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Full Length Article

Transgene Insertion Stability and Aluminum Tolerance Candidate Gene Expression in T_3 Generation of Transgenic Tobacco

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Abstract

An aluminum (Al) tolerance gene candidate, called *GERLP* gene, has been successfully isolated from an Indonesian local rice cv. Hawara Bunar, and transformed into tobacco plants under CaMV 35S strong promoter. The genetic stability of the gene insertion and its expression in the transgenic plant needs to be verified. This study was conducted to analyze the genetic stability of the *GERLP* gene insertion and the expression of the *GERLP* gene and two Al related genes in T_3 generation of the transgenic tobacco. Putative transgenic seeds were selected on hygromycin containing MS media for 28 days. Hygromycin resistant plants were then verified with PCR analysis using primers developed from the *GERLP* gene sequence. The expressions of the *GERLP* gene and two Al related genes were analyzed using qPCR technique. The result showed that 24 lines out of 26 T_2 lines were able to grow on selective medium with ratio of 3:1 for hygromycin resistant to sensitive. The other two lines showed consistently complete germination on selective medium until T_3 generation, which indicates that both lines were homozygous for *hpt* gene. Since the hpt gene is located near left border downstream the *GERLP* gene in the recombinant plasmid construct, it suggests that both lines were also homozygous for the GERLP gene.. Genetic stability analysis in T_2 and T_3 transgenic tobacco plants revealed that the transgenic tobacco plants carried both *GERLP* and *hpt* genes. The gene expression analysis shows that the increasing expression of the *GERLP* gene in the transgenic tobacco is followed by the increase of the *STOP1* and *ALMT1* gene expressions, which indicates that there is a regulation relationship between these three genes. This finding suggests that the *GERLP* gene expression regulates the expression of the *STOP1* and *ALMT1* genes.

Keywords: Aluminum tolerance; Gene expression; Genetic stability; Transgenic tobacco

Introduction

Aluminum (Al) is an abundant metal in the soil. When the soil pH < 5, Al^{3+} is dominant in soil and toxic to crops. Al toxicity is one of major limiting factor for plant growth and production in acid soils (Samac and Tesfaye, 2003). Delhaize and Ryan (1995) stated that mineral nutrition like P can be chelated by Al and will not be available for root, causing P deficiency and plant growth inhibition. Other researchers reported that Al toxicity inhibits cell divisions and roots elongation (Kochian, 1995; Ma and Hiradate, 2000). Those inhibitions relate to the ability of Al interaction to cell wall components of the root, which could then stop the mitotic process (Matsumoto, 2000). Al toxicity may damage the root system, which makes the plants sensitive to drought and mineral deficiency (Kochian, 1995; Samac and Tesfaye, 2003; Kochian *et al.*, 2004).

Fortunately, there are several Al tolerant plant species or genotypes that can be used to develop valuable Al tolerant varieties. Those plants show good root growth when grown on high Al soluble soil. Kim *et al.* (2001) consider that rice is the most Al-tolerant plant among cereals and has complex Al tolerance mechanism involving multiple genes. Wheat (*Triticum aestivum* L.) has rather simple Al tolerance mechanism, which involves secretion of organic acids such as malic acid (Delhaize *et al*., 1993) and citric acid (Li *et al.*, 2000). The secreted organic acid may chelate and detoxify Al in the rhizosphere restricting the Al ion entering the wheat root cells (Delhaize *et al.*, 1993; Sasaki *et al.*, 2002).

Aluminum activated malate transporter (*ALMT*) and multidrug and toxic compound extrusion (*MATE*) belong to gene families controlling malate and citrate efflux from rye and wheat roots (Li *et al.*, 2000; Sasaki *et al*., 2004). In certain cases, over-expression of *TaALMT1* genes in wheat (Pereira *et al*., 2010) and barley (Delhaize *et al.*, 2009) improved the tolerance to Al toxicity. Over-expression of

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MATE in wheat and barley also increase the tolerance to Al toxicity (Zhou *et al.,* 2013). Conversely, knocout of *AtALMT1* and *AtMATE* genes in *Arabidopsis* decreases Al tolerance of the plant (Liu *et al.,* 2008).

An Al resistance transcription factor 1 (*ART1*) gene is reported as a gene encoding a transcription factor that involved in Al tolerance in rice (Yamaji *et al*., 2009). The gene regulates the expression of *OsFRDL4* that controls citrate secretion in rice (Yokosho *et al.,* 2011). The *ART1* homolog gene in *Holcus lanatus*, *HlART1*, also regulates the expression of *HlALMT1,* the gene encoding malate secretion from *H. lanatus* (Chen *et al*., 2013). The ART1 protein is homolog to STOP1 protein in *Arabidopsis*. *AtSTOP1* gene regulates the expresssion of *AtMATE1* and *AtALMT1* in *Arabidopsis* (Sawaki *et al.,* 2009) similar to the *ART1* gene role in rice and *H. lanatus*.

Roslim (2011) has isolated an Al tolerance candidate gene, called *GERLP* (Gene Encoding Ribsomal L32-Like Protein) gene, from an Indonesian local rice cv. Hawara Bunar that is tolerant to Al stress. The gene has been successfully introduced into tobacco plants under strong promoter 35S CaMV through *Agrobacterium*-mediated transformation. Genetically stable transgenic plants that carry a transgene that is inherited to the next generation are required (Jones and Shewry, 2009; Kempken and Jung, 2010) in order to be used as a model plant to study the role of the gene in Al tolerance mechanism. The stability test to transgenic tobacco plants carrying the *GERLP* gene had indicated that the first generation of transgenic tobacco plants (T_1) carried the *GERLP* gene. The stability test of the *GERLP* gene integration into the genome of transgenic tobacco until T_3 generation and selection of the seeds to obtain homozygous lines for the *GERLP* alleles need to be done in order to obtain a stable homozygous transgenic plant for further *GERLP* gene function analysis.

Function analysis of the *GERLP* gene in T_1 transgenic tobacco showed that the gene increased Al tolerance in transgenic tobacco, which suggests that the gene has function in Al tolerance mechanism (Roslim, 2011). However, the *GERLP* gene expression in the transgenic tobacco has not been analyzed. It is not known whether the *GERLP* gene directly invloved in Al tolerance mechanism or indirectly through regulation of other Al related gene expression. One of the approaches to answer the question is by analyzing the expression of the *GERLP* gene and other Al related genes in the transgenic tobacco uder Al stress condition.

Bioinformatic analysis of the predicted GERLP protein suggests that the GERLP protein is a transcription factor that regulates the expression of other gene/s. Since the *GERLP* gene is able to increase the T_1 transgenic tobacco tolerance to Al stress, it is hypothesized that the expression of the *GERLP* gene has regulation coordination with other Al related genes. This paper reports the genetic stability of the *GERLP* transgene insertion and the expression pattern of the *GERLP* gene and other Al related genes in T_3 generation of transgenic tobacco. The objectives of the study were to evaluate the stability of *GERLP* transgene insertion in the T_2 and T_3 transgenic tobacco plants and to analyze the expression of the *GERLP* gene and two other Al related genes, *ALMT1* and *STOP1*, under Al stress.

Materials and Methods

Plant Materials

Transgenic tobacco seeds generation T_2 and T_3 were used in this research. The seeds were derived from T_1 seeds that produced from *Agrobacterium*-mediated transformation using *GERLP* gene construct in pGWB5 under strong promoter 35SCaMV (Roslim, 2011; Fig. 1).

Transgenic Seed Selection and Production of T_3 Generation Seeds

The T_2 transgenic seeds were selected on the MS (Murashige and Skoog, 1962) selection media MS + 112 mg/L Vitamin B5 containing 50 µg/mL hygromycin at pH 5.8. The seeds were grown at 25°C, 12 h light a day for 28 days. The resistant seedlings were then transferred to bottles containing MS + Vitamin B5 media and maintained in the same condition for 14 more days (Roslim, 2011). The selected seedlings were then transferred to acclimatization media containing sand and placed in room temperature (25°C) with 12 h light a day for 14 days. The viable seedlings were then planted in pots containing soil and compost mixture with the ratio of 3:1 (w/w) and grown in greenhouse to produce T_3 generation seeds. The T_3 seeds were then selected on hygromycin selection media as previous T_2 selection procedure.

Al Stress Treatment

The selected T_3 seeds were grown on half strength MS media (Murashige and Skoog, 1962) at 25°C 12 h light a day for 7 days and then treated with 300 µM Al in liquid nutrient medium followed Delhaize and Ryan (1995) for 21 days. Shoot and root length, fresh and dry weight were observed.

Transgene Stability Analysis of Transgenic Plants

Analysis of transgene stability was carried out using PCR technique. Total DNA was isolated from 0.2 g fresh leaves of transgenic tobacco using CTAB method (Saghai-Maroof *et al.,* 1984). The DNA was then used as a template for PCR amplification using primers GERLP_EXP (Forward: 5'-CACCATGGCGGCGGC GGCTTGGTCGGG-3', Reverse: 5'-GCTTGATGATTAGCCGTCGTTCCCGGGT-3') and HPT (Forward: 5'-GATGTTGGCGACCTCGTATT-3', Reverse: 5'-GATGTAGG GCGTGGAGGATA-3') primers.

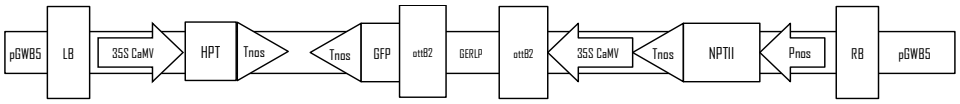

Fig. 1: The linear map of recombinant pGWB5 with *GERLP* gene (Roslim, 2011)

The PCR reactions were prepared using KAPA2G ™ Fast Mix kit (Kapa Biosystems, USA). The PCR program consisted of pre-denaturation step at 95°C 3 min; followed by 35 cycles of denaturation at 95°C 15 sec; annealing at 65°C for GERLP primers and 60°C for HPT primers 15 sec; elongation at 72°C 15 sec; followed by final extension at 72°C 10 min. The amplification products were then migrated on 1% agarose gel in 1X TBE buffer, at 65 volts for 45 min. The gel was stained with 5 µg/mL of ethidium bromide dye and visualized on UV transiluminator (WiseUv wuv-M20, Daihhan Sci. South Korea) and recorded using a gel photo (WiseDoc © Gel Documentation System, Daihhan Sci. South Korea).

Total RNA Isolation

A total of 0.1 g root tips (<1 cm) was cut and put in a fresh and sterile 2 mL microtube and grinded using stainless steel stick while occasionally dipped into liquid nitrogen. RNA isolation was performed using Trizol reagent following the manufacturer's instructions (*Invitrogen*®, USA). The total RNA was then treated with DNaseI enzyme to remove DNA contamination. The reaction was consisted of 10x DNaseI buffer, 1 µg total RNA, 1 U DNaseI and 0.01% DEPC (diethyl polycarbonate) treated water to a total volume of 10 µL. The reaction mixture was then incubated at 37°C for 30 min followed by adding 1 µL 50 mM EDTA pH 8.0 and incubated at 65°C for 10 min to inactivate the DNAseI enzyme. Tube was immediately placed on ice for 30 min. The RNA was electrophoresed on a 1% agarose gel in RNAse free 1x TBE buffer at 65 volts for 1 h.

cDNA Synthesis

Synthesis of cDNA from total RNA used *SuperscriptIII First-Strand Synthesis System* kit (Invitrogen®, USA) with oligo d(T)-21 as primer. The reaction composition was as followed: 1.5 µg RNA, 1 µL 50 µM primer oligo d(T)-21, 1 µL 10 mM dNTP and RNAse free H_2O to a total volume of 13 µL. The reaction mixture was then incubated at 65°C for 5 min and then quickly chilled on ice for not less than 1 min in order to primer remain attached. Another reaction mixture was made with composition of 4 µL 5 x buffer, 1 µL 0.1 mm DDT, 1 µL enzyme *SuperscriptIII*. Both rections were mixed and then incubated in a PCR machine with the following program: 50°C for 45 min, followed by 70°C for 15 min. The cDNA was then stored at -20°C until used.

Quantitative Real-Time Polymerase Chain Reaction

The cDNA was used for Quantitative Real Time PCR (qPCR) template using three primer sets i.e., GERLP_EXP primers (Forward: 5'-CACCATGGCGGCGGCGGGTTGTC-3', Revese: 5'-TTATGAGCTTGAGTCGCCGGGGTTCCCT-3'), STOP1 (Forward: 5'-CAAAGCCGTCTTCATCTGGC-3', Revese: 5'-CCTCCTT CTCCAACTGCAGA-3'), and ALMT (Forward: 5'-GACACACTTGGGAAAG GAGT-3' Revese: 5'-ATGTAGA TCTTCACCAGCCC-3'). As internal control of endogenous gene expression, actin gene was amplified using primers developed from actin gene sequence (Forward: 5'-CCTCTTAACCCGAAGGCTAA-3', Revese: 5'-GAAGGTTGGAAAAGGACT-3'). Quantitative PCR (qPCR) reaction was prepared using the KAPA SYBR® FAST qPCR Kit with composition of 10 ng cDNA template, 10 µL 2x KAPA SYBR® FAST qPCR master mix, 0.4 µL 10 µM each forward and reverse primers, 0.4 µL 50x ROX Reference Dye High (fluorescence detection) and PCR grade water until volume 20 µL. qPCR program was initiated with 95°C 3 min, followed by 40 cycles of denaturation 95°C 3 sec; annealing 64°C (*GERLP* and STOP1), 60°C (ALMT), 56°C (Actin) for 30 sec.

Standard curves for both normalizer gene (houskeeping gene) and target gene of interest were made using the same sample to determine an acceptable range of deviation. ΔCt (Ct normalizer-Ct target) was plotted against dilution or input amount of RNA to obtain a slope. While a perfectly slope = 0 indicates identical efficiency across all input concentration, a slope of <0.1 is generally considered acceptable when employing the ΔΔCt. For example, a ΔCt range from 2.0 - 2.5 with a slope of 0.0393 is considered acceptable when employing the ΔΔCt.

Data Analysis

Relative expression of the gene of interest was analyzed based on comparative quantification with ΔΔCt method. Ct (Cycle threshold) was the number of cycles required for the flourecent signal to croos the threshold, while ΔCt was the difference Ct value between both gene of interest (GOI) and normalizer gene. The ΔΔCt method compared ΔCt from target gene with both ΔCt calibrator (wild type sample) and ΔCt normalizer (housekeeping gene). Ct values for the GOI in both target samples and calibrator sample are adjusted in relation to a normalizer (norm) gene Ct from the same samples. The resulting ΔΔCt value is incorporated to determine the fold difference in expression as the following formula:

$$\Delta\Delta Ct = \Delta Ct_s - \Delta Ct_c$$
$$\Delta Ct_s = Ct_{GOI}{}^s - Ct_{norm}{}^s$$
$$\Delta Ct_c = Ct_{GOI}{}^c - Ct_{norm}{}^c.$$

Results

Antibiotic Selection of Transgenic Tobacco

In this research, transgenic seed selection was carried out using higromycin antibiotic, which was one of the selectable markers in recombinant plasmid pGWB5_*GERLP* (Fig. 1). The higromycin concentration applied for the selection was determined based on the preliminary experiment with several concentrations of antibiotic applied to the wild type plants. The application of 50 µg/mL hygromycin for 3-4 weeks to the wild type indicated sensitivity evident i.e., the plants produced short roots (< 1 cm) and only 2 small green leaves in 3 weeks, and the leaf colour changed into white in following 4 week (Fig. 2A-B). The whole plants became white and die after 7–8 weeks antibiotic treatment (Fig. 2C-D). In contrast, the transgenic plants exhibited resistance to hygromycin after 8 weeks treatment, which were indicated by root elongation to more than 1 cm, and produced more than 2 green leaves. Therefore, we used 50 µg/mL hygromycin as selective concentration for selection purposes.

The selection analysis results showed that among 26 T_2 lines selected, 24 T_2 lines segregated for the character with the ratio of 3 to 1 for hygromycin resistant to sensitive (χ^2 calculation $_{(3:1)(db=1;\alpha=0,05)} < \chi^2$ table $_{(3:1)(db=1;\alpha=0,05)}$, 3.841) in each line, while two other T_2 lines showed no segregation or homozygous resistant to hygromycin. The segregation of hygromycin resistant character continued in T_3 generation. Among 17 T_3 lines analyzed, 8 T_3 lines segregated for the character with the ratio of 3 to 1 for hygromycin resistant to sensitive (χ^2 calculation $_{(3:1)(db=1;\alpha=0,05)} < \chi^2$ table $_{(3:1)(db=1;\alpha=0,05)}$, 3.841) in each line, while 9 other T_3 lines indicated homozygous resistant to hygromycin.

Transgene Stability of T_2 and T_3 Generation

All T_2 and T_3 transgenic lines that resistant to hygromycin were PCR analyzed using primers developed from the *GERLP* and *HPT* gene sequences to confirm the presence of transgene in the transgenic lines. The result showed that all analysed transgenic lines produced the GERLP and HPT band size as previously predicted, which were approximately 340 and 570 bp, respectively (Fig. 3 and 4). There was no corresponding PCR band in the wild type for both GERLP and HPT bands.

Growth Responses of Transgenic Tobacco to Al Stress

One of the T_3 transgenic lines, 15-11-3 and its wild type was grown on half strength of MS media for 7 days, followed with 300 µM Al treatment in nutrient culture media (Delhaize and Ryan, 1995) to evaluate the growth responses to Al stress. After being Al stressed for 14 days the wild type showed severe growth inhibition in all parameters observed i.e., root and shoot length and biomass fresh and dry weight, whereas the transgenic tobacco grew better in both Al and no Al nutrient culture (Fig. 5 and 6).

When the wild type and transgenic line were grown on nutrient culture without Al, the shoot and root length were not different between both genoypes. However, treatment with Al stress to both genotypes significantly reduced the shoot and root length of the wild type, whereas the Al stress only less affected both parameters in the transgenic laine. The similar response was also observed in both dry and fresh weight of biomass. The reduction in biomass fresh and dry wieght was much higher in the wild type than that in the transgenic line (Fig. 5).

Analysis of Gene Expression in T_3 Generation of Transgenic Tobacco Plants

The expression of the *GERLP* gene and two other Al related genes, *STOP1* and *ALMT1*, were analyzed in both wild type and transgenic line using qPCR. The wild type plant did not express the GERLP gene in both Al-stressed and unstressed plants. However, the Al-stressed transgenic line expressed the *GERLP* gene 5.4 fold higher than that of unstressed tansgenic line (Fig. 7).

The expression of the *STOP1* gene showed similar pattern with the expression of the *ALMT1* gene. The expression of the *STOP1* and *ALMT1* genes were 2.9 and 3.3 fold higher in the transgenic line than that in the wild type, respectively. When the wild type was stressed with 300 µM Al for 24 h, the expression of both genes were induced up to 5.6 and 7.2 fold, which indicated that the expression of both genes were upregulated by Al stress. In the same Al stress condition, the transgenic line expressed both genes even higher than that in the wild type and unstressed transgenic lain (Fig. 7).

Discussion

The selection of transgenic tobacco seeds was conducted to obtain T_2 and T_3 seeds that consistently carried the *GERLP* gene insert. This selection used hygromycin antibiotic, which is highly toxic to plants. Hygromycin poisoning plants by inhibiting the protein synthesis through bothering tRNA to stick to the ribosome. The hygromycin resistance character is controlled by *hpt* gene, which is one of the antibiotic markers contained in the recombinant plasmid *GERLP* carrier. Plants containing the *hpt* gene, such as the transgenic tobacco used in this study, has the ability to produce *hygromycin phosphotrasferase* (HPT), an enzyme that phosphorylates hygromycin causing the antibiotic becomes inactive (Pardon *et al.*, 1985; McCoy *et al.*, 2011), therefore the plants become resistant to hygromycin and grow well in hygromycin containing media. Effective concentration of hygromycin used for selection is the lowest concentration that can suppress the growth of sensitive cells but do not cause a negative effect on the growth of transformed cells (Bashir *et al.*, 2004).

Fig. 2: The performance of tobacco seedlings on antibiotic selection media (MS+VitB5+50 µg/mL hygromycin). The pictures show four week-old transgenic tobacco (A) and its wild type (B), as well as eight week-old transgenic tobacco (C) and its wild type (D)

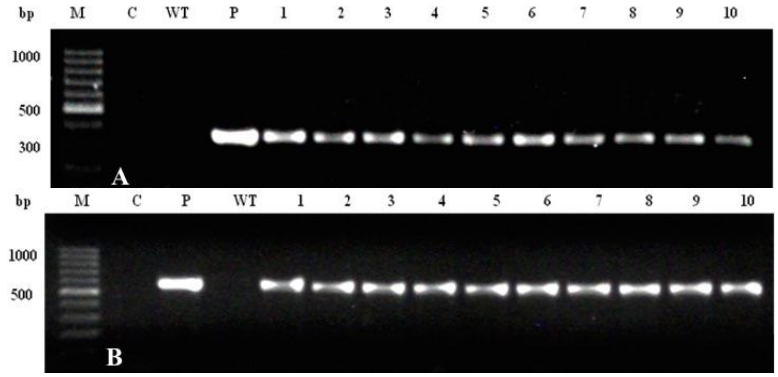

Fig. 3: Eletrophoregram of PCR products in hygromycin resistant T_2 transgenic lines using GERLP (A) and HPT (B) primers. M: marker 100 bp, C: control without DNA template, P: recombinant pGWB5_*GERLP*, WT: wild type, 1-10: T_2 transgenic lines

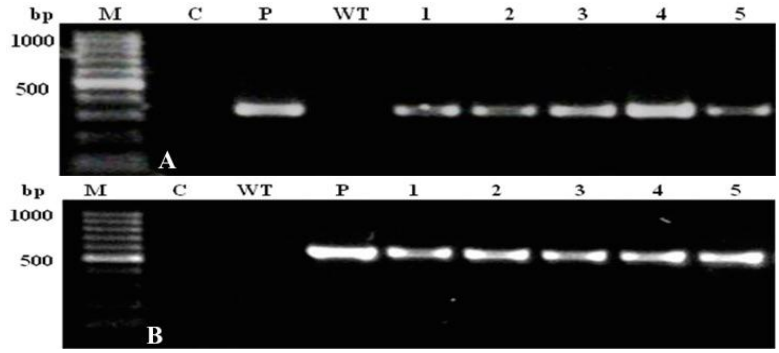

Fig. 4: Eletrophoregram of PCR products in hygromycin resistant T_3 transgenic lines using GERLP (A) and HPT (B) primers. M: marker 100pb, C: control without DNA template, P: recombinant pGWB5_*GERLP*, WT: wild type, 1-5: T_3 transgenic lines

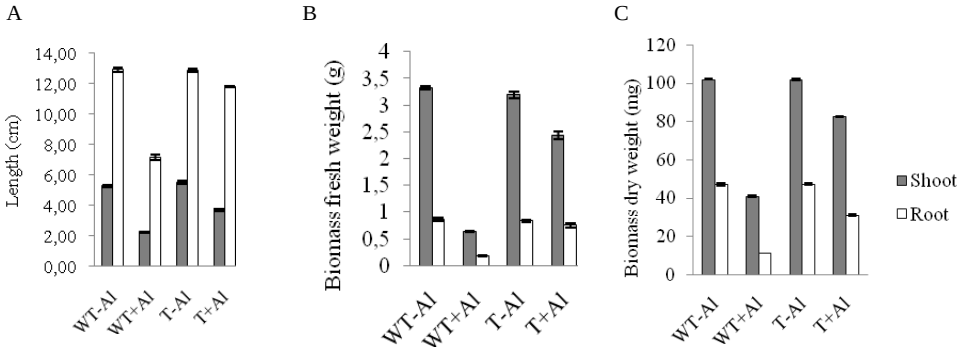

Fig. 5: The plant length (A), biomass fresh weight (B), and biomass dry weight (C) as aresponses of transgenic tobacco (T) and wild type (WT) to 300 µM at pH 4.1 for 14 days

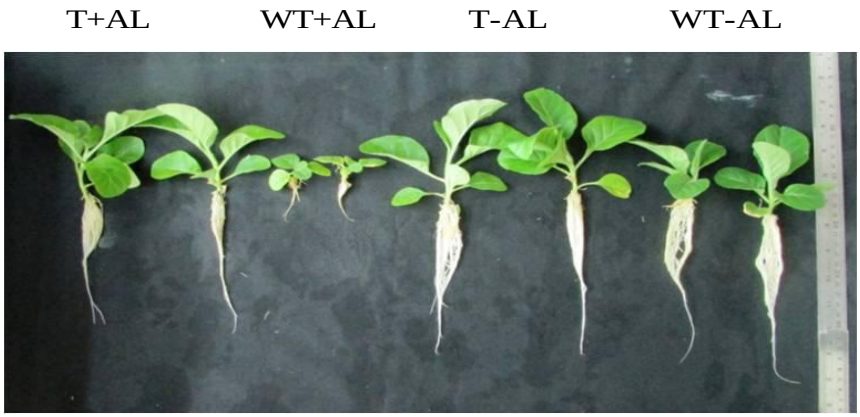

Fig. 6: Performance of transgenic tobacco (T) and its wild type (WT) grown on nutrient culture with 300 μM Al for 21days

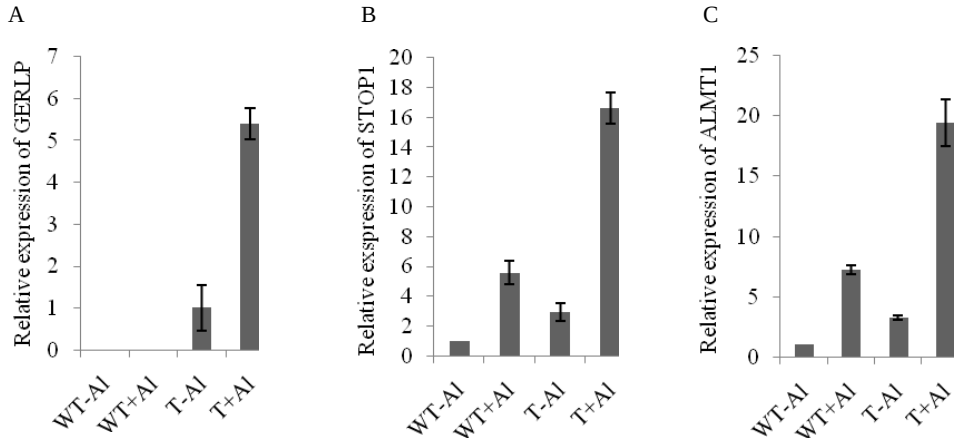

Fig. 7: The relative expression of the *GERLP* (A), *STOP1* (B), *ALMT1* (C) genes, in transgenic tobacco (T) and its wild type (WT) under 300 μM Al stress for 24 Hours

The evidences that all hygromycin-resistant transgenic tobacco lines grew well in the antibiotic selection media and contained positive GERLP and HPT bands indicates that antibiotic selection with 50 μg/mL hygromycin was effective to select transgenic tobacco.

The results of antibiotic selection indicated that the transgene was succesfully inserted and maintained in the genome of transgenic tobacco in most selected lines. The transgene was inherited into T_2 and T_3 generation, with most of the lines still showed segregation ratio of 3:1 for resistant to sensitive to hygromycin in T_2 generation. The segregation was also observed in transgenic lines in T_3 generation. The segregation ratio of 3:1 for resistant to sensitive suggested that the gene encoding hygromycin resistance was a single copy. Several lines of T_2 and T_3 generation show 100% resistant to hygromycin indicated that those lines has been homozygous for the *hpt* gene, which suggested that those lines were also homozygous for the transgene.

The evidence of transgene insertion and stability in transgenic tobacco plants was detected by PCR technique. The profile of PCR result supported the stability of the *GERLP* gene insertion in T_2 and T_3 generations. Interestingly, there was no observed GERLP bands in the wild type plants suggested that the *GERLP* gene might not be found in tobacco plants or the corresponding primer sequences was missing in the *GERLP* orthologous gene in tobacco. This occurrence needs further verification.

The growth analysis of transgenic plants under Al stress indicated that the transgenic tobacco was more tolerant to Al stress than that of its wild type. Conversely, the wild type plant showed severe growth inhibition under Al stress. It suggests that the Al tolerance character in transgenic tobacco related to the expression of the *GERLP* gene. Al stress in plants damages root cap and inhibit root cell elongation in the elongation zone, which ultimately inhibit root elongation. The inhibition of root elongation in plants can also be caused by the failure of cell division (Panda *et al*., 2009). Aluminum can interact with pectin of the cell wall and plasma membrane outer layer through electrostatic bonds with the carboxyl or phosphate residua.

Cosequently, the cell walls become stiff due to the decrease of cell wall plasticity and membrane permeability. In addition, Al can strongly bind the plasma membrane lipid components causing the membrane plasma becomes rigid (Jones and Kochian, 1997). Those conditions cause the cells fail to divide and elongate.

One of the Al tolerance mechanisms in plants is through the inhibition of trivalent Al ions accumulation in the symplast and/or minimized the interaction between Al and the cell wall and/or plasma membrane or other target in the apoplast. This mechanism could be facilitated by root exudate secretion in form of organic anion, such as malate, for Al chelation (Delhaize *et al*., 2001). We expected that the *GERLP* gene affected the expression of genes encoding organic anion secretion, such as *ALMT1* gene, or other Al related genes.

Analysis of the gene expression showed that no *GERLP* gene expression in the wild type plant. There were two possible explanations for this phenomenon. First, the *GERLP* gene ortholog might not be present in the wild type tobacco. Second, since the primers used for DNA amplification was specific primers designed from the *GERLP* sequences of rice, the corresponding primer sequences might be missing in the *GERLP* orthologous gene in tobacco.

The expression of the *GERLP* gene in the transgenic line under Al stress was higher than that in the transgenic line without Al stress. It was surprising because the *GERLP* gene was constructed under constitutitve promoter 35S CaMV, which should not be significant different in the gene expression between Al-stressed and unstressed transgenic line. Carefull experiment had been carried out to avoid experimental error and bias data between Al treated and non treated plants. The experiment was conducted with three biological replications and three technical replications for each biological replication. Without ignoring the evidence that still leave the question, the expression data produced from the experiment indicated that the expression of the *GERLP* gene was influenced or induced by Al and might contribute to Al tolerance in transgenic line. Genes whose expression is induced by Al, and its expression is higher in tolerant plants than that of sensitive plants indicates that the gene is involved in Al tolerance (Ezaki *et al*., 2000).

The expression of the *STOP1* gene in the wild type under Al stress was higher than that of the wild type without Al stress. It indicates that the *STOP1* gene was regulated by Al stress. The *STOP1* gene was also highly expressed in the transgenic line under Al stress compared to both wild type and unstressed transgenic line, suggesting that the *STOP1* gene involved in Al tolerance. The knockout of *STOP1* gene in *Arabidopsis* showed hypersensitivity to Al^{3+} rhizotoxicity (Luchi *et al.*, 2007). In this research, the expression of the *STOP1* gene was also in line with the increased expression level of the *GERLP* gene under Al stressed. The expression of *STOP1* gene in transgenic tobacco plants under Al stress is increased 5.7 fold higher than that of unstressed transgenic line. The tremendeous increase in expression of the *STOP1* gene might be due to the increase of the *GERLP* expression. The fact that the *STOP1* gene was expressed 2.9 fold higher in unstressed transgenic line than that in unstressed wild type supports the evidence that the *GERLP* gene regulates the expression of the *STOP1* gene.

Similar expression pattern with the *STOP1* gene also occured with the expression of the *ALMT1* gene. The expression of the *ALMT1* gene in the wild type under Al stress was 7.2 fold higher than that in unstressed wild type. The *ALMT1* gene was also highly expressed in the transgenic line under Al stress compared to both wild type and unstressed transgenic line, suggesting that the *ALMT1* gene also involved in Al tolerance. The *ALMT1* was reported to contribute to Al detoxification in several plants species (Ma *et al.*, 2014). *ALMT1* is a gene encoding malate transporter that responsible for malate secretion. In wheat and barley, knockout and knockdown the gene exhibit lower tolerance to Al. Conversely, the overexpression of the gene are able to increase the plant tolerance to Al stress (Delhaize *et al.*, 2004; Pereira *et al.*, 2010; Zhou *et al.*, 2013). The expression of the *ALMT1* gene in transgenic line under Al stress increased near 6 fold higher than that in transgenic line without Al stress. This was similar with the increased expression level of the *GERLP* and *STOP1* genes, suggesting that those three genes were related each other. The fact that the expression of the *ALMT1* gene in transgenic line is higher than that of the wild type supports the notion that the expression of the *GERLP* gene regulates the expression of the *ALMT1* gene expression.

The research showed that high expression of the *GERLP* gene was followed by increased expression of *STOP1* and *ALMT1* genes. Liu *et al.* (2008) and Sawaki *et al.* (2009) reported that STOP1 regulates the expression of the *ALMT1* gene in *A. thaliana*. The knockdown the *STOP1* gene reduces the *ALMT1* gene expression in tobacco (Ohyama *et al.*, 2013). The STOP1 protein is homolog to ART1 protein in rice, which regulates the expresssion of downstream genes such as the gene controling organic anion secretion (Yamaji *et al.*, 2009; Yokosho *et al.*, 2011). Based on the sequential regulation between *STOP1* and *ALMT1* genes and the evidence found in this research, it can be concluded that the *GERLP* gene expression regulates the *STOP1* gene expression, and ultimately the later regulates the *ALMT1* gene expression. Those genes contributed to Al tolerance in transgenic tobacco.

Conclusion

Transgenic tobacco stably carries the *GERLP* gene and it is inherited to T_3 generation. The expression of the *GERLP* gene enhanced Al tolerance in tobacco through

the up regulation of the *STOP1* and *ALMT1* gene expression.

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