

# Agronomic characteristics and genetic relationship of putative transgenic rice lines of cv. Fatmawati with the *Glu-1Dx5* transgene

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**Abstract.** Carsono N, Prayoga GI, Sari S, Rachmadi M. 2021. Agronomic characteristics and genetic relationship of putative transgenic rice lines of cv. Fatmawati with the *Glu-1Dx5* transgene. *Biodiversitas* 23: 291-298. Transgenic rice lines cv. Fatmawati with the *Glu-1Dx5* transgene (encoding high molecular weight glutenin sub-unit Dx5 from bread wheat) has been produced in order to improve dough functionality of rice flour. These lines have reached T<sub>1</sub> (transformant-1) dan T<sub>2</sub> (transformant-2) generations. Some transgenic rice plants obtained from *in vitro* culture and transformation events frequently show phenotypic and genotypic variations from the original plants, thus evaluation of agronomic traits of these transgenic rice is highly important in order to obtain rice genotypes with the best agronomic traits that will be integrated into rice breeding program. Nine transgenic rice lines and two check lines, i.e. seed-derived and callus-derived rice lines, were used in this experiment. A comparison between transgenic rice traits with those of non-transgenic checks was done by student's t-test and relationship among transgenic rice lines was evaluated by UPGMA (Unweighted Pair Group Method with Mean Arithmetic) method using NT Sys (Numerical Taxonomy and Multivariate Analysis System). Results indicated that agronomic traits of transgenic rice lines were similar to those of non-transgenic check except for number of productive tillers (T<sub>2-11</sub>, T<sub>2-12</sub>, T<sub>2-20</sub>), the maximum number of tillers (T<sub>2-7</sub>, T<sub>2-11</sub>, T<sub>2-12</sub>, T<sub>2-20</sub>), number of filled grains (T<sub>1-45</sub>) and days to maturity (all T<sub>2</sub> lines). It suggests that somaclonal variations, gene insertional effect, genetic and epigenetic mutations might occur. Genetic relationship between putative transgenic and non-transgenic checks was closely related, although somaclonal variants were found. The selected transgenic rice lines will be incorporated into rice breeding programs.

**Keywords:** Callus-derived plants, somaclonal variation, transformation effect, transgenic rice

## INTRODUCTION

Plant breeding has been known as a key discipline that plays significant role in improving and utilizing genetic diversity of crops for the benefit of humankind. The main objective of plant breeding is to improve and enhance the genetic potential of plants, thus to obtain new genotypes or cultivars with superior traits. Some superior traits for rice that are expected by consumers and farmers are as follows: high yielding (>7 ton. ha<sup>-1</sup>; Khan et al. 2015), resistance to major pests and diseases, tolerant to abiotic stresses (Ansari et al. 2015), high grain quality traits (Bressegello and Coelho 2013; Kordrostami et al. 2020) and high palatability (Ohtsubo and Nakamura 2016; Kim et al. 2019). There are many ways to improve rice plants with the objective as mentioned previously. To improve the genetic potential of plants, conventional and modern approaches which include recombinant DNA (deoxyribonucleic acid) technology can be applied. In modern biotechnology, method that is commonly used in plant biotechnology, is genetic transformation or gene transfer (Sah et al. 2014). By using this technique, improvement in the rice flour quality can be possibly achieved. Weaknesses of rice flour, especially on dough elasticity and functionality, can be improved by inserting a gene from wheat that contains gluten into the rice genome.

The *Glu-1Dx5* gene, an allele that encodes a high molecular weight glutenin subunit Dx5 and is responsible for dough elasticity and functionality of bread wheat, has been successfully transferred into the genome of rice variety Fatmawati, by using particle bombardment (Wada et al. 2009). The gene was isolated from bread wheat cv. Cheyenne (Anderson et al. 2002). This effort was intended to improve the quality of rice flour so that it can be used as a substitute for wheat flour in the food industry. However, before this special transgenic rice be integrated into conventional rice breeding programs, an evaluation should be conducted to characterize agronomical traits for selecting its resemble by comparing it with its origin or wild type (cv. Fatmawati).

Jiang et al. (2000) discovered transgenic rice with dwarf phenotype, over-tillering, and high grain sterility. Meanwhile, Jeong et al. (2013) found transgenic rice with reduced number of grains and yield, then Bollinedi et al. (2017) discovered cv. Swarna derived from marker-assisted backcrossing of GR2-R1 (Golden Rice-R1) event, showing dwarf with pale green leaves and short panicle length, low grain number and yield. More recently, Chairunisa et al. (2020) found that plant height, leaf length and width, stem diameter, tiller number and productive tiller numbers of six transgenic rice lines that they produced are generally less than those of cv. Rojolele (wild-type). These authors

confirmed the phenotypic variations on agronomic traits of transgenic rice lines they produced. Some important agronomic traits showed significant differences between transgenic versus non-transgenic, for example, panicle fertility, number of productive tillers, yield and yield component traits. This is because of the instability of the genome of transgenic plants which had been *in vitro* cultured and the effect of gene transfer (Wei et al. 2016) which may affect the phenotypic of transgenic rice plants including important agronomic traits (Jiang et al. 2000; Jeong et al. 2013; Fan et al. 2020). Thus, evaluation of agronomic characteristics of transgenic rice with the *Glu-1Dx5* transgene derived from biolistic transformation event and callus culture regeneration, is a prerequisite before they are integrated into breeding programs. In addition, measuring agronomic traits of this transgenic versus non-transgenic origin could be treated as preliminary biosafety assessment in terms of substantial equivalence on agronomic characteristics, which means that transgenic rice carrying the *Glu-1Dx5* transgene has to be similar to those of the non-transgenic plant except for the traits that were improved, inserted or removed through genetic modification (Garcia-Alonso 2013).

Evaluation of agronomic characteristics of this transgenic rice is an important task as many researchers reported above. The *Glu-Dx5* transgenic rice lines that have been developed have the possibility of phenotypic changes in appearance due to the influence of environmental factors, transformation events, and also due to somaclonal variation as reported by Moghaieb et al. (2009), where seven rice genotypes derived from *in vitro* culture showed a close genetic relationship with genetic similarity values ranged from 82 to 91%. However, until now there is limited reports regarding the genetic relationship among transgenic rice lines obtained. Chaerunisa et al. (2020) mentioned that one (W3 line), out of six transgenic rice lines, showed a close agronomic characteristic with cv. Rojolele wild-type. In this study, a comparison of agronomic characteristics (transgenic versus non-transgenic wild-type) and estimation of genetic relationship is not only with wild-type cv. Fatmawati was developed from the seed, but also with cv. Fatmawati was regenerated from the callus cell culture.

The genetic relationship analysis can be used as a basis for making decision on which rice line (s) will be further investigated as promising parents for the next breeding programs and as valuable genetic rice lines. To the best of our knowledge, this is the first report informing the evaluation of agronomic characteristics of transgenic rice with the *Glu-1Dx5* transgene and assessment of genetic relationship among putative transgenic rice lines and the origin cv. Fatmawati developed from seed and callus culture, thus this will open the possibility to find transgenic rice lines with the best agronomic traits. The rice cv. Fatmawati was used in this experiment due to its high regeneration capacity (Carsono and Yoshida 2006) and it did not significantly induce somaclonal variants (Carsono and Yoshida 2007). This study is also expected to promote the development of some important transgenic crops as a

promising approach for improving productivity and quality of valuable crops in Indonesia.

## MATERIALS AND METHODS

The experiment was conducted in the Ciparanje Experimental Station, Faculty of Agriculture, Universitas Padjadjaran, Jatinangor Campus, Sumedang Regency, West Java, Indonesia. No experimental design was applied for this study due to the genetic composition of rice plants which are in the state of segregated generation. The materials used in this experiment were eleven putative transgenic rice lines (T<sub>1</sub>-11, T<sub>1</sub>-12, T<sub>1</sub>-45, T<sub>2</sub>-7, T<sub>2</sub>-11, T<sub>2</sub>-12, T<sub>2</sub>-20, T<sub>2</sub>-40, and T<sub>2</sub>-45) with *Glu-1Dx5* transgene and two other rice plants as the check: rice genotype which derived from seeds and callus cells of cv. Fatmawati. These callus-derived plants were T<sub>2</sub> generation of rice plants derived from different populations with the T<sub>1</sub> generation in this experiment.

Rice seeds were germinated in a plastic tray containing soil and organic fertilizer (50:50). After three weeks, rice plants were transferred and grown in the greenhouse with appropriate cultivation management. The plants were grown into a permanent box with dimensions 3 x 1.5 x 1 m. The 25 x 25 cm spacing was applied for optimized growth, with one seedling per hole. Growing media was prepared into a permanent box with a soil layer of approximately 70 cm, and it is mixed between soil (Inceptisols) and compost (sheep dung). For supporting rice growth and development, nitrogen fertilizer (Urea) was applied per hole at a dose of 1.8 g, phosphorus (SP-36) 0.6 g, and potassium (KCl) 0.6 g. These fertilizers were applied twice (two weeks after planting and nine weeks after planting or at final vegetative phase). Weeding was done manually. Insecticide (Profenofos 500 g/l) and fungicide (Propinep 70%) were applied when needed. Intermittent watering was done and these rice plants were then manually harvested with high care. Rice seeds were naturally dried under the sunlight.

Confirmation of the insert (*Glu-1Dx5*) transgene was performed by using PCR (Polymerase Chain Reaction; Eppendorf Thermal Cycler, Germany) in the Laboratory of Plant Breeding, Faculty of Agriculture, Universitas Padjadjaran. Leaves from each line of young rice plants were sampled at seedling stage. Genomic DNA was isolated from young leaves of 148 plants (146 putative transgenic and 2 checks) by using *Genomic DNA isolation kit* from Fermentas. After RNase treatment, the genomic DNA concentrations were estimated by using a spectrophotometer (Rayleigh UV-9200) and electrophoresed on an agarose gel, then stored at -20°C. PCR reaction was performed in 20 µL volume containing 1 µL genomic plant DNA (20-50 ng), 2 µL dNTP, 15 µL Go Taq® Green Master Mix (Promega, USA), 1 µL (0.1 µM) each specific primer for detecting the *Glu-1Dx5* transgene: Forward 5'-CCTCTTTGTGGCGGTAGTCGTC-3' and Reverse 5'-AGTTCTATCACTGGCTGGCCGA-3'. PCR programs consisted of: 94°C for 3 min. (denaturation), 59°C for 1 min. (annealing) and 72°C for 5 min. (elongation) for

thirty cycles and 1 cycle for 7 min. at 72°C was used for final extension and 4°C as the latest program for storage.

PCR product (2.5 kb) of coding region of the *Glu-1Dx5* transgene was electrophoresed in 0.8-1.0 % agarose gel with 1x TBE buffer solution for 30-60 min. at the voltage around 80-100 volt. For DNA standard, 2-log DNA ladder (New England Biolabs) was used. Agarose gel then was immersed in EtBr (Ethidium bromide) solution for 10-15 min. for gel staining. DNA fragments were visualized by using *Gel documentation Box Syngene Type G-Box* (UV transilluminator), and estimation of DNA fragment size was aided by the *Genetools* software. Amplified PCR products were stored at -20°C for further use.

The phenotypic evaluation was performed for agronomic characteristics according to Bioversity International, International Rice Research Institute (IRRI), and Warda (2007). Characters observed were the maximum number of tillers, number of productive tillers, days to harvest, harvest index (the ratio of grains to total shoot dry matter), grain dry weight (at 14% water content), number of filled grains, and the number of empty grains. Eleven transgenic rice genotypes from two generations ( $T_1$  and  $T_2$ ) and two check genotypes were observed. Comparison between traits of transgenic versus non-transgenic rice was analyzed by student's t-test (Mishra et al. 2019) with considering homogeneity of variance by *Levene's test*. The test was performed by using SPSS ver 23.0 software. Estimation of genetic relationship among transgenic rice lines and the check plants was performed using the UPGMA (Unweighted Pair Group Method with Mean Arithmetic) method by NTSYS v.2.0 software.

## RESULTS AND DISCUSSION

### Molecular confirmation of the *Glu-1Dx5* transgene

The result of molecular characterization utilizing PCR for putative transgenic rice is presented in Figure 1. By using PCR, the *Glu-1Dx5* transgene was detected in some putative transgenic rice lines as mentioned:  $T_2$ -7: 26 plants,  $T_2$ -11: 12 plants,  $T_2$ -12 : 3 plants,  $T_2$ - 40 : 3 plants, and  $T_2$ -45: 5 plants. In total, 49 transgenic rice lines with the *Glu-1Dx5* transgene (out of 146 plants, 33.56%), were detected. The number of transgenic rice lines with the *Glu-1Dx5* transgene was higher in the  $T_2$ -7 rice line compared to others.

Detection of the *Glu-1Dx5* gene on 146 putative transgenic rice plants revealed that some rice lines contained the inserted gene and some did not. This is due to

self-pollination that results in genotypic segregation, in which some plants contained the *Glu-1Dx5* transgene, but some did not. This is because that transgene segregation may occur after consecutive self-pollination as reported by Wang et al. (2012) for *Uda* gene (GUS) and *cry1Ab* transgene (Wang et al. 2012; Lohn et al. 2020). This data confirms that the *Glu-1Dx5* transgene has been integrated into the rice genome of cultivar Fatmawati. Furthermore, genetic transformation of the *Glu-1Dx5* transgene with rice callus cells as target tissues by using particle bombardment has been successfully achieved. Further assessment on transgene expression and stability and the efficacy of the *Glu-1Dx5* transgenic selected lines study should also be performed.

### Evaluation of agronomic characteristics of transgenic rice

Table 1 shows that the genotype  $T_1$ -45 differed significantly with both checks (cv. Fatmawati developed from seed/FS, and regenerated from callus/FC) on the number of filled grains and all  $T_2$  genotypes differed significantly with both checks on days to harvest. Meanwhile, other genotypes were shown to be different with cv. Fatmawati developed from seed were genotyped  $T_2$ -11,  $T_2$ -12, and  $T_2$ -40 for the number of productive tillers,  $T_2$ -7,  $T_2$ -11,  $T_2$ -12,  $T_2$ -40 for the number of maximum number of tillers, and  $T_1$ -45 for the number of filled grains. In addition, eight genotypes showed differences with Fatmawati derived from callus, i.e.,  $T_2$ -7 and  $T_2$ -45 for the number of productive tillers,  $T_2$ -20 for maximum tiller number,  $T_1$ -11,  $T_1$ -12,  $T_1$ -45 for days to harvesting, and  $T_1$ -12 and  $T_1$ -45 for number of empty grains (Table 1). These data suggest that changes in phenotypic of some transgenic rice traits might occur.

Phenotypic alteration of rice traits derived from transformation event and *in vitro* culture during transgenic development as we found in this experiment, i.e., number of filled grains, and days to harvest, number of tillers have been reported to occur by some researchers (Jiang et al. 2000; Jeong et al., 2013; Fan et al. 2020; Racheal et al. 2020). This phenomenon is known as somaclonal variation, which is a genetic alteration that is generated through tissue culture (Larkin and Scowcroft 1981; Scowcroft et al. 1985; Zhang et al. 2014). The phenomenon is estimated to be one that causes this difference. Somaclonal variation can occur as a result of injuries on the target gene (wounding) by the transformation, tissue culture media, and tissue culture environment (Wei et al. 2016).



**Figure 1.** PCR detection for the *Glu-1Dx5* transgene on  $T_2$  line #7. Remarks: C- (negative control, not transformed rice plant), C+ (positive control with plasmid pK+Dx5), M (Marker 2-Log Ladder), 1-23: plant number. Arrow corresponds to the *Glu-1Dx5* transgene (2.5 kb coding region)

**Table 1.** Comparison for agronomic traits of transgenic rice lines with their checks

Genotype	No. of plants	No. of productive tillers	No. of maximum tillers	Days to harvest (dap)	Harvest index	Grain dry weight per panicle (g)	No. of filled grains	No. of empty grains
FS (a)	32	18.27 ± 2.46	18.30 ± 1.09	123.60 ± 0.63	0.68 ± 0.11	31.00 ± 5.72	189.04 ± 32.83	138.07 ± 20.09
FC (b)	20	11.80 ± 1.14	11.60 ± 1.09	116.10 ± 0.10	0.51 ± 0.12	32.28 ± 5.04	126.44 ± 21.01	165.38 ± 11.33
T <sub>1-11</sub>	3	13.50 ± 5.50 ns	13.50 ± 5.50 ns	125.00 ± 0.00*b	0.28 ± 0.05 ns	22.45 ± 5.05 ns	127.10 ± 40.90 ns	136.20 ± 12.60 ns
T <sub>1-12</sub>	7	11.83 ± 2.25 ns	12.00 ± 2.46 ns	125.00 ± 0.00*b	0.56 ± 0.14 ns	35.90 ± 11.49 ns	150.83 ± 15.95 ns	123.00 ± 11.11 *b
T <sub>1-45</sub>	4	10.75 ± 1.65 ns	11.00 ± 1.63 ns	125.00 ± 0.00*b	0.26 ± 0.09 ns	8.720 ± 3.21*ab	62.30 ± 19.15 *a	98.45 ± 34.88 *b
T <sub>2-7</sub>	35	18.33 ± 2.73*b	18.33 ± 2.73 *a	127.20 ± 0.87*ab	0.50 ± 0.11 ns	29.42 ± 7.11 ns	151.08 ± 12.86 ns	186.50 ± 15.74 ns
T <sub>2-11</sub>	33	12.09 ± 1.24 *a	12.18 ± 1.24 *a	130.00 ± 0.00*ab	0.52 ± 0.54 ns	24.01 ± 2.97 ns	168.38 ± 26.92 ns	124.77 ± 16.27 ns
T <sub>2-12</sub>	15	9.08 ± 0.72*a	9.16 ± 0.77 *a	126.10 ± 0.56*ab	0.46 ± 0.05 ns	23.53 ± 1.84 ns	148.60 ± 17.70 ns	167.32 ± 25.64 ns
T <sub>2-20</sub>	12	15.33 ± 2.06 ns	17.16 ± 1.72 *b	130.00 ± 0.01*ab	0.56 ± 0.40 ns	36.18 ± 8.13 ns	143.70 ± 27.82 ns	192.40 ± 30.72 ns
T <sub>2-40</sub>	17	10.75 ± 0.64*a	11.62 ± 0.59*a	126.10 ± 0.91*ab	0.46 ± 0.07 ns	29.80 ± 5.57 ns	141.62 ± 6.82 ns	159.02 ± 13.38 ns
T <sub>2-45</sub>	20	18.75 ± 2.74 *b	18.25 ± 2.83 ns	127.20 ± 0.46*ab	0.62 ± 0.18 ns	51.20 ± 9.18 ns	125.27 ± 7.20 ns	129.40 ± 11.99 *b

Note: Data show mean values with standard error of the means. Data were analyzed by *student's t-test* at 0.05 with considering homogeneity of variance by *Lavene's test*; ns= non-significant with both checks; \*a= significant with cv. Fatmawati developed from the seed (FS); \*b= significant with cv. Fatmawati regenerated from callus (FC).

The significant differences between traits of transgenic and those of non-transgenic rice genotypes can also be caused by environmental influences as explained by Amaya et al. (2019) that the quantitative agronomic characters were observed visually influenced by the environment, and each genotype has a different response to the environmental carrying capacity. Biotic and abiotic environmental factors such as pests, diseases and temperature can also affect the filling of grains. Temperature is above 35°C during the anthesis to flowering period can induce spikelet sterility (Hakata et al. 2017) and affect grain weight and grain quality (Chen et al. 2017). In addition, the panicle that is formed at the later tillers are usually small and too late to be ripened, so that the grain is only half-full or empty at the harvest time (Arraudeau and Vergara 1988). Grist (1965) stated that the number of productive tillers in rice plants is affected by genetic and environmental factors. The lost number of productive tillers was affected by environmental factors caused by competition of tillers and nitrogen deficiency (Arraudeau and Vergara 1988). However, interaction between genetic and environmental factors could also cause differences in agronomic characteristics, such as reduced tillers number (Arraudeau and Vergara 1988), and decreased plant height (Patnaik et al. 1999), productive tillers number (Grist 1965), and harvest age (Jiang et al. 2000).

Other possible causes of this phenotypic alteration of transgenic rice plants are activation of *Tos17* retrotransposon (Hirochika et al. 1996; Cho and Paszkowski 2017), *mPing* transposable genetic element (Park et al. 2019), the single-nucleotide polymorphisms (Hoai et al. 2014), and insertions and deletions (Miyao et al. 2012; Qin et al. 2018) that have been known to contribute to the somaclonal variations from *in vitro* culture. During transgenic development, calluses as starting materials that were induced, sub-cultured/multiplied and regenerated which are not the same as in natural conditions or these calluses were in the state of stressed conditions, would activate many retrotransposons (Hirochika et al. 1996; Zhang et al. 2014; Park et al. 2019). This condition makes these transposons are very active when callus cells are cultured *in vitro*, making random insertions in any sites of chromosomal DNA of rice, thus genetic and phenotypic variations may occur as we observed in this experiment. It is estimated that around 32 retrotransposon families are found in 3000 rice genome datasets by using *Trackposon* software as reported by Carpentier et al. (2019). These authors also pointed out that retrotransposon activation in rice may be triggered by external stimuli, rather than by the alteration of genetic factors involved.

Among retrotransposons, three rice long terminal repeats (LTR) retrotransposons (*Tos10*, *Tos17*, and *Tos19*) are activated to transpose, with significant increase in the copy number of *Tos17* with prolonged *in vitro* culture (Hirochika et al. 1996) and *ONSEN* (Masuta et al. 2016). From this point of view, it is clear that *in vitro* culture of rice callus cells applied during the *Glu-1Dx5* gene transformation effort possibly activated some retrotransposons in rice and produced many genetic and phenotypic variations as found in this experiment. In the

case of the *Glu-1Dx5* transgenic rice production, it required about 2 to 3 months accompanied by six steps consisting of callus induction, gene construct preparation, gene transfer using particle bombardment (Helios gen gun from Biorad Inc.), cell callus selection, shoot differentiation and plant regeneration and acclimatization in the greenhouse. Genetic transformation by using particle bombardment discovers unintended effects on high copy number and mutation translocation, thus it might induce genetic mutation in rice's genome due to insertional effect which may occur in random and uncontrolled manner (Wilson et al. 2013). Fu et al. (2019) studied the unintended effect of eight varieties of transgenic rice lines that have been developed in China. They found that 2892-8758 differentially expressed genes (DEGs) and 7-50 metabolites at significant levels between transgenic rice lines and their isogenic counterparts, which were far fewer than that between traditional rice varieties. DEGs shared among eight transgenic rice samples found were less than 1% of the genes in the genome. The insertion effect on the nearby gene expression and the associated metabolism is only restricted to 50 genes. These results provide analysis of unintended effects of genetic transformation especially by using gene gun particle bombardment.

Concerning callus culture may induce phenotypic and genotypic variations, it has been well known that longer callus induction time could increase mutations, which was far below natural mutation rate as reported by Qin et al. (2018). They proved that molecular spectrum of mutations in regenerated rice by genome sequencing of genotype Dongjin is higher than of natural mutation rate as revealed by resequencing (Qin et al. 2018). It is, therefore, to distinguish transgene insertional mutations and somaclonal variations induced by tissue culture would be very essential in confronting debates on biosafety of transgenic crops. Recently, DNA polymorphisms of transformants or regenerants derived from several treatments such as variable *Agrobacterium* strain, T-DNA transformation approach, and selection condition had been studied by Wei et al. (2016). They found that regenerants and transformants showed relatively low mutation rates compared to those of T-DNA tagged mutants and *Tos17* lines under different treatments collectively. And also, they could not conclusively differentiate SNPs (single nucleotide polymorphisms) and InDels (insertions and deletions) caused by *Agrobacterium* transformation and callus-induced somaclonal variations. Thus, unintended genomic changes may be interpreted to have occurred in some rice genotypes/germplasms.

Meanwhile, other researchers are concern about epigenetic phenomena which may contribute to phenotypic alteration in transgenic rice (Smulders and deKlerk 2011; Fujimoto et al. 2013; Stroud et al. 2013; Wang et al. 2013; Deng et al. 2016; Fan et al. 2020). Epigenetics refers to the study of heritable phenotype changes that do not involve DNA alterations in DNA sequence or without genetic alterations. DNA methylation (Stroud et al. 2013) and histone modifications (Smulders and deKlerk 2011; Fujimoto et al. 2013; Deng et al. 2016) are well-known epigenetic modifications. Other, changes in chromosome

structure and number have been reported in barley. Ploidy level in transgenic barley is much higher compared to non-transgenic. Twenty-seven (46%), out of 59 independent transgenic events are tetraploid ( $2n=2x=28$ ) or aneuploid around the tetraploid level (i.e., 26, 27, 29 and 30 chromosomes; Choi et al. 2000). However, all transgenic rice lines that express human lactoferrin have diploid ( $2n=2x=24$  chromosomes; Rachmawati et al. 2014). Contradiction results between these two transgenic experiments can be attributed to the differences in plant type, callus age, callus culture condition, transformation method, insertional effect, retrotransposon activation, and other possible cause for this condition.

The non-significant differences in traits between transgenic and non-transgenic rice plants were shown by harvest index on all transgenic genotypes, number of productive tillers ( $T_{1-11}$ ,  $T_{1-12}$ ,  $T_{1-45}$ ,  $T_{2-20}$ ), number of maximum tillers ( $T_{1-11}$ ,  $T_{1-12}$ ,  $T_{1-45}$ ,  $T_{2-45}$ ), grain dry weight per panicle, number of filled grains ( $T_{1-11}$ ,  $T_{1-12}$ ,  $T_{2-7}$ ,  $T_{2-11}$ ,  $T_{2-12}$ ,  $T_{2-20}$ ,  $T_{2-40}$ ,  $T_{2-45}$ ), and number of empty grains ( $T_{1-11}$ ,  $T_{2-7}$ ,  $T_{2-11}$ ,  $T_{2-12}$ ,  $T_{2-20}$ ,  $T_{2-40}$ ) (Table 1). Harvest index, number of productive tillers, number of maximum tillers, grain dry weight per panicle, number of filled grains and number of empty grains in rice are affected by genetic, environment and genetic-environment interactions. Saito et al. (2021) found two novel QTLs (quantitative trait loci) controlling harvest index in rice. Harvest index is also affected by Phosphorous and Zinc concentration (Amanullah and Imanullah, 2016). Meanwhile, Huang et al. (2015) pointed out that high harvest index of high yielding rice cv. Guiliangyou 2 (GLY 2) was mostly demonstrated by large sink size, high remobilization of stored reserves, and maintained biomass production after heading. In addition, Nitrogen fertilizer plays significant role in determining number of tillers in rice. The application of Nitrogen fertilizer highly affected number of tillers in two rice cultivars with different yielding capacities. However, early emerging tillers had significant number of spikelets and more grain filling in all Nitrogen fertilizer levels (Wang et al. 2017). Nitrogen limitation and high density resulted in decreased shoot fresh weight, tiller number, plant height and chlorophyll content as studied by Misyura et al. (2014). Among environmental factors, solar radiation during heading date affected yield in tropical conditions (Garces-Varon and Restrepo-Diaz 2015; Amaya et al. 2019), altitude has high contribution to the variation of yield-related traits, including number of productive tillers, panicle length, number of filled grains, number of empty grains, weight of 1000 gains, and total grain weight per plant (Hasan et al. 2020).

Concerning other yield-related traits, i.e., grain weight, Katsura and Nakaide (2011) pointed out that greater grain weight was achieved in aerobic during ripening period than under flooded culture. In addition, one QTL cluster (*qTGW2*), located in the terminal region of chromosome 2, had significant effects on 1000-grain weight, grain length

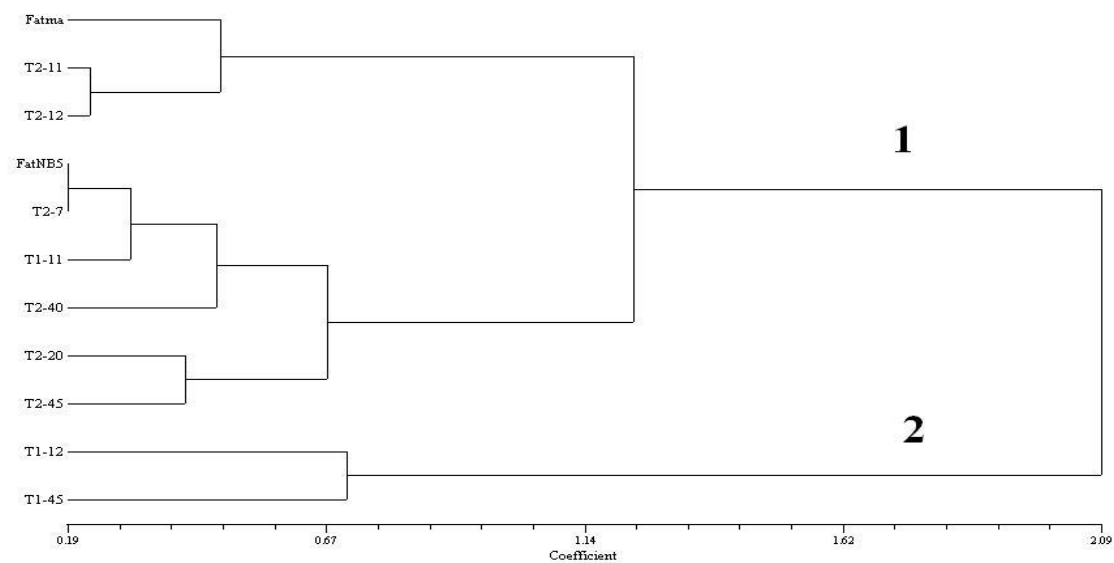
and grain width (Zhang et al. 2020). The development of grain weight is affected by molecular and genetic aspects that lead to dynamic changes in cell division, expansion and differentiation. Furthermore, several important biological pathways contribute to grain weight, such as ubiquitination, phytohormones, G-proteins, photosynthesis, epigenetic modifications and microRNAs (Chen et al. 2021). From these data, it suggests that grain weight is strongly affected by genetic, environmental, and genetic by environmental interaction.

### Genetic relationship analysis

The dendrogram (Figure 2) shows that the *Euclidian coefficient* was relatively short, which ranged from 0.19 to 2.09. For details, the dendrogram is divided into two main clusters, namely: (i) *Cluster I*: Fatmawati (developed from seed),  $T_{2-11}$ ,  $T_{2-12}$ , Fatmawati NB<sub>5</sub> (regenerated from callus cells),  $T_{2-7}$ ,  $T_{1-11}$ ,  $T_{2-40}$ ,  $T_{2-20}$ , dan  $T_{2-45}$ . (ii) *Cluster II*:  $T_{1-12}$  and  $T_{1-45}$ .

The genetic relationship among transgenic rice lines with the check plants was closely related because all genotypes have the same genetic background i.e., Fatmawati. Based on the dendrogram, it could be seen that there were two transgenic rice genotypes ( $T_{1-12}$  and  $T_{1-45}$ ) located in different clusters with the check genotypes. The genetic relationship among putative transgenic rice lines with their check was closely related. This is because all genotypes have the same genetic background, i.e., Fatmawati. Based on the dendrogram presented in Figure 2, it could be seen that there were two transgenic rice genotypes ( $T_{1-12}$  and  $T_{1-45}$ ) located in different clusters with the check genotypes. It could be possibly explained that although the plant has the same genetic background, the effect of transgene insertional, somaclonal variation, and activation of retrotransposon allows broadening the genetic base of rice. Transgenic rice lines which showed differences in agronomic traits with their checks were shown on productive tillers number (genotype  $T_{2-11}$ ,  $T_{2-12}$ ,  $T_{2-20}$ ), maximum tillers number ( $T_{2-7}$ ,  $T_{2-11}$ ,  $T_{2-12}$ ,  $T_{2-20}$ ), number of filled grains ( $T_{1-45}$ ), days to harvest on all  $T_2$  transgenic rice lines. Although it is not mentioned how close the relationship between transgenic and non-transgenic traits is, Chaerunisa et al. (2020) discovered one (W3 line) transgenic rice line, out of six, has a close agronomic characteristic with cv. Rojolele wild type. Further study on the molecular level is highly required to clarify the genetic relationship. It is suggested to utilize transgenic rice lines ( $T_{2-11}$  and  $T_{2-12}$ ) which are in the same cluster with the genotypes developed from the seeds.

In conclusion, transgenic rice plant with *Glu-1Dx5* transgene has been successfully developed and they have been selected based on a close genetic relationship of agronomic characteristics with its wild type. The selected genotypes will be further developed and assessed for the rice breeding program.



**Figure 2.** Dendrogram of genetic relationship among 11 genotypes of transgenic rice lines with the check plants based on agronomic characters. Remarks: T with additional number was transgenic rice lines, while Fatma: cv. Fatmawati developed from seed, while FatNB5: cv. Fatmawati regenerated from callus

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