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Original research article

Overexpression of *B11* Gene in Transgenic Rice Increased Tolerance to Aluminum Stress

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ABSTRACT

Rice cultivation on acid soils is mainly constrained by aluminum (Al) toxicity. However, rice has tolerance mechanism to Al stress, which is controlled by many genes. *B11* gene is one of the Al- tolerance gene candidate isolated from rice var. Hawara Bunar. It has not been known whether overexpression of the gene in Al-sensitive rice is able to increase Al tolerance. The research objective was to analyze root morphological and physiological responses of transgenic rice overexpressing *B11* gene to Al stress. The experiment was carried out using five rice genotypes including two varieties (Hawara Bunar and IR64) and three T4 generation of transgenic lines, that are T8-2-4, T8-12-5, and T8-15-41. All rice genotypes were grown in nutrient solution for 24 h (adaptation period), and then were exposed to 15 ppm Al for 72 h (treatment period) and recovered in normal nutrient solution for 48 h (recovery period). The result showed that the overexpression of the *B11* gene in T8-2-4, T8-12-5, and T8-15-41 transgenic lines improved tolerance to Al stress based on root growth characters, accumulation of Al, root cell membrane lipid peroxidation, and root tip cell structure.

1. Introduction

The effort to increase national rice production can be carried out through extensification of rice cultivation by using marginal land such as acid soils. The high solubility of aluminum (Al) in the form of Al^{3+} in acid soils is the main constraint for rice cultivation because of its toxicity to the plant roots (Kochian 1995; Kochian *et al.* 2005). The toxicity symptoms can be observed through root development (Matsumoto and Motoda 2012). According to Horst *et al.* (2010), root apex is among the area mostly affected by Al toxicity. The major site of Al accumulation and toxicity is root meristem cells. Meristem cells were actively dividing, expanding, and also the most sensitive area to environmental changes (Yamamoto *et al.* 2001). An exposure of the root cells to Al could alter cytosolic Ca^{2+} and pH level (Ma *et al.* 2002), which could inhibit the root growth.

There are several mechanisms of Al-tolerance in plants, among them are an exclusion of Al from root apices by organic acid secretion from the roots, Al-binding in the root cell walls (Delhaize *et al.* 1993). The exclusion of Al by organic acid secretion was showed in rice (Yokosho *et al.* 2011), wheat (Ryan *et al.* 2009), common bean (Rangel *et al.* 2010), and maize (Wang *et al.* 2004). The research of Yang *et al.* (2008) showed in rice the tolerance level of Al related to Al content in the cell wall. Wang *et al.* (2004) found the evidence for Al-binding in cell walls. More than 85% Al content was detected in the cell wall and the root apoplast from the root tip of maize.

The tolerance level of Al stress in plant can be observed from the root characters. The characters that have been known distinguished the tolerant character from Al stress was relative root elongation (RRE) (Kim *et al.* 2001; Doncheva *et al.* 2004), root regrowth relative (RGR) from the longest root is commonly used to assess Al-tolerance in cereals (Famoso *et al.* 2010; Roslim 2011).

The Al-tolerance character in rice is suggested to be controlled by many genes. Several genes that involved in Al-tolerance in rice and have been identified are *STAR1 and STAR2* (Huang *et al.* 2009), *OsFRDL4* (Yokosho *et al.* 2011), *ALMT1* (Sasaki *et al.* 2004), *ASR5* (Arenhart *et al.* 2014), and *ART1* (Yamaji *et al.* 2009). The later is considered to be the main regulator of the other Al-tolerance genes in rice; however, the *ART1* gene is not regulated by Al stress.

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Therefore, it should be some other genes that regulated the expression of the *ART1* gene.

Rice was known as a cereal crop that is tolerant to Al stress; however, the tolerance level in rice shows variation among genotypes and varieties. Roslim (2011) has successfully isolated *B11* gene from Indonesian local rice Hawara Bunar using rye/rice synthetic approach (Miftahudin *et al.* 2005). The gene has already been characterized and is suggested to be an Al-tolerance gene. Zulkifli (2015) and Ratnasari (2015) have proved that the *B11* gene increased the tolerance of transgenic tobacco overexpressing the gene against Al stress based on physiological and morphological characters. The expression analysis of the *B11* gene in transgenic tobacco shows that the high expression of *B11* gene is followed by the high expression of *STOP1* and *ALMT1* genes under the Al stress treatment of transgenic tobacco (Ratnasari *et al.* 2016); *STOP1* gene is an ortholog gene of the *ART1* gene (Ohyama *et al.* 2013), whereas the *ALMT1* gene is an aluminum malate transporter that involves Al-tolerance in several cereal species (Sasaki *et al.* 2004), which suggests that the *B11* gene might regulate both Al-tolerance genes.

Pambudi (2012) has developed transgenic rice with overexpressed *B11* gene derived from rice var. IR64, which was expected to be more tolerant to Al than that of var. IR64, and still retain good agronomic characters under acid soil. However, the response of transgenic rice under Al stress, especially the morphological and physiological responses of the transgenic root has not been carried out. Therefore, the objective of the research was to study the responses of root morphology and physiology of transgenic rice carrying overexpressed *B11* gene to Al stress.

2. Materials and Methods

2.1. Plant materials

Rice seeds var. Hawara Bunar (Al-tolerant variety), IR64 (Al-sensitive variety as the wild type of transgenic lines), and three T4 generations of transgenic lines (T8-2-4, T8-12-5, and T8-15-41) were used in this experiment. The transgenic lines are rice var. IR64 carried *B11* gene originated from Hawara Bunar that overexpressed *B11* gene under CaMV35S constitutive promoter with the following construction (Figure 1).

2.2. Verification of *B11* gene insertion in transgenic lines

Verification of *B11* gene insertion was carried out using polymerase chain reaction (PCR) method. DNA total was isolated by Hexadecyltrimethylammonium bromide (CTAB) method (Saghai-Maroof *et al.* 1984), then the DNA was used as a template for PCR amplification using primer 35S Nakajima F (5′-GAT-GTG-ATA-TCT-CCA-CTG-ACG-TAA-G-3′) and primer B11 check-R (5′-GAA-CGA-TTG-GGC-CTC-TGT-GA-3′). The PCR program followed the method of Ratnasari *et al.* (2016).

2.3. Aluminum treatment

The rice seeds were sterilized using 0.5% (v/v) NaOCl for 15 min, rinsed with distilled water three times, and then soaked in distilled water for 48 h. The seeds were germinated in dark room at 27°C for 24 h. The seedlings with the root length of 0.5–1 cm (20 seedlings per genotype) were planted to plastic net floating with the minimum nutrient culture media (Miftahudin *et al.* 2002) without an addition of Al at pH 5.8 and 4.0 for 24 h (adaptation period). The nutrient culture media was replaced with fresh media with the addition of 15 ppm $AlCl_3 \cdot 7H_2O$ and the seedlings were grown for 72 h, and then followed by recovery period for 48 h. The treatments were carried out in the growth chamber with a controlled temperature of 29–31°C and relative humidity of 80% with 12/12 h lighting dark/light. The solution was aerated and changed daily to maintain the pH 4 of the solution. Each treatment was repeated three times and each replication consisted of 20 seedlings that were used for root growth and physiological analysis.

2.4. Root growth analysis

The characters of root morphology observed in this experiment were the length of the main root, the number of adventitious roots, number of lateral roots, length of adventitious roots, length of lateral root, and the total root length. The analysis of all root morphologies was conducted in Al 15 ppm treatment. The roots were scanned using Epson Perfection Photo V370 scanner with the transparent mode to produce a black and white image, then the images were measured using IJ Rhizo software (Pierret *et al.* 2013). Root growth responses were expressed as relative root elongation (RRE), root growth inhibition (RGI), and root re-growth relative (RGR) calculated using the following formulas adopted from Roslim (2011):

$$\mathrm{RRE} = \frac{\Delta \text{ treatment}}{\Delta \text{ control}}$$

$$\mathrm{RGI} = \frac{\Delta \text{ control} - \Delta \text{ treatment}}{\Delta \text{ control}} \times 100$$

Δ control: the differences of main root length between stress period and adaptation period without Al stress.
Δ treatment: the differences of main root length between stress period and adaptation period with 15 ppm Al.

$$\mathrm{RGR} = \frac{\Delta \text{ treatment}'}{\Delta \text{ control}'}$$

Δ control′: the differences of main root length after recovery period with stress period without Al treatment.
Δ treatment′: the differences of main root length after recovery period with stress period with 15 ppm Al treatment.

2.5. Aluminum accumulation in the root tips

Qualitative analysis of Al accumulation in the root tips was performed using Ehrlich's aluminum hematoxylin method (Ehrlich 1886) with some modification. After Al treatment, the roots were soaked in 0.6% (w/v) hematoxylin for 2 min, and then rinsed with distilled water. Quantitative analysis of Al concentration in the roots was performed by following the method of AOAC (2012) using

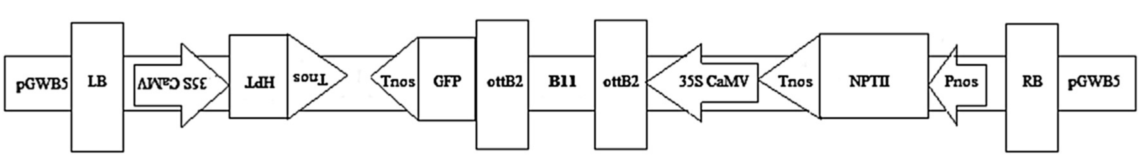

Figure 1. Linear mapping of T-DNA plasmid pGWB5 with *B11* gene (Roslim 2011).

Figure 2. Electrophoregram of PCR product of transgene insertion in transgenic lines using *B11* and 35S primer combination. C = control without DNA template; IR64 = Al-sensitive rice; M = marker 100 bp; P = recombinant pGWB5-*B11*; T8-2-4, T8-12-5, and T8-15-41 = T4 generations of transgenic lines IR64.

atomic absorption spectrophotometer Agilent Technologies 200 Series AA Systems (Agilent, CA, USA).

2.6. Root cell membrane lipid peroxidation analysis

Root cell membrane damage due to Al toxicity was detected through qualitative and quantitative analyses. *Schiff's* staining method following the method of Yamamoto *et al.* (2001) was applied to qualitatively measure the level of lipid peroxidation of root cell membrane. Quantitative analysis of lipid peroxidation was carried out based on malondialdehyde (MDA) concentration measured using spectrophotometer by following the method of Meriga *et al.* (2010).

2.7. Root tip cell structure observation

Aluminum treated and untreated roots from all genotypes were rinsed with distilled water, then cut along 1.5 mm from the root tip, and then were subsequently fixed in 2.5% (v/v) glutaraldehyde and 0.1 M cacodylate buffer + sucrose 3% (w/v), dehydrated in ethanol series, infiltrated with ethanol absolute: propylene oxide series, and embedded in *Spurr's* resin. The embedded roots were excised to obtain 70 nm thick sample size, and then observed under transmission electron microscope (TEM) type JEM1010-JEOL (JEOL, Tokyo, Japan) at 80 kV.

2.8. Data analysis

Data were analyzed using one-way analysis of variance, and continued with Duncan multiple range test to the test of differences among treatment at significance level of $\alpha = 0.05$ using SPSS version 16.0 (IBM SPSS, USA). The classification of rice genotypes into their tolerance level to Al stress was carried out with principal component analysis (PCA) using PAST 3.06 program (Hammer *et al.* 2001).

3. Result

Verification of the *B11* gene insertion in transgenic rice using PCR analysis showed that the PCR produces only single DNA band (342 bp) in positive control (pGWB5-11) and six of seven tested transgenic lines, whereas the wild type, IR64, did not produce PCR band (Figure 2). Among the six transgenic lines, there were T8-2-4, T8-12-5, and T8-15-41 that were used in this research, which confirmed that the genome of the transgenic lines used in the research contain the *B11* gene that overexpressed under promoter CaMV 35S.

3.1. Root growth under aluminum stress

Root growth responses to low pH and Al stress were observed based on qualitative and quantitative measurements. Based on Figure 3, the main root length of all genotypes was similar under pH 5.8. The decrease of pH medium from 5.8 to 4.0 caused the decrease of main root length of all genotypes (Figure 3A and B), suggesting that low pH until 4.0 inhibits the root growth of all genotypes. However, the main root length of rice var. Hawara Bunar (Al-tolerant), transgenic lines T8-2-4, T8-12-5, and T8-15-41 were

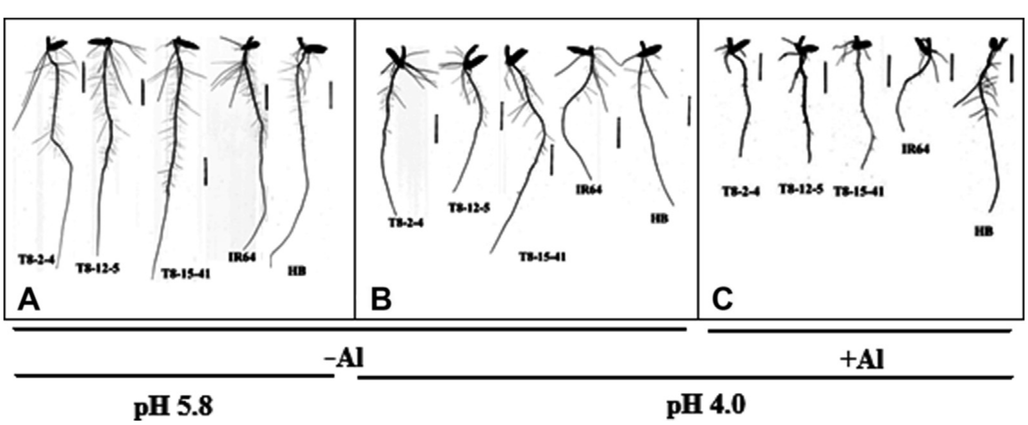

Figure 3. Root growth responses of five rice genotypes to low pH and 15 ppm Al stress. Rice seedlings were grown on nutrient solution (A) pH 5.8, (B) pH 4.0, and (C) pH 4.0 + 15 ppm Al.+Al = 15 ppm Al; −Al = 0 ppm Al (control); HB = Al-tolerant rice; IR64 = Al-sensitive rice; T8-2-4, T8-12-5, and T8-15-41 = T4 generations of transgenic lines IR64. Bar = 1000 µm.

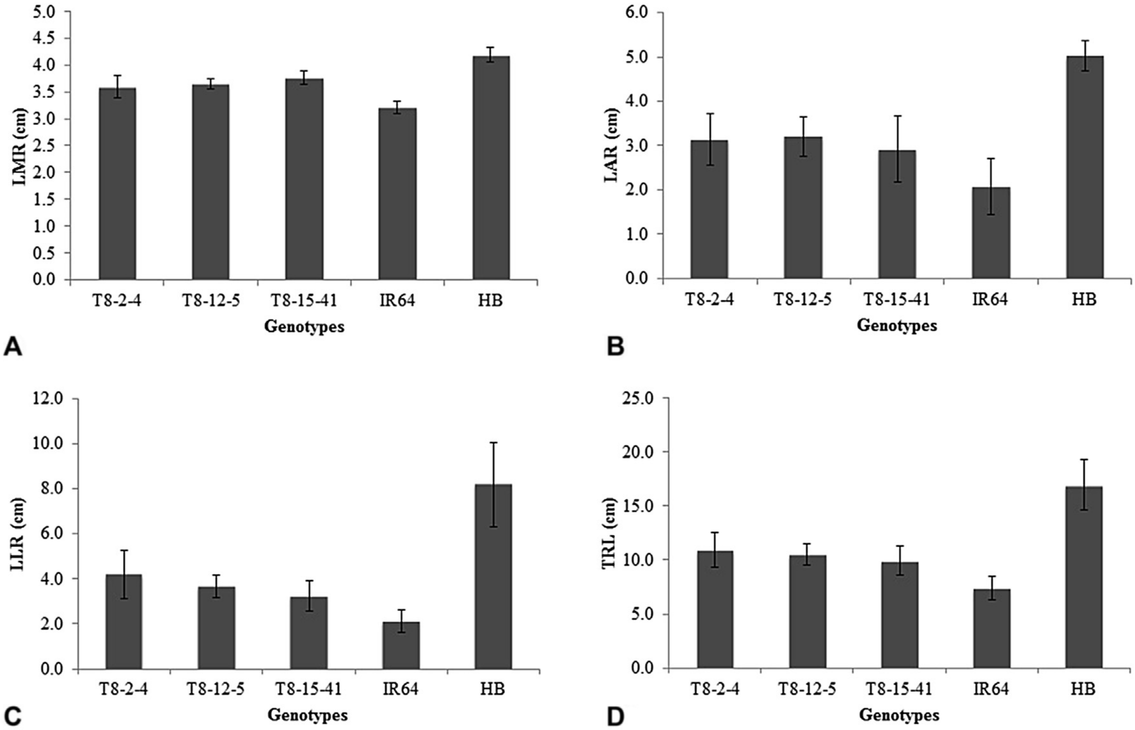

Figure 4. The root length of five rice genotypes treated with 15 ppm Al. (A) LMR, (B) LAR, (C) LLR, and (D) TRL. HB = Al-tolerant rice; IR64 = Al-sensitive rice; LAR = length of the adventitious roots; LLR = length of the lateral roots; LMR = length of the main root; T8-2-4, T8-12-5, and T8-15-41 = T4 generations of transgenic lines IR64; TRL = total root length. Bar = standard error.

longer than that of IR64 (Al-sensitive) under pH 4.0. The result also showed different inhibition effect among the treatments on root length (Figure 3). The root growth of all rice genotypes was even more inhibited when treated with Al as compared to the control (pH 4.0) treatment. However, rice var. Hawara Bunar was the only genotype that had the longest main root.

To support the qualitative data, we analyzed root characters after being treated with 15 ppm Al for 72 h. All transgenic lines showed similar root length (Figure 4) and number of the roots (Figure 5). Both root characters of the transgenic lines were higher than that of IR64, which indicated that the transgenic lines were more tolerant to Al than that of the wild type. Compared with var. Hawara Bunar, the transgenic lines have higher value in number of the roots. However, the length of the root of transgenic lines was lower than that of var. Hawara Bunar. Al treatment not only affected the inhibition of the main root, but also decreased the value of all root characters.

Aluminum-tolerance parameters in rice could also be expressed as RRE, RGI, and/or RGR of the main root (Table 1). Transgenic lines T8-2-4 and T8-12-5 have higher value of RRE and RGR than that of IR64, but both transgenic lines have lower value of RGI than that of IR64. Transgenic line T8-15-41 has not significantly different than that of IR64 in RRE, RGR, and RGI. Based on those three Al-tolerance parameters, the transgenic lines T8-2-4 and T8-12-5 were suggested as more tolerant to Al stress than that of its wild type (IR64).

3.2. Aluminum accumulation in the root tips

Qualitative analysis of Al accumulation in the root tips with hematoxylin staining showed the absence of purple color in the root tips of plants without Al treatment, and the presence of purple color in the root tips of Al-treated plant with different color intensity among genotypes, which indicated the differences in Al accumulation. The purple color was clearly visible in the root tip of

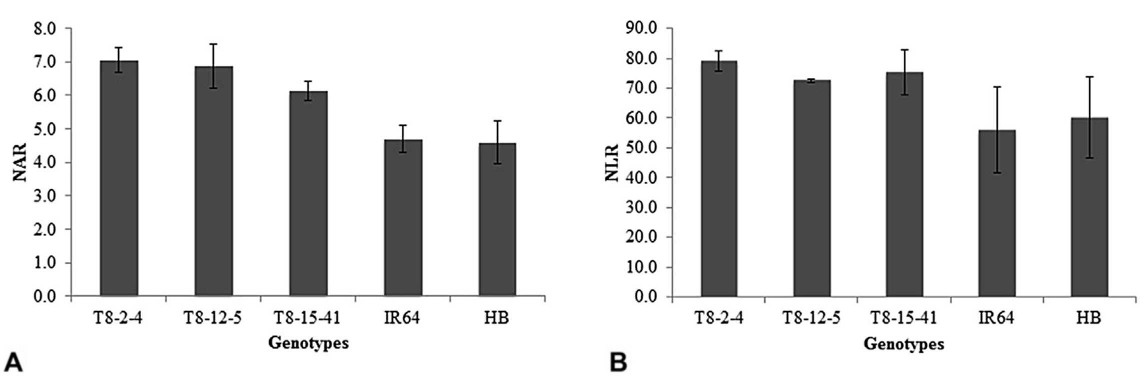

Figure 5. The number of root of five rice genotypes treated with 15 ppm Al. (A) NAR and (B) NLR. HB = Al-tolerant rice; IR64 = Al-sensitive rice; NAR = number of adventitious roots; NLR = number of lateral roots; T8-2-4, T8-12-5, and T8-15-41 = T4 generations of transgenic lines IR64. Bar = standard error.

Table 1. The RRE, RGI, and RGR characters of five rice genotypes under 15 ppm of Al

Genotypes	The growth characters of the main root		
	RRE	RGI	RGR
T8-2-4	0.34^{ab}	65.45^{ab}	0.49^{bc}
T8-12-5	0.42^{b}	58.16^{a}	0.26^{ab}
T8-15-41	0.26^{a}	73.51^{b}	0.08^{a}
IR64	0.26^{a}	73.25^{b}	0.06^{a}
HB	0.44^{b}	55.35^{a}	0.67^{c}

The same letter following the number in the same column showed no differences based on DMRT ($\alpha = 0.05$).
DMRT = Duncan multiple range test; RGI = root growth inhibition; RGR = root regrowth relative; RRE = relative root elongation.

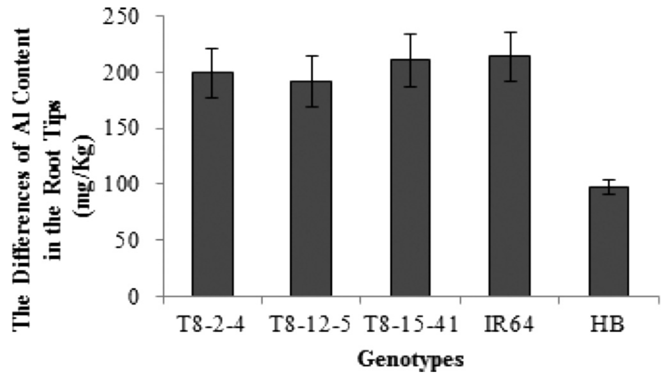

Figure 7. The differences of Al content in the root tips between without Al pH 4.0 and with 15 ppm Al treatment. HB = Al-tolerant rice; IR64 = Al-sensitive rice; T8-2-4, T8-12-5, and T8-15-41 = T4 generations of transgenic lines IR64. Bar = standard error.

IR64 and followed by T8-15-41 transgenic line, but less intense purple color in the root tips of T8-2-4 and T8-12-5 transgenic lines, even almost clear in Hawara Bunar (Figure 6).

Aluminum content in the root tips was analyzed using atomic absorption spectrophotometer (AAS). The results showed different Al content among genotypes, with the lowest content was Hawara Bunar followed by T8-12-5. Three other genotypes, IR64, T8-15-41, and T8-2-4 accumulated higher Al in the root tips (Figure 7).

3.3. Root cell membrane lipid peroxidation

Lipid peroxidation of root cell membranes was detected through qualitative and quantitative analyses. Qualitative analysis of lipid peroxidation was carried out using *Schiff's* staining method (Yamamoto *et al.* 2001). The presence of lipid peroxidation was indicated by pink color of the root tip tissue. The more intense color showed the higher level of lipid peroxidation of the cell membrane. The result showed that there was different intensity of pink color between the Al-treated and -untreated root tips. The pink color intensity was found in root tips of IR64 and T8-15-41 transgenic line (Figure 8).

Quantitative measurement of lipid peroxidation of the cell membrane can be described as the production of MDA in the cell. The MDA analysis showed that the level of MDA differs among genotypes and increased in Al-stressed roots (Figure 9). The highest MDA concentration was found in the root of IR64 (426.52 nmol/g FW) and the lowest was found in the root of the transgenic line T8-2-4 (111.11 nmol/g FW).

3.4. Root tip cell structure under aluminum stress

Root tip cell structure was observed with TEM. Under control condition without Al stress, the root tip cells of transgenic lines and IR64 have complete organelles with good cell wall (Figure 10A, B, D and E). However, when the transgenic lines and IR64 were treated with Al, the appearance of the root tip cell structure between transgenic line and IR64 was different (Figure 10C and F). The transgenic root tip cell has complete organelles (nucleus, mitochondria, cytoplasm, cell wall), which was similar to the root tip structure in control condition, but the IR64 root tip cells showed high damaged cell with the cell wall became folded with irregular cell form and lost its organelles (Figure 10C).

3.5. Rice tolerance level to Al stress

PCA based on 11 quantitative characters could group the rice genotypes into three groups of tolerance level to Al stress (Figure 11). The first two principal components explained 99.79% variation, and the main characters contributed to the grouping were Al and MDA content in the root tips. Rice var. Hawara Bunar and transgenic lines T8-2-4 and T8-12-5 were classified as Al-tolerant genotypes, whereas transgenic line T8-15-41 and var. IR64 were grouped as Al-moderate tolerant and -sensitive genotypes, respectively.

4. Discussion

In this study, we showed the different response of main root to Al treatment. The main root treated with 15 ppm Al was shorter than that of the untreated one (Figure 3). The inhibition of the root length was an initial detection of Al toxicity in plants. The inhibition

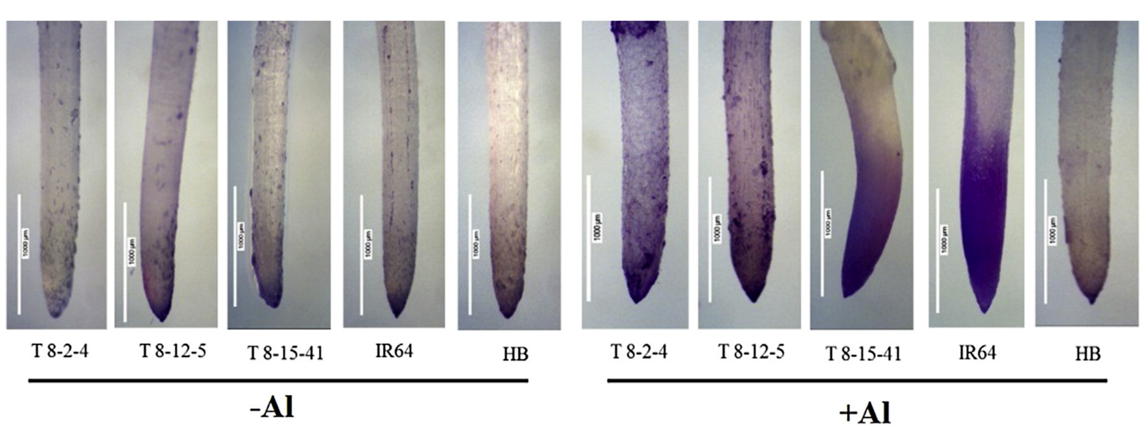

Figure 6. Hematoxylin staining of the root tips after being stressed without Al pH 4.0 and with 15 ppm of Al for 72 h. +Al = 15 ppm Al; −Al = 0 ppm Al (control); HB = Al-tolerant rice; IR64 = Al-sensitive rice; T8-2-4, T8-12-5, and T8-15-41 = T4 generations of transgenic lines IR64 = . Bar = 1000 μm.

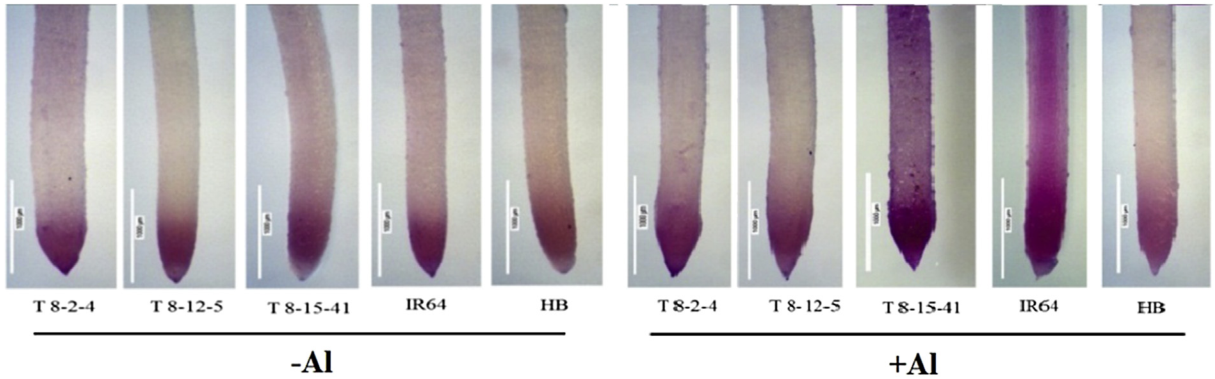

Figure 8. *Schiff's* staining of the root tips of rice genotypes after being stressed without Al pH 4.0 and with 15 ppm of Al for 72 h. +Al = 15 ppm Al; −Al = 0 ppm Al (control); HB = Al-tolerant rice; IR64 = Al-sensitive rice; T8-2-4, T8-12-5, and T8-15-41 = T4 generations of transgenic lines IR64. Bar = 1000 μm.

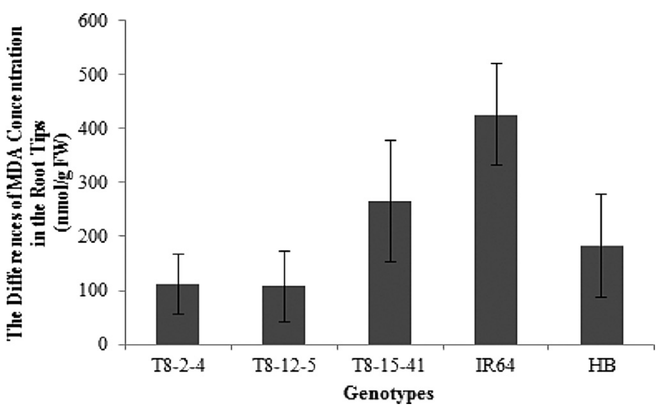

Figure 9. The differences of MDA content of the root tips of rice between without Al pH 4.0 and with 15 ppm Al treatment. HB = Al-tolerant rice; IR64 = Al-sensitive rice; MDA = malondialdehyde; T8-2-4, T8-12-5, and T8-15-41 = T4 generations of transgenic lines IR64. Bar = standard error.

of the root growth occurred because of an accumulation of Al in the root tip region (root tip, meristem cell, and elongation zone; Matsumoto 2000) even at micro-molar concentrations (Matsumoto and Motoda 2012). Based on the result, the main root of var. IR64 showed the highest inhibition of the main root length (Figure 3C), or in other words, it showed the shortest main root length compared with transgenic main root length. According to Doncheva *et al.* (2004), the presence of Al in the root could change the root architecture. Al toxicity also inhibits basipetal auxin transport in the root, which is one of the Al toxicity mechanisms in the root cell (Kollmeier *et al.* 2000).

Analysis of the root morphology under Al stress showed that all transgenic lines have the higher number and root length than that of var. IR64 (Figures 4 and 5). The transgenic lines also have higher root number, but shorter roots when compared with rice var. Hawara Bunar, the Al tolerant variety. The results indicated that transgenic lines still have root characteristic of IR64, but it was also influenced by overexpression of *B11* gene that induced root number and length with less Al inhibition.

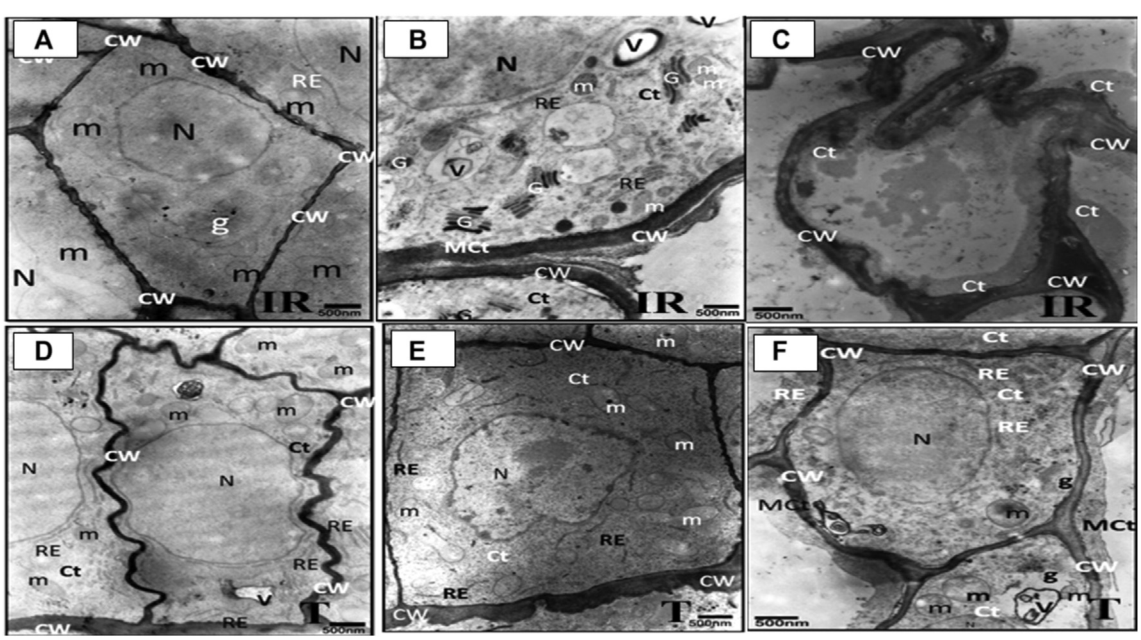

Figure 10. Root tip cell structure after treated with and without 15 ppm Al treatment for 72 h using TEM. (A and D) Control treatment without Al pH 5.8; (B and E) control treatment without Al pH 4.0; and (C and F) treatments with 15 ppm Al pH 4.0. Ct = cytoplasm; Cw = cell wall; G = golgi apparatus; IR = IR64; M = mitochondria; MCt = membrane of cytoplasm; N = nucleus; RE = reticulum of endoplasm; T = transgenic rice; TEM = transmission electron microscope; V = vacuole. Magnification 10,000×. Bar = 500 μm.

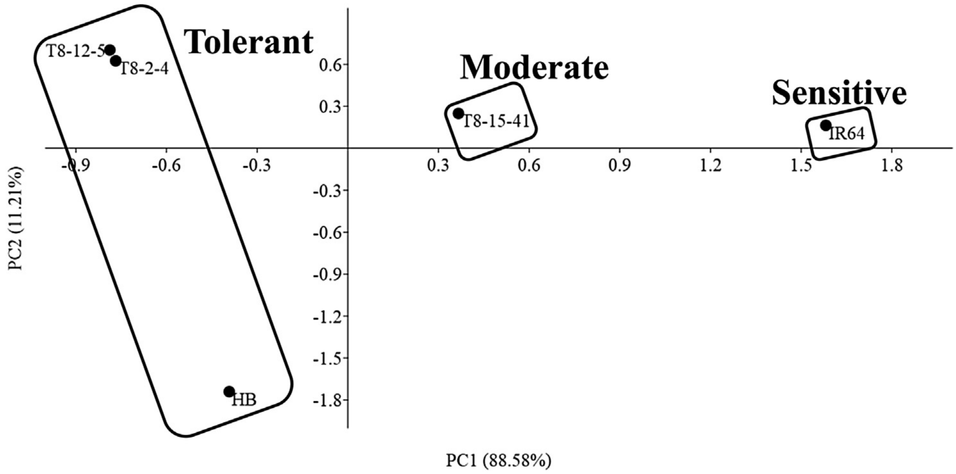

Figure 11. Principal component analysis of five rice genotypes based on 11 quantitative characters related to Al-tolerance parameters in rice. PC = principal component.

This study also observed RRE, RGI, and RGR characters as Al-tolerance parameters (Table 1). The results showed that all those three parameters in the transgenic lines T8-2-4 and T8-12-5 were not significantly different with that of Hawara Bunar, an Al-tolerant genotype where the *B11* gene is isolated. The RRE and RGR of the transgenic lines T8-2-4, T8-12-5, and var. Hawara Bunar were higher than that of IR64 and transgenic line T8-15-41, indicating that the transgenic lines T8-2-4, T8-12-5, and var. Hawara Bunar were more tolerant than IR64 and the transgenic line T8-15-41. The RRE character could be used to distinguish between Al-sensitive and -tolerant variety in maize (Doncheva 2004), and the RGR character has been used as Al-tolerance parameter in rice (Roslim 2011). The higher value of RRE and RGR showed the higher level of tolerance to Al stress. Conversely, the RGI character was the highest in IR64 and the transgenic line T8-15-41, indicating the highest inhibition of the root growth by Al stress in both genotypes. The high inhibition of the root growth will affect the limitation of nutrient absorption by roots, because it has been known that Al toxicity damages root system and inhibits water and mineral uptake from the soil (Barceló and Poschenrieder 2002). The root inhibition was related to Al-induced changes in Ca^{2+} concentration in the cytosol (Zhou *et al.* 2011), and also inhibited Ca^{2+} and K^{+} transport (Kochian *et al.* 2005). Root exposure to Al ion is also related to Ca^{2+} and Mg^{2+} deficiencies (Ridolfi and Garrec 2000). For water and nutrient absorption, the inhibition of the root growth must be decreased (Azura *et al.* 2011) and tolerant genotypes are expected to be able to alleviate Al toxicity with better grow of its roots.

The research also showed that pH can also be a limiting factor for the root growth of rice. The root growth of all rice genotypes was inhibited at pH 4.0. This phenomenon is also found in tobacco (Zulkifli 2015) and maize (Yan *et al.* 1992). There was a linear correlation of the root growth of maize and net H^{+} release between pH 3.5 and 6.5, reduction of net H^{+} release is related to the reduction of nutrient uptake, cell wall loosening, and cytoplasmic pH regulation, which consequently will reduce the root growth.

Aluminum-sensitive plant could be indicated by the high accumulation of Al in the root tip (Miftahudin *et al.* 2007; Matonyei *et al.* 2014), which can be detected qualitatively using hematoxylin staining method. A purple color will be produced in the tissue that is stained with hematoxylin. The more intense purple color indicated the higher accumulation of Al in the tissue. The results showed that Al-stressed IR64 and T8-15-41 root tips produced more intense purple color than that of transgenic lines T8-2-4, T8-12-5, and Hawara Bunar (Figure 6). The results were in agreement with Miftahudin *et al.* (2007) and Jumiati (2016) that showed darker root tip color of IR64 and less intense root tip color of Hawara Bunar after stained with hematoxylin. This indicated that IR64, an Al-sensitive genotype, accumulated more Al than that of the Al-tolerant genotype Hawara Bunar. It has been considered that tolerant genotype accumulates less Al in the root tips than that of sensitive genotype (Piñeros *et al.* 2005).

Quantitative analysis of Al accumulation in the root tips showed slightly different with staining method. All transgenic lines and IR64 accumulated Al in the root tips were higher than that of Hawara Bunar. However, although there was no significant different in Al accumulation between transgenic lines and IR64, transgenic lines T8-2-4 and T8-12-5 tend to accumulate Al less than that of IR64 and T8-15-41, which indicates that both transgenic lines tend to be more tolerant than IR64.

Aluminum toxicity causes damage to cell membranes through lipid peroxidation. It can be detected through either *Schiff's* staining method or MDA accumulation. The functional aldehyde of lipid peroxidation can be detected by *Schiff's* solution (Pompella *et al.* 1987). The color intensity produced from the staining and MDA concentration in the cell of the target tissue indicates the level of lipid peroxidation of the cell membrane (Choudury and Panda 2004). The superoxide anion was the main cause of reactive oxygen species (ROS) that was synthesized by several plant cell organelles including mitochondria, chloroplast, and membrane plasma (Matsumoto and Motoda 2012). Subsequently, ROS could induce lipid peroxidation of the cell membrane, and both are related to environmental stress including Al stress (Yin *et al.* 2010; Hamim *et al.* 2017). The result showed that transgenic lines T8-2-4, T8-12-5 and Hawara Bunar produced less pink color of *Schiff's* staining than that of IR64 and the transgenic line T8-15-41 (Figure 8). The more intense pink color the higher level of lipid peroxidation, which means the more sensitive root to the Al stress. From the result, it could be concluded that transgenic lines T8-2-4 and T8-12-5 were more tolerant than that of IR64.

Response to Al toxicity could be observed in the cell levels. Cell wall integrity under Al stress has a relation with Al tolerance in rice (Wu *et al.* 2014). The analysis of cell ultrastructure using TEM showed that the cell wall of var. IR64 under 15 ppm Al was damaged (Figure 10C), this indicates that transgenic line was more tolerant to Al stress than that of var. IR64. The tolerance level to Al was also related to Al-binding in cell wall component that could disturb the function of apoplast and symplast as a factor related to RGI (Horst *et al.* 2010). There was a change in the cell wall as a response to Al-binding in tolerant plant. Al stress for 6 h in wheat causes the cell wall to become rigid and significant decrease in root length (Tabuchi & Matsumoto 2001). Panda *et al.* (2008) suggest that the analysis of the ultrastructure of tobacco stressed with $AlCl_3$ showed shrinkage of cell structure and organelles that lead to the cell death. Li and Xing (2011) suggest that ROS production caused by Al could affect the dysfunction of cell organelles, which lead to the changes of the organelles.

The present study showed different tolerance level of five rice genotypes to Al stress. Based on the morphological and physiological responses of root to Al stress and PCA analysis (Figure 11), the five rice genotypes could be grouped into three groups of Al tolerance level, that is, rice var. Hawara Bunar and transgenic lines T8-2-4 and T8-12-5 were Al-tolerant genotypes, transgenic line T8-15-41 was Al-moderate tolerant genotype, and var. IR64 was Al-sensitive genotype. It was suggested that the increase of the tolerance level of transgenic rice T8-2-4, T8-12-5, and T8-15-41 from its wild type (IR64) was due to the overexpression of *B11* gene in rice var. IR64 that is sensitive to Al.

Conflict of interest

The authors do not have any conflict of interest.

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