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High contents of proline and anthocyanin increase protective response to salinity in *Oryza sativa* L. spp. *indica*

Sutee Chutipaijit[1], Suriyan Cha-um[2*] and Kanokporn Sompornpailin[1,3]

[1]College of Nanotechnology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, 10520, Thailand
[2]National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, 113 Thailand Science Park, Klong Luang, Pathumthani, 12120, Thailand
[3]Thailand Center of Excellence in Physics, CHE, Ministry of Education, Bangkok,10400, Thailand

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

Abstract

Pigmented rice cultivar is a good candidate for abiotic tolerance especially salt tolerant trait. Physiological and growth parameters of eight *indica* rice genotypes under control or salt stress conditions were subjected to Hierarchical cluster analysis. From cluster ranking, rice genotypes were classified into two groups, salt-tolerant (Sangyod, Khao Dang, Kulab Dang and TD49) and salt-sensitive genotypes (Klum Sakol, Klum Khonkaen1, Klum Khonkaen2 and Black Sticky Rice). In salt stress (100 mM NaCl), the salt-tolerant genotypes showed the high percentages of proline and anthocyanin more than in the salt-sensitive genotypes. In addition, the photosynthetic pigment stabilizations of stressed-seedlings were positively correlated to the proline (R^2 = 0.98) and anthocyanin (R^2 = 0.70) accumulations. While, the malondialdehyde (MDA) contents of salt-stressed seedlings were negatively correlated to the proline (R^2 = 0.63) and anthocyanin (R^2 = 0.51) accumulations. Moreover, the total antioxidant activity of extraction from stressed-seedlings was assessed using the ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) method. The higher percentages of total antioxidant activity were determined in salt-tolerant (125-199%) than salt-sensitive genotypes (106-113%). From the results it can be concluded that accumulation of above mentioned metabolites is associated with cellular protection and salt detoxification in salinity rice seedlings.

Keywords: anthocyanin, *indica* rice, lipid peroxidation, photosynthetic pigments, proline, salt stress.

Abbreviations: ABTS_2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; APX_ascorbate peroxidase; BSR_Black Sticky Rice; Car_total carotenoids; CAT_catalase; Chl_A_chlorophyll a; Chl_B_chlorophyll b; DW_dry weight; FW_fresh weight; KD_Khao Dang; KK1_Klum Khonkaen1; KK2_Klum Khonkaen2; KLD_Kulab Dang; KS_Klum Sakol Nakorn; MDA_malondialdehyde; PPF_photosynthetic photon flux; ROS_reactive oxygen species; SOD_superoxide dismutase; SY_Sangyod.

Introduction

Salinity is one of the most important abiotic stresses affecting plant growth and development, as well as productivity, especially in rice crops. Under saline conditions, the plant cell is confronted by osmotic stress, ionic toxicity and nutritional disorder (Patade et al., 2008). The defense response signals inside cell are regulated downstream, and the cell consequentially generates secondary messengers, including calcium and reactive oxygen species (ROS). Undue amounts of ROS may lead to metabolic imbalance and cause cell damage by a lipid peroxidation mechanism, resulting in oxidative stress. In the lipid peroxidation reaction, malondialdehyde (MDA) is the first product formed during free radical induced damage and the decomposition of polyunsaturated fatty acids in membranes. Thus, the determination of membrane function by measuring MDA production is frequently performed to identify oxidative damage in cells (Sheokand et al., 2008). Salt-tolerant species have many defense mechanisms which cope with stress. In stressed plant cells, various compatible solutes i.e. polyamine and proline are commonly accumulated and function as osmotic adjustment. Many plant species, such as maize, wheat and rice, accumulate high levels of proline when exposed to saline stress (Neto et al., 2004; Demiral and Türkan, 2005; Tammam et al., 2008). In higher plants, proline is biosynthesized by either the glutamate or the ornithine pathway. The glutamate pathway is considered the major route, especially in response to osmotic stress (Kishor et al., 2005). Generally, proline protects plants from stress through different processes, including by the adjustment of cellular water, detoxification of ROS, protection of membrane integrity, and stabilization of enzymes and proteins, thus, it can be beneficial to plants in adapting to stress (Maggio et al., 2002; Trovato et al., 2008). ROS scavenging systems in higher plants consist of several antioxidant enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT), as well as some low molecules of non-enzymatic antioxidants, such as flavonoids, ascorbic acid, and α-tocopherol. These molecules are generally functioned when plant exposed to abiotic stresses (Kartashov et al., 2008). Flavonoids derived from the phenylpropanoid biosynthetic pathway are known to be responsive to biotic and abiotic stress situations (Treutter, 2006). Flavonoids are well known for a range of biological functions, including powerful antioxidant activities.The antioxidant capacity of each flavonoid, including anthocyanin, is related to their chemical structure (Wu et al., 2010). Therefore, the objective of this study was to classify the salt-tolerant group of pigmented rice genotypes based on the accumulation of proline and anthocyanin antioxidants, as well as photosynthetic pigment stabilization, and growth performance in salt stressed seedlings.

Results

Morphological characters and cluster ranking

Morphological, physiological and biochemical characteristics of eight rice genotypes were evidently degraded when seedlings were exposed to 100 mM NaCl salt stress for 4 days. Multi-parameters of growth reduction (FW and DW), photosynthetic pigment degradation, proline accumulation, MDA enrichment and the total antioxidant activity in salt-stressed rice seedlings were subjected to cluster analysis. The results showed that rice genotypes were divided into two groups, salt-tolerant genotypes, SY, KD, KLD and TD49, and salt-sensitive genotypes, KS, KK1, KK2 and BSR (Fig. 1). The growth characters, fresh weight and dry weight, in both salt-tolerant and salt-sensitive genotypes were reduced when exposed to salt stress. Growth performance in salt-tolerant genotypes was maintained better than in salt-sensitive genotypes (Table 1). The reduction in FW and DW of salt-tolerant genotypes was about 5.5-9.5 and 2.5-4.0%, respectively. While, FW and DW reduction in salt-sensitive genotypes was about 8.5-23.5 and 7.5-13.0%, respectively (Table 1).

Physiological characters

Photosynthetic contents in salt-stressed seedlings were significantly reduced, especially in salt-sensitive genotypes. Chl_A, Chl_B and Car contents were 30-50%, 40-50% and 30-37% of the control, respectively. In contrast, those parameters were maintained in salt-tolerant genotypes by 87-95%, 89-93% and 89-97%, respectively (Table 2). This means that the photosynthetic pigments in salt-tolerant seedlings grown under salt stress stabilized better than those in salt-sensitive seedlings.

Biochemical characters

Proline accumulation in salt-stressed seedlings increased to a significantly higher degree than in seedlings without salt stress, especially in salt-tolerant genotypes. The proline content in the stressed leaves of salt-tolerant genotypes was enriched by 1.64-1.78 times when compared to seedlings without salt stress (Fig. 2A). On the other hand, proline content in salt-sensitive genotypes increased slightly in response to salt stress by 1.09-1.20 times. In addition, anthocyanin content in salt-tolerant genotypes was accumulated greatly and higher than those in salt-sensitive genotypes under salt treatments (Fig. 2B). The highest rate of increase in anthocyanin accumulation (3.86 times) was demonstrated in SY salt-stressed seedlings while the lowest rate was represented in BSR salt-stressed seedlings (1.09 times) in comparison with non-stressed seedlings. An increase in MDA content in rice genotypes was generally induced by salt stress treatment. Under this treatment, MDA content in salt-sensitive rice seedlings (131-157%) was significantly higher than salt-tolerant seedlings (119-127%; Fig. 3). In this study, the positive relationships between proline accumulation and photosynthetic pigment stabilization (R^2 = 0.98), and between anthocyanin accumulation and photosynthetic pigment stabilization (R^2 = 0.70) were demonstrated (Fig. 4). In addition, the relationship between MDA and proline accumulation (R^2 = 0.63) as well as MDA and anthocyanin accumulation (R^2 = 0.51) were negatively correlated (Fig. 5). The scavenging ABTS free radicals ($ABTS^{\bullet+}$) can be used to estimate the total antioxidant activity of extracted solution. The total antioxidant activity of anthocyanin extraction from salt-stressed seedlings was expressed as µM of Trolox by ABTS method. The total antioxidant activity result of stressed-seedlings extraction was presented in Fig. 6. The total antioxidant activity of salt-tolerant extractions was 125-199% higher than that of salt-sensitive extractions (106-113%) when compared to seedlings without salt stress.

Discussion

Different genetic resources revealed varying defensive responses to stress, as determined by multivariate physiological parameters, and were classified as salt-tolerant (SY, KD, KLD and TD49) or salt-sensitive (KS, KK1, KK2 and BSR) genotypes. Both physiological and biochemical characters in rice seedlings grown under saline stress were significantly degraded, especially in the salt-sensitive genotypes. The multivariate cluster analysis for salt tolerance has been utilized to classify the group of salt-tolerant rice including upland rice (Cha-um et al., 2010) and breeding lines (Cha-um et al., 2009a). The severity of antagonism to the plant growth caused by salinity, as indicated by FW and DW measurements, was lower in salt-tolerant rice, implying that NaCl has less influence on the growth of these rice seedlings (Table 1). The chlorophyll pigment in rice is very sensitive to salt stress, especially in salt susceptible genotypes (I Kong Pao) (Lutts et al., 1996). This study also indicated that the content of photosynthetic pigments in the salt-sensitive group, when exposed to salt stress, was less stable than in the salt-tolerance group. The restriction of water supply, occurring due to salinity, has been shown to reduce plant biomass in relation to genotype (Cha-um et al., 2004; Feizi et al., 2010). In previous reports, it was shown that chlorophyll degradation in salt-stressed rice is positively related to reduction of net photosynthetic rate (Cha-um et al., 2009b; Hasaneem et al., 2009), or CO_2 assimilation, because of the limitation of leaf stomatal closure and transpiration rate (Yamane et al., 2003; Cha-um et al., 2004). In addition, rice adapted in response to salt stress by dangerously increasing ROS production, and this response affected rice biomass (Wanichananan et al., 2003; Cha-um et al., 2004).Stress signaling, including salinity may cause an imbalance between light absorption and the use of light energy. Oxygen is converted to different ROS, especially singlet oxygen ($^1O_2$), which is mainly generated within chloroplasts (Jogeswar et al., 2006). Chlorophyll concentration can be used as a sensitive indicator of the cellular metabolic state; thus, its decrease signifies toxicity in tissues due to the accumulation of ions. In this study, Chl_A, Chl_B and Car contents decreased in NaCl treated rice seedlings (Table 2), so growth efficiency also declined. Several reports have shown that $^1O_2$ ROS generated under stress conditions is rapidly amplified and spreads throughout the cell (Sharma et al., 2005; Djanaguiraman et al., 2006). It can react rapidly with unsaturated lipids present in plant organelles (chlorophyll and mitochondria) and intact membranes (Demiral and Türkan, 2005). The MDA content in rice seedlings represents the peroxidation level of membrane lipids, which is used as an effective index of cellular damage (Sharma et al., 2005). The

Table 1. Fresh weight (FW) and dry weight (DW) of *indica* rice genotypes cultured in 100 mM NaCl or without NaCl for 4 days

Genotypes	NaCl (mM)	FW (mg)	DW (mg)
KS	0	218.82 b (100 %)	32.68 b (100 %)
	100	200.16 a (91.47 %)	30.32 a (92.77 %)
BSR	0	200.62 b (100 %)	27.92 b (100 %)
	100	153.50 a (76.51 %)	22.92 a (88.15 %)
KK1	0	256.38 b (100 %)	37.58 b (100 %)
	100	210.26 a (82.01 %)	32.70 a (87.01 %)
KK2	0	192.92 b (100 %)	30.40 b (100 %)
	100	175.78 a (91.12 %)	27.56 a (90.66 %)
SY	0	176.26 b (100 %)	25.86 a (100 %)
	100	166.68 a (94.56 %)	25.20 a (97.45 %)
KD	0	170.54 b (100 %)	21.76 b (100 %)
	100	160.26 a (93.97 %)	21.10 a (96.97 %)
KLD	0	129.66 b (100 %)	26.64 b (100 %)
	100	121.02 a (93.34 %)	25.72 a (96.55 %)
TD49	0	183.96 b (100 %)	26.74 b (100 %)
	100	168.54 a (91.62 %)	25.68 a (96.04 %)

Means in each genotype followed by the same letter are not significantly different at $P \leq 0.05$.

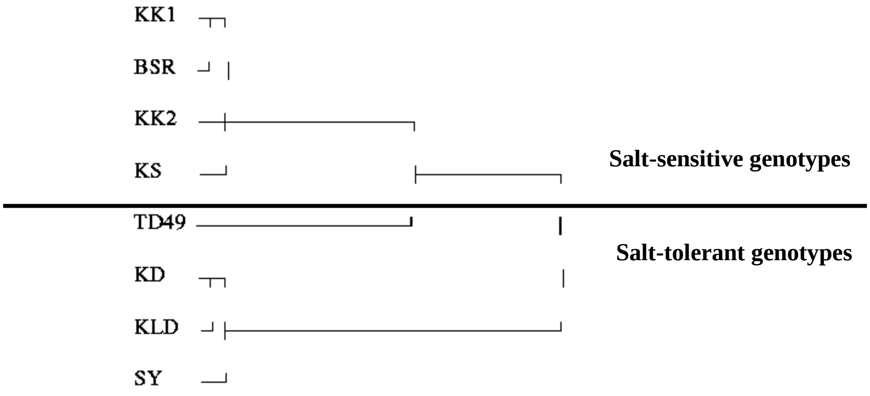

Fig 1. Characterization of cluster in the fresh weight, dry weight, photosynthetic pigments, MDA contents and total antioxidant activity of rice seedlings cultured in 100 mM NaCl for 4 days by Hierarchical cluster analysis in SPSS software

results showed that MDA levels in the salt-sensitive group cultured under salt stress conditions were higher than in the salt-tolerant group. This is a similar pattern to previous studies in salt-tolerant rice genotypes, CT6748-8-CA-17, Pokkali and Lunishree and salt-susceptible genotypes, INIAP12, IR28, Pusa Basmati 1 and Begunbitchi (Demiral and Türkan, 2005; Khan and Panda, 2008) as well as studies into sesame (Koca et al., 2007) and mungbean genotypes (Sumithra et al., 2006). The lower levels of lipid peroxidation in salt-tolerant seedling genotypes under saline conditions suggest that they may have better protection against damage. Various kinds of plant metabolites have been shown to be induced in response to stress, *via* various signals, including ROS (Mandhania et al., 2006; Khan and Panda, 2008). Genotypic variations in proline accumulation during salt stress have been remarked on a variety of crop species and several reports have demonstrated the alteration of proline content in various plant species in response to salinity (Neto et al., 2004; Sohn et al., 2005; Martino et al., 2006). Under saline conditions, these experimental results showed a significant increase in proline accumulation in rice seedlings, with the highest rate in the SY genotype (salt-tolerant). Proline accumulation was positively related to photosynthetic pigment stabilization (R^2 = 0.98). The contradictory results showed that the proline content in the roots of salt-tolerant cv. Pokkali under salt-stress is less than in cv. IR-28 which is salt susceptibile (Demiral and Türkan, 2005). The connection between stress tolerance and the accumulation of proline is either species-specific, or, it can be changed by experimental conditions and may not be universal (de-Lacerda et al., 2003; Ashraf and Foolad, 2007). Proline accumulation normally occurs in the cytosol where it contributes substantially to cytoplasmic osmotic adjustment and has been shown to be correlated with stress tolerance. Proline concentration has been shown to be generally higher in stress-tolerant plants than in stress-sensitive plants when under stress. This has been found to be the case in many plant species, such as mangroves (Datta et al., 2009), wheat (Martino et al., 2006) and rice (Hsu et al., 2003; Sohn et al., 2005). Moreover its osmolyte function, proline has been reported to detoxify free radical activity by scavenging two ROS (OH^o and $^1O_2$) in certain *in vitro* detection systems (Blokhina et al., 2003). From these results, the endogenous pool of proline might help to explain the relatively high overall osmotic and antioxidant tolerance of rice seedlings. The result was a positive relationship between the percentage

Table 2. Chlorophyll a (Chl_A), chlorophyll b (Chl_B) and total carotenoids (Car) contents of *indica* rice genotypes cultured in 100 mM NaCl or without NaCl for 4 days

Genotypes	NaCl (mM)	Chl_A ($\mu g\ g^{-1}$ FW)	Chl_B ($\mu g\ g^{-1}$ FW)	Car ($\mu g\ g^{-1}$ FW)
KS	0	667.18 b	244.90 b	247.21 b
	100	465.03 a	135.31 a	157.16 a
BSR	0	748.55 b	174.79 b	275.56 b
	100	415.60 a	100.98 a	175.81 a
KK1	0	863.50 b	220.98 b	279.31 b
	100	503.42 a	133.08 a	194.51 a
KK2	0	700.04 b	190.37 b	238.69 b
	100	351.77 a	91.21 a	162.14 a
SY	0	708.70 b	170.19 b	260.55 a
	100	672.71 a	157.86 a	252.41 a
KD	0	659.13 b	169.07 b	245.62 b
	100	587.99 a	153.17 a	227.00 a
KLD	0	824.49 b	210.15 b	308.68 b
	100	740.58 a	188.54 a	283.54 a
TD49	0	805.13 b	222.95 b	295.66 b
	100	697.58 a	197.44 a	263.88 a

Means in each genotype followed by the same letters are not significantly different at $P \leq 0.05$.

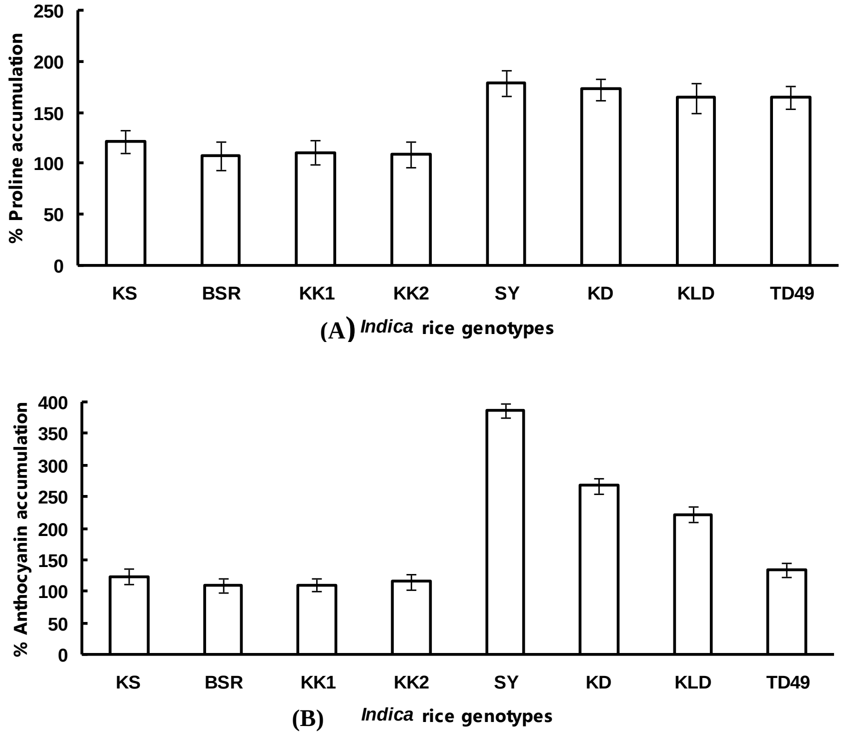

Fig 2. Proline (A) and anthocyanins (B) accumulation of *indica* rice genotypes cultured in 100 mM NaCl (salt stress) for 4 days when compared to control treatment (without NaCl) set as 100%. Error bars represent by ±SE (n=5).

increment of flavonoid substance (anthocyanins) and photosynthetic pigment stabilization (R^2 = 0.70). Salt-tolerant seedlings treated with NaCl had a greater increase in the percentage of accumulated anthocyanins than salt-sensitive seedlings when compared with the control. The results accord with many reports of increased levels of flavonoid and anthocyanins during abiotic stress, including salinity, in various plant species (Tattini et al., 2004; Treutter, 2006). In this research, the result in Fig. 6 showed the high radical scavenging activity correlated with level of stress tolerance in each genotype. During the adaptation of rice seedlings to stress conditions, ROS rapidly amplifies for use in several biological activities. A ROS scavenger should be developed in order to minimize the cytotoxic effects of ROS within the cell. Flavonoids have been reported extensively in terms of their antioxidant activity, which is a result of ROS scavenging (Pourcel et al., 2006). Many reports have mentioned that flavonoids are not only predominantly distributed in the vacuole of most species, but also have been detected in the chloroplast of several species (Tattini et al., 2004; Agati et al., 2007). Flavonoids associated with the chloroplast envelop can potentially help to protect the photosynthetic apparatus from the photo–induced generation of $^1O_2$ ROS (Kong et al., 2003). Moreover, in *Arabidopsis*, anthocyanin-deficient mutants grown under strong light showed more lipid peroxidation than wild-type plants (Agati et al., 2007). Thus, anthocyanins may affect stress defense mechanisms.

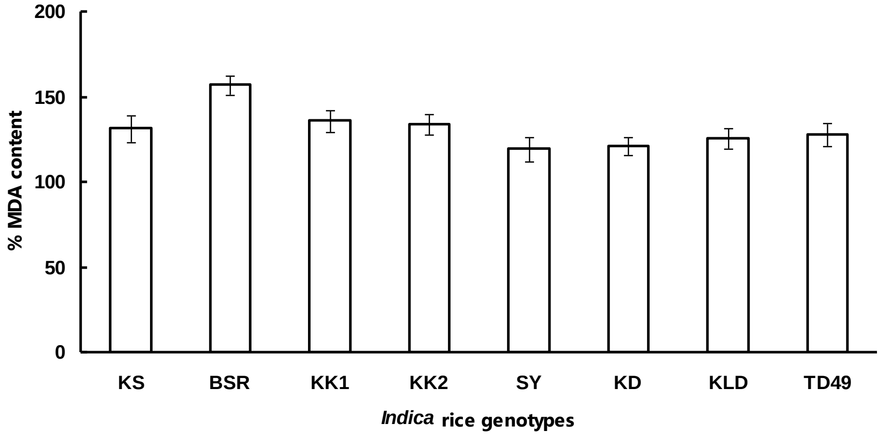

Fig 3. MDA content of *indica* rice genotypes cultured in 100 mM NaCl (salt stress) for 4 days when compared to control treatment (without NaCl) set as 100%. Error bars represent by ±SE (n=5).

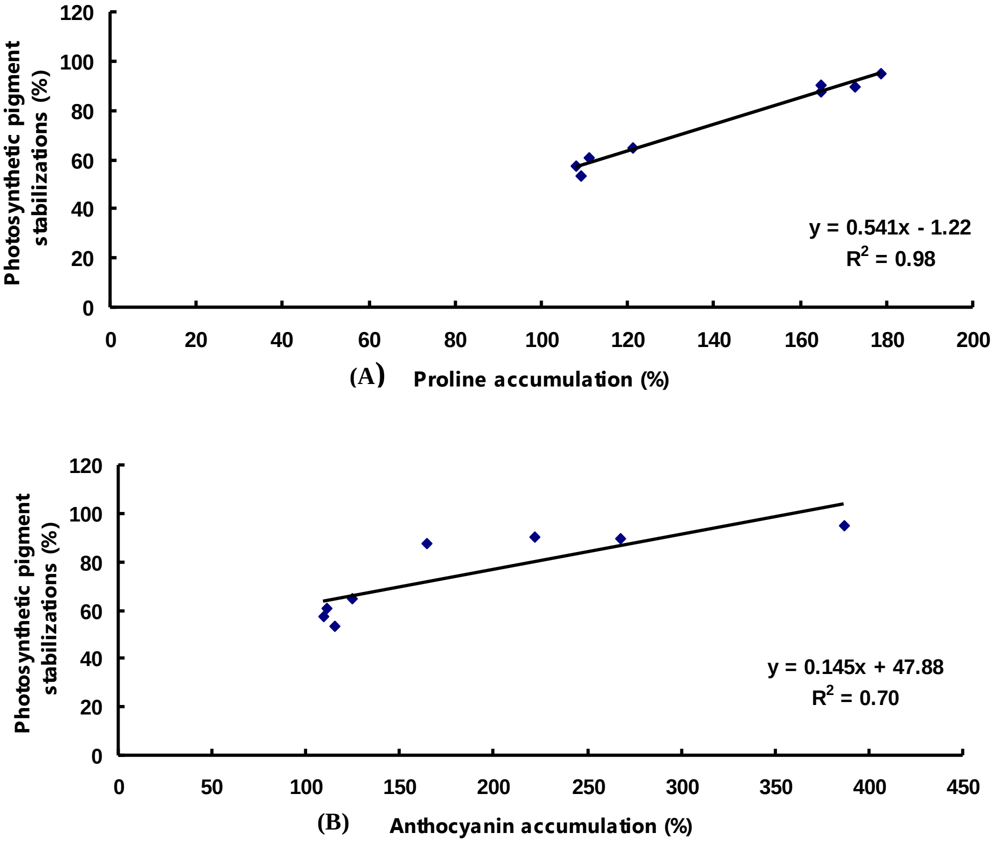

Fig 4. Relationship between proline accumulation and total photosynthetic pigment stabilization (A), anthocyanin accumulation and total photosynthetic pigment stabilization (B) of *indica* rice genotypes cultured in 100 mM NaCl for 4 days.

Materials and methods

Plant materials and treatments

Eight rice genotypes [*Oryza sativa* L. cv. Sangyod (SY), Khaodang (KD), Kulabdang (KLD), TD49, Klumsakolnakorn (KS), Klumkhonkaen1 (KK1), Klumkhonkaen2 (KK2) and Black Sticky Rice (BSR)] were obtained from the Pathumthani and Sakolnakorn Rice Research Center (Rice Research Institute, Department of Agriculture, Ministry of Agriculture and Cooperative, Thailand). Dehusked seeds were surface-disinfected with 70% ethanol for 2-3 min, 5% Clorox® (5.25% sodium hypochlorite, The Clorox Co., USA) for 40 min, 30% Clorox® for 30 min and then washed thoroughly with distilled water. The surface-sterilized seeds were germinated on NB medium (Li et al., 1993) containing 3% sucrose and 0.8% agar and were grown in 25±2°C air temperature, 85-

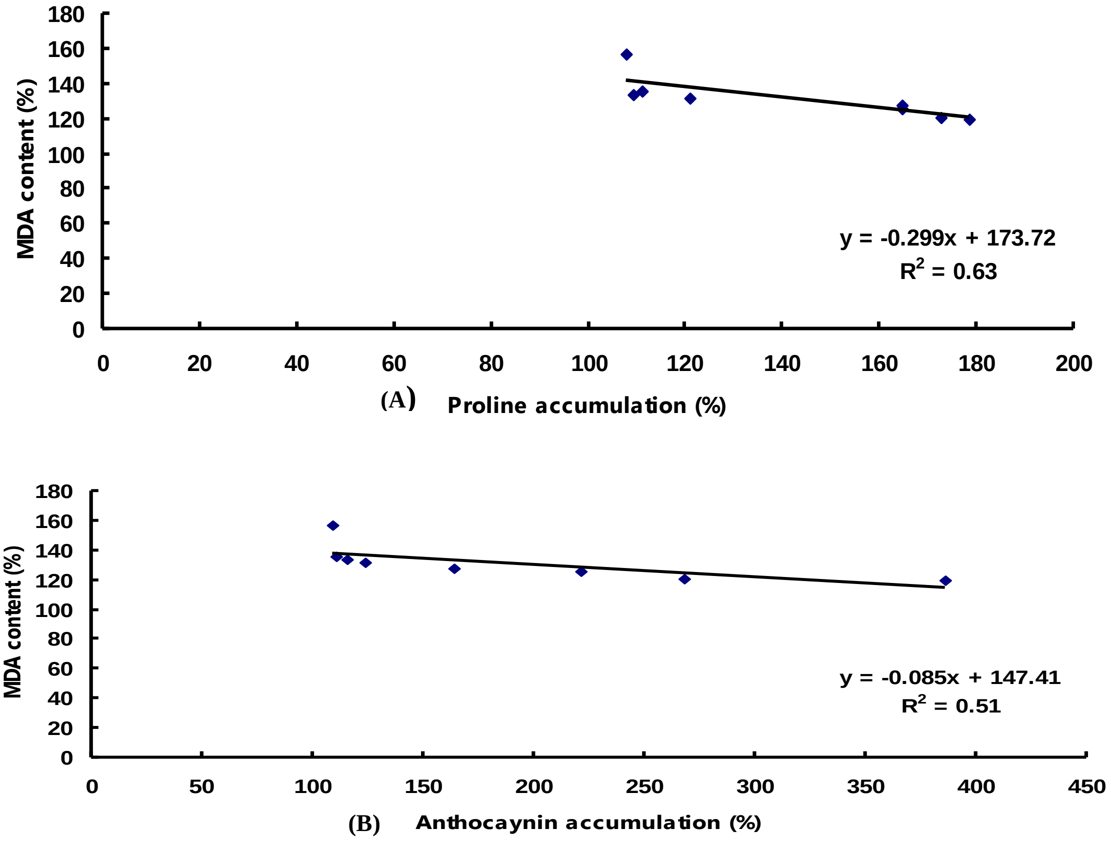

Fig 5. Relationship between proline accumulation and MDA percentage (A), anthocyanin accumulation and MDA percentage (B) of *indica* rice genotypes cultured in 100 mM NaCl for 4 days.

90% relative humidity (RH) and 60 ±5 $\mu molm^{-2}s^{-1}$ photosynthetic photon flux (PPF) with 16 h d^{-1} photoperiod. After one week of germination, seedlings were aseptically transferred to NB-liquid medium under photoautotrophic conditions (CO_2 as a carbon source) using vermiculite as support. The number of air-exchanges in the photoautotrophic system was adjusted to 2.32 µmol CO_2 h^{-1} by punching a hole in the plastic cap (1 cm diameter) and covering the hole with a microporus filter (0.20 µm pore size). After 7 days of growth in the photoautotrophic system, the NaCl concentration (salinity) of the medium was adjusted to 0 (control) or 100 mM NaCl (salt stress) for 4 days. Rice seedlings were frozen in liquid nitrogen (-196°C) and stored at -80°C prior to analysis.

Growth parameters

Five seedlings from control and stress treatments were sampled randomly on the fourth day of NaCl treatment. The fresh weight (FW) and dry weight (DW) of seedlings were recorded. For DW determination, samples were oven dried at 70°C for 24 h and then weighed.

Photosynthetic pigments

Chlorophyll a (Chl_A), chlorophyll b (Chl_B) and total carotenoids (Car) contents were analyzed following the methods of Lichtenthaler (1987) and Shabala et al. (1999).

Lipid peroxidation

The levels of lipid peroxidation in rice seedling samples were determined in term of malondialdehyde (MDA) content according to the method of Hodges et al. (1999).

Proline Content

Free proline content was determined according to Gilmour et al. (2000).

Anthocyanin level

Total anthocyanin contents were determined according to Harborne (1998) and Reddy et al. (1995).

Total antioxidant activity assay

The total antioxidant activity of anthocyanin extraction from rice seedlings was determined using ABTS method according to Re et al. (1999) expressed as µM of Trolox equivalent antioxidant capacity.

Experimental design

The experiment was designed as a Completely Randomized Design (CRD) with five replicates (n=5). The mean values were compared by *t*-test and analyzed using SPSS software (SPSS for Windows version 15, SPSS Inc., Chicago, USA).

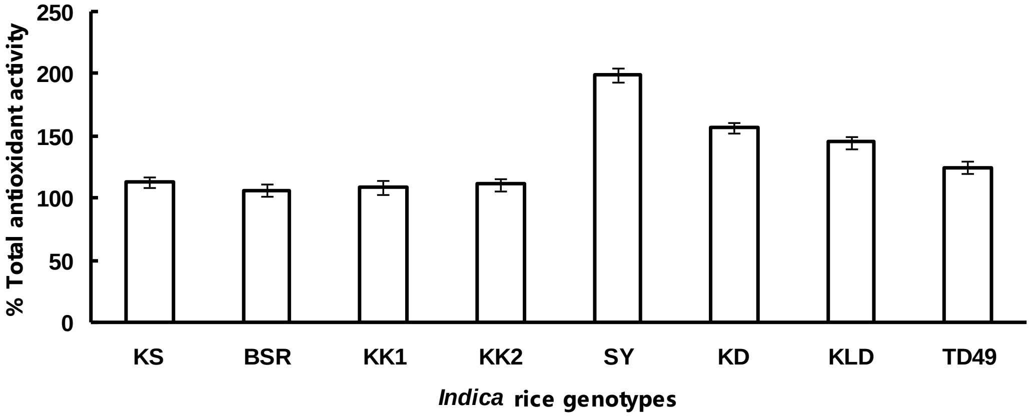

Fig 6. The total antioxidant activity of *indica* rice genotypes cultured in 100 mM NaCl (salt stress) for 4 days when compared to control treatment (without NaCl) set as 100% by ABTS method. Error bars represent by ±SE (n=5).

The degradation of growth characters and accumulation of proline and anthocyanin in salt stressed seedlings were calculated and subjected to classify the group using Hierarchical cluster analysis.

Conclusion

Different genotypes of *indica* rice showed variation in biomass, photosynthetic pigment contents, MDA contents, proline accumulation, anthocyanin accumulation and total antioxidant activity when exposed to saline stress. Multivariate parameters played a role as effective criteria to classify the rice genotypes into salt-tolerant and salt-sensitive clusters. Under saline conditions, salt-tolerant genotypes for example SY, which showed higher percentage increases of proline and anthocyanins, demonstrated better biomass and stability of photosynthetic pigments than salt-sensitive genotypes. Both substances are relative important in plant defense mechanisms against salt stress.

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