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QTL FOR ALUMINUM TOLERANCE ON RICE CHROMOSOME 3 BASED ON ROOT LENGTH CHARACTERS

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SUMMARY

The association between aluminum (Al) tolerance trait and quantitative trait loci (QTL) on chromosome 3 needs further research. Our study aimed to identify QTL for Al tolerance trait based on root length characters under Al stress in a rice F9 recombinant inbred lines (RILs) population derived from a cross between IR64 x Hawara Bunar. The mapping population consisted of 256 RILs. Genotyping was performed using SSR and SNPB11 markers, whereas phenotyping of the RILs was based on the root length characters, i.e. total root length (TRL), primary root length (PRL), lateral root length (LRL), and lateral root count (LRC). QTL analysis was performed based on linkage analysis using QTL IciMapping software. The results showed that root length characters TRL, PRL, LRL, and LRC were associated with Al tolerance trait in rice and the QTL for Al tolerance based on those root length characters was located on chromosome 3 between markers RM545 and RM14543, with the peak located at SNPB11 marker with LOD score > 2.5. The PRL and TRL characters can be used to predict Al tolerance in rice. Epistatic gene action controls the PRL character, whereas the TRL character is controlled by additive gene action.

Key words: Aluminum tolerance, quantitative trait loci, recombinant inbred lines, molecular marker

Key findings: QTLs for Al tolerance were found on rice chromosome 3, particularly in the region between RM545 and RM14543 with the peak located at SNPB11 marker. The markers can be used for screening aluminum tolerance trait. Primary root length and total root length can be used to predict Al tolerance in rice.

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INTRODUCTION

Aluminum (Al) is one of the major plant-growth–limiting factors in acidic soils. The pH of acidic yellow-red podzolic soil tends to be <5; at such low pH values, Al is present in its dissolved form (Al^{3+}) and is toxic to plants and reportedly limits plant growth (Kochian *et al.,* 2015). In particular, Al^{3+} can inhibit cell division, terminate mitotic processes, inhibit root elongation, and damage root caps (Kochian, 1995; Matsumoto, 2000; Ma *et al.*, 2014). Furthermore, it can damage the architecture of the root system, causing dramatic nutrient deficiency symptoms and decreasing rice production (Kochian *et al.*, 2004).

One way to overcome Al stress in rice grown in acidic soils is to develop Al-tolerant rice varieties with high productivity. Rice is the most Al-tolerant of all cereal crops (Kim *et al.*, 2001). In rice, Al tolerance is a quantitative phenomenon that involves many genes or quantitative trait loci (QTL) (Wu *et al.*, 2000; Nguyen *et al.*, 2001; Ma *et al.*, 2014); hence, its underlying mechanism is complex. Yamaji *et al.* (2009) reported at least 31 Al tolerance genes, the expression levels of which are regulated by aluminum resistance transcription factor 1 (ART1). The genes regulated by ART1 include *STAR1, STAR2, OsFRDL4, OsALS1, OsCDT3*, and *Nrat1*. STAR1 and 2 proteins, as characterized by Huang *et al.* (2009), form the ABC transporter protein that transports UDP-glucose to modify the cell wall, thereby reducing Al accumulation therein. Yokosho *et al.* (2011) reported that the protein OsFRDL is involved in citric acid secretion in rice. Further, OsALS1 reportedly plays a critical role in the influx of Al^{3+} from the cytoplasm into vacuoles (Huang *et al.*, 2012). Xia *et al.* (2010) described the protein Nrat1 as a member of the NRAMP family that is involved in the detoxification of Al in vacuoles. In addition to the genes regulated by ART1 transcription factors, several other genes, such as *ASR1* (Arenhart *et al.*, 2016), *ASR5* (Arenhart *et al.*, 2014), *WRKY46* (Ding *et al.*, 2013), and *STOP1* (Iuchi *et al.*, 2007), play roles in Al tolerance. Mao *et al.* (2004) also identified approximately 19 gene loci in rice plants, the expression of which was induced by Al stress. Thus, Al tolerance in rice is controlled by many genes or by QTL. Miftahudin *et al.* (2005) reported homology between the Alt3 regions (the gene that controls Al tolerance) in rye and rice chromosome 3 BAC clones. Screening of rye markers based on chromosome 3 BAC sequences revealed two selected markers, i.e. B11 and B26, which showed co-segregation with the Alt3 locus, indicating that Alt3 was very close to the two markers. Therefore, Alt3 can be located between B11 and B26 or even at B11 or B26. Based on the Alt3 homology markers, Roslim (2011) isolated the B11 region from Hawara Bunar, a local Indonesian rice that is tolerant to Al. The B11 region was then used as a candidate marker to screen the genes controlling Al tolerance (Roslim, 2011). In previous studies on QTL, Miftahudin *et al.* (2008) and Akhmad (2009) crossed the IR64 and Hawara Bunar of rice to produce F2 and BC2F1 populations and to identify the location of QTL for Al tolerance, which is flanked by the markers RM489 and RM517 on chromosome 3. The F2 population was assessed by Roslim (2011); as utilized the marker B11 to differentiate Al-tolerant and sensitive genotypes. The BC2F1 population was used by Akhmad (2009) and later by Wijayanto (2013) for studies on Al

tolerance related to QTL. Of note, Wijayanto (2013) increased the densities of molecular markers on chromosome 3 in the backcross populations of BC2F1 and BC2F2 by crossing IR64 and BC1F1. In addition, we developed F2 rice seeds into a collection of recombinant inbred lines (RILs) to generate F9 populations through single seed descent method. However, data regarding Al tolerance parameters related to QTL on chromosome 3 with rice cv. IR64 and Hawara Bunar background are still lacking. According to Wijayanto (2013), the parameters of root growth inhibition (RGI) and root re-growth (RRG) have relatively low logarithm of odds (LODs) in markers between RM489 and RM517. In addition, it was difficult to use RRG, RGI, or relative root length (RRL) to clearly distinguish between several genotypes of B11-transgenic and wild-type IR64 rice (Siska *et al.*, 2017). However, total root length (TRL), primary root length (PRL), lateral root length (LRL), and lateral root count (LRC) appear to be able to differentiate among rice genotypes according to Al tolerance (Siska *et al.*, 2017). Furthermore, the B11 region of chromosome 3 has not yet been genetically mapped. Therefore, the present study is important to determine how exactly root length can be used to predict Al tolerance in rice, to map the *B11* locus of chromosome 3 based on linkage recombination mapping, and to verify the usage of B11 markers (B11CAPS and SNPB11) in RIL populations. The present study therefore aimed to analyze QTL related to Al tolerance trait based on root length in a Rice RIL F9 population derived from the cross between the IR64 and Hawara Bunar rice and to determine SNPB11 marker linkage mapping on chromosome 3 between the markers RM489 and RM517.

MATERIALS AND METHODS

Plant materials

The samples used in this study were seeds and DNA obtained from the parental plants IR64 and Hawara Bunar and from 256 recombinant inbred F9 lines derived by crossing the IR64 and Hawara Bunar rice cultivars. IR64 is an indica rice cultivar that is sensitive to Al (Siska *et al.,* 2017; Fendiyanto *et al.,* 2019) and Hawara Bunar is a tropical japonica rice cultivar that is tolerant to Al (Hairmansis *et al*., 2005; Fendiyanto *et al.,* 2019). The seeds and DNA of the RILs were obtained from the collection of the Plant Physiology and Molecular Biology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Bogor, Indonesia.

Al stress treatment

Rice seeds were sterilized in 0.5% (v/v) NaOCl for 15 min and then rinsed thrice in distilled water. The rinsed seeds were soaked in distilled water for 24 h at room temperature and then allowed to germinate on moist paper for 3–4 d at room temperature in a dark place (Jumiati, 2016; Umaiyah, 2016; Wijayanto, 2013). After germination, rice seedlings with a root length of 1 mm were planted on a plastic net floated on minimum nutrient culture medium without Al at pH 4.0 with aeration (Miftahudin *et al.*, 2002). The plants were allowed 24 h for adaptation. Al stress treatment was then conducted by administering 15 ppm Al^{3+} in the form of $AlCl_3{\cdot}6H_2O$ for 72 h. After 72 h, the nutrient

culture was replaced with minimum nutrient culture medium without Al and the plants were grown for 48 h to allow for recovery (Miftahudin *et al.*, 2002; Wijayanto, 2013). Adaptation, Al stress treatment, and recovery were performed in a growth chamber at room temperature with regular photoperiodism, i.e., 12h day and 12h night (Wijayanto, 2013). The minimum nutrient culture medium was replaced daily to maintain a pH of 4.0.

Phenotyping of RIL populations

Phenotyping analysis in RIL populations was performed using PRL, TRL, LRL, LRC, and seminal root length (SRL). Root measurements were taken after the recovery phase by scanning the root using an Epson Scanner; the images were analyzed using RootReader2D (Clark *et al*., 2013).

DNA isolation

DNA isolation was performed using the fast isolation technique (Miftahudin *et al.*, 2002). DNA was isolated from young leaves of rice plants that were approximately 3–4 weeks old and had at least three leaves. First, 1–2 fresh leaf tissue from each RIL were crushed in liquid nitrogen. Then, lysis buffer (SDS, 2%; glycine, 0.1 M; NaCl, 0.05 M; EDTA, 0.01 M; pH 8) was added, and the mixture was centrifuged at 13,000 rpm for 10 min. The supernatant was pipetted out, and a phenol/chloroform/isoamyl alcohol (25:24:1) mixture was added and centrifuged at 13,000 rpm for 10 min. The supernatant was again pipetted out, and then a chloroform/isoamyl alcohol (24:1) mixture was added and centrifuged at 13,000 rpm for 10 min. The supernatant was pipetted out once more and isopropanol was added, and then the mixture was centrifuged at 15,000 rpm for 5 min. DNA pellets were rinsed with 70% ethanol. The isolated DNA quantity was measured using UV spectrophotometry, whereas quality was observed using electrophoresis on a 1% agarose gel in 1× TBE buffer (Tris-Borate-EDTA; pH 8.0) at 85 V for 90 min. The obtained DNA was dissolved in sterile TE solution and stored at −20°C.

Allele-specific PCR for SNPB11

Allele-specific PCR techniques were applied as described by Bui *et al.* (2017). This method involved a forward primer in which the 3′ end was specific to the single nucleotide polymorphism (SNP) on the Al-tolerant B11 allele and a reverse primer that was general to both alleles. In addition, each allele-specific primer contained a base mismatch at the 3′ end to destabilize the region hybridized with the target sequence and increase allele specificity (Chiapparino *et al.*, 2004). Each specific allele contained five random base extensions at the 5′ end. SNPB11 primer was designed based on the B11 sequences of the IR64 and Hawara Bunar using Primer3 in the U-Gene application (Okonechnikov *et al*., 2012). The primer sequences used in this study were: 5′-TTCTTTCAATGTCT AAAAAG-3′ and 5′-AATGCTTTCGGT CCATTC-3′; the resulting amplicon size was 300 bp. PCR consisted of 12.5 µL MyTaq mix (Bioline, Australia), 1.25 µL of 0.2 µM primers (forward and reverse, respectively), 1 µL of 50 ng genomic DNA, and 10 µL of ddH_2O to make the total reaction volume of 25 µL. DNA was amplified at following conditions: initial denaturation at 95°C for 10 min, denaturation at 95°C for 15 s, annealing at 55°C for 15 s, extension at 72°C for 15 s, and post-extension at 72°C for 5 min and

4°C for 10 min. The program from denaturation to extension was repeated for 30 cycles. PCR products were analyzed on a 1.5% agarose gel in 1x TBE buffer and visualized using PegGreen dye (Thermo Scientific, USA) under UV light.

Genotyping, linkage mapping, and QTL analysis

The molecular markers used in the present study were simple sequence repeat (SSR) rice plant obtained from the Gramene website (http://www.gramene.org). In addition, B11CAPS (Roslim, 2011) and SNPB11 (Fendiyanto *et al.*, 2019) markers were also used; these were designed based on *B11* sequence region of the rice chromosome 3. Linkage mapping and QTL analysis were performed using QTL IcimMapping software version 3.3 (Meng *et al.*, 2015).

Data analysis

Data were analyzed using the analysis of variance test. If significant differences were detected, further testing was conducted using the Tukey Test with a significance level of $\alpha = 0.05$. Pearson correlations were constructed using R version 3.5.1 (https://cran.r-project.org/) (Lander 2014).

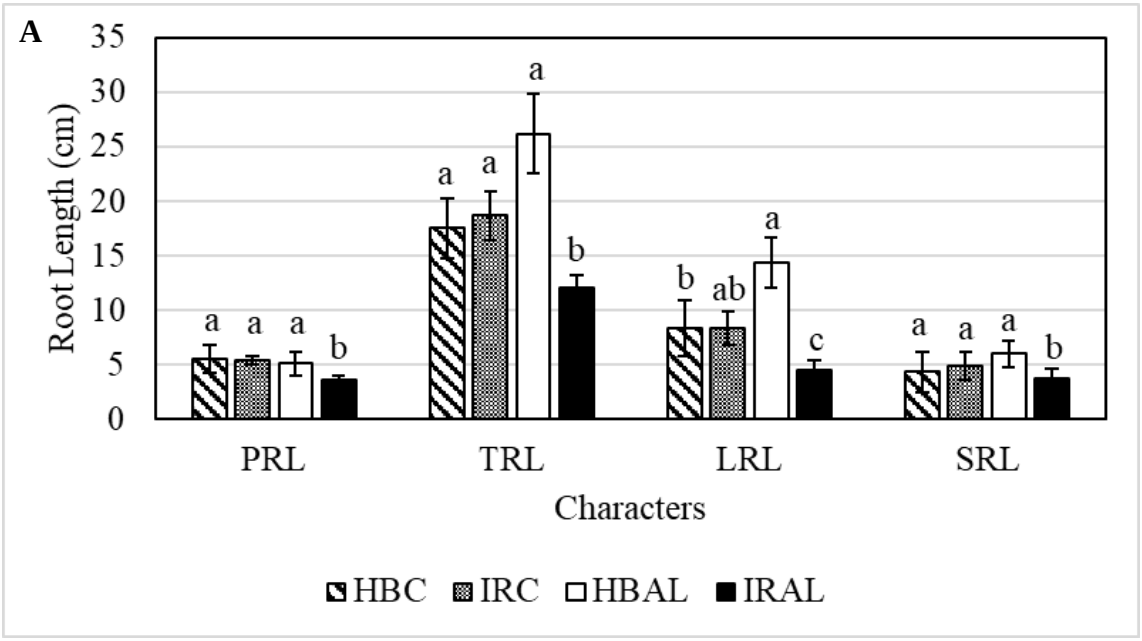
RESULTS

Phenotyping of the parental and rice RIL population

Rice cv. IR64 and Hawara Bunar were the parental of the RIL population that were used in this study. The root length characters that were used to evaluate the Al tolerance level of both rice parents and the RIL population were PRL, TRL, SRL, LRL, and LRC. The Al tolerance level of both parents and the RIL frequency distributions of the Al tolerance are shown in Figure 1 and 2. In general, the Al tolerance level of Hawara Bunar based on PRL, TRL, SRL, LRL, and LRC characters were higher than that the IR64. Root growth characters of rice cv. Hawara Bunar under 15 ppm Al stress condition were no significant different with the root growth characters under normal condition. Conversely, the root growth characters of rice cv. IR64 showed significant decrease when stressed with 15 ppm Al. Thus, rice cv. Hawara Bunar is considered as an Al-tolerant rice, whereas rice cv. IR64 is an Al-sensitive rice (Figure 1). The frequency distribution of each root growth characters in the RIL population showed that the characters distributed normally, which indicates that those characters are polygenic controlled or a quantitative trait (Figure 2). The Figure 2 also shows that Al tolerance level of rice cv. Hawara Bunar and IR64 are distinctly separated.

SNPB11 is a molecular marker developed from B11CAPS

Markers B11CAPS (Roslim, 2011) and SNPB11 (Fendiyanto *et al.*, 2019) are molecular markers developed based on DNA sequence in the area of the B11 locus in the rice chromosome 3, which is a candidate locus for Al tolerance trait in rice. According to that information, we used both markers for genotyping and QTL analysis. To verify that the B11CAPS marker is related to root length characters, we analyzed the correlation between B11CAPS (and/or other markers) and total root length (Figure 3).



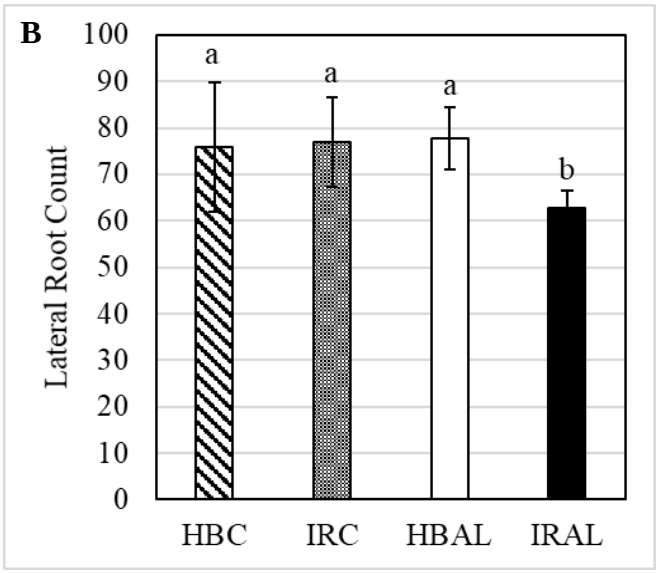


Figure 1. Aluminum (Al) tolerance level between Hawara Bunar and IR64 parental lines using root length (a) and lateral root count (b) characters. The rice was treated using minimum nutrient culture with 15 ppm Al for 72 hours and pH 4. For control/normal condition, the rice was treated using minimum nutrient culture without Al for 72 hours and pH 4. PRL: primary root length, TRL: total root length, LRL: lateral root length, SRL: seminal root length, LRL: lateral root count, HBC: Hawara Bunar in Control, IRC: IR64 in Control, HBAL: Hawara Bunar with Aluminum treatment, IRAL: IR64 with Aluminum treatment. The data obtained were average ± SD. Different letter indicate significantly different using Duncan test ($P < 0.05$).

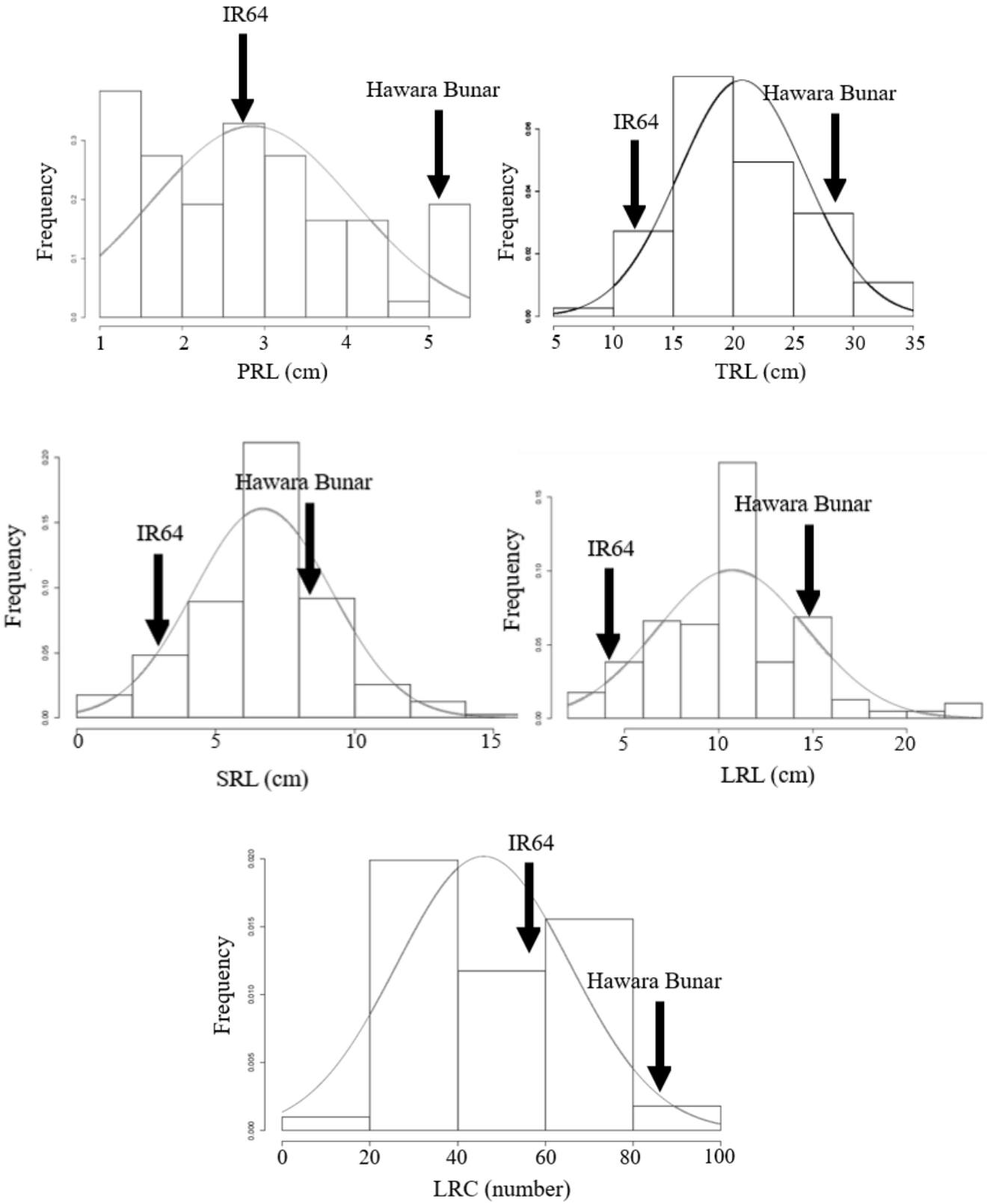


Figure 2. Frequency distribution of primary root length (PRL), total root length (TRL), seminal root length (SRL), lateral root length (LRL), and lateral root count (LRC) in the RIL population derived from the cross between rice cv. IR64 and Hawara Bunar under Al stress condition.

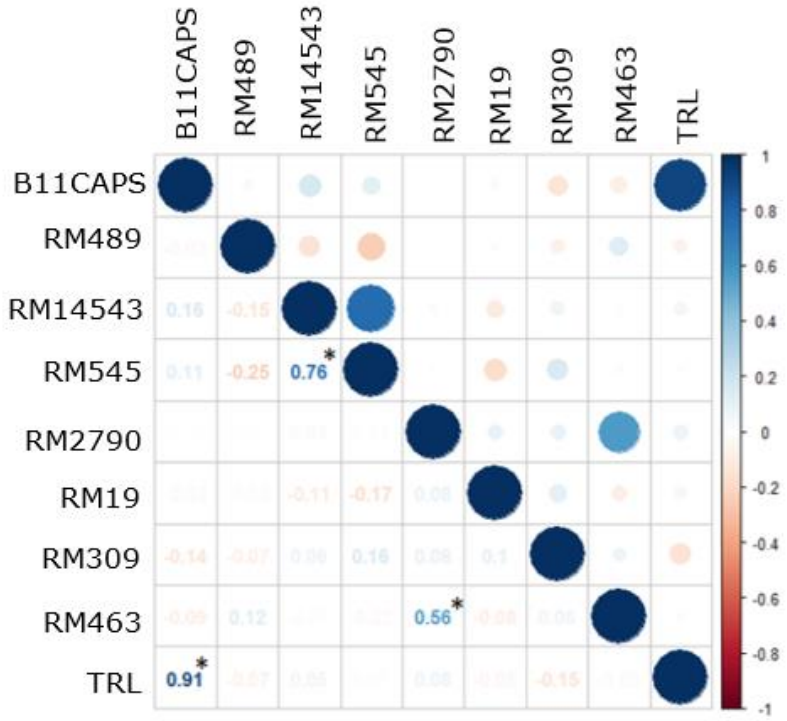


Figure 3. Pearson correlation between the total root length (TRL) character and B11CAPS markers and other markers using Pearson correlation. Testing of B11CAPS markers correlation with SSR markers and TRL characters was conducted using 256 recombinant inbred lines (RILs). Asterisks indicate statistically significant differences ($P < 0.05$). The number in the box of lower diagonal shows a correlation value (R^2).

Based on the Pearson correlation results shown in Figure 3, the molecular marker B11CAPS is closely associated with the total root length characters. Both markers showed relatively similar correlation values to other root length characters (PRL, TRL, LRL, SRL and LRC) and have a high Pearson correlation value (0.94) in randomly selected RILs. Thus, both B11CAPS and SNPB11 are molecular markers that can be used to predict Al tolerance based on root length characters. To identify the type of root length characters associated with the markers, Pearson correlation between the B11CAPS marker (and/or the SNPB11 marker) and various root length measurements, i.e. PRL, TRL, LRL, SRL, and LRC was also analyzed (Figure 4). The results showed that the SNPB11 marker is highly correlated with PRL and TRL characters under Al treatment. Therefore, it is necessary to perform QTL analysis in the region around SNPB11 on chromosome 3 to verify the presence of Al tolerance loci in the region based on the root length character. The SNPB11 is a molecular marker that has been used by Fendiyanto *et al.* (2019) to differentiate the Al tolerance level among the natural genotypes of upland rice. Based on the B11 sequence information, we designed primers in the SNP region on the introns to produce specific SNP-dominant markers of the *B11* allele.

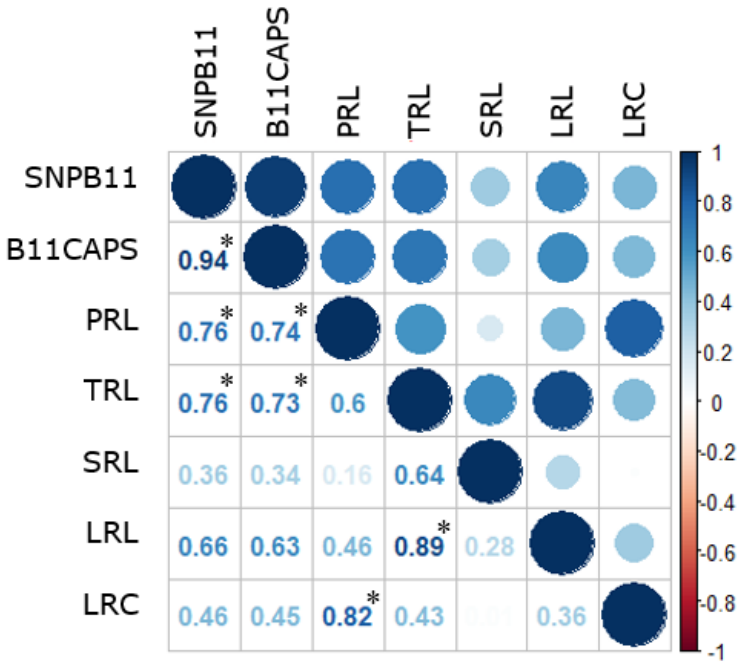


Figure 4. Relationship among SNPB11, B11CAPS and root length characters after Al stress using Pearson correlation. The character of the root length was calculated after a recovery period of six times of a biological replication in each RIL number. (*P < 0.05). Primary root length (PRL), total root length (TRL), seminal root length (SRL), lateral root length (LRL), and number of lateral roots (LRC). Pearson correlation was performed using corrplot packages in R program. The number in the box of lower diagonal shows a correlation value (R^2).

The results showed that the PCR-based SNPB11 marker was successfully used in the rice RIL population and could distinguish between rice cv. IR64 (Al-sensitive), Hawara Bunar (Al-tolerant) as well as among Al-tolerant and -sensitive lines of the RIL populations.

Polymorphism verification of the molecular markers SSR, SNPB11, and B11CAPS

Polymorphisms of the SSR markers were verified using the IR64 and Hawara Bunar Cultivars. The SSR primers were selected only from the SSRs that mapped in the area between RM489 and RM517 on chromosome 3 because according to Miftahudin *et al.* (2008) and Wijayanto (2013), there is a putative QTL for Al tolerance in that region. The markers RM19, RM309, and RM463 were used for comparison. Of 113 SSR markers from Wijayanto's (2013) genetic map, eight SSR markers showed polymorphisms between the IR64 and Hawara Bunar cultivars. In addition, the SNPB11-specific allele markers were also polymorph between the parental cultivars. A total of 10 markers were successfully amplified by PCR and scored against the RIL population. Then, the scoring values of the 256 RIL lines were used for linkage mapping and QTL analysis.

Genotyping, linkage mapping, and QTL analysis

Genetic maps were constructed according to the principles of recombination among markers using SNPB11, RM489, RM2790, RM545,

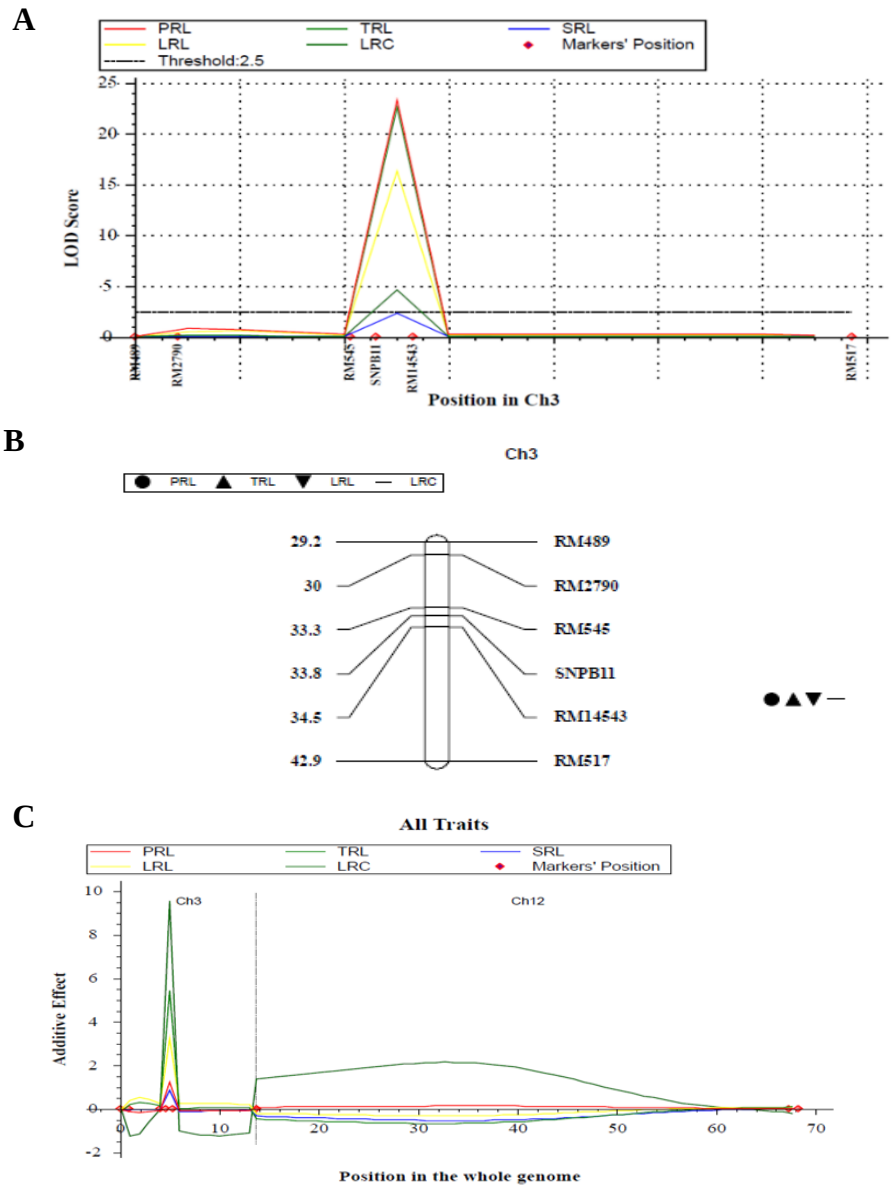


Figure 5. LOD values of root length characters on chromosome 3 (A), position of QTL for root length characters on chromosome 3 (B), and additive effects of five root length characters on SNPB11 molecular markers (C). Molecular Mapping uses frequency distribution of the primary root length (PRL, red) phenotype characters, total root length (TRL, black), seminal root length (SRL, blue), lateral root length (LRL, yellow), and lateral root count (LRC, green) in the rice population of RIL Hawara Bunar (Al-tolerant line)/IR64 (Al-sensitive line). A total of 256 rice RILs were adapted in Minimum Nutrient without Al for 24 h, then Al (15 ppm) was held for 72 h and recovered in the minimum media without Al for 48 h (Miftahudin *et al.*, 2002). The characters of PRL, TRL, LRL, LRC, and SRL were used to evaluate the level of rice line tolerance to Al. Additive effects are significant in the characters of TRL, LRC, and LRL in the SNPB11 marker area. Genetic distance follows McCouch *et al.,* (2002), CIAT, (2006), and linkage mapping from IcimMapping ICIM QTL using the Haldane function with default settings.

RM14543, RM19, RM309, RM463, and RM517 markers. Based on the linkage group analysis in the RIL population, as many as seven markers (RM489, RM2790, RM545, SNPB11, RM14543 and RM517) were located in one linkage group, whereas three other markers (RM19, RM309, and RM463) on chromosome 12 were located in the other linkage group (Figure 5). The first linkage group (on chromosome 3) shows that the farthest linkage distance from the telomere was 42.9 cM and the nearest was 29.2 cM, therefore the farthest distance between two markers was 13.7 cM or the average distance between two adjacent markers was 2.28 cM (Figure 5). Because the SNPB11 has a recombinant distance of 33.8 cM from the telomere, then we placed the SNPB11 in between markers RM545 and RM14543. In the other group, the farthest linking distance from the telomere was 75.5 cM and the nearest was 20.9 cM, therefore the farthest distance between two markers was 54.6 cM or the average distance between two adjacent markers was 18.2 cM (Figure 5).

Based on QTL analysis, we found that four characters of Al tolerance, i.e., PRL, TRL, LRL, and LRC were linked to the SNPB11 marker (Figure 5) and those characters can be classified into major QTL based on PVE and LOD values (Table 1). In this study, there are four characters of root length, i.e., TRL, PRL, LRL, and LRC that have LOD values above the threshold and have R^2 values higher than that of SRL (Table 2). The four characters of root length, which are used as parameters of plant tolerance to Al stress, can be classified into additive and epistasis action modes. The highest additive effect value among the four characters is TRL at 9.5, while the lowest is PRL at 1.25 (Figure 5). Because PRL has the lowest value of additive effect (lower among major QTL on chromosome 3), it could be classified into epistasis action modes (Table 1). To convince our study, we also compared QTL position in the present study with other QTL previously reported using different rice background and molecular markers (Figure 6). The result shows that the position of QTL for Al tolerance trait in the rice chromosome 3 is similar.

DISCUSSION

Rice cv. Hawara Bunar shows a higher Al tolerance level than that of cv. IR64 (Figure 1). Phenotypic analysis of the 256 RILs showed that the root length characters of the RIL population normally distributes from Al-sensitive to -tolerant (Figure 2) although for certain characters are rather skewed toward Al-sensitive lines, indicating that the characters are a quantitative trait. The root length characters have been used as Al tolerance parameters in plants including rice. Previous studies involving the measurement of root length characters in rice after treated with 15 ppm Al for 72 h followed by recovery period for 48 h in minimum nutrient medium with pH 5.8 showed that root length characters, i.e. root re-growth (RRG), relative root length (RRL), and root length inhibition (RLI) inconsistently distinguish Al-tolerance and -sensitive rice as well as between Al-tolerant rice transgenic and its Al-sensitive wild type (Roslim, 2011; Jumiati, 2016; Siska *et al.*, 2017; Lestari, 2017). In addition, when Wijayanto (2013) used the RRG dan RRL characters as Al tolerance parameters to find QTL in rice chromosome 3, it shows the QTL position with low LOD value in the area between markers RM489 and RM517. Therefore, it is necessary to use other root length

characters that can accurately be used as Al tolerance parameters in rice. Thus, the root growth characters, i.e., PRL, TRL, LRL, SRL, and LRC were used to phenotype the RIL population used in this study.

Table 1. Putative QTLs detected on rice chromosome 3 for primary root length (PRL), total root length (TRL), lateral root length (LRL), lateral root count (LRC), seminal root length (SRL) using RIL IR64/Hawara Bunar population.

QTL	Chr	Trait	Left Marker	Right Marker	Position (cM)	LOD	PVE (%)	AE	Type of QTL
qPRL	3	PRL	SNPB11	RM14543	34.2	23.0205	55.6568	1.2111	Major
qTRL	3	TRL	SNPB11	RM14543	34.2	22.6453	55.5642	5.4393	Major
qLRL	3	LRL	SNPB11	RM14543	34.2	16.2787	42.4747	3.2456	Major
qLRC	3	LRC	SNPB11	RM14543	34.2	4.2498	13.8698	9.2115	Major
qSRL	3	SRL	SNPB11	RM14543	34.2	2.5322	7.7409	0.8645	Minor

Notes: QTL = Quantitative Trait Locus; Chr = Number of Chromosome, LOD = Logaritm of Odds, PVE = Percentage of variance explained by the QTL; AE = Additive effects

Table 2. Significant ($P < 0.05$) associations between molecular markers and aluminum tolerance.

QTL	Marker	Trait	R^2(%)	Intercept	*P*-value (MLM)
qPRL	SNPB11	PRL	57.4071	3.6921	0.0005
qTRL	SNPB11	TRL	57.4061	23.5634	0.0007
qLRL	SNPB11	LRL	42.8622	12.5672	0.0023
qLRC	SNPB11	LRC	20.2994	52.4744	0.0073
qSRL	SNPB11	SRL	12.2108	7.3199	0.0178

Notes:R^2 (%) Percentage of phenotypic variance explained by the relevant marker.

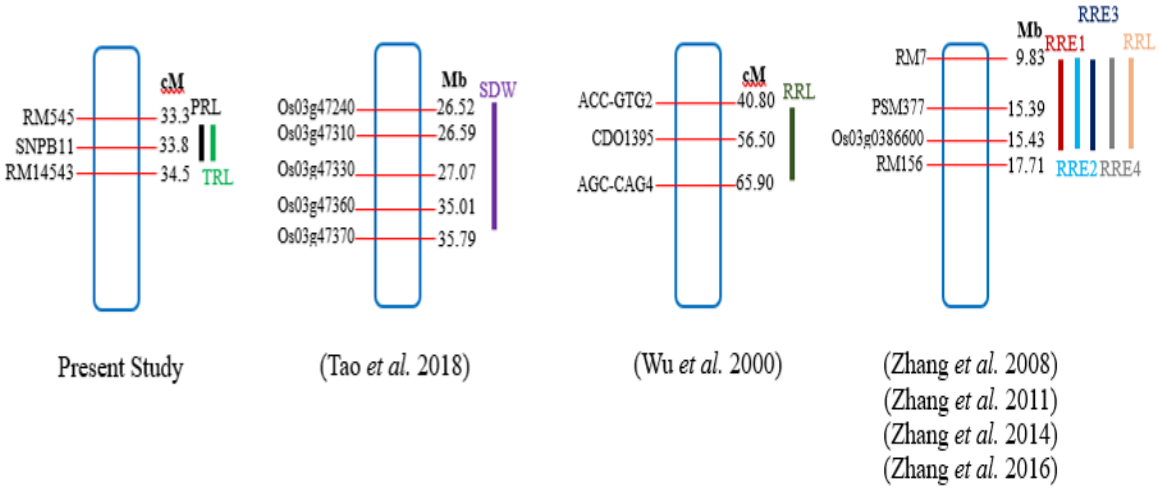


Figure 6. Comparative studies of QTL on chromosome 3 for aluminum tolerance in rice. PRL: primary root length; TRL: total root length; SDW: shoot dry weight; RRL: relative root length; RRE1: relative root elongation 1; RRE2: relative root elongation 2; RRE3: relative root elongation 3; RRE4: relative root elongation 4.

Simple sequence repeat (SSR) marker is a codominant marker that can be used to genotype the segregation population, such as RILs, used in the genetic mapping study or QTL identification. In the present study, we used SSR markers, i.e. RM14543, RM545, RM517, RM2790, RM489, RM19, RM309, and RM463 that previously have been mapped on rice chromosome 3 using the rice BC_2F_3 population derived from the cross between rice cv. IR64 x Hawara Bunar (Wijayanto 2013). Besides the SSR markers, we also used a SNP marker derived from a CAPS marker, i.e. B11CAPS that was previously developed by Roslim (2011). The molecular marker B11CAPS was developed based on polymorphisms in the *AluI* restriction enzyme site in B11 DNA region on rice chromosome 3. Roslim (2011) reported that the segregation of B11CAPS in the F2 rice population follows single-gene inheritance. Thus, the molecular marker B11CAPS shows potential as a selection tool for rice breeding programs that aim at generating Al-tolerant rice genotypes (Roslim, 2011). The B11CAPS marker is relatively impractical and costlier than other Al tolerance markers because it requires a restriction enzyme after the amplification of the 300 bp of B11 DNA region. Recently, researchers have used allele-specific SNP markers based on PCR that tend to be cheaper, more efficient, and can reliably distinguish particular characters (Bui *et al.*, 2017). Therefore, in the present study, the molecular marker B11CAPS was further developed into a SNPB11 marker. Both the B11CAPS and SNPB11 markers carry the 300 bp nucleotides derived from B11 sequence.

The SNPs are markers used for genetic analyses in many organisms, including plants and animals (Ganal *et al.*, 2012). SNP markers can be combined in SNP detection and

genotyping, such as in genotyping by sequencing (GBS), to obtain high-precision data (Andrews *et al.*, 2016). However, using such combinations is generally costly. SNP markers can also be developed by designing SNPs to match restriction enzyme sites. This PCR-based SNP marker approach is relatively easy to use, effective, and inexpensive (Gaudet *et al.*, 2009) when compared to SNP arrays, SNP–GBS combinations, and CAPS. The present study uses two B11 sequence-based markers- one SNP (SNPB11) and one CAPS (B11CAPS). We successfully developed and amplified the SNPB11 marker based on Fendiyanto *et al.* (2019) information using allele-specific PCR in RIL population. The Al-tolerant lines produced a 300 bp DNA band, similar to rice cv. Hawara Bunar. Other Al-sensitive lines have no DNA band, similar to rice cv. IR64. Before we used the markers for genotyping and genetic mapping, we analyzed the correlation among the markers and also between SNPB11 marker and all root length characters (Figures 3 and 4). The result shows a close relation among the markers and also between markers and root length characters, therefore we used all eight markers to genotype the 256 lines of the RIL population. Based on the linkage map data, the SNPB11 markers are located in the same linkage group as the RM14543, RM545, RM2790, and RM517 markers (Figure 5). This supports the results of linkage mapping analysis, which corroborates those of McCouch *et al.* (2002) and Wijayanto (2013) who grouped the RM14543, RM545, RM2790, and RM517 markers into one linkage group (chromosome 3) with close genetic distances. Gene or locus discovery for specific traits could be performed with two approaches: QTL mapping and genome-wide association studies (GWAS). Linkage recombination mapping is the basis for QTL identification to find markers related to certain phenotypes. GWAS uses linkage disequilibrium as the basis for QTL identification using the association between genotype and phenotype in the natural population (Singh and Singh, 2015). QTL analysis can be performed by creating segregating populations in the form of RILs, BC1F2 backcrossing populations, or near-isogenic lines, whereas GWAS analysis can be performed using natural populations. The present study discovered QTL using RIL F9 population derived from crossing the IR64 and Hawara Bunar rice cultivars. RILs are a perpetual population with a homozygous genetic composition of lines; the population creating process is performed without selection and can be used for QTL mapping (Singh and Singh, 2015). QTL analysis to find the QTL area for Al tolerance was conducted in several stages, including reviewing of molecular marker polymorphisms, genotyping analysis, phenotyping analysis, creating linkage maps based on recombination values, and finding QTL for Al tolerance trait (McCouch *et al.*, 2002; Wijayanto, 2013).

Previous QTL research conducted by Akhmad (2009), Hariyanto (2009) and Wijayanto (2013) showed that all four SSR markers that were mapped to the position between markers RM489 and RM517 on rice chromosome 3 (i.e. markers RM2790, RM14535, RM14543, and RM14552 used to amplify DNA from the F2 population) comprised an Al tolerance area of QTL. DNA scoring data from the four SSR markers together with markers

RM489, RM545, and RM517 were performed in this study using RIL population. The QTL areas for TRL, PRL, LRL, and LRC characters appeared in the area between the markers RM545 and RM14543 and peaked in the SNPB11 area. The nature of SRL does not give rise to the QTL area on chromosome 3 (Figure 5). The positions of QTL TRL, PRL, LRL, and LRC appear in the area around the SNPB11 markers because they have LOD values >2.5 (Figure 5). The highest LOD value (i.e. 23) is owned by TRL and PRL, followed by LRL and LRC with LOD values of 16 and 4.75, respectively. The LOD values of TRL, PRL, LRL, and LRC characters obtained from the QTL mapping in this study was relatively higher compared with the LOD RRG and RRL values in the areas around the markers RM489 and RM517, as reported by Wijayanto (2013). The different results between Wijayanto (2013) and this study occur due to the root length characters and population differences. This study performed QTL using TRL, PRL, LRL, and LRC characters and the F9 RIL population. In contrast, Wijayanto (2013) used RRG and RRL characters and a population of BC2F1 to BC2F3 to analyze QTL in Al tolerance.

In our knowledge, mapping of the QTL for Al tolerance in rice using the root length characters i.e.: of TRL, PRL, LRL, and LRC after treatment with 15 ppm Al for 72 h followed by a recovery period for 48 h has never been performed. Mapping of QTL for Al tolerance on rice chromosome 3 area has previously been performed based on other root growth characters, including three root length characters, i.e., RRG, RRL, and RGI. However, these three characters have relatively lower LOD values (Wijayanto, 2013), and after further analysis to determine Al tolerance, the characters tend to be unstable to distinguish between Al-tolerant and -sensitive rice cultivars (Siska *et al.*, 2017). There were two root length characters obtained in this study, i.e.: TRL and PRL that had the highest LOD value and could be used as a parameter for Al tolerance in future studies (Figure 5). Miftahudin *et al.* (2008), Akhmad (2008), and Wijayanto (2013) reported that there was an area of QTL tolerance for Al using RRG, RRL, and RGI characters in the area between the markers RM489 and RM517 on rice chromosome 3. The region between the markers is thought to have contained QTL for the Al tolerance trait. The estimation was proven after performing linkage mapping in this study, where *B11* allele-specific markers, SNPB11, were very close to the RM545 and RM14543 markers (Figure 5). The SNPB11 marker is located in between both markers with the distance of 0.5 and 0.7 cM, respectively. Other markers have the same genetic distance as reported by McCouch *et al*. (2002) i.e., RM489, RM2790, RM545, RM14543, and RM517 are 29.2, 30, 33.3, 34.5, and 42.9 cM away, respectively. Based on the linkage mapping data, two linkage groups 1 (LG1) and group 2 (LG2) were obtained. The LG1 group consists of markers RM489, RM2790, RM545, RM14543, and RM517, located on chromosome 3; the LG2 group contains markers RM19, RM309, and RM463, are located on another chromosome (data is not shown).

Genetic influences on a plant's tolerance to Al stress can also be influenced by additive or epistasis effects. Additive effects are a genetic influence transmitted from parents to their offspring. In contrast, the

epistasis effect is a genetic influence that is not transmitted into among the phenotype and the genotype of offspring in RIL populations (Wu *et al.*, 2000). In the present study, there were four characters of root length, including TRL, PRL, LRL, and LRC with LOD values above the threshold. The four root length characters that were used as parameters for plant tolerance to Al stress can be reclassified into additive and epistasis action modes. TRL had the highest additive effect value among the four characters at 9.5, and PRL had the lowest value at 1.25 (Figure 5). We map the marker region on chromosome 3 and narrow down the QTL region because the chromosome 3 region is an area where QTL has been found but has not been fine-mapped (Figure 6).

In addition, it is reported that there is a SNPB11 region on chromosome 3 which is proven to be able to distinguish Al tolerance level in natural populations (Fendiyanto *et al.*, 2019). Based on comparative results in Figure 6, QTL for Al-tolerance in rice was found on chromosome 3 using the characters of shoot dry weight (SDW), relative root length (RRL) and relative root elongation (RRE) (Wu *et al*., 2000; Tao *et al*., 2018; Zhang *et al.*, 2008; Zhang *et al.*, 2011; Zhang *et al.*, 2011; Zhang *et al*., 2014; Zhang *et al*., 2016). Therefore, in this research, we focus on analyzing the QTL region in rice chromosome 3 using different root growth characters using the RIL population derived from the cross between rice cv. IR64 and Hawara Bunar.

In general, we found four major QTL on rice chromosome 3, i.e. qPRL, qTRL, qLRL, and qLRC. Determination of major QTL of Al tolerance trait in the rice chromosome 3 is based on the LOD and PVE values. The qPRL and qTRL were the two QTLs with the highest LOD scores and PVE values (Table 1), which indicate that both QTL are the major QTL. However, the qPRL has low AE value, suggesting that the QTL has epistatic gene action. The other three QTL have high AE value indicating that those QTL has additive gene action. According to correlation analysis, qPRL and qTRL showed a close association with the SNPB11 marker (Table 2), meaning that the SNPB11 marker can be used as a marker for Al tolerance trait, which in this case masker for the qPRL and qTRL. The PRL (primary root length) and TRL (total root length) are the root growth characters that can be a representative Al tolerance trait in rice.

CONCLUSION

There are four QTLs for aluminum (Al) tolerance in the region between the markers SNPB11 and RM14543 on rice chromosome 3. The QTLs are related to the total root length (TRL), primary root length (PRL), lateral root length (LRL), and lateral root count (LRC) characters of Al-stressed rice. Those root characters linked to the SNPB11 marker, which suggests that the SNPB11 marker can be used as a molecular marker for Al tolerance trait in rice.

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