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Salt tolerance enhancement in *indica* rice (*Oryza sativa* L. spp. *indica*) seedlings using exogenous sucrose supplementation

Kongake Siringam[1], Niran Juntawong[1], Suriyan Cha-um[2*] Thanapol Boriboonkaset[3] and Chalermpol Kirdmanee[2]

[1]Department of Botany, Faculty of Science, Kasetsart University, Chatuchak, Bangkok 10900, Thailand
[2]National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency (NSTDA), Klong Luang, Pathumthani 12120, Thailand
[3]Department of Biotechnology, Faculty of Science, Mahidol University, Payathai, Bangkok 10400, Thailand

***Corresponding author: suriyanc@biotec.or.th**

Abstract

Salt-affected soil is an abiotic stress which is an acute problem, interrupting the metabolic processes of plants, resulting in reduced growth and productivity. The objective of this study was to enhance the salt tolerance ability of Pathumthani 1 (PT1) sensitive rice cultivars using exogenous sucrose. Fourteen-day-old seedlings of PT1, along with Homjan (HJ), salt-tolerant (positive control), were cultured in MS liquid medium supplemented with 0.0, 29.2, 58.4 or 116.8 mM sucrose, then exposed to 0.0 or 342.0 mM NaCl. An osmotic potential (Ψ_s) in the leaf tissues of rice seedlings dropped significantly when subjected to 342 mM NaCl. Exogenous sugar application in the culture medium was directly absorbed and enriched in rice seedling, leading to soluble sugar accumulation and played a key role as osmoregulation of salt defense mechanism. The increase in Ψ_s in the leaf tissues of salt stressed seedlings directly caused damage to the ultrastructure of chloroplstic organelles, as well as to photosynthetic pigments i.e. chlorophyll a, chlorophyll b and total chlorophyll. Chlorophyll content and chlorophyll fluorescence parameters were stabilised in salt-stressed rice seedlings pre-treated with 58.4 mM sucrose, resulting in enhanced growth performance. A positive correlation between photosynthetic pigment degradation and chlorophyll fluorescence diminution was demonstrated. Exogenous application of 58.4 mM sucrose to the culture medium may play a role as an alternative way to enhance salt tolerance in rice, especially in PT1, a salt-susceptible cultivar.

Keywords: chlorophyll fluorescence, fructose, glucose, photosynthetic pigments, salt stress, osmotic potential.

Abbreviations: Chl_a_chlorophyll a; Chl_b _chlorophyll b; C_{x+c}_total carotenoids; CW_cell wall; DW_dry weight; F_v/F_m_maximum quantum yield of PSII; FW_fresh weight; GR_granum; IN_inner membrane; LA_leaf area; OT_outer membrane; P_n_net photosynthetic rate; PPFD_photosynthetic photon flux density; qP_photochemical quenching; RH_relative humidity; SL_stroma lamellae; ST_stroma; TC_total chlorophyll; TEM_transmission electron microscope; Φ_{PSII}_photon yield of PSII; Ψ_s_osmotic potential.

Introduction

Salinity is a cause of abiotic stress, resulting in reduced growth and loss of productivity in crop species. Excessive amounts of salts, especially sodium chloride (NaCl), in the soil induce osmotic and ionic effects, leading to changes in plant metabolism (Hasegawa et al., 2000; Qadir et al., 2008) and reduced plant growth and development in many crop species (Cha-um et al., 2004; Singh et al., 2007; de Lacerda et al., 2005; Chen et al., 2007). Salt-stress defence mechanisms, including ion regulation and compartmentalisation, antioxidant systems, plant hormones and osmoregulation, are well established (Hasegawa et al., 2000; Parida and Das, 2005). The osmoregulation defence mechanism is one of the best candidate functions with which to adjust the cell osmotic pressure using low-molecular-mass compounds, for example carbohydrate, proline, glycine betaine and sugar alcohol (Hasegawa et al., 2000), without biochemical interference. Sugar may play key role in salt defence mechanisms, including membrane stability, *via* interaction with phospholipid head groups and reactive oxygen species detoxification (Bohnert and Jensen, 1996; Bentsink et al., 2000; Roy et al., 2005). Moreover, soluble sugar, produced by photosynthesis in higher plants and a major energy source, also plays a critical role in signal transduction in primary and secondary metabolites, including the building blocks of macromolecules in the developmental processes of plants (Smeekens, 2000; Price et al., 2004). An accumulation of sugars in salt stressed plants has been demonstrated in rice (Cha-um et al., 2009a; Pattanagul and Thitisaksakul, 2008), wheat (Tammam et al., 2008), barley (Ahmad et al., 2006), *Lactuca sativa* (Hasaneem et al., 2009) and tomato (Mohamed and Ismail, 2011). However, photosynthetic pigment degradation, chloroplast destruction, chlorophyll fluorescence diminution and net photosynthetic rate reduction in salt stressed plants have been reported (Yamane et al., 2008; Cha-um et al., 2009a, b). So, increasing sugar levels in crop plants, using exogenous sucrose application, is being investigated, especially in plant tissue

Table 1. Sucrose, glucose and fructose concentration (µmol g^{-1} FW) in root and leaf tissues of *indica* rice pre-treated with 0.0, 29.2, 58.4 or 116.8 mM sucrose and subsequently exposed to 0 or 342 mM NaCl for 4 days.

Rice varieties	Sucrose (mM)	NaCl (mM)	Roots		Leaves	
			Glucose	Fructose	Glucose	Fructose
HJ	0.0	0	53.7 c	50.0 c	155.9 g	133.5 f
HJ	0.0	342	48.8 e	49.7 c	369.0 e	397.0 d
PT1	0.0	0	47.8 f	48.4 d	194.5 f	193.9 e
PT1	0.0	342	37.4 g	39.6 e	411.0 d	430.2 c
PT1	29.2	342	51.5 d	40.7 e	481.7 c	446.1 c
PT1	58.4	342	92.2 b	84.1 b	989.5 b	995.7 a
PT1	116.8	342	242.0 a	237.0 a	1084.8 a	619.4 b

Means with the different letters are significantly different at $P \leq 0.01$ (**) by Duncan's New Multiple Range Test.

culture (Chen et al., 2003; Javed and Ikram, 2008; Unno and Maeda, 2008).

Rice is major grain crop and carbohydrate source, supplying the necessary daily calories for more than half the world's population (Dawe, 1999; Khush, 2005). It has been predicted that the demand for rice in the world will increase from 560 million tons to 780 million tons by the year 2020 (Shabbir et al., 2001). However, environmental stress is a serious issue confronting rice production, especially the problem of salinity (Yokoi et al., 2002; Zeng et al., 2003). The aim of this investigation was to enhance salt-tolerance in the salt-sensitive rice cultivar PT1 in equivalent to salt tolerant cultivar HJ, by exogenous sucrose in the culture medium.

Results

Osmotic potential and soluble sugar

Osmotic potential (Ψ_s) in the root tissues (-0.57 to -1.13 MPa) was maintained better than in the leaf tissues (-1.53 to -5.74 MPa) in both rice cultivars when subjected to 342 mM NaCl. The Ψ_s in the salt stressed leaves increased to -5.74 MPa, while it was maintained at -4.35 MPa in seedlings pre-treated with 58.4 mM sucrose in the culture medium (Fig. 1). Soluble sugars, including glucose and fructose, in the root tissues of salt stressed seedlings dropped significantly, except in the case of fructose in HJ. In contrast, glucose and fructose content in the leaf tissues of salt stressed seedlings increased considerably. On the other hand, the sugar content in the salt-stressed PT1 rice seedlings increased, relative to the exogenous sucrose application (Table 1). Sugar accumulation in salt stressed seedlings may play a role in osmotic adjustment, as demonstrated by low Ψ_s. In salt-stressed PT1seedlings, a positive relationship between Ψ_s and total soluble sugar in the root tissues was demonstrated (Fig 2A), whereas, there was a negative correlation in the leaf tissues (Fig 2B).

Chloroplast ultrastructure and photosynthetic pigments

Chloroplast ultrastructure, including stroma, granum and chloroplast membrane, in the leaf tissues of salt stressed HJ was maintained better than in PT1 (Fig 3). Swelling and the destruction of the chloroplast ultrastructure including stroma (ST) and stroma lamellae (SL) in PT1 plants under salt stress may cause photosynthetic inactivation and reduction in photosynthesis (Fig 3). Reduction of osmotic potential in the leaf tissues of salt stressed seedlings caused damage to the photosynthetic pigments, including chlorophyll a (Fig 4A) and total chlorophyll (Fig 4B). In general, the photosynthetic pigment content in the leaves of salt stressed rice seedlings decreased.

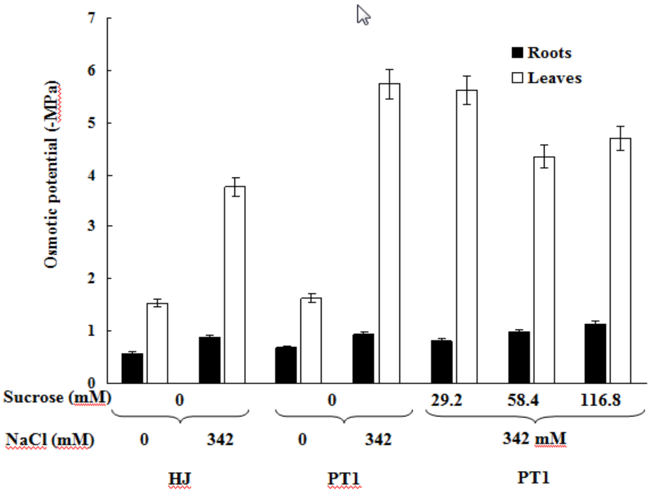

Fig 1. Osmotic potential in the root and leaf tissues of *indica* rice pre-treated with 0.0, 29.2, 58.4 or 116.8 mM sucrose and subsequently exposed to 0 or 342 mM NaCl for 4 days.

In the present study, Chl_a, Chl_b and TC content in salt stressed HJ seedlings were degraded by 12.32, 19.75 and 14.60%, whereas in salt stressed PT1 seedlings they dropped by 19.93, 55.66 and 30.81% respectively. In contrast, the C_{x+c} content increased in seedlings subjected to 342 mM NaCl (Table 2). The photosynthetic pigments in PT1 rice seedlings pre-treated with exogenous sucrose application were maintained better than in plants not treated with sucrose, when exposed to salt stress, especially in 58.4 mM sucrose (degraded by 12.64, 6.33 and 10.63%). The degradation of Chl_a and TC in salt stressed seedlings was positively related to F_v/F_m (Fig. 5A) and Φ_{PSII} diminution (Fig 5B).

Photosynthetic abilities and growth performances

Chlorophyll fluorescence parameters, including F_v/F_m, Φ_{PSII}, and qP in the leaves of salt stressed plants diminished by 10.88, 14.02 and 6.14% in the HJ salt-tolerant cultivar and by 13.02, 28.62 and 20.82% in PT1, salt-susceptible (Table 3). Moreover, the diminution of F_v/F_m, Φ_{PSII}, and qP in PT1 salt-susceptible, pre-treated with exogenous sucrose, was suppressed in plants exposed to salt stress, for example in 58.4 mM sucrose application (diminution by 6.34, 20.10 and 8.50% respectively). The photosynthetic abilities of salt-stressed seedlings of PT1 (salt-susceptible) pre-treated with exogenous sucrose improved to a greater extent than in plants without sucrose application, leading to enhanced growth performance, including root and shoot weight. In salt stress

Table 2. Chlorophyll a (Chl_a), chlorophyll b (Chl_b), total chlorophyll (TC) and total carotenoids (C_{x+c}) concentration in the leaf tissues of *indica* rice pre-treated with 0.0, 29.2, 58.4 or 116.8 mM sucrose and subsequently exposed to 0 or 342 mM NaCl for 4 days.

Rice varieties	Sucrose (mM)	NaCl (mM)	Chl_a ($\mu g\ g^{-1}$ FW)	Chl_b ($\mu g\ g^{-1}$ FW)	TC ($\mu g\ g^{-1}$ FW)	C_{x+c} ($\mu g\ g^{-1}$ FW)
HJ	0.0	0	155.0 a	62.8 ab	218.5 a	24.1 c
HJ	0.0	342	135.9 b	50.4 d	186.6 d	28.2 b
PT1	0.0	0	156.5 a	66.3 a	223.0 a	24.1 c
PT1	0.0	342	125.3 c	29.4 e	154.3 e	25.0 c
PT1	29.2	342	138.5 b	51.7 d	190.2 cd	27.8 b
PT1	58.4	342	137.0 b	62.1 b	199.3 b	34.2 a
PT1	116.8	342	139.2 b	55.7 c	194.0 c	27.1 b

Means with the different letters are significantly different at $P \leq 0.01$ (**) by Duncan's New Multiple Range Test.

Table 3. Maximum quantum yield of PSII (F_v/F_m), photon yield of PSII (Φ_{PSII}) and photochemical quenching (qP) in the leaf tissues of *indica* rice pre-treated withy 0.0, 29.2, 58.4 or 116.8 mM sucrose and subsequently exposed to 0 or 342 mM NaCl for 4 days.

Rice varieties	Sucrose (mM)	NaCl (mM)	F_v/F_m	Φ_{PSII}	qP
HJ	0.0	0	0.901 a	0.585 b	0.651 b
HJ	0.0	342	0.803 c	0.503 c	0.611 c
PT1	0.0	0	0.914 a	0.622 a	0.682 a
PT1	0.0	342	0.795 c	0.444 e	0.540 d
PT1	29.2	342	0.804 c	0.460 d	0.561 d
PT1	58.4	342	0.856 b	0.497 c	0.624 c
PT1	116.8	342	0.857 b	0.494 c	0.542 d

Means with the different letters are significantly different at $P \leq 0.01$ (**) by Duncan's New Multiple Range Test.

conditions, root fresh weight, root dry weight and shoot fresh weight of both HJ and PT1 seedlings decreased significantly. The exception was shoot dry weight, which was unchanged (Table 4). Interestingly, the overall growth performance of sugar pre-treated seedlings improved significantly when subjected to salt-stress, especially in 58.4 and 116.8 mM sucrose, which performed better than the control seedlings (without sucrose application).

Discussion

In rice, soluble sugar content in HJ (salt-tolerant) was significantly greater than in PT1 (salt-sensitive) in plants exposed to salt stress (Cha-um et al., 2009a). At 100 mM salt treatment, total soluble sugars in the IR651 salt-tolerant variety accumulated to a higher level than in IR29 (salt-susceptible) (Nemati et al., 2011). In contrast, the concentration of sugar in Pokkali (salt-tolerant) was unchanged in plants subjected to salt stress, while in the KDML 105 salt-sensitive variety it was enriched, relating to salt concentration (Pattanagul and Thitisaksakul, 2008). Similarly, the levels of sugar in the salt-tolerant rice cultivars CSR-1 and CSR-3 were lower than in "Ratna" and "Jaya" (salt-sensitive) when exposed to 14 dS m^{-1} NaCl salt stress (Dubey and Singh, 1999). Soluble sugar may play a key role in osmotic adjustment at the cellular level of plants under salt stress (Gupta and Kaur, 2005). In the present study, the soluble sugar content in salt-stressed seedlings of the PT1 salt-sensitive cultivar was enriched, using exogenous sucrose application to the culture medium, leading to a reduction in osmotic potential, especially in the leaf tissues. Exogenous sucrose application to the culture medium has been previously investigated in rice (Boriboonkaset et al., 2007), perennial ryegrass (Unno and Maeda, 2008) and *Microcoleus vaginatus* (Chen et al., 2003). Total carbohydrate, intracellular sucrose and water soluble sugar in the cells of sucrose pre-treated plants increased, while a low level of sodium ions in the cells was observed in plants exposed to

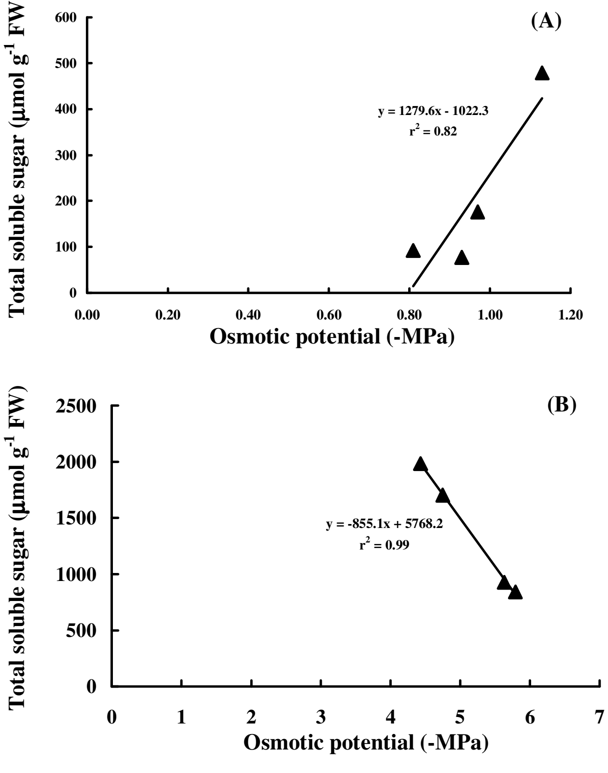

Fig. 2 Relationship between osmotic potential and total soluble sugar contents in roots (A) and leaves (B) of *indica* rice seedlings pre-treated with 0.0, 29.2, 58.4 or 116.8 mM sucrose and subsequently exposed to 0 or 342 mM NaCl for 4 days.

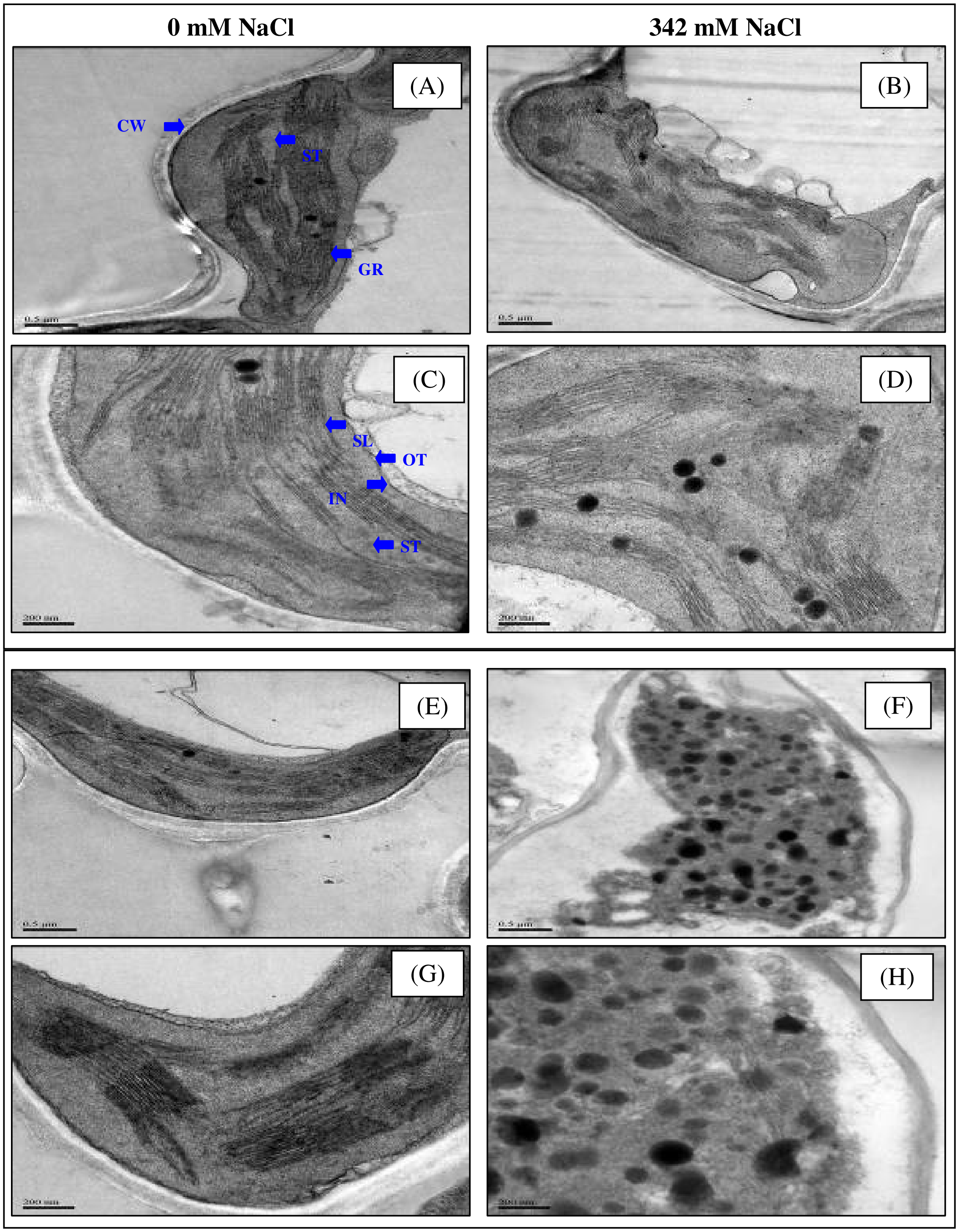

Fig 3. Chloroplast ultrastructure in the leaf tissues of HJ; (A) chloroplastic organ of HJ without NaCl or (B) with 342 mM NaCl (a bar = 0.5 µm), (C) chloroplastic organ of HJ without NaCl or (D) with 342 mM NaCl (a bar = 200 nm) and PT1; (E) chloroplastic organ of PT1 without NaCl or (F) with 342 mM NaCl (a bar = 0.5 µm), (G) chloroplastic organ of PT1 without NaCl or (H) with 342 mM NaCl (a bar = 200 nm) rice cultivars grown under 0 and 342 mM NaCl for 4 days. CW, cell wall; GR, granum; IN, inner membrane; OT, outer membrane; SL, stroma lamellae; ST, stroma.

Table 4. Fresh weight (FW) and dry weight (DW) in root and shoot organs of *indica* rice pre-treated with 0.0, 29.2, 58.4 or 116.8 mM sucrose and subsequently exposed to 0 or 342 mM NaCl for 4 days.

Rice varieties	Sucrose (mM)	NaCl (mM)	Roots		Shoots	
			FW (mg)	DW (mg)	FW (mg)	DW (mg)
HJ	0.0	0	48.3 a	4.5 c	257.5 a	38.6 b
HJ	0.0	342	24.0 c	3.1 de	212.5 b	37.4 b
PT1	0.0	0	49.1 a	4.0 cd	201.4 b	33.1 b
PT1	0.0	342	15.7 d	1.8 e	154.0 c	31.7 b
PT1	29.2	342	40.4 b	5.1 c	159.2 c	35.4 b
PT1	58.4	342	41.3 b	9.2 a	219.2 ab	50.8 a
PT1	116.8	342	40.6 b	6.4 b	231.6 ab	56.5 a

Means with the different letters are significantly different at $P \leq 0.01$ (**) by Duncan's New Multiple Range Test.

200 mM NaCl (Chen et al., 2003). In the present study, the chloroplast organelle in HJ salt-tolerant was conserved better than in PT1 when subjected to 342 mM NaCl for 4 days. Chloroplast ultrastructure i.e. stromal thylakoids, granum and chloroplast membrane, is one of the most abundant organelles that is susceptible to salt stress, especially in the salt-sensitive cultivar 'Nipponbare'. For example, swollen thylakoids (17% of control) are evidently demonstrated in rice exposed to 200 mM NaCl for 3 days (Yamane et al., 2008). Photosynthetic pigments and their activities were significantly degraded in salt-sensitive rice cultivars, relating to growth retardation. In rice, chlorophyll stabilisation and chlorophyll fluorescence, as well as net photosynthetic rate are exhibited better in HJ, salt-tolerant, than in PT1 salt-sensitive (Cha-um et al., 2009a). In addition, F_v/F_m and photosynthetic oxygen evolution in sucrose pre-treated cells (29.2 mM) were enhanced to a greater degree than those in plants without sucrose treatment when exposed to 200 mM NaCl (Chen et al., 2003). In IR29, salt-sensitive, survival percentage, growth performance and root cortex thickness in sucrose pre-treated seedlings (116.8 mM) improved in plants subjected to 342 mM NaCl for 7 days (Boriboonkaset et al., 2007). Alternatively, the exogenous application of mannitol (Seckin et al., 2009) and trehalose (Garcia et al., 1997) sugar classes has been well established and has been demonstrated to improve salt-tolerance in rice.

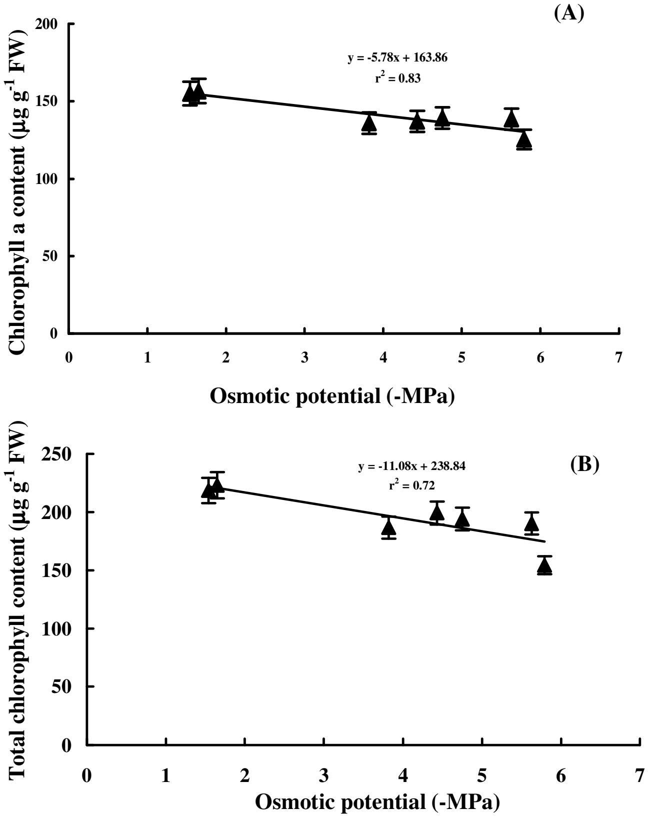

Fig. 4 Relationships between osmotic potential and chlorophyll a content (A), osmotic potential and total chlorophyll content (B) in the leaf tissues of *indica* rice seedlings pre-treated with 0.0, 29.2, 58.4 or 116.8 mM sucrose and subsequently exposed to 0 or 342 mM NaCl for 4 days.

Materials and methods

Plant materials and salt stress treatment

Seeds of salt-tolerant (Homjan; HJ) and salt-sensitive (Pathumthani 1 rice; PT1) rice (*Oryza sativa* L. subsp. *indica*) varieties provided by the Pathumthani Rice Research Centre (Rice Research Institute, Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand) were de-husked and rinsed with 70% ethanol for 1 min, disinfected once in 5% (v/v) Clorox® (5.25% (w/v) sodium hypochlorite solution, Clorox Co. Ltd., Oakland, CA, USA) with 0.1% Tween 20® (Merck, Germany) for 12 h, once in 25% (v/v) Clorox® for 30 min, then rinsed with sterile distilled water. Surface sterilised seeds were germinated on 25 mL MS-solidified (Murashige and Skoog, 1962) supplemented with 3% sucrose (w/v) added to the culture medium (photomixotrophic conditions) in a 250 mL glass vessel. The medium pH was adjusted to 5.7 before autoclaving. Rice seedlings were cultured *in vitro* in a culture room for 14 days, under conditions of 25±2°C air temperature, 60±5% relative humidity (RH), 60±5 µmol $m^{-2} s^{-1}$ photosynthetic photon flux (PPF) provided by fluorescent lamps (TLD 36 W/84 Cool White 3350 Im, Philips, Thailand) with a 16 hd^{-1} photoperiod. Fourteen-day-old seedlings of HJ and PT1 rice were aseptically transferred to 60 mL MS liquid medium supplemented with 0.0 (control), 29.2, 58.4 or 116.8 mM sucrose with vermiculite used as a supporting material and grown on for 14 days. The air exchange rate in the glass vessels was adjusted to 2.32 µmol CO_2 h^{-1} by punching a hole in the plastic cap (∅ 1 cm) and covering the hole with gas permeable microporous polypropylene film (0.22 µm pore size, Nihon Millipore Ltd., Tokyo, Japan). Sodium chloride in the culture medium was adjusted to 0.0 (control) or 342.0 mM NaCl (salt stress) for 4 days. Soluble sugar content, water potential, photosynthetic pigment content, chlorophyll fluorescence and growth characters were evaluated.

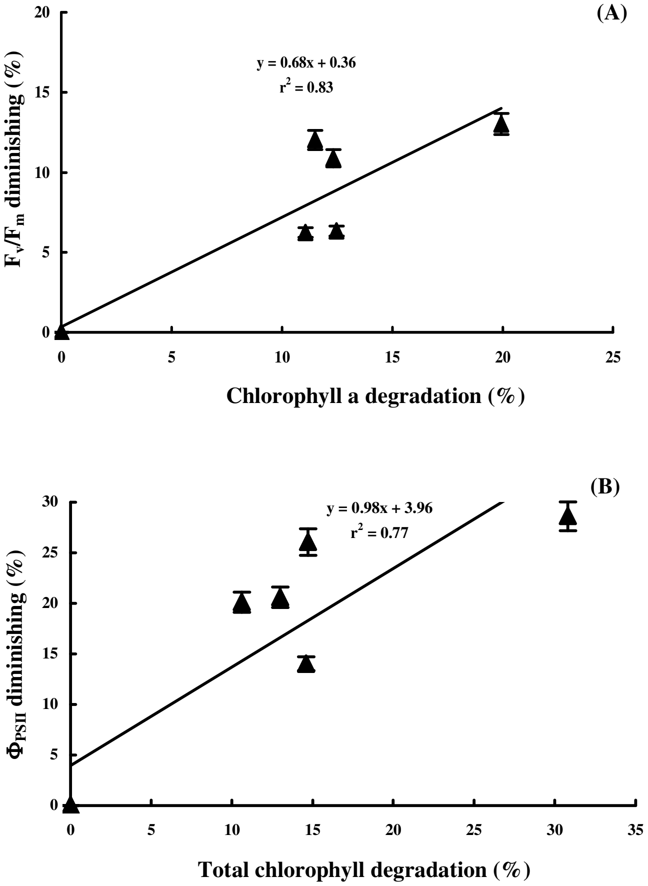

Fig 5. Relationships between chlorophyll a degradation and maximum quantum yield diminishing (F_v/F_m) (A), total chlorophyll degradation and photon yield of PSII (Φ_{PSII}) (B) in the leaf tissues of *indica* rice seedlings pre-treated with 0.0, 29.2, 58.4 or 116.8 mM sucrose and subsequently exposed to 0 or 342 mM NaCl for 4 days.

Data collection

Measurement of soluble sugar

Glucose and fructose content in the roots and leaves of both HJ and PT1 were measured using the modified Karkacier et al. (2003) method.

Measurement of osmotic potential

Osmotic potential in the roots and leaves of HJ and PT1 rice seedlings was measured according to the method of Lanfermeijer et al. (1991) using a vapour pressure osmometer (5520 Vapro®, Wescor, Inc., USA).

Measurement of photosynthetic pigment

Chlorophyll a (Chl_a), chlorophyll b (Chl_b), total chlorophyll (TC) and total carotenoid (C_{x+c}) concentrations were determined following the methods of Shabala et al. (1998) and Lichtenthaler (1987), respectively.

Measurement of chlorophyll fluorescence

Chlorophyll fluorescence emission from the adaxial leaf surface was monitored with a Fluorescence Monitoring System (FMS 2; Hansatech Instruments Ltd., Norfolk, UK) in the pulse amplitude modulation mode, as previously described by Loggini et al. (1999) and Maxwell and Johnson (2000).

Chloroplast ultrastructure observation

Electron-microscopic studies were performed using the middle section of a fully expanded leaf of a rice seedling following Yamane et al. (2003). Leaf samples (0.20 × 0.20 cm^2) were fixed in Karnovski's fixative solution (4% paraformaldehyde and 5% glutaraldehyde in 50 mM phosphate buffer, pH 7.2), then transferred to 2% osmium tetroxide in 50 mM phosphate buffer. The leaf samples were dehydrated in a series of gradient ethanol and propylene oxide, embedded in Araldite epoxy and polymerised at 70°C for 24 h. Ultra-thin sections (70-90 mm) were cut with a diamond knife and placed on 150 mesh copper grids. The grids were stained with 2% uranyl acetate for 20 min, followed by lead citrate for 5 min. The grid samples were observed by transmission electron microscope (TEM) (TECNAI 20 Philips®, The Netherlands) at 80 Kv.

Measurement of growth characters

The fresh weight of roots and shoots were rapidly obtained using a digital balance. Fresh samples were then dried in a hot-air oven (Memmert, Model 500, Germany) at 80°C for 3 days and incubated in a desiccators for dry weight measurement.

Statistical analysis

The experiment was designed as a completely randomised design (CRD) with eight replicates (n = 8). Analysis of variance (*ANOVA*) was analysed using SPSS software (SPSS for Windows, SPSS Inc., USA). Mean values in each treatment were compared using Duncan's New Multiple Range Test (DMRT).

Conclusion

In conclusion, soluble sugar content in leaf tissues of salt stressed seedlings was enriched, relating to negative water potential and sucrose concentration in the culture medium. Increased levels of soluble sugar in salt stressed seedlings alleviated the disruption to photosynthetic pigments and their activities, leading to maintained growth abilities. Exogenous sucrose application (58.4 mM) to PT1 salt-susceptible, may play a role as an alternative way to improve salt-tolerance.

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