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Effect of Salinity on Biochemical and Physiological Characteristics in Correlation to Selection of Salt-tolerance in Aromatic Rice (*Oryza sativa* L.)

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ABSTRACT: To investigate the influence of rice line, salt concentration and salt exposure time on proline accumulation, 23 indica rice lines were cultured on Murashige and Skoog (MS) medium supplemented with 0, 171, 342, 513 and 684 mM NaCl for 10 days. All three factors, especially rice line, significantly affected the proline accumulation. Among the salt-tolerant rice lines, Leuang Tang Mo showed the highest proline content. This line was then cultured under high salt concentration to determine the correlation between proline accumulation and chlorophyll index (CI). The addition of NaCl and exposure time significantly influenced both the proline accumulation and the CI. The proline content positively correlated with the CI. Consequently, the CI measurement was performed as a rapid method to select the salt-tolerance of Thai aromatic rice. To select for salt-tolerance by this developed method, 230 Thai aromatic rice lines were cultured on MS medium containing 513 mM NaCl for 8 days. The Hawm Naipon line manifested the highest CI and survival percentage, followed by Hawm Durian and Hawm Pae-palo, respectively. The survival percentage also significantly correlated with the CI.

KEYWORDS: Chlorophyll index, *Oryza sativa* L., Proline accumulation, Salt-tolerant rice, Salinity stress.

INTRODUCTION

Rice (*Oryza sativa* L.) is an important crop, which more than 700 million people consume as their main food.[1] The production and cultivation areas of rice are greatly menaced by soil [2] salinity. The area affected by soil salinity is 10^8 to 10^9 ha, three-times larger than the area currently used for agriculture.[3]

Reports of high quality rice, which is characterized by its aroma, low amylose, and diseases- and pest-resistance with salt-tolerance are very few. Salt-tolerant rice lines have often been investigated through the non-aromatic Pokkali and Nona Brokra rice lines,[4-6] while the genetic diversity of salt-tolerant aromatic rice has never been reported. The improvement of rice salt-tolerance is urgently required.[7]

Plant cells accumulate proline as an osmo-protectant to conserve osmotic stability and to prevent damage.[8-10] Plants cultured under salt stress show high proline accumulation. Plants also show the high chlorophyll degradation symptom, chlorosis, as a common morphological and physiological characteristic in response to salt stress. The occurrence of high proline content coupled with the reduced chlorophyll content under salt stress condition was reported by Harinasut *et al.*[11] However, the correlation between proline content and the chlorophyll index (CI) is still unclear. The objective of this investigation was to elucidate the correlation between proline content and CI, and to develop a rapid method for selection of the salt-tolerant aromatic rice.

MATERIALS AND METHODS

Plant Materials

Effect of salinity on the proline content.

Rice (*Oryza sativa* L. subtype *indica*) seeds, including 23 lines of Abhaya, Deang Dawk Kok, Leuang Tang Mo, FR 13A, IR 5882, NSG 19, PTT, KDML 105, KDML 01197, KDML 01223, KDML 01614, KDML 01625, KDML 01678, KDML 06723, KDML 06750, KDML 09909, KDML 13743, KDML 13744, KDML 16007, KDML 18431, KDML 19669, KDML 22789 and KDML 22791, were obtained from Rice Unit, Kasetsart University, National Center for Genetic Engineering and Biotechnology, National

Science and Technology Development Agency, Thailand. Thai aromatic rice, KDML 105, was used as a control line. The rice seeds were hand dehusked and then sterilized by the following steps; dipping in 70% (v/v) ethanol for 5 min, soaking in 5% (v/v) Clorox® (5.25% w/v sodium hypochlorite, Clorox Co., Ltd., USA) with 0.1% Tween 20® (Merck, Germany) for 1 h, soaking in 30% Clorox® with 0.1% Tween 20® for 30 min and rinsing with sterile-distilled water for three times. The rice seeds were germinated on Murashige & Skoog (MS, 1962) medium[12] supplemented with 0.25% Phytagel® (Sigma, USA) as a supporting material. Culture condition was 25±2°C air tempera-ture, and 16-h photoperiod of 60±5 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPF) provided by fluorescent lamps (TLD 36W/84, Cool White, Philips, Thailand). After culturing for 12 days, the medium was adjusted to 0, 171, 342, 513 and 684 mM NaCl under aseptic conditions. All leaves were collected and measured for proline content after treating with NaCl for 0, 2, 4, 6, 8 and 10 days.

Development of the rapid method for selection of salt-tolerant lines.

Seeds of Leuang Tang Mo, line acquired from Rice Unit, Kasetsart University, were germinated on MS medium as described above. After culturing for 12 days, the medium was adjusted to 0, 171, 342, 513 and 684 mM NaCl under aseptic conditions. All leaves, in each treatment, were collected and measured for proline content and CI, after treating with NaCl for 0, 2, 4, 6, 8 and 10 days. These plantlets were utilized to evaluate the correlation between proline content and CI.

Selection of the salt-tolerant rice.

The 230 rice lines from Rice Gene Bank, Patumthani Rice Research Center were germinated on MS medium as described above. After culturing for 12 days, the medium was adjusted to 513 mM NaCl under aseptic condition. All lines of Thai aromatic rice were selected for the salt-tolerant ability by measuring their CI.

Determination of the Proline Content

Proline was extracted according to the method by Bates.[13] Whole plantlets, 250 mg fresh weight, were ground in a motar with liquid nitrogen. The homogenate was mixed with 5 ml aqueous sulfo-salicylic (3% w/v) acid and filtered through Whatman® #1, 110 mm diameter filter paper (Whatman, England). The filtrate, 1 ml, was reacted with an equal volume of glacial acetic acid and ninhydrin reagent (1.25 mg of ninhydrin, 30 ml of glacial acetic acid and 20 ml of 6 M H_3PO_4) and incubated for 1 h at 100°C in boiling water. The reaction was terminated by placing the test tubes in an ice bath. The reaction mixture was vigorously mixed with 2 ml toluene. After warming at 25°C, the chromophore was measured for proline at 520 nm with a DR/4000 Spectrophotometer (HACH, USA). The proline content was determined using a standard curve of 0 to 512 μmol of L-proline (Fluka, Switzerland).

Measurement of the Chlorophyll Index

Chlorophyll content (CC) was measured by Chlorophyll meter (Minolta, SPAD-502, Japan). The 2nd leaf from the shoot apex were measured at 4 positions for the CC at the beginning of experiment and after eight days of NaCl treatment. The CC was calibrated with a standard curve of total chlorophyll concentration according to the method by Shabala *et al*.[14] The CI was calculated by the following equation:

$$CI = \frac{\text{CC at day } 8^{th}}{\text{CC at day } 0^{th}}$$

Correlation between proline content and the CI was shown in scatter charts (Figs 4, 5). A trend line was created by using a linear equation:

$$y = ax + b$$

Where y represents the proline content (mmol g^{-1} fresh weight), x represents the CI value, and a and b are calculated constants. The R-squared value (r^2) was calculated to represent that proportion of the total variability of the y-values that is accounted for by the independent variable x.

Determination of the Survival Percentage

The survival percentage was observed from the rice plantlets after treating with salinity. Plantlets with chlorosis at the basal area of stem and leaf blade were scored as dead plantlets.

Correlation between the CI and survival percentage was shown in the scatter chart (Fig. 7). The trend line was created by using a polynomial equation:

$$y = a_1x + a_2x^2 + b$$

Where y represents the survival percentage, x represents the CI value, and a_1, a_2 and b are the calculated values. Again, the R-squared value (r^2) represents that proportion of the total variability of the y-values, that is accounted for by the independent variable x.

Statistical Analysis of Data

Effect of salinity on the proline content.

Data from the 23 rice lines were evaluated by analysis of variance (ANOVA) according to a 23× 5× 6 factorial experiment in a Randomized Completely Block design (RCB) for four replications. Means were statistically compared among treatments by Duncan's Multiple Range Test (DMRT) at the $P \leq 0.01$ level.

Development of the rapid method for selection of salt-tolerant lines.

Data of the Leuang Tang Mo line were analysed by the Pearson's correlation for four replications. Means were statistically compared by DMRT at the $P \leq 0.01$ level.

Selection of the salt-tolerant rice

Data of 230 rice lines were analysed by ANOVA according to RCB for four replications. Means were statistically compared by DMRT at the $P \leq 0.01$ level.

RESULTS

Effect of Salinity on the Proline Content

To investigate the influence of salinity on the proline accumulation, rice seeds were germinated and grown for 10 days on MS medium containing 0, 171, 342, 513 and 684 mM NaCl. Most seedlings grown on the presence of 513 mM NaCl for 8 days exhibited the greatest proline content. Therefore, 23 rice lines were classified into 3 groups according to the proline content level after culturing on MS medium with 513 mM NaCl for 8 days. The Leuang Tang Mo, KDML 22789, NSG 19, KDML 22791, IR 5882 and KDML 105 lines were classified in the high level of proline, 40-70 μmol g^{-1} fresh weight (FW, Fig 1). The FR 13A, Abhaya, PTT, Daeng Dawk Kok, KDML 18431 and KDML 09909 lines were classified in the middle level of proline, 10-40 μmol g^{-1} FW (Fig 1). The KDML 19669, KDML 01197, KDML 01223, KDML 01614,KDML 01625, KDML 01678, KDML 06723, KDML 06750, KDML 13743, KDML 13744,and KDML 16007, lines were classified in the low level of proline, lower than 10 μmol g^{-1} FW.

The proline content of rice seedlings was affected by the presence of NaCl in the growth medium. For the high level of proline, the increment of NaCl concentration from 0 to 513 mM raised the proline levels significantly, by more than a 8-fold increase. There was a reduction of proline content in three rice lines, Leuang Tang Mo, KDML 22789 and KDML 22791, in the presence of 684 mM NaCl (Fig 2 A). In contrast, for the middle level of proline, PTT, KDML 18431 and KDML 09909 lines showed high proline

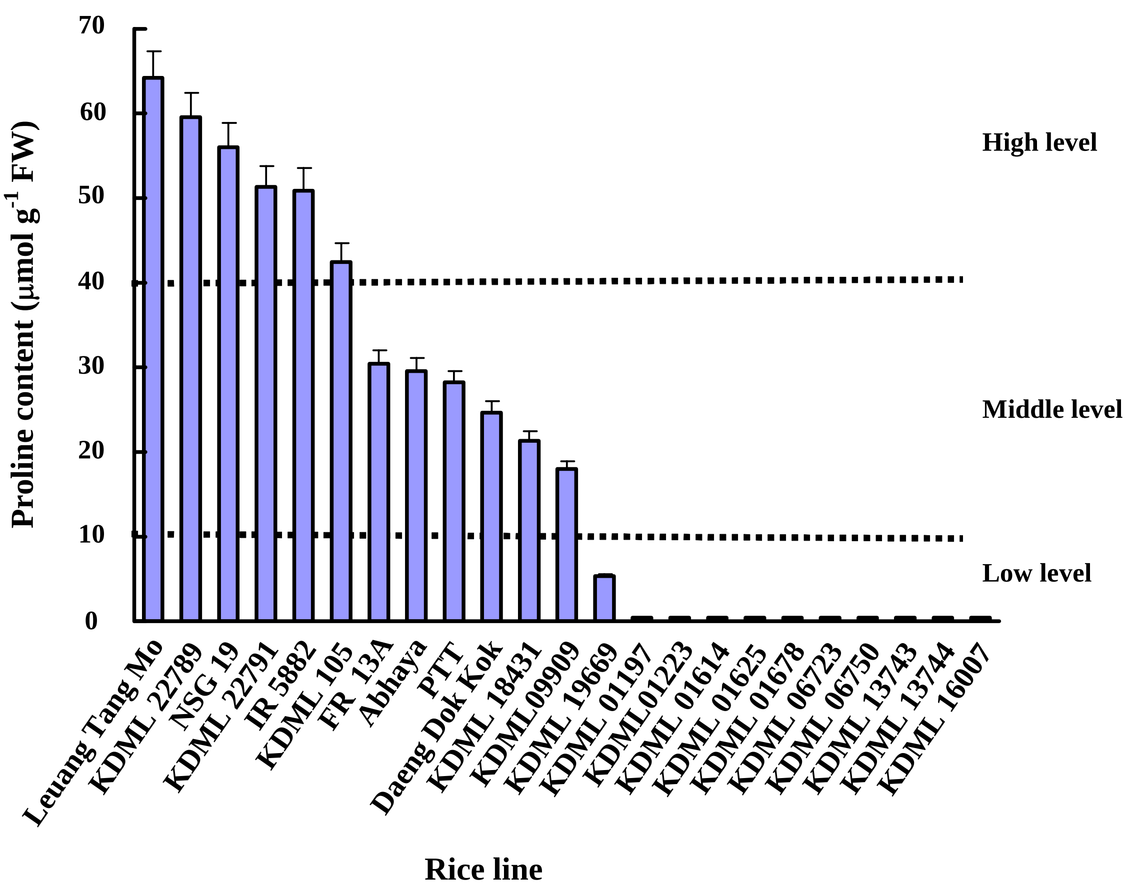

Fig 1. Proline accumulation classified into three levels of 23 rice lines after exposure to 513 mM NaCl for 8 days. Leaves were collected and measured for the proline content. Vertical bars represented standard errors (± SE).

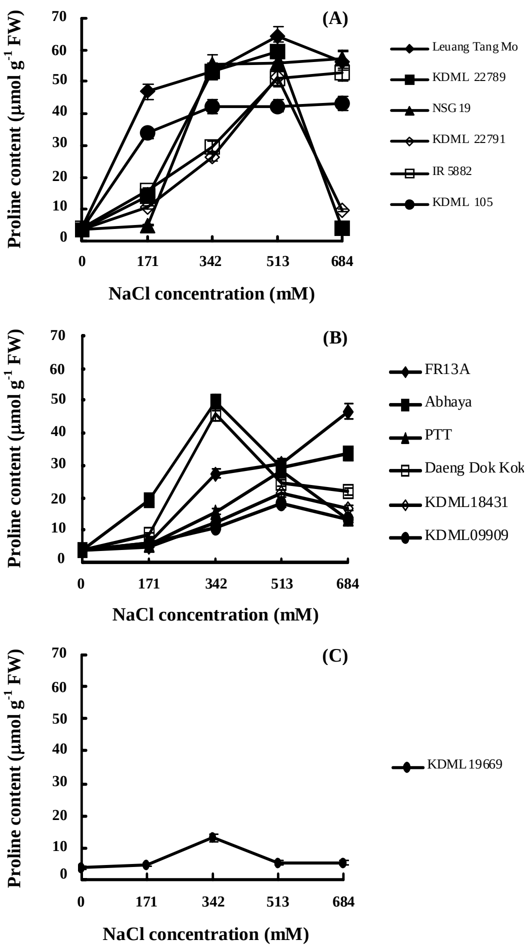

Fig 2. Effect of NaCl on proline content in 6 rice lines (Leuang Tang Mo, KDML 22789, NSG 19, KDML 22791, IR 5882 and KDML 105) (**A**), 6 rice lines (FR 13A, Abhaya, PTT, Daeng Dok Kok, KDML 18431 and KDML 09909) (**B**) and KDML 19669 line (**C**), classified in the high, middle and low level of proline content, respectively. After 8 days of exposure to 0, 171, 342, 513 and 684 mM NaCl, rice leaves were collected and measured for proline content. Vertical bars represented standard errors (± SE).

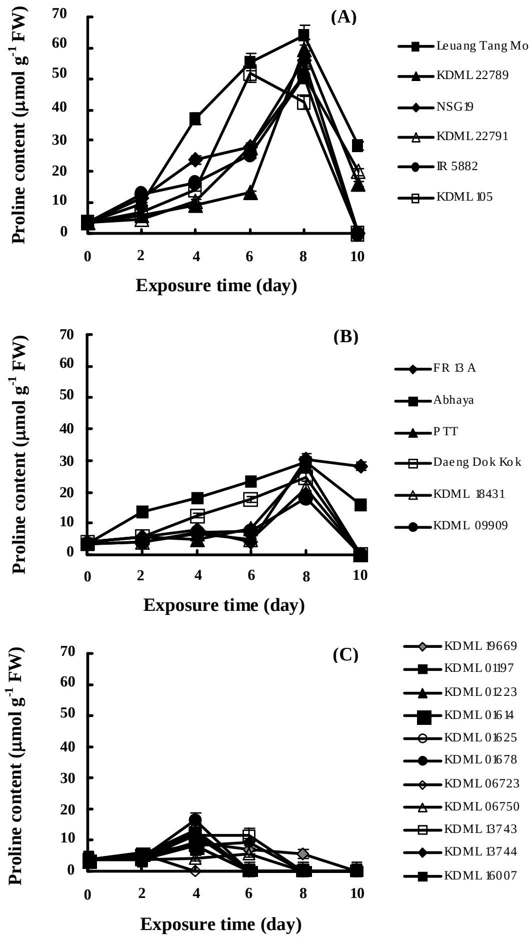

Fig 3. Effect of exposure time to salt stress on proline content in high(**A**), middle(**B**), and low(**C**) level of proline accumulation rice lines. After 0, 2, 4, 6, 8 and 10 days of exposure to 513 mM NaCl, rice leaves were collected and measured for the proline content. Vertical bars represented standard errors (± SE).

content at 513 mM NaCl, whereas both Abhaya and Daeng Dok Kok lines showed a 9-fold increase in proline content at 342 mM NaCl. The proline accumulation of the FR 13A line increased with increasing NaCl concentration and reached the highest level at 684 mM NaCl (Fig 2 B). For the low level of proline, the greatest proline content of the KDML 19669 line was 13 μmol g^{-1} FW when 342 mM NaCl was added to the medium. The average proline content of the remainder was 5 μmol g^{-1} FW (Fig 2 C). From these results, 513 mM NaCl induced highest proline content in the Leuang Tang Mo line, therefore, 513 mM NaCl was chosen for the experiments on the effect of exposure time.

Exposure time in the presence of 513 mM NaCl also affected the proline content in all rice lines. All of the rice lines in the high level of proline content, except the KDML 105 line, showed an increase in proline content of more than 10-fold during the first 8 days of salinity exposure. By 10 days of the salinity treat-ment, the rice lines exhibited a considerable reduction in proline content to lower than 30 μmol g^{-1} FW (Fig 3 A). All rice lines ceased to grow after 10 days treatment with 513 mM NaCl. Likewise in the middle level, the rice lines with salinity treatment contained significantly high proline level during the first 8 days of the salinity exposure (Fig 3 B). In contrast, for the low level group, the KDML 19669, KDML 01197, KDML 01223, KDML 01614, KDML 01625, KDML 01678 and KDML 16007 lines showed high proline content during the first 4 days of salinity exposure, whereas the KDML 06750, KDML 13743 and KDML 13744 lines revealed high proline content during the first 6 days of salinity exposure. Only KDML

06723 showed a slight increase of proline content during the first 2 days and then decreased after that (Fig 3C).

The interaction among 3 factors, rice lines × NaCl concentration, rice lines × exposure time, NaCl concentration × exposure time and rice lines × NaCl concentration × exposure time, significantly increased with increasing proline content in each rice line (r^2 = 0.76).

Development of the Rapid Method for Selection of Salt-Tolerant Lines

NaCl concentration and exposure time affected both proline content and CI of the Leuang Tang Mo line. The proline content of Leuang Tang Mo line was significantly increased with increasing NaCl concentration from 0 to 513 mM NaCl, then slightly decreased from 513 to 684 mM NaCl (Fig 2A). The proline content was significantly increased with increasing NaCl exposure time from day 1 to day 8, then considerably decreased from day 8 to day 10 after salt stress (Fig 3A). The CI of the Leuang Tang Mo line positively correlated with the proline content (Figs 4, 5). The Leuang Tang Mo line seemed to show not only the high salt-tolerance, but low chlorophyll degradation as well.

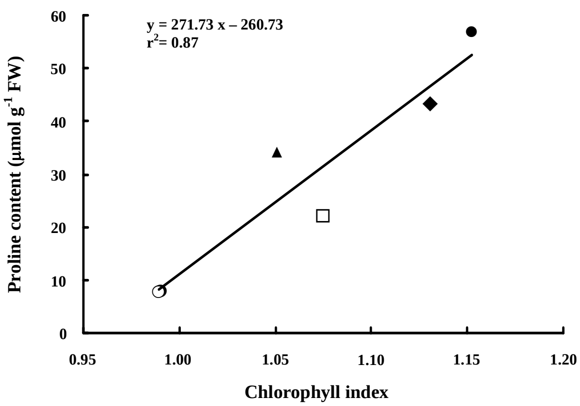

Fig 4. Correlation between proline content and chlorophyll index of the Leuang Tang Mo line, influenced by 0 (❍), 171 (☐), 342 (◆), 513 (●) and 684 (▲) mM NaCl for 8 days.

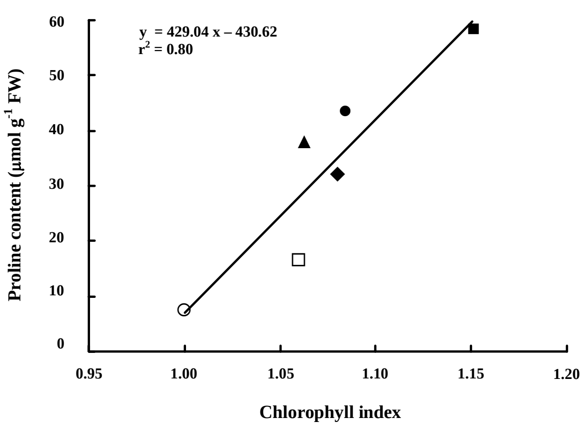

Fig 5. Correlation between proline content and chlorophyll index of the Leuang Tang Mo line, influenced by 0 (❍), 2 (☐), 4 (◆), 6 (●), 8 (■) and 10 (▲) days exposure to 513 mM NaCl. Chlorophyll index was determined as chlorophyll content at each respective day of salt treatment divided by chlorophyll content before salt treatment.

Selection of Salt-Tolerant Rice

The rapid technique, CI measurement, was used to select the salt-tolerant rice. The criteria for selection were 1) those with a CI higher than 1.0 and 2) those with the highest survival percentage, i.e. 100%. The 230 lines of Thai aromatic rice exhibited different CI. The CI of 11 of the 230 rice lines was increased with increasing exposure time (above 1.0), while the CI of the others was decreased (data not shown). The Hawm Naipon line showed the highest CI followed by Hawm Durian, Hawm Pae-palo, Hawm Phrae, Hawm Thong, Hawm Tang, Hawm Jampa, Hawm Nang Nuan, Hawm Sa-dung, Hawm and Hawm Mae-jan, respectively (Fig 6).

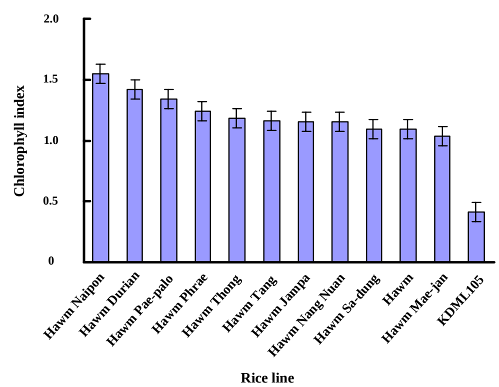

Fig 6. Chlorophyll index of 11 salt-tolerant rice lines, and KDML 105 as a control, under salt stress condition (513 mM NaCl at day 8). Vertical bars represent standard errors (± SE).

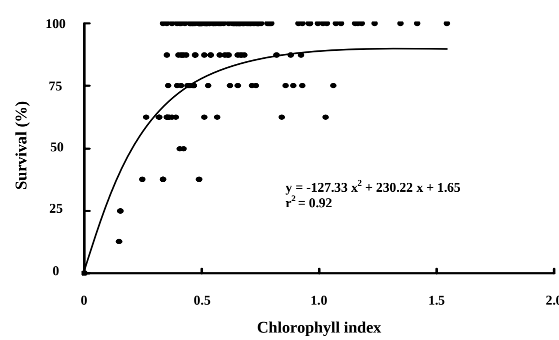

Fig 7. Correlation between chlorophyll index and survival percentage among 230 rice lines after culturing on the medium containing 513 mM NaCl for 8 days.

The survival percentage of 230 lines was observed at the 8th day after salt stress. The rice lines exhibited different survival percentages. Only 95 lines survived. The survival percentage of the rice lines strongly correlated with the CI (Fig 7).

Discussion

Soil salinity, one of the most serious problems on planting areas, has the mast obstructive impact on crop production in the world. This crisis problem attracts many scientists to overcome this obstruction by improving salt-tolerant lines.

Indica rice is an important crop in the world. There are many varieties of this subspecies distributed in several countries. One of the highly desired qualities of rice is aroma. Although KDML 105 is well known as aromatic rice with soft texture, this rice has low salt tolerance. Presently, the production and planting area of rice are greatly menaced by soil[2] salinity. Therefore, a broad genetic diversity of Thai aromatic rice was evaluated for salt-tolerant rice lines that may be able to grow in saline soil.

Proline accumulation was firstly observed in wilted rye grass.[15] Proline is well known as an osmo-regulatory solute in plants subjected to hyperosmotic stress.[8] The hyperosmotic stress from drought and salinity is the most important factor limiting plant growth and crop production.[16] The effect of salt stress on proline accumulation was reported in many plant species such as *Beta vulgaris* L.,[9] *Lycopersicon esculentum*,[17] *Brassica napus* L.,[18] *Oryza sativa* L.[5,6] and *Morus alba* L. cv. Khonpai.[11] The understanding of the role of proline accumulation in salt-tolerant rice under salt stress is still unclear.[5,6] Several reports showed that proline accumulation in salt-tolerant rice lines was lower than that in salt-sensitive rice lines.[5,6] In contrast, results herein showed that the proline accumulation of the salt-tolerant rice line, Leuang Tang Mo, was higher than that of the other 22 lines in the initial screen. The Leuang Tang Mo line showed high salt-tolerance and high chlorophyll index, whereas the others showed significantly lower chlorophyll indices and wilting. The discrepancy of these results might be due to differences in the rice lines.

Accumulation of proline in the cytoplasm is accompanied by a reduction in the concentrations of less compatible solutes, e.g. K^+ and glutamate, and an increase in cytosolic water volume.[19] It has been reported that the accumulation of proline occurred up to 3 days after treatment with 200 mM NaCl in tobacco[10] and up to 10 days with 100 mM NaCl in rice.[4,5] This study showed that the accumulation of proline occurred up to 8 days after treatment with 513 mM NaCl. *In vitro* studies indicated that proline was much less inhibitory than equivalent concentrations of NaCl to enzymes and protein synthesis machinery and may also function as a hydroxyl radical scavenger.[19] Proline may play a role to protect chlorophyll, a photosynthetic pigment of the chloroplast. Under salt stress, the CO_2 assimilation rate is reduced. As a result, chloroplasts are subjected to excess excitation energy leading to an increased rate of formation of reactive oxygen intermediates.[20]

Chlorophyll, found in all photosynthetic plants, is the principal pigment for light absorption in photosynthesis.[21] Chlorophyll degradation is induced by many stresses, leading to changes of certain enzyme activities, photosynthetic electron transport, carbon metabolism and photophosphorylation in photo syn-thesis. During salt stress, salt-sensitive plants clearly showed chlorophyll degradation and growth reduction.[11,22] The measurement of chlorophyll content, CC, is very simple and rapid, and has been applied in rice as an index for fertilizer management and salt tolerance screening.[23,24] The chlorophyll index, CI, was calculated by the ratio of CC at day 8 of salt stress to CC at day 0 (control). A high CI value means that the salt stress did not have much effect on chlorophyll content of the plants. This led to an increased photosynthetic rate, much more dry matter production and higher productivity. Therefore, the CI was used to select for salt-tolerance in aromatic rice.

The results indicated that the CI of the Leuang Tang Mo line gradually increased during salt stress. The Leuang Tang Mo line also showed a high proline content, which positively correlated with the increase of the CI. The CI may be involved with the high salt-tolerance of this line that accumulates proline to a high level to prevent damage from osmotic stress. Since the result showed significant correlation between the CI and the proline content, the CI should be used as an index to select salt-tolerant rice lines. However, the CI and proline content of the Leuang Tang Mo line were greatly decreased after treatment with 684 mM NaCl for 8 days and 513 mM NaCl for 10 days, respectively (Figs 4, 5). Leaves of the Leuang Tang Mo line gradually changed from green to yellow and subsequently died. This confirmed the previous report showing that high concentration of NaCl and long exposure time strongly reduced growth and survival of rice seedlings.[25]

To find salt-tolerant rice from among the genetic diversity of Thai aromatic rice, 230 rice lines were selected through the CI (Fig 7). Eleven of the 230 lines, Hawm Naipon, Hawm Durian, Hawm Pae-palo, Hawm Phrae, Hawm Thong, Hawm Tang, Hawm Jampa, Hawm Nang Nuan, Hawm Sa-dung, Hawm and Hawm Mae-jan, showed the higher CI (Fig 6) than the other lines (data not shown) including KDML 105. During the salt-tolerance selection, the growth of the rice plantlets was observed. The highly salt-tolerant rice lines such as Hawm Naipon and Hawm Durian lines grew better than the other lines. The salt-sensitive rice lines showed significant reduction of growth and subsequently died.

The chlorophyll degradation in rice plantlets occurred from tip of leaf blade to base, then also occurred from base to tip of leaf sheath. Moreover, the accuracy of the CI method can be improved via testing several positions of leaf.

In conclusion, the main factors, rice line, NaCl concentration and exposure time, strongly affected the proline content. The CI positively correlated with the proline content in the Leuang Tang Mo line, and was performed as a rapid method for selection of salt-tolerance in Thai aromatic rice lines. However, the salt-tolerance selection with the CI under the high concentration of sodium chloride (513 mM) with long exposure time (8 days), may inevitably lead to over-looking other salt-sensitive and moderately salt-tolerant rice lines that may otherwise possess several good phenotypes.

This investigation reported the group of salt-tolerant rice lines from selection, it will be necessary to further investigate their productivity in saline fields. The highly salt-tolerant rice lines will be utilized as a genetic resource to discover the function of salt-tolerant genes.

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