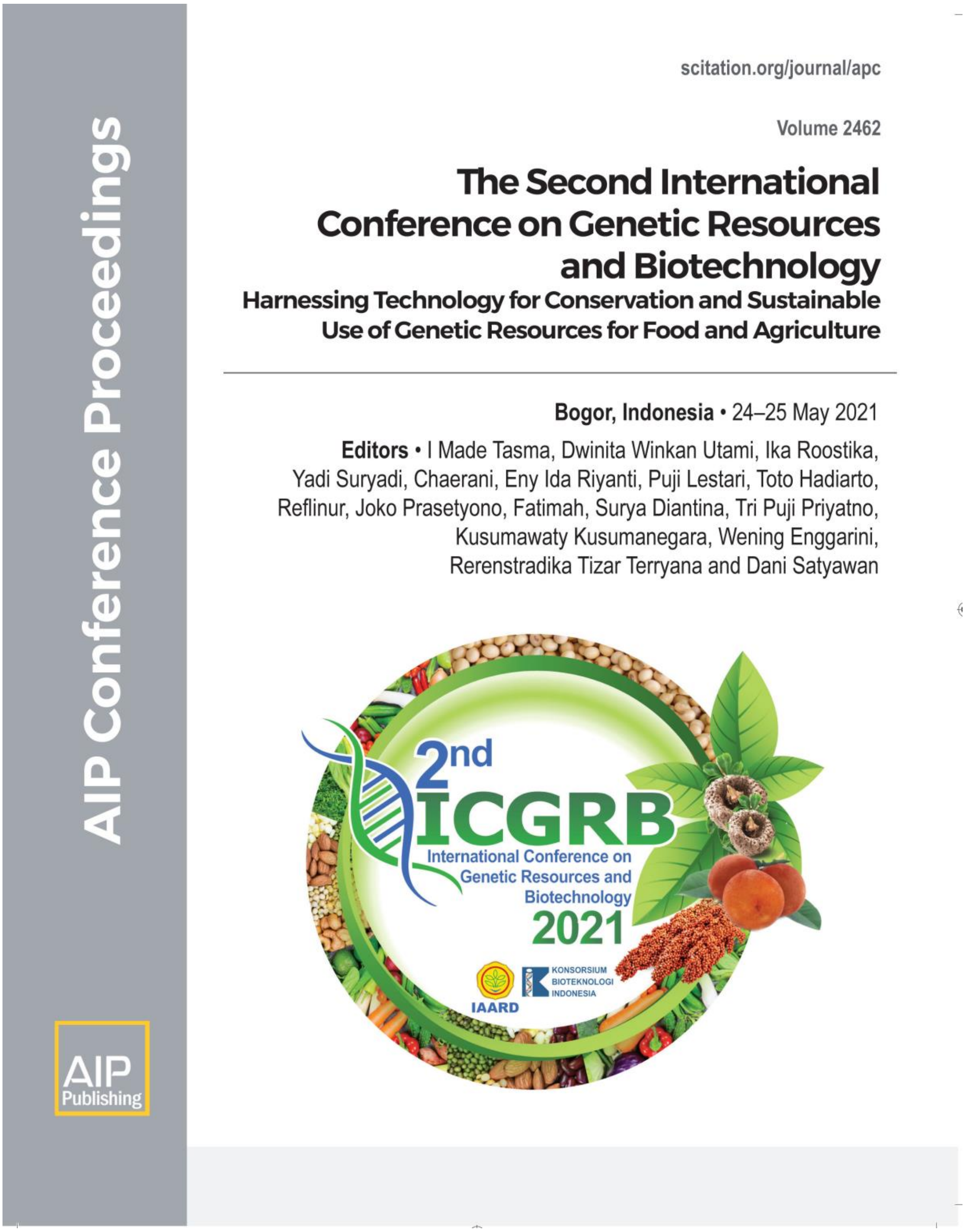

January 2022

THE SECOND INTERNATIONAL CONFERENCE ON GENETIC RESOURCES AND BIOTECHNOLOGY: Harnessing Technology for Conservation and Sustainable Use of Genetic Resources for Food and Agriculture

Committees: The Second International Conference on Genetic Resources and Biotechnology

Cite as: AIP Conference Proceedings **2462**, 010002 (2022); https://doi.org/10.1063/12.0008934
Published Online: 19 January 2022

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Cite as: AIP Conference Proceedings **2462**, 010001 (2022); https://doi.org/10.1063/12.0006897
Published Online: 19 January 2022

Preface: The Second International Conference on Genetic Resources and Biotechnology

The Second International Conference on Genetic Resources and Biotechnology, which is the continuation of the first event held in 2018, focuses on topics related to advances in biotechnology to create more opportunities for effective conservation and sustainable utilization of genetic resources for food and agriculture. This year conference's theme is Harnessing Technology for Conservation and Sustainable Use of Genetic Resources for Food and Agriculture. The conference was organized by Indonesian Agency for Agricultural Research and Development (IAARD), Ministry of Agriculture, Indonesia, in collaboration with Indonesian Biotechnology Consortium and held on 24^{th}-25^{th} of May 2021virtually due to the pandemic of COVID-19.

The conference aims to share and exchange current scientific information and technological developments on biotechnology and their applications for conservation and sustainable use of genetic, to encourage and promote quality, efficiency, and modernization of management and utilization of genetic resources, and to facilitate national and international collaboration among participants. There are five scopes discussed in this conference. They are effective management of conservation and sustainable use of genetic resources for food and agriculture, application of genomics and molecular markers for genetic resource conservation and crop adaptation to climate change, application of innovative crop improvement techniques for conservation and sustainable use of plant genetic resources for food and agriculture, plant cell and tissue culture for conservation and effective utilization of genetic resources, and the use of microbial genetic resources as biological control agents of agricultural pests and diseases, and for soil bioremediation.

Five speakers from the United States of America, Japan, India and Indonesia were invited to discuss about their expertise and knowledge on relevant subjects in the plenary sessions. This conference was attended by more than 100 participants including 75 presenters and 44 listeners worldwide. They came from diverse governmental, private, or academic institutions and also scientific communities. The presented materials have undergone peer review processes and only qualified papers were selected. Furthermore, all papers were subjected to double blind peer-review and expected to meet the scientific criteria of significance and academic excellence to be published in a conference proceedings indexed in a well-known, reputable service.

We would like to express our sincere gratitude to our speakers, presenters and all participants for their contributions in this conference. We would also like to express our appreciation for the generosity of our sponsors that support this conference: PT CropLife, PT ITS Science Indonesia, PT Fajar Mas Murni and PT Prima Instrument Analitika. Lastly, special thanks to all committee members for their exceptional work and contributions in the conference and publication.

Chair of Organizing Committee

Dr. Toto Hadiarto

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Location: Bogor, Indonesia
ISBN: 978-0-7354-4172-9
Editors:
I Made Tasma, Dwinita Winkan Utami, Ika Roostika, Yadi Suryadi, Chaerani, Eny Ida Riyanti, Puji Lestari, Toto Hadiarto, Reflinur, Joko Prasetyono, Fatimah, Surya Diantina, Tru Puji Priyanto, Kusumawaty Kusumanegara, Wening Enggarini, Rerenstradika Tizar Terryana and Dani Satyawan
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Location: Bogor, Indonesia
ISBN: 978-0-7354-4172-9
Editors:

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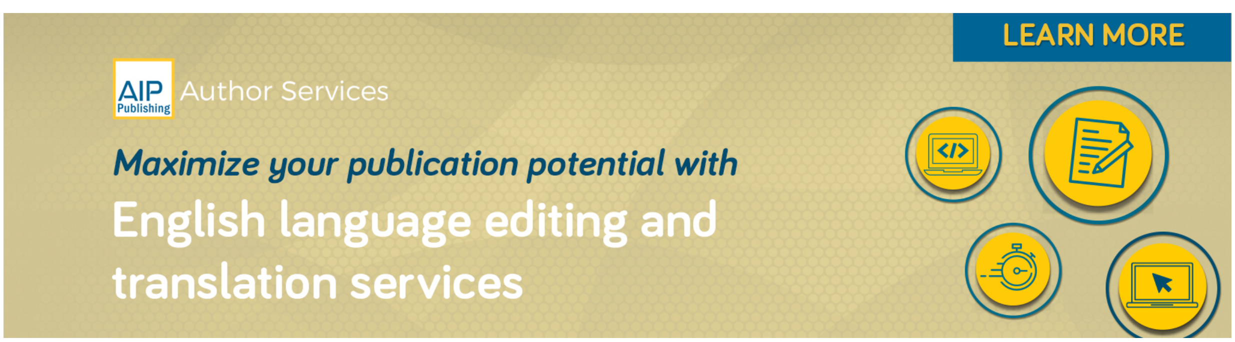
Cite as: AIP Conference Proceedings **2462**, 040003 (2022); https://doi.org/10.1063/5.0075458
Published Online: 19 January 2022

Atmitri Sisharmini, Aniversari Apriana, Intan Kamila, et al.



Construction and Introduction of OsAER1::LeAlaAT Cassette to Improve the Nitrogen Use Efficiency in Rice cv. Mekongga

Atmitri Sisharmini[1, a)], Aniversari Apriana[1], Intan Kamila[1], Aqwin Polosoro[1], Wening Enggarini[1], Tri Joko Santoso[2], Toto Hadiarto[1], Bahagiawati A. Husin[1] and Kurniawan Rudi Trijatmiko[1]

[1]*Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development, IAARD, Jln. Tentara Pelajar No. 3A, Bogor 16111, West Java, Indonesia*
[2]*Indonesian Industrial and Beverage Crops Research Institute, IAARD, Jln. Raya Pakuwon Km. 2 Parungkuda, Sukabumi 43357, West Java, Indonesia*

[a)]Corresponding author: mitri.bm38@gmail.com

Abstract. Nitrogen use efficiency (NUE) in rice can be improved by developing a variety that absorbs and use nitrogen (N) more efficiently. We have successfully isolated the *Lycopersicon esculentum alanine aminotransferase* (*LeAlaAT*) gene from tomato, which is responsible for improved efficiency of N use in plants, and *Oryza sativa alkenal reductase* (*OsAER1*) promoter from rice cv. Awan Kuning. These gene and promoter can be used to develop NUE rice through the genetic engineering approach. The purposes of this study were to construct an OsAER1::LeAlaAT expression plasmid cassette, to introduce the constructed plasmid cassette into genome of rice cv. Mekongga, and to test the transgenic rice lines at several levels of N concentration. The cassette was constructed by replacing the *β-glucuronidase* (GUS) gene derived from the pCAMBIA1300_OsAER1::GUS cassette with *LeAlaAT* gene. The recombinant plasmid was then transformed into *Agrobacterium tumefaciens* vector strain LBA4404 and introduced into rice cv. Mekongga. The plant transformation technique was carried out at with immature embryos as explants. Molecular analysis was performed using PCR to evaluate the presence of targeted gene construct introgression in the rice lines and the efficacy test of NUE was carried out hydroponically to determine the performance of lines at several levels of N concentrations. The results showed that the *OsAER1* promoter and *LeAlaAT* gene were successfully constructed in pCAMBIA1300 vectors and introduced to *Agrobacterium* vector. Rice transformation cv. Mekongga resulted in 50 transgenic rice lines (T_0). Hydroponic rapid screening of NUE in 50 T_1 transgenic rice lines using Yoshida solution with ¼ strength of N, resulted in five selected rice lines based on superior biomass dry weight compared to control, namely M40, M41, M45, M50, and M54 lines. The T_1 generation of rice lines was tested hydroponically to evaluate the response and plant performance at several N concentrations treatment. Compared to the control, the transgenic lines showed the best performance at ¼ strength of N concentration. Further NUE testing is required to determine the performance of selected rice lines in greenhouses and confined field trials.

INTRODUCTION

Nitrogen (N) is one of the essential nutrients that is needed for plant growth and development. The application of N fertilizer in agriculture is one reason that crop production has been able to sustain food demands associated with a quickly increasing human population [1]. The application of accurate synthetic N fertilizers at an appropriate time can significantly improve yields and quality of crops [2]. N fertilizer represents a significant cost for the farmer and may also have environmental impacts. Plants are only able to use about 30–40% of the available N in the soil. The remaining N about 60% has been lost from farmlands through erosion, surface run-off, leaching, and gaseous emissions (NH_3, N_2O, NO, NO_2, or N_2) or ground-water pathway. This loss of N negatively impacts the environment

The Second International Conference on Genetic Resources and Biotechnology
AIP Conf. Proc. 2462, 040003-1–040003-7; https://doi.org/10.1063/5.0075458
Published by AIP Publishing. 978-0-7354-4172-9/$30.00

by causing eutrophication of lakes, nitrate contamination of groundwater, increased greenhouse gas levels, and depletion of stratospheric ozone [3, 4, 5].

Environmental improvement and economic concern associated with excessive use of N fertilizers can be achieved by increasing the efficiency of nitrogen use efficiency (NUE) in crops [6]. Breeding of N-efficient cultivars is one effort to decrease N fertilizer inputs while maintaining adequate yields [7]. Genetic engineering techniques can contribute to improving important agronomic traits in rice and are a supporting technology for conventional breeding. The genetic engineering technique can be applied to develop transgenic plants if source of the genes of interest for sexual hybridization does not exist or has not been found in the germplasm collection.

Alanine aminotransferase (AlaAT) is an enzyme that catalyzes the reversible reaction between pyruvate and glutamate into alanine and oxoglutarate which linked primary to carbon (C) metabolism with the synthesis of various amino acids. Alanine can act as intercellular N and C shuttle which plays an important key in the metabolism of N and C in plants [8, 9].

The use of the *AlaAT* gene from barley has been reported on *Brassica napus* using the root-specific promoter *btg26*, which showed an increase in biomass and yields in low N conditions, both in laboratory and field tests [10]. The results of this study indicated a reduction in the amount of N fertilizer applications by 40%. Overexpression studies of the barley *AlaAT* gene have also been carried out in rice using the *OsAnt1* tissue-specific promoter. This modification succeeded in significantly increasing biomass and grain yield compared to control plants when the plants were supplied with sufficient N [11]. Overexpression of the *AlaAT* gene from the cucumber using the root-specific promoter *OsAnt1* has been carried out in rice plants. Transgenic rice events had a significantly higher grain yield than the wild type control under N application of 90 kg/ha. The increased yield was positively correlated with an increasing of tiller number. The transgenic rice plants showed significant increases in NUE [12].

ICABIOGRAD research team has succeeded in isolating the *Lycopersicon esculentum alanine aminotransferase* (*LeAlaAT*) gene from tomato [13] and the *Oryza sativa alkenal reductase* (*OsAER1*) promoter from rice cv. Awan Kuning [14]. This gene and promoter can be used to develop a transformation vector construct and applied to create NUE rice through the genetic engineering approach.

In the previous study, it was confirmed that the transgenic rice cv. Fatmawati introduced with the *LeAlaAT* gene driven by the root-specific promoter *OsAnt1* could improve the NUE [13]. *OsAER1* is a root-specific promoter [14] may be useful in the development of transgenic rice with improved root function to nutrient absorption and N use efficiently. The *AlaAT* gene isolated from tomato plants is expected to have the same effect as other *AlaAT* genes from other species, such as barley [11] and cucumber [12], and thus can be utilized as an alternative gene for the development of NUE rice transgenic. The purposes of this study were to construct an OsAER1::LeAlaAT expression plasmid cassette, to introduce the constructed plasmid cassette into genome of rice cv. Mekongga, and to test the transgenic rice lines at several levels of N concentration.

MATERIALS AND METHODS

Construction of OsAER1::LeAlaAT Cassette

The full-length coding region of *LeAlaAT* gene was amplified from the root tissue of tomato cv. Intan [13]. Tomato plants were subjected to hypoxia conditions [15, 16] by submerging plants of 10-day-old tomato seedlings in flasks containing Yoshida solution covered by about 2 cm layer of oil to prevent gas exchange previous to RNA isolation. Total RNA was isolated from the hypoxia treatment tomato plants using RNeasy Plant Mini Kit (QIAGEN). Amplification of *LeAlaAT* gene was performed using a mixture of FastStart Taq DNA Polymerase (Roche) and KAPA HiFi HotStart DNA Polymerase (Kapa Biosystems) and oligonucleotides LeAlaAT-F (5'-GCGGATCCGTGCGCTCGATTTATGCGAA-3') and LeAlaAT-R (5'-GCGGTACCTACCCCCAGACTCC-TTAGCC-3'). The oligonucleotides contained additional sequences of *Bam*HI and *Kpn*I restriction sites (underlined) to the amplified fragments at their 5′ and 3′, respectively. The PCR profile used consisted of an initial denaturation step at 94°C for 5 min, followed by 35 cycles consisting of denaturation at 94°C for 60 sec, annealing at 55°C for 45 sec, and elongation at 72°C for 1.5 min. Amplicon of *LeAlaAT* were ligated to the pGEM®-T Easy Vector and subsequently sequenced before digestion and ligation to the binary vector. Gene fragments with appropriate compatible cohesive ends were ligated to the *Bam*HI-*Kpn*I digested pCAMBIA1300int-OsAER1-GUS-tNOS binary vector [14] to create pCAMBIA1300int-prOsAER1-LeAlaAT-tNOS. The binary vector was introduced into *Agrobacterium tumefaciens* strain LBA4404 [17] using the freeze-thaw method [18].

Generation of Transgenic Rice and Molecular Analysis

Plant genetic transformation of rice cv. Mekongga was performed following the method described by Slamet-Loedin *et al*. [19]. Immature embryos were co-cultivated with *Agrobacterium* containing pCAMBIA1300int-OsAnt1-LeAlaAT-tNOS construct for 7 days. Immature embryos that showed elongated shoots were transferred to the resting medium for 5 days, followed by transfer to a selection medium containing hygromycin 30 mg/l for 10 days. To induce calli, the embryos were sub-cultured three times with a time interval of 10 days. The resistant calli were obtained at the third selection, and they were then transferred to pre-regeneration medium and incubated for 10 days. At this stage, proliferating calli with green spots were transferred to regeneration medium. Transformant plantlets were transferred to a rooting medium and incubated for 14 days. Plant acclimatization was carried out after the formation of vigorous roots to the soil medium and planted in the greenhouse.

Direct PCR with leaf disc as the source of template DNA was performed to confirm the presence of OsAER1::LeAlaAT construct in transformed plants using KAPA 3G Plant PCR Kits (Kapa Biosystems) and oligonucleotides LeAlaAT-F2 (5'-TTGGAGGGAGTAACATGCAA-3') and tNOS-R (5'-ATTGCCAAATGTTT-GAACGA-3'). The PCR profile used in this study was initial denaturation at 94°C for 10 min, followed by 35 cycles consisting of denaturation at 94°C for 60 sec, annealing at 60°C for 30 sec, and elongation at 72°C for 45 sec.

Screening of Transgenic Rice Events on Yoshida Solution Containing Low Nitrogen

T_0 generation of 50 transgenic rice lines containing the gene construct were grown in a greenhouse to produce T_1 seeds. T_1 seeds were used to test the efficacy of NUE. Screening of T_1 seeds of 50 transgenic rice events was conducted on Yoshida solution containing low N concentration. The ¼ strength of N concentration of NH_4NO_3 on Yoshida solution was used. Each T_1 of rice seed was germinated in a petri dish for 2 days. The rice seedlings were then transferred to pots containing sand soil in the nursery. Direct PCR of 1-week-old of rice seedling was carried out using the same primer at the molecular analysis to select the T_1 transgenic rice seedlings. PCR positive transgenic rice seedlings were used for hydroponic testing at low N concentration.

Ten-day-old rice seedlings were transferred into a hydroponic container and tested with each treatment in three replications. The rice seedlings in the container were randomized for each treatment. Forty-five-day-old rice seedlings were harvested for evaluation on root dry weight and shoot dry weight and used as the criterion to select the lead events.

RESULTS AND DISCUSSIONS

Construction of OsAER1::LeAlaAT Cassette

In the previous study, fragment of the *LeAlaAT* gene was successfully amplified from tomato root cDNA, inserted into pGEM®-T Easy Vector Systems, and sequenced [13]. The gene with correct sequence was digested with *Bam*HI and *Kpn*I, and successfully ligated into a binary vector pCAMBIA1300int-prOsAER1-tNOS to produce a recombinant vector pCAMBIA1300int-pOsAER1-LeAlaAT-tNos (Fig. 1). This construct was introduced into *A. tumefaciens* strain LBA4404 and used for the rice transformation. As commonly known that in the field of plant genetic engineering, one important component required to develop is the expression vector. The vector should have a set of genetic elements that make it suitable for transformation and selection in the host organism. In this study, the constructed expression vector contained a tomato *AlaAT* cDNA under the control of a tissue-specific promoter (*OsAER1*).

(a)

(b)

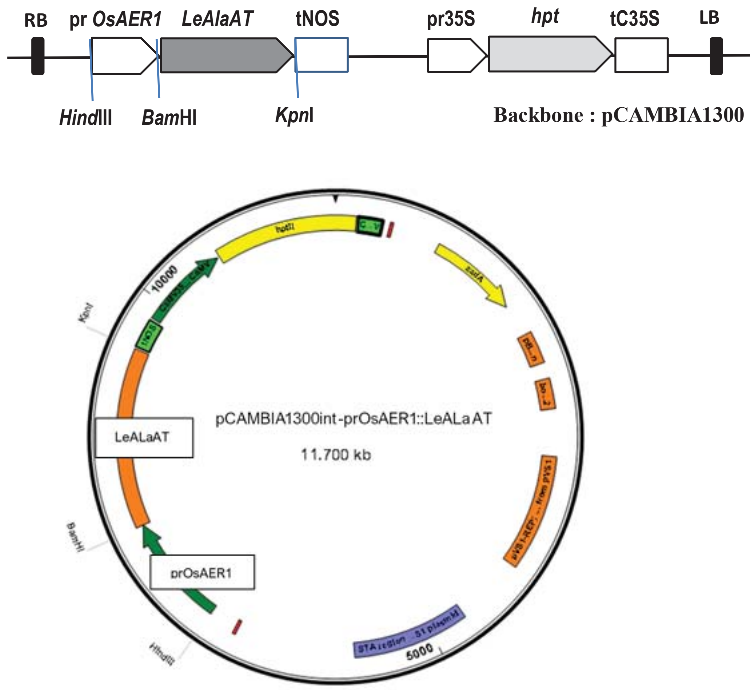


FIGURE 1. Construct of OsAER1::LeAlaAT cassette. (a) Schematic diagram of the T-DNA construct OsAER1::LeAlaAT. RB = right border, prOsAER1 = *alkenal reductase 1* promoter, LeAlaAT = *Lycopersicum esculentum alanine aminotransferase*, tNOS = nopaline synthase terminator, pr35S = *Cauliflower mosaic virus* (CaMV) 35 S promoter, *hpt* = *hygromycin phosphotransferase*, tC35S = CaMV 35 S terminator, LB = left border. (b) Genetic map of pCAMBIA1300int-pOsAER1-LeAlaAT-tNos.

Generation of Transgenic Rice and Molecular Characterization

The transformation of the pCAMBIA1300int-pOsAER1-LeAlaAT-tNos construct into rice cv. Mekongga was carried out using immature embryos based on the method of Slamet Loedin *et al*. [19]. Five transformations were carried out with a total of 2,695 immature embryos used as explants (Table 1). In callus induction step, 66.86% from the number of immature embryos successfully formed. The calli were then cultured on selection medium containing selection agent of hygromycin. After third sub-cultured on selection media, we obtained 427 (15.84%) of selected calli. The calli were then transferred for regeneration. Proliferating of calli with green spots resulted in 73 (2.70%). Of these, only 57 (2.07%) calli could develop into plantlets. A total of 57 independent lines has been produced. All the process of the rice genetic transformation is presented in Fig. 2.

TABLE 1. The result of the total number of the independent rice lines cv. Mekongga produced after genetic transformation using pCAMBIA1300int-pOsAER1-LeAlaAT-tNos construct into rice cv. Mekongga.

Number of immature embryos	Number of induced calli	Number of resistant calli	Number of regenerant calli	Number of independent lines	Number of PCR positive lines
2,695	1,802 (66.86%)	427 (15.84%)	73 (2.70%)	57 (2.07%)	50 (1.86%)

Number in parentheses is the percentage of number calli or lines divided by the number of immature embryos.

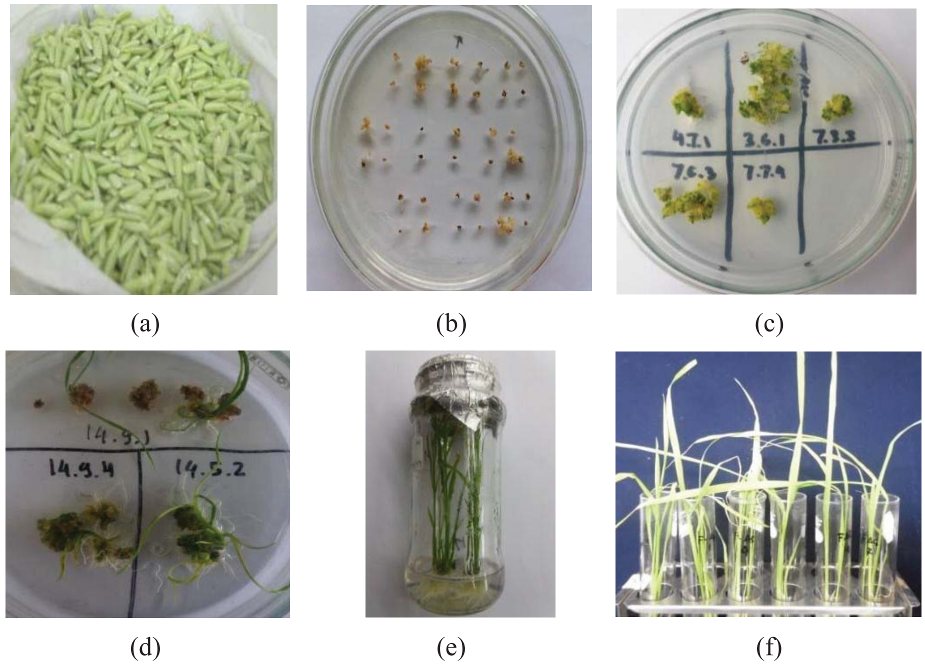


FIGURE 2. Different stages of the transformation of the pCAMBIA1300int-pOsAER1-LeAlaAT-tNos construct into rice cv. Mekongga. (a) Immature rice seeds as explants. (b) Hygromycin resistant calli. (c) Proliferating of hygromycin resistant calli with green spots on the pre-regeneration media. (d) Transformant shoots on regeneration media. (e) Transformed plantlets on rooting media. (f) Acclimatization stage of transformant plantlets in water media.

Molecular analysis using PCR technique was performed to confirm the integration of transgene in the transformed rice line. In this study, we used a pair of primer which amplify the downstream part of *LeAlaAT* gene and the upstream of tNos terminator. The results showed that out of 57 rice lines cv. Mekongga (2.07% of total immature embryo), there were 50 positive lines (1.86% of total immature embryo) that positively contained the construct pCAMBIA1300int-pOsAER1-LeAlaAT-tNos transgene. The positive PCR lines was indicated by amplicon of 355 bp in the downstream *LeAlaAT* gene and the upstream tNos terminator which is size about 355 bp (Fig. 3).

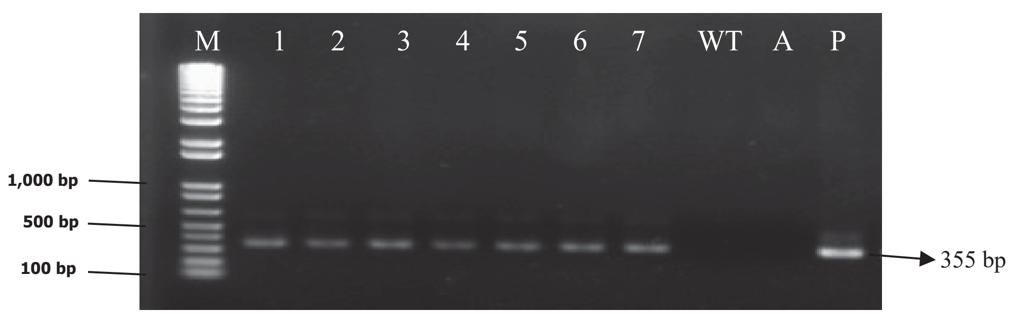


FIGURE 3. An example of PCR amplification result of seven T_0 transgenic rice lines cv. Mekongga. M = 1 Kb Plus DNA Ladder, 1–7 = transgenic rice lines, WT = wild type (cv. Mekongga nontransgenic), A = water, P = plasmid pCAMBIA1300-prOsAER1-LeAlaAT-tNOS.

Screening of Transgenic Rice Events on Yoshida Solution Containing Low Nitrogen

Rapid screening for a superior trait was carried out by growing hydroponically the transgenic and nontransgenic rice lines cv. Mekongga in Yoshida's solution at ¼ strength of N and evaluating its root dry weight. The fifty lines of T_1 PCR positive transgenic rice cv. Mekongga were used for this screening test.

The results of the efficacy screening test of N use efficiency showed that there were five lines had higher root and shoot dry weight compared to the nontransgenic Mekongga (wild type). They were M40, M41, M45, M50, and M54 lines (Fig. 4). This result indicated that all five lines increased its biomass compare with control. Previous report showed that plant growth and developmental processes, including root architecture, leaf development, seed dormancy, and flowering, can be affected by the amount of N supplied to plants [20]. Reduced levels of available N generally lead to reduced root growth, loss of lateral root initiation, increases in the C:N ratio within the plant and decrease of photosynthesis [21]. In this study, transgenic rice plants that were given low N actually showed higher biomass and better root architecture than control plants.

The existence of a good root system of plants will significantly help plants to absorb nutrients, one of which is N and is used to support their growth. The root system is the key place for nutrient and water acquisition and its ability on soil exploring is the major determinant of N uptake efficiency [22]. Overexpression of the *AlaAT* gene isolated from tomato plants showed better growth indicated by biomass and dense root. The vigorous and dense root system in transgenic rice indicates that expression of *AlaAT* gene may function for better uptake and use N efficiently [11, 23]. The transgenic rice lines will then be tested for their efficacy against the N use efficiency using soil media with different levels of N fertilizer concentration. *AlaAT* gene isolated from tomato plants is expected to have similar effect as other *AlaAT* genes, such as barley [11] and cucumber [12], and therefore, can be an alternative gene source for developing N-efficient transgenic rice.

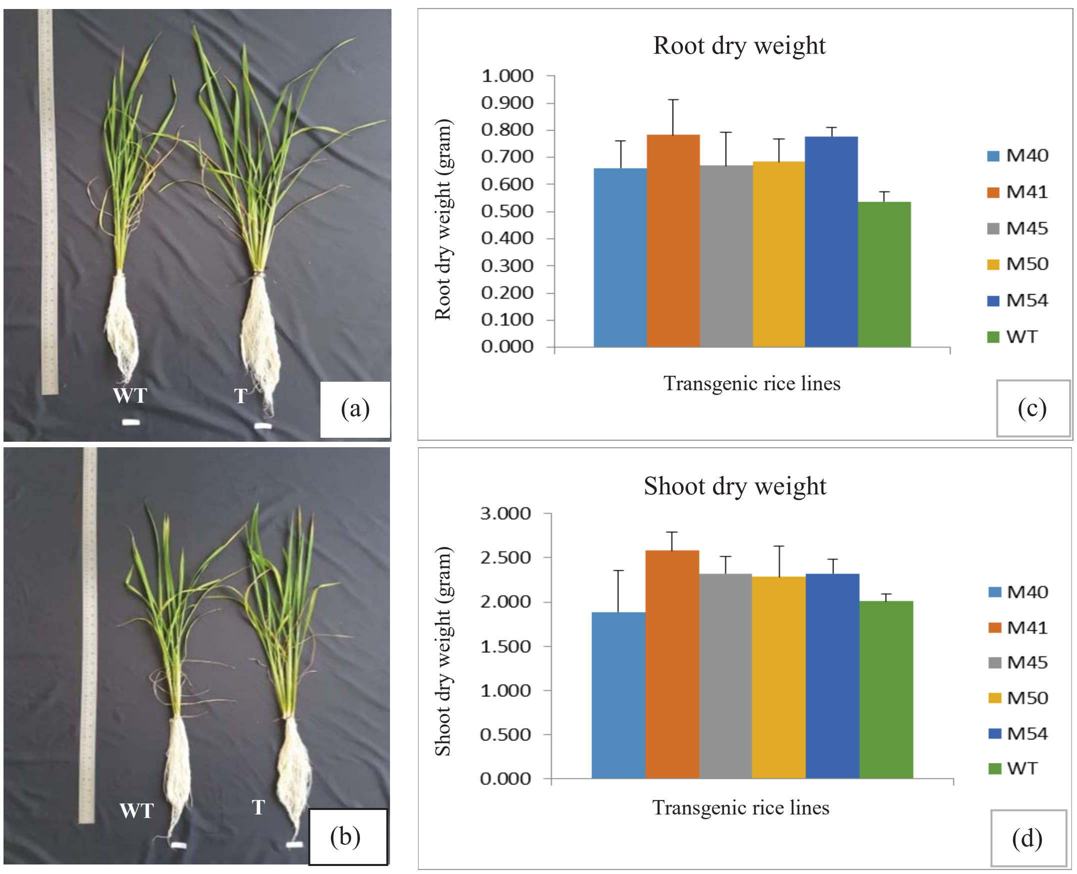


FIGURE 4. The comparison of plant performance and shoot dry weight between transgenic rice lines cv. Mekongga and wild type in hydroponic rapid screening using Yoshida's solution at low N conditions. (a) Plant performance of M41 transgenic line vs wild type line at 45 days after planting (DAP). (b) Performance of M54 line at 45 DAP. (c) Histograms of root dry weight of the five selected transgenic rice lines. (d) Histogram of shoot dry weight of the five selected transgenic rice lines. WT = wild type (nontransgenic), T = transgenic.

CONCLUSION

The expression vector containing a tomato *AlaAT* cDNA under the control of a tissue-specific promoter (*OsAER1*) named pCAMBIA1300-prOsAER1-LeAlaAT-tNos was successfully constructed. Genetic transformation of rice cv. Mekongga mediated by *A. tumefaciens* containing the construct produced 50 independent lines that were PCR positive. Hydroponic rapid screening of NUE in the 50 transgenic rice lines using Yoshida solution with ¼ strength of N resulted in five rice lines with superior biomass dry weight, namely M40, M41, M45, M50, and M54 lines. These preliminary results are expected to become a reference for genetic engineering research to develop rice that is efficient in using N. Further NUE testing is required to determine the performance of the selected rice lines in greenhouses and confined field trials.

ACKNOWLEDGEMENTS

This research was funded by grant from Indonesian Agency for Agricultural Research and development (IAARD) to Indonesian Center for agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD).

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