Physio-biochemical traits in improved ‘KDML105’ jasmine rice lines containing drought and salt tolerance gene under drought and salt stress

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Received: 22 July 2021; Accepted: 17 November 2021; doi:10.4067/S0718-58392022000100097

ABSTRACT

Drought and salinity are important abiotic stress factors negatively affecting productivity of the world-renowned jasmine rice (Oryza sativa L. subsp. indica Kato) ‘KDML105’ in the northeast Thailand. Two chromosome segment substitution lines (CSSLs) of ‘KDML105’ which were introgressed with drought tolerance quantitative trait loci (DT-QTLs), designated CSSL94 and CSSL103, and two improved ‘KDML105’ lines, designated RGD1 and RGD4, carrying the salt tolerant gene (SKC1) were used for evaluation of growth, physiological and biochemical traits in response to drought and salt stress conditions in comparison with ‘KDML105’ (sensitive parental cultivar) and DH103 (DT-QTL donor). Seedlings were grown for 21 d in hydroponic solutions and then subjected to drought stress (20% polyethylene glycol 6000) or salt stress (150 mM NaCl) for 10 d. Under both stresses, all four improved rice lines showed lower percent reduction in growth compared with ‘KDML105’. Under drought stress, CSSL94 and CSSL103 had significantly higher relative water content (88% and 89%) compared with that of ‘KDML105’ (82%). These lines accumulated 42% and 45% lower hydrogen peroxide, resulting in 25% and 32% lower malondialdehyde, and less membrane damage (24% lower electrolyte leakage). On the other hand, better growth of RGD1 and RGD4 under salt stress, compared with ‘KDML105’, was associated with an efficient maintenance of ion homeostasis (57% and 69% lower Na+/K+), 34% and 43% lower hydrogen peroxide, and 54% lower electrolyte leakage. The introgression of DT-QTL conferred not only drought but also salt tolerance to the recipient genotype. Likewise, introgression of SKC1 conferred both salt and drought tolerance.

Key words: Aromatic rice, antioxidant enzymes, chromosome segment substitution lines, drought tolerance QTL, SKC1 gene.

INTRODUCTION

Abiotic stress particularly drought and salinity are major stress factors that limit growth and productivity of rice especially in rainfed lowland areas. In Thailand, the largest rice (Oryza sativa L.) planting areas are in the northeastern region characterized by drought due to low precipitation and fluctuating rainfall patterns, poor soil quality, and salinity problems. Farmers in this region grow ‘Khao Dawk Mali 105’ (‘KDML105’) rice as a majority. This cultivar, also known as Thai jasmine rice (Oryza sativa L. subsp. indica Kato), is the most popular aromatic rice because of its good cooking/eating
quality of grains with jasmine-like aroma. However, ‘KDML105’ has several disadvantages including its susceptibility to drought and salinity conditions. Efforts have been made in improving rice cultivars to better resist biotic or abiotic stress through molecular breeding. Previously, the quantitative traits loci associated with drought tolerance (DT-QTLs) on chromosomes 1, 3, 4, 8 and 9 in rice were identified (Lanceras et al., 2004). In order to improve drought tolerance of ‘KDML105’, chromosome segment substitution lines (KDML-CSSLs) carrying different segments of DT-QTL controlling drought resistance from chromosome 8 of the drought resistance donor, DH103, were developed. These CSSLs were demonstrated to produce better yield than ‘KDML105’ under drought and well-watered conditions (Kanjoo et al., 2012). In addition, Siangliw (2015) and Punyawaew et al. (2016) have developed ‘KDML105’ backcross introgression lines (KDSKC1-FL) with increased salt tolerance ability by introgressing SKC1 (gene regulating K+/Na+ in shoots under salt stress) from chromosome 1 of the salt tolerant donor, FL530. The improved KDSKC1-FL lines were reported to perform considerably better than ‘KDML105’ exhibiting a lower Na+/K+ ratio and higher yield under salt stress at 10-12 dS m⁻¹ (Punyawaew et al., 2016).

Plant responses to drought and salinity are complex and have much in common. Early responses of plants to drought and salinity are similar since they induce osmotic stress which reduces the ability of plants to absorb water leading to cell dehydration, growth reduction, stomatal closure, reduced photosynthesis, nutrient deficiency, and alterations of metabolic pathways (Ma et al., 2020). However, with longer period of stress exposure, salinity also induces ionic stress due to accumulation of Na⁺ to a toxic level which provokes a wide variety of physiological and biochemical alterations that inhibit plant growth and production (Munns and Tester, 2008). Drought and salinity stresses also induce an overaccumulation of reactive oxygen species (ROS) such as superoxide anion (O₂⁻), singlet oxygen (¹O₂), hydroxyl radical (OH•) and hydrogen peroxide (H₂O₂), which damage essential macromolecules leading to inhibition of enzyme activity, lipid peroxidation, loss of membrane integrity, and eventually cell death (Huang et al., 2019). A number of plant species have evolved osmotic adjustment (OA) as an important mechanism to protect cells from dehydration due to drought- and salt-induced osmotic stress. Osmotic adjustment involves an accumulation of inorganic (notably K⁺ in the vacuoles) and organic (sugars, proline, glycine betaine, etc.) compatible solutes in the cytosol (Blum, 2017). It was reported that rain-fed paddy rice varieties which lack the ability for extensive root growth tend to possess good OA capacity (Nguyen et al., 1997). Tolerance mechanisms to reduce adverse impacts of ROS accumulation are also similar for plants affected by drought and salinity stress. Plants use antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), and ascorbate peroxidase (APX) as well as non-enzymatic antioxidants (such as ascorbic acid, carotenoids, glutathione and phenolic compounds) to scavenge ROS in plant cells (Lee et al., 2013). Specific mechanisms that plants have evolved to reduce adverse effects of salt stress involve ion exclusion and tissue tolerance (Munns and Tester, 2008). Ion exclusion involves exclusion of Na⁺ by root cells, retrieval of Na⁺ from xylem sap minimizing Na⁺ transport to shoots, maintaining low shoot Na⁺/K⁺ ratio, exclusion of Na⁺ by shoot and leaf cells and efflux of Na⁺ back to roots and then to the soils (Roy et al., 2014). Tissue tolerance involves compartmentation of Na⁺ in the vacuoles preventing toxic Na⁺ from inhibiting cytosolic enzyme activity, and maintaining structural integrity of chloroplast, mitochondria, and plasma membrane (Chakraborty et al., 2020). Due to the overlapping mechanisms of drought and salt stress response, some CSSLs of ‘KDML105’ carrying DT-QTLs not only had higher level of drought tolerance compared with ‘KDML105’ but also performed better under salt stress (Nounjan et al., 2018). However, whether or not the integration of SKC1 gene could raise drought stress tolerance of the backcross introgression lines has not been elucidated.

Detailed information on physiological and biochemical response mechanisms of rice under drought and salt stress is necessary for improving the efficiency of selection process in a breeding scheme. The objective of this study was to evaluate physiological and biochemical attributes of the backcross introgression lines of ‘KDML105’ carrying DT-QTLs, and those carrying SKC1 gene in response to polyethylene glycol (PEG)-induced drought and NaCl-induced salt stress. The results from this study are expected to provide a valuable insight into the beneficial roles of DT-QTL on alleviating salt stress effects, and conversely the roles of SKC1 gene on mitigating drought stress effects. These data may provide useful information for genetic improvement of jasmine rice for multiple or combined abiotic stress tolerance.
**MATERIALS AND METHODS**

**Rice materials and treatments**
This experiment included six jasmine rice lines/cultivar (*Oryza sativa* L. subsp. *indica* Kato) including ‘KDML105’ that is susceptible to drought and salt stress, four improved ‘KDML105’ rice lines (CSSL94, CSSL103, RGD1 and RGD4), and DH103 that is tolerant to both drought and salt stress. Seeds of all rice genotypes were obtained from Rice Gene Discovery Unit, BIOTEC, Thailand. The lines CSSL94 and CSSL103 are backcross introgression lines of ‘KDML105’ carrying quantitative traits loci associated with drought tolerance (DT-QTLs) located on chromosome 8 (Kanjoo et al., 2012). These chromosome segment substitution lines (CSSLs) are BC$_3$F$_5$ carrying different fragments of DT-QTLs on chromosome 8 introgressed from the donor parent DH103, a drought and salt-tolerant line (Table 1). The lines RGD1 and RGD4 are backcross introgression lines of ‘KDML105’ containing a salt tolerance gene (*SKC1*) located on chromosome 1 (Table 1). These lines were BC$_2$F$_4$ derived from the cross between ‘KDML105’ and FL530, a salt-tolerant recombinant inbred line (RIL) derived from a cross IR29 × Pokkali (Siangliw, 2015). Sterilized seeds were germinated on wet filter papers. After 3 d, germinated seeds were transferred to plastic containers each containing 15 L nutrient solution pH 5.0 (Yoshida et al., 1976). In each container, 20 germinated seeds of each of the six lines/cultivar were randomly arranged, and seedlings allowed to grow for 21 d in the greenhouse (Faculty of Agriculture, Khon Kaen University) under natural light conditions. The nutrient solutions were refreshed every 4 d and the pH was regularly monitored. The plants were then divided into three groups: control (normal nutrient solution), drought stress (nutrient solution containing 20% polyethylene glycol PEG 6000), and salt stress (nutrient solution containing 150 mM NaCl). The salt solution used had the same osmotic potential (-0.7 MPa) as that of the PEG 6000 solution. The growth, physiological and biochemical parameters were determined at 10 d after drought and salt treatment.

**Growth and ion concentration**
Rice seedlings were divided into shoots and roots, and root fresh weight (RFW), shoot fresh weight (SFW), root dry weight (RDW) and shoot dry weight (SDW) were determined. For measurement of Na$^+$ and K$^+$ content, shoots of plants were dried at 70 °C for 5-7 d. Approximately 0.1 g powdered samples were subjected to chemical analyses by digesting in 10 mL nitric acid mixture at 300 °C, 5 mL perchloric acid at 200 °C and 20 mL 6 M hydrochloric acid. The concentrations of Na$^+$ and K$^+$ were analyzed using an atomic absorption spectrophotometer (932AAA, GBC Scientific, Braeside, Victoria, Australia).

**Relative water content (RWC)**
Leaf RWC was determined by cutting 2 cm segments which were immediately placed in a pre-weighed micro-tube, and weighed to obtain the fresh weight (FW). The leaf segments were then floated on deionized water, under fluorescent light for 4 h at 25 °C. Then, leaves were evaluated for turgid weight (TW) and dried in a hot air oven at 80 °C for 48 h for determination of dry weight (DW). The RWC was calculated following Larkunthod et al. (2018) using the equation:

\[
RWC (\%) = \left[\frac{(FW - DW)}{(TW - DW)}\right] \times 100.
\]

| Lines/cultivar | Generation | RM310 | RM3845 | RM615 | RM447 | RM3480 | RM4153 | SKC1 |
|---------------|------------|-------|--------|-------|-------|--------|--------|------|
| CSSL94        | BC$_3$F$_5$ | -     | -      | -     | -     | +      | -      | -    |
| CSSL103       | BC$_3$F$_5$ | +     | +      | +     | +     | +      | -      | -    |
| RGD1          | BC$_2$F$_4$ | -     | -      | -     | -     | -      | -      | +    |
| RGD4          | BC$_2$F$_4$ | -     | -      | -     | -     | -      | -      | +    |
| ‘KDML105’     | BC$_3$F$_5$ | +     | +      | +     | +     | +      | +      | +    |
| DH103         | -          | +     | +      | +     | +     | +      | +      | -    |

*Table 1. Genotypic information of rice lines/cultivar used in this study.*
**Electrolyte leakage (EL)**

Electrolyte leakage was determined following the method described by Ghoulam et al. (2002) with some modifications. The fully expanded leaf sample of four plants for each treatment was used. Leaf samples were cut to small pieces (2-3 cm) and placed in closed tubes containing 10 mL deionized water for 2 h at 32 °C. Electrical conductivity (EC) was measured by using electrical conductivity meter (PL-700PCS GOnDO, Taipei City, Taiwan) and recorded as EC1. The leaf tissue sample in tubes were then boiled at 100 °C for 30 min and cooled down at room temperature; EC was measured and recorded as EC2. The percentage of EL was calculated using the following equation: EL (%) = (EC1/EC2) × 100.

**Proline content**

Proline content was analyzed by the modified method from Bates et al. (1973). Briefly, leaf samples (0.1 g) were soaked with 5 mL 3% aqueous sulfosalicylic acid for 3 h. The extract (2 mL) was treated with 2 mL glacial acetic acid before adding 2 mL acid-ninhydrin solution and heated in a water bath at 100 °C for 1 h. After immediately cooling down on ice, the solution was extracted with 4 mL toluene and then absorbance was determined at 520 nm by using spectrophotometer (Model i3, Hanon, Shandong, China). The contents of proline were evaluated based on a standard curve and are expressed as μg g⁻¹ FW.

**Malondialdehyde (MDA) and hydrogen peroxide (H₂O₂) content**

Lipid peroxidation was determined by estimating MDA content according to Velikova et al. (2000). Leaf sample (0.1 g) was added with 1.5 mL distilled water and 1.5 mL 0.5% 2-thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA). The solution was heated at 95 °C for 25 min, and quickly cooled in an ice bath for 5 min. Then, the absorbance of supernatant was read at 532 and 600 nm. Calculation of MDA was performed using an extinction coefficient of 155 mM⁻¹ cm⁻¹. For measurement of H₂O₂, fresh leaf tissue (0.1 g) was extracted with 3 mL TCA (0.1% w/v) in an ice bath and centrifuged at 12 000 × g for 15 min and 0.5 mL of the supernatant was added to 0.5 mL 10 mM potassium phosphate buffer (pH 7.0) and 1 mL 1 M potassium iodide (KI). The absorbance of supernatant was read at 390 nm. The content of H₂O₂ was determined by a standard curve (Velikova et al., 2000).

**Protein and antioxidant enzyme activities**

For enzyme extraction and measurement of enzyme activities, 0.5 g leaf tissue was homogenized in 5 mL of grinding buffer (10 mM potassium phosphate buffer pH 7.0 and 4% polyvinylpyrrolidone). The homogenate was centrifuged at 12000 × g at 4 °C for 15 min. The supernatants were immediately used for estimation of enzyme activity. All steps in preparation of the enzyme extract were performed at 4 °C. The supernatant of crude extract was used to determine the protein content by the method of Bradford (1976). The determination of activities of antioxidant enzymes was performed as described by Nounjan and Theerakulpisut (2012). For superoxide dismutase (SOD) activity, the reaction mixture contained 1.8 mL 50 mM potassium phosphate buffer (pH 7.8), 50 μL 0.4 M methionine, 50 μL 16 mM ethylenediaminetetraacetic acid (EDTA), 100 μL 2 mM nitroblue tetrazolium chloride (NBT), and 50 μL enzyme extract. The reaction started when adding 20 μL 1 mM riboflavin, the reaction mixture was placed under fluorescence lamps for 10 min. Then, the absorbance of reaction mixture was measured at 560 nm. The reaction mixture with no enzyme extracts developed maximum color due to maximum rate of NBT reduction. One unit of SOD was determined as the amount of enzyme that produced 50% inhibition of NBT reduction. The activity was expressed as unit mg⁻¹ protein. For catalase (CAT) activity, the reaction mixture (1.8 mL 50 mM potassium phosphate buffer pH 7.0 and 100 μL 0.5 M H₂O₂) was mixed with 100 μL enzyme extract. The absorbance was determined at 240 nm with a spectrophotometer every 1 min for 3 min after the addition of H₂O₂. The CAT activity was expressed as μmol H₂O₂ decomposed mg⁻¹ protein min⁻¹. For ascorbate peroxidase (APX) activity, the reaction mixture containing 1.7 mL 50 mM potassium phosphate buffer pH 7.0, 100 μL 20 mM ascorbic acid, 50 μL 0.5 M H₂O₂ and 50 μL 16 mM EDTA was mixed with 100 μL enzyme extract. The absorbance of the mixture was determined at 290 nm with a spectrophotometer every 30 s for 2 min. The APX activity was expressed as μmol ascorbic acid decomposed mg⁻¹ protein min⁻¹.
Experimental design and statistical analysis
The experimental design was performed as randomized complete block with four replicates for each treatment. For statistical analysis, data were subjected to ANOVA, and means were compared using Tukey’s test at significant difference p ≤ 0.05. Comparison of means between the control and stress condition of each line/cultivar was done using Student’s t-test. Correlations among investigated parameters were calculated using Pearson’s correlation. All statistical analysis were performed using SPSS Statistics 19.0 (IBM, Armonk, New York, USA).

RESULTS

Effects of drought and salt stresses on growth
Drought and salt stresses inhibited growth resulting in the reductions in RFW, SFW, RDW and SDW of all rice lines/cultivar to different degrees (Tables 2 and 3). Under drought and salt stresses, CSSL94, CSSL103, RGD1 and RGD4 showed slightly lower growth reduction than ‘KDML105’. Under drought stress, ‘KDML105’ exhibited a significant reduction in RFW (22.35%), SFW (39.74%), and SDW (26.01%) whereas DH103 showed nonsignificant reduction in those parameters. The improved lines, CSSL94 and CSSL103, showed lower percent growth reductions than ‘KDML105’. Under salt stress, RGD4 demonstrated the lowest percent reduction in RFW (13.55%), SFW (21.40%), RDW (16.21%) and SDW (11.90%) whereas ‘KDML105’ showed the highest percent reduction in RFW (34.52%), SFW (49.14%), RDW (31.65%) and SDW (35.78%).

Effects of drought and salt stresses on Na+, K+ and Na+/K+ ratio
The sodium ion (Na+) concentration and Na+ to K+ ratio (Na+/K+ ratio) were not different in all rice lines/cultivar under the control condition (Figures 1a and 1c). Salt stress markedly increased Na+ concentration in rice shoots, i.e., 28-, 33-, 21-, 16-, 26- and 46-fold increase in CSSL94, CSSL103, RGD1, RGD4, DH103, and ‘KDML105’, respectively (Figure 1a). Similar trend was found in Na+/K+, which showed 33-, 37-, 23-, 17-, 28-, and 54-fold increase in CSSL94, CSSL103, RGD1, RGD4, DH103, and ‘KDML105’, respectively (Figure 1c). For K+ concentration, drought stress caused significant increases in K+ (6% to 25%) in all rice lines/cultivar (except ‘KDML105’), whereas K+ significantly decreased after salt stress in all rice lines/cultivar (Figure 1b).

Table 2. Fresh and dry weight of roots, and percent reduction in weight under stress compared with that in the control.

| Lines/cultivar | Root fresh weight | Root dry weight |
|---------------|-------------------|----------------|
|               | Control | Drought stress | Reduction | Salt stress | Reduction |
| CSSL94        | 2.00b    | 1.77a          | 11.75     | 1.51b       | 24.90     |
| CSSL103       | 1.85b    | 1.64a          | 11.01     | 1.55b       | 16.12     |
| RGD1          | 2.38b    | 2.08a          | 12.24     | 2.02b       | 14.82     |
| RGD4          | 2.43b    | 2.14a          | 11.91     | 2.10b       | 13.55     |
| ‘KDML105’     | 2.08b    | 1.61a          | 22.35     | 1.36a*      | 34.52     |
| DH103         | 1.13c    | 1.08b          | 4.29      | 0.96a*      | 15.10     |
|               | g       | g              | %         | g           | %         |
| CSSL94        | 0.22b   | 0.19ae         | 11.41     | 0.17a       | 20.12     |
| CSSL103       | 0.23b   | 0.21ae         | 8.48      | 0.19a       | 19.23     |
| RGD1          | 0.26a   | 0.22a          | 13.17     | 0.21a       | 17.75     |
| RGD4          | 0.26a   | 0.23a          | 10.27     | 0.22a       | 16.21     |
| ‘KDML105’     | 0.22a   | 0.18a          | 15.29     | 0.15a*      | 31.65     |
| DH103         | 0.12b   | 0.12           | 6.08      | 0.10a       | 19.23     |

Means in the same column followed by the same letter are significantly different at the 5% level by Tukey’s test. *, **Significant difference (p ≤ 0.05, p ≤ 0.01, respectively) in the mean values between control and stress treatment for each line/cultivar.
Table 3. Fresh and dry weight of shoots, and percent reduction in weight under stress compared with that in the control.

| Lines/cultivar | Control | Drought stress | Reduction | Salt stress | Reduction |
|---------------|---------|----------------|-----------|------------|-----------|
| Shoot fresh weight | g     | g             | %         | g          | %         |
| CSSL94        | 5.83a  | 4.94ab        | 15.37     | 4.29b**   | 26.45     |
| CSSL103       | 5.48ab | 4.65b*        | 15.07     | 3.96b**   | 27.69     |
| RGD1          | 6.24b  | 5.00b**       | 19.98     | 4.75b**   | 23.96     |
| RGD4          | 6.71a  | 5.52b         | 17.75     | 5.28b     | 21.40     |
| ’KDML105’     | 5.93ab | 3.58b**       | 39.74     | 3.02b**   | 49.14     |
| DH103         | 2.22c  | 1.97b         | 11.16     | 1.73b     | 21.84     |
| Shoot dry weight | g     | g             | %         | g          | %         |
| CSSL94        | 1.14ab | 0.99b         | 14.10     | 0.88b*    | 22.67     |
| CSSL103       | 1.04b  | 0.89b*        | 14.66     | 0.80b*    | 22.95     |
| RGD1          | 1.39b  | 1.15b         | 16.71     | 1.17b     | 15.57     |
| RGD4          | 1.39b  | 1.16b         | 16.58     | 1.23b     | 11.90     |
| ’KDML105’     | 1.11ab | 0.82b*        | 26.01     | 0.71b**   | 35.78     |
| DH103         | 0.35c  | 0.31d         | 13.41     | 0.29d*    | 17.28     |

Means in the same column followed by the same letter are significantly different at the 5% level by Tukey’s test.

*, **Significant difference (p ≤ 0.05, p ≤ 0.01, respectively) in the mean values between control and stress treatment for each line/cultivar.

Figure 1. Concentration of Na⁺ (a), K⁺ (b), and Na⁺/K⁺ ratio (c) in rice shoots after 10 d under salt and drought stress.

Means with different letters within each treatment group are significantly different according to Tukey’s test (p ≤ 0.05).

*, **Significant difference (p ≤ 0.05, p ≤ 0.01, respectively) in the mean values between control and stress (drought or salt stress) conditions of each line/cultivar.
Effects of drought and salt stresses on RWC and EL

Under the control conditions, all rice lines/cultivar had similar leaf RWC ranging from 94% to 97% (Figure 2a). Drought and salt stresses resulted in reductions in RWC in all rice lines/cultivar. The RWCs were in the range of 81%-91% and 87%-93% under drought and salt stresses, respectively. Leaves of ‘KDML105’ showed the lowest RWC in both types of stresses which were significantly lower than all other genotypes (Figure 2a). Under drought stress, DH103 had the highest RWC of 91% which was not significantly different from those of CSSL94 and CSSL103, but significantly higher than RGD1 and RGD4. In contrast, under salt stress all of these five genotypes had similar RWC values. For EL, under control conditions, EL from leaf tissues of all rice lines/cultivar were in the range of 9.05%-10.30% which was not significantly different (Figure 2b). In response to drought stress the EL increased in all rice lines/cultivar. The percentage of EL was found to be significantly increased in all rice lines/cultivar (12.08% in CSSL94, 12.31% in CSSL103, 13.36% in RGD1, 13.19% in RGD4, 13.03% in DH103, and 15.88% in ‘KDML105’) (Figure 2b). Under salt treatment, the EL increased dramatically in all rice lines/cultivar, especially in ‘KDML105’. The percentage increase in EL were 25.67%, 27.33%, 24.25%, 23.99%, 25.56% and 44.87% in CSSL94, CSSL103, RGD1, RGD4, DH103, and ‘KDML105’, respectively (Figure 2b).

Effects of drought and salt stresses on proline and protein content

Leaf proline contents in all rice lines/cultivar were significantly increased in response to drought (3.0- to 4.7-fold increase) and salt stress (4.4- to 7.7-fold increase). In response to drought stress, DH103 accumulated the highest amount of proline (333.11 μg g\(^{-1}\) FW) while the lowest was found in ‘KDML105’ (215.23 μg g\(^{-1}\) FW). All four improved lines had significantly higher concentrations of proline than ‘KDML105’, and those in the CSSLs were higher than the RGDs (Figure 2c). On the contrary, under salt stress, the highest proline content was observed in ‘KDML105’ (7.7-fold increase from 70.20 to 537.80 μg g\(^{-1}\) FW) while the lowest was in RGD4 (5.40-fold increase from 64.12 to 323.39 μg g\(^{-1}\) FW). Leaf protein contents under drought and salt stresses were significantly decreased when compared to control plants in all cases (except DH103 under drought stress) (Figure 2d). Salt stress imposed more severe reduction in protein content (39.4% to 49.48%) than did drought (12.1% to 27.51%). Under both stresses ‘KDML105’ had significantly lower protein contents than the improved lines.

Means with different letters within each treatment group are significantly different according to Tukey’s test (p ≤ 0.05).

*Significant difference (p ≤ 0.05, p ≤ 0.01, respectively) in the mean values between control and stress (drought or salt stress) conditions of each line/cultivar.
Effects of drought and salt stresses on H$_2$O$_2$ and MDA

The amount of H$_2$O$_2$ content in all rice lines/cultivar significantly increased when exposed to drought and salt stresses as compared with the control plants (Figure 3a). The contents of H$_2$O$_2$ in ‘KDML105’ (1.545 and 1.757 μmol g$^{-1}$ FW under drought and salt stress, respectively) were markedly higher than those of the other genotypes. Under drought stress, DH103 had the lowest H$_2$O$_2$ content of 0.77 μmol g$^{-1}$ FW. It was noted that the CSSLs with the introgressed DT-QTLs exhibited significantly lower H$_2$O$_2$ (0.84 and 0.94 μmol g$^{-1}$ FW) than the RGD lines (1.12 and 1.15 μmol g$^{-1}$ FW) which carried the salt tolerance genes. On the other hand, under salt stress, the RGD lines accumulated significantly lower H$_2$O$_2$ (1.13 and 1.01 μmol g$^{-1}$ FW) than the CSSLs (1.32 and 1.35 μmol g$^{-1}$ FW). The MDA contents of all rice lines/cultivar under control conditions were not significantly different (Figure 3b). Under drought stress, the MDA content was highest in ‘KDML105’ (3.53 μmol g$^{-1}$ FW), lowest in DH103 (1.84 μmol g$^{-1}$ FW), and moderate in the four improved lines (2.41 to 2.79 μmol g$^{-1}$ FW). Salt stress caused highest lipid peroxidation in ‘KDML105’ leaves (5.17 μmol MDA g$^{-1}$ FW). Similar to changes in H$_2$O$_2$, the amount of MDA in the RGD lines were significantly lower than those in the CSSLs (Figure 3b).

Effects of drought and salt stresses on antioxidant enzyme activities

The activities of antioxidant enzymes in rice lines/cultivar exhibited pronounced increases under stress, generally with stronger increases under salt than drought stress (Figures 4a, 4b, and 4c). The comparative activities of all three enzymes under drought stress were similar with ‘KDML105’ showing the lowest, DH103 the highest, and all four improved lines the moderate activities. Nonsignificant differences in enzyme activities were apparent among the four improved lines. Under salt stress, the patterns of SOD and CAT activities were more or less similar with RGD4 exhibited the highest activities (93.86 and 235.47 μmol H$_2$O$_2$ decomposed mg$^{-1}$ protein min$^{-1}$ for SOD and CAT, respectively) which were significantly higher than those in the CSSLs and DH103. Notably, ‘KDML105’ had very high activity of SOD (87.28 μmol H$_2$O$_2$ decomposed mg$^{-1}$ protein min$^{-1}$, Figure 4a) but extremely low activity of CAT (132.41 μmol H$_2$O$_2$ decomposed mg$^{-1}$ protein min$^{-1}$; Figure 4b). Interestingly, under salt stress ‘KDML105’ exhibited prominently high activity of APX (7.54 μmol ascorbic acid decomposed mg$^{-1}$ protein min$^{-1}$), while the other genotypes had similar activities (4.53 to 5.53 μmol H$_2$O$_2$ decomposed mg$^{-1}$ protein min$^{-1}$).

Correlation among growth, physiological and biochemical traits

Under drought stress (Figure 5), the RWC had strong negative correlations with EL, H$_2$O$_2$ and MDA while significant positive correlations were found with proline and activities of all three antioxidant enzymes. Proline content was negatively correlated with EL, H$_2$O$_2$ and MDA while it had positive correlations with RWC and antioxidant enzymes activities. In addition, H$_2$O$_2$ was negatively correlated with activities of all three antioxidant enzymes. Under salt stress (Figure 6), the Na$^+$/K$^+$ ratio was negatively correlated with RDW, SDW, protein, and antioxidant enzymes activities but it was positively correlated with EL, proline, H$_2$O$_2$, and MDA. Significantly negative correlations were observed between the EL
and RWC, protein, and antioxidant enzymes activities. In contrast, EL showed positive correlations with proline, H$_2$O$_2$, and MDA. Proline was negatively correlated with protein and antioxidant enzymes activities whereas it was positively correlated with H$_2$O$_2$ and MDA. Moreover, negative correlations were presented between H$_2$O$_2$ and activities of all three antioxidant enzymes while positive correlation was found between H$_2$O$_2$ and MDA.

**DISCUSSION**

Drought and salt stresses are known to cause many adverse effects on growth, development, and yield in crop plants. In this study, drought and salt stress significantly decreased plant growth (Tables 2 and 3) compared with the controls. Iso-osmotic concentrations of PEG and NaCl were applied in this study to create equal intensity of osmotic stress (-0.7 MPa), therefore could differentiate between osmotic and ionic effects of NaCl stress. Thus, PEG solutions imposed only osmotic stress throughout the treatment period while NaCl initially exerted the osmotic effect on growth and later (after several days) an ion toxicity effect (Munns and Tester, 2008). It was evident from Tables 2 and 3 that salt stress resulted in more growth reduction than drought stress due to the additive effects of osmotic and ion toxicity stress. Similar observations were reported in sugarcane (Patade et al., 2011) exposed to iso-osmotic concentrations of PEG and NaCl.
Figure 5. Heat map showing Pearson’s correlation coefficients (r values) among 12 physio-biochemical and growth parameters in rice seedlings exposed to drought stress.

Each square indicates the Pearson’s correlation coefficient of a pair of parameters.

*, **Significant differences at p ≤ 0.05 and p ≤ 0.01 level, respectively.

RDW: Root dry weight; SDW: shoot dry weight; Na+/K+: ratio between sodium and potassium ions; RWC: relative water content; EL: electrolyte leakage; H$_2$O$_2$: hydrogen peroxide; MDA: malondialdehyde; SOD: superoxide dismutase; CAT: catalase; APX: ascorbate peroxidase.

Figure 6. Heat map showing Pearson’s correlation coefficients (r values) among 12 physio-biochemical and growth parameters in rice seedlings exposed to salt stress.

Each square indicates the Pearson’s correlation coefficient of a pair of parameters.

*, **Significant differences at p ≤ 0.05 and p ≤ 0.01 level, respectively.

RDW: Root dry weight; SDW: shoot dry weight; Na+/K+: ratio between sodium and potassium ions; RWC: relative water content; EL: electrolyte leakage; H$_2$O$_2$: hydrogen peroxide; MDA: malondialdehyde; SOD: superoxide dismutase; CAT: catalase; APX: ascorbate peroxidase.
Mechanisms underlying drought and salinity responses are complex and overlapping including cell dehydration, inhibition of cell elongation, stomatal closure, inhibition of photosynthesis, osmotic and ionic imbalance, synthesis of osmolytes, excessive accumulation of ROS, and membrane damage (Ma et al., 2020). However, as evidenced in this study, the extent to which drought and salt stress induced the physiological and biochemical responses leading to growth inhibition differed depending on plant genotypes. Under both the stresses, the susceptible genotype ‘KDML105’ suffered the greatest growth reduction. The introgression of DT-QTLs and SKC1 successfully conferred drought and salt tolerance, respectively, to all four improved lines compared to that of the recipient ‘KDML105’ as indicated by lower percent growth reduction (Tables 2 and 3). The positive effects of DT-QTLs on growth and yield enhancement under drought stress of the ‘KDML105’ introgression lines have been reported by Kanjoo et al. (2012). Moreover, under salt stress, the presence of DT-QTLs also promoted seedling growth (Nounjan et al., 2018) of the introgression lines compared to the recipient ‘KDML105’. The positive roles on seedling growth under salt stress of the introgressed Saltol QTL containing SKC1 in the genetic background of ‘KDML105’ and ‘RD6’ rice were demonstrated by Punyawaw et al. (2016) and Thanasrilungura et al. (2020), respectively. However, the beneficial roles of SKC1 under drought stress are reported for the first time in this study.

For plants to survive under abiotic stress, particularly drought and salt stress, it is crucial for the plants to maintain water uptake and minimize water loss so as to achieve optimum water content to support normal cellular functions. One of the first detectable symptoms of drought is dehydration of plant tissues which can be reliably monitored by a reduction in RWC (Larkunthod et al., 2018). In this study, maximum decrease in RWC was observed in ‘KDML105’ compared to other rice lines/cultivar (Figure 2a). Meanwhile, CSSL94 and CSSL103, which received DT-QTLs from chromosome 8 of DH103, were able to maintain similar RWC as that of DH103. This result confirmed the observation that DT-QTLs from chromosome 8 of DH103 conferred drought tolerance to the improved CSSLs compared with ‘KDML105’ resulting in better yield in a field experiment (Kanjoo et al., 2012). Similarly, introgression of DT-QTL from chromosome 1 of DH212 into the genetic background of ‘KDML105’ has been shown to raise drought tolerance levels of two improved lines at seedling stage by maintaining significantly higher RWC under PEG-induced water deficit which was related to better osmotic adjustment contributed by higher concentrations of proline (Larkunthod et al., 2018). Under salt stress, all improved rice lines and DH103 maintained the same levels of RWC as those in the controls, while salt-stressed ‘KDML105’ had significantly reduced RWC. The observation that PEG-induced drought stress caused significant reduction in RWC while iso-osmotic NaCl solutions did not affect RWC (with the exception of ‘KDML105’ as shown in Figure 2a) could be related to certain disadvantages of PEG, for instance, the viscosity of the PEG solution could lead to root damage in addition to the osmotic effect (Osmolovskaya et al., 2018) further exacerbated the ability of roots to absorb water. Moreover, accumulation of Na+ in salt-stressed plant cells (Figure 1a) could partly contribute to osmotic adjustment (Munns et al., 2020) which consequently facilitated water absorption from the external saline solution.

Osmotic adjustment involves both the accumulation of inorganic ions such as Na+ and Cl− in the vacuoles, and small organic molecules (referred to as compatible solutes such as proline, glycine betaine and sugars) in the cytosol (Roy et al., 2014). Stronger accumulation of compatible osmolytes such as proline has been reported to be associated with greater tolerance in rice under drought (Larkunthod et al., 2018) and salt stress (Reddy et al., 2017). Significant increase in proline was found in all rice lines/cultivar under both stresses, although higher concentrations were found in the salt-stressed plants (Figure 2c). Under drought stress, proline concentrations were positively correlated to RWC (Figure 5), and tightly associated with growth; the higher the proline content (Figure 2c), the lower the percent growth reduction (Tables 2 and 3). These results corroborated the evidence on the roles of proline in maintaining tissue water status and growth in transgenic rice with enhanced proline synthesis (Zarifith et al., 2020). In contrast, proline concentrations under salt stress were negatively correlated with RWC (Figure 6), and genotypes with higher proline tended to show more growth reduction (Tables 2 and 3). In case of salt stress, there have been mixed results with regard to the relationship between proline and stress tolerance depending on plant genotypes, stress levels, and duration (Dar et al., 2016). Therefore, the roles of proline in response to salt stress are complex and need to be further explored.
While higher proline concentrations under drought stress was highly positively correlated with RWC (Figure 5), proline was negatively correlated with RWC when plants were subjected to salt stress (Figure 6). Moreover, higher proline contents in salt-stressed plants were related to greater degrees of cellular damage including lower protein content (Figure 2d), more membrane damage (Figure 2b), higher H$_2$O$_2$ and MDA content (Figure 3). Among genotypes under salt stress, the most sensitive genotype ‘KDML105’ accumulated huge amount of proline and expressed greater damage in all physiological attributes. It was suggested that proline accumulation in salt-sensitive rice genotypes could be a symptom of stress injury rather than a component of salt resistance associated with osmotic adjustment (Lutts et al., 1999). However, proline in moderate concentrations in less sensitive genotypes, DH103, and the four improved lines, could play roles in protection against salt-induced damages including ROS detoxification, protection of membrane integrity, stabilizing protein structure, and maintaining the balance between NADPH/NADP+ (Moukhtari et al., 2020).

Physiological mechanisms conferring Na$^+$ exclusion and selectivity for K$^+$ and Na$^+$ have been reported to be the most crucial for salt tolerant ability of plant (Roy et al., 2014). In this study, Na$^+$ accumulation in all rice lines/cultivar were increased under salt treatment. In comparison with ‘KDML105’, both RGD1 and RGD4 showed lower Na$^+$ and higher K$^+$ concentrations, hence lower Na$^+$/K$^+$ ratio (Figures 1a, 1b, 1c). The SKCI gene was found to encode a Na transporter gene (OsHKT1;5) which controls Na$^+$/K$^+$ homeostasis in rice shoots by unloading Na$^+$ from the xylem sap minimizing the entry of Na$^+$ to shoots (Thomson et al., 2010). This observation indicated that the SKCI gene introgressed from FL530 in the ‘KDML105’ genetic background (Siangliw, 2015; Punyawaew et al., 2016) was responsible for the maintenance of ionic homeostasis (lower shoot Na$^+$/K$^+$ ratio) of RGD1 and RGD4.

In addition to osmotic and ion toxicity stress, drought and salinity also induce formation of ROS. Reactive oxygen species, particularly hydroxyl radicals, attacks membrane lipids through lipid peroxidation resulting in an increase in membrane fluidity causing membrane to be leaky, eventually leading to cell death (Das and Roychoudhury, 2014). Previous studies revealed that H$_2$O$_2$ and MDA (a product of lipid peroxidation) contents are considered to be reliable indicators of oxidative stress, and could be used to discriminate between sensitive and tolerant plant genotypes (Asaeda et al., 2018). Likewise, EL as a measure of membrane injury has been reported as an efficient selection criterion for drought (Tripathy et al., 2000) and salt tolerance (Mansour, 2013) in many plants including rice. In this study, the relationships among ROS, lipid peroxidation and membrane injury under both drought and salt stress were clearly demonstrated by strong positive correlations between H$_2$O$_2$ and MDA, EL and H$_2$O$_2$, and EL and MDA (Figures 5 and 6). Introggression of DT-QTL and SKCI conferred greater ability of the improved lines to reduce ROS and lipid peroxidation, leading to better maintenance of membrane stability under both stresses (Figures 2c and 3) compared to that of ‘KDML105’ . Previous reports indicated that more tolerant rice genotypes were better able to maintain membrane integrity under drought stress (Swapna and Shylaraj, 2017) and salt stress (Nounjan et al., 2018).

In addition, drought and salt stresses also caused a reduction in total protein content, in which the extent of reduction was more substantial under salt than drought stress, probably due to ion toxicity effects (Figure 2d). A reduction in protein content was previously observed in rice under salt stress (Hakim et al., 2014). It was proposed that the decreased amount of protein was attributed by a reduction in protein synthesis rates, together with an increase in proteolytic activity and protein oxidation by ROS (Parida et al., 2007). Protein contents of all four improved lines were significantly higher than that in ‘KDML105’ which supported previous reports that more tolerant cultivars were better able to protect against protein degradation than the sensitive genotypes (Hakim et al., 2014).

The enhanced activities of antioxidant enzyme under abiotic stress, like drought and salinity are considered to be important defense mechanisms against oxidative stress in plants. The crucial roles of antioxidant enzymes were clearly demonstrated in this study that H$_2$O$_2$ and MDA contents were negatively correlated with all three antioxidant enzymes under both stresses (Figures 5 and 6). In response to drought, SOD tended to have higher activities than CAT and APX, and higher negative correlation was found between SOD and H$_2$O$_2$. The sensitive ‘KDML105’ had the lowest activities of all three enzymes compared with the improved lines and DH103 resulting in the highest H$_2$O$_2$. However, salt-stressed rice tended to have more enhanced activities of CAT and SOD than APX, and among genotypes more pronounced CAT activity was expressed in the more salt tolerant genotypes (RGD1, RGD4 and DH103). The results in this study were supported by previous reports that tolerant genotypes had higher activities of antioxidant enzymes than sensitive genotypes (Lee et al., 2013), and enhancement of these enzymes through genetic engineering and conventional breeding is considered as promising strategies for development of stress-tolerant rice.
CONCLUSIONS

In conclusion, all improved rice lines (CSSL94, CSSL103, RGD1 and RGD4) showed higher degrees of tolerance to drought and salt stresses compared with the parental ‘KDML105’. The higher tolerance ability of these lines was indicated by a lower inhibition of growth, high relative water content, good osmolyte accumulation, lower level of drought- and salt-induced hydrogen peroxide as a result of higher activity of antioxidant enzymes, and less lipid peroxidation leading to greater ability to maintain the membrane stability. In addition, RGD1 and RGD4 were found to maintain good ionic balance when exposed to salinity stress. Therefore, these lines can serve as useful genetic resources for breeders interested in further developing drought and salt tolerance in rice.

ACKNOWLEDGEMENTS

This work was supported by a grant from Thailand Agricultural Research Development Agency (ARDA) (Project code PRP5805021550). DP was supported by Science Achievement Scholarship of Thailand (SAST). The authors also wish to thank the National Research Council of Thailand (NRCT) for additional support through the Senior Research Scholar Project of Prof. Dr. Piyada Theerakulpisut (Project Nr NRCT813/2563).

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