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Assessment of zygosity and nitrogen used efficacy in Mekongga transgenic rice

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Abstract. *Aini MQ, Suharsono, Apriana A, Sisharmini A, Santoso TJ, Trijatmiko KR. 2022. Assessment of zygosity and nitrogen used efficacy in Mekongga transgenic rice. Biodiversitas 23: 4040-4046.* Nitrogen (N) is a vital macronutrient that can be a limiting factor for rice plant growth. Nitrogen-efficient plants can be assembled by modifying nitrogen-related metabolic pathways to improve Nitrogen Use Efficiency (NUE) in plants. T_0 generation of transgenic Mekongga rice plants containing the *LeAlaAT* gene was obtained. In transgenic plant research, zygosity test was desirable to ensure the integration and inheritance stability of transgene. This study aimed to assess zygosity and nitrogen use efficacy of two transgenic Mekongga rice lines, namely M41 and M50. This research involved three main activities, i.e. detection of flanking sequence for T_0 generation by TAIL-PCR method, zygosity test for T_1 generation, and efficacy test for T_2 generation of Mekongga transgenic lines in response to different N fertilizer rates of 0, 60, 90, and 120 kg ha^{-1}. Zygosity analysis showed that six out of 14 M41 and three of 13 M50 transgenic lines were homozygous for *LeAlaAT* transgene. The efficacy test showed that homozygous transgenic lines had higher tiller numbers, grain numbers, and biomass compared to non-transgenic ones under the same nitrogen level. The notable discrepancies of the transgenic plants can also be recognized in the absorption, agronomical, and grain NUE of the studied plants.

Keywords: *LeAlaAT*, Mekongga transgenic rice, NUE, TAIL-PCR, zygosity

Abbreviations: *LeAlaAT*: *Lycopersicon esculentum alanine aminotransferase*; NUE: Nitrogen Use Efficiency, TAIL-PCR: Thermal Asymmetric Interlaced - Polymerase Chain Reaction

INTRODUCTION

The improvement of national crop production is still faced with a variety of obstacles, including both biotic and abiotic stresses. One of the latter constraints often encountered by farmers is the lack of nutrients in paddy fields. N deficiency can lead to non-optimum growth, while excess levels of nitrogen may cause environmental pollution (Huang et al. 2018). Moreover, plants uptake only about 40% of the available nitrogen in the soil, and the rest 60% is estimated left as residue (Good et al. 2007). Excessive application of nitrogen fertilizer is risky and may not result in yield improvements and the remaining N being dissolved in the irrigation canal and evaporating into NO (Nitrooxide), a greenhouse gas (Andrews et al. 2013). Therefore, the efficiency of such a high amount of N application will decrease due to an increase in the rate of N fertilizer per plant.

Along with increasing N fertilizer costs, more nitrogen used efficient (NUE) crops, which are more able to uptake, utilize, and remobilize the available nitrogen were needed to be developed (McAllister et al. 2012). These processes can be applied as approaches to improve plant effectiveness in utilizing N. Alanine aminotransferase, an enzyme encoded by the *AlaAT* gene that is involved in the conversion of alanine and α-oxoglutarate to pyruvate and glutamate in plastids or chloroplasts (Xu et al. 2017). Tiong et al. (2021) reported the introduction of barley *alanine aminotransferase* (*HvAlaAT*) in transgenic rice upregulated carbon metabolisms, such as glycolysis and TCA cycle. The increase of these two processes could result in higher energy production and than increasing biomass production. Another research conducted by Selvaraj et al. (2017) showed that overexpression of *HvAlaAT* gene in African transgenic rice reported a significant increase in plant biomass and grain yield of the transgenic events in comparison to that of the non-transgenic under limited N supply. This research also revealed that the *HvAlaAT* gene could improve NUE in rice without causing undesirable growth phenotypes.

AlaAT genes from tomato (*LeAlaAT*) and cucumber (*CsAlaAT2*) have been successfully isolated by Sisharmini

et al. (2020). The transformation of the *LeAlaAT* and *CsAlaAT2* genes under the control of tissue-specific promoter *OsAnt1* improved the NUE of transgenic rice. Apriana et al. (2019) isolated a root-specific rice promoter, *OsAER1*, and then constructed the *LeAlaAT* gene into *pCambia1300-prOsAER1:LeAlaAT*. Sisharmini et al. (2022) introduced this construct into the Mekongga rice (an *indica* rice group that has better transformation efficiency than others and is one of the prominent rice varieties for Indonesian farmers).

The combination of the *OsAER1* with the *AlaAT* gene is expected to increase plant metabolism in root tissue due to the *AlaAT* ability to encode an enzyme catalyzing the conversion of the amino acids. The NUE performance of T1 generation of transgenic Mekongga has been evaluated by Yulita et al. (2021). In order to know the genetic stability of the T_2 generation of transgenic Mekongga-*LeAlaAT*, several analyses should be carried out. This study aimed to assess the zygosity in the progeny of transgenic Mekongga rice lines carrying *LeAlaAT* gene and to observe the efficacy of using N nutrients over the phenotype of the product component and the NUE value in the transgenic Mekongga rice plants. It is expected that Mekongga transgenic rice carrying the tomato *AlaAT* gene evaluated in the present study can express the ameliorated NUE traits as previously successfully expressed in plants containing the *AlaAT* gene from barley.

MATERIALS AND METHODS

Plant materials

Two transgenic Mekongga rice lines, namely M41 and M50 from Sisharmini et al. (2022) and their wildtype (Mekongga) as a control were used as plant genetic materials. This research was conducted at the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD), Bogor from March 2019 until October 2020.

Procedures

Left flanking sequence detection on the T_0 generation

Total genomic DNA of T_0 generation Mekongga has extracted in the previous study by the CTAB (cetyltrimethylammonium bromide) method (Doyle and Doyle 1987). TAIL-PCR method was used to detect the left flanking sequence of the LeAlaAT cassette. The TAIL-PCR included primary, secondary, and tertiary steps according to the procedures by *Zhang et al. (2018)*. We designed nested specific primers, namely TDNA-LB3 (5'-CAGTACTAAAATCCAGATCCCCCGAAT-3') and LB4 (5'-ACGTCCGCAATGTGTTATTAAGTTGTC-3') according to the left border sequence of T-DNA. TDNA-LB3 primer was used for primary TAIL-PCR while, TDNA-LB4 primer was used for secondary and tertiary TAIL-PCR. A short arbitrary degenerate (AD) primer (5'-AGWGNAGWANCAWAGG-3') was used in all TAIL-PCR steps. The PCR mixtures were performed in a volume of 20 µL containing 11.6 µL nuclease-free water, 2 µL Buffer + $MgCl_2$, 0.4 µL dNTP mix, 0.8 µL LB primer, 3 µL AD primer, 0.2 µL Taq Pol, and 2 µL DNA template. The product from the primary TAIL-PCR step was used as a template for secondary TAIL-PCR, and the product from the secondary TAIL-PCR was used as a template for the tertiary TAIL-PCR step. The tertiary TAIL-PCR products were separated on 1% agarose gel and the amplicons were isolated from agarose gel and subjected to a sequence step by PT. Genetika Science, Indonesia. To determine the T-DNA insertion location, the fragment sequences were then subjected to BLAST search service in the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Zygosity test on the T_1 generation

Two specific primers, coded as P2 and P3 used for the zygosity test were designed based on the flanking sequence position using PrimerPlus (Hung and Weng 2016). (https://www.bioinformatics.nl/cgibin/primer3plus/primer3plus.cgi). TDNA-LB4 primer was used as a forward primer for the zygosity test, called P1 primer (Figure 1). The DNA was then used as a template to amplify the T-DNA clamping region using specifically designed primers, namely P1, P2, and P3. T_2 generation seeds from each of the homozygous lines carrying the T-DNA region were subsequently planted in the greenhouse.

The efficacy test of nitrogen used in the homozygous transgenic Mekongga T_2 generation

A total of two transgenic Mekongga rice lines (M41 and M50) and wildtype Mekongga were planted in pots containing 10 kg of soil each to analyze the agronomic characteristics. The planting process was conducted in the greenhouse of the biological molecular laboratory, ICABIOGRAD. The soil was incubated for two weeks inside the greenhouse before cultivating. The experiment was arranged in a square layout divided into four replications. The main plot contained the dose of applied N fertilizer, i.e. N0: 0 kg ha^{-1}, N1: 60 kg ha^{-1} (0.6667 g urea/pot), N2: 90 kg ha^{-1} (1 g urea/pot), dan N3: 120 kg ha^{-1} (1.3333 g urea/pot). The fertilizer was applied 3 times at 5 DAP, 13 DAP, and 33 DAP (Days After Planting into the Pot) as recommended by (Selvaraj et al. 2017). The phenotypic evaluation was then analyzed based on the response of agronomic characters related to the potential plant product including plant height (cm) and tiller numbers which were observed every 2 weeks until the plant reached 84 DAP (Triadiati et al. 2012). Grain numbers of panicle contents, and dry weight of the shoot and root biomass (g) were observed after harvesting. The N content in the roots and shoot was then analyzed using Kjeldhal method by Bogor Soil Research Centre, the Indonesian Ministry of Agriculture. The Nitrogen Use Efficiency (NUE) in each plant was calculated using the following formulas:

i. Absorption Value of NUE (aEPN) (Dobermann 2005)

$$\text{aEPN} = \frac{\text{Total of N absorbed by the plants (g)}}{\text{Total of applied N (g)}}$$

ii. Agronomic Value of NUE (agEPN) (Samborski et al.

2008)

$$\text{agEPN} = \frac{\text{Total biomass of dried plants (g)}}{\text{Total of applied N (g)}}$$

iii. Grain Value of NUE (gEPN) (Good et al. 2007)

$$\text{gEPN} = \frac{\text{Weight of filled seed per clump (g)}}{\text{Total of applied (g)}}$$

Data analysis

Observed data derived from all agronomic characters were statistically analyzed using 5% variance (ANOVA) on Microsoft Excel. This analysis was performed to determine the effect of the two factors (3 plant lines and 4 levels of fertilizer) on the observed phenotype, as well as the effect of the combination of the two treatments. If the statistical analysis showed the influence of the line on the phenotypic character, then the results were compared with the molecular analysis data. Furthermore, if the ANOVA analysis showed a significant effect of the treatment, it would subsequently proceed to the DMRT test at the 5% level and correlation analysis.

RESULTS AND DISCUSSION

Left flanking area of T-DNA on the T_0 generation of transgenic Mekongga

Transgene's activity in plant cells is modulated by modifications of nucleic acids or the physical packaging of the chromatin in which it is embedded. So, the transgene integration site in the plant genome must be detected (Rajeevkumar et al. 2015). Detection of the T-DNA flank area on transgenic events conducted in the present study was indicated by the presence of DNA fragments in the two transgenic lines, whereas no amplification product in wildtype. Amplicons of T-DNA left flanking sequence were the band that does not show in amplification product of wildtype Mekongga template. Tertiary TAIL-PCR products of the M41 line produced two bands with an estimated size of lower than 500 bp, while the M50 line was about 500-750 bp (Figure 2).

Based on sequence alignment results between two amplicons against rice genome sequence available in the gene bank, the two transgenic Mekongga lines were 100% similar to the third chromosome of the rice genome of the Shuhui498 cultivar Indica group (accession number CP018159.1). This finding indicated that the *LeAlaAT* gene construct in each line is on chromosome three with different locus. To determine the T-DNA locus integrated with the genome, TAIL-PCR has been widely used to amplify the flanking sequence adjacent to the insertion sites due to its simplicity, sensitivity, low cost, and high-throughput screening (Fujimoto et al. 2016; Zhang et al. 2018). However, this method tended to produce false positive and small bands which usually provide little information about the flanking sequences (Wu et al. 2015). AD primer used in this procedure had a melting temperature of about 45°C, thus the relative amplification efficiencies of non-target products can be thermally controlled.

Zygosity of *LeAlaAT* gene on the T_1 generation of transgenic Mekongga

The primer design generated on Primer3Plus software was able to provide recommendation data for several pairs of primers as well as its forward and reverse primer base sequences, melting temperature, and the size of the amplification product of these primers (Table 1). Based on the zygosity tests conducted in this study, three banding types were observed as presented in Figure 3. The first amplicon produced a band of 373 bp for the M41 and 329 bp in size for the M50 lines. These amplicons were derived from the P2 and P3 primers amplification on each line. Genomic DNA without the presence of T-DNA (wildtype) fragments could be amplified by those primers. In contrast, the second amplicon was yielded from the P1 and P2 primers amplification of genomic DNA containing T-DNA in both (homozygous). The homozygous band of the M41 line was indicated by amplicon with an estimated size of about 200-300 bp, whereas M50 line was about 500 bp. The third amplicon produced in this study was known as heterozygous allele (containing T-DNA construct in one of its loci).

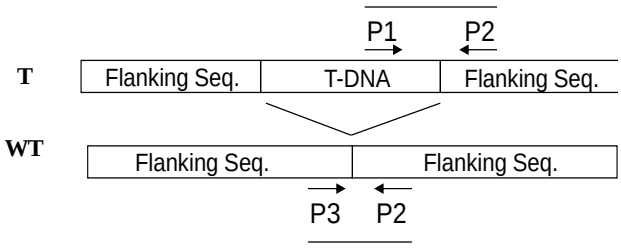

Figure 1. Scheme of primer position in Mekongga transgenic lines genome for zygosity test. T: transgenic events, WT: wildtype

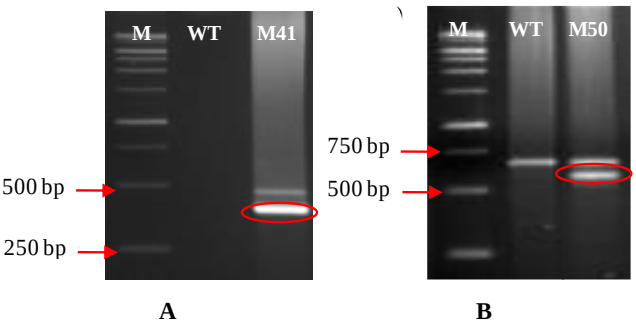

Figure 2. Electropherogram of tertiary TAIL-PCR. A. M41 line of T_0 generation transgenic Mekongga, B. M50 line. M: 1 Kb marker, WT: wildtype

Table 1. List of primers used for zygosity test on T_1 generation Mekongga transgenic rice

Lines	Primers	Sequence	Melting point (°C)	Product size
M41	M41-P2	5'- GCTTACGAGAAAGCGACCGA -3'	60.5	373 bp
	M41-P3	5'- TGGCTATCTGGAAATTTCAGCA -3'	58.0	
M50	M50-P2	5'- CTCAGAGCAGCCATTGTCAG -3'	59.7	329 bp
	M50-P3	5'- ATCCAAACAGCAGGACAGGT -3'	59.6	

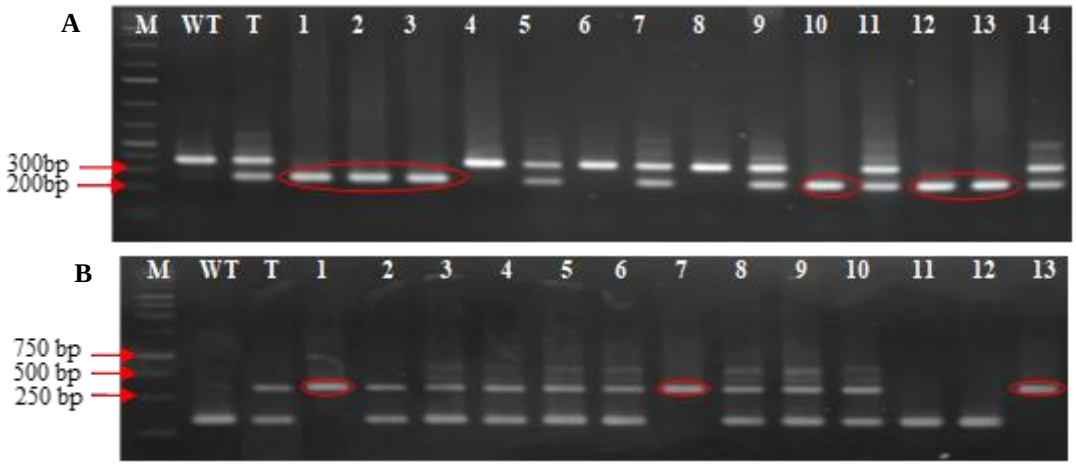

Figure 3. Electropherogram of zygosity test of the T_1 generation of Mekongga transgenic plants. A. M41 line of T_1 generation transgenic Mekongga, B. M50. M: Kb marker, WT: wildtype, T: T_0 generation from each transgenic line (heterozygous control), 1-14: T_1 generation of individual numbers from each transgenic line

Based on this amplification result, the M41 transgenic Mekongga line at T_1 generation consisted of six plants coded by numbers 1, 2, 3, 10, 12, and 13 were identified as homozygous individual plants carrying the given transgene. On the other hand, the M50 line possessed 3 homozygous individuals, coded by numbers 1, 7, and 13. The seeds of these selected homozygous plants were harvested and subsequently planted in a greenhouse to produce T_2 generation. In this generation, plant materials were subjected to both their phenotypic and plant efficacy analyses against nitrogen use efficiency.

A diploid organism such as plant is called homozygous for transgene when both alleles at a given locus are similar (dominant or recessive). The homozygous plant maintains a high degree of consistency for a particular character determined by the gene throughout the subsequent generations, which has advantages for plant breeding (Passricha et al. 2015). DNA of the T_0 generation transgenic Mekongga in the present study was used as the heterozygous control, because transgenic plants generated immediately after process of genetic transformation is hemizygous for the gene of interest (Passricha et al. 2016). This transgene generally inherits a dominant trait, follows the Mendelian inheritance and produces homozygous and heterozygous plants after segregation.

Efficacy of nitrogen use on the T_2 generation of homozygous transgenic Mekongga

The introduction of the *LeAlaAT* gene in Mekongga rice does not only provide genetic alteration but also influences nitrogen metabolism which leads to phenotype modification. In this study, the phenotype discrepancy between transgenic and wildtype was observed by treatment of both groups with 4 doses of N fertilizer (N0: 0 kg N ha^{-1}, N1: 60 kg N ha^{-1}, N2: 90 kg N ha^{-1}, and N3: 120 kg N ha^{-1}) application in each plant line.

In general, both transgenic lines showed significant differences from those of wildtype plants (Table 2). The homozygous Mekongga transgenic rice showed the highest number of tillers at a dose of 90 kg ha^{-1} (N2) fertilizer. Of these, the homozygous-M41 line indicated the best tillering performance with an average tiller number value of 22.7 (42% higher than wildtype). In contrast to the number of tillers, observations of plant height showed that the transgenic line was shorter than that in the wildtype. The homozygous-M50 line exhibited the biggest number of seed contents, with an average grains value of 150.3 for each tiller (36% higher than wildtype) under 60 kg ha^{-1} of N fertilizer application (N1). The shoot and root dry weights of the two transgenic lines were also higher than those in the wildtype. Regarding the homozygous-M41 line performance, the largest shoot and root dry weights of 65.1 g and 9.1 g, respectively were observed. This result indicated that homozygous Mekongga transgenic lines possessed higher shoot and root dry weight traits of 14% and 62.5%, respectively than those in wildtype.

According to Beatty et al. (2013), the transgenic rice lines had increased shoot root biomass as compared to controls. While, in an African rice (NERICA) background, the *OsAnt*1:*HvAlaAT* transgenic lines showed no or limited

increase in biomass of 43-day-old plants under low N conditions (Selvaraj et al. 2017). Tiong et al. (2021) reported low significant differences in the root dry weight of rice overexpressing the *HvAlaAT* gene. 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.

The tiller and grain numbers of T_1 generation of Mekongga transgenic lines were increased by 36% and 30% higher than those of wildtype (Yulita et al. 2021). Similarly, both tiller and grain number traits in the T_2 generation of homozygous Mekongga transgenic lines were also improved in the present study in comparison to those in the T_1 generation which was not homozygous yet. The M41 line displayed the best-given product for the character of the number of tillers and canopy biomass when a fertilizer dose of 90 kg ha^{-1} (N2) was applied. Meanwhile, the M50 line revealed the highest shoot biomass and grain contents at a nitrogen fertilizer dose of 120 kg ha-^{1} (N3) (Figure 4). These results were in accordance with Sisharmini et al. (2019) reported an increase in grain yield and tiller numbers was observed in transgenic events at 90 kg ha^{-1} N.

Table 2. Performance of phenotypic traits observed from T_2 generation homozygous transgenic Mekongga lines along with the wildtype under three N fertilizer application

N level	Line	Tiller numbers	Plant height (cm)	Grain numbers	Shoot dry weight (g)	Root dry weight (g)
N0	WT	15.7^{b}	118.2^{a}	124.7^{bc}	50.0^{cd}	5.6^{b}
	M41	20.7^{ab}	110.8^{de}	140.0^{ab}	59.5^{abcd}	9.1^{a}
	M50	16.7^{b}	111.9^{de}	139.3^{ab}	52.4^{bcd}	4.8^{b}
N1	WT	17.3^{ab}	118.2^{a}	126^{bc}	48.4^{d}	6.0^{b}
	M41	20.0^{ab}	112.9^{bcde}	145.7^{a}	60.5^{abc}	7.3^{ab}
	M50	15.7^{b}	115.5^{abcd}	148.7^{a}	50.6^{bcd}	5.3^{b}
N2	WT	16.0^{b}	116.8^{abc}	120.0^{c}	57.0^{abcd}	4.8^{b}
	M41	22.7^{a}	112.3^{cde}	139.3^{ab}	65.1^{a}	6.7^{ab}
	M50	18.3^{ab}	111.1^{de}	141.3^{ab}	54.1^{abcd}	5.9^{b}
N3	WT	16.3^{b}	117.4^{ab}	110.3^{c}	59.6^{abcd}	5.8^{b}
	M41	17.7^{ab}	110.2^{e}	142.0^{ab}	62.0^{ab}	5.1^{b}
	M50	16.0^{b}	110.6^{e}	150.3^{a}	56.3^{abcd}	4.4^{b}
Fertilizer level (N)		ns	ns	ns	ns	ns
Genotype (G)		**	**	**	**	*
N × G		ns	ns	ns	ns	ns

Note: Numbers followed by the same letter in the same column showed no significant difference observed between traits based on the DMRT test at the 5% level. *significant difference on the 5% level (P<0.05), **significant difference on the 1% level (P<0.01)

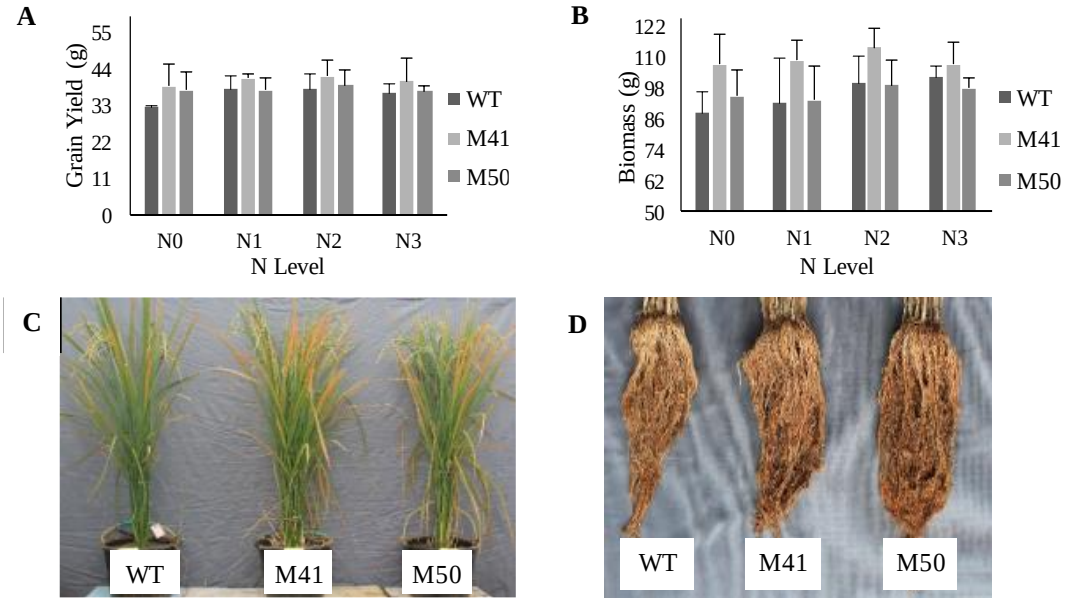

Figure 4. The comparison of plant performance between transgenic Mekongga rice lines and wildtype. A. Histograms of grain yield of homozygous transgenic lines, B. Histograms of total plant biomass of homozygous transgenic lines, C. Shoot performance of homozygous transgenic line under 90 kg ha^{-1} (N2), D. Root performance of homozygous transgenic line under 90 kg ha^{-1} (N2). WT: wildtype

Good et al. (2007) and (Shrawat et al. 2008) reported that overexpression of the *AlaAT* gene could increase not only phenotypic performances but also NUE of plants even though the alanine aminotransferase enzyme is not a key enzyme in nitrogen metabolism. NUE can be used for minimizing nutrient losses and the negative impact on the surrounding environment, as well as reducing costs associated with excessive fertilizer inputs (Galloway et al. 2014). Most NUE indices relied on ratios or proportions of crop yield vs fertilizer N sources within the spatial bounds of experimental plots (Congreves et al. 2021). The uptake of N by the root was influenced by the availability of soil N and controlled by plant N assimilation processes and N metabolite levels as well as the N demand of the plant (Stahl et al. 2019). Absorption NUE can be determined by calculating the nitrogen content in the total plant biomass per the amount of the applied nitrogen fertilizer (Dobermann 2005). Nitrogen absorbed by plant roots must also be mobilized to all parts of the plant (Masclaux-Daubresse et al. 2010). Nitrogen mobilization in plants can be expressed from the agronomical and the seed NUE.

In the present study, the NUE value of each tested line showed a significant effect on the genotype, while the fertilizer dose only affected the agronomical NUE. The transgenic Mekongga lines showed better performance for the NUE values trait in comparison to the wildtype. Furthermore, the M41 line showed the best NUE at a dose of N1 fertilizer (60 kg ha^{-1} N). Regardless of the absorption NUE parameters, the M41 line showed an average value of 0.66, agronomical NUE 101.74, and grain NUE 60.56, reflection 14%, 25%, and 7% higher values than those of wildtype, respectively. Similarly, the M50 line also showed better NUE at a level of N1 fertilizer, although with a lower NUE increase (3-12%). Although in general the increase in NUE of transgenic lines at N1 (60 kg ha^{-1}) kg/ha was higher than at N2 (90 kg ha^{-1}) (Table 3). The increase in grain yield and total plant biomass of transgenic events at N2 was higher than at N1 (Figure 4). These results may indicate that 90 kg ha^{-1} nitrogen was an optimum N fertilizer dose to reach a good balance with the doses of other fertilizers, such as phosphorus and potassium, that were used in this experiment (normal doses). It has been shown previously in rice that the relationship between N fertilizer level and grain yields is not linear (Zhang et al. 2013).

Several phenotype characters, such as the number of tillers, root dry weight, and absorption NUE observed in the present study were positively correlated with the agronomic NUE of the plant (Table 4). The N absorbed by plants can increase the number of tillers and root dry weight which later produce high biomass plants. The seed NUE was positively correlated with root dry weight as well as absorption and agronomic NUE. Thus, it can be concluded that the N absorbed through the roots was metabolized and mobilized to increase the production of transgenic Mekongga rice.

Table 3. Response of the homozygous transgenic Mekongga lines against nitrogen fertilizer applications

N Level	Line	aNUE	agNUE	gNUE
N1	WT	0.58^{ab}	81.35^{b}	56.64^{a}
	M41	0.66^{a}	101.74^{a}	60.56^{a}
	M50	0.65^{ab}	83.82^{b}	56.29^{a}
N2	WT	0.65^{ab}	61.79^{cd}	37.89^{b}
	M41	0.54^{ab}	71.75^{bc}	41.79^{b}
	M50	0.58^{ab}	59.92^{cd}	39.09^{b}
N3	WT	0.50^{ab}	49.05^{d}	27.47^{c}
	M41	0.44^{b}	50.32^{d}	27.65^{c}
	M50	0.44^{b}	45.48^{d}	27.84^{c}
Fertilizer dose (N)		ns	*	ns
Genotype (G)		*	**	**
N × G		ns	ns	ns

Note: Numbers followed by the same letter in the same column showed no significant difference observed between traits based on the DMRT test at the 5% level. *significant difference on the 5% level (P<0.05), **significant difference on the 1% level (P<0.01)

Table 4. Correlation among phenotype characters

Phenotype	Tiller numbers	Plant height	Grain numbers	Shoot dry weight	Root dry weight	aEPN	agEPN	bEPN
Tiller numbers	-							
Plant height	-	-						
Grain numbers	-	-0.41*	-					
Shoot dry weight	0.68**	-	-	-				
Root dry weight	0.78**	-	-	0.5**	-			
aEPN	-	-	-	-	-	-		
agEPN	0.46*	-	-	-	0.64**	0.59**	-	
gEPN	-	-	-	-	0.55**	0.41*	0.9**	-

Note: *significant difference on the 5% level (P<0.05), **significant difference on the 1% level (P<0.01)

In conclusion, the M41 and M50 lines were known to carry a transgene on the third chromosome with different locus. Homozygous T_1 generation was chosen by zygosity test using specifically designed primers. The T_2 generation of the homozygous transgenic lines showed better phenotype performances than the non-transgenic Mekongga including tiller numbers, grain numbers, and dry weight of roots and shoots traits at the same dose of fertilizer. The *LeAlaAT* gene in homozygous transgenic Mekongga rice resulted in the improvement of the NUE value in plants. However, further studies are required to verify the phenotype performance of selected transgenic lines in the field.

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