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Variation in tolerance to salinity stress at the reproductive stage in a large fast neutron mutant rice *(Oryza sativa* L.) population

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Abstract Rice is very sensitive to salinity stress during the reproductive stage and salinity stress causes reduced grain yields. The salinity concentration, critical point and phenotypic parameter of the reproductive stage were identified for screening a large fast neutron mutant population (M4). The results showed a 150 mM NaCl treated in irrigated water at the early booting stage (R2) had the greatest effect on flag leaf and panicle damage, and it also reduced seed setting, especially in susceptible genotype. However, seed setting was a suitable parameter for screening salinity tolerance in the large mutant population. Then, the 9,000 JHN mutant lines were screened for salinity tolerance under 150 mM NaCl at early R2 - R9. The result showed 5,397 mutant lines failed to produce seed setting and found 20 lines produced seed setting above 60% in the preliminary screening. In repeated screening, the 20 lines (M_5) were confirmed a potential to produce seed set under salinity stress and found M1145 produced the highest seed set. Finally, the validated screening, M1145 showed a stable tolerance under salt stress and produced the highest seed setting at 48%, which was not significantly different from the standard salinity tolerant (Pokkali, FL496 and FL530). In conclusion, seed setting during salinity stress at reproductive stage was representative of salinity tolerance and can be used as a screening parameter for salinity tolerance on a large scale. In addition, the fast neutron mutant population showed variations in salinity tolerance levels, and M1145 was identified as a salinity tolerant and is a potential genetic stock for use in a salinity tolerance breeding program.

Keywords: rice (*Oryza sativa* L), salinity stress, salinity tolerance, seed setting, screening, large mutant population

Introduction

One of the crucial limitations affecting crop yield global is soil salinity. Rice is an important crop that in sensitive to soil salinity. Approximately 20% of Earth's land area and 50% of irrigated land worldwide including about 30% of rice areas are salinized (Wang *et al*., 2012). Rice is most sensitive to salinity

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at level above 3 dSm^{-1} (USDA, 2013). At an EC as low as 3.5 dSm^{-1}, the grain yield loss can be approximately 10% depending on varied differences (Gregorio *et al*., 1997), and when salinity is at the most severe level (> 10 dSm^{-1}), the rice yield can decrease more than 50% (Dobermann and Fairhurst, 2000). In the northeast of Thailand, approximately 1.84 M ha or 16% of the lowland rainfed area is classified as an inland saline area ranging from 11 to 35 dS m-1 (Im-Erb *et al*., 2013; Arunin and Pongwichian, 2015). Therefore, breeding programs for salinity tolerance can help poor farmers grow rice in high-salinity areas.

Salinity stress affects seed germination, seedling growth, leaf size, shoot growth, shoot and root length, shoot dry weight, shoot fresh weight, number of tillers per plant, flowering stage, spikelet number, percent of sterile florets and productivity (Zeng and Shannon, 2000; Lauchli and Grattan, 2007; Moradi and Ismail, 2007; Munns and Tester, 2008; Ashraf, 2009; Hakim *et al*., 2010; Gupta and Huang, 2014). However, rice is very sensitive at the early seedling stage and reproductive stage (Singh and Flowers, 2010). It can be suggested that these two sensitive stages are independent of each other. Thus, tolerance at the reproductive stage is not correlated with tolerance at the seedling stage (Ahmadizadeh *et al*., 2017). This means that a genotype with seedling stage salinity tolerance is not automatically going to be tolerant at reproductive stage as well.

At the flowering stage, a salinity level from 2.5 to 4.0 dSm^{-1} seriously affected to rice at the booting stage. Salinization affects primary branch and spikelet formation, pollination and germination of the pollen, reduces the number of effective spikelets/panicles and increases the unfilled grain ratio (Singh *et al*., 2008). Salinity also reduces panicle length, grains/panicle and 1,000 grain weight, resulting in reduced rice productivity (Akbar and Yabuno, 1974; Ota and Yasue, 1962). Another research reported that salinity stress at 8 dS/m^{-1} in the flowering stage caused a reduction in the overall vigor of rice, especially in pollen germination, fertilization and grain yield (Sen *et al*., 2017). In addition, high salt reduces pollen viability at the flowering stage, which in turn determines grain yield (Khatun and Flowers, 1995; Singh and Flowers, 2010; Singh *et al*., 2004; Calapit-Palao *et al*., 2013; Hossain *et al*., 2015). However, the growth differences among various genotypes in response to salinity are dependent on the salt concentration and the degree of salt tolerance (Eynard *et al*., 2005). Therefore, screening for salt tolerance at the reproductive stages has been considered to be more useful than screening at other stages.

Extensive germplasm collection provides a useful source of the genetic diversity used to find salt tolerant genotypes. Therefore, the choice of germplasm is most crucial for the success of breeding (Reddy *et al*., 2017). Ideally, germplasms should differ as much as possible for the traits to be

improved. Thus, the application of mutation breeding to identify abiotic stress tolerant mutants has been successful in some crop plants, including rice (Ahloowalia *et al*., 2004; Lee *et al*., 2003) and this procedure can be used to explore the salt tolerance genotype.

There are few studies on reproductive-stage salinity tolerance mainly because of the lack of reliable reproductive stage-specific phenotyping techniques and incomplete knowledge of the stage-specific mechanisms of salinity tolerance. The first important step in the selection of salinity tolerant rice varieties is screening in the greenhouse. It helps shorten the time and lower the cost of the selection process before production in the field and simultaneously uses as a scientific basis to select the primary materials for breeding. The aims of this study were to (1) find techniques that are suitable and stable for a large population screening of salinity tolerant at the reproductive stage and (2) identify a parameter to use for screening salt tolerance at reproductive stage and (3) discovery a new genetic donors for salt tolerance from 9,000 mutant population (M_4) of Jao Hom Nin (JHN) comparing with IR29 (susceptible) and Pokkali (tolerance) under salt stress at reproductive stage from booting to harvesting stage.

Materials and Methods

Plant materials and growth conditions

The experiments were conducted from 2014 to 2016 at the Rice Science Centre at Kasetsart University, Nakhon Patom, Thailand. A total of 9,000 lines of JHN mutants (M4) were screened for salt tolerance. The population arose from 100,000 breeder seeds of the JHN wild type, and the mutations were induced using 33 Gy fast neutrons (FN). The successive generations from M_1-M_4 and the family history were traceable from individual M_1 plants. Due to abnormal mutations affecting the seed set, several families were terminated, leaving only 21,024 mutant families at M_4 that formed the base population for genetic screening (Ruengphayak *et al*., 2015).

The 9,000 M_4 mutant lines were seeded in a field nursery. After 30 days, the rice seedlings were transferred into plastic pots with 1 plant/pot (30 cm in height and 25 cm in diameter with 8 kg of sieved sandy loam soil). The soil contained 5.57% organic matter, 0.332% total N, 111.56 mg/kg of available (avail) P, 558 mg/kg of exchangeable (exch) K, 1882.3 mg/kg of exchangeable Ca, and 118 mg/kg of exchangeable Mg. Other management activities followed a conventional high-yielding cultivation approach.

Greenhouse setup

The capacity of the greenhouse was 640 m^2, which consisted of 2 ponds (40 m x 8 m). The greenhouse was controlled for temperatures between 32-35 ℃ with a relative humidity of 50%-60% RH and a daytime light intensity of 900-1,000 µM/m^2s (Jagadish *et al*., 2008). The micro-climate data were recorded by using data loggers (WatchDog 1000 series Micro Stations) at 3 positions (10 m distance), and the carbon dioxide concentration in the greenhouse, which was approximately 390 µmol-1, was also monitored with a HUATO 653 series detector.

Determination of the optimum NaCl concentration for screening salt tolerance

To determine the suitable concentration of NaCl that can be used to screen salt tolerance at the reproductive stage, a split-plot with a CRD with 4 replications was performed. The seeds of the JHN wildtype as well as the standard salt tolerant varieties (Pokkali, FL496, FL530) and standard susceptible varieties (IR29 and KDML105) were grown under normal conditions from seed germination to the booting stage (R2). After that, all rice plants were treated with NaCl 0, 50, 75, 100 and 100 mM in a circular irrigated water pond (pH 7) until physiological maturity (R9; 27 days after treatment). A waterproof EC tester (EC Testr11) was used to check the NaCl concentration in the hydroponic solution every 7 days. The salinity symptoms were scored, including the percentage of flag leaf damage and panicle damage, seed set, seed weight/panicle and 20-seed weight. In addition, after selecting the NaCl concentration for screening, the pollen viability, pollen germination, and anther dehiscence were examined only in the JHN wild type.

Determination of the optimum period in the reproductive stage for screening salt tolerance

The JHN wild type was used to observe the severity of salt stress at the reproductive stage. A CRD with 4 replications was used in this experiment. Salt stress at 150 mM NaCl was induced at different growth stages, including 65 (panicle initiation; PI), 70 (R1), 75 (early R2), 80 (late R2), 85 (R3), 90 (R4), 95 (R5), 100 (R6) and 105 (R7) days after germination (DAG). These starting points cover the reproductive stage from PI until grain filling (R5-R7) (Counce *et al*., 2000; Endo *et al*., 2009). The rice plants that grew in the normal solution were used as a control. All of the treatments were left in 150 mM NaCl for 10

days and then moved to the normal solution. The pollen viability, panicle length, seed set and 20-seed weight were observed.

Screening the large JHN mutant population for salt stress

The experiment was conducted in an augmented design with 2 replications during the rice growing season from November 2013 to December 2014. First, 9,000 lines from the JHN mutant (M4) population were planted in pots (4 pots per line) and maintained under natural conditions from the seedling (VE) to the booting stage (R2). After that, all of the mutant lines were transferred to a 150 mM NaCl solution until R9. The concentration of NaCl was tested every 7 days to maintain the salt concentration. However, the greenhouse had a sufficient capacity to screen 1,500 lines (6,000 pots)/crop. Thus, the screening for salt tolerance had to be performed as a preliminary screening for six crops. The JHN wild type and FL530 were used as a control. The spikelet fertility was investigated at the maturity stage (Sen *et al*., 2017).

Repeated screening and validation of salt tolerance

For the repeat screening, 13 lines of JHN mutants (M5) that showed high spikelet fertility (>60%) were treated with a 150 mM NaCl and normal solution (0 mM) under the same method as in the preliminary screening. The experiment began in 2015 (April-May), and it was set up in a CRD split-plot design 4 replications (5 pots/rep).

The candidate lines (M6) validated for salt tolerance with the JHN wild type, IR29 and FL530 lines were treated with a 150 mM NaCl and normal solution (0 mM) under the same method as in the previous work. The experiment began in 2015 (July-August), and it was set up in a CRD split-plot design with 4 replications (5 pots/rep).

Data collection

Panicle traits; for the preliminary screening, repeat screening and validation screening at panicle emergence (R4), the first panicles in each pot were selected. The data for plant height, panicle length, number of filled and unfilled grains per panicle, seed weight/panicle and 20-seed weight were recorded. The spikelet fertility was estimated as the ratio of the number of filled grains to the total number of spikelets and expressed as a percentage. The number of filled grains included both completely and partially filled grains.

However, due to the limited standard for salt tolerance for seed setting the standard for heat tolerance (IRRI, 1996) was applied in this research.

Pollen viability; for the study of the salt stress period in the reproductive stage, pollen viability was estimated using 1% IKI stain. The pollen grains that stained uniformly were considered viable. For pollen viability, 10 anthers from different plants were collected early in the morning before anthesis, and the anthers were opened with a needle. The pollen grains were immediately brushed onto a glass slide and covered with a drop of IKI. Pollen viability was estimated as the ratio of the number of stained pollen grains to the total number of pollen grains and expressed as a percentage (Prasad *et al*., 2006).

Statistical Analysis

All the data were analyzed using the R program to test the significance of the results for the NaCl treatments. The means were separated using Tukey's least significant difference (LSD) test at an alpha level of 0.05. If there was no significant difference among the experiments for a given parameter, then the values from all of the experiments for that parameter were used to obtain the mean and error. The standard errors of the means were also calculated, and they are presented in the graphs as error bars.

Results

The damage to the flag leaf under salt stress

The flag leaf in all cultivars developed normally in the nutrient solution until 12 days after treatment with NaCl. The results showed that salt stress at every concentration did not affect Pokkali, FL496, FL530 (tolerance cultivars) and KDML105 (sensitive cultivar). However, the flag leaf of IR29 was 4, 25, 38 and 75% damaged by salt stress at 50, 75, 100 and 150 mM respectively. On the other hand, the wild type JHN flag leaves were also slightly damaged by salt stress at 50, 75, 100 and 150 mM with 2, 2, 3 and 21% damage, respectively (Figure 1a).

Then, the rice plants continued to grow in salt solution until 27 days after treatment with NaCl, and the levels of flag leaf damage were increased in every cultivar, especially in IR29, which showed severe flag leaf damage compared to the other cultivars at every NaCl concentration. It showed 10, 63, 79 and 100% damage in 50, 75, 100 and 150 mM NaCl, respectively, while FL496, FL530, Pokkali, KDML105 and JHN wild type showed severe flag leaf damage at 150 mM NaCl with 58, 76, 40, 40 and 41% damage, respectively (Figure 1b).

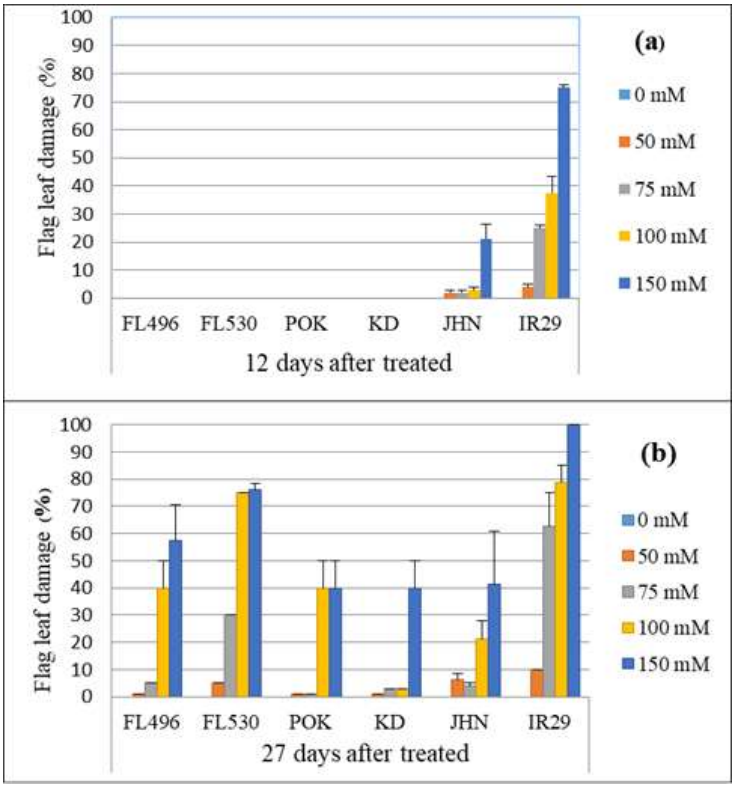

Figure 1. The percentage of flag leaf damage at 12 and 27 days after treatment with 0, 50, 75, 100 and 150 mM NaCl

The damage to the panicle under salt stress

The evaluation of panicle damage due to salt stress is one parameter that can be used to screen salt tolerance in a large population. If Na^+ can be translocated from the root to the panicle, the panicle will be damaged, and the florets cannot be fertilized. The result showed that at 12 days after treatment with 150 mM NaCl, only IR29 was damaged by salt stress with 21% damage. Then, the rice plants continued to be under salinity stress for 27 days. The percentage of panicle damage of IR29 was increased to 60%, and the panicle of JHN was damaged at 50%. On the other hand, the panicles of Pokkali, FL496, FL530 and KDML105 appeared normally.

The ability for seed setting under salt stress

In this experiment, we found that the increase in NaCl concentration caused a decrease in the seed setting on the panicle. The JHN wild type treated

in NaCl at 50, 75 and 100 mM produced seed settings at 95, 75 and 64%, respectively. In addition, the JHN wild type, IR29 and KDML105 at 150 mM showed severe decreases in seed setting to 9, 10 and 10%, respectively. However, the tolerant cultivars, including FL496, Pokkali and FL530, showed a high seed setting of 38, 44 and 46% at 150 mM NaCl, respectively (Figure 2). Thus, 150 mM NaCl can be used to screen the mutant population in the next experiment. After that, Pokkali, JHN wild type and KDML were used to examine pollen viability and anther dehiscence in 150 mM NaCl. The pollen viability of the JHN wild type decreased to 35.8% (60% viability) in 150 mM NaCl, while the pollen viability of Pokkali and KDML105 decreased slightly by 12.3% (85% viability) and 13.5% (74% viability), respectively. However, the percentage of pollen viability of JHN and KDML still reached up to 60%, which was in the opposite of seed setting. Thus, the low seed setting in JHN and KDML might be affected by the failure of anther dehiscence and pollen fertilization (data not shown).

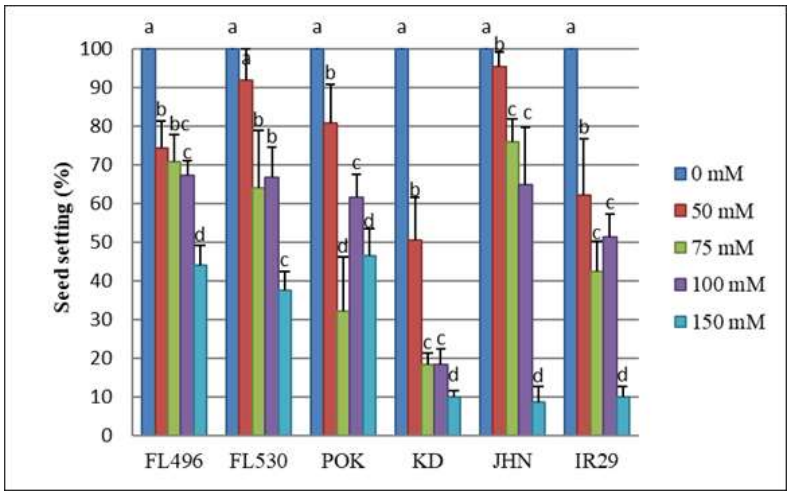

Figure 2. The percentage of seed setting at the R9 stage under 0, 50, 75, 100 and 150 mM NaCl

Critical phase of salt stress at the reproductive stage

The JHN wild type was used to determine the sensitive period of the reproductive stage by inducing salt stress at 150 mM NaCl at 9 different reproductive stages and leaving the rice plants in the solution for 10 days. These periods covered panicle initiation (PI) through the seed development stages (R7).

The results showed that salt stress significantly affected some traits at different periods in the reproductive stage ($p<0.05$), and the critical reproductive period under salt stress in the JHN wild type was 75 DAG or the early booting stage (R2), which had the lowest seed setting (17.68%) and 20-seed weight (0.309 g) compared with those of the JHN wild type under the control conditions (0 mM NaCl), 79.05% seed setting and 0.497 g of 20 seeds (Figure 3b and c). However, when the JHN wild type was treated with salinity stress after 90 DAG (R4), the difference in seed setting was not significant ($p>0.05$), with plants at 95, 100, 105 DAG and under control conditions producing a seed setting of 71.18, 69.02, 83.34, 83.67 and 79.05%, respectively. This result suggests that salt stress at 150 mM NaCl can severely affect spikelet development. Thus, seed setting decreased dramatically. In addition, salt stress affected panicle development but not for seed setting at 65 (PI) and 70 (R1) DAG (Figure 3a). In the first part of the experiment, the JHN wild type was the most sensitive to salt stress at 150 mM NaCl and at the booting stage (R2), and this condition was used to screen the JHN mutant population using seed setting as a parameter and the JHN wild type as a control.

Screening a large JHN mutant population for salt tolerance

The preliminary screening that included 9,000 lines of irradiated M_4 JHN plants was conducted for 6 crops, including 725, 1221, 1234, 1524, 1985 and 1635 lines/crops. The total number of mutant lines that underwent screening was 8324 lines because some lines did not germinate or died after screening. The salt tolerance screening was controlled at 150 mM NaCl from R2 – R9 by using seed setting as the parameter. The results of the preliminary screening showed that salt stress decreased the number of spikelets. The results showed that the seed setting produced by the 8,324 lines ranged from 0-76%. Most mutant lines (5,397 lines) were sensitive to salt stress and could not produce a seed set (0%). A total of 2,388 lines showed seed setting from 1% to 20%; 63 lines showed 21% to 30%; 91 lines showed 31% to 40%; and 51 lines showed 51% - 60%. A seed setting from 61% to 70% under salt stress was classified as salt tolerant, met by 11 lines. A seed setting greater than 70% was classified as highly salt tolerant, met by only 5 lines (Figure 4). When the seed settings among the JHN wild type and mutant populations were compared, 5,397 mutant lines showed lower seed settings than the JHN wild type, and 2,451 lines showed a similar seed set to the JHN wild type. In addition, 246 mutant lines showed a higher seed setting than the JHN wild type.

Therefore, the mutant lines that showed a seed setting over 60% including M395, M1145, M1389, M1724, M1846, M2168, M2265, M2289, M2849,

M2957, M3053, M3318, M3353, M3475, M3662, M5899, M6671, M6753, M6780 and M7376 were used for repeat screening to confirm their salt tolerance.

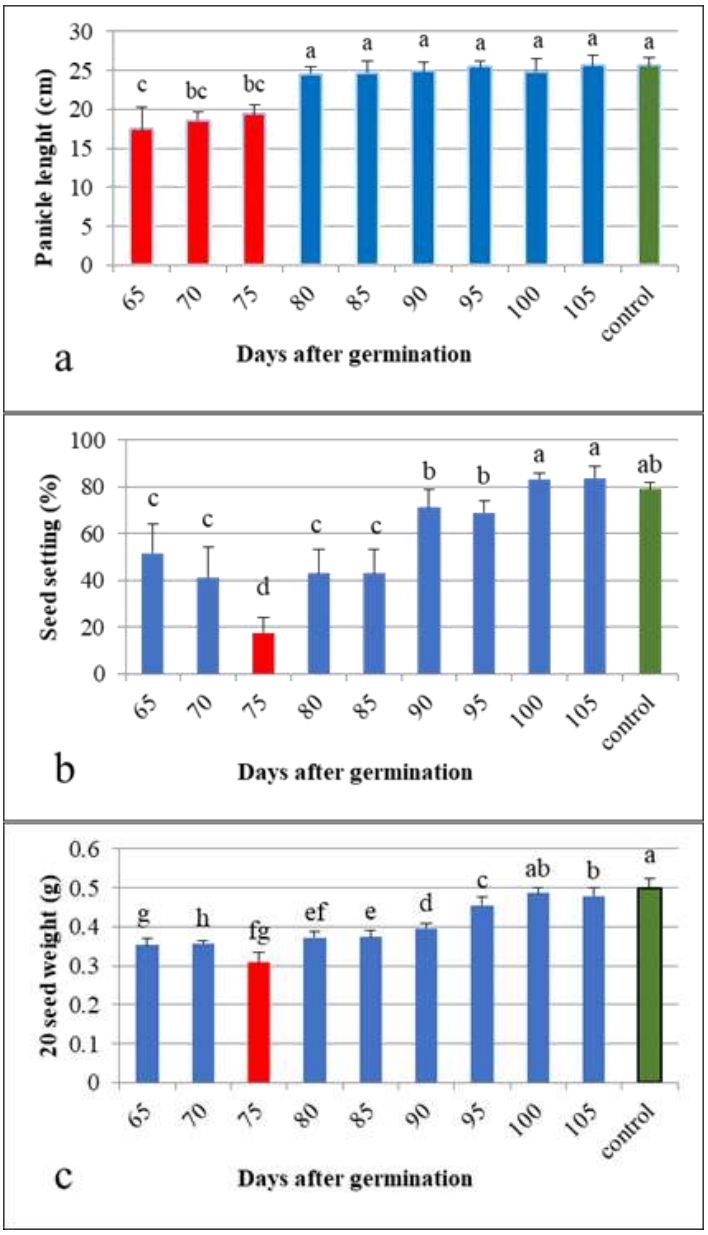

Figure 3. The panicle length, seed setting and 20-seed weight of JHN treated with 150 mM NaCl at various phases of the reproductive stage

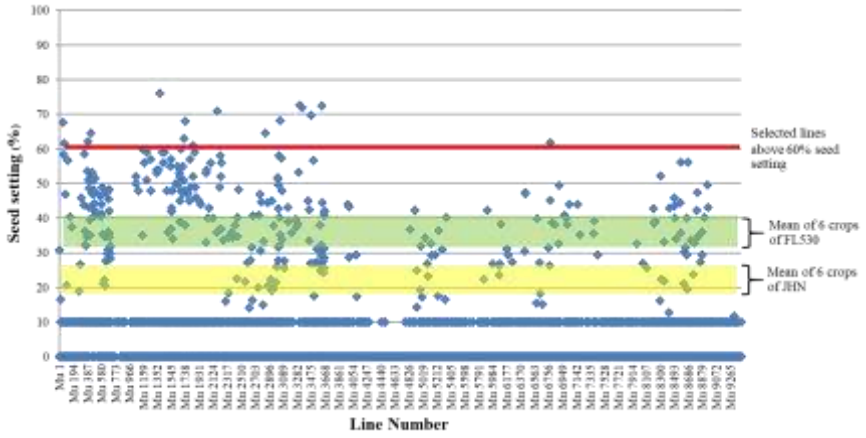

Figure 4. The preliminary screening of 9,000 M_4 mutant lines of 6 crops, the mutant lines that showed a seed setting over 60% were selected for repeated screening

Repeated screening

The 20 lines (M_5) selected from 8,324 lines of M_4 that exhibited a seed setting above 60% in the preliminary screening were investigated and compared with the JHN wild type, FL496, FL530 and IR29 in the same conditions as first screening. However, lines M395, M6771 and M7376 did not germinate. Thus, the total number of lines that underwent repeated screening was 17 mutant lines. The seed set of 13 mutant lines was still greater than JHN wild type (12.3% seed setting). However, the seed set of 19 mutant lines was less than first screening (Figure 5a). This may show that the accuracy of the repeated screening was greater than the first screening or the M_5 generation was still segregated. The mutant line that showed the highest seed setting under salt stress was M1145 (41.2% seed setting). The seed set of this line also did not significantly differ from Pokkali and FL530 ($P>0.05$), which gave 45.2 and 42.8%, respectively. Therefore, the ability of M1145 to set seed was investigated again in the validation screening.

The seed weight/panicle showed the same result as the seed setting. The salinity stress reduced seed weight (Figure 5b). However, the panicle length should not be a factor in the reduction of seed weight because there was no difference in panicle between stressed and non-stressed plants ($P>0.05$) (Figure 5c). This means that the florets per panicle of both conditions should be the same. Pokkali, FL530 and M1145 showed the highest seed weight/panicle under salt stress at 0.71, 0.78 and 0.66 g, respectively. On the other hand, JHN and IR29 had a seed weight/panicle of 0.26 and 0.25 g, respectively.

The other trait that was affected by salinity stress was flag leaf damage. This is also related to seed setting and seed weight/panicle. Pokkali, FL496 and M1145 showed low levels of flag leaf damage, at 38.5, 37.7 and 40.0%, respectively, while IR29 showed the highest rate of damage to the flag leaf at 89.2% (Figure 5d). However, JHN wild type (44.5% damage) was not significantly different from M1145. It might be suggested that flag leaf damage is not a clear parameter for screening in the reproductive stage.

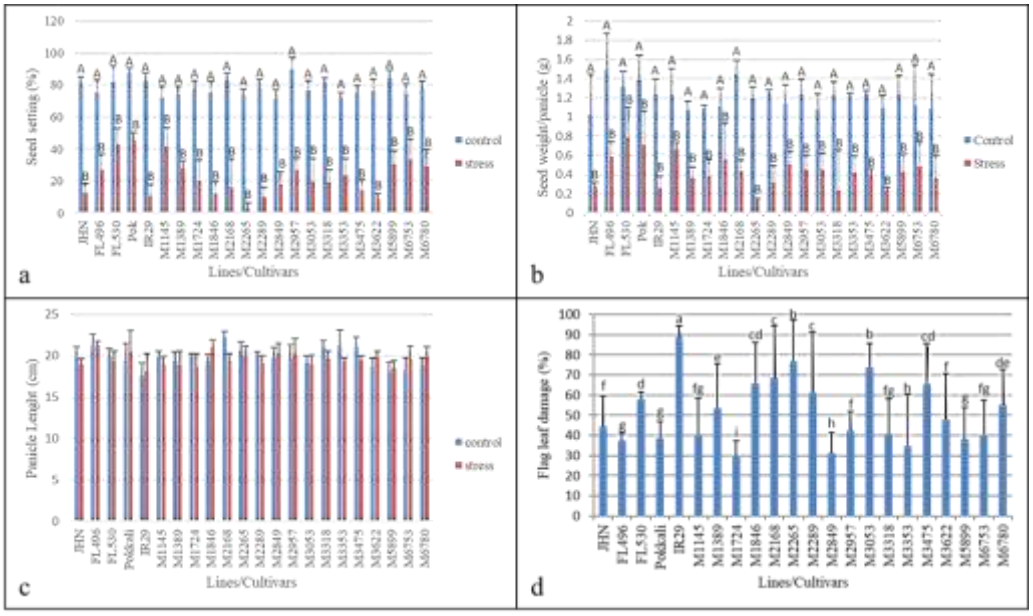

Figure 5. The repeated screening of 17 M5 mutant lines showed (a) seed setting, (b) seed weight/panicle, (c) panicle length and (d) percentage of flag leaf damage

Validation screening

The seeds (M_6) from four plants of M1145 (M_5) were treated with 150 mM NaCl at R2-R9 and compared with the JHN wild type, IR29, Pokkali and FL530 to confirm their potential for seed setting and any variations under salt stress at the reproductive stage. The results showed that M1145-1, -2, and -3 under salinity stress gave a seed set of 41.7, 41.7, and 48.3% ($P>0.05$), respectively, but M1145-4 showed a significant reduction in seed setting at 39.0% ($P<0.05$). While Pokkali, FL530, JHN and IR29 had 40.2, 31.9, 25.0, and 21.0% seed settings, respectively (Table 1). This suggests that M1145 still has some variation but should be confirmed to be a salt tolerance line similar to Pokkali (seed was not significantly different from M1145-1, -2 and -3) and M1145 gave

Table 1. Agronomic traits of four progeny lines (M_6) of M1145 compared with JHN, IR29, FL530 and Pokkali

Lines/Cultivar	Plant Height (cm)		Panicle Length (cm)		Seed Setting (%)		Pollen Viability (%)		Seed Weight/Panicle (g)		20-Seed Weight (g)	
	0 mM	**150 mM**	**0 mM**	**150 mM**	**0 mM**	**150 mM**	**0 mM**	**150 mM**	**0 mM**	**150 mM**	**0 mM**	**150 mM**
M1145-1	102.5aA	97.0aA	24.6aA	22.1bA	77.8bA	41.7cdB	79.0bcA	41.2dB	1.81bA	1.08eB	0.30bcA	0.30bcB
M1145-2	102.0aA	99.3aA	24.8aA	22.4bA	80.5abA	41.7cdB	89.2abA	33.1eB	2.09abA	1.15eB	0.37bA	0.31bcB
M1145-3	100.0aA	97.3aA	23.8abA	23.6abA	82.3aA	52.3cB	88.9abA	38.1deB	1.67cA	1.19eB	0.36bA	0.31bcB
M1145-4	101.3aA	98.5aA	23.7abA	23.5abA	77.2bA	39.2dB	88.6abA	35.4deB	1.75bcA	1.10eB	0.34bcA	0.30bcB
JHN	100.4aA	97.1aA	23.9abA	23.8abA	85.0aA	25.0efB	89.4abA	58.6bcB	1.80bA	1.30dB	0.36bA	0.32bcB
IR29	81.0bA	83.3bA	21.6cA	22.7bA	89.9aA	21.0fgB	92.0aA	34.8deB	1.86bA	0.79efB	0.33bcA	0.25dB
FL530	106.0aA	99.1aA	25.7aA	23.7abA	72.9cA	31.9eB	98.0abA	64.7bcB	2.85aA	1.32dB	0.43aA	0.41aB
Pokkali	105.5aA	100.2aA	22.5bA	21.9bcA	87.5aA	46.8cB	90.1abA	60.6bcB	2.10abA	1.31dB	0.40aA	0.37bB
CV	18.56		15.79		20.27		36.8		12.65		9.83	
F-test (0.05)	*		*		*		*		*		*	
Line x condition (0.05)	ns		ns		*		*		*		*	

seed set higher than FL530. However, the pollen viability of JHN (58.6%), Pokkali (60.6%) and FL530 (64.7%) was higher than M1145-1, -2 and -3 (41.2%, 33.1% and 38.1%), but the seed setting of JHN was low (25.0%) (Table 1). JHN showed the same result as some genotypes such as IR66946-3R-178-1-1 and IR65858-4B-11-1-2 that have been categorized as sensitive at the flowering stage due to reduced grain yield, although high pollen viability occurred (Reddy *et al*., 2014).

Moreover, this result was consistent with previous results showing that there was no correlation between pollen viability and seed setting in KDML105. This finding suggests that salt stress in the reproductive stage could affect pollen viability and anther non-dehiscence. Therefore, the fertilization process fails and cannot produce seeds on the panicle. However, susceptible check IR29 had affected to salinity stress both on pollen viability and seed set.

Discussion

The present study was conducted in two phases. First, it set the criteria for the salinity concentration and duration of treatment at the reproductive stage that are suitable, easy, cost-saving and accurate. Second, screening salt tolerant from a large mutant population by using phenotyping assessments under salt stress.

The suitable and stable screening method for a large population at the reproductive stage

This study grew rice plants in pots that rice plants were easily maintained from the seedling to the booting stage before treatment with NaCl. Then, the rice plants were placed in irrigated water with different NaCl levels. However, the NaCl from solution spread to the soil in the pot, and the final NaCl absorbed by the rice plants was 6.0 dS m^{-1} for 150 mM NaCl when soil was measured EC by waterproof EC tester (EC Testr11) (data not shown). Similar methods were disclosed to others research, which used levels of salinity from 2 dS m^{-1} to 6 dS m^{-1} to assure salt tolerance selection through screening mutant lines (Saleem *et al*., 2005; Singh, 2009; Stephen *et al*., 2002). Moreover, salinity stress at ECe 6.0 dS m^{-1} in the reproductive stage clearly affected spikelet fertility (Sen *et al*., 2017; Rad *et al*. 2012) than flag leaf damage or pollen viability. Thus, salinity with 150 mM and seed setting were used to screen the mutant population.

The damage to the flag leaf and panicle from salt stress was depended on the concentration of NaCl and the duration of stress. Rice plants control the

transport of salts initially via selective uptake by root cells, and the ions enter the root along with water through symplastic and apoplastic routes (Das *et al*., 2015). This means that NaCl can be translocated from the root to the top of rice plants and cause leaf damage (Zhang *et al*., 2015). However, Na^+ accumulation was comparatively less in saline-tolerant genotypes like Pokkali (Mondal *et al*., 2013). In addition, many studies have suggested that the top three leaves are mainly associated with contributions to grain yield (Yoshida, 1981). This means that the flag leaf is the closest source of panicles that significantly reduces the translocation of soluble sugars to spikelets and inhibits starch synthetase activity during grain development, which are the main reasons behind lower rice grain yield under salt stress (Abdullah *et al*., 2018). In this study, the assessment of NaCl content in leaf and panicle was not observed but the panicles of Pokkali, FL496, FL530 and KDML105 appeared normally while, the percentage of panicles damage in IR29 and JHN were increased dramatically in 27 days after treated with NaCl. This suggests that the tolerant cultivars have an osmotic mechanism to prevent NaCl transportation up to the panicle. In addition, the panicle length is not affected to salinity stress after treated with NaCl in R2 stage in all screening steps because the development of panicle already finished (Counce *et al*., 2000). However, salinity stress affected panicle development at panicle initiation (PI) and R1 stages. Then salinity in these stages could be affected panicle development and finally caused to reduce the panicle length. This research showed the same result as screening of heat stress at reproductive stage by using the same mutant population (Cheabu *et al*., 2019).

The final yield of grain is dependent on spikelet fertility and is severely affected by salinity, thus, seed setting decreased in the panicle due to salinity stress (Hasamuzzaman *et al*., 2009; Cui *et al*., 1995; Khan *et al*., 1997). The results of this study showed that the R2 stage is the most critical phase for salinity stress. The seed setting decreased dramatically due to pollen viability. It means that the few days before the pollen development process (gametophytic stage) are the most appropriate time to impose salt stress (Ahmadizadeh *et al*., 2017).

Thus, the salinity at early R2 (75 DAG) showed the lowest seed setting, while before early R2 (PI and R1) and late R2 to R3, the seed setting decreased slightly under salinity stress. On the other hand, salinity stress was not reduced in the seed setting after R4 stage (pollination). The mature pollen grains were not affected by prolonged periods of salinity stress, which proves the effect of salinity on the pollen grains depending on the developmental stage of the anthers (Sadeghirad *et al*., 2017). Thus, salinity stress after late R2 (complete pollen development) may not be severely spikelet fertility in rice.

Screening for salinity tolerant from large mutant population

In rice, a tremendous number of genotypes have been screened for salt tolerance. Some seedling evaluation methods have been used for mass screenings of seedlings at the International Rice Research Institute (Gregorio *et al*., 1997), while the lack of an effective evaluation method for salt tolerance in the screening process at the reproductive stage is one of the reasons for the limited success of conventional breeding. Therefore, the percentage of flag leaf damage, percentage of panicle damage, seed setting, pollen viability and seed weight were studied in relation with salt stress in the reproductive stage. The results showed that seed setting was easily observed in rice and can be used to identify salt tolerance in the reproductive stage. Furthermore, wide genotypic differences in relative salt tolerance can be used with spikelet fertility to identify the salt tolerant genotypes in the reproductive stage (Zeng *et al*., 2002). However, the use of visual damage as an evaluation for salt tolerance is not always applicable because symptoms such as chlorosis and leaf rolling are not always easily observed in rice at low or moderate salinity (Yeo *et al*., 1990).

The production of mutants to identify abiotic stress-tolerant mutants has been successful in some crop plants, including rice (Ahloowalia *et al*., 2004; Lee *et al*., 2003; Cheabu *et al*., 2019), and induced mutations and *in vitro* techniques have been employed to induce salt tolerance in Basmati rice (*Oryza sativa* L.) (Reddy *et al*., 2014). For the salt tolerance screening in this research, 9,000 M_4 lines were used in an initial screening under 150 mM NaCl from R2 to R9. Based on the seed setting, we selected 20 tolerant M_5 lines to confirm their potential for seed setting. The repeated screening showed different seed settings when compared with the first screening. It was clear from the first screening of germplasm for salinity tolerance at the reproductive stage in large populations of different crops did not account for factors such as interactions between cropping and genetic variation with in genotype (M_4) and lacked replication. Therefore, screening of salinity tolerance in breeding programs has been suggested in two steps: (1) screening salinity tolerance for large segregating populations under controlled conditions; and (2) testing salinity tolerance of promising lines from the first round screening. In this study, when M1145 was compared with the JHN wild type under salt stress, the seed setting of M1145 was higher than wild type, despite of the pollen viability of M1145 was less than JHN. When considering the anthers, it was found that the anthers of KDML were not opened, while the color of the anthers was still yellow (data not shown). It means that anther growth is not normal under saline conditions and some anthers are wrinkled and small under these conditions. The early destruction of the anther wall, a reduction in size of the pollen grains, and the

formation of pollen with abnormal shapes and properties prove that salinity stress reduces the yield by affecting the development of the male gametophyte of plants (Sadeghirad *et al*., 2017). In addition, M1145 showed the highest of seed set both in the repeated and validation screening and it showed a stable tolerance under salt stress at the reproductive stage in validation screening, similar to Pokkali in terms of seed setting and seed weight/panicle. The Pokkali has been found to be comparatively tolerant at the flowering stage due to better viability of pollen, moderate of seed set and grain yield (up to 40%) under salinity stress (Ren *et al*., 2005; Yousaf *et al*., 2004). It was confirmed that M1145 has been recognized for providing more salt tolerance when compared with Pokkali that has been frequently used as a donor of salt tolerance genotype in breeding programs.

Thus, M1145 is another choice of Thai salinity tolerant germplasm that can be used as a highly potential salt tolerant donor to improve the salt tolerance at the reproductive stage of future Thai rice varieties. However, M1145 should be investigated more details of genetic, physiology, biochemistry and morphology to further our understanding of the mechanism of salt tolerance.

Conclusion

The salinity condition used for screening the JHN mutant population was 150 mM NaCl (6.0 dS m^{-1}) from the early booting stage (R2) to physiological maturity (R9). The seed setting was the major parameter used to screen salinity tolerant lines at the reproductive stage. This parameter is easier to use in large screenings. However, flag leaf damage and pollen viability can also be used as minor parameters. The salinity tolerant line found in this study was M1145. This line produced a seed setting under salt stress similar to Pokkali and FL530. Therefore, M1145 will be investigated in more detail to determine the physiological mechanisms of salinity stress and identify genes or markers that are related to salinity tolerance. In addition, it can be used for breeding programs. Moreover, M1145 must also be tested in the field to confirm its potential for salt tolerance.

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