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MOLECULAR CHARACTERIZATION AND N USE EFFICIENCY OF *LeAlaAT* 'MEKONGGA' TRANSGENIC RICE LINES

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SUMMARY

Genetic engineering is one of the strategies for developing nitrogen (N)-use-efficient rice (*Oryza sativa*) varieties. One gene that plays an indirect role in N metabolism is *alanine aminotransferase* (*AlaAT*). It can efficiently increase N content and crop yield. In a previous study, the tomato *AlaAT* gene (*LeAlaAT)* was successfully isolated and introduced into 'Mekongga' rice. The present research was conducted during 2018 and 2019 at the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD), Bogor, Indonesia. The objectives of the present study were to perform the molecular characterization of *LeAlaAT* 'Mekongga' rice lines on the basis of the *hpt* marker gene, the direct PCR of the *LeAlaAT* fragment, and the phenotypic evaluation of the selected *LeAlaAT* T1 'Mekongga' rice lines in response to different N fertilizer rates (0 kg ha^{-1} [control] and 60, 90, and 120 kg ha^{-1}). This research involved three activities, namely (1) Southern blot analysis, (2) direct PCR, and (3) N use efficiency (NUE) test of 'Mekongga' transgenic lines. Southern blot analysis revealed that in T0 transgenic lines, the copy number of the *hpt* marker gene varied from 1 to 3. Direct PCR confirmed the presence of the *AlaAT* fragment in the T1 generation of five 'Mekongga' transgenic lines. The five transgenic lines showed high panicle number, biomass weight, shoot dry weight, and total grain weight under 120 kg ha^{-1} nitrogen. The high agronomical NUE of transgenic lines under 120 kg ha^{-1} N implied that the transgenic rice lines have the potential for efficient N use at a certain minimum level of N (120 kg ha^{-1} of nitrogen) and should be further evaluated at high N levels.

Keywords: Ma *Alanine aminotransferase, LeAlaAT* transgenic rice lines, Mekongga, Southern blot, direct PCR, N use efficiency

Key findings: This research confirmed the integration of the *LeAlaAT* gene into five T0 and T1 generation 'Mekongga' transgenic lines. All transgenic lines showed better yield and agronomic NUE (agNUE) than the 'Mekongga' wild type under 120 kg ha^{-1} N. M40 and M50 were identified as potential promising lines for improved agNUE.

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INTRODUCTION

Rice (*Oryza sativa*) has an essential role in the development of agriculture in Indonesia because it is the most consumed staple food of Indonesian people. The need for nitrogen (N) as an essential nutrient is higher than that for other nutrients. The lack of N decelerates plant growth, and excessive N use can cause environmental pollution (Duan *et al.,* 2007). The application of N fertilizer has become one of the main requirements for rice crop cultivation in Indonesia. However, the use of N fertilizer itself remains inefficient. An understanding of N use efficiency (NUE) is needed to increase crop yield, reduce environmental pollution due to the excessive use of N fertilizers, and achieve sustainable agriculture (Kant *et al.,* 2011; Triadiati *et al.,* 2012; Haegele *et al.,* 2013; Ju *et al.,* 2015; Chen *et al.,* 2016).

The efficient use of fertilizers plays a vital role in increasing farmers' earnings and is also related to the sustainability of production systems, the environment, and energy resource conservation. In Indonesia, farmers commonly apply N fertilizer in the form of urea at the rates of 200–500 kg ha^{-1}, which exceed the recommended rates of 200–300 kg ha^{-1} of urea (Rachman, 2009). On the other hand, many reports have revealed that N rates of 200–250 kg ha^{-1} result in higher rice grain yields than rates of 200 kg N ha^{-1} and above 250 kg N ha^{-1} (Che *et al*., 2015). Genetic engineering is one of the strategies for improving the NUE of rice. One gene that can influence NUE is *alanine aminotransferase* (*AlaAT*). *AlaAT* encodes the alanine aminotransferase enzyme, a pyridoxal phosphate-dependent enzyme that is found in all plant parts. *AlaAT* catalyzes the reversible reaction of alanine and oxoglutarate into pyruvate and glutamate in plastids or chloroplasts (Rocha *et al.,* 2010). The glutamate produced from this reaction is then transferred from the roots to the leaves and mobilized from the leaves to other plant organs, including seeds, that need it.

Selvaraj *et al.* (2016) stated that the development of N-use-efficient transgenic rice plants is important for sustainable agriculture. They performed the field evaluation of transgenic 'New Rice for Africa 4' (NERICA4) rice harboring *AlaAT* from barley (*HvAlaAT*) under the control of the stress-inducible promoter from rice (*pOsAnt1*) during three growing seasons in two rice-growing ecologies (lowland and plateau). They reported that under different N application rates, the grain yields of transgenic *pOsAnt::HvAlaAT* rice plants were significantly higher than those of null and wild-type plants. The field results showed that this genetic modification could significantly increase dry biomass and grain yield. An increase in transgenic rice yield had a positive correlation with an increase in tiller number and high panicle number under field evaluation. This study also demonstrated that *HvAlaAT* gene expression could increase the NUE of rice without causing undesired growth phenotypes.

NUE technology can considerably reduce the need for N fertilizer while simultaneously increasing food security and reducing greenhouse gas emissions from the rice ecosystem (Sapkota *et al.,* 2021). A recent study by Tiong *et al.* (2021) on rice genetic transformation by using *HvAlaAT* under a stress-inducible promoter *OsAnt1* reported that *OsAnt1:HvAlaAT* lines exhibited increased above-ground biomass with a negligible change in nitrate and ammonium uptake rates. The mechanism involved was related to the drastic alteration of *OsAnt1:HvAlaAT* lines to increase metabolic turnover. Moreover, the upregulated genes were involved in the glycolysis and TCA cycle. The increased activity of these two processes

drove increased energy production and N assimilation then consequently increased biomass production. The involvement of phytohormonal responses and secondary metabolite alteration may contribute to NUE.

Sisharmini (2018) successfully isolated the *AlaAT* gene from tomato (*LeAlaAT*) and cucumber (*CsAlaAT2*). The cucumber *AlaAT* gene (*CsAlaAT2*) has been successfully cloned and constructed into *pCAMBIA1300* plant expression vectors under the control of the tissue-specific promoter *OsAnt1*. The transformation of the *CsAlaAT2* gene with root-specific expression improved the NUE of transgenic rice (Sisharmini *et al.* 2019). Given that the NUE value of the *LeAlaAT* and *CsAlaAT2* genes is equivalent to that of the *HvAlaAT* gene, they can be applied to develop N-use-efficient transgenic rice plants. Apriana *et al.* (2019) isolated a root-specific rice promoter, *OsAER1*, and then constructed the *LeAlaAT* gene into *pCambia1300-prOsAER1::LeAlaAT*. Trijatmiko *et al.* (2018) introduced this construct into the 'Mekongga' rice variety (one of the popular and prominent rice varieties for Indonesian farmers) and produced 50 putative transgenic lines. From these 50 lines, five lines were selected after hydroponic screening and demonstrated the best growth under low N conditions. This research aimed to identify the number of *hpt* marker gene copies integrated into T0 *LeAlaAT* 'Mekongga' rice, analyze the presence of the *LeAlaAT* transgene in the T1 generation by direct PCR, and evaluate the phenotypic performance of the selected *LeAlaAT* T1 'Mekongga' rice lines in response to different N fertilizer rates to determine their NUE.

MATERIALS AND METHODS

The research was conducted during 2018 and 2019 at the Contained Use Facilities (Laboratory and Greenhouse) for Genetically Modified Plants, Molecular Biology Division, Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD), Bogor, West Java, Indonesia.

Plant material

The T0 plants of the 'Mekongga' transgenic lines were produced in the previous study of Sisharmini *et al*. at Molecular Biology Division, ICABIOGRAD, Bogor, West Java, Indonesia (unpublished). The genetic construct used to produce T0 transgenic 'Mekongga' plants was pCAMBIA1300int-pOsAER1-LeAlaAT-tNos. The T-DNA of the genetic construct consisted of two genes: the gene of interest, namely, *LeAlaAT* controlled by the *OsERA1* promoter, and the *hpt* gene selection marker controlled by the 35S promoter. The five T0 generations of transgenic 'Mekongga' rice (M40, M41, M45, M50, and M54) that were used in this study were selected on the basis of the highest biomass weight identified by previous studies on hydroponic N efficacy testing under several N levels (unpublished). T1 generations from the five lines were evaluated for their growth and yield in the greenhouse for NUE calculation.

Molecular characterization of T0 'Mekongga' transgenic rice lines

Southern Blot analysis of the hpt marker gene in T0 LeAlaAT 'Mekongga' transgenic rice lines

Genomic DNA for Southern blot analysis was isolated from the leaf tissue of T0 'Mekongga' transgenic rice lines (M40, M41, M45, M50, and M54) by using the CTAB procedure (Murray and Thompson, 1980). Southern blot analysis was performed in accordance with the methods described by Trijatmiko *et al.* (2018). DNA was digested with the *EcoRI* restriction enzyme, then electrophoresed and blotted on Hybond-N+ nylon membranes. The blots were probed with the digoxigenin (DIG)-labeled *hpt* gene. Anti-DIG antibody conjugated to alkaline phosphatase was added, and CDP star solution was used as

the chemiluminescent substrate for alkaline phosphatase. The generated light emission was recorded on X-ray film. Gene copy number was estimated on the basis of the number of bands generated from Southern blot analysis. Each band represented a copy of the *LeAlaAT* gene in the T0 transgenic rice lines.

Direct PCR for the molecular analysis of T1 LeAlaAT 'Mekongga' rice lines

Five *LeAlaAT* 'Mekongga' transgenic rice lines from the T1 generation (M40, M41, M45, M50, and M54) and 'Mekongga' wild type (MWT) were selected for direct PCR analysis. A total of 60 seeds from each T1 line were germinated in a Petri dish for 3 days then transferred into a box container with sand medium in the greenhouse. The leaves from 2-week-old seedlings (40 seedlings per line) were used for direct PCR. The presence or absence of the *LeAlaAT* transgene was confirmed by using direct PCR, a method that amplifies DNA directly from a plant tissue sample without DNA isolation and purification. PCR for target gene detection was carried out by using a specific pair of primers designed from the downstream part of the *LeAlaAT* gene (LeAlaAT-F: 5'–TTGGAGGGAGTAACATGCAA–3') and the upstream part of the tNOS terminator (tNOS-R: 5'–AATGCCAAATGTTTGAACGA–3'). The PCR reaction mixture consisted of 5 µl of KAPA 2G mix, 0.3 µl of forward primer, 0.3 µl of reverse primer, 4.4 µl of ddH_2O, and plant tissues. PCR analysis was performed with the PCR program as follows: initial denaturation at 95 °C for 5 min, followed by 34 cycles consisting of denaturation at 95 °C for 20 s, annealing at 60 °C–72 °C for 15–30 s, and elongation at 72 °C for 2 min. The PCR products were then electrophoresed to visualize the presence of the DNA bands of the target genes. PCR provided a positive result if the sizes of the DNA bands were similar to those of the DNA band of the control plasmid. Four positive PCR samples from each line were further used for the NUE test.

NUE test of the T1 generation of *LeAlaAT* 'Mekongga' rice lines

NUE was evaluated in the greenhouse by following the method described by Selvaraj *et al.* (2016) with modifications. This test used T1 seedlings from five selected transgenic 'Mekongga' lines (M40, M41, M45, M50, and M54) that were PCR-positive for the *LeALaAt* gene and MWT as the control. Two-week-old seedlings were transplanted from the sand medium into a pot container with 10 kg of mixed medium (soil and animal manure mixed at a v/v of 3:1). The medium was fertilized with TSP equivalent to 125 kg ha^{-1} and KCl fertilizer equivalent to 100 kg ha^{-1}. N fertilizer (in the form of urea) was applied at different rates, i.e., 0.665, 1.00, and 1.335 g pot^{-1} equivalent to 0 (control), 60, 90, and 120 kg ha^{-1}, respectively. N fertilizer was applied at 0, 10, and 30 days after transplanting (DAT).

The experiment used a split-plot design with N fertilizer rates as the main plot and rice lines (MWT, M40, M41, M45, M50, and M54) as the subplots. Each N treatment consisted of four replications for each line with one plant sample per replication. Agronomic traits, namely, plant height and tiller number, were observed every 2 weeks up to 84 DAT (Triadiati *et al.,* 2012). The harvest, number of panicles, panicle length, number of seeds, shoot dry weight, root dry weight, shoot-root ration, and weight of seeds per hill were measured during harvest. The N content of plant tissue was measured by using the Kjeldahl method. NUE was calculated in accordance with the formulas from Li *et al.* (2016) and Sisharmini *et al.* (2019) and included the absorption NUE (aNUE) and agronomic NUE (agNUE) as given below:

$$aNUE = N\ content \times Biomass\ Weight$$

$$agNUE = (Total\ grain\ weight\ with\ N\ fertilization - Total\ grain\ weight\ without\ N\ fertilization)/N\ rate\ fertilizer$$

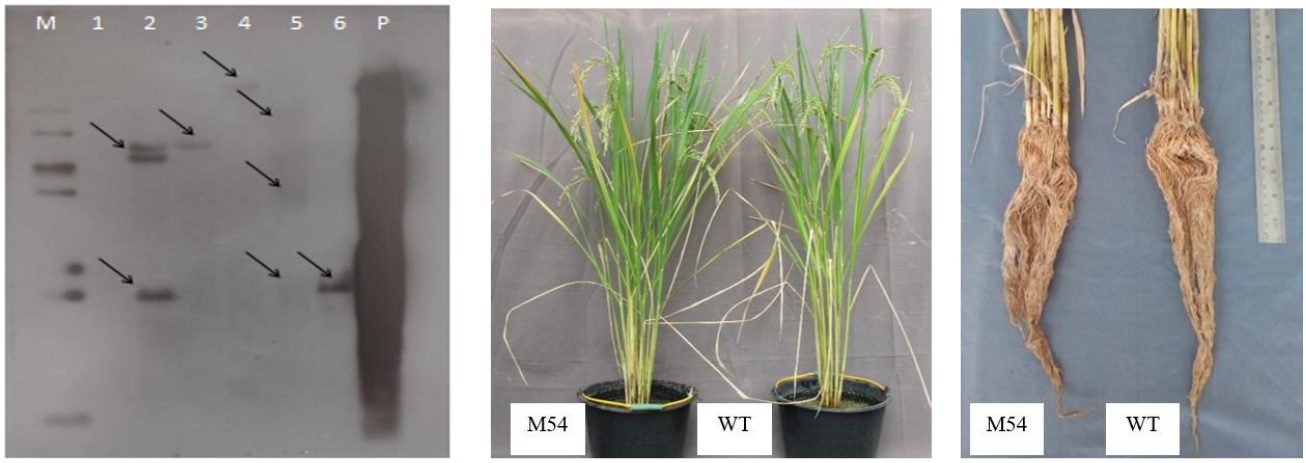

Figure 1. Southern blot analysis and comparison of the M54 *LeAlaAT* 'Mekongga' transgenic line and MWT. (a) Southern blot analysis of *LeAlaAT* 'Mekongga' transgenic rice lines with the *hpt* probe. M, 1 kb ladder marker. Lane 1: WT 'Mekongga' rice, Lane 2–6: *LeAlaAT* 'Mekongga' transgenic rice lines (M40, M41, M45, M50, and M54). P: plasmid control; (b) Comparison of the effects of 120 kg ha^{-1} (N3) on the shoots of the M54 *LeAlaAT* 'Mekongga' transgenic line and MWT, (c) Comparison of the effects of 120 kg ha^{-1} (N3) on the roots of the M54 *LeAlaAT* 'Mekongga' transgenic line and MWT.

Data analysis

Agronomic data were analyzed by using ANOVA at the test level of 0.05 and SAS software (SAS Institute Inc., Cary, NC, USA). If the ANOVA results showed significance, the test was continued with the Duncan multiple range test. Regression analyses were conducted to evaluate the lines' responses to N levels.

RESULTS AND DISCUSSION

Southern Blot analysis of the *hpt* marker gene in T0 *LeAlaAT* 'Mekongga' transgenic rice lines

Southern blot analysis was conducted on five selected transgenic lines, namely, M40, M41, M45, M50, and M54, to determine the copy numbers of the *hpt* gene. The *hpt* gene in the *prOsAER1::LeAlaAT* construct was used as the probe. It was labeled with DIG by using the PCR technique with *pCambia1300-prOsAER1::LeAlaAT* as the template. Genomic DNA from five putative 'Mekongga' transgenic lines and wild-type plants were successfully digested with the *EcoR1* restriction enzyme.

The number of integrated *hpt* gene copies could be used to predict that of *LeAlaAT* gene copies because these two genes are located in one T-DNA. Southern blot analysis showed that the copy number of the *hpt* gene varied among the five transgenic lines tested in this study and ranged from 1 to 3 (Figure 1A). A single copy of the *hpt* gene was confirmed in the M41, M45, and M54 lines, whereas three copies were integrated into the M40 and M50 lines. Wild-type plants (control) did not show a hybridization signal. However, the performances of the transgenic lines, for example, M54, and MWT showed no difference as illustrated in Figures 1B and 1C.

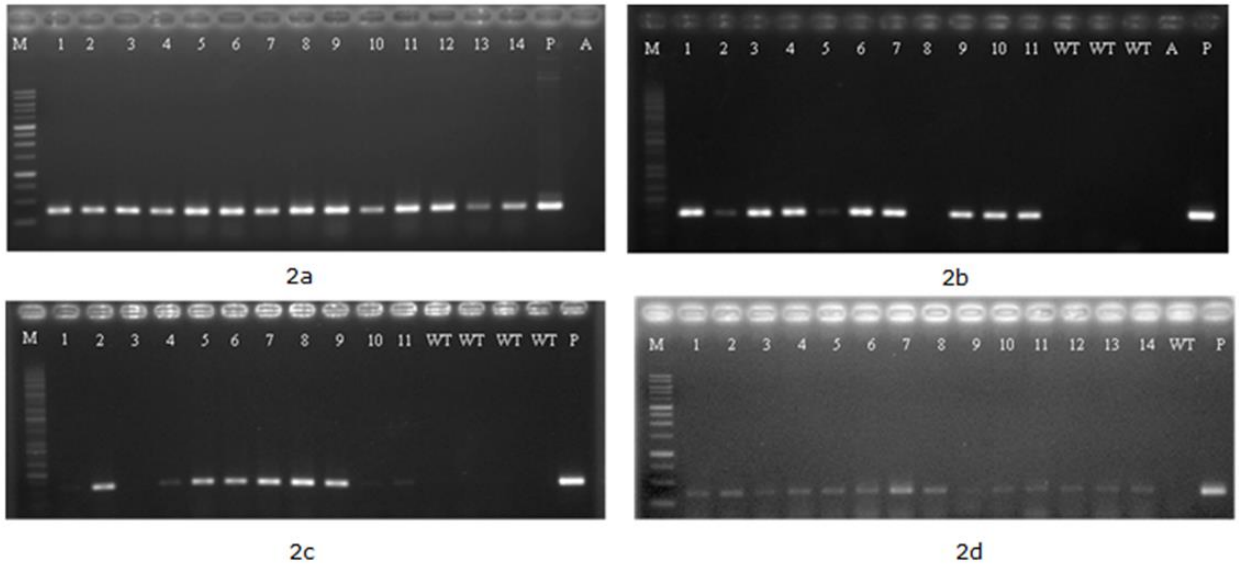

Figure 2. Direct PCR of the *LeAlaAT* gene fragment from several T1 transgenic lines. **(a)** M: 1 kb ladder marker, 1–5: T1 generation of the M54 transgenic line, 6–14: T1 generation of the M40 transgenic line, P: positive control (plasmid), A: negative control (water); **(b)** M: 1 kb ladder marker, 1–11: T1 generation of M50, WT: negative control (wild type), A: negative control (water), P: positive control (plasmid), **(c)** M: 1 kb ladder marker, 1–11: T1 generation of M45, WT: negative control (wildtype), P: positive control (plasmid), **(d)** M: 1 kb ladder marker, 1–14: T1 generation of M41, WT: negative control (wild type), P: positive control (plasmid).

Direct PCR for the molecular analysis of T1 *LeAlaAT* 'Mekongga' Rice Lines

Positive transgenic lines in the T1 generation of 'Mekongga' were confirmed through direct PCR. The PCR results showed that T1 plants positively produced approximately 250 bp-sized amplicons of the *LeAlaAT* gene fragment (Figures 2A–2D). Among the 40 plants from each line tested, as many as five T1 plants of M54, nine T1 plants of M40, 10 T1 plants of M50, seven T1 plants of M45, and 14 T1 plants of M41 produced a DNA band with a size that was similar to that of the DNA band produced by the positive control. These results proved that the target gene was inherited by a limited number of T1 plants, whereas numerous T1 plants were identified as negative in the PCR test. Eight MWT plants did not produce the aforementioned DNA band.

Direct PCR analysis was performed to verify that the T1 generation inherited the *LeAlaAT* gene. The absence of the *LeAlaAT*gene in some T1 plants could be caused by 1) the escape of nontransformed plants, 2) unsuccessful gene target amplification, and 3) the failure of the gene to cointegrate into the rice genome due to invalid *Agrobacterium* T-DNA transfer. Chan *et al.* (1993) transformed *japonica* rice (*O. sativa* L. cv. 'Tainung') with an A*grobacterium* strain carrying a plasmid harboring the chimeric genes of β-glucuronidase (uidA) and neomycin phosphotransferase (nptlI). They found that PCR-amplifiied GUS DNA fragments from the DNA of 13 out of 18 R1 plants presented a 3.15:1 ratio of GUS DNA-positive versus GUS DNA-negative progeny. This result suggested that GUS DNA segregation in the R1 progeny of transgenic T1 plants was consistent with the predicted 3:1 Mendelian inheritance pattern of a heterozygous × heterozygous cross. Our present results did not exhibit Mendelian inheritance likely because of unsuccessful gene target amplification as a result of some technical reasons.

Efficacy test for the NUE of the T1 transgenic generation containing *LeAlaAT* gene

NUE could be simply defined as the yield of grain, forage, or fruit per unit of N available in the soil (Rios *et al.*, 2010). A good understanding of the function and regulatory mechanism of the critical components involved in N acquisition, transport, assimilation, and signal transduction is essential for improving the NUE of crops (Xu *et al.,* 2012). One constraint in the genetic improvement of fertilizer NUE is the poor characterization of the phenotype and genotype for crop N response and NUE (Sharma *et al.,* 2018). The present study described several vegetative and generative traits of *AlaAT* transgenic rice lines under different N fertilizer rates.

Table 1 shows the performance of the agronomic traits of MWT and five transgenic lines under different N fertilizer rates. In general, under 0 kg ha^{-1} N, all of the characters of the all transgenic lines showed no significant difference from those of MWT plants. By contrast, under 60 kg ha^{-1} N, M50 produced a lower number of tillers, panicles, and shoot dry weight than MWT and other transgenic lines. Under 90 kg ha^{-1} N, M41 presented the highest number of tillers, whereas M45 revealed the lowest number of tillers and shoot and root dry weights. Conversely, under 120 kg ha^{-1} N, all lines showed no statistically significant differences in most of their agronomic traits, except for the number of tillers. All transgenic lines showed a significantly higher number of tillers than MWT. The transgenic lines produced approximately 15 tillers on average, whereas MWT produced approximately 11 tillers. The higher number of tillers of all transgenic lines may have contributed to the higher biomass weight and total grain weight of transgenic lines than those of MWT (Table 2). The biomass weight of MWT was approximately 70 g, and that of transgenic lines was 78–86 g. The total grain weight of MWT was approximately 23 g, whereas that of transgenic lines was 30–31 g. These data indicated that the number of tillers, biomass weight, and total grain weight of transgenic lines were 36%, 13%–22%, and 30% higher than those of MWT, respectively. Table 2 shows that among the five transgenic lines, M40 produced the highest biomass, followed by M54, M41, M45, and M50. All transgenic lines seemed to have similar total grain weights (30–31 g).

Under all N rates, nonsignificant differences in root dry weights were found between MWT and transgenic lines, except for M45 under 90 kg ha^{-1} N. M50 showed a lower root dry weight than MWT under the control (without N) and 60 kg ha^{-1} N fertilizer rate. Root-to-shoot ratio did not significantly differ between the MWT and all transgenic lines under each N rate (Table 2). Similarly, Tiong *et al.* (2021) found low significant differences in the root dry weight of rice overexpressing the *HvAlaAT* gene. By contrast, Shrawat *et al*. (2008) and Beatty *et al.* (2013) found that the shoot and root biomasses of *OsAnt1:HvAlaAT* rice lines were higher than those of the control. Selvaraj *et al.* (2016) discovered a limited or no increase in the biomass of the 43-day old plants of *OsAnt1:HvAlaAT* transgenic lines with a NERICA4 background under low N conditions. These results implied that during vegetative growth, the biomass of the transgenic lines may moderately and nonsignificantly increase and is then accumulated throughout the growth stages, resulting in a significant increase in biomass and seed yield (Tiong *et al.*, 2021).

The biomass weight and total grain weight showed a linear response to the N rate (Figures 3A–3F and 4A–4F). The wild-type and transgenic lines presented increased growth and yield with the increase in N rate. However, the transgenic (M40, M45, and M50) lines produced significantly higher yields than the wild-type line. Regression analysis revealed that 60 and 90 kg ha^{-1} N were the suboptimal levels of N. At the suboptimal level, MWT and all transgenic lines would not show different agronomic performances. The rate of 120 kg ha^{-1} N

was suggested as the lower optimal level at which *LeAlaAt* 'Mekongga' transgenic rice exhibited a positive response in biomass and total grain weight. Under this N rate, the *LeAlaAt* transgenic rice showed higher biomass production efficiency and total grain weight than the MWT.

Table 2 shows the biomass weight, total grain weight, N content, aNUE, and agNUE of each line under every N fertilizer rate. The aNUE was calculated by multiplying N content by biomass weight, whereas the agNUE was calculated as the delta between total grain weight under N rate treatment and control (without N) then divided by N rate treatment. The lack of a significant difference in the N content of all rice lines (MWT and all transgenic lines) under each N fertilizer rate indicated the lack of differences in the capacity for N absorption among the lines. The N contents of MWT and all *LeAlaAT* transgenic lines increased with the elevation in N rate as shown by their linear response in regression analysis. However, under 120 kg ha^{-1} N, the N content of M54 was higher than that of MWT and other transgenic lines. These results indicated that the transgenic lines showed strong absorption under the optimal N rate (minimum at 120 kg ha^{-1} N). M54 also had the highest aNUE. MWT and all *LeAlaAt* transgenic lines did not show significantly different agNUE.

Table 1. Number of tillers (NOT), number of panicles (NOP), root dry weight (RDW), shoot dry weight (SDW), and root:shoot ratio (RSR) from greenhouse experiment of the wild type (MWT) and T1 *LeAlaAT* 'Mekongga' transgenic rice lines under different N fertilizer rates.

N Level (kg ha^{-1})	Lines	NOT	NOP	RDW (g)	SDW (g)	RSR
0	MWT	10.25 a	7.50 a	3.54 a	30.86 a	-
	M40	10.75 a	8.50 a	3.31 a	25.03 ab	-
	M41	10.50 a	8.50 a	3.15 a	25.57 ab	-
	M45	11.50 a	9.00 a	3.41 a	25.80 ab	-
	M50	10.25 a	7.75 a	3.98 a	24.49 b	-
	M54	10.75 a	7.00 a	3.51 a	25.86 ab	-
60	MWT	13.00 a	11.50 ab	4.03 a	35.29 a	11.34 ab
	M40	13.50 a	11.25 ab	4.35 a	35.73 a	12.02 ab
	M41	13.50 a	11.50 ab	3.85 a	33.77 ab	11.30 ab
	M45	14.50 a	12.75 a	3.37 a	33.85 ab	9.97 ab
	M50	10.75 b	10.50 b	3.34 a	29.20 b	11.21 ab
	M54	13.25 a	12.00 ab	4.12 a	33.59 ab	12.09 ab
90	MWT	13.25 a	12.00 ab	3.95 ab	37.24 ab	10.55 ab
	M40	15.25 a	13.00 ab	3.86 ab	39.80 a	9.78 ab
	M41	15.25 a	14.00 a	3.93 ab	36.28 ab	10.67 ab
	M45	13.00 a	11.25 b	2.86 b	33.42 b	12.94 a
	M50	14.50 a	12.50 ab	3.78 ab	34.49 ab	11.11 ab
	M54	13.75 a	11.75 ab	4.85 a	37.71 ab	8.52 b
120	MWT	19.25 a	11.25 b	4.98 a	42.46 a	11.39 ab
	M40	21.25 a	15.75 a	5.68 a	48.58 a	11.63 ab
	M41	20.00 a	15.75 a	5.53 a	47.03 a	11.68 ab
	M45	19.00 a	15.50 a	4.75 a	42.91 a	11.11 ab
	M50	16.75 a	14.75 a	4.45 a	42.70 a	10.41 ab
	M54	18.00 a	15.50 a	6.05 a	47.32 a	12.90 a

NOT: number of tillers, NOP: number of panicles, RDW: root dry weight, SDW: shoot dry weight, RSR: root:shoot ratio

Numbers in the same column followed by the same letter in each N level indicate no statistical difference ($P < 0.05$) in accordance with Duncan's multiple range test.

Table 2. Biomass weight (BW), total grain weight (TGW), N content, absorption N use efficiency (aNUE), and agronomic N use efficiency (agNUE) of T1 *LeAlaAT* 'Mekongga' transgenic rice lines under different N fertilizer rates in the greenhouse efficacy test.

N Level (kg ha^{-1})	Lines	BW (g)	TGW (g)	N content* (%)	aNUE**	agNUE***
0	MWT	49.99	15.59	1.24 b	0.63 gh	-
	M40	43.09	14.75	1.32 ab	0.60 h	-
	M41	44.71	15.98	1.22 b	0.57 h	-
	M45	45.60	16.39	1.21 b	0.57 h	-
	M50	43.17	14.69	1.39 ab	0.60 h	-
	M54	42.04	12.67	1.51 ab	0.67 gh	-
60	MWT	62.76	23.43	1.22 b	0.77 efgh	12.07 ab
	M40	62.90	22.82	1.19 b	0.78 efgh	11.76 ab
	M41	58.26	20.64	1.24 b	0.72 efg	8.70 abc
	M45	62.52	25.29	1.37 ab	0.86 efg	13.84 ab
	M50	52.85	20.30	1.47 ab	0.86 efg	11.84 ab
	M54	61.02	23.30	1.15 b	0.73 fgh	10.76 abc
90	MWT	67.17	25.97	1.35 ab	0.89 defg	10.20 abc
	M40	67.97	24.32	1.36 ab	0.95 def	9.09 abc
	M41	65.06	24.85	1.25 b	0.85 efg	9.71 abc
	M45	58.62	22.34	1.39 ab	0.85 efg	4.73 c
	M50	61.44	23.17	1.55 ab	0.96 def	8.00 bc
	M54	67.18	25.25	1.48 ab	1.01 cde	11.69 ab
120	MWT	70.45	23.00	1.50 ab	1.24 abc	10.03 abc
	M40	86.21	31.94	1.48 ab	1.35 ab	14.94 a
	M41	82.96	30.41	1.50 ab	1.25 ab	12.86 ab
	M45	79.38	31.72	1.48 ab	1.12 bcd	13.88 ab
	M50	78.89	31.75	1.48 ab	1.22 abc	14.38 ab
	M54	83.72	30.35	1.71 a	1.43 a	12.35 ab

The mean of each line in each N level followed by the same letter indicate no statistical difference ($P < 0.05$) in accordance with Duncan's multiple range test.
N test results using the Kjeldahl method. aNUE was calculated by multiplying N content by biomass weight; agNUE*** was calculated by reducing total grain weight with N fertilization by total grain weight without N fertilization and divided by N fertilizer rate.

However, under 120 kg ha^{-1} N, all transgenic lines exhibited higher agNUE (12–14 g) than MWT (10 g). Furthermore, among the five *LeAlaAT* transgenic rice lines, M40 and M50 exhibited the highest agNUE. This result revealed that both lines showed improved performance in directing every unit of N to grain filling.

The biomass response to N rate may be affected by the environmental culture condition. Garnett *et al.* (2013) found no biomass differences among wild-type and maize *AlaAT* transgenic plants in a hydroponic system under different N rates (0.5 and 2.5 mM N). Tiong *et al.* (2021) found similar results for rice plants expressing *OsAnt1:HvAlaAT* grown in a hydroponics system. This similarity suggested that hydroponic systems provide steady N supplies even at low concentrations.

The results of the regression analysis on the relationship among the fertilizer rate, biomass weight, and total grain weights showed a linear pattern (Table 3, Figures 3 and 4). These results indicated that increasing N fertilizer rates could increase the availability of nutrients in the soil, promote the high absorption of nutrients by rice, and increase biomass weight and total grain weight. Although the N contents under each N rate did not significantly differ (Table 2), the higher R^2 values of all transgenic lines (0.6–0.8 for

Table 3. Regression analysis of the effects of N fertilizer rate on the biomass and total grain weight of MWT and T1 *LeAlaAT.*

Lines	Biomass weight		Total grain weight	
	Y	R^2	Y	R^2
MWT	0.1733X + 50.90	0.3307	0.0716X + 17.17	0.1895
M40	0.3387X + 42.12	0.8294	0.1343X + 14.39	0.7205
M41	0.3003X + 42.48	0.8083	0.1170X + 15.07	0.7595
M45	0.2463X + 44.91	0.6424	0.1108X + 16.46	0.4988
M50	0.2811X + 40.12	0.6948	0.1326X + 13.53	0.6992
M54	0.3316X + 41.11	0.8832	0.1437X + 13.20	0.8530

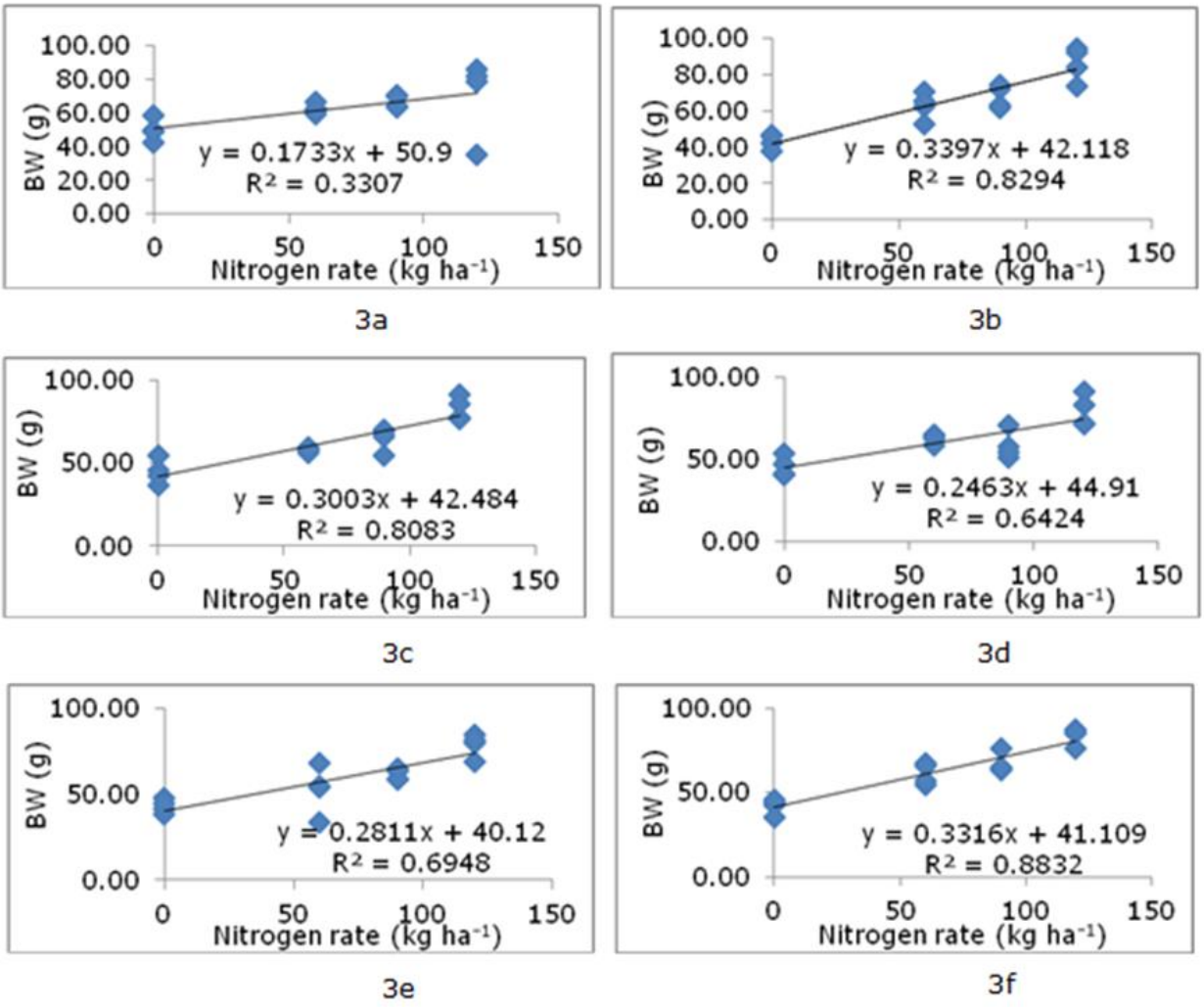

Figure 3. Regression analysis of the effects of N fertilizer rates on biomass weight (BW): (a) MWT, (b) M40, (c) M41, (d) M45, (e) M50, and (f) M54 lines.

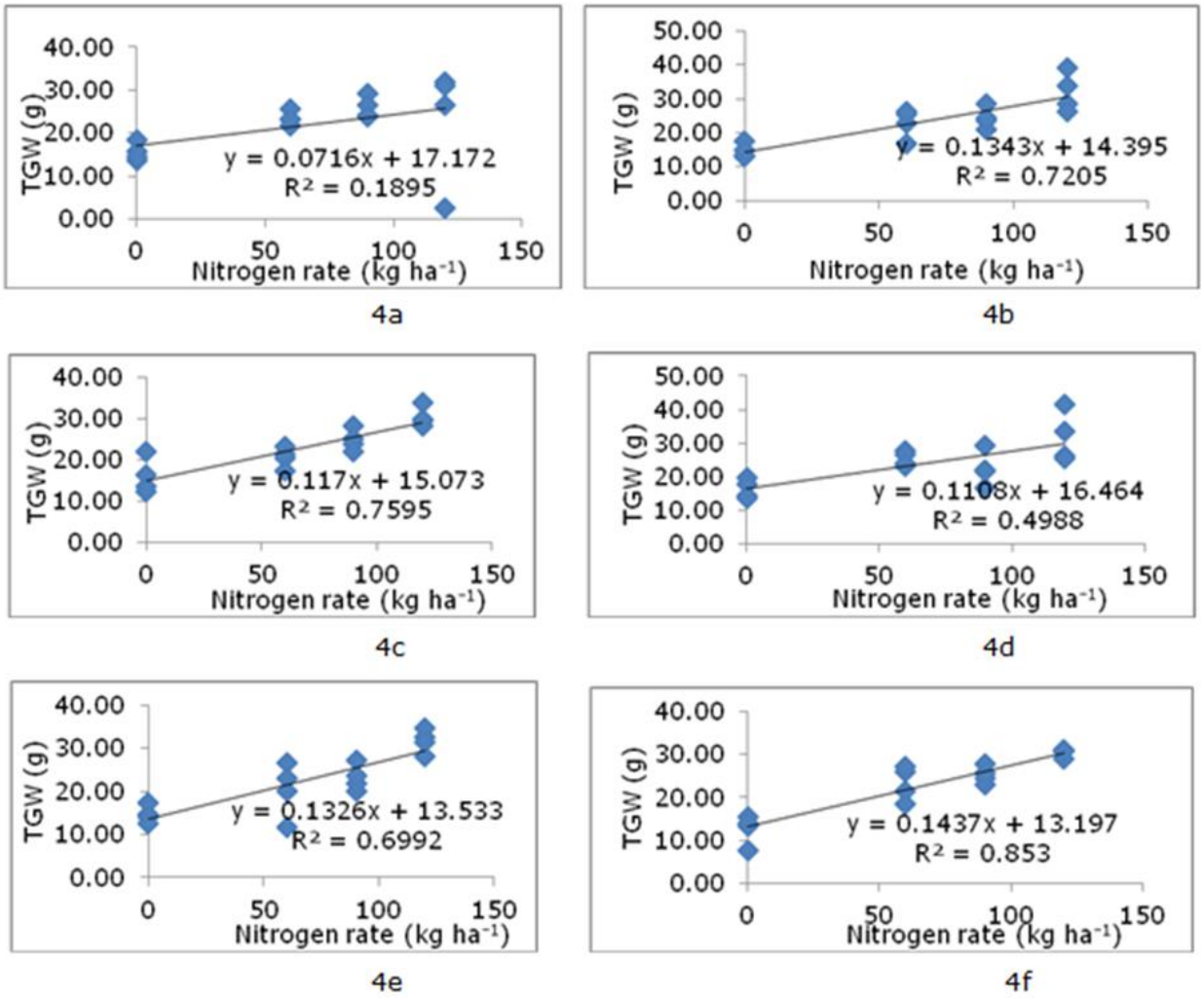

Figure 4. Regression analysis of the effects of N fertilizer rates on total grain weight (TGW): (a) MWT, (b) M40, (c) M41, (d) M45, (e) M50, and (f) M54 lines.

biomass weight and 0.4–0.8 for total grain weight) than those of MWT (0.3 for biomass weight and 0.2 for total grain weight) implied that the transgenic *LeAlaAT* lines were more responsive to the increase in N rate. The increase in biomass and grain weight should be considered as a result of the efficiency of N metabolism in plant cells. N metabolism in plants includes assimilation and translocation/remobilization (Masclaux-Daubresse *et al.,* 2010; Li *et al.,* 2017). *AlaAT* is involved in the assimilation step. It catalyzes the reversible conversion of pyruvate and glutamate into alanine and α-oxoglutarate, thus connecting the metabolism of carbon with the synthesis of various amino acids (Rocha *et al.,* 2010; McAllister *et al.,* 2012). It is part of the downstream N metabolism, and its sensing mechanism may not tightly regulate its substrates and products (Good and Beatty, 2011). Hence, the genetic manipulation of *AlaAT* may provide a viable option for altering the biochemical balance of N and C metabolism to produce a plant with NUE (Good and Beatty, 2011). In poplar, the expression of *AlaAT* genes is induced by exogenous N application and is regulated by glutamine and its metabolites in roots (Xu *et al.,* 2017). Sisharmini *et al*. (2019) showed an increase in N content in transgenic rice with *CsAlaAT* genes from cucumber.

The presence of the *LeAlaAt* transgene in five T1 lines of 'Mekongga' had been confirmed by PCR. However, further research is needed to evaluate *LeAlaAT* gene expression through RT-PCR, enzyme activity analysis, or Western blot analysis. All gene expression indicators were the higher in transgenic plants of *Brassica napus* with *HvAlaAT* (Good *et al*., 2007). Tiong *et al.* (2021) found that *AlaAT* transgene overexpression in rice did not affect the genes related to N uptake and assimilation and that a putative nitrate reductase (LOC_Os02g53130) and a putative nitrite reductase (LOC_Os02g52730) in shoots and root were downregulated. These results implied that *AlaAT* genes affect N absorption and assimilation and improve NUE through a complicated mechanism.

Southern blot analysis confirmed the numbers of *hpt* gene copies that were integrated into five T0 'Mekongga' transgenic lines: a single copy of the *LeAlaAT* gene was integrated into M41, M45, and M54; two copies were integrated into M40; and three copies were integrated into M50 lines. Direct PCR proved the presence of *LeAlaAt* transgene in the T1 generation of five 'Mekongga' *LeAlaAT* lines. Under N fertilization at each rate, the N content of MWT did not significantly differ from that of all transgenic lines. All transgenic lines showed increased yield (total grain weight) under 120 kg ha^{-1} N. M40 and M50 had a higher agNUE than other lines and thus could be the promising lines that need further evaluation.

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