Munns 2002) and increases production of reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\bullet}$), which leads to oxidative stress and cell death (Gill and Tuteja 2010). Plants possess efficient antioxidant enzymes that function as ROS scavengers to protect cells against oxidative damage (Foyer and Harbison 1994). For example, superoxide dismutase (SOD; EC1.15.1.1) dismutates $O_2^{\bullet-}$ to H_2O_2 and O_2. Hydrogen peroxide is scavenged by catalase (CAT; EC 1.11.1.6) which hydrolyzes H_2O_2 to H_2O and O_2 (Gill and Tuteja 2010). Salinity stress causes an imbalance between ROS generation and scavenging that leads to oxidative damage. Thus, increased antioxidant activity is an important mechanism plants use to enhance tolerance to salt stress (Gill and Tuteja 2010).

Excluding Na^+ ions and maintaining a low Na^+ content in roots and shoots is another important mechanism which plants use to tolerate a high salinity (Golldack *et al.* 2003, Ren *et al.* 2005, Munns and Tester 2008). Many studies have reported the crucial role of certain Na^+ transporters in maintaining a low shoot tissue

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Abbreviations: AsA - ascorbate; APX - ascorbate peroxidase; CAT - catalase, DAB - 3,3-diaminobenzidine; F_v/F_m - variable to maximum chlorophyll *a* fluorescence ratio; GR - glutathione reductase; H_2O_2 - hydrogen peroxide; NBT - nitroblue tetrazolium; NB - Nona Bokra; $OH^{\bullet}$ - hydroxyl radical; ROS - reactive oxygen species; SOD - superoxide dismutase; *shs1 - salt hypersensitive 1*; $O_2^{\bullet-}$ - superoxide; TNG67 - Tainung 67.

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Na^+ content under a high salinity in *Arabidopsis*, durum wheat, bread wheat, and rice (Shi *et al.* 2002, James *et al.* 2006, Martinez-Atienza *et al.* 2007, Munns *et al.* 2012).

Plants have evolved complex salt-responsive signaling and metabolic processes at cellular, organ, and whole plant levels to cope with salt stress. Understanding the genetic control mechanisms underlying salt tolerance greatly facilitates the development of salt-tolerant cultivars. Mapping studies have identified quantitative trait loci of several physiological traits related to salt tolerance at various growth stages in rice (Sabouri *et al.* 2009, Thomson *et al.* 2010, Islam *et al.* 2011, Zhou *et al.* 2013). Cytosolic *ASCORBATE PEROXIDASE 2* (*OsAPX2*) and chloroplast/mitochondrion co-localized *GLUTATHIONE REDUCTASE 3* (*OsGR3*) have been showed to play an important role in rice protection against abiotic stresses by scavenging ROS (Zhang *et al.* 2013, Wu *et al.* 2015). A salt-induced receptor-like kinase OsRMC acts as negative regulator of salt stress response in rice (Serra *et al.* 2013). Transgenic rice over-expressing the *Na^+/H^+ antiporter 1* (*OsNHX1)* exhibits a higher salt tolerance (Fukuda *et al.* 2004). A *drought and salt tolerance* (*dst*) mutant isolated from an ethyl methanesulfonate-mutated M2 population of the *japonica* variety Zhonghua 11 shows an enhanced tolerance to drought and salt *via* a stomatal aperture control (Huang *et al.* 2009). The rice salt sensitive 1 (RSS1) protein is key factor in maintaining meristematic cell division in both shoots and roots by regulating cell cycle under salt stress (Ogawa *et al.* 2011).

The aim of this research was to identify the *salt-hypersensitive 1* (*shs1*) rice mutant by a forward genetic screening sodium-azide (NaN_3)-mutated *Oryza sativa* L. cv. Tainung 67 (TNG67) and follow its growth and development under a salt stress. In addition, photosynthetic efficiency, Na^+ content, and activities of antioxidant enzymes SOD and CAT were compared with the wild type.

Materials and methods

Salt-sensitive rice mutants were screened from a sodium azide (NaN3) induced *Oryza sativa* L. cv. TNG67 mutant (M_{10}) population comprising 460 mutant lines treated with 150 mM NaCl, and severity of damage was scored (Gregorio *et al.* 1997). One of the salt-hypersensitive mutants, *shs1*, was further investigated. After screening another eight generations by the pedigree method, the M_{18} pure line of *shs1* was used for physiological and biochemical assays. Surface-sterilized seeds were germinated on water-soaked filter paper (*Whatman No. 1*). The seedlings were grown hydroponically in a half-strength Kimura B solution (Yoshida *et al.* 1972) in a phytotron (Agricultural Experimental Station, National Taiwan University, Taipei) with a natural irradiance, day/night temperatures about 30/25 °C, and a 90 % relative humidity. For the test of salt sensitivity of the wild-type TNG67 and *shs1*, the rice salt-tolerant cultivar Nona Bokra and salt-sensitive cultivar IR28 (Igarashi *et al.* 1997) were used as controls. Two-week-old seedlings were used in all experiments.

For growth analysis, two-week-old seedlings underwent various NaCl treatments (0, 100, 150, 200, and 250 mM) for 3 d. The shoot and root lengths of the seedlings were measured with a ruler and changes were calculated as percentage of the length of the control plants treated with the hydroponic solution only. For biomass analysis, shoots and roots were divided for measurement of fresh mass at the end of the treatments. For dry mass estimation, tissues were dried at 70 °C for 2 d. For germination assay, at least 100 surface-sterilized seeds were germinated on water-soaked filter paper. Seeds were considered as germinated when at least 0.5 cm of coleoptiles and radicles had emerged.

For survival rate assay, two-week-old seedlings underwent the NaCl treatment (150 mM) for 0, 3, 5, and 7 d and a recovery with a half-strength Kimura B solution for 7 d. Surviving shoots showed a green colour. Photosynthetic efficiency was evaluated according to a variable to maximum chlorophyll *a* fluorescence ratio (F_v/F_m) according to (Kitajima and Butler 1975). After the seedlings underwent the NaCl treatments (0, 100, 150, 200, and 250 mM) for 3 d, the 2nd leaves were darkened for at least 10 min before measurement of F_v/F_m using a *Junior-PAM* fluorometer (Walz, Germany).

For Na^+ and K^+ determinations, shoot and root tissues were dried at 70 °C for 2 d, incubated with 70 % (v/v) HNO_3 and 70 % (v/v) $HClO_4$ at 200 °C for 1 h and then dissolved in distilled water (Yoshida *et al*. 1972). Content of Na^+ and K^+ was quantified by use of flame photometry (*Model M410*, *Sherwood Scientific*, Cambridge, UK).

Hydrogen peroxide was visually detected in the 2nd leaves with 3,3-diaminobenzidine (DAB) used as substrate (Orozco-Cárdenas and Ryan 1999). The leaves were cut and incubated with a DAB (1 mg cm^{-3}) solution for 12 h under an irradiance of 50 μmol m^{-2} s^{-1} and a temperature of 27 °C, then in 95 % (v/v) ethanol for 12 h which decolorized the leaves except for a brown polymerization product produced by DAB with H_2O_2. H_2O_2 staining was repeated 4 times with similar results. Hydrogen peroxide content in leaves of TNG67 and *shs1* was estimated according to Patterson *et al.* (1984). A reaction mixture consisted of 2 cm^3 of a 50 mM phosphate buffer (pH 6.8), 0.5 cm^3 of a plant extract and 1 cm^3 of a reagent [0.1 % (v/v) $TiCl_4$ in 20 % (v/v) H_2SO_4]. The plant extract was prepared from the 2nd leaves of two-week-old rice seedlings. Leaf tissue was ground in liquid nitrogen and extracted with a 50 mM phosphate buffer. Absorbance was measured at 410 nm. The amount of H_2O_2 was calculated using a standard curve. In ascorbate (AsA) pretreatment, the 2nd leaves were incubated in a 2 mM AsA containing hydroponic solution for 6 h before the salt treatment. After the AsA pretreatment, the rice seedlings were moved into a hydroponic solution with or without 150 mM NaCl for H_2O_2 content determination, and the 2nd leaves were cut for DAB staining.

For extraction of enzymes, leaf tissue was homogenized with a 0.1 M sodium phosphate buffer (pH 6.8) using a chilled pestle and mortar. Superoxide dismutase activity was determined as described by Giannopolitis and Ries (1977); one unit of SOD activity was defined as the amount of the enzyme that inhibited the rate of nitroblue tetrazolium (NBT) photoreduction by 50 %. The activity of CAT was estimated by monitoring a decrease in absorbance at 240 nm due to H_2O_2 reduction (coefficient of absorbance of 39.4 M^{-1} cm^{-1}) according to Roland and Irwin (1951). One enzyme unit was defined as the amount of H_2O_2 consumed per min. Values were corrected by subtracting those obtained in the absence of substrate or enzymatic extracts. Protein content of the enzyme extracts was measured by the Bradford (1976) method.

Data are expressed as means ± SEs. Statistical differences were analyzed by Student's *t* test or Duncan's multiple range test. A value $P < 0.05$ was considered as statistically significant.

Results

Screening 460 NaN_3-induced rice mutants associated with salt tolerance revealed 8 mutant lines exhibiting a severe injury under the high salt stress as compared with the wild type. The mutants were named *salt hypersensitive* (*shs*). One of the mutants, SA0604, also termed as *shs1*, was selected for morphological and functional characterization. The salt hypersensitivity of the mutant was tested in every generation to confirm mutant purity. Segregation analysis of the F_2 population from a cross between *shs1* and TNG67 plants revealed a digenic segregation with a 9:7 (152:132) ratio for salt sensitivity:salt tolerance. Thus, the salt sensitivity in *shs1* was caused by duplicate recessive epistasis, and the salt hypersensitivity was likely caused by mutation at two different loci. The *shs1* was screened for another eight generations by the pedigree method, and the M_{18} homozygous lines of *shs1* were used for physiological and biochemical assays.

To determine the effect of *shs1* mutation on growth and development of rice, changes in germination rate, shoot height, root length, and survival were examined under the control and NaCl-stress conditions. The *shs1* displayed salt sensitivity at the germination and seedling stages. In the control and NaCl-stressed plants, seed germination started within 24 h of imbibition for the TNG67 seeds and after 24 h for the *shs1* seeds (Fig. 1*A*). The TNG67 and *shs1* seeds exhibited 100 and 45 % germination rates, respectively, on day 3 under the control conditions. The *shs1* achieved a 100 % germination on day 6 under the control conditions (Fig. 1*A*). Under the high NaCl concentrations, germination rates decreased in both the TNG67 and *shs1* seeds to 65 and 10 % on day 3, 90 and 50 % on day 6, and 97 and 67 % on day 10, respectively, as compared to the control conditions (Fig. 1*A*). Thus, compared to TNG67, the *shs1* seeds showed a reduced germination rate under the salt stress until day 10 and a delayed germination under both the control and NaCl-stress conditions. The shoot height and root length (Fig. 1*B*) gradually decreased with the increasing NaCl concentration in the two-week-old TNG67 and *shs1* seedlings. The shoot height was more inhibited in the *shs1* than TNG67 seedlings with a NaCl concentration > 200 mM. The root length did not differ between *shs1* and TNG67 (Fig. 1*C*). When the seedlings were treated with 150 mM NaCl for 5 d, only

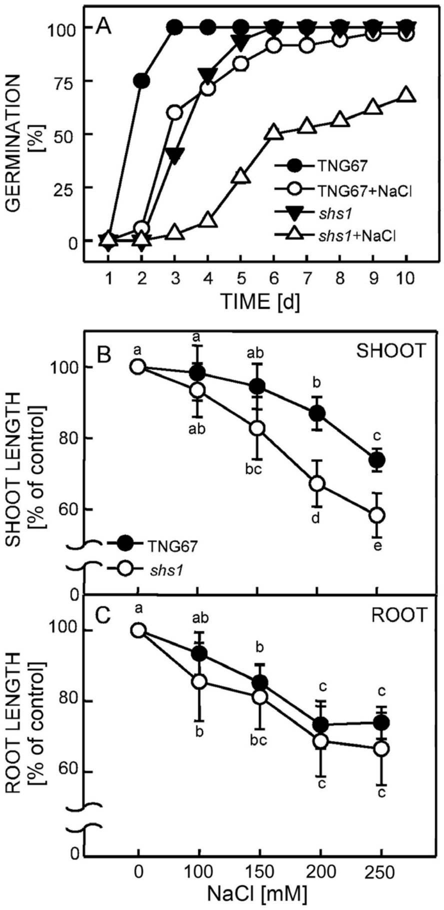

Fig. 1. Effects of NaCl on seed germination and growth of rice seedlings. *A* - Quantification of germination rate of TNG67 and *shs1* with or without 150 mM NaCl. Each measurement consisted of at least 100 seeds. *B* - Shoot height represented as percentage of control plants in two-week-old TNG67 and *shs1* after 3-d treatment with indicated NaCl concentrations. *C* - Root length represented as percentage of control plants in two-week-old TNG67 and *shs1* after 3-d treatment with indicated NaCl concentrations. Data are means ± SE ($n = 10$). Points with different letters are significantly different at $P < 0.05$.

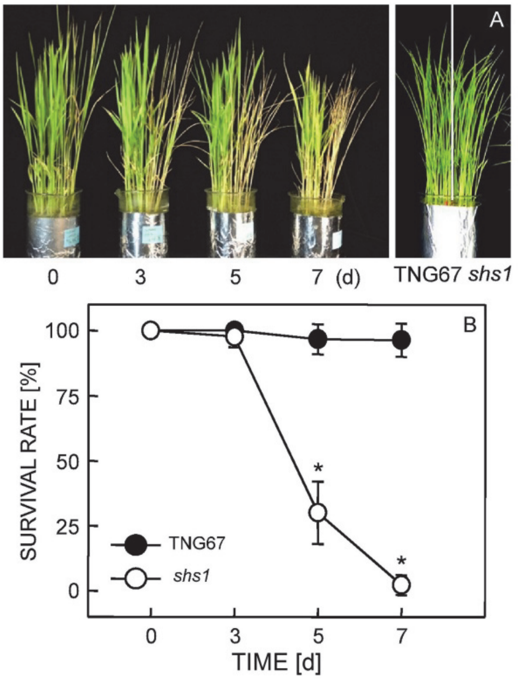

Fig. 2. Survival of TNG67 and *shs1* seedlings under NaCl treatment. *A* - Two-week-old TNG67 and *shs1* seedlings grown in the same pots (as indicated in right panel) were treated with 150 mM NaCl for different periods and then recovered for one week before pictures were taken. *B* - Quantification of survival. Data are means ± SE (n = 50). * - Significant differences between TNG67 and *shs1* at $P < 0.05$.

25 % of the *shs1* seedlings survived, and no seedlings survived after 7 d of the treatment (Fig. 2*A,B*). In contrast, TNG67 was barely affected by the salt stress. Hence, *shs1* exhibited a more rapid and more severe wilting symptoms than the wild-type plants. Because of growth and developmental defects exhibited by *shs1* under the salinity, it was considered as hypersensitive salt-stress mutant.

To understand the salt-sensitivity differences of TNG67 and *shs1*, we performed a physiological analysis comparing TNG67 and *shs1* with a salt-tolerant rice cultivar Nona Bokra (NB) and a salt-sensitive cultivar IR28. All cultivars showed similar morphologic features under the control conditions (Fig. 3*A*). However, under the NaCl treatment, the IR28 and *shs1* plants showed wilting symptoms unlike NB and TNG67 which displayed tolerance to the salt stress (Fig. 3*A*). The IR28 and *shs1* showed a gradual decrease in dry mass with the increasing NaCl concentration as compared with TNG67, with a similar decrease in both the cultivars (Fig. 3*B*). The decrease in dry mass production caused by salinity was less severe in NB and TNG67 than in IR28 or *shs1*, which further confirms that the two former cultivars were salt-tolerant. Moreover, the dry mass inhibition was less pronounced for TNG67 than NB, so TNG67 was more tolerant to the salt stress than NB (Fig. 3*B*).

Photosynthetic efficiency was determined in leaves of NB, IR28, TNG67, and *shs1* by analysis of the F_v/F_m ratio. With the increasing NaCl concentration, the IR28 and *shs1* plants showed a rapid decrease in photosynthetic efficiency but no change was noticed in the NB and TNG67 plants (Fig. 3*C*). Also, comparison of germination rates showed no significant change between IR28 and *shs1* under the control conditions. Under the salt stress, the germination rate decreased in IR28 and *shs1* as compared to the control plants; *shs1* exhibited a more pronounced decrease in germination rate as compared with IR28 (Fig. 3*D*). Therefore, TNG67 was more tolerant to the salt stress, and *shs1* exhibited a salt-sensitivity phenotype analogous to IR28.

To understand whether salt hypersensitivity is due to the overaccumulation of Na^+ ions, we determined Na^+ content in the shoots of the TNG67 and *shs1* plants grown under 0 and 150 mM NaCl for 72 h. Both the TNG67 and *shs1* plants showed an increased Na^+ content under 150 mM NaCl (Fig. 4*A*). However, after 9 h of the NaCl treatment, the *shs1* plants showed a more pronounced accumulation of Na^+ ions as compared with the TNG67 plants (Fig. 4*A*). The *shs1* showed a lower K^+ content as compared with TNG67 under the control conditions (Fig. 4*B*). The *shs1* plants showed an increased accumulation of K^+ ions from 3 to 24 h of the NaCl treatment and a higher K^+ content than TNG67 from 6 to 12 h of the NaCl treatment. After 12 h of the NaCl treatment, *shs1* showed a sudden decrease in K^+ content which was lower than that in TNG67 after 48 h (Fig. 4*B*). The *shs1* showed a slightly greater Na^+/K^+ ratio under the control conditions, a lower Na^+/K^+ ratio from 6 to 12 h after the NaCl treatment, and a greater Na^+/K^+ ratio after 24 h of the NaCl treatment than the TNG67 plants (Fig. 4*C*). In contrast, these plant types did not differ in accumulation of Na^+ in roots (data not shown). Therefore, Na^+ overaccumulation in shoots might be responsible for the salt-hypersensitivity phenotype of *shs1*.

Changes in H_2O_2 content are indicators of the ROS scavenging capacity of plants under oxidative stress. To monitor the efficiency of ROS scavenging capacity in TNG67 and *shs1*, we estimated H_2O_2 content in the seedlings during exposure to 150 mM NaCl for 3 d. The TNG67 plants showed a steady-state content of H_2O_2 regardless of a number of days of the NaCl treatment (Fig. 5*A*). In contrast, the *shs1* plants showed a significantly increased H_2O_2 content with the salt stress. The H_2O_2 content increased four-fold in *shs1* as compared with TNG67 under the high salt stress, which indicates that H_2O_2 scavenging was more efficient in TNG67 (Fig. 5*A*). Surprisingly, H_2O_2 content was two-fold higher in *shs1* than in TNG67 even under the control conditions, which might explain the salt-hypersensitivity of *shs1*. To confirm that the hypersensitivity of *shs1* under the salt stress could be due to H_2O_2 hyper-accumulation, we pretreated the plants with AsA which impairs H_2O_2 accumulation in plants. Under both the control conditions and the salt stress conditions, H_2O_2 accumulation decreased in *shs1* (Fig. 5*B*). These results suggest that H_2O_2 hyperaccumulation participated in salt hypersensitivity of *shs1*.

Because H_2O_2 content varied significantly between TNG67 and *shs1*, we determined the activities of CAT, SOD, ascorbate peroxidase (APX), and glutathione reductase (GR) in the TNG67 and *shs1* seedlings under 150 mM NaCl for 3 d. Both TNG67 and *shs1* showed an increase in SOD activity with the salt stress; however, the SOD activity increased 1.5 times in the *shs1* plants at day 1 and relatively less in the TNG67 plants (Fig. 6*A*). The CAT activity decreased in both TNG67 and *shs1* with the NaCl stress. The CAT activity was reduced to almost 50 % of the non-treated control in the *shs1* plants, but in the TNG67 plants, it was less reduced. Interestingly, the CAT activity was lower in both the non-stressed seedlings and the stressed seedlings of *shs1* than of TNG67 (Fig. 6*B*). In contrast, the APX and GR activities did not change in both TNG67 and *shs1* with the salt stress (data not shown). Thus, *shs1* showed H_2O_2 hyperaccumulation, a decreased CAT activity, and an increased SOD activity under the salt stress.

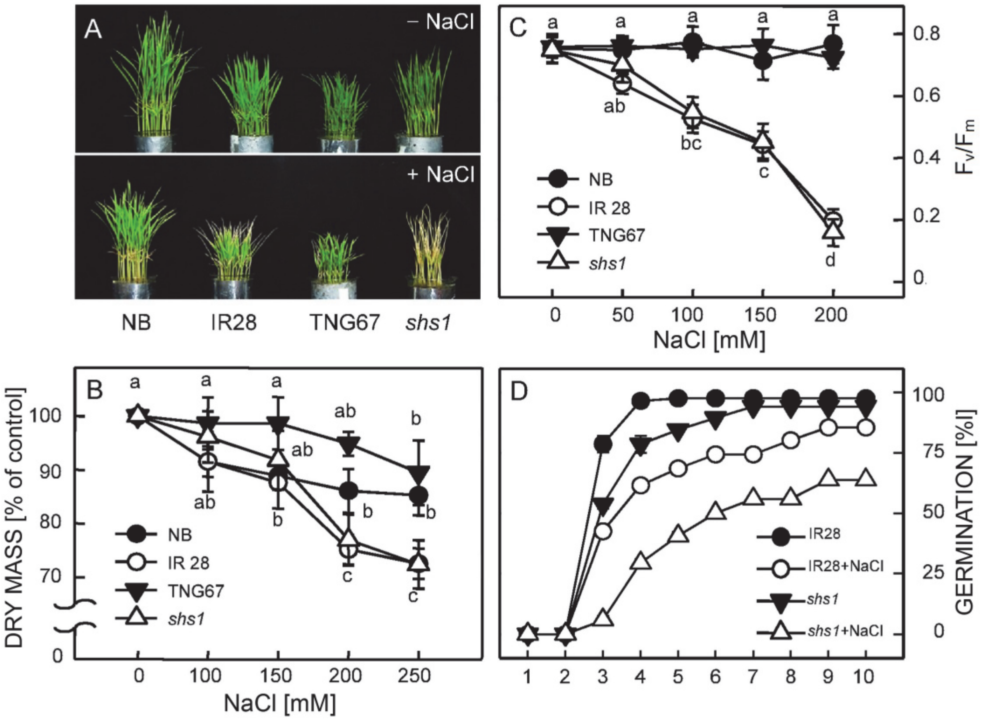

Fig. 3. Comparison of TNG67 and *shs1* to salt-tolerant and salt-sensitive rice cultivars. *A* - Phenotypic responses of Nona Bokra (NB, salt-tolerant cultivar), IR28 (salt-sensitive cultivar), and TNG67 and *shs1* under the NaCl treatment. Two-week-old hydroponically cultivated seedlings were treated with or without 150 mM NaCl for 3 d and recovered for 5 d. *B* - The inhibition percentage of dry mass. Each measurement consisted of 50 seedlings. Data are means ± SE. *C* - F_v/F_m of seedlings under NaCl treatment. Two-week-old seedlings were treated with 150 mM NaCl for 3 d, and 2nd leaves were used for F_v/F_m analysis. Data are means ± SE ($n = 4$). *D* - The germination rate and vigor of IR28 and *shs1* treated with or without 150 mM NaCl for 10 d. Each measurement represents at least 100 seeds. Points with different letters are significantly different at $P < 0.05$.

Discussion

A high Na^+ content within a plant is highly toxic and greatly affects growth, development, and survival. In this study, we isolated a mutant *shs1* that was hypersensitive to the salt stress both morphologically and physiologically. The *shs1* plants showed severe growth and developmental defects under the high NaCl concentrations along with a decreased seed germination rate (Figs. 1,2). Wilting symptoms appeared quickly and were severe, which indicated that the plants were extremely sensitive to the stress. In comparison of germination rate, dry mass, and photosynthetic efficiency with a salt-tolerant rice cultivar NB and a salt-sensitive cultivar IR28, the mutant *shs1* was similar to IR28, which further confirms the salt-hypersensitivity of *shs1* (Fig. 3). Similar morphological defects have been reported for other rice salt-sensitive mutants. A rice salt-sensitive mutant *rss1* shows an extreme dwarf and short-root phenotype under a high salinity (Ogawa *et al.* 2011). Similarly, another salt-sensitive mutant *rss2* shows severe wilting after exposure to NaCl (Zhou *et al.* 2013). The salt sensitivity of *rss2* is caused by over-accumulation of Na^+ in shoots. Over-accumulation of Na^+ ions in leaf blades is a major physiological consequence of high salt stress in salt-sensitive plants and leads to a reduced total photosynthetic leaf area (Munns 2002, Davenport *et al.* 2005, Munns and Tester 2008). We found a higher Na^+

accumulation in the *shs1* than TNG67 plants under the NaCl treatment (Fig. 4*A*). A salt-sensitive plant differs from a salt-tolerant one by its inability to prevent salt from accumulating to toxic levels in transpiring leaves (Munns *et al.* 2006). For instance, the salt-sensitive cv. IR29 accumulated a 5- to 10-fold greater content of Na^+ than salt-tolerant lines BK or Pokkali (Golldack *et al.* 2003). Therefore, the salt-hypersensitivity phenotype of *shs1* was partially due to over-accumulation of Na^+ ions. The decrease in the survival of *shs1* under the salt stress could be explained by the over-accumulation of Na^+ and a reduced photosynthetic efficiency.

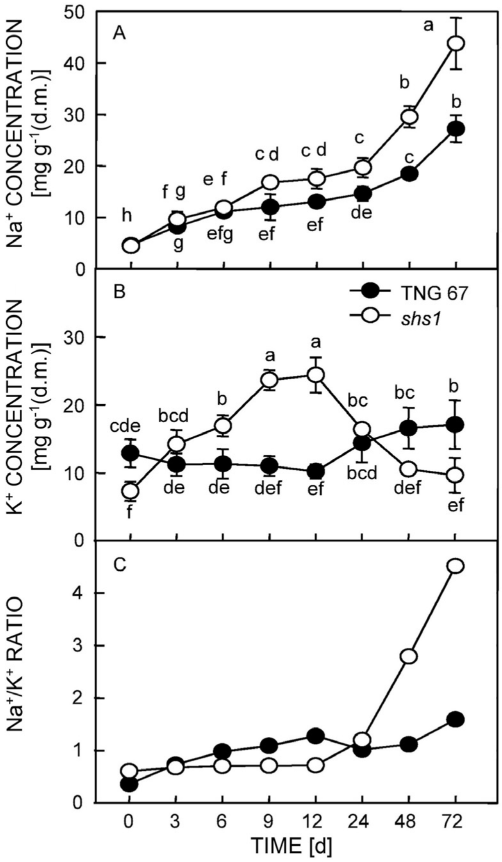

Fig. 4. Na^+ (*A*) and K^+ (*B*) content and an Na^+/K^+ ratio (*C*) in shoots of two-week-old TNG67 and *shs1* seedlings treated without or with 150 mM NaCl for 0 to 72 h. Means ± SE ($n = 4$). Points with different letters are significantly different at $P < 0.05$. Na^+/K^+ ratios are significantly different only after 48 and 72 h.

Because the gradual accumulation of Na^+ causes tissue injury and premature death of older leaves (Flowers *et al.* 1991, Munns 1993), the exclusion of Na^+ from shoot tissue and maintenance of a low Na^+/K^+ ratio is an important mechanism for salt tolerance in plants (Ren *et al.* 2005). We observed a significantly greater Na^+/K^+ ratio in the *shs1* than TNG67 plants after 24 h of the salt stress, which indicates the salt-sensitivity of *shs1* (Fig. 4*C*). Interestingly, the Na^+/K^+ ratio was higher in the *shs1* than TNG67 plants under the control conditions. During 6 to 12 h of the salt treatment, the K^+ content was higher, and the Na^+/K^+ ratio was lower in the *shs1* than TNG67 plants (Fig. 4*A,C*). However, a reason behind these changes is not known. Transcriptomic and proteomic analyses have revealed changes in sodium and potassium channels/transporters, such as a plasma membrane Na^+/H^+ antiporter, salt overly sensitive 1 (SOS1) (Shi *et al.* 2002), a high-affinity potassium transporter (OsHKT) (Ren *et al.* 2005, Sunarpi *et al.* 2005, James *et al.* 2006, Davenport *et al.* 2007, Yao *et al.* 2010, Waters *et al.* 2013), AKT family channels, and KUP/HAK/KT family (HAK) transporters (Bañuelos *et al.* 2002, Amrutha *et al.* 2007), involved in compartmentation, exclusion, and transportation of Na^+ and K^+. These findings may explain the changes in the ion accumulation between the *shs1* and TNG67 plants.

Increased ROS production under salt stress is represented by H_2O_2 accumulation both inside and outside the cell (Yang *et al.* 2007). The H_2O_2 content was

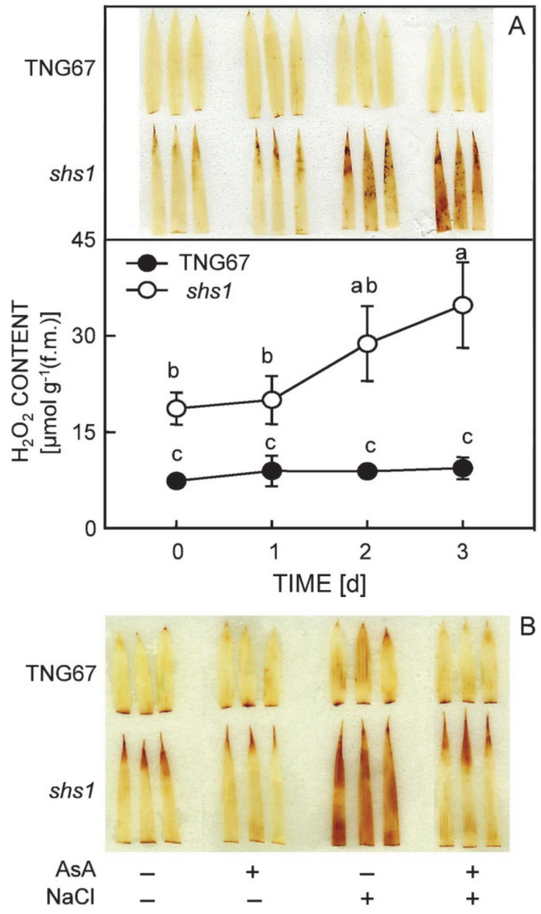

Fig. 5. H_2O_2 content in TNG67 and *shs1* seedlings under NaCl treatment. *A* - Histochemical detection of H_2O_2 with 3,3-diaminobenzidine (DAB) staining (the *upper part*) and H_2O_2 content (the *lower part*) in the 2nd leaves of TNG67 and *shs1* seedlings after treatment with 150 mM NaCl for up to 3 d. Means ± SE ($n = 4$). Points with different letters are significantly different at $P < 0.05$. (*B*) - DAB-staining in the 2nd leaves pretreated with 2 mM ascorbate and exposed to 150 mM NaCl.

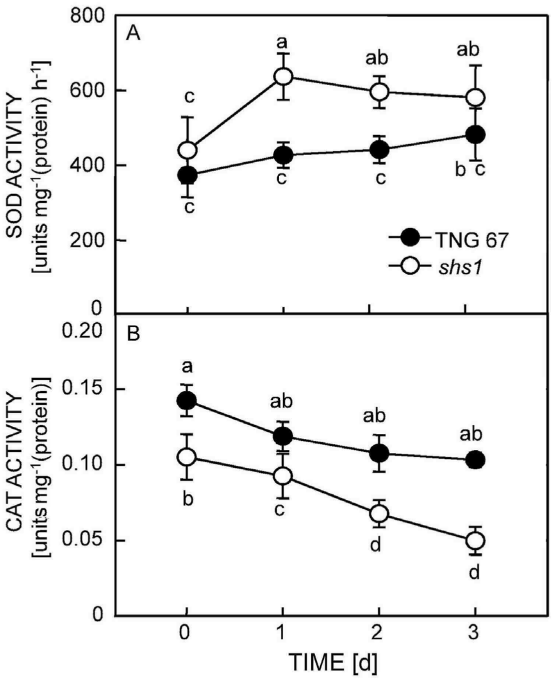

Fig. 6. Antioxidant enzyme activities of TNG67 and *shs1* seedlings under NaCl treatment. Superoxide dismutase (SOD) activity (*A*) and catalase (CAT) activity *(B)* in shoots of NaCl (150 mM) treated two-week-old seedlings. The CAT and SOD activities were determined 1, 2, or 3 d after the addition of NaCl. Means ± SEs (*n* = 4). Points with different letters are significantly different at $P < 0.05$.

greater in the *shs1* than in TNG67 plants under the salt stress, which indicates a greater oxidative stress damage to *shs1*.

The pretreatment with AsA effectively reduced the accumulation of H_2O_2 in *shs1*, so the hypersensitivity of *shs1* to the salt stress might result in part from an inefficient antioxidant defense system (Fig. 5). The analysis of the antioxidant enzyme activities showed an increased SOD activity and a decreased CAT activity in *shs1* under the salt stress (Fig. 6), which is probably the main cause of the H_2O_2 over-accumulation in *shs1* shoots under the high NaCl concentration. Apart from SOD and CAT, H_2O_2 production due to salt stress depends on NADPH oxidase in rice (Yang *et al.* 2007, Hong *et al.* 2009). Thus, the accumulation of H_2O_2 in *shs1* might have several explanations including dysfunctional enzymatic or non-enzymatic ROS scavenging systems (Asada 1999) or disordered ROS generators such as NADPH oxidase (Hossain *et al.* 1984), amine oxidase (Alessandra *et al.* 2006), and glutathione peroxidase (Miao *et al.* 2006).

The *shs1* showed an extreme salt sensitivity and an impaired growth under the salt stress. The salt hypersensitivity of *shs1* might be caused by an imbalanced Na^+ homeostasis and/or impaired ROS-scavenging antioxidant enzyme activities. Considering its role in growth and development under salt stress, *SHS1* identification will be highly beneficial for future breeding programs to generate salt-tolerant rice cultivars. Map-based gene cloning could be used to identify and characterize the function of candidate gene(s) associated with the *SHS1* locus.

References

Alessandra, C., Giuseppina, R., Riccardo, A., Rodolfo, F., Paraskevi, T.: Functions of amine oxidases in plant development and defense. - Trends Plant Sci. **11**: 80-88, 2006.

Amrutha, R.N., Sekhar, P.N., Varshney, R.K., Kishor, P.: Genome-wide analysis and identification of genes related to potassium transporter families in rice (*Oryza sativa* L.). - Plant Sci. **172**: 708-721, 2007.

Asada, K.: The water-water cycle in chloroplasts: scavenging of active oxygen and dissipation of excess photons. - Annu. Rev. Plant Physiol. Plant mol. Biol. **50**: 601-639, 1999.

Bañuelos, M.A., Garciadeblas, B., Cubero, B., Rodríguez-Navarro, A.: Inventory and functional characterization of the HAK potassium transporters of rice. - Plant Physiol. **130**: 784-795, 2002.

Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Chikelu, M., Rownak, A., Shri, M.J., Glenn, B.G., Francisco, J.Z.A.: Induced mutations for enhancing salinity tolerance in rice. - In: Jenks, M.A., Hasegawa, P.M., Jain, S.M. (ed.): Advances in Molecular Breeding Towards Drought and Salt Tolerant Crops. Pp. 413-454. Springer, Dordrecht 2007.

Davenport, R., James, R. A., Zakrisson-Plogander, A., Tester, M., Munns, R.: Control of sodium transport in durum wheat. - Plant Physiol. **137**: 807-818, 2005.

Davenport, R. J., Muñoz-Mayor, A., Jha, D., Essah, P. A., Rus, A., Tester, M.: The Na^+ transporter AtHKT1 controls xylem retrieval of Na^+ in *Arabidopsis*. - Plant Cell Environ. **30**: 497-507, 2007.

Foyer, C.H., Harbison, J.: Oxygen metabolism and the regulation of photosynthetic electron transport. - In: Foyer, C.H., Mullineaux, P. (ed.) Causes of Photo-oxidative Stresses and Amelioration of Defense Systems in Plants. Pp. 1-42. CRC Press, Boca Raton 1994.

Flowers, T.J., Hajibagheri, M.A., Yeo, A.R.: Ion accumulation in the cell walls of rice plants growing under saline conditions: evidence for the Oertli hypothesis. - Plant Cell Environ. **14**: 319-325, 1991.

Fukuda, A., Nakamura, A., Tagiri, A., Tanaka, H., Miyao, A., Hirochika, H., Tanaka, Y.: Function, intracellular localization and the importance in salt tolerance of a vacuolar Na^+/H^+ antiporter from rice. - Plant Cell Physiol. **45**: 149-159, 2004.

Giannopolitis, C.N., Ries, S.K.: Superoxide dismutases: I. Occurrence in higher plants. - Plant Physiol. **59**: 309-314, 1977.

Gill, S.S., Tuteja, N.: Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. - Plant Physiol. Biochem. **48**: 909-930, 2010.

Golldack, D., Quigley, F., Michalowski, C.B., Kamasani, U.R., Bohnert, H.J.: Salinity stress-tolerant and sensitive rice (*Oryza sativa* L.) regulate AKT1-type potassium channel transcripts differently. - Plant mol. Biol. **51**: 71-81, 2003.

Gregorio, G.B., Senadhira, D., Mendoza, R. D.: Screening Rice for Salinity Tolerance. - IRRI Discussion Paper No. 22. International Rice Research Institute, Manila 1997.

Hong, C.Y., Chao, Y.Y., Yang, M.Y., Cho, S.C., Kao, C.H.: Na^+ but not Cl^- or osmotic stress is involved in NaCl-induced expression of glutathione reductase in roots of rice seedlings. - J. Plant Physiol. **166**: 1598-1606, 2009.

Hossain, M.A., Nakano, Y., Asada, K.: Monodehydroascorbate reductase in spinach chloroplast and its participation in regeneration of ascorbate for scavenging hydrogen peroxide. - Plant Cell Physiol. **11**: 351-358, 1984.

Huang, X.Y., Chao, D.Y., Gao, J.P., Zhu, M.Z., Shi, M., Lin, H.X.: A previously unknown zinc finger protein, DST, regulates drought and salt tolerance in rice via stomatal aperture control. - Genes Dev. **23**: 1805-1817, 2009.

Huang, Y.G., Zhang, F., Wu, J., Chen, J., Zhou, M.: Differences in physiological traits among salt-stressed barley genotypes. - Commun. Soil Sci. Plant Anal. **37**: 557-570, 2006.

Igarashi, Y., Yoshiba, Y., Sanada, Y., Yamaguchi-Shinozaki, K., Wada, K., Shinozaki, K.: Characterization of the gene for Δ^1-pyrroline-5-carboxylate synthetase and correlation between the expression of the gene and salt tolerance in *Oryza sativa* L. - Plant mol. Biol. **33**: 857-865, 1997.

Islam, M.R., Salam, M.A., Hassan, L., Collard, B.C.Y., Singh, R.K., Gregorio, G.B.: QTL mapping for salinity tolerance at seedling stage in rice. - J. Food agr. Sci. **23**: 137-146, 2011.

James, R.A., Davenport, R.J., Munns, R.: Physiological characterization of two genes for Na^+ exclusion in durum wheat, Nax1 and Nax2. - Plant Physiol. **142**: 1537-1547, 2006.

Kitajima, M., Butler, W.L.: Quenching of chlorophyll fluorescence and primary photochemistry in chloroplasts by dibromothymoquinone. - Biochim. biophys. Acta **376**: 105-115, 1975.

Martinez-Atienza, J., Jiang, X., Garciadeblas, B., Mendoza, I., Zhu, J.K., Pardo, J.M., Quintero, F.J.: Conservation of the salt overly sensitive pathway in rice. - Plant Physiol. **143**: 1001-1012, 2007.

Mahajan, S., Tuteja, N.: Cold, salinity and drought stresses: an overview. - Arch. Biochem. Biophys. **444**: 139-158, 2005.

Miao, Y., Dong, L., Wang, P., Wang, X.C., Chen, J., Miao, C., Song, C.P.: An *Arabidopsis* glutathione peroxidase functions as both a redox transducer and a scavenger in abscisic acid and drought stress responses. - Plant Cell **18**: 2749-2766, 2006.

Munns, R.: Physiological processes limiting plant growth in saline soil: some dogmas and hypotheses. - Plant Cell Environ. **13**: 143-160, 1993.

Munns, R.: Comparative physiology of salt and water stress. - Plant Cell Environ. **25**: 239-250, 2002.

Munns, R., James, R.A., Lauchli, A.: Approaches to increasing the salt tolerance of wheat and other cereals. - J. exp. Bot. **57**: 1025-1043, 2006.

Munns, R., James, R.A., Xu, B., Athman, A., Conn, S.J., Jordans, C., Byrt, C.S., Hare, R.A., Tyerman, S.D., Tester, M., Plett, D., Gilliham, M.: Wheat grain yield on saline soils is improved by an ancestral Na^+ transporter gene. - Nat. Biotechnol. **30**: 360-364, 2012.

Munns, R., Tester, M.: Mechanisms of salinity tolerance. - Annu. Rev. Plant Biol. **59**: 651-681, 2008.

Ogawa, D., Abe, K., Miyao, A., Kojima, M., Sakakibara, H., Mizutani, M., Takeda, S.: RSS1 regulates the cell cycle and maintains meristematic activity under stress conditions in rice. - Nat. Commun. **2**: 1-11, 2011.

Orozco-Cárdenas, M.L., Ryan, C.: Hydrogen peroxide is generated systemically in plant leaves by wounding and systemically in via the octadecanoid pathway. - Proc. nat. Acad. Sci. USA **96**: 6553-6557, 1999.

Patterson, B.D., Macrae, E.A., Ferguson, I.B.: Estimation of hydrogen peroxide in plant extracts using titanium (IV). - Anal. Biochem. **139**: 487-492, 1984.

Prasad, S.R., Bagali, P.G., Shailaja, H., Shashidhar, H.E., Hittalmani, S.: Molecular mapping of quantitative trait loci associated with seedling tolerance to salt stress in rice (*Oryza sativa* L.). - Curr. Sci. **78**: 162-164, 2000.

Ren, Z.H., Gao, J.P., Li, L.G., Cai, X.L., Huang, W., Chao, D.Y., Zhu, M.Z., Wang, Z.Y., Luan, S., Lin, H.X.: A rice quantitative trait locus for salt tolerance encodes a sodium transporter. - Nat. Genet. **37**: 1141-1146, 2005.

Roland, F.B., Irwin, W.S.: A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. - J. biol. Chem. **195**: 133-140, 1951.

Sabouri, H., Rezai, A.M., Moumeni, A., Kavousi, A., Katouzi, M., Sabouri, A.: QTLs mapping of physiological traits related to salt tolerance in young rice seedlings. - Biol Plant. **53**: 657-662, 2009.

Serra, T.S., Figueiredo, D.D., Cordeiro, A.M., Almeida, D.M., Lourenço, T., Abreu, I.A., Sebastián, A., Fernandes, L., Contreras-Moreira, B., Oliveira, M.M.: OsRMC, a negative regulator of salt stress response in rice, is regulated by two AP2/ERF transcription factors. - Plant mol. Biol. **82**: 1-17, 2013.

Shi, H., Quintero, F.J., Pardo, J.M., Zhu, J.K.: The putative plasma membrane Na^+/H^+ antiporter SOS1 controls long-distance Na^+ transport in plants. - Plant Cell **14**: 465-477, 2002.

Sunarpi Horie, T., Motoda, J., Kubo, M., Yang, H., Yoda, K., Horie, R., Chan, W.Y., Leung, H.Y., Hattori, K., Konomi, M., Osumi, M., Yamagami, M., Schroeder, J.I., Uozumi, N.: Enhanced salt tolerance mediated by AtHKT1 transporter-induced Na unloading from xylem vessels to xylem parenchyma cells. - Plant J. **44**: 928-938, 2005.

Thomson, M.J., Ocampo, M., Egdane J., Rahman M.A., Sajise, A.G., Adorada, D.L., Tumimbang-Raiz, E., Blumwald, E., Seraj, Z.I., Singh, R.K., Gregorio, G.B., Ismail, A.M.: Characterizing the *saltol* quantitative trait locus for salinity tolerance in rice. - Rice **3**: 148-160, 2010.

Waters, S., Gilliham, M., Hrmova, M.: Plant high-affinity potassium (HKT) transporters involved in salinity tolerance: structural insights to probe differences in ion selectivity. - Int. J. mol. Sci. **14**: 7660-7680, 2013.

Wu, T.M., Lin, W.R., Kao, C.H., Hong, C.Y.: Gene knockout of glutathione reductase 3 results in increased sensitivity to salt stress in rice. - Plant mol. Biol. **87**: 555-564, 2015.

Yao, X., Horie, T., Xue, S., Leung, H.Y., Katsuhara, M.,E. Brodsky, D., Wu, Y.I., Schroeder, J.: Differential sodium and potassium transport selectivities of the rice OsHKT2;1 and OsHKT2;2 transporters in plant cells. - Plant Physiol. **152**: 341-355, 2010.

Yang, Y.L., Xu, S.J., An, L.Z., Chen, N.L.: NADPH oxidase-dependent hydrogen peroxide production, induced by salinity stress, may be involved in the regulation of total calcium in roots of wheat. - J. Plant Physiol. **164**: 1429-1435, 2007.

Yoshida, S., Forno, D.A., Cock, J.H., Gomez, K.A.: Laboratory Manual for Physiological Studies of Rice. 2nd Ed. - The International Rice Research Institute, Los Baños 1972.

Zhang, Z., Zhang, Q., Wu, J., Zheng, X., Zheng, S., Sun, X., Qiu, Q., Lu, T.: 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, 2013.

Zheng, L., Shannon, M.C., Lesch, S.M.: Timing of salinity stress affects rice growth and yield components. - Agr. Water Manage. **48**: 191-206, 2001.

Zhou, J., Wang, F., Deng, P., Jing, W., Zhang, W.: Characterization and mapping of a salt-sensitive mutant in rice (*Oryza sativa* L.). - J. Integr. Plant Biol. **55**: 504-513, 2013.