5_Artikel_Biodiversitas_Pak_Yogi.

by Suprayogi Suprayogi

Submission date: 03-Mar-2023 12:21PM (UTC+0700)
Submission ID: 2027721585
File name: 5_Artikel_Biodiversitas_Pak_Yogi.pdf (447.74K)
Word count: 5087
Character count: 26390

BIODIVERSITAS
Volume 23, Number 9, September 2022
Pages: 4517-4523

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d230918

Marker validation for salt tolerance in Indica rice

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Manuscript received: 31 May 2022. Revision accepted: 30 August 2022.

Abstract. *Suprayogi, Dewi PS, Oktaviani, E, Aisya AW. 2022. Marker validation for salt tolerance in Indica rice. Biodiversitas 23: 4517-4523.* The development of salt-tolerant rice variety is of importance with concern to the use of the coastal area for rice production and, consequently, the development of a rapid and accurate method of variety selection. Molecular markers associated with Quantitative Trait Loci (QTL) for salt tolerance in rice have been reported elsewhere. This research aimed to identify molecular markers associated with salt tolerance in rice. The research was carried out from September 2020 to February 2021 at Plant Breeding and Biotechnology Laboratory, Faculty of Agriculture, Jenderal Soedirman University, Indonesia. Nine Simple Sequence Repeat (SSR) markers and one Sequence Tagged Site (STS) marker were evaluated for their association with salt tolerance. The rice varieties consisted of three salt-tolerant varieties, one salt-sensitive variety, and six commercial varieties. Nona Bokra PCR amplicon was used as a positive control for DNA band scoring. Binary data of the amplicon banding pattern were analyzed using Principal Component Analysis (PCA) to determine the grouping of the evaluated varieties about their tolerance to assigned markers. The result demonstrated that the evaluated rice varieties fell into four groups of marker resemblances. Among the evaluated SSR markers, RM 3412, RM 342, RM 8094, and Wn 11463 significantly contributed to the grouping. The present study confirmed that Wn 11463 is a strong marker for salt tolerance in rice. However, validation needs to be made on a broader range of genotypes to see the significance of RM 8094 and RM 3412 as salt tolerant markers.

Keywords: Molecular marker, Principal Component Analysis, salt tolerance, SSR, STS

INTRODUCTION

Rice is a major staple food crop of most Indonesian people. However, the effort to maintain national self-sufficiency in rice production has been challenged by the decreasing area of arable paddy fields due to land conversion to non-agriculture purposes (Suprayogi et al. 2019), which is estimated not less than 38.000 ha year^{-1} (Nafisah et al. 2017). To date, the rice production area should be extended to marginal land that includes coastal areas, which during the dry season, are affected by seawater intrusion resulting in saline conditions. Salinity represents any unfavorable condition of excessive salt accumulation (Sunusi et al. 2018). Salt stress promotes osmotic stress, ionic toxication, and nutrient deficiencies. Rice plants grown in saline condition experience growth retardant expressed in inhibition of root growth, plant height, number of tillers, promotes infertile spikelets, and ultimately, reduced yield (Qin et al. 2020). Among the solution to the salinity problem of rice production in the coastal areas is using salt-tolerant rice varieties (Nafisah et al. 2017; Suprayogi et al. 2019).

Breeding for salt-tolerant rice variety has been hindered by the limited availability of salt-tolerant germplasm, limited information on the genetic control of salt tolerance, and a lack of information on the relation between salt tolerance traits and specific agronomic characters (Sunusi et al. 2018). Slow progress in the breeding of salt-tolerant rice is also due to inadequate information on salt tolerance mechanisms, less information on screening techniques, inefficient selection techniques, and the complexity of effects of genetic x environment interactions (Lang et al. 2017).

Salt tolerance in rice is controlled by many genes (Ismail and Horie 2017). Recent development in molecular marker analysis has enabled rapid and accurate identification of individual genes or otherwise Quantitative Trait Loci (QTL) controlling a trait of interest. QTL mapping approach has been used to describe the genetic architecture of salt tolerance in rice using a mapping population derived from the cross of salt sensitive and salt tolerant varieties (Cheng et al. 2015; Liu et al. 2019; Nakhla et al. 2021). Some of the major salt tolerance QTLs included *qSKC-1* and *qSNC-7*. These QTLs are responsible for the regulation of K^+/Na^+ homeostasis under salt stress, which explains phenotypic variance of 48.5% and 40.1%, respectively; *qSKC-1* encodes a major QTL for Shoot K^+ concentration and qSNC-7 for shoot Na+ concentration; *Saltol* which is responsible for maintaining low Na+, high K+, shoot Na^+/K^+ homeostasis; *qSE3* encodes the gene for K^+ transport; *OsHAK21* encodes gene responsible for seed germination and seedling establishment under salt stress, *qST1* and *qST3* conferred salt tolerance at the young seedling stage (Qin et al. 2020). *qST1.1* was reported to be responsible for salt tolerance in SR86 and explained a phenotypic variance of 62.6% (Wu et al. 2020)

Simple Sequence Repeat (SSR, microsatellite) marker has been used to identify salt tolerance or introgress genes to develop new cultivars (Mizan et al. 2015). Using SSR markers RM 3412, RM 510, and RM 336, Islam et al.

(2015) identified five salt tolerant varieties out of the 25 evaluated varieties. Ali et al. (2014) identified four salt tolerant varieties out of 33 varieties using morphological markers and SSR markers RM 8046, RM 336, and RM 8094. Emon et al. (2015) Reported STS (Sequence Tagged Sites) marker Wn 11463 in marker-assisted selection (MAS) for rice with salt tolerance gene *SKC1*. Using 17 SSR markers, including a significantly associated salinity tolerance marker Wn11463 (https://archive.gramene.org/markers/microsat/all-ssr.html), saline tolerant variety Inpari Unsoed 79 Agritan could be separated distantly from the susceptible variety IR 29 (Suprayogi et al. 2021).

This study aimed at identifying and validating molecular markers for salt tolerance in several commercial indica rice varieties. The validated markers will later be used for rapid and accurate screening techniques for salt-tolerant rice genotypes.

MATERIALS AND METHODS

Materials

Eleven indica rice varieties were used as DNA sources. These varieties included salt-tolerant rice varieties Nona Bokra, Inpari Unsoed 79 Agritan, Atomita 2; a salt-sensitive rice variety IR 29; and common commercial rice varieties Cisadane, Dendang, Inpago Unsoed 1, Lambur, Pelopor, Siak Raya, Unsoed Parimas. Alluvial soils, organic manure, and rice husk were used as growth media. The chemicals used for markers analysis were Cetyl Trimethyl Ammonium Bromide (Amresco), NaCl (Merck), HCl, Tris base (Merck), Na EDTA, NaOH, polyvinyl Pyrrolidine (Amresco), β-Mercapto-ethanol (Merck), aquades (H_2O), liquid nitrogen, chloroform (Merck), ammonium acetate (Merck), isopropanol (Merck), ethanol 70%, ethanol 96% (Merck), RNAse (Geneaid), nuclease-free water (Thermo Fisher Scientific), agarose, TBE (Tris-Boric-EDTA) 1x, DNA ladder 100 bp (Bioline), loading dye (Thermo Fisher Scientific), ethidium bromide (Invitrogen), 10 primer pairs (Table 1), and Dream Taq PCR Master Mix (Thermo Fisher Scientific).

Methods

DNA extraction and quantification

DNA extraction was carried out using CTAB method with modification (Zannati et al. 2015). Leaves samples of two-week-old seedlings were ground using a mortar and pestle in the presence of liquid nitrogen. Powdery leaves sample was put into a 2 mL Eppendorf tube, added with 1000 µL of CTAB Buffer mix, and homogenized by vortexing for 5 seconds, followed by incubation in a 65ºC water bath and homogenized carefully every 10 minutes. Chloroform of 500 µL was added to the tube and centrifuged for 7 minutes. Next, the supernatant was transferred to a 1.5 mL Eppendorf tube and added with 500 µL chloroform, followed by centrifugation for 7 minutes. This step was repeated twice to allow better-quality DNA. Next, the supernatant was transferred to a new 1.5 mL Eppendorf tube. Ammonium acetate (0.8 µlL1 µL) and cold isopropanol (5,4µL/10µL) were then added, followed by homogenization. The tubes were stored in the refrigerator at 4ºC for 24 hours. After overnight precipitation, the tube was allowed to stand at room temperature. After 7 minutes of centrifugation, the supernatant was discarded, and the DNA pellet was allowed to settle in the bottom of the tube. The DNA pellet was then washed with 70% Ethanol (700 µL) and precipitated by centrifugation for one minute. The supernatant was discarded, and the pellet was allowed to dry. The pellet DNA was then washed with 90% ethanol (700 µL), centrifuged for 1 minute, and let the pellet dry. Tris-EDTA (TE) buffer (30 µL) and RNAse (1 µL) were added to dilute the DNA pellet. The DNA solution was then incubated at 37°C for 1 hour. The DNA was stored in a -20ºC freezer to keep the DNA from degradation.

The concentration and purity of DNA were measured using a nanophotometer. The DNA was diluted 100x using Tris-EDTA (TE) as a solvent. A total of 3 µL of diluted DNA was used to determine the purity and concentration of the DNA.

Marker amplification

Nine SSR markers and one STS marker Wn 11463 (Table 1) were evaluated for their possible association with salt tolerance.

A total volume of 25 μL PCR-mix solution was used for PCR reaction. The PCR mix comprised 1 μL (50 ng) DNA samples. 1 μL forward primer, 1 μL reverse primer, 12.5 μL Dream Taq PCR Master-Mix, and 9.5 μL nuclease-free water. The PCR followed the program as seen in Table 2.

Amplicon visualization

The DNA quality and amplicon visualization was checked by Agarose Gel electrophoresis. First, the DNA ladder was loaded in the first well. Meanwhile, 3 μL of PCR product mixed with 2 μL of loading dye was loaded into each well. Electrophoresis was carried out at 50 volts for 30 minutes. For DNA visualization, the electrophoretic gel was immersed in EtBr for 20 minutes, then seen under UV-transilluminator.

Data analysis

Polymorphic Information Content (PIC) value was used to evaluate each genetic marker based on the amplified DNA bands. The PIC value is obtained by calculating the number of amplified alleles using the application on https://gene-calc.pl/pic. The value of PIC was grouped into three classes, namely: PIC>0.5: highly informative, 0.25>PIC>0.5: moderately informative, and PIC<0.25: poorly informative (Carsono et al. 2014). PCR amplicon (DNA bands with different molecular weights) was converted into binary data by scoring. Notation 1 was given to DNA bands with similar molecular weight to Nona Bokra, 0 was given to DNA bands that are not similar to the molecular weight of Nona Bokra. The binary data were then used for PCA analyses using XLSTAT 2020 to group the evaluated varieties (Vidal et al. 2020).

Table 1. List of primers used in this study

Classi-fication	Primer name		Nucleotide sequences	Size of amplicon (bp)	Ta (°C)	Traits related	Literature review	Source of information
SSR	RM 212	Forward	CCACTTTCAGCTACTACCAG	136	53.5	Drought tolerance	Roychowdhury et al. (2013)	archive.gramene.org
		Reverse	CACCCATTTGTCTCTCATTATG					
SSR	RM 8264	Forward	ACGCTCCTCGCTTTCTAC	206	55.5	Agronomic traits (total of secondary branch and *seed-set ratio)*	Kebriyaee et al. (2012); Ma et al. (2019)	archive.gramene.org
		Reverse	GCACCTCACACCAGTAATTC					
SSR	RM 342	Forward	CCATCCTCCTACTTCAATGAAG	141	58	Aromatic and drought tolerance	Shams et al. (2012); Iqbal et al. (2015)	archive.gramene.org
		Reverse	ACTATGCAGTGGTGTCACCC					
SSR	RM 225	Forward	TGCCCATATGGTCTGGATG	140	53	Length of grain	Khatibani et al. (2019)	archive.gramene.org
		Reverse	GAAAGTGGATCAGGAAGGC					
SSR	RM 222	Forward	CTTAAATGGGCCACATGCG	213	56.3	Agronomic trait (numbers of tillers)	Chaudari (2018)	archive.gramene.org
		Reverse	CAAAGCTTCCGGCCAAAAG					
SSR	RM 224	Forward	ATCGATCGATCTTCACGAGG	157	56.3	Blast disease	Zarbafi et al. (2019)	archive.gramene.org
		Reverse	TGCTATAAAAGGCATTCGGG					
SSR	RM 223	Forward	GAGTGAGCTTGGGCTGAAAC	165	55	Not linked to specific trait	Supari et al. (2015)	archive.gramene.org
		Reverse	GAAGGCAAGTCTTGGCACTG					
SSR	RM 8094	Forward	AAGTTTGTACACATCGTATACA	209	55	Salinity tolerance	Ali et al. (2014)	archive.gramene.org
		Reverse	CGCGACCAGTACTACTACTA					
SSR	RM 3412	Forward	AAAGCAGGTTTTCCTCCTCC	211	55	Salinity tolerance	Saha et al. (2016)	archive.gramene.org
		Reverse	CCCATGTGCAATGTGTCTTC					
STS	Wn 11463	Forward	TCCTCCTTCTCTCGCAAC	120	54.9	Salinity tolerance	Emon et al. (2015)	Emon et al. (2015)
		Reverse	GATCCACTCGTCACAGG					

Table 2. PCR program

Stage	Temp. (°C)	Time (minutes)	Cycle
Pre-denaturation	95	5	1
Denaturation	95	1	35
Annealing	53.5-58	1	35
Extension	72	1	35
Final-extension	72	5	1
Refrigeration	4	∞	

RESULTS AND DISCUSSION

Polymorphic Information Content (PIC) value

PIC value describes the level of informativeness of a marker. A marker with a high PIC value is recommended for molecular screening as this marker could better differentiate different genotypes. PIC analysis on the amplified alleles (Figure 1) shows that the PIC value of evaluated markers ranged from 0.1516 to 0.6781 (Table 3). Followings Carsono et al. (2014), the evaluated markers could be grouped into three classes, i.e.: RM 223, RM 3412, RM 342 as highly informative markers, RM 212, RM 222, RM 223, RM 224, RM 225, RM 8264, Wn 11463 as moderately informative markers, and RM 8094 is poorly informative marker.

PCA Biplot analysis of degree of resemblance

Analysis of the degree of resemblance based on ten molecular markers was carried out using PCA Biplot analysis. Varieties are considered to have similar characteristics when they are at two adjacent points. The closer the distance between two points, the higher the similarity between two investigated varieties based on a marker. Figure 2 shows the result of Biplot analysis in which the evaluated varieties fell into four quadrants. Cisadane, Unsoed Parimas, and Siak Raya are in one quadrant group characterized by RM 224. No information on salt tolerance has been reported for these three varieties. Cisadane is a lowland rice variety that does not have resistance to any environmental stress conditions. Unsoed Parimas is a drought and aluminum-tolerant variety, which is also resistant to *Pirycularia oryzae* race 0733 and moderately resistant to *P. oryzae* race 133 (Agricultural Research and Development Agency 2019). Siak Raya is a tidal swamp rice variety that is tolerant Fe and Al (Dirgasan et al. 2019). Siak Raya is a moderately tolerant variety to salinity stress at the seedling stage (Safitri et al. 2017).

Lambur, Inpago Unsoed 1, and Nona Bokra varieties were in one group and marked by RM 222, RM 342, RM 3412, RM 8264, and RM 8094. Among these SSR markers, RM 8094 was reported to be linked with salt tolerance (Ali et al. 2014). This result may suggest that Lambur and Inpago Unsoed may also be tolerant to salinity stress. Lambur is somewhat tolerant to salt, Al, and Fe. Inpago Unsoed 1 is drought tolerant and moderately tolerant to iron (Fe) toxicity (Agricultural Research and Development Agency 2019).

Atomita 2 is in the same group as Inpari Unsoed 79 Agritan, marked by RM 223, RM 212, RM 225, and Wn 1146. Inpari Unsoed 79 Agritan is known to be tolerant to salt stress of 12 dSm^{-1} at the seedling stage (Prasetia et al.

2022). Atomita 2 is a gamma-ray mutant rice variety known to be tolerant to the salinity of 4-6 mhos/cm (Dewi et al. 2020). The similarity in the characteristics of these two varieties is also due to the fact that Atomita 2 is the parent of the salt-tolerant donor for Inpari Unsoed 79 Agritan. Wn 11463 marker was closely linked to the *SKC1* salt tolerance gene (Emon et al. 2015). Atomita and Inpari Unsoed 79 Agritan is also in the same quadrant group with STS marker Wn 11463.

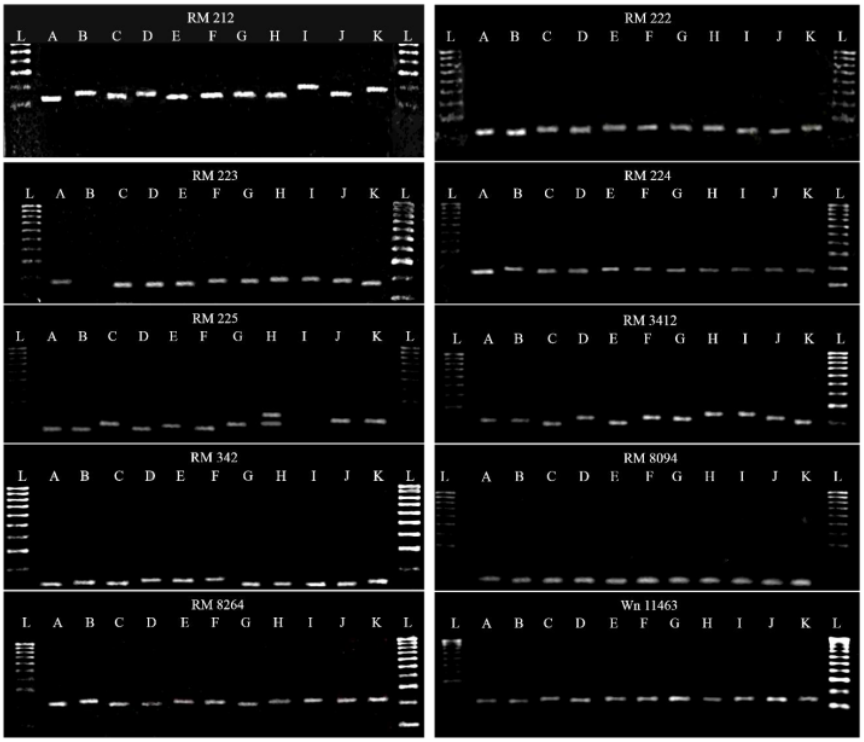

Figure 1. DNA band in 2% gel agarose. A: Nona Bokra, B: Inpari Unsoed 79 Agritan, C: Cisadane, D: Atomita 2, E: Pelopor, F: Dendang, G: Lambur, H: Siak Raya, I: Unsoed Parimas, J: Inpago Unsoed 1, K: IR 29, L: Ladder 100 bp

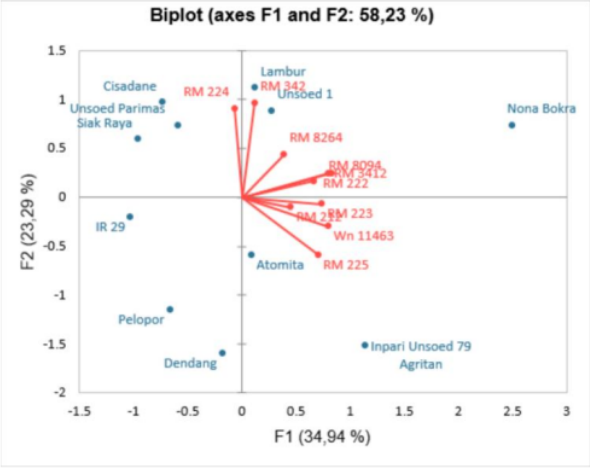

Figure 2. Varietal grouping based on PCA Biplot analysis

Table 3. Chromosome location, number of alleles, and PIC of the evaluated markers

Marker	Chromosome location	Number of alleles	PIC
RM 212	1	3	0.4732
RM 222	10	2	0.3557
RM 223	8	3	0.5632
RM 224	11	2	0.318
RM 225	6	3	0.4762
RM 3412	1	4	0.6781
RM 342	8	3	0.5262
RM 8094	1	2	0.1516
RM 8264	8	2	0.3557
Wn 11463	1	2	0.318

Table 4. Contribution of the marker in the grouping of varieties (%)

Marker	1st Dimension (F1)	2nd Dimension (F2)
RM 212	5.384	0.398
RM 222	11.443	1.110
RM 223	14.337	0.186
RM 224	0.107	32.463
RM 225	12.924	13.511
RM 3412	16.813	2.346
RM 342	0.387	36.585
RM 8094	17.944	2.342
RM 8264	4.025	7.695
Wn 11463	16.635	3.362

IR 29, Pelopor, and Dendang were in the same quadrant with no marker. Interestingly, PCA Biplot analysis placed IR 29, Pelopor, and Dendang in the opposite quadrant of Nona Bokra. IR 29 is a salt-sensitive variety (Ferreira et al. 2015), and consequently, Pelopor and Dendang, as being in the same quadrant, could be considered salt sensitive. Pelopor was previously reported as a salt-sensitive variety (Zannati et al. 2015). Dendang, however, is tidal swamp rice that is reported to be quite tolerant to Fe, Al, and salinity (Suprayogi et al. 2019). The disagreement between our result and that of (Zannati et al. 2015) on the grouping of Dendang still needs to be investigated further using more salt tolerance markers published elsewhere.

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Grouping of the evaluated varieties, as shown in the PCA Biplot demonstrates that markers used in this study could differentiate salt tolerant varieties from the sensitive ones. This is because each marker relates to a different variety in a different quadrant (Figure 2). Table 2 shows that in the F1 (first) dimension, RM 8094 markers contributed the highest in the grouping of varieties, followed by RM 3412, and Wn 11463. Meanwhile, in F2 (second) Dimension, RM 342 had the highest contribution, followed by RM 224.

Analysis of contributing markers to the grouping of varieties (Table 4) and PCA Biplot (Figure 2) demonstrated that RM 8094, RM 3412, Wn 11463, and RM 342 significantly related to salt-tolerant varieties of Nona Bokra, Atomita-2, and Inpari Unsoed 79 Agritan. This result follows the previous report (Emon et al. 2015). RM 3412 and RM 8094 were linked to Saltol QTL, located on chromosome 1 (Geetha et al. 2017). The main genes for salt tolerance traits (*Saltol*) are mapped on chromosomes 1 and 8 (Lang et al. 2017). The STS marker Wn 11463 is significantly linked to the salt-tolerant gene, *SKC 1* (Emon et al. 2015). The RM 342 marker determines salinity tolerance at the reproductive stage and is located on chromosome 8 (Iqbal et al. 2015). The salt tolerance gene on chromosome 8 controls tolerance at the vegetative phase at EC (electrical conductivity) below 10 dSm^{-1} (Lang et al. 2017).

This study also found that the drought-tolerant rice variety Inpago Unsoed 1 fell in the same quadrant as the salt-tolerant variety Nona Bokra (Figure 2). This may suggest that drought tolerance and salt tolerance share some common mechanisms in the plant. This argument is based on (Li et al. 2013) that *DST* gene of salt tolerance also relates to drought tolerance. This gene is located on chromosome 3 and controls stomata's movement during drought and high salt conditions through the regulation of genes involved in ROS (Reactive Oxygen Species) homeostasis (Ar-Rafi et al. 2017).

Apart from marker-based genotypes grouping, validation was carried out for the salt tolerance markers Wn 11463 (Emon et al. 2015), RM 8094, and RM 3412 (Lang et al. 2017). In practice, the marker validation could be made by observing the presence/absence of specifically related salt tolerance marker bands in the evaluated genotypes. However, such a method could only be applied for the validated dominant-type molecular marker or a specific gene sequence. Therefore, for co-dominant-type markers, such as STS marker Wn 11463, and SSR markers RM 8094 and RM 3412, validation should be based on molecular size and its consistency across salt tolerant genotypes.

In the present study, polymorphic bands of Wn 11463 with consistent molecular size could be seen across the reference tolerant genotypes Nona Bokra, Inpari Unsoed 79 Agritan, Atomita-2, Dendang, Lambur, and Siak Raya (Figure 1). This result confirms that Wn 11463 is a strong rice salt tolerance marker (Emon et al. 2015). RM 8094 was polymorphic, but the tolerant genotypes did not necessarily have the same molecular size. RM 3412 produced 3 (three) polymorphic bands (Table 3) with inconsistent molecular size across salt tolerant genotypes. In contrast with Lang et al. (2017), the present study

suggests that validation needs to be made on a broader range of genotypes to see the significance of RM 8094 and RM 3412 as salt tolerant markers.

ACKNOWLEDGEMENTS

Jenderal Soedirman University Grant funds this research through Development Research Scheme 2020 with contract number T/366/UN23.18/PT.01.03/2020. The author thanks the Head of the Institute of Research and Social Extension for the funding support for this research, Dean of Agriculture faculty for permission to use lab facilities, and Alwa Widi Aisya for her assistance in the molecular works.

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