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Evaluation of Na^+ enrichment and expression of some carbohydrate related genes in *indica* rice seedlings under salt stress

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Abstract

Salt-affected soil is one of the most important abiotic stresses, leading to reduce rice productivity in many regions of the world. The objective of this investigation was to determine the Na^+, soluble sugar and starch contents and expression of some starch-related genes in two genotypes, Pokkali (salt tolerant) and IR29 (salt sensitive), grown under 200 mM NaCl. Three-week-old rice seedlings cvs. Pokkali and IR29 were treated with 0 or 200 mM NaCl subsequently Na^+, starch content, soluble sugar and growth characters were evaluated. The Na^+ concentration in salt-stressed seedlings cv. IR29 was reached following leaf sheath>leaf blade>root tissues. Na^+ accumulation in leaf blade and leaf sheath was positively related to soluble sugar enrichment (r^2>0.68). In starch biosynthesis, *OsAGPS2b* mRNA expression in leaf blade of rice seedlings cv. Pokkali was up-regulated for 66%, when exposed to 200 mM NaCl for 48h, leading to starch accumulation. Soluble starch content in salt-stressed seedlings was peaked to 68.84 μg g^{-1} FW in the leaf blade of cv. Pokkali and 165.83 μg g^{-1} FW in leaf sheath of cv. IR29 which was confirmed by iodine dye staining. In cv. Pokkali, soluble starch in the leaf blade of salt-stressed seedlings was enhanced and correlated with Na^+ gathering CoroNa green emission. Growth performances of *indica* rice cv. IR29 seedlings were significantly declined when subjected to salt stress for 4 d. Na^+ absorption by root tissue was greater in IR29 than that in Pokkali. The starch concentration in salt-stressed seedlings of cv. Pokkali was the maximum to 68.8 μg g^{-1} FW in the leaf blade, and it related to up-regulated levels of *OsAGPS2b* mRNA and *OsGPL1*. The study concludes that the regulation of carbohydrate metabolism in salt tolerant cultivar of rice may play a key role as a major salt defense mechanism when seedlings subjected to 200 mM NaCl.

Keywords: rice; salt stress; sodium ion; soluble sugar; specific organs; soluble starch.
Abbreviations: AGPL_ADP-glucose pyrophosphorylase large subunit; AGPS_ADP-glucose pyrophosphorylase small subunit; BAM_β-amylase; DMSO_dimethyl sulphoxide; DPE_disproposionating enzyme; FBPase_fructose-1,6-bisphosphatase; GBSS_granule-bound starch synthase; GWD_glucan water dikinase; HPLC_high performance liquid chromatography; ISA_isomerase; PCR_polymerase chain reaction, PPFD_photosynthetic photon flux density; PWD_phosphoglucan water dikinase; RH_relative humidity; SBE_starch branching enzyme; SDE_starch debranching enzyme; SS_starch synthase.

Introduction

Rice, a glycophytic species, is the major crop that provides carbohydrate to the world population (Khush, 2005). The seedling stage of rice, especially the root tissues, is very sensitive to salt stress (Zeng et al., 2001; Yazdani and Mahdieh, 2012). Likewise, studies have demonstrated that fertility, seed set and grain production in rice were inhibited under salinity stress (Abdullah et al., 2001). Accordingly, studies have been conducted in the past to investigate salt-tolerant genotypes of rice crop, i.e. Pokkali, FL478 (isogenic line derived from Pokkali×IR29), HJ, IR651 and CSR-1, as model objects (Dubey and Singh, 1999; Pattanagul and Thitisaksakul, 2008; Cha-um et al., 2009; Ferdose et al., 2009; Nemati et al., 2011; Platten et al., 2013; Sarkar et al., 2013). Previously, we have investigated carbohydrate-related gene(s) expression (*m*RNA), biochemical, physiological and morphological changes in photoautotrophically grown rice seedlings exposed to NaCl stress under *in vitro* conditions (Cha-um et al., 2009; Theerawitaya et al., 2012). Likewise, the loss of productivity in the salt stressed plants of salt sensitive rice cvs. IR29 and PT1 grown in pot culture under glasshouse conditions has been reported (Boriboonkaset et al., 2012; Boriboonkaset et al., 2013). Salt defense mechanisms, i.e. ion homeostasis, osmoregulation and antioxidant systems, in plant species have been widely investigated issues (Hasegawa et al., 2000; Munns and Tester, 2008). Na^+ influx from medium solution to root tissues of rice crop *via* apoplastic route has been well-known (Krishnamurthy et al., 2009; Krishnamurthy et al., 2011; Kavitha et al., 2012). Likewise, $^{22}Na^+$-radioactive labeling (Cotsaftis et al., 2012) and CoroNa green labeling (Kanai et al., 2007) have been used as good indicators to monitor the Na^+ localization in different organs of salt-stressed plants. Alternatively, soluble

sugars, including monosaccharides, disaccharides, and sugar alcohols, have been identified as a member of osmolytes to play a key role as osmotic adjustment under salt stress (Gupta and Kaur, 2005; Cha-um et al., 2009). Sugar is a primary product of photosynthesis in the leaf tissues (source) and then transferred to other organs (sink) using phloem loading, which is negatively affected by salt stress, resulting in low sucrose levels in the root tissues (Suwa et al., 2008; Lemoine et al., 2013). In a previous study, we found that soluble sugar level increased in salt-stressed seedlings of rice cultivars in relation to fructose-1,6-bisphosphatase (FBPase) activity and FBP-related gene expression (Cha-um et al., 2009).

In starch metabolism, there are several key enzymes that regulate both starch biosynthesis and degradation. For example, ADP-glucose pyrophosphorylase (AGP), starch synthase (SS), granule-bound starch synthase (GBSS), starch branching enzyme (SBE), and isomerase (ISA) in starch biosynthesis, and glucan water dikinase (GWD), phosphoglucan water dikinase (PWD), starch debranching enzyme (SDE), β-amylase (BAM) and disproportionating enzyme (DPE) in starch degradation have been proposed (Deschamps et al., 2008; Kötting et al., 2010; Weise et al., 2011). In common reed plants exposed to 100 mM NaCl starch granules accumulated in shoot base, stem and leaves, whereas Na^+ accumulation occurred in shoot base. Na^+-binding starch granule hypothesis in parenchyma cells has been proposed as a novel salt defense mechanism (Kanai et al., 2007). In Tainung 67, GBSS gene expression and GBSS activity, and AGPase activity of salt-stressed seedlings (200 mM NaCl for 1 d) declined significantly in relation to low of starch content (Chen et al., 2008). Previously, we demonstrated that the *OsAGP* large subunit related *m*RNA is up-regulated in salt-stressed seedlings (200 mM for 4 d) of cv. Pokkali, leading to soluble starch accumulation (Theerawitaya et al., 2012). In rice cv. IR28 (salt susceptible), SS activity in salt-stressed plants of developing rice grain significantly declined, resulting in low starch content (Abdullah et al., 2001). In reproductive stage, soluble starch in the flag leaf was transferred to rice grain during heading period as sink-source transition (Ishimaru et al., 2004; Hirose and Terao 2004) subsequently declined in relation to the activities of AGPase, SS and BE enzymes (Hirose et al. 2006). Under salt stress (160 mM NaCl), AGPS1, AGPL1, and AGPase activities at the fruit developmental stage in tomato are up-regulated during early salt exposure and then dropped subsequently, thereby leading to reduced starch concentration (Yin et al., 2010). However, the qualitative and quantitative information pertaining to transitory starch localization in different organs of rice under salt stress is largely lacking. Thus, the objective of the present study was to investigate the contents of Na^+, soluble sugars and starch in different organs of two rice genotypes, cvs. Pokkali (salt tolerant) and IR29 (salt intolerant), grown under salt stress (200 mM NaCl) for 4 d.

Results

Growth performances of rice seedlings

In vitro photoautotrophic growth of salt stressed rice seedlings is demonstrated in Fig 1a. Under salt stress (200 mM NaCl), the leaf blade was burned in the tip zone and the chlorosis was observed in cv. IR29 (salt susceptible). In contrast, the seedlings of cv. Pokkali were healthy with green leaves even after 4 d of salt stress (Fig 1a). The shoot height, root length, fresh weight and dry weight in salt stressed seedlings of cv. IR29 were significantly declined by 25.3%, 25.6%, 20.2% and 28.3%, respectively, while these parameters did not change significantly in salt tolerant cv. Pokkali (Fig 1b and 1c). Overall, the growth characters in salt-stressed seedlings of rice genotypes played a role as salt responsive indices, especially in cv. IR29.

Sodium and potassium ions

Sodium ion (Na^+) in salt stressed seedlings of cvs. Pokkali (salt tolerance) and IR29 (salt sensitive) was reached in leaf sheath > root ≥ leaf blade, depending on the time course of salt treatment. In the present study, the accumulation of Na^+ in salt stressed seedlings of cv. IR29 was greater than that in cv. Pokkali (Fig. 2). The accumulation site of Na^+ was distributed on leaf sheath organ, especially in cv. IR29 (Fig. 2). In cv. Pokkali, Na^+ concentration in salt stressed seedlings increased in relation to the increase in salt exposure period but this is not true for leaf blade and root. Na^+ accumulations in leaf blade, leaf sheath and root tissues of both Pokkali and IR29 after treatment with 200 mM NaCl for 24h were confirmed by CoroNa Green staining (Fig. 3). The concentration of potassium ion (K^+) in salt-stressed seedlings of cv. Pokkali declined rapidly in leaf blade and root organs, whereas it increased in leaf sheath organ during the initial periods of salt exposure (1-2 d) and then dropped during the later period (4 d) (Fig. 2). In contrast, the K^+ concentration in salt-stressed seedlings of cv. IR29 declined in leaf sheath and root. In addition, a positive relation between Na^+ and soluble sugar enrichment in leaf blade and leaf sheath organs of salt-stressed seedlings was demonstrated in both IR29 and Pokkali (Fig. 4). Na^+ content in the salt-stressed roots of cv. IR29 was negatively related to soluble sugar concentration, whereas that in cv. Pokkali was uncorrelated (Fig. 4).

mRNA expression of starch metabolism-related genes

In starch biosynthesis, *OsAGPL1* gene expression in the leaf blade of IR29 and Pokkali rice seedlings unchanged when exposed to 200 mM NaCl for 24 h and then up-regulated only in cv. IR29 grown under salt stress for 48 h (Fig. 5a). In the root tissues, *OsAGPL1* mRNA in salt-stressed seedlings of cv. IR29 for 24 h was up-regulated by 3.5 fold of the control and then decreased after 48 h. In contrast, *OsAGPL1* mRNA in root tissues of cv. Pokkali did not change during the early hours of salt exposure (12 h) and then up-regulated (3.5 fold of the control) during later periods of salt stress (48 h) (Fig. 5a). *OsAGPS2b* mRNA in the leaf sheath of cv. Pokkali declined after 48 h. Interestingly, *OsAGPS2b* mRNA expression in the root tissues of cv. IR29 increased by 1.8 fold during early salt stress and then declined by 66.67% of control. In cv. Pokkali, *OsAGPS2b* mRNA in the root tissues of salt-stressed seedlings decreased in both early- and late-salt exposure periods (Fig. 5b). *OsGWD* mRNA in the leaf blade of rice seedlings declined in both IR29 and Pokkali cultivars when exposed to 200 mM NaCl for 24 and 48 h. The expression of *OsGWD* mRNA was unaffected in the leaf sheath of salt-stressed seedlings. In the root tissues, *OsGWD* mRNA in salt-stressed seedlings reduced, especially during the late salt exposure times (Fig. 5c). *OsPWD* expression in the leaf blade of salt-stressed seedlings was down-regulated when exposed to 200 mM NaCl for 24 and 48 h. Moreover, *OsPWD* mRNA in leaf sheath of salt-stressed seedlings unchanged. In the root tissues, *OsPWD* mRNA in salt-stressed seedlings of cv. IR29 increased by 2.0 folds in both early- and late salt exposures, whereas it was unchanged in cv. Pokkali (Fig. 5d).

Table 1. Glucose (Gluc), fructose (Fruc) and sucrose (Suc) concentrations (µg g^{-1} DW) in leaf blade, leaf sheath and root of indica rice cvs. IR29 and Pokkali after treated with 0 or 200 mM NaCl for 0, 1, 2 and 4 days.

Day	Cultivar	NaCl	Leaf blade			Leaf sheath			Root		
		(mM)	Gluc	Fruc	Suc	Gluc	Fruc	Suc	Gluc	Fruc	Suc
0	IR29	0	8.89	8.67	1.11	2.36	1.50	0.00	0.26	0.57	0.00
	Pokkali	0	4.37	4.61	0.00	2.33	1.45	0.00	0.81	0.69	0.00
1	IR29	0	11.28a	10.85a	1.10c	0.98c	0.83c	0.16	0.17b	0.21c	0.00
		200	8.08b	8.17b	1.68b	3.71a	3.11a	0.00	0.18b	0.35b	0.00
			(-)	(-)	(+)	(+)	(+)	(0)	(0)	(+)	(0)
	Pokkali	0	3.62d	3.06d	0.00d	2.50b	1.22b	0.00^{NS}	0.52a	0.59a	0.00^{NS}
		200	5.17c	5.07c	3.36a	3.68a	3.04a	0.00	0.46a	0.62a	0.00
			(+)	(+)	(+)	(+)	(+)	(0)	(0)	(0)	(0)
2	IR29	0	10.94a	10.94a	0.83c	2.67d	3.71a	0.00c	0.19c	0.34c	0.00
		200	10.53a	11.38a	9.50a	4.12a	1.90c	0.59a	0.43b	0.74a	0.01
			(0)	(0)	(+)	(+)	(-)	(+)	(+)	(+)	(0)
	Pokkali	0	7.44b	7.07b	0.00d	3.04c	1.48c	0.00c	0.13c	0.44b	0.00^{NS}
		200	7.46b	6.71b	4.72b	3.71b	2.89b	0.29b	0.69a	0.49b	0.00
			(0)	(0)	(+)	(+)	(+)	(+)	(+)	(0)	(0)
4	IR29	0	9.94a	10.03a	4.39b	2.15b	1.45c	0.00c	0.16c	0.30d	0.00
		200	9.14a	9.08a	16.95a	3.65a	3.40a	0.82a	0.23b	0.46c	0.00
			(0)	(0)	(+)	(+)	(+)	(+)	(+)	(+)	(0)
	Pokkali	0	5.02b	4.61b	0.00c	3.73a	2.72b	0.62b	0.56a	0.67a	0.00^{NS}
		200	9.65a	10.23a	5.65b	2.36b	1.49c	0.00c	0.26b	0.57b	0.00
			(+)	(+)	(+)	(-)	(-)	(-)	(-)	(-)	(0)

Mean in each row followed by different letters in each column are significantly different at $p \leq 0.01$ by Turkey's HSD test. ^{NS} represented non-significant; (-) inhibit response; (0) unmovable response; (+) exhibit response.

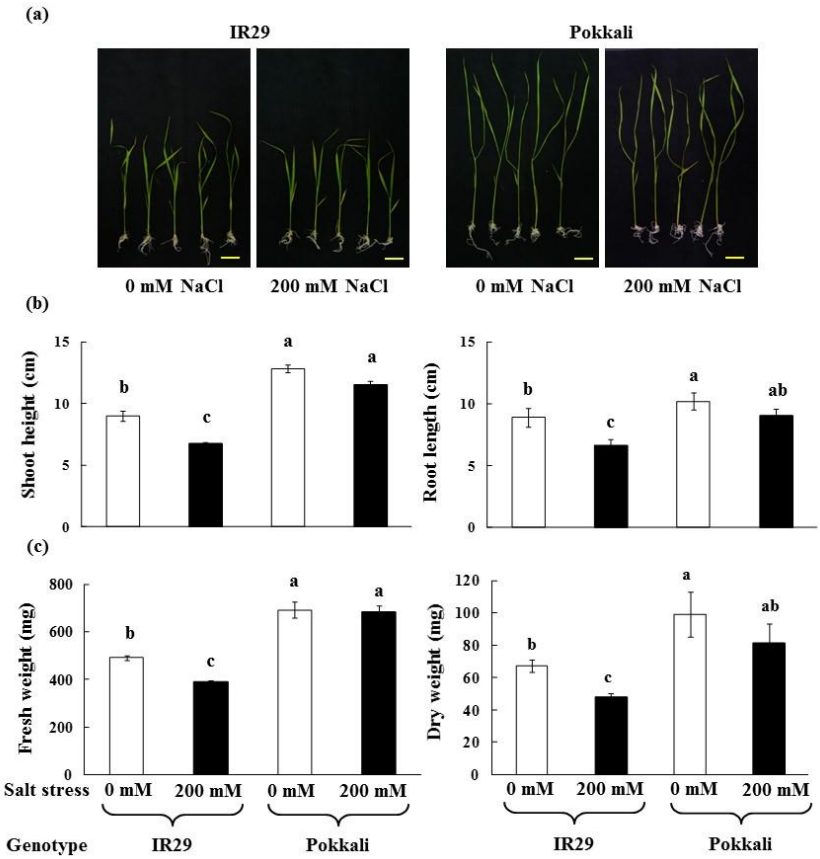

Fig 1. Morphological characters (a) and growth performance i.e. shoot height, root length, fresh weight and dry weight (b) of indica rice cvs. IR29 and Pokkali, after treatment with 0 or 200 mM NaCl for 4 days. Error bars represent ±SE. Different letters above bars are significantly different at $p \leq 0.01$ according to Tukey's HSD test. Scale bar represents 2 cm.

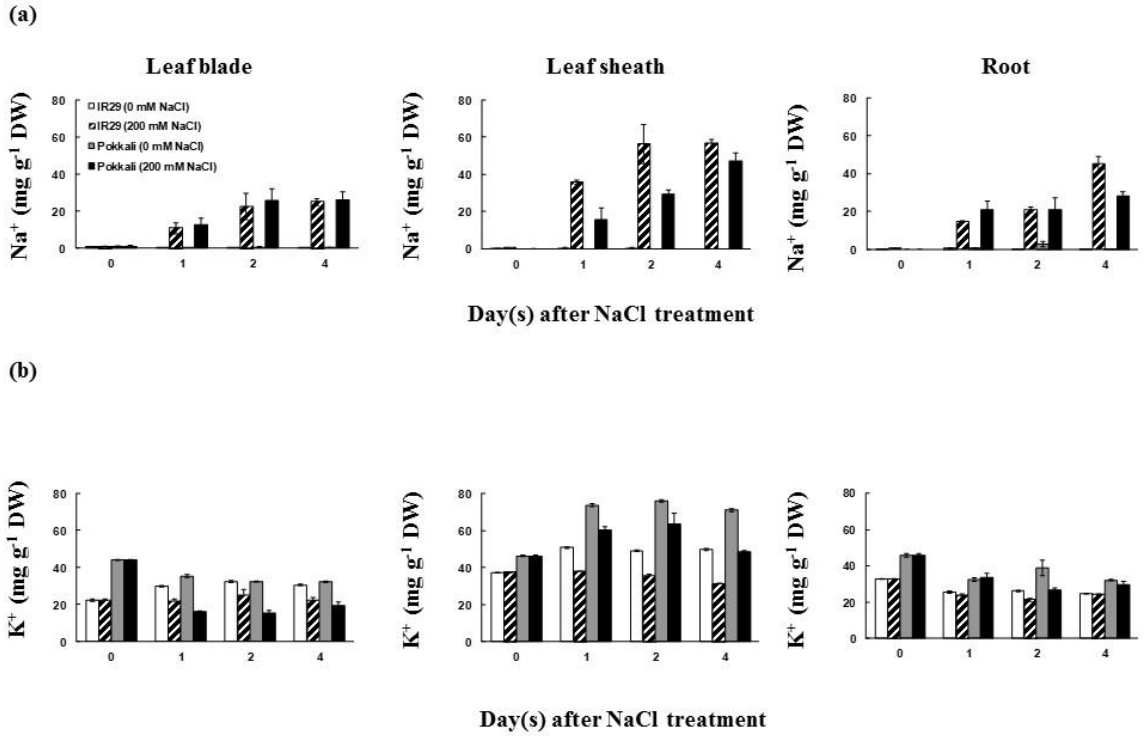

Fig 2. Na^+ (a) and K^+ (b) in leaf blade, leaf sheath and root of indica rice cvs. IR29 and Pokkali after treatment with 0 or 200 mM NaCl for 0, 1, 2 and 4 days. Error bars represent ±SE.

Starch content and distribution

Soluble and insoluble starches accumulated in rice seedlings of IR29 and Pokkali cultivars, depending on the salt stress exposure times and plant organs. The enrichments of soluble and insoluble starches in salt-stressed seedlings were observed in the order: leaf blade>leaf sheath>root tissues (Fig. 6). In cv. IR29, insoluble starch peaked in leaf blade. The X scale should be changed when exposed to 200 mM NaCl for 1 d and then declined in the late exposure periods. Soluble starch in leaf blade enriched when rice seedlings were exposed to 200 mM NaCl for 2 d. On the contrary, soluble and insoluble starches in rice seedlings of cv. Pokkali enhanced after treated with 200 mM NaCl for 4 d. It was confirmed by the starch localization indicated by blue color of iodine starch staining in the leaf blade (Fig. 7). Soluble and insoluble starch in the leaf sheath of cv. IR29 in rice seedlings subjected to 200 mM NaCl for 4 d were peaked by 9.1 and 5.7 fold, respectively. This was confirmed by distribution with iodine dye stain (Fig. 7). Starch accumulation in the leaf blade of salt stressed seedlings of cv. Pokkali was evidently demonstrated (Fig. 7). The soluble and insoluble starch contents in the root tissue of salt-stressed seedlings (4 days) of Pokkali genotype were accumulated (Fig. 6).

Soluble sugar enrichment

Sucrose concentration in the leaf blade of rice seedlings enhanced in cvs. IR29 and Pokkali grown under salt stress for 1, 2 and 4 d. Glucose and fructose concentrations in leaf blade of cv. Pokkali were enriched when exposed to 200 mM NaCl for 1 and 4 d, whereas these declined in cv. IR29 (Table 1) but not on all days. In leaf sheath, glucose and fructose concentration in salt stressed seedlings of cv. Pokkali was increased initially (1-2 d) and then declined (at 4 d). In contrast, glucose concentration in leaf sheath of cv. IR29 was enhanced at all salt exposure periods. Fructose and sucrose contents in leaf sheath of cv. Pokkali was enriched when exposed to 200 mM NaCl for 2 d, and then declined (at 4 d). In addition, glucose and fructose in root tissues of IR29 genotype grown under salt stress for 2 and 4 d increased, while in cv. Pokkali subjected to salt stress for 4 d, these were reduced. In the present study, sucrose was undetected in the root tissues of both salt- and without salt-stressed seedlings (Table 1). The amount of total soluble sugar in rice seedlings was reached following leaf blade>leaf sheath>root tissues (Fig. 8). Total soluble sugar in leaf blade seedlings of IR29 and Pokkali cultivars grown under salt stress increased with salt stress and salt exposure times. The soluble sugar in leaf blade was 35.2 (1.4 fold of control) and 25.5 μg g^{-1} DW (2.6 fold of control) in IR29 and Pokkali genotypes, respectively, when exposed to 200 mM NaCl for 4 d (Fig. 8). In addition, the soluble sugar in leaf sheath followed a trend similar to that in the leaf blade, while in the root tissue, it was in a very low concentration and did not change significantly.

Discussion

Leaf burn and chlorosis in salt stressed seedlings of salt sensitive rice cv. IR29 were evidently demonstrated. It is in conformity with similar toxic symptoms observed in three-week-old seedlings of cv. IR29 grown under ½ MS supplemented with 100 mM NaCl for 3 d (Lee et al., 2013). Growth performance measured in terms of shoot height, root length, fresh weight and dry weight in salt stressed seedlings of cv. IR29 was significantly dropped, whereas in cv. Pokkali, these growth performance parameters were retained. These observations are corroborated by previous similar findings in other cultivars / genotypes of rice (Zhao et al., 2014). For

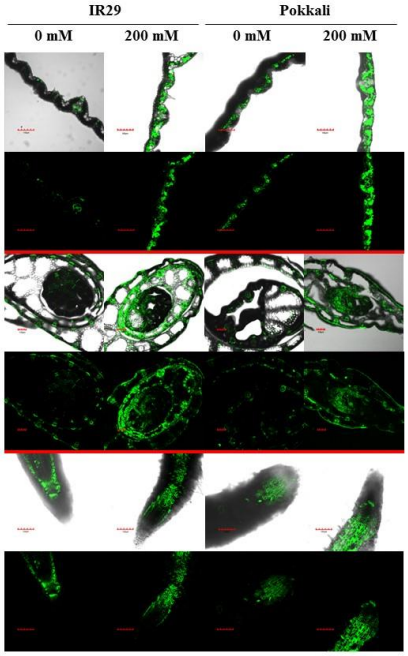

Fig 3. CoroNa Green staining in leaf blade, leaf sheath and root of *indica* rice cvs. IR29 and Pokkali after treatment with 0 or 200 mM NaCl for 24 h. Upper panel: bright field; Lower panel: dark field. Scale bar represents 100 µm.

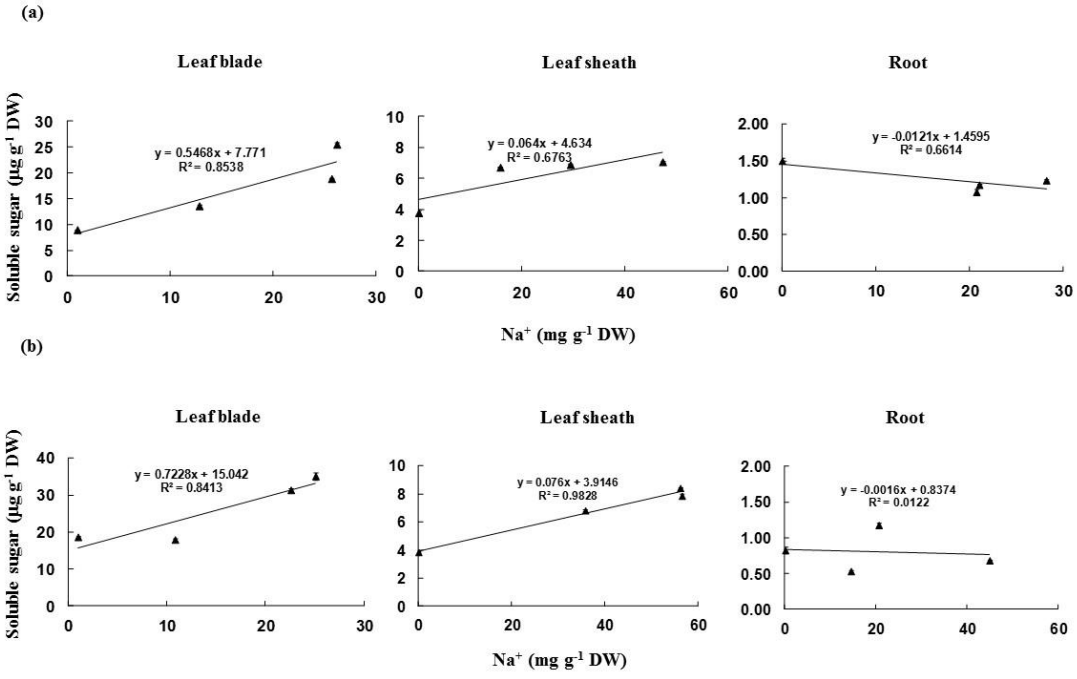

Fig 4. Relationship between $Na^+$and total soluble sugar in leaf blade, leaf sheath and root of *indica* rice cvs. IR29 (a) and Pokkali (b) after treatment with 200 mM NaCl for 0, 1, 2 and 4 days. Error bars represent ±SE.

example, shoot height, root length, fresh- and dry-weight in rice seedlings of cv. IR29 declined by 19.6%, 37.1%, 26.2% and 24.1%, respectively, when subjected to 200 mM NaCl under photoautotrophic growth conditions for 8 d (Theerawitaya et al., 2012). Cha-um et al. (2009) reported 10.30%, 30.8%, 12.2% and 9.1% decline in shoot height, root length, fresh- and dry-weight in salt stressed seedlings of rice cv. PT1 (342 mM NaCl for 7d). Likewise, leaf dry weight, root dry weight and total dry weight in hydroponically grown salt stressed seedlings of rice cv. IR29 declined by 38.5%, 27.1% and 46.1% of control seedlings, respectively (Nemati et al., 2011). Shoot height and root length of salt intolerant rice cv. IR26 reduced 33.42% and 63.83%, respectively, when plants were exposed to 100 mM NaCl for 10 d, as compared to salt-tolerant rice cv. Jiucaiqing (Wang et al., 2011). Na^+ accumulations in different parts of salt stressed

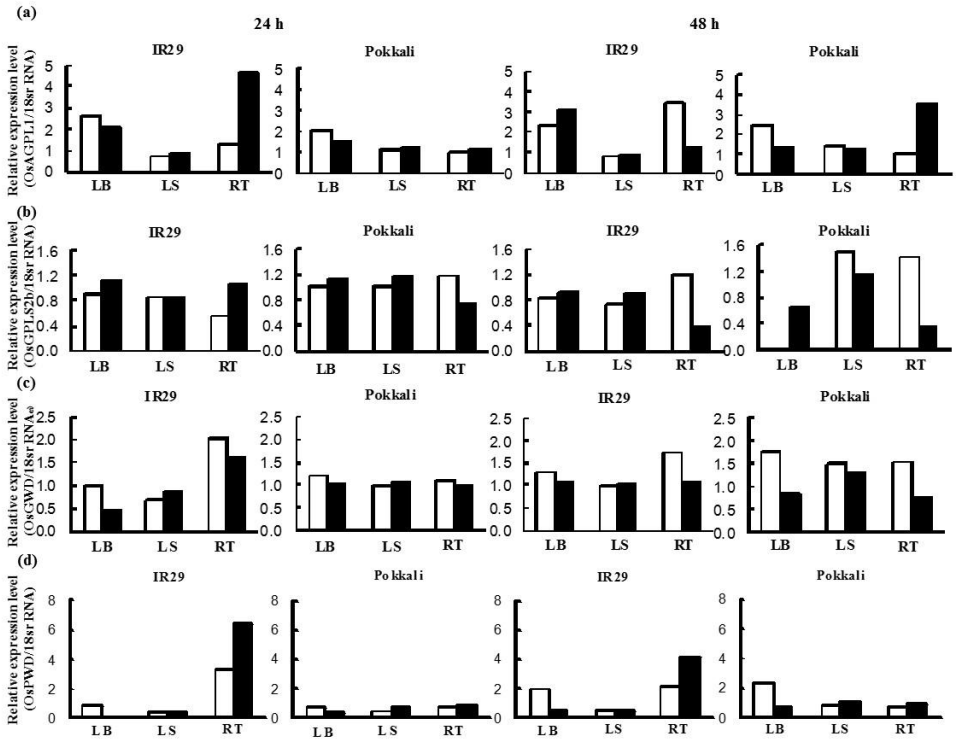

Fig 5. Relative expression of *OsAGPL1* (a), *Os AGPS2b* (b), *OsGWD* (c) and *OsPWD* (d) in *indica* rice cvs. IR29 and Pokkali at 24 and 48 h after treatment with 0 (light bar) or 200 mM NaCl (dark bar).

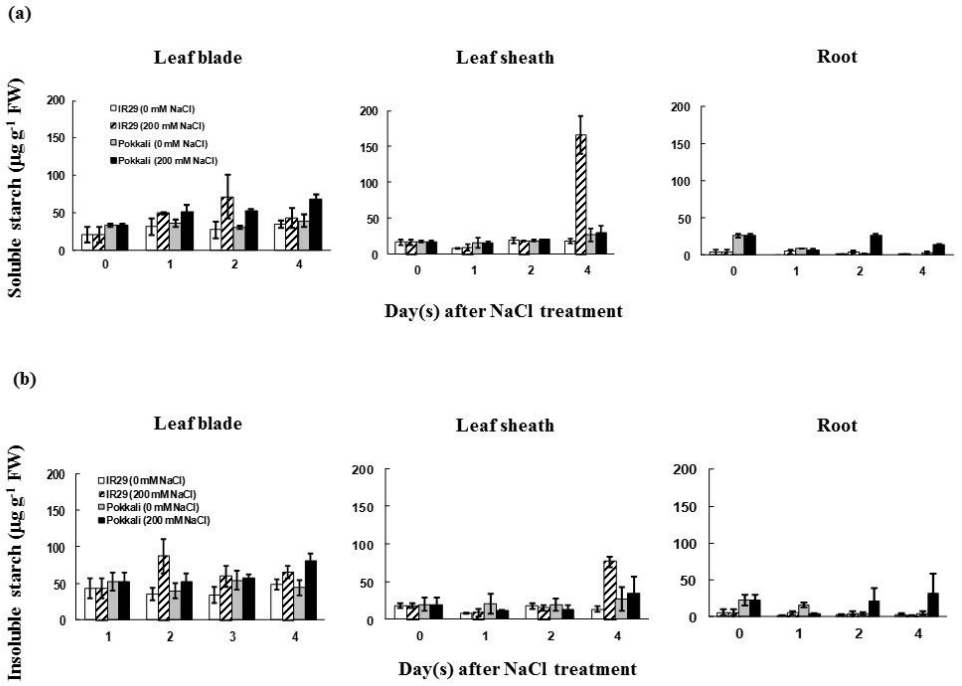

Fig 6. Soluble (a) and insoluble starch (b) in leaf blade, leaf sheath and root of *indica* rice cvs. IR29 and Pokkali after treatment with 0 or 200 mM NaCl for 0, 1, 2 and 4 days. Error bars represent ±SE.

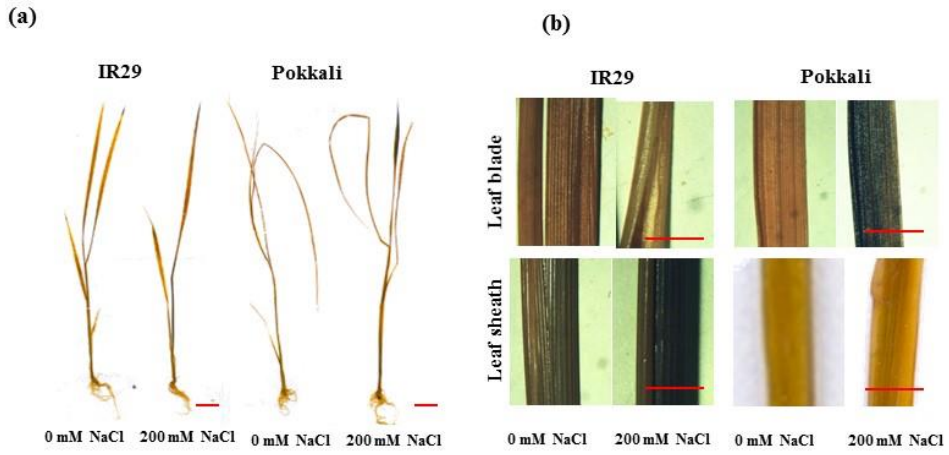

Fig 7. Iodine staining in *indica* rice cvs. IR29 and Pokkali in whole seedlings (a) and specific organs (b) after treatment with 0 or 200 mM NaCl for 4 days. Scale bar represents 2 cm.

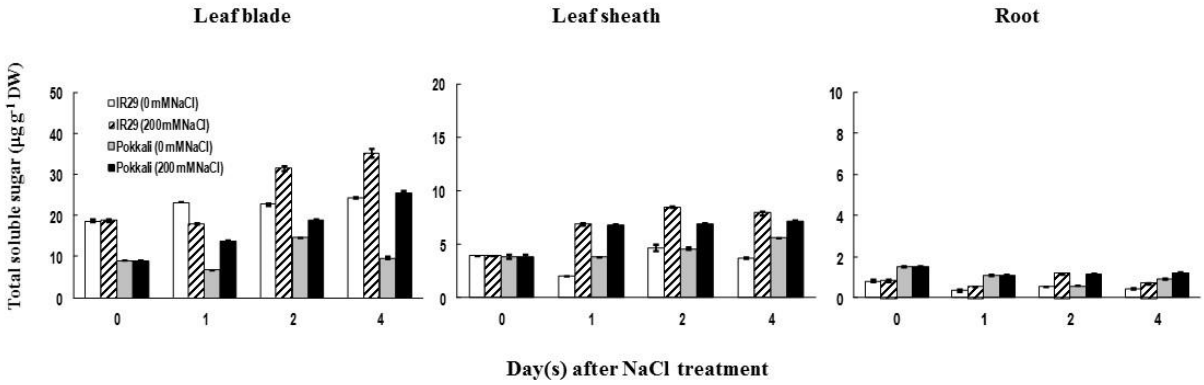

Fig 8. Total soluble sugar content in leaf blade, leaf sheath and root of *indica* rice cvs. IR29 and Pokkali after treatment with 0 or 200 mM NaCl for 0, 1, 2 and 4 days. Error bars represent ±SE.

seedlings of rice cultivars were in the order: leaf sheath>root>leaf blade and were related to the period of salt exposure. Similar result has been purposed on salt stressed seedlings of rice cv. FL478 (NILs of Pokkali × IR29), where Na^+ accumulation was in the order leaf sheath>root>leaf blade (Platten et al. 2013). Previously, the accumulation of Na^+ in IR29 (salt susceptible) and IR651 (salt tolerant) cultivars was in the order root>leaf sheath>youngest leaf (Nemati et al. 2011) and it was dependent on salt exposure period (100 mM NaCl for 3 and 7 d). In Nipponbare *japonica* rice (salt susceptible) subjected to 150 mM NaCl for 11 d, Na^+ accumulation in the order leaf sheath>leaf blade>root tissues was demonstrated (Platten et al., 2013). In our study, Na^+ accumulation in the rice cv. Pokkali was lesser than that in cv. IR29. Previously, Cotsaftis et al. (2011) reported that Na^+ in the shoot organ of salt-stressed (70 mM NaCl for 5 d) seedlings of cv. IR29 was 24.1 mM while that in cv. Pokkali was only 6.7 mM. Similarly, Na^+ content in the salt-stressed seedlings of IR20 and Nonaboka rice was greater than that in Pokkali (Krishnamurthy et al., 2009; Ghosh et al., 2011; Krishnamurthy et al., 2011; Kavitha et al., 2012). Na^+ straining by CoroNa Green dye fluorescence is a novel technique to determine the ion localization in different organs of higher plant species (Kanai et al., 2007; Park et al., 2009). It has been observed that CoroNa Green Na^+ reporter in salt-stressed root protoplasts of rice increased depending on NaCl salt concentrations and it was enriched in cv. IR20 more than that in cv. Pokkali (Kavitha et al., 2012). K^+ in salt-stressed seedling of rice declined in both IR29 and Pokkali genotypes. In the shoot organ, K^+ content in salt-stressed rice cv. IR42 (12 dS m^{-1} or 103 mM NaCl for 5 d) was significantly dropped, while it unchanged in cv. Pokkali (Sarkar et al. 2013). Likewise, K^+ in the root tissues of rice cvs. IR20 and Pokkali reduced when subjected to 100 mM NaCl for 7 d (Krishnamurthy et al., 2011). In rice cv. IR20, K^+ in leaf and root tissues was significantly decreased when exposed to 50 mM NaCl for 7 d (Djanaguiraman et al., 2006). These observations are paralleled by similar findings in other salt-susceptible rice cvs. Khazar (100 mM NaCl for 4 h; Yazdani and Mahdieh, 2012), and Jiucaiqing (120 mM NaCl for 9d; Wang et al., 2012). *OsGPL1* and *OsGPS2b* expression in the root organ of rice cv. IR29 was up-regulated by 3.45 and 1.83 fold, respectively, when subjected to 200 mM NaCl for 24 h. In rice crop, ADP-glucose pyrophosphorylase large subunit (OsAGPL) and ADP-glucose pyrophosphorylase small subunit (OsAGPS) have been identified as key enzymes (glucose 1-phoshate → ADP-glucose) for starch biosynthesis, especially in the seed endosperm development (Lee et al., 2007; Yu et al., 2011). In cv. Pokkali, *OsAGPL1* expression

in the root tissues and *OsAGPS2b* expression in the leaf blade increased when exposed to salt stress. These findings are paralleled by our previous study demonstrating the up-regulation of the expression levels of *OsAGPL1* and *OsGPS2b* mRNA in the flag leaf of salt-stressed rice cv. Homjan (HJ) under 150 mM NaCl (Boriboonkaset et al., 2012). Moreover, *OsAGPL1* gene in the leaf tissues of rice cv. Pokkali significantly expressed under salt stress at seedling (Theerawitaya et al., 2012) and reproductive stages (Boriboonkaset et al., 2013). In salt-stressed tomato, the expression of *OsAGPL1* enhanced by 160 mM NaCl for 10-22 d (Yin et al., 2010). In addition, AGPase activity in rice cv. Tainung 67 decreased when subjected to 200 mM NaCl for 24 h (Chen et al., 2008). In quinoa- a halophytic plant, the activity of ADPases in cotyledons of seedlings declined when exposed to 200 mM NaCl for 4-12 d (Rosa et al., 2009). In the present study, *OsGWD* expression levels in both leaf blade and root tissues in rice cvs. IR29 and Pokkali were down-regulated under salt stress. Previously, the mRNA appearance of *OsGWD* in flag leaf of *indica* rice cvs. PT1 (Boriboonkaset et al., 2012) and Pokkali (Boriboonkaset et al., 2013) significantly declined when plants exposed to salt stress, 150 mM NaCl and 13.25 dS m^{-1} EC, respectively. In contrast, transcriptional regulation of *GhGWD1* in salt-stressed seedlings (200 mM NaCl for 10 d) of cotton cv. Coker 312 was up-regulated (Zhu et al,. 2013). Moreover, the transcriptional level of *SEX1* (α-glucan/water dikinase) and relative GWD activity in the leaf tissues of *Arabidopsis* regulated when seedlings were incubated at 2°C for 6-48h (Yano et al., 2005). *OsPWD* mRNA expression in the leaf blade of rice cultivars was significantly decreased. Similarly, the down regulation of *OsPWD* in salt-stressed flag leaf of HJ rice genotype has been previously demonstrated (Boriboonkaset et al., 2012). The transcriptional level of *OsPWD* in the salt-stressed root tissues increased, leading to low transitory starch. Alternatively, *PWD*, correlated GWD function for phosphorylation on C3 position of glucose residues to be quickly hydrolyzed in starch degradation process has been well established (Deschamps et al., 2008; Kötting et al., 2010). In our study, under salt stress, soluble and insoluble starch contents in rice seedlings were accumulated in the order: leaf blade>leaf sheath>root organs. Previously, starch contents of the shoots of salt-stressed (14 dS m^{-1} for 20 d) seedlings of rice cvs. CSR-1 (salt tolerant) and Jaya (salt susceptible) were greater than those in the root tissues, by 2.31 and 3.65 fold, respectively. In contrast, the starch contents declined in the root tissues of salt-stressed seedlings of rice cvs. Ratna, Jaya, CSR-1 and CSR-2 (Dubey and Singh, 1999). In Taipei 309 *japonica* rice, starch level in the salt-stressed leaves was enhanced in relation to salt concentration (10–20 mM NaCl) in the media (Wankhade and Sanz, 2013). Besides, starch content in salt susceptible cv. IR29 declined in the long-term salt exposure (200 mM NaCl for > 2 d). Correspondingly, the starch contents of the leaf tissues of rice cv. IR20 *indica* and cv. Tainung 67 *japonica* declined depending on the salt exposure period and salt concentration (Djanaguiraman et al., 2006; Chen et al., 2008). In salt sensitive tomato cv. Volgoradskij, starch contents in basal leaves and young leaves declined with increased salt concentrations (Khelil et al., 2007). In contrast, soluble and insoluble starch contents in salt-stressed seedlings of rice cv. Pokkali were enriched on long term salt stress (2-4 d). Previously, studies have demonstrated that soluble starch accumulation in salt tolerant genotypes, Pokkali and CSR-1 was greater than that in salt sensitive cvs. IR29 and Jaya, respectively (Dubey and Singh, 1999; Boriboonkaset et al., 2013).

Soluble sugar levels in salt-stressed seedlings of rice genotypes enhanced in the order: leaf blade>leaf sheath>root tissues. These observations are in conformity with earlier findings reporting reduced sugar content (shoot>roots) in salt-stressed seedlings (7-14 dS m^{-1} NaCl for 20 d) of rice cvs. CSR-1 (salt tolerant) and Jaya (salt susceptible) (Dubey and Singh, 1999). In contrast, soluble sugar contents in rice cv. IR651 were in the order: root>leaf sheath>leaf blade, when plants were grown under 100 mM NaCl for 10 d (Nemati et al., 2011). Likewise, soluble sugar accumulation in salt-stressed plants of rice cvs. IR20 (Djanaguiraman et al., 2006) and Taipei 309 (Wankhde and Sanz, 2013) was dependent on salt treatment and salt exposure time. In our study, glucose, fructose and sucrose contents in the leaf blade of salt-stressed seedlings of rice cv. Pokkali increased, whereas these were decreased in cv. IR29. Glucose and fructose in roots and leaves of salt-stressed seedlings (342 mM NaCl for 7 d) of rice cv. HJ (salt tolerant) were enriched (Cha-um et al., 2009). In reproductive stage, sucrose, glucose and fructose in the flag leaf tissues of rice cv. Pokkali increased when exposed to 13.3 dS m^{-1} NaCl stress for 3 d (Boriboonkaset et al., 2013). In rice cv. INIAP12 (chilling sensitive), glucose and fructose contents were enhanced when seedlings were subjected to 100 mM NaCl for 4 d (Morsy et al., 2007). Similarly, sucrose in salt-stressed developing fruit of tomato cv. Micro-Tom was the lowest when compared with glucose and fructose (Yin et al. 2010). Alternatively, the salt inhibitory effects on sucrose translocation from leaf blade and leaf sheath via phloem loading are well established, leading to low sucrose partitioning in the roots (Suwa et al., 2008). In quinoa halophyte, sucrose, fructose and glucose levels were peaked in the cotyledons of seedlings grown under 200 mM NaCl for 4 d (Rosa et al., 2009). Moreover, soluble sugar enrichment in the leaf blade and leaf sheath of salt-stressed seedlings was positively correlated with Na^+ contents. Soluble sugars have been suggested to play a major role in osmotic adjustment in the cellular level in salt defense mechanism in salt tolerant plants (Ghosh et al., 2001; Gupta and Kaur, 2005).

Materials and Methods

Plant materials and salt treatment

Seeds of rice cv. Pokkali (salt tolerant) and cv. IR29 (salt susceptible) were manually dehusked, sterilized in 5% (*v/v*) Clorox® [0.05% (*v/v*) sodium hypochlorite, *a.i.*] for 12 h, then in 25% (*v/v*) Clorox® for 30 min, and rinsed thrice with sterile distilled water. Surface-disinfected seeds were germinated on 0.25% Phytagel®-solidified MS medium (Murashige and Skoog, 1962) supplemented with 3% (*w/v*) sucrose in a 250 mL glass vessel. The pH of culture medium was adjusted to 5.7 before autoclaving. In vitro growth conditions were established as 25±2°C ambient temperature, 60±5% relative humidity (RH) and 16 h d^{-1} photoperiod of 60±5 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) provided by fluorescent lamps. Fourteen-day-old seedlings were aseptically transferred to MS-liquid sugar-free media using vermiculite as supporting material for 14 d. Air exchange in the glass vessels was adjusted to 2.32 μmol CO_2 h^{-1} by punching a hole in the plastic cap (ϕ 1 cm) and covering the hole with a gas-permeable microporous polypropylene film (0.22 μm pore size, Nihon Millipore Ltd., Japan). After acclimatizing the seedlings for 7 d, the sodium chloride (NaCl) concentration in the culture medium was adjusted to 0 (control) or 200 mM (salt stress) for 0, 1, 2, and 4 days. The experiment was arranged as 2×2 factorial in a Completely Randomized Block Design (CRBD) with six replicates (n = 6). The mean values obtained were compared

using Tukey's HSD and analyzed by SPSS software version 11.5.

mRNA extraction and cDNA preparation

Leaf blade, leaf sheath and root tissues from the rice seedlings were collected at 0, 1, 2 and 4 d after salt stress treatment and immediately frozen at −80°C, prior to total RNA extraction. Total RNA in rice seedlings was extracted by the guanidine hydrochloride method (Sambrook et al., 1989). Ground tissues of rice seedlings were homogenized in guanidinium thiocyanate solution (0.75 M NaCitrate at pH 7.0, 10 % Sarcosyl and 2 M 2-*β*-mercaptoethanol), NaAcetate (pH 4.0) and phenol-chloroform solution. After chilling on ice for 15 min, the homogenate was centrifuged at 10,000×g for 20 min at 4°C. The aqueous phase was separated and mixed with isopropanol (1× vol), then kept at −20°C for 1 h before centrifuging at 10,000×g for 15 min at 4°C. The pellet was completely dissolved in 0.3 mL guanidinium thiocyanate solution and precipitated with ethanol. Contaminant DNA in the RNA preparations was removed with RQ1 RNase-Free DNase (Promega) and total RNA was purified by phenol-chloroform extraction. First-strand DNA was synthesized with 3 µg total RNA per sample, using ImPromp-II ™ Reverse Transcriptase (Promega) and oligo-dT15 primer.

Semi-quantitative PCR

The PCR reaction was performed using a Veriti® Thermal Cycler (Applied Biosystems, CA, USA). Primer sequences were as follows; ADP-glucose pyrophosphorylase large subunit I (F)ATGCTGGCCCAGACACTCT and (R)GCTC-GACTCTCTCCAACAGG (*OsAGPL1*; Accession number AK103906), ADP-glucose pyrophosphorylase small subunit 2b (F)CTCTGGGTGCCAACTACAGG and (R)CTTTTG-CCCTCACGTCGTC (*OsAGPS2b*; Accession number D50317), glucan-water dikinase (F)AGTGGCACCAGAAA-CTGCAC and (R)AGGAGAGCCAAGGAGCAAAG (*OsGWD*; Accession number AK103463), phosphoglucan-water dikinase (F)GTCCCTTCTGGTGCTGTGAT and (R)GACCTCAGCCTGGACAACC (*OsPWD*; Accession number FN179404), 18S rRNA (F)GTGCAACA-AACCCCGACT and (R)GCTGCTGGCACCAGACTT (Accession number AK105009). The PCR reaction was performed with 70–100 ng total RNA, 10 pM primer and EmeraldAmp® GT PCR Master Mix (Takara, Japan). The PCR conditions were as follows: 94°C for 3 min, 18–37 cycles of 94°C for 30 s, 56–67°C for 30 s, 72°C for 30 s and 72°C for 5 min. The DNA obtained was subjected to agarose gel electrophoresis and stained with ethidium bromide. The signal intensity of the stained bands was photographed with a Gel Doc image analysis system (Bio-Rad, Hercules) and the data were analyzed using GeneTools™ (Syngene, Cambridge, UK) analysis software.

Na^+ and K^+ assay

Na^+ and K^+ were assayed following the modified method of Tanaka et al. (1999) and Hossain et al. (2006). In brief, leaf blade, leaf sheath and root tissues of salt stressed seedlings were collected. The samples were washed with deionized water to remove surface contaminating Na^+. The tissues were ground into a powder in liquid nitrogen, extracted with boiling distilled water, and centrifuged at 10,000×g for 10 min. The supernatant was dried and dissolved in distilled water. Cellular Na^+ and K^+ concentrations were determined using HPLC coping with 432 Conductivity Detector and WATER IC-PACK™ ion exclusion column.

CoroNa-Green Na^+ localization assay

Histochemical localization to reveal Na^+ content was performed as described (Oh et al., 2009). Briefly, fresh leaf blade, leaf sheath and root tissues of rice seedlings were sliced and incubated in chilled medium A (0.5 M sorbitol, 1 mM $CaCl_2$, 0.20% poly-vinyl-pyrrolidone and 5 mM Tris-MES pH 5.5) containing 10 µM CoroNa Green AM (Molecular Probes, Eugene, OR, USA) and 0.02% Pluromic F-127 for 1h. Thereafter, excess CoroNa Green was removed by washing with medium A twice, and the explants were immediately observed under Confocal Lazor Scanning Microscope (FV 1000-D, Olympus, Japan) at excitation and emission wavelengths of 488 nm and 516 nm, respectively.

Starch and total soluble sugars determination

The soluble and insoluble starch contents in the leaf blade, leaf sheath and root tissues were determined with an EnzyChrom assay kit (BioAssay Systems, Hayward, CA), using an enzymatic colorimetric method that quantifies the concentration of both soluble and resistant starch (McCleary and Monaghan 2002). Briefly, a plant sample of one hundred-milligram was ground and the sugars were extracted with 1 mL of 90% ethanol at 60°C in water bath for 5 min with triple repeats. Then, the ethanol was removed by evaporation method. Soluble and insoluble starch was dissolved by 0.5 mL distilled water at 60°C in water bath for 5 min, then 0.2 mL DMSO (dimethyl sulphoxide) was added at 60°C for 5 min. Concentrations of soluble and insoluble starch were determined by colorimetric measurement of glucose residue using amyloglucosidase and α-amylase digestion. Total soluble sugars (sucrose, glucose and fructose) in the flag leaf tissues were analysed according to the modified method of Karkacier et al. (2003). In a pre-cooled mortar, 100 mg tissue was ground with liquid nitrogen, extracted with 1 mL nanopure water, vigorously shaken for 15 s, sonicated for 15 min and then centrifuged at 12,000 rpm for 15 min. The supernatant was filtered through a 0.45 µm membrane filter (VertiPure™, Vertical®) and stored at −20°C prior to the measurement of total soluble sugars content using high performance liquid chromatography (HPLC). A volume of 40 µL crude extracts was automatically injected into a Waters HPLC (Waters Associates, Millford, MA, USA) fitted with a Waters 600 pump using a MetaCarb 87C column equipped with a guard column. Deionised water was used as the mobile phase at the flow rate of 0.5 mL min^{-1}. The online detection was performed using a Waters 410 differential refractrometer detector and the data were analyzed by Empower® software. Sucrose, glucose and fructose (Fluka) were used as the reference standards.

Iodine staining for starch localization assay

Starch localization was done using iodine staining method described by Caspar et al. (1991) and modified by Yu et al. (2001). Briefly, whole seedlings of rice crop were incubated in killing solution (composed of 80% ethanol and 5% formic acid in water) for 10 min. Then, the plant samples were bleached in 80% ethanol at 80ºC for 5 min prior to staining in Lugol's solution for 3 min. The excess Lugol's solution was removed by washing with distilled water at 80ºC for 15 min and at 4ºC for 15 min, respectively. The explants were then

imaged under light stereo microscope (Stemi SV6, Zeiss, Germany).

Conclusion

In conclusion, the growth performance of *indica* rice cv. IR29 seedlings significantly declined when subjected to 200 mM NaCl for 4 d. Na^+ absorption by root tissues of rice seedlings was in the order: cv. IR29 > cv. Pokkali in qualitative assay using HPLC and quantitative determination by CoroNa green labeling. Leaf sheath of IR29 (salt intolerant) was the major organ to accumulate Na^+ and starch. A positive correlation between Na^+ and soluble sugar content was observed in leaf blade and leaf sheath.

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