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Research Article

Changes in Anthocyanin Content and Expression of Anthocyanin Synthesis Genes in Seedlings of Black Glutinous Rice in Response to Salt Stress

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Abstract

Background and Objective: Anthocyanins have been implicated to offer some protective roles for vegetative and reproductive tissues under abiotic stresses. In this study, the effects of salt stress on physiological responses including anthocyanin accumulation and the expression of anthocyanin biosynthetic genes in seedlings of two cultivars of black glutinous rice (*Oryza sativa* L.) differing in the levels of salinity tolelance and leaf anthocyanin were investigated. **Materials and Methods:** Two rice cultivars namely Niewdam Gs. No. 00621 (salt-tolerant, deep purple leaf colour) and KKU-LLR-039 (salt-sensitive, greenish purple leaf color) were grown in a hydroponic culture until 21 days old. Thereafter, they were treated with 0, 75 and 150 mM NaCl for 0, 2, 4, 6 and 8 days. Leaf tissues were collected for physiological determination and expression analysis of anthocyanin biosynthesis genes. **Results:** The growth parameters, total chlorophyll and chlorophyll fluorescence ratio (F_v/F_m) were progressively reduced while the electrolyte leakage rate increased with increasing NaCl concentrations and the length of time of salinity treatments. For all physiological parameters, the cultivar Niewdam Gs. No. 00621 was less afftected under salt stress. The highest anthocyanin content was attained after 2 and 4 days of salt stress in KKU-LLR-039 and Niewdam Gs. No. 00621, respectively; followed by a reduction in both cultivars until 8 days of salt stress. Real-time PCR analysis showed that the patterns of expression of anthocyanin biosynthesis genes Phenylalanine Ammonia Lyase (PAL), chalcone synthase (CHS), dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase (ANS) were related with variations in anthocyanin contents. The maximum expression levels of CHS, DFR and ANS compared to those of non-stressed controls were observed after 2 and 4 days of stress for KKU-LLR-039 and Niewdam Gs. No. 00621, respectively, thereafter the expression dramatically reduced after 6-8 days of stress. The anthocyanin content and relative expression of the four genes was higher in the leaves of Niewdam Gs. No. 00621 than in KKU-LLR-039. **Conclusion:** Higher accumulation of anthocyanins may be one of the protective physiological traits under salt stress. Therefore, the rice cultivar with deep purple leaf color suffered from less severe cellular damages and lower growth reduction under salt stress compared with the one with less intense anthocyanins.

Key words: Anthocyanins, black glutinous rice, chlorophyll, electrolyte leakage, gene expression, salinity stress, real-time PCR, rice seedlings

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Salinity is a major abiotic stress factor affecting growth and productivity of crop plants in many areas of the world[1]. Salt stress is an important environmental stress that causes a low soil water potential, accumulation of toxic sodium ions, nutritional imbalance and subsequently led to an over-accumulation of Reactive Oxygen Species (ROS)[2] which cause a whole range of cellular oxidative damages. Plant responses to high salinity are rather complicated, involving multiple processes and mechanisms including increased amount of anthocyanins associated with increased salt tolerance[3,4]. Recently, it was proposed that anthocyanin synthesis regulated by sucrose-signaling pathway under various types of abiotic and biotic stress not only serves as direct antioxidants but also as secondary signals involved in counteracting multiple stresses in plants[5].

Rice is an important crop and a major food source for more than half of the world population. In recent years, black glutinous rice has received growing attentions due to its high nutritional values. Pigmented rice grains contain a group of purple water soluble substances called anthocyanins which are the primary pigments in the red and black rice grains[6]. The major anthocyanin components in colored rice are cyanidin-3-glucoside and peonidin-3-glucoside[7].

Anthocyanins are plant secondary metabolites synthesized by flavonoid biosynthesis pathway using phenylalanine as the precursor. In the first 3 steps, phenylalanine is converted to 4-coumaroyl-CoA via the activity of Phenylalanine Ammonia Lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumaroyl: CoA-ligase (4CL). Anthocyanins are then synthesized by the activity of chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol reductase (DFR) and anthocyanidin synthase (ANS)[8,9]. Flavonoids compounds particularly anthocyanins have been reported to play important roles in defense under abiotic and biotic stress and serve essential functions in plant reproduction and protect plant cells against damage by reactive oxygen species[10,11].

Anthocyanin accumulation has been reported to be a protective mechanism for plants under various abiotic stresses including salinity stress[2,12]. Co-ordination between elevated expression of anthocyanin biosynthesis genes and accumulation of anthocyanin has been demonstrated in pigmented rice seeds cv: Heuglinju[13], purple storage roots of sweet potato[14], red cabbage seedlings[15] and wheat leaves under drought stress[16]. The flavonoid pathway leading to the biosynthesis of anthocyanins in rice is induced by multiple regulatory genes as well as various developmental, physiological and environmental signals. However, little is known about the anthocyanin accumulation and roles of the gene expression in the anthocyanin biosynthesis pathway in response to salt stress in the purplish leaves of black glutinous rice. Therefore, it was investigated that the effects of salt stress on physiological responses, anthocyanin accumulation and the expression of four genes involved in anthocyanin biosynthesis (PAL, CHS, DFR and ANS) in leaves of two cultivars of black glutinous rice with contrasting salt tolerance and different intensities of leaf anthocyanin.

MATERIALS AND METHODS

Plant materials and growth conditions: Seeds of 2 cultivars of black glutinous rice including, Niewdam Gs. No. 00621 (deep purple leaf color) and KKU-LLR-039 (greenish purple leaf color) were obtained from Faculty of Agriculture, Khon Kaen University. Based on the modified Standard Evaluation Scores (SES) of salt injury at seedling stage[17], Niewdam Gs. No. 00621 was previously evaluated as being more tolerant to salt stress[18] than KKU-LLR-039. Seeds were surface-sterilized using 4% (v/v) sodium hypochlorite solution for 10 min and then rinsed 3 times with sterile distilled water, thereafter they were sown on moistened filter papers placed in petri plates. Germinated seedlings were transferred to a nutrient solution[19] and the plants were grown until 21 days old, thereafter the seedlings were subjected to salinity stress. Sodium chloride (NaCl) was added to the nutrient solution to obtain 75 and 150 mM NaCl, four plastic containers were used for each treatment with 20 plants per container. Randomly sampled plants were taken for analysis after 0, 2, 4, 6 and 8 days of stress. The length of each seedling and fresh weights of shoots were measured at 0, 4 and 8 days after salt treatment. For dry weight determination, plants were dried for 48 h at 80°C until a constant weight was attained. The youngest fully expanded leaves were collected and either immediately analyzed for Electrolyte Leakage (EL) or stored at -80°C for analysis of total chlorophyll content, total anthocyanin content and gene expression.

Analysis of chlorophyll fluorescence: Chlorophyll fluorescence was measured on the youngest fully expanded leaves of rice plants after 0, 2, 4, 6 and 8 days of stress between 11:00-13:00 using a chlorophyll fluorescence meter (Handy-PEA; Hansatech, Kings Lynn, UK). The minimal (F_0) and maximal fluorescence (F_m) were assessed after 30 min of dark adaptation and the maximal quantum efficiency of PSII chemistry was calculated as:

$$\frac{F_v}{F_m} = \frac{F_m - F_0}{F_m}$$

where, F_v is a variable fluorescence yields in the dark-adapted state.

Analysis of electrolyte leakage: Electrolyte leakage was determined using the method described by Lutts *et al.*[20]. Fresh leaf samples were cut into small pieces (about 5 mm long) and then immersed in 10 mL of deionized water and incubated in a water bath at 25°C. After 2 h, the initial electrical conductivity (EC1) was measured. Subsequently, samples were heated at 100°C for 20 min to release all electrolytes, cooled to 25°C and the final electrical conductivity (EC2) was measured. The electrolyte leakage expressed in percentage (%) of total electrolytes was calculated by using the equation:

$$EL = \frac{EC1}{EC2} \times 100$$

Analysis of total chlorophyll: Total Chlorophyll (TC) was determined according to a modified method outlined by Arnon[21]. Total chlorophyll was extracted from leaf samples (0.1 g) by soaking them in 10 mL of 80% acetone solution for 72 h in the dark. After this period, the absorbance at 645 and 663 nm was recorded and the calculation of chlorophyll concentration was carried out using the following equation:

$$\text{Total chlorophyll content (mg g}^{-1}) = \left(20.2\,(A_{645}) + 8.02\,(A_{663})\right) \times \left(\frac{V}{1000\,W}\right)$$

where, A_{645} and A_{663} represent absorbances of TC extract at 645 and 663 nm, respectively, V is the total extract volume and W is the leaf fresh weight. The results are expressed as mg g^{-1} fresh weight.

Analysis of total anthocyanin content: Total anthocyanin content was analyzed by the modified procedure of Abdel-Aal and Hucl[22]. Leaf sample (0.1 g) was soaked in 10 mL of a mixture of ethanol and 1 N HCl (85:15 v/v) for 72 h at 4°C. The crude extracts were filtered through a 0.45 µm syringe filter prior to measurement of total anthocyanin content at 535 nm. The content was expressed as mg g^{-1} fresh weight.

RNA extraction and cDNA synthesis: Total RNA was prepared from leaf samples (0.1 g) using TRIZOL reagent (Invitrogen, USA), following the manufacturer's instructions and treated with DNaseI (Invitrogen, USA) prior to cDNA synthesis. The concentration of total RNA was measured with a nanodrop spectrophotometer (Maestrogen, Taiwan) and quality of the total RNA was determined by 1% (w/v) agarose gel electrophoresis containing SYBR gold dye and visualized under UV illumination. First-strand cDNA was synthesized using the transcriptor first strand cDNA synthesis kit (Qiagen, USA) according to the manufacturer's instructions. The cDNA stock was stored at -80°C until needed and diluted 10 folds prior to use.

Primer design for real-time PCR analysis: Specific primers for PAL, CHS, DFR and ANS genes were designed on the basis of the genomic sequences of *Oryza sativa*: GenBank accession: X16099, X89859, Y07956, Y07955 for PAL, CHS, DFR and ANS, respectively, based on the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). All primers were designed using IDT Primer-quest^{SM} software and synthesized by IDT (Integrated DNA technologies, Belgium) and were adjusted to a melting temperature (Tm) varying between 58 and 62°C with fragments in the 112-182 bp range. Specificity of the designed primers was confirmed by BLAST analysis at the NCBI. Primer design specifications and amplification size of real-time PCR products are listed in Table 1.

Real-time PCR analysis: Real-time analysis of gene expression involved in the anthocyanin pathway (PAL, CHS, DFR and ANS) and the housekeeping gene (Actin) was performed using the Light Cycler® 480 instrument with software version 1.5 (Roche Diagnostic GmbH, Germany). All real-time PCR runs were performed in duplicate. A PCR master mix was filled in the 96 well plate. Real-time PCR was conducted in a 15 µL reaction volume containing, 0.2 µL primers (final concentration 5 µM), 4 µL PCR grade water, 7.6 µL SYBR green real-time PCR master mix (Roche, Germany) and 3 µL of the diluted synthesized cDNA template (1:10 ratio). The PCR

Table 1: Primer sequences for real-time PCR analysis of anthocyanin synthesis gene expression in rice seedlings under salt stress

Gene	Primer	Primer sequence (5'→3')	Product size (bp)
PAL	Forward	AGCTCCGTCAAGAACTGCGTC	112
	Reverse	CGATGGCGGTGAGGAGGT	
ANS	Forward	AGCTGCTCGCCATCCTCTCC	182
	Reverse	GCTGACGTCGGTGTGTGCC	
CHS	Forward	GTTCACACCGCTGGGGATCT	113
	Reverse	TCCTCTCCTTGTCCAGCCCA	
DFR	Forward	GACATCGACTTCTGTCGCCG	121
	Reverse	TGACGCTGATGAGGTCCAGC	
Actin	Forward	TGACGGAGCGTGGTTACTCA	127
	Reverse	GAGGAGCTGGTCTTGGCAGT	

reaction was performed as follows: Initial denaturation at 95°C for 5 min followed by 45 cycles of denaturation at 95°C for 10 sec, annealing at 65°C for 10 sec and extension at 72°C for 10 sec, followed by melting curve analysis: 72°C for 1 min.

Experimental design and data analysis: The experiments followed a completely randomized factorial design with two levels of NaCl and four replicates. The data were analyzed using the SPSS for windows (SPSS for windows version 16). Mean differences were analyzed using Duncan's Multiple Range Test (DMRT) for separations of means significantly differing at $p<0.05$. Gene expression data were analyzed by Light Cycler® 480 software (Roche, Germany). The expression of genes was calculated by the method of relative quantification with automatically set baseline and manually adjusted fluorescence threshold used for determination of target gene in comparison with the reference gene, representing the endogenous reference which was used for normalization of data.

RESULTS

Effect of NaCl on growth: Shoot length, fresh and dry weights of shoots were reduced with increasing salinity (Fig. 1). Under the longest period (8 days) of stress, both concentrations of NaCl (75 and 150 mM) caused a significant reduction in shoot length (i.e., 10.48 and 23.20% reduction for Niewdam Gs. No. 00621 and 15.81 and 30.21% for KKU-LLR-039). Treatments with 75 and 150 mM NaCl resulted in 20.36 and 111.66% reductions in fresh weights of Niewdam Gs. No. 00621 seedlings and 39.66 and 130.56% for those of KKU-LLR-039. For Niewdam Gs. No. 00621 salinity

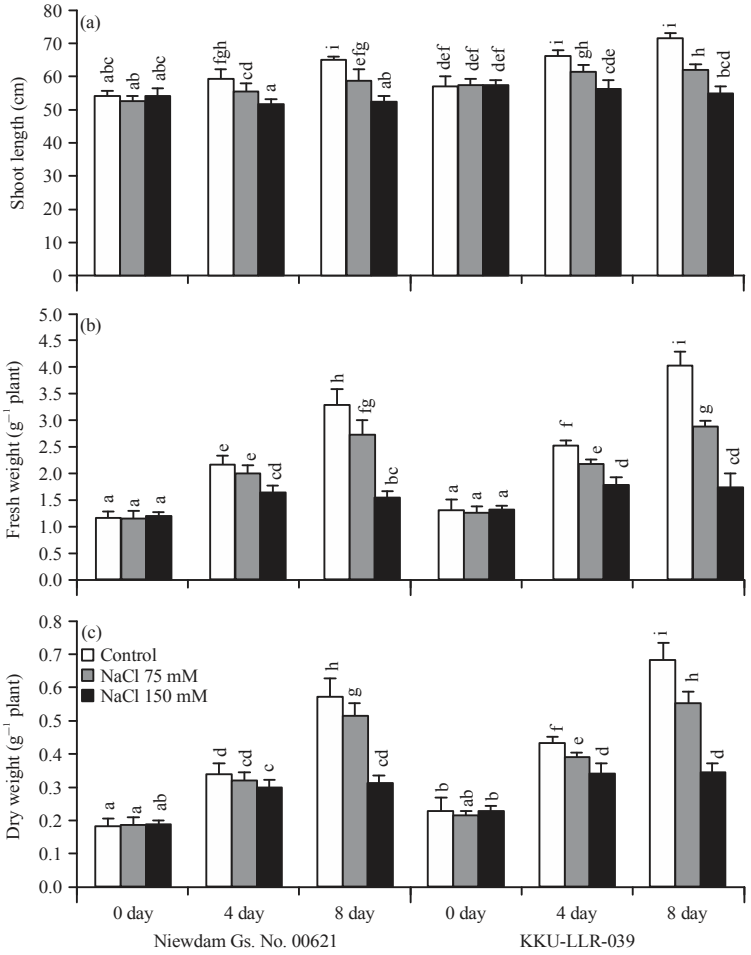

Fig. 1(a-c): Effect of salinity on (a) Shoot length, (b) Fresh weight and (c) Dry weight of two rice cultivars which were subjected to 0, 75 and 150 mM NaCl for 0, 4 and 8 days. Values represent the Mean±SD, means with different letters are significantly different according to Duncan's multiple range test ($p<0.05$)

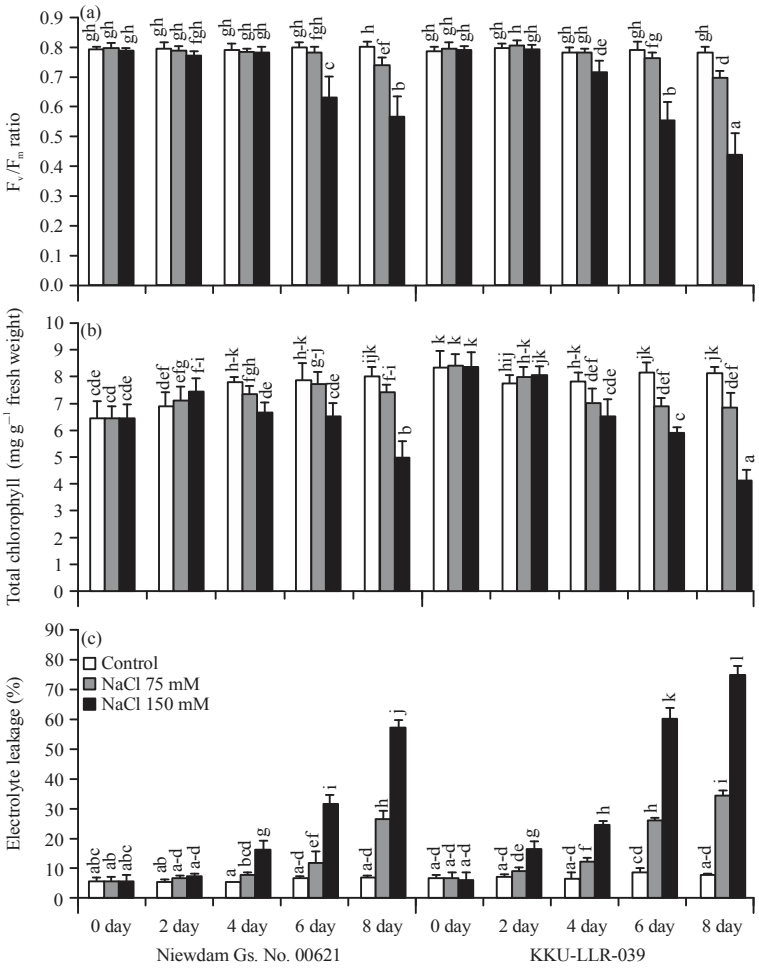

Fig. 2(a-c): Effect of salinity on (a) F_v/F_m ratios, (b) Total chlorophyll and (c) Electrolyte leakage of two rice cultivars which were subjected to 0, 75 and 150 mM NaCl for 0, 2, 4, 6 and 8 days. Values represent the Mean±SD, means with different letters are significantly different according to Duncan's multiple range test ($p<0.05$)

stress at 75 and 150 mM NaCl resulted in 12.12 and 83.91% reduction in seedling dry weights, whereas the reduction percentages for KKU-LLR-039 were 24.06 and 98.49%.

Effect of NaCl on chlorophyll fluorescence and total chlorophyll: The maximum quantum efficiency of PSII photochemistry (F_v/F_m) in both cultivars were not yet affected after two days of salt treatments (Fig. 2a). After 4 days of exposure to 150 mM NaCl, however, the F_v/F_m ratios significantly fell from 0.783-0.714 in leaves of the sensitive cultivar KKU-LLR-039, while it remained at the controlled level in salt resistant Niewdam Gs. No. 00621. After 6 days of exposure to 150 mM F_v/F_m ratios of both cultivars were significantly lower than those of the controls. The F_v/F_m ratios after 8 days of stress at 150 mM NaCl were reduced by 42.17 and 78.18% for Niewdam Gs. No. 00621 and KKU-LLR-039, respectively.

The total chlorophyll contents of Niewdam Gs. No. 00621 and KKU-LLR-039 treated with 0, 75 and 150 mM NaCl for 0, 2, 4, 6 and 8 days are shown in Fig. 2b. The total chlorophyll content in stressed seedlings slightly increased during the first two days of salt treatments in both rice cultivars. However, after 4-8 days of stress the total chlorophyll was progressively reduced in a time and dose-dependent manner. At 150 mM NaCl the total chlorophyll contents after 4, 6 and 8 days of stress were reduced by 17.54, 21.33 and 61.84%, respectively in Niewdam Gs. No. 00621 and 19.77, 37.81 and 97.94% for KKU-LLR-039.

Effect of NaCl on electrolyte leakage: Membrane damage was evaluated through electrolyte leakage. The electrolyte leakage from rice leaves which indicates the degree of cell membrane damage was significantly higher in salt-treated plants compared with the control plants as shown in Fig. 2c.

After 2 days of salt stress (150 mM NaCl) electrolyte leakage of the salt-sensitive KKU-LLR-039 was significantly higher than that of the control plants indicating membrane damage, whereas cellular membranes of the salt-resistant Niewdam Gs. No. 00621 had not yet been damaged. At 4, 6 and 8 days after exposure to 150 mM NaCl, the dramatic increase in the electrolyte leakage was found in KKU-LLR-039 (3.82, 7.52 and 9.99 folds increase compared with the controls), while less membrane damage was recorded in Niewdam Gs. No. 00621 (3.43, 4.82 and 8.61 folds increase).

Accumulation of total anthocyanins in response to salt stress: The total anthocyanins content in leaves of Niewdam Gs. No. 00621 and KKU-LLR-039 treated with 0, 75 and 150 mM NaCl during at 0, 2, 4, 6 and 8 days is shown in Table 2. At 2 and 4 days after NaCl treatment, the anthocyanin contents of the salt-tolerant Niewdam Gs. No. 00621 were 7.01 and 8.76% (for 75 mM NaCl) and 12.87 and 11.24% (for 150 mM NaCl) higher than those of the control plants. Anthocyanin contents in the KKU-LLR-039 increased after 2 days of salt treatments (8.68 and 10.76% for 75 and 150 mM NaCl) but did not increase over the controls after 4 days. At 6 and 8 days of stress, the concentrations of anthocyanin in response to salt concentration were negatively affected by both levels of NaCl. An exposure to 150 mM NaCl for 6 and 8 days resulted in reductions in total anthocyanins by 21.06 and 36.59% in KKU-LLR-039 and 17.50 and 21.41% for Niewdam Gs. No. 00621 as shown in Table 2.

Expression of anthocyanin biosynthesis genes in rice leaves: Results of the analysis of anthocyanin biosynthetic gene expression in response to NaCl relative to the expression in the control plants using quantitative real-time PCR are shown in Fig. 3. The result showed a varied expression pattern of all genes in comparison to control. The expression of anthocyanin-specific pathway steps, DFR and ANS were dramatically increasing after 2-4 days after NaCl treatment then decreased thereafter for Niewdam Gs. No. 00621 especially for the 150 mM NaCl groups. The level of expression of DFR and ANS in KKU-LLR-039 increased sharply only after 2 days of stress then progressively declines thereafter. On the day of maximum expression, the number of folds increase in expression of CHS, DFR and ANS were much greater in Niewdam Gs. No. 00621 (day 4) than KKU-LLR-039 (day 2). After 8 days of stress the expression of both DFR and ANS was depressed in both cultivars compared with the controls especially for the case of 150 mM NaCl. The PAL gene showed varying pattern of expression in response to NaCl treatments, rising sharply after 2 days, declining after 4 days, abruptly increasing again after 6 days and decreasing to the levels below the controls after 8 days. The levels of CHS expression were tremendously increased over the controls only on day 4 and 2 after NaCl treatment for Niewdam Gs. No. 00621 and KKU-LLR-039, respectively; thereafter the expressions were comparable to (for Niewdam Gs. No. 00621) or lower than the controls (for KKU-LLR-039).

Table 2: Amount of total anthocyanins in the leaves of two rice cultivars treated with 0, 75 and 150 mM NaCl for 0, 2, 4, 6 and 8 days and the percentage increase (+) or decrease (-) under salt stress compared with the controls

		Total anthocyanins (mg g^{-1} fresh weight)			
Days	NaCl (mM)	Niewdam Gs. No. 00621	(±%)	KKU-LLR-039	(±%)
0	0	1.62		1.46	
	75	1.58	-2.08	1.48	+1.34
	150	1.58	-2.50	1.47	+0.18
2	0	1.69		1.61	
	75	1.81	+7.01	1.77	+8.68
	150	1.93	+12.87	1.81	+10.76
4	0	1.79		1.69	
	75	1.96	+8.76	1.71	+1.34
	150	2.02	+11.24	1.68	-0.44
6	0	2.08		1.76	
	75	1.93	-8.07	1.62	-8.94
	150	1.77	-17.50	1.46	-21.06
8	0	2.03		1.70	
	75	1.85	-9.96	1.48	-14.96
	150	1.67	-21.41	1.24	-36.59

DISCUSSION

In general, salinity decreases the plants's ability to take up water and causes reductions in plant growth rate[23]. In the present study, growth parameters (shoot length, fresh and dry weights of shoot) were negatively affected by salinity. The salt-tolerant cultivar, Niewdam Gs. No. 00621 showed lower percentages of reduction in all growth traits than those for salt-sensitive, KKU-LLR-039 (Fig. 1). The salt-tolerant cultivar appeared to be better able to maintain their growth than the salt-sensitive cultivar under salt stress. These results are in agreement with those reported by Pattanagul and Thitisaksakul[24], Amirjani[25] and Saleethong *et al.*[26]. Moreover, Niewdam Gs. No. 00621 was reported to have higher K^+/Na^+ ratio, lower ROS (H_2O_2) and higher activities of antioxidant enzymes CAT under salt stress[18].

Leakage of electrolytes from cells as a result of membrane damage is one of the most important physiological symptoms caused by salt stress[27]. In several plant species, electrolyte leakage has been suggested as a valuable criterion for selecting stress resistant cultivars[28,29]. Electrolyte leakage can be used as a sensitive indicator of stress tolerance in pigmented rice with purplish leaves in this study as well as

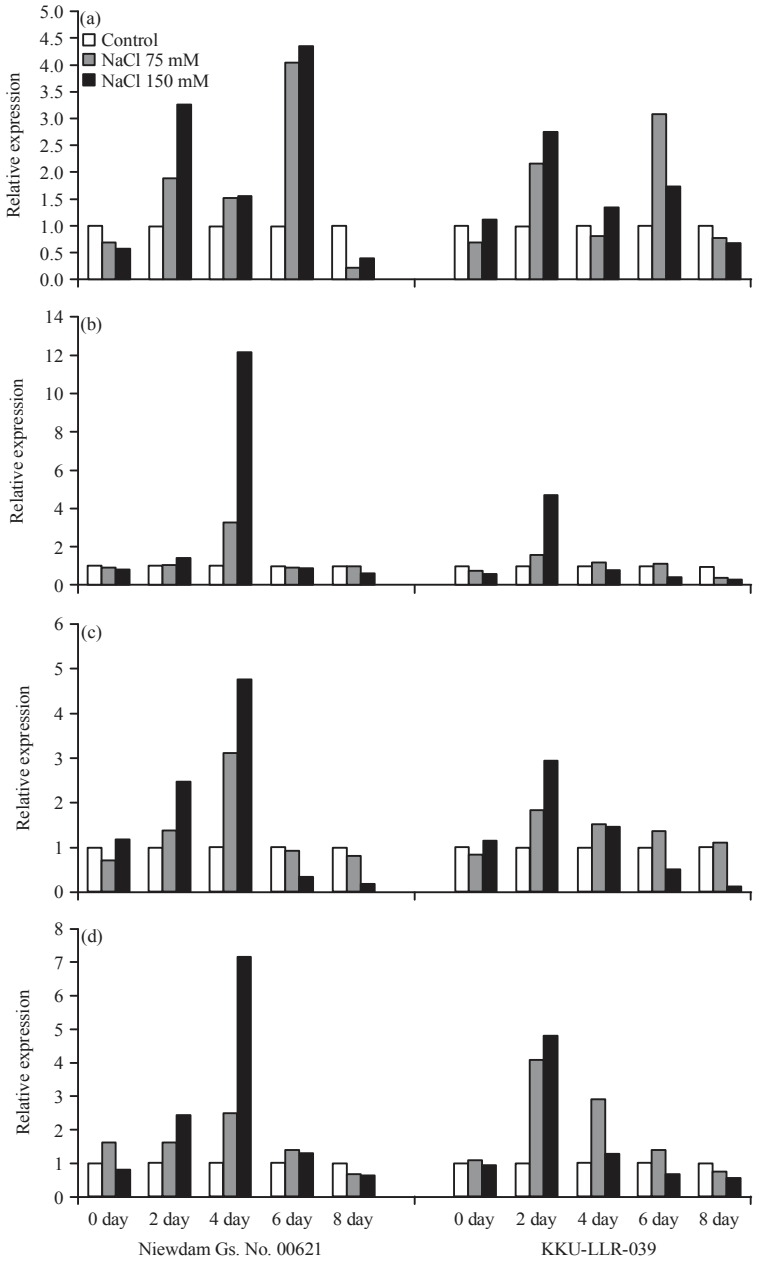

Fig. 3(a-d): Relative expression of anthocyanin-biosynthetic genes in two rice cultivars, (a) Phenylalanine ammonia lyase (PAL), (b) Chalcone synthase (CHS), (c) Dihydroflavanol reductase (DFR) and (d) Anthocyanidin synthase (ANS). Total RNA was isolated from leaves of rice subjected to 0, 2, 4, 6 and 8 days after salt treatment. Transcript levels for each gene are indicated as relative to those from rice actin gene

white rice with green leaves[26]. In the present study, salinity stress caused a marked reduction in both chlorophyll content and the maximum efficiency of PSII photochemistry (F_v/F_m) after 8 days of salt stress in both rice cultivars. The chlorophyll content is usually measured in plants to evaluate the effect of environmental stresses, as changes in concentration of pigments are conjugated to visual injury symptoms of plant damage[30] and can be used as an index of salt tolerance[20]. Salt-induced decrease in chlorophyll content was more prominent in the salt-sensitive KKU-LLR-039 than that in the salt-tolerant Niewdam Gs. No. 00621. Better protection of chlorophyll in salt-tolerant than in salt-sensitive cultivars has been reported in rice[26] and wheat[31].

Chlorophyll fluorescence attributes are also excellent measures of stress-induced damage to photosystem II (PSII) and an effective approach toward understanding of the

inhibitory effects of salt stress on photosynthetic apparatus[32]. Similar to our results, Ashraf and Ashraf[31] observed that the wheat cultivar with higher level of salt tolerance was able to maintain higher F_v/F_m under salt stress than the less tolerant one relating to better protection of chlorophyll loss. Moreover, anthocyanin-rich leaves of rice were reported to maintain higher radical scavenging activities, less membrane leakage and higher efficiency of PSII photochemistry under photooxidation treatments compared with the green rice leaves[33].

Anthocyanins are a diverse group of secondary metabolites that can be produced in response to oxidative stress and have crucial roles in stress protection[34]. The presence of phenyl groups on flavonoids including anthocyanins contributed to increase salt-tolerance in sugarcane by protecting cells from ion-induced oxidative damage by binding with the toxic ions[4]. In NaCl-exposed leaves of the tolerant cultivar, the ability of adaptation to salt stress is associated with higher concentrations of total anthocyanins (Table 2). However for the sensitive cultivar KKU-LLR-039 the accumulation of anthocyanin was enhanced only after a short-term exposure to salt stress (2 days after salt treatment). After 4, 6 and 8 days of stress total anthocyanins were markedly reduced in this cultivar. Accordingly, KKU-LLR-039 cannot accumulate high enough anthocyanin content to protect cells against damage due to ion toxicity and free radicals and hence it suffered from more severe membrane damage leading to higher electrolyte leakage rate (Fig. 2). Anthocyanin accumulation has been reported in several plants exposed to salt stress such as rice[35,36], tomato and red cabbage seedling[3] and transgenic potato[37]. In each case higher content of anthocyanin under stress was related to higher antioxidant potentials and salt tolerance.

The mechanisms by which anthocyanins provide a better protection against salt stress for the high-anthocyanin rice Niewdam Gs. No. 00621 in comparison to KKU-LLR-039 may be attributable to effects of anthocyanins as (1) ROS scavenger molecules[38,39] to alleviate salt-induced oxidative damage to leaves leading to better protection of chlorophyll and membrane stability and (2) As molecules which bind toxic ions as previously documented[4,40]. Emerging evidence also suggested intricating connections between stress-induced sucrose-specific signaling pathways leading to anthocyanin accumulation under stress which in turn act as "secondary" signals or stress modulators effective in counteracting multiple stresses[5,41].

Salinity stress causes major alteration in the expression pattern of genes involved in diverse physiological processes in plant and regulatory pathways in contrasting rice genotypes at different developmental stages[42]. The expression patterns of early (CHS) and late (DFR and ANS) genes of the anthocyanin synthesis pathway were generally related to the variation in total anthocyanins content in both rice cultivars under salt stress (Fig. 3, Table 2). These observations suggested that salt plays a regulatory role in anthocyanin synthesis pathway. Increased expression of CHS, DFR and ANS has been reported to be related to anthocyanin accumulation in rice leaves and seeds[13]. Close relationships between transcript levels of anthocyanin biosynthesis genes were also reported in red cabbage seedlings[15], grapevine[43] and plant species in Caryophyllales[11]. Higher expression of anthocyanin biosynthesis genes coinciding with increased anthocyanin contents under abiotic stress has been documented by Ma *et al*.[16] in wheat and Yuan *et al*.[44] in *Scutellaria baicalensis* roots. Rice OsDfr and OsAns genes were induced by dehydration, high salt and ABA and their expression was found to be activated by a transcription activator, OsC1-MYB[45]. The gene ANS which encodes anthocyanidin synthase, the enzyme for the committed step in anthocyanin synthesis, was overexpressed in transgenic rice[39] and resulted in an increasing amount of anthocyanins and accompanying increase in antioxidant activities. Shimada *et al*.[11] showed that expression levels of DFR and ANS were closely related to anthocyanin accumulation in leaves and seeds of Caryophyllales. Yuan *et al*.[44] reported that water deficit increased the expression of CHS both in leaves and roots of *Scutellaria baicalensis*. However, expression of anthocyanin genes can be varied depending on the developmental stages at which stresses were imposed, the intensity of stresses, the types of organs, tissues and cells investigated. Information on association between salt tolerance and higher intensity of purple color in rice leaves with higher expression of anthocyanin synthesis genes is useful for screening of indigenous colored rice and rice breeding programs aiming at improving stress tolerance of highly nutritious pigmented rice.

CONCLUSION

Salt stress induced greater accumulation of anthocyanins in the leaves of salt-tolerant rice cultivar (having deep purple leaf color) than the salt-sensitive one (having less intense purple leaf color). Salt-induced fluctuations in the anthocyanin contents were closely related to changes in the transcript levels of anthocyanin biosynthesis genes. The results obtained suggested that higher salt-response anthocyanin production in rice seedlings with purplish leaf color was one of the

mechanisms responsible for protection of seedlings from salt-induced damage leading to higher salt tolerance ability.

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