B I O D I V E R S I T A S
Volume 10, Number 2, April 2009
Pages: 63-69

ISSN: 1412-033X (printed edition)
ISSN: 2085-4722 (electronic)
DOI: 10.13057/biodiv/d100202

Genetic Transformation of Transcription Factor (35S-*oshox4*) Gene into Rice Genome and Transformant Analysis of *hpt* Gene by PCR and Hygromycin Resistance Test

ENUNG SRI MULYANINGSIH[1,♥], REGY HERMAWAN[2], INEZ HORTENZE SLAMET-LOEDIN[1]
[1] Research Centre for Biotechnology, Indonesian Institute of Sciences (LIPI), Cibinong-Bogor 16911
[2] Department of Biochemistry, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University (IPB) Bogor 16680.

Received: 8 th November 2008. Accepted: 20th December 2008.

ABSTRACT

Global warming, climate change and crop extensification in marginal dryland areas are related to long dry season and water deficit. The water availability is an important factor in improving plant production. Application of drought tolerant rice cultivars is one of several options that might be used. Genetic engineering at the level of transcription factors is particularly promising strategy to develop drought tolerant rice varieties. Transcription factors regulate a wide range of target genes in which of them contribute to stress tolerance. HD Zip genes are transcription factor that potential in the adaptation of plants to some environment stresses including water deficit. HD-ZIP *oshox4* (*oryza sativa homeobox*) gene controlled by 35S promotor is inserted into pCAMBIA 1300 vector with *hpt* (hygromycin) gene as a selectable marker. The aim of this research is to obtain transgenic rice plant from transformation with *35S-oshox4* plasmid, segregation analysis of marker gene (*hpt*) by PCR method at T_0 and T_1 generation, and hygromycin resistance analysis of seeds. Recombinant plasmid was transformed into rice genome of IRAT 112 and rojolele cultivars using *Agrobacterium tumefaciens*. The results showed that transformation efficiency of IRAT 112 was 5.7-13.6% and 26-66,7% for rojolele. While regeneration efficiency for IRAT 112 is 4.7-43.7% and 23-44.1% for rojolele. The result of hygromycin resistance test at T_1 seeds were obtained 14 lines cv. rojolele segregation Mendelian for *hpt* gene. The PCR analysis using specific primers for *hpt* gene at the parent (T_0) from 14 lines showed that 7 lines contain the gene. At the second generation (T_1), PCR analysis using *hpt* primers showed that 3 from 4 lines were followed Mendelian segregation pattern by the presence of specific band.

Key words: HD-Zip *oshox* (*oryza sativa homeobox*), *35S-oshox4*, *hpt*, *Agrobacterium tumefaciens,* PCR.

INTRODUCTION

Global warming, unpredictable climate change and crop extensification in marginal dryland area are related to long dry season and deficit water. Water availability is a major limiting factor for increase of food production. According to Gorantla et al*.* (2005), rice crop consume about 90% from total water that is utilized for agricultural need at Asian. About 80% of total rice crop need water because most of rice crops are irrigated rice field (55%) and rain field (25%). Water deficit caused stress for plant. Water deficit will be major problem which influence global productivity. The drought stress effect could be lost of productivity until more than 70% (Bray et al*.*, 2000).

In attempt to dissolve of that problem, drought tolerance plant was used. Drought tolerance plant could be obtained by some technique, such as genetic transformation and plant breeding. The important thing in creating this plant is understanding drought tolerant. Drought tolerance character is encoded by many genes. Shinozaki and Yamaguchi Shinozaki (1997, 2007) classified this into two groups; first, protein that most probably function in drought (protection factors of macromolecules, enzymes for osmolyte biosynthesis, detoxification enzymes, water channel, transporter); and second is regulatory protein (protein kinase, protein phosphatase, enzyme phospholipid metabolism and transcription factors).

Genetic Transformation at transcription factor level will enable to obtain of drought tolerant rice, because transcription factor regulate other genes that responsible to drought tolerance. Some of transcription factors have been characterized, for example DREB (*dehydration responds element binding)* (Yamaguchi Shinozaki and Shinozaki, 2001; Sakuma et al*.*, 2006), SNAC (*stress responsive NAC1)* (Hu et al*.*, 2006), and HD Zip (*Homeodomain leucine zipper)* (Meijer et al*.,* 1997; Deng et al*.,* 2002). The over expression of these gene in some plants

♥ **Corresponding address:**
Jl. Raya Bogor Km.46 Cibinong-Bogor 16911
Tel. +62-21-8754587; Fax. +62-21-8754588
e-mail: enungf@yahoo.com

caused increase of drought tolerance (Scarpella et al., 2005; Hu et al., 2006).

HD Zip genes were reported to be related with adaptation and development of plants under stressful environment. HD Zip genes in rice plant are classified into family I, II, III that consist of 33 HD Zip genes *oshox* (*oryza sativa homeobox)* which is spread in 12 chromosomes. There are only two genes *oshox* have been identified which is *oshox1* (HD Zip II) and *oshox4* (HD Zip I) that regulate by drought stress. The expression of genes are up and down regulated under drought situation and depend on plant sensitivity to drought (Purwantomo, 2007; Agalou et al*.,* 2008).

Research Centre for Biotechnology LIPI have obtained first generation (T_0) of putative transgenic rice from rojolele and IRAT 112 cultivars that transformed with transcription factor HD Zip *oshox4* gene. This gene is controlled with constitutive promotor CaMV 35S (*cauliflower mozaic virus*). *Cassette* 35S *oshox4* and terminator were inserted in pCAMBIA 1300 plasmid on Hind*III* site. *Hygromycin phosphotransferase* (*hpt*) gene to generate hygromycin antibiotic resistance for transformant selection. The recombinant plasmid was designated as 35S *oshox4* and transformed into javanica cultivar (rojolele) and indica cultivar (IRAT 112) using *Agrobacterium tumefaciens* (*A. tumefaciens)*.

The aim of research is to obtain transgenic rice plant transformed with 35S-*oshox4* plasmid, to evaluate lines containing *hpt* gene based on PCR analysis, to evaluate expression and segregation pattern of *hpt* using bioassay hygromycin seeds.

MATERIALS AND METHODS

Rice cultivar of javanica rojolele and indica IRAT 112 transformed by 35S-*oshox4* plasmid were made available Dr. A.H Meijer and Dr Pieter B.F. Ouwerkerk (Institute of Molecular Plant Science, Leiden University). Strain *A. tumefaciens* using EHA 105 resistance to rifampycin antibiotic. Analysis transformant at first (T_0) and second ($T_{1)}$ generation plant population that property RC Biotechnology LIPI. Materials for DNA isolation comprise: isolation buffer [*lysis buffer* (Tris HCl pH 7.5 0.2 m, EDTA 0.05 M, NaCl 2 m, and CTAB 2%)]; extraction buffer [*extraction buffer* (sorbitol 0.35 m, Tris HCl pH 7.5 0.1 m, and EDTA 5 mM); sarkosil 5%], N_2 liquid, chloroform: isoamylalcohol (24:1), isopropanol, ethanol 70%, TE buffer (Tris HCL pH 8.0 10 mM and EDTA PH 8.0 1 mM). Material used for PCR reaction: plant DNA, PCR buffer, dNTPs, primary *gos-5 forward* and *reverse* (5'CCGACCTCGAGGACATCGG CAACAG 3') and (5' GCCGAGAGCAGCAGGAACTT GACAGG 3'), primary *hpt forward* and *reverse* (5' GATGCCTCCGCTCGAAGTAGCG 3') and (5' GCATCTCCCGCCCGTGCAC 3 '), dH_2O, agarose gel and Tris Boric Acid EDTA buffer (TBE). Material those were utilized for hygromycin test: 50 mg/mL hygromycin, sterile aquadest, sterile filter paper, alcohol 70%, benlate 3%, and NH_4ClO 2%.

Genetic transformation into rice genome

Recombinant plasmid 35S-*oshox4* was inserted into *A. tumefaciens* strain EHA 105 by electroporation method. Bacteria were cultured in solid AB medium which contained rifampycin 20 mg/L and kanamycin 50 mg/L. Bacteria culture were then incubated at 28°C for 3 days. This culture was used for co-cultivation.

Transformation into embriogenic callus using *A.tumefaciens* was perfomed following methods as described elsewhere (Hiei et al., 1994). *A. tumefaciens* was subcultured in AAM liquid medium without antibiotic and shaked to 1-2 hours until $OD_{600:}$ 0,4-0,5. The embryogenic callus from cv. rojolele and cv. IRAT 112 were induced in IK3 medium (LS media that contains 2,4-D 2,5 mg/L and solidified with phytagel 0,2%) (Slamet-Loedin et al*.,* 1997). Callus induction was done for 2 weeks in dark room. For infection, callus is soaked in *A. tumefaciens* culture up to 30 minutes. Callus and bacteria at co-cultivation in IK3-AS medium (IK3 contain of acetosyringone 100 mM) and incubation on 25°C for 3 days in dark. After co cultivation, callus washed by cefotaxime 400 mg/L and cultured at $IK3C_{250}\,H_{50}$ as selection medium (IK3 contain of cefotaxime 250 mg/L and hygromycin 50 mg/L) for 2 weeks. Callus were subculture at second selection medium for two weeks $IK3C_{50}\ H_{50.}$ The resistance callus to hygromycin were subculture into R3B medium for regeneration (LS + IAA 0,5 mg/L + BAP 0,3 mg/L and phytagel 0,5%) (Slamet-Loedin et al*.,* 1997). Obtained plantlets were moved into MS medium without hormone. The normal plantlets were acclimatized into soil medium at greenhouse.

DNA isolation for PCR

DNA Isolation for PCR from leaf (2-3 weeks plant). Isolation method as follows: leaf 5 cm length was inserted put into microtube 1.5 mL, add with N_2 liquid then grinded and added with 750 μL isolation buffer. The mixtures were then incubated at 65°C for 1 hour. Later tube is added 750 μL chloroform: isoamylalchohol (24:1) and centrifuge 5 minutes at 12.000 rpm, 4°C. The supernatant was transferred into new tube and added with 400 μL isopropanol (cold), then centrifuge for 6 minutes at 12.000 rpm on 4°C. Supernatant was discarded and pellet was washed by ethanol 70% then centrifuge at 12.000 rpm on 4°C for 3 minutes. Supernatant is discarded and pellet in tubes dried. Palette from DNA was dissolved by TE 50 μL and stored at 20°C.

PCR analysis at T_0 plant

Plants DNA were obtained from T_0 and T_1 population. PCR reaction is enclosed with primer *gos-5 I* as internal rice gene. Negative control using DNA plant controls (without transformation) and water,

while positive control uses 35S-*oshox4* plasmid. PCR reaction mixtures were as follows: 1x PCR buffer, dNTP 0.05 mM, Taq Polymerase 0.05 u, primer 2. 5 ng/µL *gos*-5 *reverse* and *forward*, primer 2.5 ng/µL *hpt reverse* and *forward*. PCR temperature and cycle it one denaturation cycle (95°C, 10 minutes): 40 amplification cycles [denatures 95°C 1 minute, *annealing* 55°C 1 minute, synthesis 72°C 1 minute]; 72°C 10 minutes (final elongation); 4°C (store).

Hygromycin test

As many as 76 lines rice seed T_1 (planted 50 seeds for each line). Seeds was husked, washed with sterile aquadest and followed with alcohol 70% for 1 minute and washed with sterile aquadest. Seeds were then soaked with benlate 3% solution for 30 minutes and washed with sterile distilled water until clear. Finally, seeds were washed by 2% NH_4ClO for 30 minutes and rinsed with sterile distilled water for 5 minutes 10 times repeatedly at *laminar air flow*. Planted seed on sterile filter paper already been damped by 50 mg/L hygromycin. As for control, filter paper was just soaked with distilled water. Seed were placed in dark room to stimulate germination for 3 days, then moved under lamplight. The observation for seedling growth was carried out 2 weeks after plantation under following classification: normal seedling, abnormal and not grown (total number of normal seedling as amount of plant individual that using chi square to observe segregation pattern hpt gene). With opportunity point 5% and degree of freedom 1, therefore point chi square shall be smaller than F table (3,84). It means pattern of segregations follow Mendelian which is 3: 1. Equation for tests chi square as follows:

$$\chi^2 = \frac{(O_i - E_i)^2}{E_i}$$

O i = phenotype's observing point goes to i.
E i = phenotype's expectation point goes to i.

PCR analysis of T_1 plant

The normal seedling based on hygromycin test and segregation follows Mendelian (base on chi square) were planted on soil medium at greenhouse. Thirty seeds for each line were planted. DNA template for PCR reaction was isolated from leaf derived from 2-3 weeks old seedling according to the method described previously. PCR analysis was performed to confirm the presence of *hpt* gene on second generation plant (T_1). PCR conditions were the same with T_0 analysis plant.

RESULT AND DISCUSSION

Transformation into rice genome

The transformation result into genome of two cultivar rice is presented at Table 1. Based on this table transformation efficiency and regeneration of cv. rojolele was higher than that of cv. IRAT 112. The rojolele cultivar was relatively easy to form embryogenic callus than IRAT 112. Successful genetic transformation is related with embryogenic callus formation and plant regeneration system. According to Ge et al. (2006) callus induction and regeneration in rice tissue culture depend on a number of factors, such as genotype of the donor plant, type and physiology status of the explants, composition and concentration of the basal salt and organic components, and plant growth regulators in the culture medium. Among these factors, genotypic difference is the most important.

IRAT 112 Cultivar consists of indica rice. Most of the indica varieties belong to the group I, which these varieties have been quite recalcitrant cultivars (difficult for regeneration and transformation) in tissue culture and genetic transformation (Wunn et al., 1996; Zhang et al., 1998). Even success of transformation in indica rice was reported, but the results showed either low transformation efficiency or success only with very specific genotype (Zhang et al., 1997; Lin and Zhang, 2005). Therefore low efficiency and regeneration on IRAT 112 suggest the genotype of plant. The lower transformation efficiency on this genotype also occur in other cultivars such as IR72 and IR 64 each by level efficiencies 4.2% and 2.5 10.1% (Aldemita and Hodges, 1996; Hiei et al., 2006; Khanna and Raina, 2002). Although recently reported that the transformation efficiency in ten indica rice cultivars could be obtained until 30% for each immature embryo with optimation things (Hiei et al., 2006). In the meantime on rojolele cultivar transformation efficiency and regeneration were higher than IRAT 112 because rojolele belong to javanica rice. This genotype (javanica) is easier for transformation and regeneration and is not a recalcitrant type.

Table 1. Summary of transformation using of recombinant plasmid 35S-*oshox4* on cv. IRAT 112 and rojolele.

Cultivars	Number of transformed callus	Number of resistance hygromycin callus	Number of regenerated callus	Transformation efficiency (%)	Regeneration efficiency (%)
IRAT112	469	64	3	13,6	4,7
	840	48	21	5,7	43,7
Rojolele	60	40	15	66,7	37,5
	923	240	106	26,0	44,1
	584	213	49	36,5	23,0

Hygromycin test on T_1 generation

Hygromycin test on seed (the seed from T_0 plant) conducted to select a lot of number from lines putative transgenic. This analysis will reduce economic cost. The selection based on selectable

marker could be an indicator for the presence other gene in the same of T-DNA. This examination is gone upon the expression of *hpt* gene as a selectable marker. With expression of *hpt* gene be provided that target gene *oshox4* have also been integrated into rice genome. It is because *hpt* gene inserted on the same T-DNA with *oshox4* gene (Figure 1).

Hygromycin is an antibiotic resistance marker commonly used for genetic transformation on monocotyledon plant especially Gramineae (Bashir et al., 2004). This antibiotic hampered synthesis of protein by troubles translocation so causes error translation on ribosome 80S (Bashir et al., 2004). Hygromycin phosphotransferase enzyme obtained from *hpt* gene could detoxificate of hygromycin B antibiotic (Rodriguez and Nottenburg, 2003) and catalyze phosphorrylation of hydroxyl group in hygromycin antibiotic so that this antibiotic not active and not poison for plant cell (Brasileiro and Aragao, 2001). The analysis result in the second generation (T_1) and lines used for hygromycin test were presented on Table 2.

Water was used as control solution to observe seed germination from each line, whereas hygromycin solution as treatment solution. Seed which germinated and form normal seedling on hygromycin solution suggest that it may contain *hpt* expressed gene. Hygromycin was degraded by this plant, therefore it was not toxic and might not caused any trouble (Figure 2). Phenomenon of which *hpt* gene is integrated in the plant, genome but failed to express called gene silencing. There are three assumption of gene silencing which are *cis inactivation*, *trans inactivation* and *co suppression. Cis-trans-inactivation* occurred at transcription. *Cis-inactivation* occurs if 1 or many gene copies integrated on one locus in or near to genome sequent with high methylation. So, integrated gene will be methylation and not expression. *Trans-inactivation* occurred when insertion gene integrated on different locus and one of its integration experience *cis- inactivation* so becomes *silencer* to the homolog. *Co- suppression* happening on post transcription, processing, localization and or mRNA degradation while affluent mRNA production under strong promoter (Taylor, 1997). Other opinion was named of *co-suppression* occurred by involves *coordinate silencing*; sometimes *coordinate reactivation* from transgenic with endogenous homology or among 2 homolog transgenic (Matzke and Matzke, 1995).

Base on this experiment, 14 lines of plant expressing *hpt* gene were obtained and the gene was segregated follows of Mendelian (3:1). These lines were from rojolele cultivar: T_1B.4.2, T_1B.6.1, T_1B.9. 3, T_1B.17. 3, T_1B. 17.4, T_1B.18.1, T_1B.28.1, T_1C.2.7, T_1C.2.10, T_1C.2.16, T_1C.2.18, T_1C.8. 1, T_1C.12.2, and T_1C.13.1. The absence of hygromycin resistance from IRAT 112 cultivar might suggest that integrated gene was difficult to obtain, or the inserted gene was not expressed. PCR analysis to evaluate parent containing hygromycin from IRAT 112 was not performed.

PCR analysis of T_0 plant

The aim of PCR analysis for *hpt* gene was to evaluate integration of the selectable marker and the segregation based on number of integrated plants. The 14 lines plant with Mendelian segregation based on hygromycin test from the second generation (T_1) were planted in soil medium in green house with 30 seedlings for each line. Parallel with this activity, PCR analysis was done for the 14 plant as the parent (T_0).

Table 2. Analysis of *hpt* gene expression on T_1 generation seed which transform with *oshox4* gene.

Lines/ cultivar	Number of germinate seeds	Chi square	Lines/ cultivar	Number of germinate seeds	Chi square	X table (α 0.05)
IRAT 112			**Rojolele**			3.84
T1.I.B.1.1	5	106.78	T1.R.B.16.6	12	64.00	
T1.I.B.2.2	12	66.67	T1.R.B.17.1	15	49.00	
T1.I.B.2.3	2	131.44	T1.R.B.17.2	7	99.23	
T1.I.B.2.4	5	109.72	**T1.R.B.17.3**	**38**	**0.03**	
T1.I.G.1.1	3	121.00	**T1.R.B.17.4**	**34**	**0.82**	
T1.I.G.1.2	3	118.02	**T1.R.B.18.1**	**41**	**2.78**	
T1.I.G.3.1	5	139.26	T1.R.B.19.1	22	23.68	
T1.I.G.4.1	7	93.44	T1.R.B.26.1	27	9.00	
T1.I.G.5.1	6	102.92	**T1.R.B.28.1**	**35**	**0.33**	
T1.I.G.6.1	0	144.00	T1.R.B.29.1	28	8.33	
T1.I.G.7.1	20	32.67	T1.R.B.29.2	5	103.84	
T1.I.G.7.2	23	22.43	T1.R.B.29.3	14	53.78	
T1.I.G.7.3	4	116.74	T1.R.B.29.4	14	61.50	
T1.I.G.7.4	5	103.84	T1.R.B.30.1	20	30.54	
T1.I.G.7.5	10	75.11	T1.R.C.2.1	2	131.44	
T1.I.G.7.7	18	38.27	T1.R.C.2.4	19	34.29	
T1.I.G.9.1	19	32.11	**T1.R.C.2.7**	**41**	**1.97**	
T1.I.G.9.2	9	78.19	T1.R.C.2.8	20	30.54	
T1.I.G.9.3	5	106.78	T1.R.C.2.9	19	29.96	
T1.I.G.9.4	7	93.44	**T1.R.C.2.10**	**35**	**0.11**	
T1.I.G.9.5	7	96.33	T1.R.C.2.11	27	10.35	
T1.I.G.11.1	9	83.82	T1.R.C.2.12	30	4.00	
T1.I.G.12.1	9	83.82	T1.R.C.2.13	26	14.11	
T1.I.G.13.1	8	92.83	T1.R.C.2.15	30	6.00	
Control IRAT	1	139.11	**T1.R.C.2.16**	**38**	**0.17**	
			T1.R.C.2.18	**37**	**0.03**	
Rojolele			T1.R.C.4.1	26	12.58	
T1.R.A.1.5	19	34.29	T1.R.C.4.2	29	5.44	
T1.R.B.2.1	31	4.51	T1.R.C.4.3	19	27.86	
T1.R.B.4.1	21	27.00	T1.R.C.6.2	24	16.00	
T1.R.B.4.2	**33**	**2.16**	T1.R.C.7.1	28	7.11	
T1.R.B.6.1	**32**	**2.46**	**T1.R.C.8.1**	**39**	**0.24**	
T1.R.B.8.1	19	25.79	T1.R.C.8.2	22	21.78	
T1.R.B.8.2	20	28.44	T1.R.C.9.1	29	6.54	
T1.R.B.8.3	17	42.46	T1.R.C.10.1	13	64.03	
T1.R.B.8.4	8	84.26	**T1.R.C.12.2**	**39**	**0.24**	
T1.R.B.9.1	28	8.33	**T1.R.C.13.1**	**43**	**3.23**	
T1.R.B.9.3	**33**	**1.53**	T1.R.C.15.1	30	4.00	
T1.R.B.11.1	16	46.86	Control rojolele	7	99.23	

Note: bold is lines with Mendelian segregation.

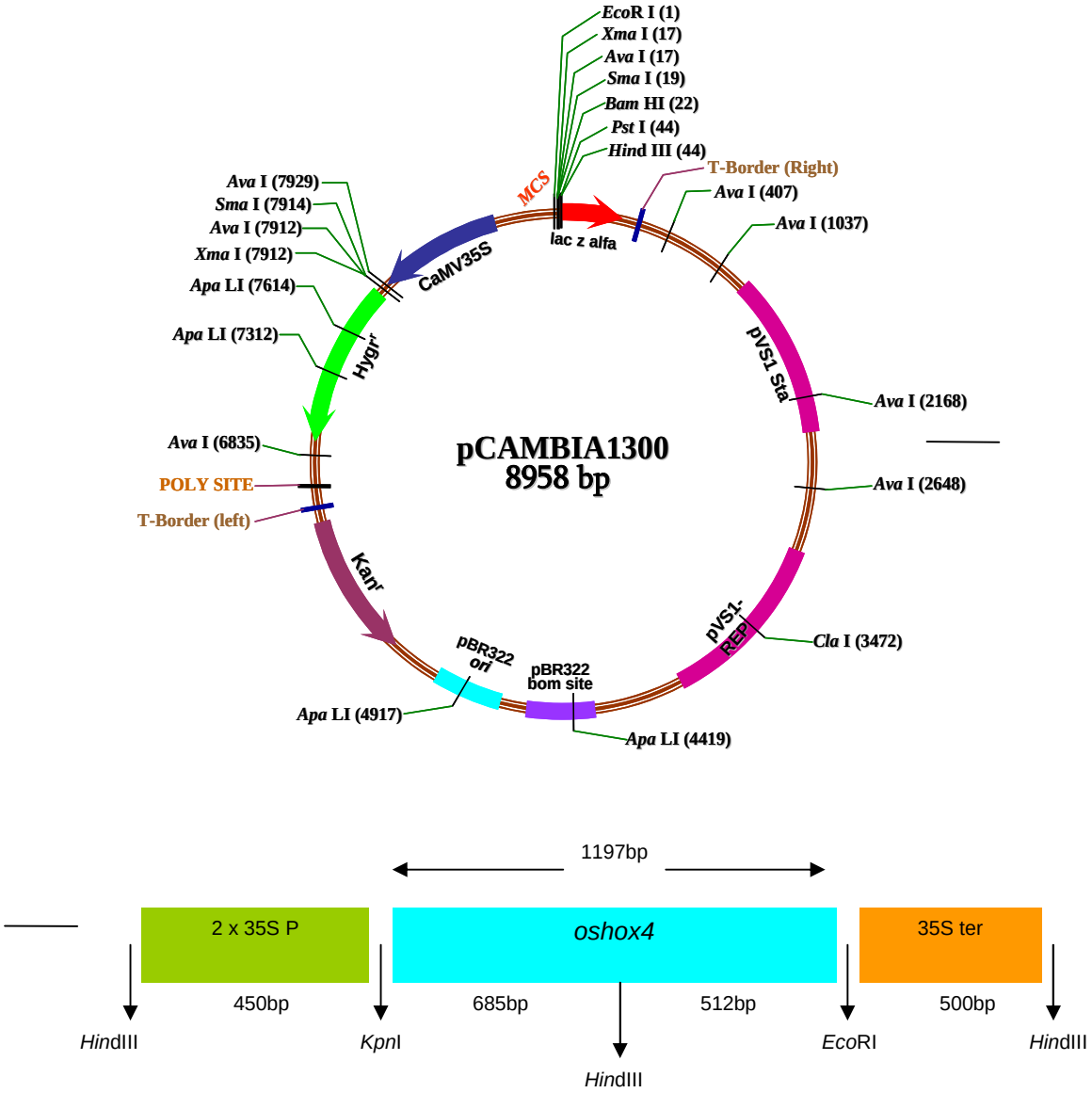

Figure 1. The recombinant vector construction *35S Oshox4* is utilized on the research.

A **B** **C** **D** **E**

Figure 2. The hygromycin test at transformant seeds (*T1*). Resistance to hygromycin solution (A), resistance to solution without hygromycin (B), transformant susceptible to hygromycin solution (C), seeds control in hygromycin solution (D), seeds control in solution without hygromycin (E).

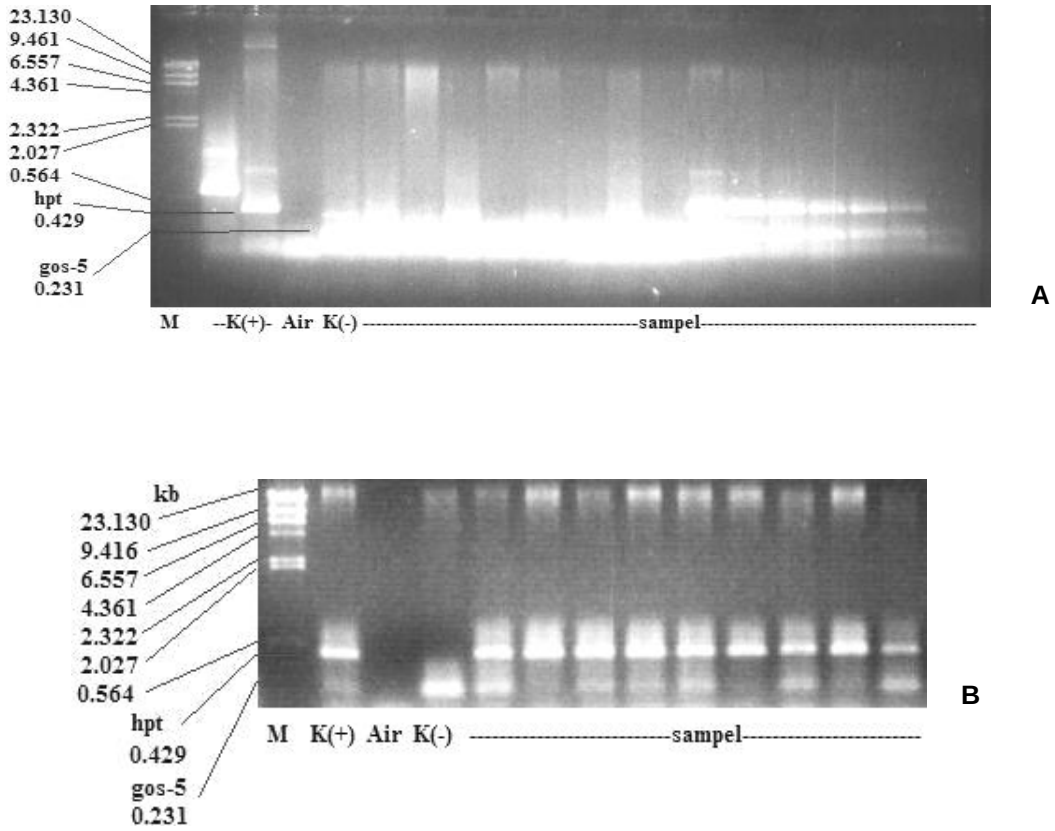

Figure 3. PCR result using *hpt* and *gos-5* primers A. first generation (*T 0)*, B. Second generation (*T1)*.

Target gene to be amplificated is *hpt* and *gos-5* with amplification product 429 and 231 bp respectively. According to Meijer et al*.* (1991) one of gene in rice genome, so called *gos-5* can be served as internal control. Internal control is subjected to confirm that DNA used fine. Proper DNA and PCR reaction were expected to give a 231 bp length of PCR product in all samples including control (non transformation). The specific primer(s) was designed for amplification of specific gene(s). PCR analysis using *hpt* and *gos-5* primers were done on 14 lines plant at first generation (*T0*). The analysis result showed that 7 lines plant T_0 contain *hpt* gene and amplification product of *gos-5* were present in all lines tested. Those are T_0C.4.2, T_0C.4.3, T_0C.8.1, T_0C.9.1, T_0C.12.2, T_0C.13.1, and T_0C.15.1. The result of PCR analysis on 14 lines (*T0*) was presented on Figure 3A.

The PCR result at second generation (*T1*) using *hpt* primers was done for 4 lines which were T_1.C.4.2, T_1.C.4.3, T_1.C.8.1 and T_1C.13.1. Tree of four lines were Mendelian segregation based on chi square tested. Those lines were T_1.C.8.1, T_1.C.13.1 and T_1.C.4.2. The Mendelian segregation means that *hpt* was integrated in rice genome and inherited on its generation. PCR result was presented on Figure 3B.

CONCLUSION

The transformation and regeneration for IRAT 112 callus cultivar using the recombinant plasmid *35S Oshox4* is lower than that of rojolele. Transformation efficiency for IRAT 112 is 5.7-13.6% whereas 26-66.7% for rojolele. Regeneration of IRAT 112 callus between 4.7-43.7% and 23- 44.1% for rojolele. Fourteen lines of rojolele cultivar at second generation (*T1*) were Mendelian segregation (3:1) for *hpt* gene. The lines are T_1B.4.2, T_1B.6.1, T_1B.9.3, T_1B.17 3, T_1B.17.4, T_1B.18.1, T_1B. 28.1, T_1C.2.7, T_1C.2.10, T_1C.2. 16, T_1C.2.18, T_1C.8. 1, T_1C. 12. 2 and T_1C.13.1. PCR analysis using *hpt* primers for 14 lines parents (*T0*) showed that 7 lines contain *hpt* genes. The lines are T_0C.4.2, T_0C.4.3, T_0C.8.1, T_0C.9.1, T_0C.12.2, T_0C.13.1, and T_0C. 15.1. Three of 4 lines at second generation (*T1*) were Mendelian segregation based on PCR analysis indicated by the presence of specific band for *hpt* gene. The third lines are T_1C.4.2, T_1C.8.1, and T_1C.13.1. Integration of *hpt* gene as selectable marker in the genome suggest that *oshox4* gene is also integrated into genome because both genes are from the same of T-DNA.

ACKNOWLEDGEMENT

The authors thank to Dr. A.H Meijer and Dr Pieter B.F. Ouwerkerk for providing recombinant plasmid, Thank to Dr. Sigit Purwantomo and Dr. Satya Nugroho for discussion, advice, and financial support.

REFERENCE

Agalou, A., S. Purwantomo, E. Overnas, H. Johannesson, X. Zhu, A. Estiati, R.J. de Kam, P. Engstom, I.H. Slamet-Loedin, Z.Zhu, M. Wang. L. Xiong, A.H. Meijer, and P.B.F. Ouwerkerk. 2008. A genome wide survey of HD-Zip genes in rice and analysis of drought responsive family members. *Plant Molecular Biology* 66: 87-103.

Aldemita, R.R. and T.K. Hodges. 1996. **Agrobacterium tumefaciens**-mediated transformation of Japonica and Indica rice varieties. *Plant*. 199: 612-617.

Bashir, K., M. Rafiq, T. Fatima, T. Husnain, and S. Riazuddin. 2004. Hygromycin based selection of transformants in a local inbred line of **Zea mays** (L). *Pakistan Journal of Biological Sciences* 7: 318-323

Brasileiro, A.C.M., and F.J.L. Aragao. 2001. Marker genes for *in vitro* selection of transgenic plants. *Journal of Plant Biotechnology* 3: 113-121.

Bray, E.A., J. Bailey-Serres, and E.Weretilniyk. 2000. Response to abiotic stresses. *In*: Gruissem, W., B.B. Buchannan, and R. Jones (eds.). *Biochemistry and Molecular Biology of Plants.* Rocvkville: American Society of Plant Physiologists.

Deng, X., J. Philips, A.H. Meijer, F. Salamini, and D. Bartels. 2002. Characterization of five novel dehydration responsive homeodomain leucine zipper genes from resurrection plant **Craterostigma plantagineum**. *Plant Molecular Biology* 49: 601-610.

Ge, X., Z. Chu, Y. Lin, and S. Wang. 2006. A tissue culture system for different germplasms of indica rice. *Plant Cell Report* 25: 392-402.

Gorantla, M., P.R. Babu, V.B.R. Lachagari, A.F. Feltus, and A.H. Peterson. 2005. Functional genomics of drought stress response in rice: Transcript mapping of annotated unigenes of an indica rice (**Oryza sativa** L. cv. Nagina 22). *Current Science* 89: 469-514.

Hiei, Y. and T. Komari. 2006. Improved protocols for transformation of Indica rice mediated by **Agrobacterium tumefaciens**. *Plant Cell Tissue and Organ Culture* 85: 271-283

Hiei, Y., S. Otha, T. Komari and T. Kumashiro. 1994. Efficient transformation of rice (**Oryza sativa** L) mediated by **Agrobacterium** and sequence analysis of the boundaries of the T-DNA. *Plant Journal* 6: 271-282.

Hu, H., M. Dai, J. Yao, B. Xiao, X. Li, Q. Zhang and L. Xiong. 2006. Over-expressing a NAM, ATAF and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. *PNAS* 103: 12987-12992

Khanna, H.K. and S.K. Raina. 2002. Elite indica transgenic rice plants expressing modified cryIA (c) endotoxin of **Bacillus thuringiensis** show enhanced resistance to yellow stem borrer (**Scirpophaga incertulas**). *Transgenic Research* 11: 411-423.

Lin, Y.J. and Q. Zhang. 2005. Optimising the tisuue culture conditions for high efficiency transformation of indica rice. *Plant Cell Report* 23: 540-547.

Matzke, M.A. and A.J.M. Matzke. 1995. How and why do plants inactivate homologous (trans) gene?. *Plant Physiology* 107: 679-685.

Meijer, A.H., E. Scarpella, E.L. Van Dijk, L. Qin, A.J.C. Taal, S. Rueb, S.E. Harrington, S.R. McCouch, R.A. Schiperoot, and J.H.C. Hoge. 1997. Transcriptional repression by *oshox1*, a novel homeodomain leucin zipper protein from rice. *Plant Journal* 11: 263-276.

Purwantomo, S. 2007. *Karakterisasi Faktor Transkripsi HD-Zip untuk Toleransi Kekeringan dan Introduksi Gen-gen Penyandi Lintasan Biosintesis Asam Salisilat untuk Ketahanan Penyakit Blas pada Tanaman Padi*. [Disertasi]. Bogor: Institut Pertanian Bogor.

Rodriguez, R.C., and C. Nottenburg. 2003. *Antibiotic Resistance Genes and their Use in Genetic Transformation Especially in Plants.* CAMBIA. http:www.cambiaip.org/whitepapers/Transgenic/Ab_resistance/ books/ whole.pdf

Sakuma, Y., K. Maruyama Y. Osakabe Y, M. Seki, K. Shinozaki, and K. Yamaguchi-Shinozaki 2006. Functional analysis of an **Arabidopsis** transcription factor, DREB2A, involved in drought responsive gene expression. *The Plant Cell* 18: 1292-1309

Scarpella, E., E.J. Simons, and A.H. Meijer. 2005. Multiple regulatory elements contribute to the vascular-spesific expression of the rice HD-Zip gene *oshox1* in **Arabidopsis**. *Plant Cell Physiology* 46: 1400-1410.

Shinozaki, K. and K.Yamaguchi-Shinozaki. 1997. Gene expression and signal transduction in water stress response. *Plant Physiology* 115: 327-334.

Shinozaki, K. and K.Yamaguchi-Shinozaki. 2007. Gene networks involved in drought stress response and tolerance. *Journal of Experimental Botany*. 58: 221-227.

Slamet-Loedin, I.H., J.L. Wibowo, S.Hutajulu, dan W. Rahayu. 1997. Penggunaan dua strain Agrobacterium tumefaciens super virulen untuk kokultivasi tanaman padi pada cv. cisadane dan rojolele. *Prosiding Konggres I dan Seminar Ilmiah PBPI*. Surabaya, 12-14 Maret 1997.

Taylor, C.B. 1997. Comprehending cosuppression. *The Plant Cell* 9: 1245-1249

Wünn, J., A. Kloti, P.K. Burkhardt, G.C.G. Biswass, K. Launis, V.A. Iglesias, and I. Potrykus. 1996. Transgenic Indica rice breeding line IR-58 expressing a synthetic cryIA (b) gene from **Bacillus thuringiensis** provides effective insect pest control. *Bio/Technology* 14: 171-176.

Yamaguchi-Shinozaki, K and K. Shinozaki. 2001. Improving plant drought, salt and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *in*: *Rice Biotechnology: Improving Yield, Stress Tolerance and Grain Quality – No. 236. (Novartis 1998. Transgenic Elite Indica Rice Varieties, Resistant to* **Xanthomonas oryzae** *pv.* **oryzae**. *Foundation Symposium).* Chichester: Wiley.

Zhang, J., R.J. Xu, M.C. Elliott, D.F. Chen. 1997. **Agrobacterium**-mediated transformation of elite indica and japonica rice cultivars. *Molecular Biotechnology* 8: 223-231

Zhang, S., W.Y. Song, L. Chen, D. Ruan, N. Taylor, P. Ronald, R. Beachy and C. Fauquet. 1998. Transgenic elite indica varieties, resistant to **Xanthomonas oryzae** pv. **oryzae**. *Molecular Breeding* 4 (6): 551-558.