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Research Article
Genetic Transformation of *Glu-1Dy10* Gene to Rice by Particle Bombardment

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Abstract

Background and Objective: *Glu-1Dy10* gene, isolated from bread wheat (*Triticum aesticum* L.), is a High Molecular Weight Glutenin Subunits (HMW-GS), which contributes to the elasticity and expanding power of wheat flour dough. Transformation of *Glu-1Dy10* into rice (*Oryza sativa* L.) cv. Fatmawati (FW) and Kitaake (KA) to obtain transgenic rice plants that contain *Glu-1Dy10* transgene. **Materials and Methods:** Eight combinations of treatment were performed with three factors: genotype (FW and KA), callus age (one and two weeks) and target distance (7 and 9 cm). Biorad PDS 1000/He was employed and evaluation of putative transgenic was carried out in T_0 rice using Polymerase Chain Reaction (PCR). **Results:** One week of Kitaake callus with a target distance of 9 cm (KA1-9) showed the best for the number of green spots, the number of shoots and plantlets obtained. Five putative transgenic rice plants (T_0) of cv. Kitaake were obtained. These plants are valuable genetic materials for further evaluation of rice dough functionality. **Conclusion:** Genetic transformation of the *Glu-1Dy10* gene has been achieved. Genotype, callus age and target distance affect transformation efficiency using this particular device.

Key words: Biorad PDS 1000/He, cv. Fatmawati, cv. Kitaake, embryogenic callus, rice

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Rice is an important food crop for more than half of the world's population of which 3.5 billion people in Asia and other countries in the world depend on rice for more than 20% of their daily calories. World production of rice was 966,879,011 t and the area harvested was 192,016,004 ha in 2019[1]. In Indonesia, rice is not only a staple food but also a source of food starch that can be used as raw material for the food industry such as rice flour. Compared with wheat flour, rice flour has a limitation in elasticity and expanding power of dough. Flour made from wheat is different from other cereal flours, including rice, because it contains gluten that gives its elasticity and extensity required for bread-making quality in bread wheat (*Triticum aesticum* L.)[2,3]. Elasticity and expanding power of flour are influenced by gluten content. Wheat is a crop that has high gluten content in the seeds. Gluten is a protein that is sticky and elastic. It is useful to bind and the gluten network is mainly responsible for the overall, viscoelastic properties of dough[4].

Gluten mostly consists of two types of seed storage proteins, the glutenins and the gliadins. Glutenins are classified into High-Molecular-Weight (HMW) subunits and Low-Molecular-Weight (LMW) subunits. They are encoded by *Glu-A1* loci, *Glu-B1* loci, *Glu-D1* loci, located in the long arms of the chromosome of homoeologous group 1[2]. The HMW glutenin has a great impact on the physical properties of flour[5,6] so that the processed food from wheat flour has good elasticity. One allele that contributes major in controlling the elasticity and expanding power of wheat flour dough, is *Glu-D1-1b*, which controls subunit *Dx5*, so-called *Glu-1Dx5*[7-9]. *Glu-1Dx5* gene was isolated from bread wheat cv. Cheyenne[10]. This gene has been transferred into the genome of rice cv. Fatmawati through particle bombardment[11] and has successfully obtained transgenic rice T_0 and T_1, also on generation T_2 and T_3 rice[12]. These transgenic rice plants had morphological and agronomical traits similar to those of origin, cv. Fatmawati. Improvement of bread-making quality from this rice (cv. Fatmawati inserted by *Glu-1Dx5* gene) has been confirmed by Wada *et al.*[13]. It was found that dough made from rice Glu-1Dx5 inserted gene was equal with those made from wheat. However, to strengthen the effect of the *Glu-Dx5* gene, the transformation of the *Glu-Dy10* gene is highly expected as practised by some researchers. Ballesteros *et al.*[14] proposed to strengthen the effect of *Dx5* by adding a *Dy10* allele in wheat-barley, while León *et al.*[15] did a cross between transgenic wheat-containing subunit *Dx5* with transgenic wheat-containing subunit *Dy10*. The result showed that the combination increases the flour dough expanding power. *Glu-1Dy10* gene is a very important allele, which is tremendously valuable if it is combined with the *Glu-1Dx5* allele for improving dough functionality. In the current research, this allele had been transferred using particle bombardment to create transgenic rice with *Glu-1Dy10*. As stated, a *Glu-1Dy10* gene, an HMW glutenin subunit that is genetically linked with *Glu-1Dx5* and located on arm chromosome group 1 genome A, B and D[2] plays important role in the dough functionality of wheat. The success of the genetic transformation is mainly affected by many factors including biological parameters, such as genotype, callus age, pre-culture, desiccation treatment, among others, meanwhile for physical parameters: helium pressure, gold particle size, gold particles per shot and plasmid-DNA per shot[16,17]. In this study, some important factors including genotype, callus age and target distance were optimized to improve transformation efficiency. Until now, there is no publication reporting the transformation of the *Glu-1Dy10* gene into the rice genome of cv. Fatmawati and Kitaake by particle bombardment (Biorad PDS-1000/He). Thus, this study will provide valuable insight for some researchers who interested in the development of plant genetic transformation techniques using this particular device.

MATERIALS AND METHODS

Study area: The experiment was carried from June, 2015 to July, 2016. These activities were callus preparation, particle bombardment experiment, callus selection (Fig. 1), plant regeneration (Fig. 2 and 3) and acclimatization and transgene detection (Fig. 4).

Embryogenic callus induction: Healthy mature dehusked embryos of rice cv. Fatmawati and Kitaake were used as explants to induce embryogenic callus using a medium containing B5 basic medium[18]. Three hundred twenty rice embryos of both genotypes were prepared for transformation. Eighty calluses for each genotype were prepared for bombardment. They were cultured in a culture room for one week and two weeks in the dark at temperature ±27°C in Table 1.

Plasmid DNA used in the bombardment: Plasmid *p*K-1Dy10 that used in this study was a kind gift from Dr A. Blechl, the Crop Improvement and Utilization, Western Regional Research Center, USDA, Albany California, USA. The plasmid was *p*K-Dy10A containing gene *Glu-D1-2b* which controls subunit *Glu-1Dy10* (9.36 kb). Another plasmid used was *p*RQ6

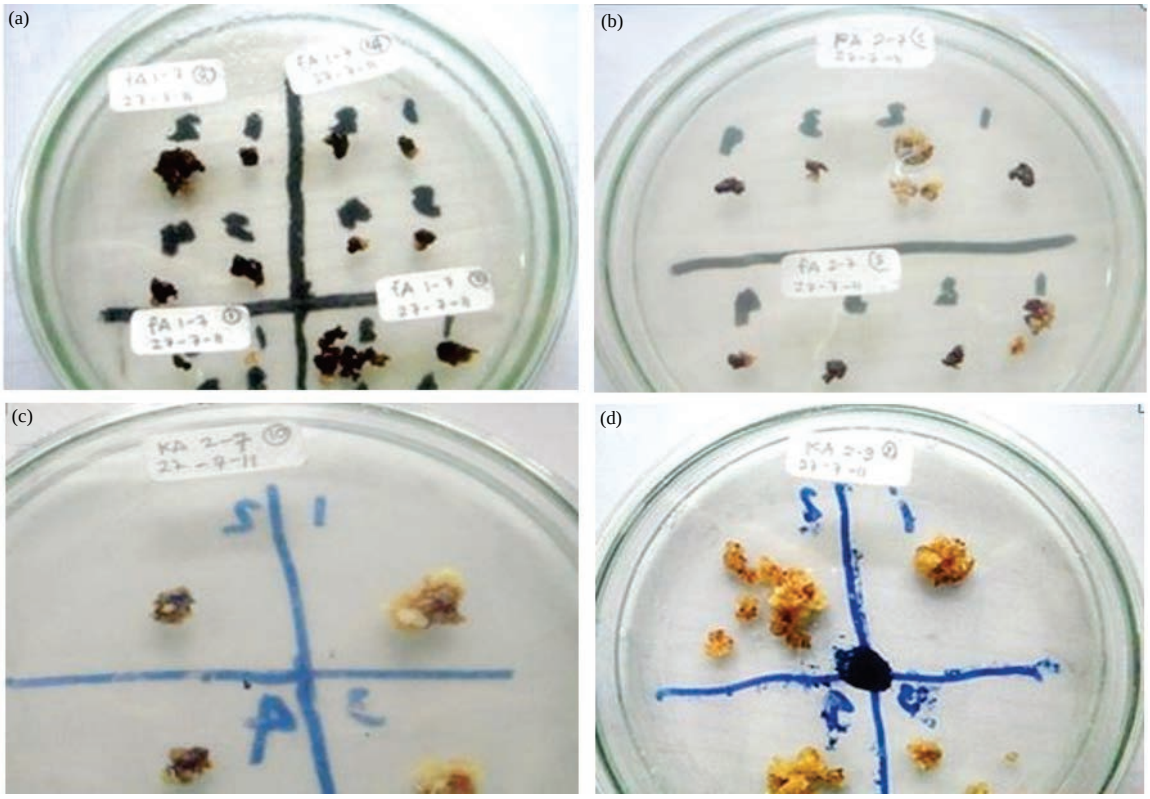

Fig. 1(a-d): *In vitro* callus appearance on hygromycin containing medium

(a) Calluses of cv. Fatmawati on hygromycin containing medium at 1 week callus age and 7 cm target distance, (b) Two-week callus age and 7 cm target distance, (c) Calluses of Kitaake on hygromycin containing medium at 2 week callus age and 7 cm target distance, (d) Two-week callus age and 9 cm target distance

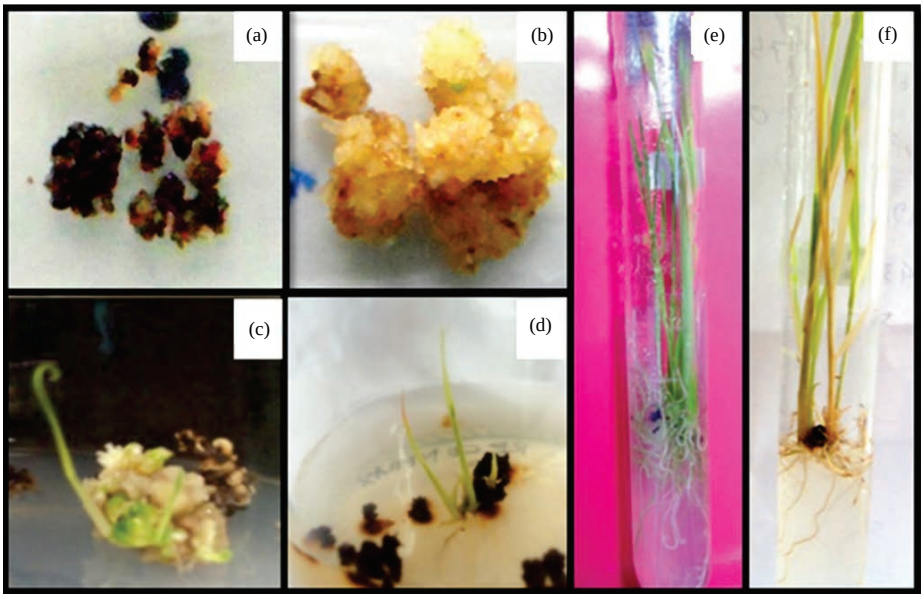

Fig. 2(a-f): Plant regeneration of cv. Fatmawati and cv. Kitaake, from calluses to plantlets

Calluses: (a) Fatmawati, (b) Kitaake , Shoots: (c) Kitaake, (d) Fatmawati, Plantlets: (e) Kitaake and (f) Fatmawati

Table 1: Culture media used (Adapted[16])

Culture media	Composition	Length of culture time	Condition
Callus Induction	Base medium B5, 3 mg L^{-1} 2,4-D, 30 g L^{-1} sucrose, 8 g agar, pH media 5.7-5.8	1 week, 2 weeks	Dark, temperature ±27°C
Pre-and post-medium after bombardment	Callus induction media, mannitol 30 g L^{-1}, sorbitol 30 g L^{-1}, pH media 5.7-5.8	4 hrs	Dark, temperature ±27°C
Recovery media	Glycine 10mL L^{-1}, glutamine 30 mg L^{-1}, casein hydrolysate, 1 g L^{-1} phytagel, 3 g L^{-1}, NAA, 10 mL L^{-1}, sucrose 5 g L^{-1}	1 week	Dark, temperature ±27°C
Proliferation	Base medium B5, 2,4-D 3 mg L^{-1}, l-proline 500 mg L^{-1}, l-glutamine 500 mg L^{-1}, 30 g L^{-1} maltose, 6 g agar, pH 5.7-5.8	1 week	Dark, temperature ±27°C
Callus selection	Base mediumB5, l-proline 500 mg L^{-1}, l-glutamine 500 mg L^{-1}, maltose 30 g L^{-1}, hygromycin 50 mg L^{-1}, 6 g agar, pH 5.7-5.8	1 week	Dark, temperature ±27°C
Regeneration	Base mediumB5, Kinetin 3 mg L^{-1}, BA 3 mg L^{-1}, IAA 0.5 mg L^{-1}, NAA 0.5 mg L^{-1}, l-proline 500 mg L^{-1}, l-glutamine 500 mg L^{-1}, casein hydrolysate 800 mg L^{-1}, maltose 30 g L^{-1}, hygromycin 50 mg L^{-1}, agar 12 g, pH 5.7-5.8	5 weeks	16 hrs light condition and 8 hrs dark, temperature ±27°C
Rooting	Media ½ MS, hygromycin 50 mg L^{-1}, +8 g agar, pH 5.7-5.8	2 weeks	16 hrs light and 8 hrs dark, temperature ±27°C
Total culture time		11-12 weeks	

(8.25 kb), which contain a *hpt* gene, harboring resistance to hygromycin, used as a selectable marker gene. Both plasmids were co-transformed with the molar ratio of 1.0:1.1 (ratio between *p*K-Dy10A and *p*RQ6).

Bombardment procedures: Bombardments were performed using protocols of Biolistic PDS-1000/He Particle Delivery System from Biorad Inc. Preparation for the bombardment was conducted by making gold suspension to coat DNA. Gold particles with a diameter of 0.6 µm as much as 3 mg were prepared for 8 times bombardment.

Selection and regeneration of transformed callus: *In vitro* selection for callus growth and development was conducted by applying hygromycin (50 mg L^{-1}). The selection was performed two until three times to obtain some promising calluses. Calluses were then regenerated in MS medium. The medium used, culture duration and incubation conditions are presented in Table 1. Some variables were recorded as follow: number of green spots, number of shoots and number of plantlets regenerated.

Detection of *Glu-1Dy10* transgene through PCR: PCR analysis for putative transgenic rice plants (T_0) to detect *Glu-1Dy10* transgene was performed. Primers designed specifically for the *Glu-1Dy10* gene was 5'ACTGACAGT CCACCGAGAT-3' (Forward) and 5-'CCTTTGTCCTGTGTGCTG-3' (Reverse). PCR reaction was performed on a thermocycler (Eppendorf) consisted of 1 cycle of 94°C for 5 min for initial denaturation, 30 cycles consisted of 94°C for 1 min for denaturation, 50°C for 1 min for annealing, 72°C for 1 min 30 sec for elongation and 1 cycle of 72°C for 5 min for final elongation. The amplification product was electrophoresed using a 1.0% agarose gel in 1×TBE at 70 volts for 60 min. DNA visualization was done by using a gel documentation system (*G-Box, Syngene*) and estimation of the DNA band size was aided by Gene tool software.

RESULTS

Transformation of *Glu-1Dy10* gene: Around 262 and 260 calluses of cv. Fatmawati and Kitaake, respectively, were bombarded. In both genotypes, the number of hygromycin-resistant callus was high, i.e., Fatmawati ranged from 23-60 calluses and Kitaake 35-60 calluses. Only in treatment 2-9 (2 week callus induction, 9 cm target distance), the number of callus drastically decreased due to hygromycin selection. Cultivar Fatmawati had 23 hygromycin-resistant

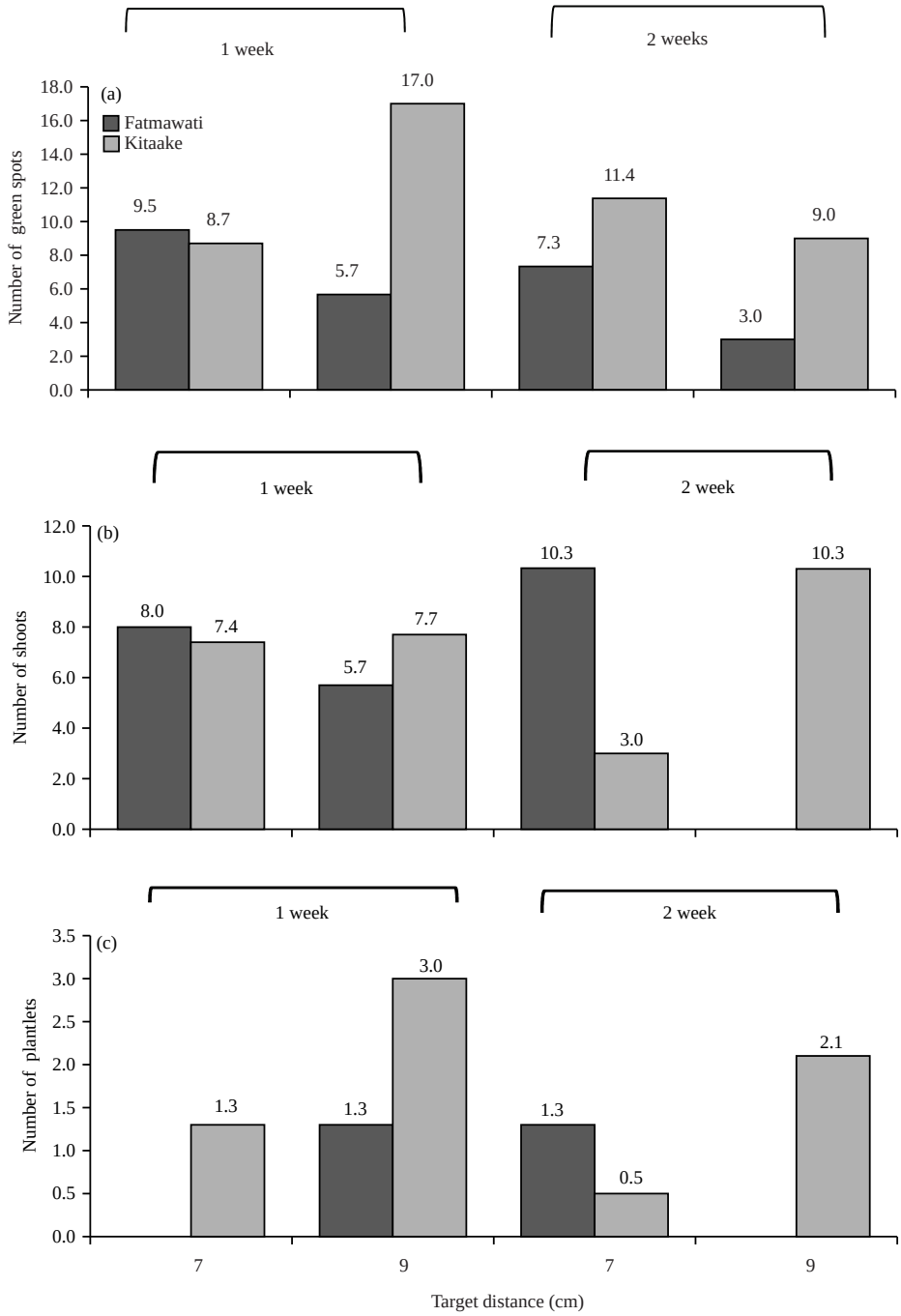

Fig. 3(a-c): Regeneration capacity of rice cv. Fatmawati and Kitaake
(a) Number of green spots, (b) Number of shoots, (c) Number of plantlets

calluses while Kitaake had as many as 35 hygromycin resistant calluses in Table 2. These callus appearances can be seen in Fig. 1, which showing calluses of cv. Fatmawati on hygromycin containing medium at 1 week callus age and 7 cm target distance in Fig. 1a and 2 week callus age and 7 cm target distance in Fig. 1b. In Fig. 1 also appearance of calluses of Kitaake on hygromycin containing medium at 2 week callus age and 7 cm target distance in Fig. 1c and at 2 week callus age and 9 cm target distance in Fig. 1d.

Table 2 showed a drastically decrease amount of callus resistance to hygromycin on regeneration media. As many as 47 calluses were resistant to hygromycin on regeneration

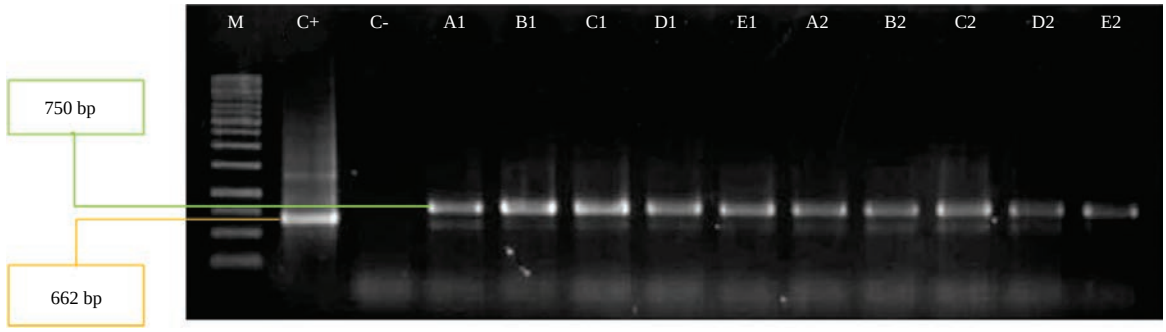

Fig. 4: PCR analysis for *Glu1-Dy10* at T_0 Kitaake (KA 1-9)
M: Marker 1 kb, C+: positive control (plasmid pK-Dy10A), C-: Negative control (wild type of Kitaake), A1-E1: Plants DNA samples (T_0), A2-E2: repetition of A1-E1

Table 2: Transformation of *Glu-1Dy10* into callus of rice cv. Fatmawati and kitaake

Treatments	No. of embryo cultured	No. of bombarded callus (A)	No. of hygromycin resistant callus on selection media (B)	No. of hygromycin resistant callus on regeneration media (C)	No. of regenerated callus (D)	Regeneration efficiency (%) (D/B)	Transformation efficiency (%) (C/A)
FA 1-7	80	64	54	3	2	3.70	4.69
FA 1-9	80	62	52	3	3	5.77	4.84
FA 2-7	80	72	60	3	3	5.00	4.17
FA 2-9	80	64	23	1	NA	NA	1.56
Total	320	262	189	10	8	4.23	3.82
KA 1-7	80	56	55	14	9	16.36	25.00
KA 1-9	80	68	60	9	6	10.00	13.24
KA 2-7	80	56	50	10	8	16.00	17.86
KA 2-9	80	80	35	14	10	28.57	17.50
Total	320	260	200	47	33	16.50	18.08

FA 1-7: Fatmawati, one week-callus age with target distance 7 cm, FA 1-9: Fatmawati, one week-callus age with target distance 9 cm , FA 2-7: Fatmawati, two week-callus age with target distance 7 cm, FA 2-9: Fatmawati, two week-callus age with target distance 9 cm , KA1-7: Kitaake, one week-callus age with target distance 7 cm, KA1-9: Kitaake, one week-callus age with target distance 9 cm, KA2-7: Kitaake, one week-callus age with target distance 7 cm, KA2-9: Kitaake, one week-callus age with target distance 9 cm, Regeneration efficiency I (%) (D/B): Number of callus regenerating/ number of callus resistant to hygromycin on selection media x 100%, Transformation efficiency (%) (C/A): Number of callus resistant to hygromycin on regeneration media/number of callus shot x 100%, NA: Data were not available

medium for Kitaake while 10 calluses of Fatmawati. The number of hygromycin resistant calluses on selection media affect transformation efficiency. Transformation efficiency was 1.56-4.84% for Fatmawati and 13.24-25.00% for Kitaake (Table 2). Inversely to the transformation efficiency, regeneration efficiency was a bit high, ranged between 16.00-28.57 and 3.70-5.77% for Kitaake and Fatmawati, respectively. Kitaake performed well in this variable. Fatmawati also showed better result because almost all transformed calluses (except FA 2-9) were able to regenerate although fewer in number compared to transformed Kitaake calluses (Table 2).

Regeneration of bombarded callus

Number of green spots: Green spot is an initial sign that transformed callus can regenerate and responsive to regeneration media content. According to Fig. 3a, at 1 week-old callus induction, a good response was given by target distance 7 cm as much as 9.5 for Fatmawati, while Kitaake bombardment by target distance 9 cm generated 17.0 green spots. The number of green spots of Kitaake was higher than those of cv. Fatmawati (Fig. 3a).

Cultivar Fatmawati at 2 week-callus induction age and target distance of 7 cm produced 7.3 green spots. Unlike Kitaake, 7 cm distance produced green spots of 11.4. When viewed holistically, KA 1-9 treatment was most responsive with the highest number of green spots (17.0). KA 1-9 produced green spots more than other treatments (Fig. 3a).

Number of shoots: Fatmawati could produce a well average number of shoots on 7 cm target distance both at one week (8.0 shots) and 2 week-callus induction (10.3 shoots). In contrast, Kitaake obtained many shoots in a 9 cm target distance i.e., 7.7 shoots at 1 week callus induction and 10.3 shoots at 2 week-callus induction. Fatmawati and Kitaake showed optimal results on 2 week-callus induction with the same result as 10.3 shoots. Fatmawati was more responsive to 7 cm shot distance, whereas Kitaake at 9 cm shoot distance in Fig. 3b.

Number of plantlets: Based on Fig. 2 and 3c, the genotype Kitaake was superior compared to Fatmawati on the number of plantlets. Kitaake (KA 1-9) treatment was optimum to produce plantlets with 3.0 plantlets per callus regenerated. A non-expected result found in Fatmawati (FA 1-7 and FA 2-9). FA 1-7 produced regenerated calluses with 19 green spots and 16 shoots but it did not produce plantlet at the rooting stage. FA 2-9 produced one-callus which produced 3 green spots, although the callus was not capable to form shoots so that the plantlets result was zero.

Detection of *Glu-1Dy10* with analysis of PCR (Polymerase Chain Reaction): Five putative transgenic plants were successfully grown and further observed by molecular analysis. DNA isolation and PCR detection were performed repeatedly on five samples to ensure the existence of band and technical error. Based on analysis using *in silico Fast PCR*, the expected product size was 662 bp. The test showed that primers specifically designed. The annealing temperature of 50°C was the optimum temperature for the amplification reaction of the *Glu-1Dy10* gene because the DNA band appeared clearly. The fifth leaf sample in Fig. 4 (marked A1-E1) and their repetition (marked: A2-E2) corresponded to the inserted gene but exceeds the expected product size (±750 bp). The clear result for positive control plasmid *p*K-Dy10A (+C) was detected in size of 662 bp whereas negative control of non-transformation Kitaake (C-) was not detected (Fig. 4).

Ten bands produced had thin bands (clear and bright band). The size of faint bands was parallel and had the same size as the positive control. This was suspected to be mispriming. Mis-priming showed primer attachment on the wrong location or found similar sequences so that it was amplified with unexpected sequences. Therefore, it is thought that the attachment of primer sequence on DNA target at annealing PCR process, the elongated sequence beyond the expected target band i.e., 750 bp.

DISCUSSION

Genetic transformation is one of the important technologies for inserting the gene of current interest. In this study, the *Glu-1Dy10* gene has been successfully transferred to rice cv. Fatmawati and Kitaake. Genotype Kitaake was excellent in producing green spots, shoots, plantlets and high in transformation efficiency. One week callus age and 7cm target distance were preferred for obtaining green spots and shoots. However, for the number of plantlets obtained, the 9 cm target distance was good than the 7 cm target distance.

In this study, the selection of promising calluses was done by adding hygromycin in the selection media. Hygromycin works by inhibiting protein synthesis with dual effects on mRNA translation. As antibiotics containing aminoglycoside, hygromycin causes mistranslation of aminoacyl-tRNA on the ribosome[19]. Hygromycin also affects the ribosome translocation process. By the presence of antibiotics mRNA often incorrectly translocate[19]. Callus cells on selection media that did not contain *hpt* (hygromycin phosphotransferase) inserted gene would die after exposure a few days. Calluses assumed dead were marked by blackened calluses but differ from browning symptoms, they tended to be dry and were not able to grow and develop.

The transformation efficiency of Fatmawati and Kitaake were lower as compared to the transformation efficiency of Damayanti *et al.*[20], the transformation of ACC oxidase genes in papaya resulted in the transformation efficiency of 6.6-53.3%. Likewise Frame *et al.*[21] on callus maize type II at 6.3-28%. Ge *et al.*[22] reported that the potential for callus induction and regeneration of rice tissue culture is highly dependent on several factors such as genotype of the donor plant, explant type, organic components and growth-regulating hormones in media. From those factors, the effect of genotype is indispensable. Though both Fatmawati and Kitaake are genotyping mostly used in regeneration study and proven to be responsive. Dewi and Purwoko[23] reported that the polyamine content of rice subspecies indica callus is lower than subspecies japonica, while the content of ACC and ACC oxidase activity of rice subspecies indica was higher. At various higher plants, it has been known that division, enlargement, elongation and rapid proliferation of cells affected by auxin application related to polyamine levels[24]. Presumed it causes rice subspecies indica is more difficult to regenerate than japonica.

It seems likely Kitaake is more responsive *in vitro* culture in terms of producing callus and green spots as obtained in this study. It is assumed that Kitaake has more the action of the genes that regulate and maintain the meristematic activity of the cells, level of hormones as well as on the activity of other genes that control different stages of plant morphogenesis[24]. Meanwhile, a 2 week callus induction time with 9 cm of target distance shows a decreased callus growth due to the presence of hygromycin. Combination of treatment between longer callus induction age and farther target distance, making plasmid (pRQ6 and pK-Dy10A) penetration is difficult to get inside the rice plant genome. Two-week callus induction age is likely to have not many young tissues, so they are difficult to be entered by particles carrying the

gene. The greater the distance the slower the firing speed of the particle, thus only some cells are transformed.

This result is consistent with those reported by Ambarwati *et al.*[25], 5 days old explants responded well to regenerate than 11-day-old explants. In general, the optimum target tissue used for transformation was a tissue that could resistant to stress or pressure during the bombardment i.e., young and actively dividing tissues.

Compared to Fatmawati, Kitaake was superior because it has good totipotency in producing a high number of plantlets. The totipotency ability of cells in each morphogenesis phase is controlled by the activity of genes to determine and maintain the meristematic activity of cells, a hormone produced, sensitivity on media and other gene activities that control various stages of morphogenesis. According to Purnamaningsih[26], the length of time (days) from callus induction until callus transfer to regeneration media can determine the frequency of regeneration. It might be due to the polyamine content and ACC oxidase activity of each genotype. Purnamaningsih[26] tested callus induction and regeneration of indica IR64 which can only survive for 30-40 days, after 40 days if not transferred to regeneration medium, then regeneration ability decrease.

Related to the sequential addition of 88 bp, another possibility of DNA contamination of superfluous DNA (excessive DNA) derived from the plasmid. DNA Integration resulted from particle bombardment is not a mechanism of T-DNA (Agrobacterium tumefaciens), so it is difficult to ascertain the exact or expected the inserted gene. As experienced by Wilson *et al.*[27] DNA sequence analysis in transgenic tobacco showed an unknown DNA fragment (previously the researcher suspected it as tobacco DNA) size of 260 bp which was a coding sequence of the E. coli chromosome. To ensure the presence of the *Glu-1Dy10* gene, it needs further confirmation by DNA sequencing.

CONCLUSION

One-week callus age is optimum for genetic transformation and regeneration of both cv. Fatmawati and Kitaake. A target distance of 7 cm is preferred for obtaining a high number of green spots, number of shoots but not for the number of plantlets. Kitaake produced a higher number of green spots, shoots and plantlets than those of Fatmawati. *Glu-1Dy10* gene has been successfully transferred by using Biolistic PDS-1000/He into rice genome and obtained five transgenic Kitaake plants.

SIGNIFICANCE STATEMENT

The transformation of *Glu-1Dy10* gene to rice genome cv. Fatmawati and Kitaake by particle bombardment can be beneficial for developing transgenic rice with other important genes. One week callus age and 7 cm target distance of Biorad PDS 1000/He is preferred and Kitaake is more responsive in obtaining green spots, shoots and plantlets and high in transformation efficiency. Thus, this experiment will provide valuable insight for some researchers who are interested in plant genetic transformation technique using this current device.

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