Agrobacterium mediated transformation of Banten local rice (cv. Pare Gajah) for Folate gene

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Abstract. Folate (vitamin B9) deficiency is a common problem that often occurs in developing countries including Indonesia. Rice which is a staple containing folate is very low. So it is necessary to find an effort so that the rice consumed can contain folate which is good for health.The aim of this study was to transform the folate gene to plant Banten local rice (*cv*. Pare Gajah) using *Agrobacterium tumefaciens*. This research was conducted from January to Mei 2019 in the Laboratory of Physiological and Plant Biotechnology, Agriculture faculty, Sultan Ageng Tirtayasa University. Transformation research used a Completely Randomized Design with two factors, the first factor is the transformation method (I) consist of 2 levels; namely in vitro and in planta. The second factor is hygromycin concentration (H) consist of 4 levels; namely 25, 50, 75, and 100 ppm. Each treatment was replicated six times. The results showed, in vitro transformation method had a lower lethal dose, causing average death of explant reaches 93,75%. In planta transformation method giving the best average efficiency transformation reach 13,33%. Hygromycin concentration 100 ppm causing the higher average death of explant reaches 98,34%. Hygromycin concentration 25 ppm causing the higher average efficiency transformation reach 17,50%.

Keywords: 2.4-D, Transformation, in vitro, in planta, hygromycin, Rice, folate, CGH1 gene.

1. Introduction

Folic acid (vitamin B9) is needed by children and adults to produce red blood cells and prevent anemia. Folic acid plays a major role in cell growth and development, and tissue formation. Folic acid deficiency, the body will be susceptible to diseases such as depression, anxiety, fatigue, insomnia, difficulty remembering, red tongue and sores to digestive disorders. Folic acid deficiency in pregnant women increases the risk of premature delivery, babies with low birth weight or with neural tube defects [1], [2] said that someone who lacks folate is more at risk of getting cancer because folate deficiency can increase chromosomal damage and incorporation of uracil into DNA.

The human body cannot synthesize the structure of folate so it requires intake from food. About 24-60% of women, both in developing countries and in developed countries experience folic acid deficiency because the content of folic acid in their daily diet is not sufficient.

To fulfill the need for folic acid, the assembly of rice varieties that can contain folic genes is needed. This is due to the high level of rice consumption in Indonesia, so that by assembling rice varieties containing folic genes can increase folic acid intake and prevent folic acid deficiency in the body. According to [3] this development effort can be carried out with plant breeding, one of which is biotechnology. Assembling rice varieties using genetic transformation techniques is expected to produce superior rice varieties with desired characteristics.

The use of local varieties of rice in breeding programs has often been advocated with the aim of broadening the genetic background of the superior varieties to be produced [4]. According to [5] Pare Gajah variety is one of the local varieties of Banten which is cultivated by the Kasepuhan community of Cisungsang Village because it is known for its long and rather large grains. By doing the breeding process to improve the weaknesses of local varieties of rice without changing other traits that have been favored such as the taste of rice and good adaptation in areas where rice is widely cultivated, local varieties can be obtained which are more beneficial while adding to its economic value.

Agrobacterium tumefaciens is one of the transformation techniques that many researchers have done and has been successful. *Agrobacterium* is a bacterium that plays a role in helping to insert genes into plant genomes. Rice is one of the agricultural crops that has been carried out a lot of genetic transformation to get superior varieties of rice. Some genes that have been successfully inserted in rice plants and are being evaluated for expression and function are genes for resistance to yellow stem borer pests, fungal disease, and drought-tolerant genes [6].

Transformation with *Agrobacterium* is divided into 2 methods, namely in vitro and *in planta*. *In vitro* transformation is a transformation method that uses tissue culture techniques, namely by callus culture or callus induction. While the *in planta* transformation method is a new method that is simpler than in vitro because it is done in ex vitro (outside the lab).

Based on the description above, it is necessary to do research on the Transformation of Folate Gene in the Banten Local Rice Varieties of Pare Gajah (*Oryza sativa* L.). The purpose of this study was to carry out the transformation of the folate gene into the genome of rice plants of the Banten local variety Pare Gajah (*Oryza sativa* L.).

2. Materials and Methods

This type of research is experimental research. This research was conducted at the Biotechnology Laboratory, Faculty of Agriculture, Sultan Ageng Tirtayasa University from November 2018 to January 2019.

2.1. Treatments

2.1.1. Callus Induction

The design used in the manufacture of callus as a preparation for transformant material consisted of one factor, namely the concentration of 2,4 Dichlorophenoxyacetate (D). The 2,4-D concentration factor consists of 3 levels, namely:

D_1 : 1 ppm
D_2 : 2 ppm
D_3 : 3 ppm

Each treatment was repeated 9 times to obtain 27 experimental units. Each experimental unit contained 6 seed explants. Then the total explants needed are 162 seed explants.

2.1.2. Folate Gene Transformation

The design used in this transformation research consisted of two factors. The first factor is the transformation method (I) and the second factor is the concentration of hygromycin (H). The first factor transformation method (I) consists of 2 levels, namely:

I_1 : *In vitro* transformation method
I_2 : *In planta* transformation method

The second factor is the concentration of hygromycin (H) consisting of 4 levels, namely:

H_1 : 25 ppm
H_2 : 50 ppm
H_3 : 75 ppm
H_4 : 100 ppm

Each treatment was repeated 6 times to obtain 48 experimental units. Each unit of experiment contained 10 callus / sprouts. Then the total explants needed are 240 callus and 240 sprouts.

2.2. Observation parameters:

2.2.1. Callus Induction

1) Time when Callus Formed (DAP */ days after planting*)
   Observation of the emergence of callus is done every day by counting how many days the callus has begun to appear or grow.
2) Number of Explants Forming Callus (%)
   Observations were made in the 3rd week after planting explants, with the following formula :

$$\% \text{ Callus} = \frac{\text{The number of explants forms a callus}}{\text{Total explants}}$$

3) Callus Texture
   Callus texture is visually observed in the appearance of callus which includes compact, intermediate or crumb callus textures. Callus texture was observed at 35 DAP (5 weeks after implantation).
4) Callus Colour
   The color of the callus is seen from the color formed. Callus color was observed at 35 DAP (5 weeks after implantation).
5) Callus Diameter (mm)
   Callus diameter is calculated by measuring the longest diameter of the side of the callus. Observation of callus diameter was carried out at the end of observation of callus induction (5 weeks after explant planting).
6) Callus Area (mm^2)
   The callus area was calculated using millimeter block. Callus area observation was carried out at the end of callus induction observation (5 weeks after explant planting).

2.2.2. Folate Gene Transformation

1) Lethal hygromycin dose
   Observation of lethal hygromycin dose test on transformation in vitro and *in planta* was done at the end of explant selection using hygromycin antibiotics, with the following formula [9]:

$$LD_{50} = a - (b/c)$$

   Notes:
   a = The smallest dose that causes the highest death of explants in one dose group.
   b = Total number of multiplications between different doses and the average mortality at the same interval
   c = The number of explants in one group
2) Efficiency of Putative Transgenic Transformation (%)
   Calculation of transformation efficiency *in vitro* and *in planta* is done using the following formula [6]:

$$\text{Transformation efficiency (\%)} = \frac{\text{The amount of hygromycin - resistant explants}}{\text{The number of initial explants of transformation}} \times 100\ \%$$

2.3. Research Implementation

2.3.1. In Vitro Transformation

1) Making Embryogenic Callus

Rice seeds are washed clean and soaked for 30 minutes in distilled water. After that the seeds are peeled and sterilized with 2 $g.L^{-1}$ detergent solution, 2 g^{-1} agrymicin, and 2 g^{-1} dithane for 15 minutes, then the seeds are rinsed with distilled water and sterilized again with 30% chlorine solution for 15 minutes in LAF. After that the seeds are rinsed with sterile distilled water 6 times, and then soaked in betadine solution for 5 minutes [8]. Then the seeds are planted on 2,4-D callus induction media. Media that has been planted with explants is then placed on a culture rack at 25-28^{o}C. Every 2 days the culture bottle is sprayed with 70% alcohol to avoid contamination. Callus began to form 2-3 weeks after planting explants in the media. The resulting callus was transferred to the same callus induction medium by cutting buds and endosperm. The first 2-week embryogenic callus can be used as an explant for transformation.

2) *Agrobacterium tumefaciens* Culture

Agrobacterium strain LBA4404 which carries the construction of the folate gene is grown on solid LB media containing 100 ppm kanamycin antibiotic and 50 ppm hygromycin antibiotic. Bacteria culture that has been distreaked on solid LB media is stored in an incubator of 28^{o}C for about 3 days or until the bacteria grows [6, 7].

3) Transformation of *Agrobacterium tumefaciens* to Embryogenic Callus

The infection solution to be used was inserted into corning as much as 15 mL, *Agrobacterium tumefaciens* culture was added to OD (optical density) 0.03-0.05 at 600 nm wave, acetosiringone was also added and allowed to stand for 15 minutes before use. The embryogenic callus that has been prepared is soaked with an infusion solution that has been added with acetosiringon first for 15 minutes for adaptation, then soaked in a bacterial suspension infectious solution for 15 minutes, after which the suspension is discarded and the callus is drained on filter paper for 10 minutes [11].

4) Co-cultivation

Co-cultivation media were made with 2 sheets of filter paper that were given liquid infection and acetosiringone that were previously made as much as 2 mL. Callus that has been infected by *Agrobacterium tumefaciens* was carried out co-cultivation in the dark for 3 days on the previously created co-cultivation media [11].

5) Selection of Transgenic Plants

Co-cultivation callus was subsequently grown on selection media with modified hygromycin concentrations of 25 ppm, 50 ppm, 75 ppm, and 100 ppm, then grown in dark conditions for 2 weeks (until the callus grows with callus enlarged, callus colorless black or necrosis) [11].

2.3.2. In planta Transformation

1) Rice Sprouts Preparation

Rice seeds used as explants are washed and soaked for 30 minutes in distilled water. After that the seeds are peeled and sterilized with 2 g/L detergent solution, 2 $g.L^{-1}$ agrymicin, and 2 $g.L^{-1}$ dithane for 15 minutes, then the seeds are rinsed with distilled water and sterilized again with 30% chlorine solution for 15 minutes in LAF. After that the seeds are rinsed with sterile distilled water 6 times, and then soaked in betadine solution for 5 minutes [8]. Furthermore, the seeds are soaked in sterile water for 2 days at 20°C. Water is replaced once during the soaking process. After 2 days of immersion, the embryo will turn white [10].

2) *Agrobacterium tumefaciens* Culture

Agrobacterium strain LBA4404 which carries the construction of the folate gene is grown on solid LB media containing 100 ppm kanamycin antibiotic and 50 ppm hygromycin antibiotic. Bacteria culture that has been distreaked on solid LB media is stored in an incubator of 28^{o}C for about 3 days or until the bacteria grows. [6, 7].

3) *Agrobacterium tumefaciens* inoculation to rice shoot

The infection solution to be used was inserted into corning as much as 15 mL, added *Agrobacterium tumefaciens* culture to OD 0,3-0,6 at 600 nm, also added acetosiringone and allowed to stand for 15

minutes before use. For bacterial inoculation of rice shoots is done by pricking the embryo (1-1,5 mm depth) with a sterile needle tip and then dipping in bacterial suspension for 15 minutes, after that the suspension is removed and rice sprouts are drained on filter paper for 10 minutes [10].

4) Co-cultivation

Co-cultivation media were made with 2 sheets of filter paper that were given liquid infection and acetosiringone that were previously made as much as 2 mL. Rice sprouts that have been infected by *Agrobacterium tumefaciens* were co-cultivated in the dark for 5 days on previously cultivated co-cultivation media [11].

5) Transgenic Plants Selection

Rice seeds were grown under sterile conditions in water (2-3 mm depth) containing hygromycin antibiotics with concentrations of 25 ppm, 50 ppm, 75 ppm and 100 ppm at 28°C. Resistance to hygromycin antibiotics was assessed after 7 days later [10].

6) DNA Isolation.

Leaves of 23 F1 from cross-bred Kewal Bulu Hideung X Mira-1 and the two parents (21 day after planting) were used to isolate the DNA with CTAB method. DNA from each samples was amplified with specific CGH1 SSR primer. The PCR product was separated using 2% agarose and visualized with Chemidoc gel system.

3. Results and Discussion

3.1. Callus Induction

The results of the variance recapitulation in Table 1 show the 2.4-D concentration significantly affected the time callus parameter (DAP) and had a very significant effect on the number of explants forming callus (%), but gave no significant effect on the callus diameter parameter (mm) and callus area (mm2).

Table 1. Recapitulation of Variance Effects of Growth Regulatory Substances 2.4-D Concentration on Banten Local Callus Pare Gajah (*Oryza sativa* L.)

No.	Observation Parameters	Concentration 2,4-D	KK (%)
1.	Time when Callus Formed (DAP)	*	6,52
2.	Number of Explants Forming Callus (%)	**	13,83
3.	Callus Diameter (mm)	tn	5,20[a]
4.	Callus Area (mm^2)	tn	5,82[a]

Notes :
tn : Have no influential effect
* : Significant influential effect at the 5% level
** : Very significant influential effect at 1% level
KK : Coefficient of Diversity
DAP : Days After Planting (*Hari Setelah Tanam)*
a : Transformed data $\sqrt{x + 0{,}5}$ once

3.1.1. Time of Callus Formation

Based on the data presented in Table 2 it was known that the 2.4-D concentration significantly affects the time parameters of callus formation. The 2.4-D concentration gives an average callus formation time at 13.37 DAP (days after planting), and the best 2.4-D concentration is produced at the D2 level (2 ppm) which is 12.67 DAP. The process of callus formation in Pare Gajah rice embryos induced using 2.4-D can be seen in Figure 1.

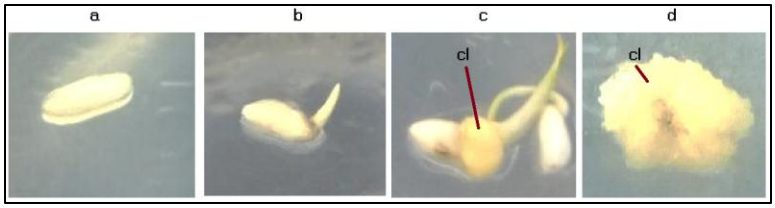

Figure 1. Process of Callus Formation in Pare Gajah Rice Embryo Induced using 2,4-D. Notes: a. Rice seeds were planted on N6 media with the addition of 2,4-D on day 0. b. Shoots on the 7th day after planting. c. Callus formed at the base of the bud of Pare Gajah rice embryo on the 12th day after planting. d. Callus shape on the 35th day. Information: (bj) seeds, (tn) shoots, and (kl) callus.

Table 2. The Effects of Growth Regulatory Substances 2,4-D Concentration towards Time when Callus Formed (DAP)

2,4-D Concentration	Time when Callus Formed (DAP)
D1 (1 ppm)	14,00c
D2 (2 ppm)	12,67a
D3 (3 ppm)	13,44b
Average	13,37

Note : the numbers that followed by the same letters in the same row or column show no significant difference according to the DMRT test of 5% level.

3.1.2. Number of Explants Forming Callus (%)

Based on the data presented in Table 3, the 2.4-D concentration at D2 level (2 ppm) is the best concentration with the percentage of explants forming callus that is 94.44%.

Table 3. The Effects of Growth Regulatory Substances 2.4-D Concentration towards Number of Explants Forming Callus (%)

2,4-D Concentration	Number of Explants Forming Callus (%)
D1 (1 **ppm**)	72,22a
D2 (2 **ppm**)	94,44b
D3 (3 **ppm**)	88,89b
Average	85,18

Note: the numbers that followed by the same letters in the same row or column show no significant difference according to the DMRT test of 5% level.

3.1.3. Callus Texture

Based on the data presented in Table 4. it is known that the three concentrations of growth regulators 2,4-D have no significant effect on the callus texture, because all three concentrations of 2,4-D produce a callus that is crumbly (friable)

Table 4. The Effects of Growth Regulatory Substances 2,4-D Concentration towards Callus Texture

2.4 D concentration	(1)	(2)	(3)
		%	
D1 (1 ppm)	0	0	100
D2 (2 ppm)	0	0	100
D3 (3 ppm)	0	0	100

Note: Scoring of callus texture : (1) solid, (2) intermediate, dan (3) friable.

3.1.4. Callus Colour

From the data in Table 5. it is known that administration of different 2,4-D growth regulators produces different callus colors. At the beginning of the formation of the callus, the resulting callus is yellowish white. But over time there are even calluses that change color to white, yellow, and even brownish white at the end of the observation (Figure 2). In the treatment D1 (1 ppm), the color of the callus is dominated by yellowish white and brownish white. Whereas the treatment of D2 (2 ppm) and D3 (3 ppm) was dominated by a yellowish white color.

Table 5. The Effects of Growth Regulatory Substances 2,4-D Concentration towards Callus Colour

2.4 D concentration	(1)	(2)	(3)	(4)	(5)	(6)	(7)
				%			
D1 (1 ppm)	22,22	11,11	0	0	33,33	0	33,33
D2 (2 ppm)	33,33	0	0	0	66,67	0	0
D3 (3 ppm)	22,22	0	0	0	44,44	0	33,33

Notes: scoring of callus colour : (1) white, (2) yellow, (3) green, (4) brown, (5) yellowish white, (6) greenish white, dan (7) brownish yellow.

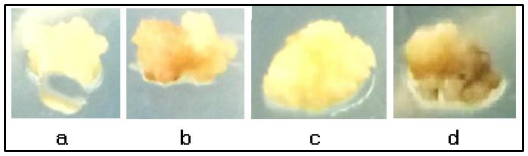

Figure 2. Callus Colour of Pare Gajah Rice on 35 DAP (days after planting). Notes: (a) White, (b) Yellow, (c) Yellowish White, and (d) Brownish Yellow

3.1.5. Callus Diameter (mm)

Table 6. Shows the average callus diameter decreases with increasing 2,4-D concentrations given. The average callus diameter tends to be longer to be found at the D1 level (1 ppm) which is 0.49 mm and which tends to be smaller at the D3 level (3 ppm) which is 0.37 mm.

Table 6. The Effects of Growth Regulatory Substances 2,4-D Concentration towards Callus Diameter (mm)

2.4 D concentration	Callus diameter (mm)
D1 (1 ppm)	0,49
D2 (2 ppm)	0,44
D3 (3 ppm)	0,37
Average	0,44

3.1.6. Callus Area (mm^2)

Table 7. The Effects of Growth Regulatory Substances 2,4-D Concentration towards Callus Area (mm^2)

2.4 D concentration	Callus area (mm^2)
D1 (1 ppm)	43,32
D2 (2 ppm)	38,14
D3 (3 ppm)	30,25
Average	37,24

The diameter of the callus is directly proportional to the area of the callus, the greater the diameter of the callus produced, the greater the callus area. Based on Table 7 it is known that the 2.4-D

concentration gives an average callus area of 37.24 mm2. Callus tends to be more widely produced at the D1 level (1 ppm) which is 43.32 mm2 and callus tends to be smaller at the D3 level (3 ppm) which is 30.25 mm2. Calculation of callus diameter and callus area using millimeter block can be seen in Figure 3.

Figure 3. Calculation of Callus Diameter and Area Using Millimeter Block.

3.2. Folate Gene Transformation

The results of the variance recapitulation in Table 8. show that the transformation method treatment significantly affected the hygromycin lethal dose parameters (LD50) and the transgenic putative transformation efficiency (%), while the hygromycin concentration treatment had a very significant effect on the parameters of lethal hygromycin dose (LD50) observations and the efficiency of putative transformation transgenic (%). transgenic putative transformation efficiency (%), but there is no interaction between the transformation method treatment and hygromycin concentration.

Table 8. Recapitulation of the Variance effects of the transformation method and Hygromycin concentration towards the results of the transformation of folate genes in local Banten rice explants Pare Gajah

No.	Observation parameters	Treatment			cv (%)
		Transformation Method (I)	Hygromycin concentration (H)	Interaction (I*H)	
1.	Lethal Hygromycin Dose (LD_{50})	*	**	ns	10,97
2.	Efficiency of Transformation (%)	*	**	ns	10,08[a]

Notes: ns : Not significant
* : Significant at 5%
** : Very significant at 1%
cv : Coefisien of variation
a : Transformed data $\sqrt{x + 0,5}$ twice

3.2.1. Lethal Hygromycin Dose

Table 9. Percentage of dead Pare Gajah Rice Explants in Hygromycin Lethal Antibiotic Test Trials on Selection Media

Hygromycin Concentration	Transformation Method		Average
	In vitro (I1)	*In planta* (I2)	
H1 (25 ppm)	83,33	81,67	82,50[a]
H2 (50 ppm)	95,00	83,33	89,16[b]
H3 (75 ppm)	96,67	85,00	90,84[b]
H4 (100 ppm)	100,00	96,67	98,34[c]
Average	93,75[b]	86,67[a]	

Notes : the numbers that followed by the same letters in the same row or column show no significant difference according to the DMRT test of 5% level.

The table shows the percentage of dead callus and dead or non-growing sprouts increasing with increasing concentrations of hygromycin antibiotics. In the in vitro transformation the lowest percentage of callus was 25 ppm which was 83,33%, while the largest percentage of callus died was 100% at 100 ppm hygromycin concentration. *In planta* transformation data obtained dead sprouts tend to be lower at 25 ppm hygromycin concentration that is 81,67% and tend to be higher 96,67% at 100 ppm.

From the table shows LD50 hygromycin using the Karber Method formula for in vitro transformation which is 29,17 ppm. This figure is smaller than the LD50 transformation *in planta* which is 35,63 ppm.

The results of transformation carried out *in vitro* and *in planta* using Agrobacterium tumefaciens were tested based on the effectiveness of hygromycin in inhibiting the growth of explants that failed to be transformed. Explants that have successfully grown and which have died in the selection media can be seen in Figure 4. and Figure 5.

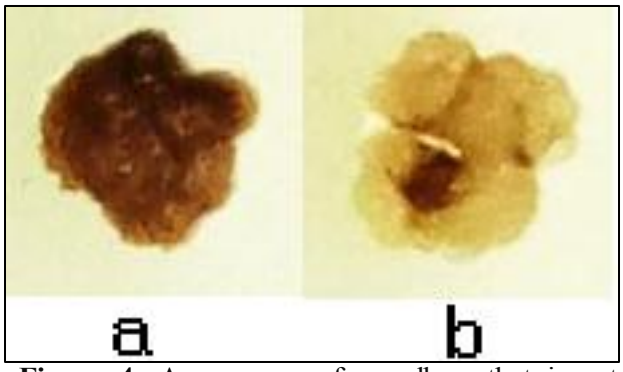

Figure 4. Appearance of a callus that is not resistant to hygromycin (a); kalus resisten terhadap higromisin (b).

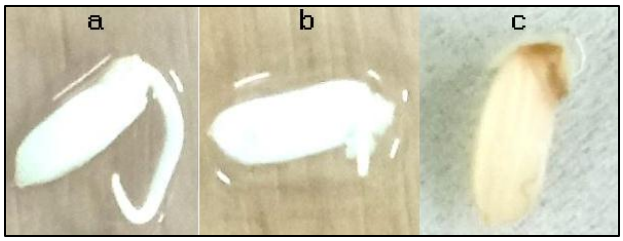

Figure 5. (a) Transgenic putative rice seeds are still able to germinate in hygromycin-containing media, (b) Non-transgenic rice seeds are germinated in hygromycin-containing media, (c) Non-transgenic rice seeds blackened after selection on hygromycin media.

3.2.2. Efficiency of Putative Transgenic Transformation (%)

Table 10. The Effect of Transformation Method and Concentration of Hygromycin towards the Efficiency of Putative Transgenic Transformation of Pare Gajah Rice Explants (%)

Hygromycin Concentration	Average Number of Callus Resistant	Average Number of Sprouts Resistant	Efficiency of *In Vitro* Transformation (%)	Efficiency of *In Planta* Transformation (%)	Average Transformation Efficiency (%)
25 ppm	1,67	1,83	16,67	18,33	17,50[c]
50 ppm	0,50	1,67	5,00	16,67	10,84[b]
75 ppm	0,33	1,50	3,33	15,00	9,16[b]
100 ppm	0,00	0,33	0,00	3,33	1,66[a]
Average			6,25[a]	13,33[b]	

Notes : the numbers that followed by the same letters in the same row or column show no significant difference according to the DMRT test of 5% level.

Based on Table 10. it is known that the transformation efficiency of putative transgenic *in planta* method is greater than *in vitro* method, which is 18.33% and the percentage of transformation efficiency of putative transgenic *in vitro* and *in planta* decreases with increasing hygromycin concentration in the selection media. Putative transgenic that pass in hygromycin media are then maintained and PCR tested with a phen-specific CG CG1 primeer. The PCR results can be seen in Figure

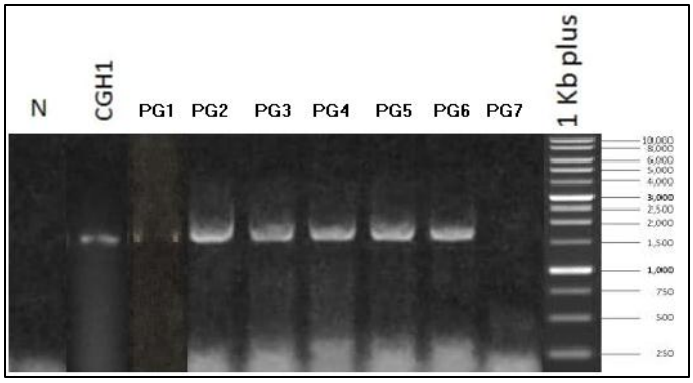

Figure 6. PCR result of putative transgenic rice folate gene

Notes:

N = Pare Gajah (Non-transgenegenic)
PG1,2,3,4,5,6,7 = Pare Gajah putative transgenic folate gene
CGH1 = *Agrobacterium tumefaciens* which contains the folate gene construct
1 kb plus: Ladder

Figure 7. Appearance of Pare Gajah transgenic rice in vegetative phase that has been inserted with the folate gene (a); starts flowering (b); starts the grain filling phase (c)

Positive rice has a 1200 bp folate gene band, treated and implanted in soil media. Based on information from PCR results it is known that putative GMOs PG1, PG2, PG3, PG4, PG5, PG 6 have a DNA band size of 1200 bp (the same as the control which is *Agrobacterium tumefaciens* which carries 'construct folate gene'). Putative transgenic samples of PG7 did not have a 1200 bp DNA band (the same as the negative control which is Pare Gajah rice that is not transformed). The appearance of the transgenic plant can be seen in Figure 7.

4. Conclusions

4.1. Callus induction

Callus induction namely: 2,4-D concentration gives a significant effect on the time parameters of callus formed (DAP) and significantly affects the number of explants forming callus (%), but not significantly on callus texture parameters, callus diameter (mm) and callus area (mm^2). Of the three concentration levels used, a 2,4-D concentration of 1 ppm produced the best callus diameter (0.49 mm) and callus area (43,32 mm^2). 2,4-D concentrations of 2 ppm for the time the callus formed (12,67 days), the number of explants formed a callus (94,44%) and the best callus color (yellowish white).

4.2. Transformation of folate genes

1. The transformation method significantly affects the lethal parameters of hygromycin dose and the efficiency of transgenic putative transformation (%). Of the two transformation methods used, the *in vitro* transformation method has a lower lethal dose (more toxic), causing an average explant mortality of 93.75% with an average transgenic putative transformation efficiency of 6,25%. In the in planta lethal method the dose is higher with an average explant mortality of 86,67%, and the best efficiency of transgenic putative transformation is 13,33%.
2. Hygromycin concentration has a very significant effect on lethal hygromycin dose and the efficiency of transgenic putative transformation (%). Of the four levels of hygromycin concentration used, the 100 ppm hygromycin concentration had the most toxic lethal dose which caused the highest mortality rate (98,34%) with the lowest average level of transgenic putative transformation efficiency (1,66%). Hygromycin concentrations of 25 ppm had lethal doses causing the lowest explant death rates (82,50%), with the highest average transgenic putative transformation efficiency (17,50%).
3. There is no interaction between the treatments of transformation method with the treatment of hygromycin concentration on the results of the transformation of folate genes in the Banten Pare Gajah local rice.
4. PCR results show DNA samples of Pare Gajah rice PG1, PG2, PG3, PG4, PG5, PG 6 have a folate gene encoding DNA band with a size of 1200 bp.

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6. Acknowledgements

Thank you for the Islamic Development Bank which has funded this research as a part of the Center of Excellent for Food Security of University of Sultan Ageng Tirtayasa.