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Original research article

Construction of RNA Interference Vector to Silence Aluminum Tolerance Gene Candidate in Rice cv Hawara Bunar

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ABSTRACT

One of the aluminum (Al) tolerance gene candidates, namely *B11* gene, has been successfully isolated from Al-tolerant rice cv Hawara Bunar. However, the role of the gene in Al tolerance in rice has not been known. RNA interference (RNAi) technique is an effective tool to examine the biological function of the target gene in plant. The objective of the research was to construct RNAi recombinant vector carrying untranslated region of the *B11* gene. RNAi recombinant vector carrying 195 bp sized 3′UTR_B11 fragment as a double-stranded RNA (dsRNA) trigger has been successfully constructed using GATEWAY™ cloning technology, pENTR™/D-TOPO® as a shuttle vector, and pANDA vector as a destination vector. RNAi construct was successfully introduced into *Agrobacterium tumefaciens* AgL0, and has been infected to rice cv Hawara Bunar. Analysis of putative transgenic rice showed eight of 20 plants were transgenic carrying the B11-RNAi construct.

1. Introduction

Rice (*Oryza sativa* L.) is one of the important food crops in Indonesia. Rice consumption continues to increase as the Indonesian population increases. There are many constraints to meet this demand, which one of them is the continuous decreasing of fertile rice field areas due to its conversion into industrial and residential areas. Maintaining and increasing rice production through rice extensification to marginal land such as acid soil are the continuous efforts that should be taken to meet the rice demand in Indonesia.

Rice farming in acid soils is experiencing many obstacles, one of them is high solubility of aluminum (Al) that can be toxic for rice root that considerably inhibits root growth and damages root tips (Kochian 1995). As a result, the plant roots will be inhibited in nutrient and water uptake that consequently will reduce plant growth and production (Delhaize & Ryan 1995).

Development of tolerant rice varieties to Al toxicity can be achieved through both conventional breeding and genetic engineering approaches. Genetic engineering technology gives possibility to manipulate the plants to produce new varieties with superior traits, including rice tolerant to Al stress. Identification of functional Al tolerance gene candidate is a preliminary step to achieve the goal. The function of a gene in plants can be studied through two approaches. First, one can increase the expression of the gene by constructing over-expression vector. Second, to stop or decrease the expression of the gene such as by constructing RNA interference (RNAi) vector. Once the gene function is characterized, it can be used either for marker assisted selection or for transformation of rice into Al-tolerant transgenic plant.

The RNAi technique is an effective way to test the biological function of mRNA target in plants. Recent developments regarding the RNAi vector, using constitutive promoter and Gateway™ cloning technique, makes it easy to construct the RNAi vector that has a double-stranded RNA (dsRNA) trigger sequences and also makes it easier to analyze the function of the target gene. RNAi causes degradation of mRNA that genes become dysfunctional.

Effort to isolate Al tolerance gene from rice has been initiated through revealing the syntenic relationship between Al tolerance locus region in chromosome 4RL in rye and a short region in short arm of rice chromosome 3 (Miftahudin *et al.* 2005). Through screening several markers in that rice region, Roslim (2011) has successfully isolated an Al tolerance gene candidate from an Indonesian local rice cv Hawara Bunar. The gene was then cloned into pGWB5 expression vector under 35S promoter and

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temporarily named as *B11* gene. This article reports our effort to characterize the Al tolerance gene candidate function through construction of RNAi vector.

2. Materials and Methods

2.1. Materials

B11 clone in pGWB5, pENTR™/D-TOPO® cloning kit (Invitrogen™, USA) and pANDA vector (Prof. Ko Shimamoto collection, NAIST, Japan) were used for cloning the 3′UTR and construction of RNAi recombinant vector. The *Escherichia coli* DH5α and *Agrobacterium tumefaciens* AgL0 were used as host for recombinant pENTR vector and recombinant pANDA vector, respectively. Rice cv Hawara Bunar seeds was used to develop transgenic plants.

2.2. Isolation of 3′UTR fragment

The 3' untranslated region of *B11* gene (3'UTR_B11) fragment was polymerase chain reaction (PCR) amplified from *B11* gene clone (Roslim 2011) using primers 3′UTR_B11-F and 3′UTR_B11-R (Table 1). To facilitate the insertion of the 3'UTR_B11 fragment in the cloning site of pENTR™/D-TOPO® vector, it is necessary to add a specific sequence CACC at the 5′ end of forward primer 3′UTR_B11-F. The 20 µL PCR reaction consisted of 100 ng vector DNA template, 1X PCR buffer, 0.2 mM dNTPs, 0.4 µM each primer and 1 U Taq polymerase. The PCR program was 94°C for 5 minutes pre-denaturation followed by 35 cycles of 94°C for 60 seconds denaturation, 56°C for 30 seconds annealing, 72°C for 30 seconds extension. The PCR was ended by 72°C for 10 minutes final extension. The PCR products were electrophoresed on 1% agarose gel (LE GQ Top Vision, Fermentas, Canada) in 1X Tris/borate/EDTA (TBE) buffer at 75 volt for 1 hour. The gel was then stained with ethidium bromide. Gel was observed and documented using WiseDoc Gel Documentation System (Daihan Scientific, South Korea). The 3′UTR_B11 fragment was eluted and purified from agarose gel following the procedure of Geneaid gel purification kit (Geneaid, Taiwan).

2.3. Gateway cloning of 3′UTR_B11

The cloning procedure of 3′UTR_B11 fragment was carried out based on Gateway Cloning System protocol (Invitrogen™, USA). First, the 3′UTR_B11 fragment was inserted into a shuttle vector pENTR™/D-TOPO® following the protocol provided in pENTR™/D-TOPO® cloning kit (Invitrogen™, USA). The ligated 3′UTR_B11-pENTR™/D-TOPO® vector was then transformed into *E. coli* DH5α using heat shock method (Sambrook *et al.* 1989). Transformant bacteria were grown in selection media Luria agar (LA) containing 50 ppm kanamycin for overnight at 37°C. Several growth colonies of bacteria were randomly selected for colony PCR following the procedure performed by Suharsono *et al.* (2008) using a primer pair of 3'UTR_B11-F and 3′UTR_B11-R. The vectors from positive colonies carrying recombinant pENTR™/D-TOPO® – 3′UTR_B11 vector were isolated using the Wizard Minicolumn Plasmid Extraction kit (Promega, USA) and then sequenced.

Second, the 3′UTR_B11 fragment was then cloned from the recombinant pENTR™/D-TOPO®-3′UTR_B11 into pANDA vector using the Gateway® LR Clonase ®II enzyme mix (Invitrogen™, USA) to obtain a recombinant expression vector (pANDA recombinant). Introduction of recombinant pANDA vector into competent *E. coli* DH5α and PCR colony followed the same procedure as described previously, except the transformants were grown on LA selection media containing 50 ppm of kanamycin and 50 ppm of hygromycin. Colony PCR test was performed using several primer combinations (Table 2).

2.4. Introduction of RNAi construct into *A. tumefaciens* AgL0

Introduction of recombinant pANDA vector (RNAi construct) into *A. tumefaciens* AgL0 was done by freeze-thaw method following the procedure performed by Xu and Li (2008). The transformants then were selected on LA medium containing 50 ppm of kanamycin, 50 ppm of hygromycin, and 25 ppm of rifampicin. Colony PCR was done using the same procedure as described previously.

2.5. Infection of rice embryo through *in-planta* transformation system

Introduction of RNAi construct into rice seeds cv Hawara Bunar was carried out using *in-planta* transformation technique following the procedure of Lin *et al.* (2009). The bacteria was infected to the embryo or the plumule apical meristem using sterile needles deeped in *A. tumefaciens* AgL0 culture. The infected seeds were then planted in pots containing soil media and grown in a green house. The putative transgenic tillers and plants were selected using PCR technique with GUS-F and GUS-R primers and HPT-F and HPT-R primers as well using the same PCR procedure as mentioned previously. The positive tillers or plants were then maintained to produce T1 seeds.

3. Results

3.1. Isolation of the 3′UTR fragment of *B11* gene

The 3′UTR_B11 fragment was successfully amplified from the 3′ untranslated region of the *B11* gene clone that previously has been cloned in pGWB5 (Figure 1A). After being visualized using agarose gel, the fragment was then purified and re-checked in agarose gel (Figure 1B). The fragment length of the unpurified and purified PCR products was the same as the expected length of the 3′UTR *B11* gene sequence, suggesting that the PCR products were the 3′UTR of *B11* gene. The fragment was then inserted in the pENTR™/D-TOPO® shuttle vector and maintained in *E. coli* DH5α.

Table 2. Primer combinations used to check insert orientation in recombinant pANDA vector

No.	Primer combination	
	Forward	Reverse
1	GUS_cek-F	3′UTR_B11-R
2	GUS_cek-F	3′UTR_B11-F
3	GUS_cek-R	3′UTR_B11-F
4	GUS_cek-R	3′UTR_B11-R

Table 1. Primers used in the research on RNAi vector construction

Primers	Sequences	Function
3′UTR_B11-F	5′-CACCCTG TTTCTCCCCAAGTTCAG-3′	Amplification of 3′UTR fragment and check on insert orientation
3′UTR_B11-R	5′-GTCACGAGTGGCATTTGAG-3′	Amplification of 3′UTR fragment and check on insert orientation
GUS-F	5′-CATGAAGATGCGGACTTACG-3′	Check on GUS linker and transgenic plant
GUS-R	5′-ATCCACGCCGTATTCGG-3′	Check on GUS linker and transgenic plant
GUS_cek-F	5′-CCGAATACGGCGTGGAT-3′	Check on insert orientation
GUS_cek-R	5′-CGTAAGTCCGCATCTTCATG-3′	Check on insert orientation

GUS_cek-F and R are the reversed primer sequence of GUS-F and R.

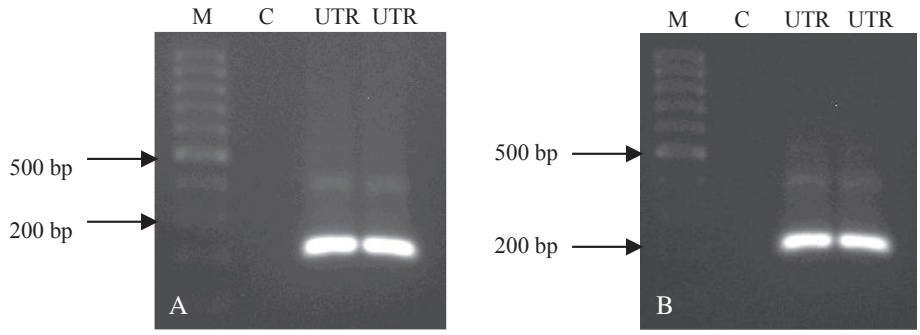

Figure 1. Electrophoregram of polymerase chain reaction (PCR) fragment amplified from *3′UTR_B11* gene (A) and purified PCR fragment (B). The fragments were electrophoresed in 1% agarose gel in 1X Tris/borate/EDTA (TBE) buffer at 75 volt for 45 minutes. C = negative control; M = 100 bp marker; UTR = 3′UTR_B11.

3.2. Recombinant pENTR-3′UTR_B11

The recombinant vector of pENTR-3′UTR_B11 was transformed into *E. coli* DH5α and subsequently grown on LA medium containing antibiotic. The transformation was successful as indicated by the colonies grew on antibiotic selection medium. The growth colonies were then verified using colony PCR and insert sequencing of the isolated recombinant vector. PCR verification of several colonies using primers 3′UTR_B11-F and 3′UTR_B11-R showed the fragments with the same length as the original size (Figure 2). Sequence analysis of the insert showed the same sequence as the original 3′UTR sequence of *B11* gene (Figure 3), which confirmed that the 3′UTR was successfully isolated from *B11* gene and cloned into pENTR shuttle vector. The recombinant vector was then isolated and used for cloning the 3′UTR into destination pANDA vector.

3.3. Recombinant pANDA-3′UTR_B11

The 3′UTR_B11 insert was cloned from the recombinant pENTR-3′UTR_B11 vector into pANDA vector using LR Clonase enzyme, which is part of the Gateway cloning system (Invitrogen™, USA). Recombination between attR1 sequence from pANDA vector and attL1 sequence from the recombinant pENTR-3′UTR_B11 vector, as well as between attR2 sequence from pANDA vector and attL2 sequence from the recombinant pENTR-3′UTR_B11 vector caused insertion of the 3′UTR_B11 fragment into pANDA vector. The region of cloning site in pANDA vector was designed such that the 3′UTR insert can be cloned in two sites with opposite orientation separated by GUS linker (Figure 4). The recombinant pANDA-3′UTR_B11 was then introduced into *E. coli* DH5α.

Introduction of the recombinant pANDA-3′UTR_B11 into *E. coli* DH5α has been successfully carried out. *E. coli* DH5α transformant could grow on LA selection media containing 50 ppm kanamycin and 50 ppm hygromycin. Randomly selected colonies were then verified with colony PCR using primers, HPT-F and HPT-R, developed from *hpt* gene (Table 1), which is included in the pANDA vector. Colony PCR result showed 574 bp sized DNA fragments (Figure 4) suggesting that the B11-RNAi construct has been successfully introduced into *E. coli* DH5α. To verify the insert and its orientation, the recombinant vector was isolated from several transformant colonies grown on LB media. Not all positive transformants of *E. coli* DH5α grow on LB media containing both kanamycin and hygromycin antibiotics. The recombinant pANDA vector could only be isolated from two colonies. The recombinant vectors were then used as template for PCR amplification of GUS linker and the 3′UTR_B11 insert to ensure that the GUS linker and the 3′UTR_B11 fragment have been inserted in recombinant pANDA vector in proper orientation arrangement. Primers used to observe the presence of GUS linker were GUS-F and GUS-R primers (collection of Prof. Ko Shimamoto, NAIST, Japan), whereas the primers used to verify the insert orientation were consisted of four primers combinations (Table 2).

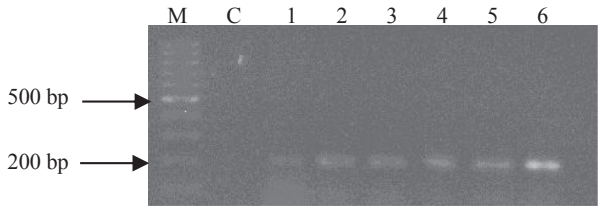

Figure 2. Electrophoregram of colony polymerase chain reaction transformation result of recombinant pENTR™/D-TOPO® vector with *Escherichia coli* DH5α bacteria which is 199 bp sized 3′UTR_B11 fragment (No. 1–6). The fragments were electrophoresed in 1% agarose gel in 1X Tris/borate/EDTA (TBE) buffer at 75 volt for 45 minutes. C = negative control; M = 100 bp marker.

The GUS linker verification showed 636 bp DNA fragment, which indicated that the GUS linker has been inserted in the recombinant pANDA vector (Figure 5). However, the GUS linker position needs to be further verified whether it resides in proper site in relation to the orientation of the 3′UTR_B11 insert. Further PCR amplification was done to confirm the 3′UTR_B11 orientation.

The PCR amplification of the recombinant pANDA-3′UTR-B11 vector using four primer combinations showed that the 3′UTR-B11 was inserted in proper orientation separated with GUS linker (Figure 6). The primer combination 1 and 4 (Table 2) identified the target insert at the right and left of GUS linker, respectively. The sizes of the DNA fragment produced from PCR amplification using both primer combinations 1 and 4 were ±517 bp (195 bp of 3'UTR_B11 + 322 bp of GUS linker) and ±436 bp (195 bp of 3'UTR_B11 + 241 bp of GUS linker), respectively. The other two primer combinations, combinations 2 and 3 (Table 2), did not produce while within the DNA fragment, because both primer combinations were designed to identify the wrong orientation of the target insert. The 3′UTR_B11 insert along with the GUS linker with proper orientation was then called as B11-RNAi construct.

3.4. Introduction of recombinant pANDA vector into *A. tumefaciens* AgL0

The introduction the recombinant pANDA vector into *A. tumefaciens* AgL0 has been successfully carried out. Colonies of transformant *A. tumefaciens* AgL0 grew on LA selection media containing 50 ppm of kanamycin, 50 ppm of hygromycin, and 25 ppm of rifampicin. Colony PCR was performed using the same primer combinations performed to identify the orientation of target gene in the recombinant pANDA vector (Table 2) and the result of colony PCR was shown in Figure 7.

>UTR – GUS – UTR
CTGTTTCTCCCCAAGTTCAGTCTGCACTCAGCACATCAAGCAATGTCTGAACACTATTCCACCATGAACGTTTTCAGA
TCTCATTTTGTGTTCGGCCGCTGTTCTGTTTTCAAGCTTTTTAATCAGTGTACACACTTTAAACCCAGCTTGGATTTC
AAAACTCCCCTTGTCAAAAACTCAAATGCCACTCGTGACATCTACCCGCTTCGCGTCGGCATCCGGTCAGTGGCAGTC
AAGGGCGAACAGTTCCTGATTAACCACAAACCGTTCTACTTTACTGGCTTTGGTCGTCATGAAGATGCGGACTTACGT
GGCAAAGGATTCGATAACGTGCTGATGGTGCACGACCACGCATTAATGGACTGGATTGGGGCCAACTCCTACCGTACC
TCGCATTACCCTTACGCTGAAGAGATGCTCGACTGGGCAGATGAACATGGCATCGTGGTGATTGATGAAACTGCTGCT
GTCGGCTTTAACCTCTCTTTAGGCATTGGTTTCGAAGCGGGCAACAAGCCGAAAGAACTGTACAGCGAAGAGGCAGTC
AACGGGGAAACTCAGCAAGCGCACTTACAGGCGATTAAAGAGCTGATAGCGCGTGACAAAAACCACCCAAGCGTGGTC
ATGTGGAGTATTGCCAACGAACCGGATACCCGTCCGCAAGTGCACGGGAATATTTCGCCACTGGCGGAAGCAACGCGT
AAACTCGACCCGACGCGTCCGATCACCTGCGTCAATGTAATGTTCTGCGACGCTCACACCGATACCATCAGCGATCTC
TTTGATGTGCTGTGCCTGAACCGTTATTACGGATGGTATGTCCAAAGCGGCGATTTGGAAACGGCAGAGAAGGTACTC
GAAAAAGAACTTCTGGCCTGGCAGGAGAAACTGCATCAGCCGATTATCATCACCGAATACGGCGTGGATACGTTAGCC
GGGCTGCACTCAATGTACACCGACATGTGGAGTGAAGAGTATCAGTGTGCATGGCTGGATATGTATCACCGCGTCTTT
GATCGCGTCAGCGCCGTCGTCGGTGAACAGGTATGGAATTTCGCCGATTTTGCGACCTCGCAAGGCATATTGCGCGTT
GGCGGTAACAAGAAAGGGATCTTCACTCGATCGAATTCCTGCAGCCCGGGGGATCCACTAGATGCATGCTCGAGCGGC
CGCCAGTGTGATGGATATCTGCAGAATTCGCCCTTATCACAAGTTTGTACAAAAAAGCAGGCTCTGTTTCTCCCCAAC
TTCAGTCTGCACTCAGCACATCAAGCAATGTCTGAACACTATTCCACCATGAACGTTTTCAGATCTCATTTTGTGTTC
GGCCGCTGTTCTGTTTTCAAGCTTTTTAATCAGTGTACACACTTTAAACCCAGCTTGGATTTCAAAACTCCCCTTGTC
AAAAACTCAAATGCCACTCGTGACACCCAGCTTTCTTGTACAAAGTGGTCCCN

Figure 3. Sequence of orientation 3′UTR_B11 fragment in pANDA recombinant vector. Yellow color shows the sequence of 3′UTR_B11. The grey color shows *GUS* gene sequence. The sequence results that the orientation of 3′UTR_B11 was inverted repeat.

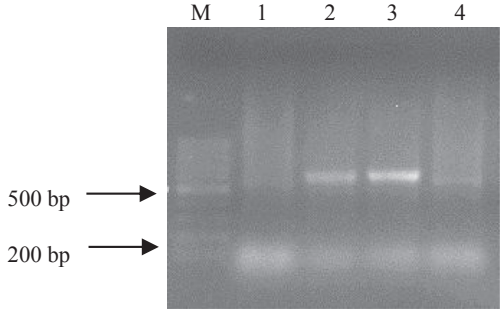

Figure 4. Electrophoregram of colony polymerase chain reaction recombinant pANDA vector using hygromycin primer with fragment size of 574 bp (No. 1–4). The fragments were electrophoresed in 1% agarose gel in 1X Tris/borate/EDTA (TBE) buffer at 75 volt for 45 minutes. M = 100 bp marker.

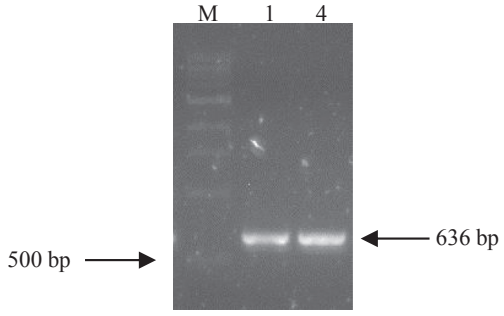

Figure 5. Electrophoregram of colony polymerase chain reaction recombinant pANDA vector using GUS-F and GUS-R primers (No. 1 and 4). The fragments were electrophoresed in 1% agarose gel in 1X Tris/borate/EDTA (TBE) buffer at 75 volt for 45 minutes. M = 1 kbp marker

Among 12 colonies PCR was tested, four colonies, namely 5, 6, 7, and 8, showed the expected size of the DNA fragment as the fragment size from the insert orientation verification. Furthermore, the colonies were grown in LB liquid selection media containing 50 ppm of kanamycin, 50 ppm of hygromycin, and 25 ppm of rifampicin, then were grown on the LA solid selection with the same antibiotics. This propagation was to create a sufficient *A. tumefaciens* AgL0 suspension to infect rice seeds.

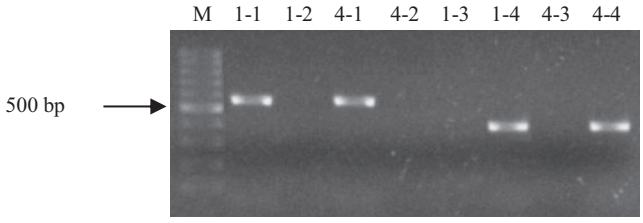

Figure 6. Electrophoregram of polymerase chain reaction result of recombinant pANDA vector using several primer combinations. First number on column number shows colony number, and second number shows primer combination. The fragments were electrophoresed in 1% agarose gel in 1X Tris/borate/EDTA (TBE) buffer at 75 volt for 45 minutes. M = 100 bp marker.

3.5. Putative transgenic plant carrying B11-RNAi construct.

The B11-RNAi construct has been successfully introduced into rice cv Hawara Bunar through *in-planta* infection. Putative transgenic plants were tested by PCR using GUS-F and GUS-R primers and expected to have 636 bp DNA fragment (Figure 8). Twenty putative transgenic rice were planted on pots in a green house and produced a total of 112 tillers. Among 20 plants, eight plants were confirmed as transgenic plants carrying the B11-RNAi construct. There were no chimera detected in each transgenic plants, which was all transgenic plants having all transgenic tillers. If the transformation efficiency was calculated based on comparison between number of positive tillers and total tiller evaluated from all transformed plants, the transformation efficiency reached 45.53% (Table 3).

4. Discussion

The 3′UTR has been successfully isolated from the *B11* Al tolerance gene candidate and cloned into pENTR™/D-TOPO® vector and subsequently into pANDA vector through the Gateway™ system. Gateway™ technology is an advance cloning technology based on site-specific recombination reaction of phage lambda. The technology is very easy to construct RNAi vector compared with conventional cloning techniques. RNAi technique is an effective way to test the function of the target mRNA in plant biology. Recent developments on the RNAi vector, which uses a constitutive promoter

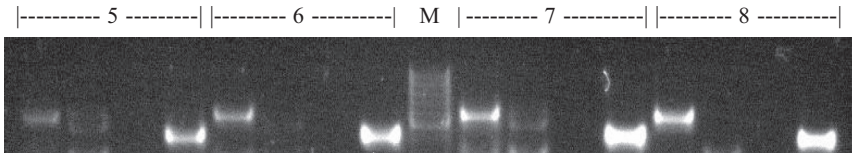

Figure 7. Electrophoregram of colony polymerase chain reaction of recombinant pANDA vector using several primer combinations. Number shows colony number, each column is primer combination. The fragments were electrophoresed in 1% agarose gel in 1X Tris/borate/EDTA (TBE) buffer at 75 volt for 45 minutes. M = 100 bp marker.

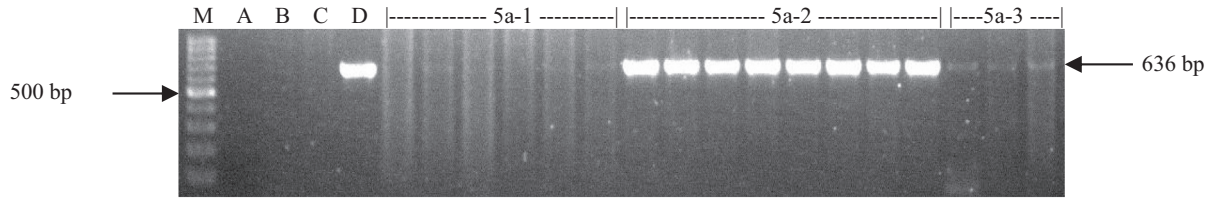

Figure 8. Electrophoregram of polymerase chain reaction transgenic plant using GUS primer A, B, C = negative control (water, IR64, Hawara Bunar WT, respectively). The fragments were electrophoresed in 1% agarose gel in 1X Tris/borate/EDTA (TBE) buffer at 75 volt for 45 minutes. D = positive control (pANDA_3′UTR_B11). M = 100 bp marker.

Table 3. Transformation efficiency of *in-planta Agrobacterium* mediated transformation technique to rice seeds cv Hawara Bunar

Sample no.	Positive tillers	Total tillers	Efficiency (%)
1b-1	4	4	100
1b-2	7	7	100
3-2	0	5	0
3-3	8	8	100
3-4	6	6	100
3-5	0	2	0
5a-1	0	8	0
5a-2	8	8	100
5a-3	0	4	0
5a-4	0	6	0
5a-5	4	4	100
5-4	0	3	0
5b-1	0	6	0
5b-2	0	8	0
5b-3	0	7	0
5b-4	0	3	0
6-1	6	6	100
6-4	0	6	0
6-5	0	3	0
8b-1	8	8	100
Total	51	112	45.53

cloning techniques and Gateway™ technologies, makes it easy to construct the RNAi vector which has a dsRNA trigger sequences and facilitates functional analysis of the target gene.

In this new technique, trigger sequence was obtained by amplification using PCR and then be cloned into RNAi vectors with the desired orientation. One of the RNAi Gateway technologies is the use of pANDA vector as a destination vector, which has a corn *ubiquitin1* promoter and GUS linker. The pANDA vector was developed by Miki & Shimamoto (2004). The vector shows high effectiveness in disrupting the expression of specific genes and specific gene family members. The expression of *phytoene desaturase (PDS)* gene and GTPase *Rac1* gene small family has been successfully suppressed using specific sequences that were cloned into pANDA vector (Miki *et al.* 2005). It is expected that B11-RNAi construct that used the 3′ UTR of *B11* gene could silence the *B11* gene in rice cv Hawara Bunar where the gene was isolated from.

RNAi is RNA that interferes existing RNA function in the cell. The presence of RNAi causes RNA in the cell to be bound by RNAi to form a dsRNA. dsRNA will be cut by the enzyme Dicer in the cytoplasm into a short dsRNA fragments size 21–26 nucleotides to the 3′ end of two nucleotide overhangs (Pickford & Cogoni 2003). One of the two strands of each fragment, which is known as the guide strand, was associated with the target mRNA (Hammond *et al.* 2000). A catalytic component of RNA-induced silencing complex called Argonaute (Ahlquist 2002), activates the function of RNA-induced silencing complex to degrade the target mRNA and suppress gene expression at various levels (Hannon 2002; Meister and Tuschl 2004).

The B11-RNAi construct has been introduced to rice cv Hawara Bunar through *in-planta Agrobacterium* mediated transformation technique. The *Agrobacterium* suspension was inoculated to the rice seeds. Targets of the *in-planta* infection in seed are apical meristem cells found in the embryo, or more precisely on the apical meristem of plumula (Lin *et al.* 2009). However, the embryo in the seed certainly has shaped organ primordia that have been first differentiated. If *A. tumefaciens* infects the area primordial that are already differentiated, then most likely generated chimera of T0 plants, therefore the T0 generation required strict selection to obtain T1 transgenic seeds. Insertion test should be performed on each tillers of transformed plants. In this research, selection was done by PCR technique using GUS-F and GUS-R primers developed from GUS linker sequence. Based on the insertion test, it could be found that one plant has transgenic and non-transgenic tillers (chimera), but there is also possibility that the whole tillers are transgenic. Careful infection technique greatly affects the success of the infection and can be one of the factors affecting variation of transformation efficiency among genotypes. In addition, different responses to the infection among plants or genotypes also contribute to the transformation efficiency. The infection result will be the best if target is on plumula meristem. If plumula is pierced directly (not plumula meristem), there will be a fatal error and seed will fail to germinate (Lin *et al.* 2009). This research has obtained eight positive transgenic plants with all transgenic tillers indicated no chimera produced. The transformation efficiency was 45.53% (Table 3).

The transgenic rice plants will then be grown to produce T1 seeds, which subsequently will be used for further Al tolerance analysis. It is expected that the transgenic plant carrying B11-RNAi construct will be susceptible to Al stress than that of wild-type cv Hawara Bunar that is originally tolerant to Al stress. If this is true then the *B11* gene is confirmed to have a role in Al tolerance mechanism in rice. It could be one of the Al tolerance genes in rice that could be used to develop Al tolerant rice cultivar.

Conflict of interest

There is no conflict of interest.

Acknowledgements

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