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Genetic variations associated with salt tolerance detected in mutants of KDML105 (*Oryza sativa* L. spp. *indica*) rice

Cattarin Theerawitaya[1], Kanokporn Triwitayakorn[2], Chalermpol Kirdmanee[3], Duncan R. Smith[2] and Kanyaratt Supaibulwatana[1,*]

[1]Department of Biotechnology, Faculty of Science, Mahidol University, Ratchathewi, Bangkok 10400, Thailand
[2]Institute of Molecular Biosciences, Mahidol University, Salaya, Nakorn Pathom 73170, Thailand
[3]National Center for Genetic Engineering and Biotechnology, 113 Phaholyothin Rd., Klong 1, Klong Luang, Pathumthani 12120, Thailand

***Corresponding author: scksp@mahidol.ac.th**

Abstract

Genetic variations in associate with salt tolerance were investigated in Khao Dawk Mali105 (KDML105), a well-known Thai rice that has high cooking quality and aroma. Forty-treatment combinations of gamma irradiation and ethyl methanesulfonate (EMS) were applied to M_0 seeds of KDML105 and micropropagated M_1 lines *in vitro* prior to cultivate in field for M_2 seeds. The M_2 seedlings were screened for salt tolerance in Hoagland solutions contained 171 mM NaCl or 342 mM NaCl. Salt tolerance (ST) lines could withstand for 15 days under salt stress up to 342 mM NaCl, whereas those of sensitive (SS) lines are drastically susceptible to condition of 171 mM NaCl after 5 days of screening. Identification of genetic variations among SS and ST populations was performed by Amplified Fragment Length Polymorphism (AFLP) using 56 primer combinations. A total of 889 polymorphic bands were detected and scored to investigate the degree of genetic change among 46 ST and 57 SS mutants. The polymorphism percentage was 92.2 and heterozygosity was 0.159 within mutant populations. The genetic distance between ST and SS populations was 0.0141, the mutants were then classified into 3 clusters as cluster I for 35 ST lines, cluster II for 37 SS lines and cluster III containing 12 ST and 11 SS lines. The 4 primers generated 5 novel fragments were obtained which gave more than 80% reliability in identifying 25.68 % of ST mutants gained form this study.

Keywords: AFLP; genetic distance; heterozygosity; jasmine rice; mutation; polymorphism.
Abbreviations: AFLP_ Amplified Fragment Length Polymorphism; EMS_ ethyl methanesulfonate; PGR_ plant growth regulator; SS_ salt sensitive mutant; ST_ salt tolerant mutant; TFPGA_ Tool for Population Genetic Analysis; UPGMA_ unweighed pair group method with arithmetical means.

Introduction

One of the most serious abiotic constraints for agricultural production is salt stress. High salinity areas increase yearly due to world environmental changes, pollution and inappropriate agricultural management. Cultivation areas of KDML105 rice (*Oryza sativa* L. sub. *indica* cv. KDML105), which is recognized worldwide as "Jasmine rice" a famous and high quality cultivar of Thailand, are mostly located in the northeastern part of Thailand, where soil salinity plays a significant role in decreasing grain productivity (Cooper et al., 1999). Improvement of rice lines to tolerate salinity is a complex subject since salt tolerance is controlled through several multigenic pathways such as the ion homeostasis, osmoregulation, antioxidant and hormonal systems (Ashraf and Akram, 2009; Vijayan 2009, Younis et al., 2009). Despite the complexity, the prospect of sustainably solving the salinity problem by the production of salt tolerant trait is particularly attractive. Although new salt tolerant lines have been achieved through conventional breeding, this approach is limited by the time consuming and labor intensive nature of the process which requires multiple crosses over several generations and intensive phenotypic selection (Peleman and van der Voort, 2003; Hwa and Yang, 2008). In addition, the lack of well characterized germplasm is a major obstacle to the success of rice breeding programs, and this issue is compounded when inbred lines are used (Pandey and Rajatasereekul, 1999; Senadhira et al., 2002). Therefore the creation of variation resources with precise changes at the genetic level is required to speed up and facilitate rice breeding programs for salt tolerance. Induced mutation using chemical and physical mutagens is frequently used as a tool to alter genes and to subsequently enhance the variation of rice genetic stock (Phanchaisri et al., 2007). Amplified Fragment Length Polymorphism (AFLP) is a DNA fingerprinting technique that has been used as a marker for agronomic traits of rice varieties (Zhu et al., 1998; Fuentes et al., 1999; Aggarwal et al., 2002; Zaka et al., 2004) or for QTL identification (Kepiro et al., 2008). AFLP is an excellent DNA fingerprinting technique to detect variation in mutants, with several advantages over other PCR-based techniques (Powell et al., 1996; Saini et al., 2004; Evaristo, 2008) that can be used to detect several mutation events with robustness and reducibility (Vos et al., 1995). As a consequence, when mutations occur at restriction sites, polymorphic patterns are generated and these regions can be marked as potential hotspots of mutation. Since AFLP can detect multiple polymorphic loci, which randomly occur at

restriction sites, it has a high potential to detect random mutations such as those occurring in response to γ-irradiation and ethyl methanesulfonate (EMS) treatment. In addition, AFLP produces more multiple polymorphic loci by a single primer pair than other marker systems (Powell et al., 1996; Saini et al., 2004, Lu et al., 2007; Evaristo, 2008) and therefore is a method of choice to detect random mutations occurring in the genome. AFLP has been reported for its potential to detect hotspots in mutant rice after exposure to the space environment (Li et al., 2007; Yu et al., 2007), the detection of genomic changes in transgenic rice produced by infecting calli (Labra et al., 2001), analysis of somaclonal variation in Arabidopsis regenerated plants (Polanco and Ruiz, 2002), micropropagated *Echinacea purpurea* (Chuang et al., 2009), rye (de la Puente et al., 2008), peach palm (Steinmacher et al., 2007) and detection of segregating populations derived from EMS generated mutants of chilli pepper (Lee et al., 2010). Therefore, AFLP analysis has considerable potential to be used as a tool to evaluate genetic differences among mutant lines. This study aimed to characterize the genetic variations in KDML105 mutants derived from gamma ray and EMS treatments and identify AFLP fragments specific to salt tolerance in the mutant population.

Results

Screening of SS and ST and analysis of polymorphism in mutant population

From 40 treatments of induced mutation, 370 lines of M_2 population were selected for salt tolerant screening. Under condition of 171 mM NaCl, 18.38 % of M_2 seedling was unable to survive, thereby they were characterized as SS. After 10 days of continuing treated with solution of 342 mM NaCl, 95 ST lines of M_2 were obtained, which was about 25.68% of total tested seedlings (data not shown). These ST seedlings could withstand in this high salt concentration and showed greenish leaves when compared with SS (Fig 1).

Genetic variation was analyzed using 56 primer pair combinations among original KDML105 and M_2 population consisting of 46 ST and 57 SS lines. A total of 898 polymorphic bands were identified with fragment sizes ranging from 80 to 800 bp. The AFLP bands were read and scored by presence or absence of the bands. The number of polymorphic fragments generated depended on the specific primer combination and varied from 3 (primer pair *Eco*-GAG/*Mse*-GGC) to 39 (primer pair *Eco*-GAC/*Mse*-GTT) with an average of 16.03 polymorphic bands per primer pair. To examine the genetic variation and the degree of polymorphism displayed by the mutants, genetic similarity was analyzed by the TFPGA program in terms of heterozygosity and percentage polymorphism (Table 1). The mean-unbiased heterozygosity according to Nei's (1978) of the SS population (0.1393) was slightly lower than that of the ST population (0.1530). Analysis of the percentage polymorphism in the individual ST and SS populations showed that the ST population was the most diverse with 88.64% polymorphism which was higher than that of the SS population (78.62%). The highest degree of polymorphism (92.2%) was observed in the population of all mutants.

Genetic clustering of mutants

To characterize genetic divergence and relatedness between control and mutants, the polymorphic AFLP data was clustered according to the UPGMA method (Fig 2). The control group was distinctively branched away from the ST and SS populations with a distance of 0.2208. The data showed that SS population was the most divergent from the control (0.2299) when compared to ST population (0.2116) as shown in Table 2. A remarkably small distance was seen between the ST and SS populations, with a distance of 0.0141 (Fig 2). Genetic characteristic of individual ST and SS lines reaffirmed their correlation on a tree plot neighbor joining tree matrix according Nei's 1978 distance (Fig 3). Amongst the mutant lines, the neighbor joining consensus tree was grouped into 3 distinct clusters. Thirty-five lines of ST mutants formed cluster I, while 37 lines of SS mutants belonged to cluster II. Cluster III was mixed, and contained 12 ST lines and 11 SS lines. The plot analysis of cophenetic correlation and similarity coefficient shows a goodness of fit with a very high similarity indices at r = 0.95951.

AFLP fragments specific to the ST population of KDML105

Amongst all of the polymorphic bands generated, five AFLP fragments from 4 primer pairs that showed 81-82 %reliability for salt tolerance were observed (Table 3). The AFLP fragments were between 123 and 254 bp in length and none of the bands were present in the parental KDML105 line. A dendogram generated according to the UPGMA method based upon the genotypic data obtained from the 5 fragments showed a distinct genetic distance between ST and SS of 0.3048 (Fig 4), which was more than 20 times higher than the genetic distance determined when all 898 polymorphic markers were included in the analysis.

Discussion

Rice is one of the most important agricultural crops in many countries of the world including Thailand which is the largest exporter of rice in the world (Ahloowalia et al., 2004; Hill, 2006). Production of the KDML105 is predominantly limited to the northeast of the country, where limitation is placed upon the available growing areas by increasing problems with salinity (Cooper et al., 1999). The KDML105 has been reported as moderate salt tolerance, it could tolerate < 200 mM NaCl salt (Prajuabmon et al., 2009). Nevertheless, the salinity stress occurred widely in rice cultivated areas and about 17% of total northeast area contains 240,000 and 590,000 ha of severe and moderate saline soils, respectively (Yuvaniyama et al., 2005). High salinity stress affected to its productivity, especially in dry condition, therefore selection of line with increased salt tolerance will increase the potential cultivatable area. In our experiment, high salt concentration (342 mM NaCl) was used to identify ST population which was higher than other existing reports. In this study, 103 mutant KDML105 lines, classified as either salt tolerant (ST) or salt sensitive (SS) were generated through induced mutagenesis with gamma-radiation and EMS treatment, and selected through their responses to saline stress. AFLP was used to investigate the genetic relatedness amongst the various lines. A total of 56 combinations of primer pairs produced 898 polymorphic fragments which were used to determine the genetic distance between the ST and SS populations. A very high value of polymorphic variation in either the ST (88.64%) or SS (78.62%) populations confirmed the randomness of the mutations occurring amongst the mutants. In addition, the higher polymorphic variation detected in the ST population suggests a greater number hot spots in the ST plants as compared to the SS plants. The polymorphic value in mutants was essentially similar that determined for rice expose to space which was

Table 1. Analysis of genetic variation as assessed by AFLP analysis.

Population	Heterozygosity [a]	% polymorphism [b]
All	0.1590	92.20
Within salt tolerance	0.1530	88.64
Within salt sensitive	0.1393	78.62

[a] Nei's 1978 unbiased heterozygosity [b] 99% criterion

Fig 1. Characteristics of M_2 seedling after culture in half-strength Hoagland solution supplemented with 342 mM NaCl for 10 days (EC > 9.9 $dS.m^{-1}$). Representative salt sensitive, S18, (left) and salt tolerant, T33 (right) plants are shown.

75- 85% (Li et al., 2007). The lower value of heterozygosity in the SS population indicates a greater similarity of genetic background. When compared to previous research that tested somaclonal variation in *Echinacea purpurea*, an out-crossing plant, somaclonal mutants showed higher distances of up to 0.9 (Chuang et al., 2009). The lower heterozygosity value observed among all populations in this study probably reflects the low genetic variation in the KDML105 germplasm, which is a consequence of KDML105 being a self-fertilizing crop, even after induced-mutation (Miller, 1997). A low genetic distance between the ST and SS populations (0.0141) confirmed the relatedness amongst the 46 ST and 57 SS lines (Table 2 and Fig 2). The mutants were clustered into 3 different groups containing different salt tolerance characteristics, and the relationship between genetic changes and phenotypic characteristics was shown in the consensus tree. Cluster 1 representing 35 ST lines was the most divergent group from cluster 3 which contained both salt tolerant and salt sensitive lines. Although cluster 3 contained different phenotypic characteristics, the tree implies closeness in the degree of genetic change between the ST and SS mutants among members in the group, implying that alteration of the same genes may lead to sensitivity or tolerance, rather than distinct genes controlling each trait. However, the distance between the SS and ST plants changed from 0.0141 to 0.3085 when only five sets of polymorphic data obtained from the E-GCC-M-GGC, E-GAC-M-GAC, E-GAT-M-GAT and E-GTG-M-GTG primers was used in the analysis. The genetic distance between the ST and SS plants was thus 20 times greater than the distance calculated from the full set of 898 polymorphic markers. Significantly, these fragments largely segregated with salt tolerance, giving >80% reliability in evaluating salt tolerance, suggesting that they are good candidates for further development as trait specific markers (Mba et al., 2007; Peters et al., 2003).

Materials and methods

Induction of mutant and identification of ST and SS populations

Seeds of KDML105 (provided by Pathumthani Rice Research Center, Rice Research Institute, Department of Agriculture, Ministry of Agriculture and Cooperative, Thailand) were irradiated with 8 doses of gamma ray (0 - 500 Gy) generated by ^{60}Co-gamma radiation at rate 6000 Gy h^{-1} (Gammabeam-650®, Office of Atomic for Peace, Ministry of Science and Technology, Thailand). Irradiated seeds were dehusked and surface sterilized by 0.3 % sodium hypochlorite before rinsed and subsequently immersed for 60 min in 5 concentrations of EMS solutions (0 - 2 mL L^{-1}). Forty-treatment combinations of gamma ray and EMS were performed with 10 seeds (replication) per treatment. They were then washed with sterile distilled water and germinated on PGR-free MS (Murashige and Skoog, 1962) agar medium containing 3 % (w/v) sucrose. Seeds were germinated under 25±2 °C, 60±5 %RH, 60±5 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPF) with 16 h d^{-1} photoperiod. Each M_1 seedlings were micropropagated for 3 replications of cloned plantlet before acclimated and transplanted into experimental field until setting M_2 seeds. About 3-4 seeds per panicle of each individual plant (30 plants per treatment) were sampling

Table 2. Genetic distance among KDML105, salt-sensitive and salt-tolerant mutants as assessed by AFLP analysis.

	KDML105	Salt tolerance	Salt sensitive
KDML105	-	0.2116	0.2299
Salt tolerance		-	0.0141
Salt sensitive			-

Table 3. Primers producing AFLP fragments segregating within salt tolerance.

Primer combination	Fragment size (bp)	%reliability	*p*-value
E-GCC-M-GGC	254	82.52	0.0000
E-GAC-M-GAC	165	82.29	0.0000
E-GAT-M-GAT	135	81.55	0.0000
E-GTG-M-GTG	123	81.55	0.0000
	125	81.55	0.0000

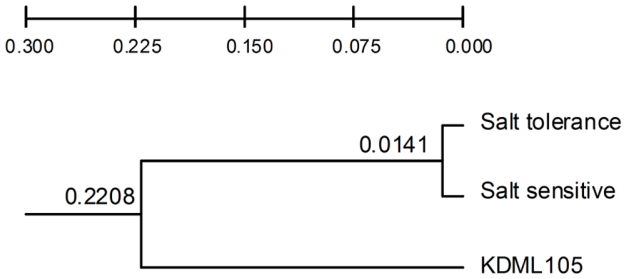

Fig 2. Dendrogram showing genetic distance amongst KDML105, salt-sensitive and salt-tolerant mutants according to the UPGMA method by application of 889 polymorphic data.

and pooled together within same treatment line for total 100 seeds per line, ten representative seeds of each M_2 line were sampling and subsequently germinated to derive M_2 plants. Screening of salt sensitive (SS) and salt tolerance (ST) in KDML105 rice was previously suggested by Theerawitaya et al. (2009). Briefly, after 13 days of germination in Hoagland solution (Macblis and Torrey, 1956), the M_2 seedlings were transferred to Hydro-set (Hygreen Hydroponics Farm, Thailand) feeding with half-strength Hoagland solution and acclimated for 4 days prior to identify the susceptible SS lines under salt stress condition at 171 mM NaCl for 5 days. The survivals were further treated with 342 mM NaCl-containing solution for 10 days; the seedlings that withstand in this condition were classified as ST.

DNA extraction and AFLP analysis

Total DNA from leaf samples of SS and ST populations were extracted using the DNeasy® Plant Mini Kit (Qiagen, Germany). AFLP analysis was modified from method described by Vos et al. (1995), a total of 500 ng of genomic DNA was double digested with 10 U *Mse*I (New England BioLabs, Beverly, MA), followed by 12 U *Eco*RI (Promega, Madison, WI) in a final volume of 30 µL. The digested-ligated DNA samples were pre-amplified with two primers which are complementary to *Eco*RI adapter (5'-CTCGTAGACTGCGTACC-3' and 3'-CTGACGCATGG-TTAA-5') and *Mse*I adapter (5'-GACGATGAGTCCTGAG-3' and 3'-TACTCAGGACTCAT -5'). The primers used in the pre-amplification were *Eco*RI (5'-GACTGCGTACCA-ATTCG-3') and *Mse*I (5'-GATGAGTCCTGAGTAAG-3'). The pre-amplification products were diluted 20 fold in sterile distilled water, and 1 µL was used for selective amplification with the *Eco*RI primer (5'-GACTGCGTACCAATTCGNN-3') and *Mse*I primer (5'-GATGAGTCCTGAGTAAGNN-3') containing an additional two selective nucleotides at the 3' end (*Eco*RI+NN and *Mse*I+NN, where N refers to either A, C, G or T). The PCR products were analyzed on 5% denaturing polyacrylamide gels that run at constant voltage (1100 V) for 2.5 h. DNA bands were visualized by the silver staining method as described by Benbouza et al. (2006). The AFLP fingerprints were then scored for polymorphisms, the presence or absence of the fragments comparing between ST and SS lines were analyzed.

Genetic clusters and genetic distance of mutant lines

Data matrices were scored as binary data, which was represented as presence (1) or absence (0) of bands. By using the Tool for Population Genetic Analysis (TFPGA) 1.3 software (Miller, 1997), genetic distances among control (KDML105) and mutants (ST and SS) were calculated according to Nei's 1978 coefficient. A total of 898 polymorphic bands were clustered and analyzed by the Unweighed Pair Group Method with Arithmetical Means (UPGMA) method in order to generate a dendrogram comparing between KDML105 and its mutants. To identify genetic variation of individual mutant, a neighbor joining consensus tree of ST and SS populations was reaffirmed by NTSYS version 2.02 (Rolf, 1997). A plot analysis of cophenetic correlation and similarity coefficients was additionally calculated to test goodness of fit and robustness of the tree. In the meantime, to identify AFLP fragments specific to ST, %reliability was used as a determining criterion. The genotypic data from ST and SS lines was analyzed by an independent sample *t*-test using the SPSS program (version 10.0). The significant data that showed $p \leq 0.05$ was consequently used to calculate percentage of reliability by following equation;

$$\text{Reliability } \% = \frac{(\text{No. of bands present in ST} + \text{No. of bands absent in SS}) \times 100}{\text{No. of total samples}}$$

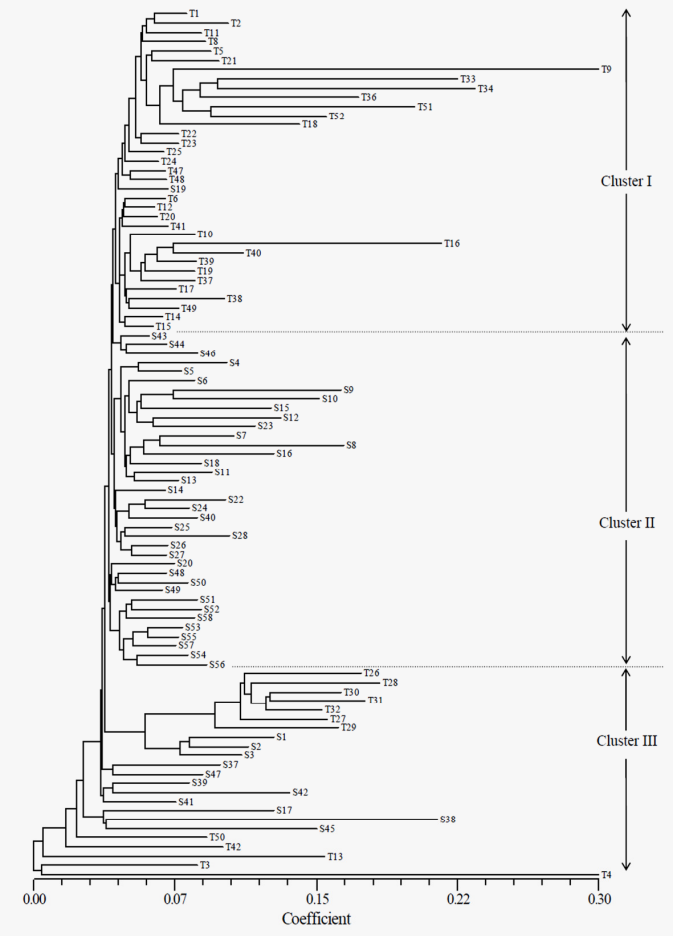

Fig 3. A neighbor joining consensus tree of individual salt tolerance (ST) and salt sensitive (SS), where T represents for tolerant and S represents for sensitive samples.

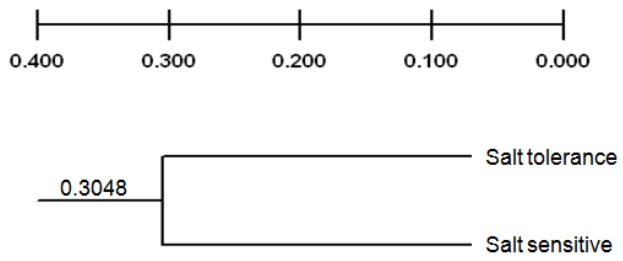

Fig 4. Dendrogram showing genetic distance between salt-sensitive and salt-tolerant mutants by application of polymorphic data derived from E16M8, E4M4, E3M3 and E10M10 primers.

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