Genetic transformation of rice cv. Ciherang using double T-DNA vector harboring *cry*1Ab gene

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Abstract

Rice stem borer (*Scirpophaga* sp.) is considered as one of the primary pests causing yield decrease in Indonesia. Genetic engineering is considered as one of the most effective way to improve the resistance of rice plants to yellow stem borer since no resistance gene has been found in rice and its wild relatives. A double T-DNA binary vector p2TDNAcryIAb wich carry two independent T-DNA was used to transform Indonesian elite rice cv Ciherang using *Agrobacterium*-mediated transformation to generate transgenic rice that free from selectable marker genes. One T-DNA containing a selectable marker *hygromycin phosphotransferase* (*hpt*) gene and the other carrying the *cry1Ab* gene. As many as 37 putative transgenic plants from 14 embryos were generated. PCR analysis demonstrated that 22 out of 37 putative transgenic rice contain *cry1Ab* gene. All Ciherang rice carrying the *cry*1*Ab* gene expressing Cry1Ab protein as shown by the immunostrip assay. Further work will be carried out to determine the transgenes copy number, to select marker free transgenic rice, and to evaluate the resistance of transgenic rice against neonates larvae of yellow stem borer.

Keyword*:* double T-DNA, transgenic rice, *Cry1Ab*, transformation

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Introduction

Rice stem borer (*Scirpophaga* sp.) is considered as one of the primary pests causing rice yield lost in Indonesia. The yellow stem borer or YSB (*Scirpophaga incertulas*) is the most damaging rice stem borer in tropical Asia including Indonesia. The caterpillars (larvae) bore into the rice stem and hollow out the stem completely causing it to break. In Indonesia, stem borers are still major threats especially in large-scale paddy fields (Rowell, 2008) which causes yield losses up to 25 % (Biro Pusat Statistik, 1996). In general, new resistant varieties can be developed by conventional breeding. However, in the case of resistance to stem borer, the problem to overcome by conventional breeding is the lack of any resistant gene available in the respective spesies and their wild relatives (Rao & Padhi, 1988). Plant genetic engineering is, therefore, one of the alternatives to answer the problem. It has some advantages such as the wider germplasm options, reduction of the number of backcrossing needed to eliminate one of the parental genetic backgrounds and the precision of transferring only the target gene without any other unknown genes introgessed (Conner, 1997).

Bt-toxin is specific insecticidal proteins, which kill lepidopteran insects by binding to and creating pores in the midgut membranes. Chemical control has proven to be ineffective because the insect larvae feed inside the stem pith and remain out of the reach of the pesticide. Moreover, the use of agrochemicals is associated with high cost and the risk of environmental and health hazards (Ho *et al.*, 2006). Genetic engineering on the other hand, offers a means to introduce into rice genom, genes which confer increased tolerance to pests and reduced environmental and health risk. However, the release of genetically modified plants are still widely debated because of concerned about negative impact of environmental release of transgenic plants.

Selectable markers and antibiotics are two component that commonly used in plant genetic transformation. Usually a selection gene is introduced together with gene of

interest to only allow the transformed cells to grow.. However, these marker genes are not needed once transgenic plants have been produced. Besides minimizing public concerns, strategies to eliminate marker genes also reduce the need for time-consuming and expensive safety evaluations (Hare & Chua, 2002). There are several methods to eliminate selectable marker genes. The methods currently employed are co-transformation, site-specific recombination and intra genomic translocation via transposable elements (Zuo *et al.*, 2001). Techniques based on DNA recombination and *Agrobacterium*-mediated co-transformation with two binary vectors in a single or two different *Agrobacterium* strains, or with super-binary vectors carrying two sets of T-DNA border sequences (twin T-DNA vectors), have been employed by researchers to produce selectable marker-free (SMF) transgenic progeny (Lu *et al.*, 2001). Rachmat (2006) has developed a double T-DNA binary plasmid (p2TDNAcryIAb) which harbored two independent T-DNA, containing *hygromycin phosphotransferase* gene (*hpt*) in one T-DNA region and gen of interest (*cry1Ab*) in another T-DNA region. This double T-DNA was used to generate selectable marker-free transgenic of Indonesian elite rice cv. Ciherang carrying *cry1Ab* by *Agrobacterium* mediated transformation. Transgene insertion and expression were confirmed by PCR analysis and biochemical immunostrip assay.

Materials and Methods

Plasmid and bacterial strain. Vector p2TDNAcryIA (Figure 1) carrying two independent T-DNA was developed by Rachmat (2006). One T-DNA containing selectable marker (*hpt*) under controlled of CaMV35S promoter and the other T-DNA carrying gen of interest (*cry1Ab*) driven by maize ubiquitin promoter. This plasmid was introduced into *Agrobacterium* strain Agl-1 was used in the transformation. *Agrobacterium* strain Agl-1 was inoculated on AB solid medium supplemented with 100 mg/L kanamycin and 10 mg/ L rifampicin for 3 days at 28^{o}C. The bacteria were collected and suspended in AAM medium containing 0.1 mM acetosyringone. This bacteria suspension was adjusted to OD_{600} =0.3.

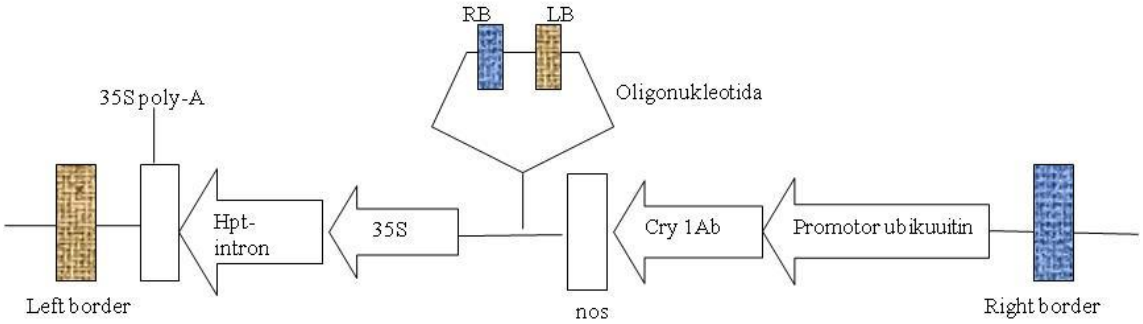

Figure 1. Double T-DNA construct on plasmid p2TDNAcryIAb

Rice transformation and regeneration. Transformation and regeneration of Ciherang calli were carried out as described by Hiei & Komari (2006) with some modifications. Immature seeds between 12-15 days after anthesis were manually dehusked and sterilized in 70% of ethanol for 1 min and in 1% of sodium hypochlorite solution containing a drop of tween 20 for 5 min. The immature seeds were rinsed several times in sterile water. Embryos were collected by squeezing the seeds using a sterile pinset in a laminar flow cabinet, blotted dry on a sterile filter paper and placed on a co-cultivation medium (N6 major salts, B5 minor salts and vitamins, 20 g/L of sucrose, 10 g/L of glucose, 2.0 mg/L of 2.4 D, 1.0 mg/L of NAA, 1.0 mg/L of BA, 0.1 mM of acetosyringone, 5.5 g/L of agarose Type I, pH 5.2). Then 5 µl of *Agrobacterium* suspension was dropped on each of the immature embryos. Infected embryos were incubated at 25°C for 7 days in the dark. After 7 days, actively growing calli were transferred on to NBM medium containing 250 mg/L cefotaxim and 100 mg/L timentin and placed at 30°C under continuous light for 10 days.

The calli were then transferred to NBM selection medium containing 30 mg/L hygromycin, 250 mg/L cefotaxim and 100 mg/L timentin for 4 weeks, which transferred on to fresh media in every two weeks. Resistant calli were transferred to pre-regeneration medium NBPR for 10 days. Calli showed green spot were transferred on RNM regeneration medium for 2 weeks and then regenerated planlets were tansferred to the rooting medium (½ MS salt contaning 30 mg/L hygromycin). Rooted plants were transferred to soil in pots and kept in the transgenic greenhouse.

PCR analysis. The genomic DNA was isolated from the leaves of putative transgenic plants T0 generation using CTAB protocol (Heusden *et al*., 2000). The PCR amplification was carried out in a 12.5 μl reaction mixture containing 1 µl of DNA sample, 2.5 µM of each primer, and 6.5 µl of Dream Taq PCR master kit (Fermentas). PCR was performed with primers specific for *cry*1Ab. Those primers are *cry* 1Ab F: 5'-CATTGTGTCTCTCTTCCC-3', and cry1Ab R: 5'-CCGTTAGAGAAGTTGAAAGG-3'. The samples were initially denatured at 95°C for 3 min, followed by 35 cycles of 1 min denaturation at 95°C, 1 min of primer annealing at 55°C and 1 min of synthesis at 72°C, with a final extension step of 72°C for 10 min. The PCR products were analysed on a 1% agarose gel.

Immunostrip assay. Immunostrip assay was done using immunostrip Bt cry1Ab from Agdia following the procedure given by the supplier (2003). The leaves of transgenic plants which positive *cry1Ab* gene confirmed by PCR analysis were assayed to detect the expression of Cry 1Ab protein.

Results and Discussion

Ciherang is a widely adopted rice variety by Indonesian farmer's today for it's good quality of rice. Now, Ciherang is in the second position after IR64 based on the planting area, and the trend has increased every year since it was released in 2000. The development of tissue culture and transformation techniques of Ciherang has been initiated with regeneration effiency ranging from 33-59% and transformation efficiency ranging from 3–12% depends on bacterial agents used (Purnamaningsih, 2006; Rahmawati et al. 2010). Transformation of Ciherang with *cry*1Ab will be very important to decrease yield loss caused by YSB in endemic areas. Part of the transformation process is presented in Figure 2.

In this experiment, Ciherang was transfomed with *Agrobacterium* harboring double T-DNA vector (p2TDNAcryIAb) carrying *cry*1Ab and *hpt* in separate T-DNA in attemps to generate-marker free transgenic rice plants. At the T0 generation the transgenic plants are still expected to have both genes with a possibility that both genes were integrated in separate different loci. In the subsequent generations we expect a segregated population, thus selection of progeny with no marker gene in its genome would be possible. The previous study have already demonstrated that the use of double T-DNA binary vector system could be succesfull in producing marker-free transgenic plants such as in barley (Matthew *et al*., 2001), tobacco (Zhou *et al*., 2003), soybeans (Xing *et al*., 2000), and rice (Lu *et al*., 2001).

In this study, regeneration of Ciherang transformed with vector p2TDNAcryIAb was low (10,22%) (Tabel 1). As many as 37 of putative transgenic rice plants were obtained from 14 hygromisin resistant calli obtained. The transformation efficiency (number of PCR positive plants divided by number of embryo inoculated) of Ciherang was low ,only 4%, (Table 1) compare to IR64, IR24 and IR72 (>50%) (Hiei & Komari, 2006). Many factors such as plant genotype, explant, vectors-plasmid, bacterial strain, addition *vir*-genes inducing synthetic phenolic compounds, culture media composition, suppression and elimination of bacterial infection after co-cultivation, and desiccation of explant (Roy *et al*., 2000; Opabode, 2006), may affecting the transformation frequency. Among these factors, genotypic difference is the most important (Ge *et al.,* 2006). Thus optimizing these factors may could enhance the transformation frequency of Ciherang.

Putative transgenic plantlets were verified for the presence of the transgenes using polymerase chain reaction (PCR) analysis. Integration of the *cry 1Ab* gene into the genome of putative transgenic T0 plantlets was confirmed by PCR using *cry 1Ab* gene

specific primers. The expected band (1012 bp) for *cry 1Ab* gene was present in the positive control but was absent in the negative control (non-transformed plants). PCR analysis was done on all 37 putative transgenic plants obtained from 14 hygromisin resistan embryos. The analysis results showed that 22 out of 37 T0 plants contain *cry 1Ab* gene. Some of the data PCR analysis was presented on Figure 3.

PCR positive plants were subjected to protein analysis, and functionally expressed proteins were detected using Immuno-Strips specific for *cry1Ab* gene products. Transgenic plants carrying *cry1Ab* gene were found expressing *Bt* toxins. The expression of *cry 1Ab* gene from PCR positive *cry 1Ab* transgenic plants were analyzed on 7 independent plants (Figure 4). Immunostrip assay of the putative transgenic lines showed that all the transgenic plants tested expressed cry 1Ab protein. It proved that *cry 1Ab* gene was expressed in the plants

In the transformation, the T-DNA inserts were integrated randomly among the chromosomes (Wang *et al*., 1995). Thus, through this transformation, both T-DNA may integrate in separate different loci and subsequently segregate in the successive generations. All PCR positive plants will be analysed further to determine the gene copy number of each transgene using Southern blot analysis. Plants with one copy of *cry* 1Ab will be selected further for segregation analysis in T1 generation to select marker free lines using PCR analysis and to evaluate the afficacy of transgenic lines against the noenate larvae of YSB. Developing marker-free transgenic plants not only decreases the risk of harm to the environment and to the human health, but also increases public acceptance of transgenic plants.

Conclusion

In this study, we obtained 22 of indica rice plants cv. Ciherang containing *cry* 1Ab gene based on PCR analysis and expressing Cry 1Ab protein based on immunstrip assay. Further studies will be conducted to determine transgene copy number, to select marker free lines, and to evaluate the resistance of marker free transgenic lines.

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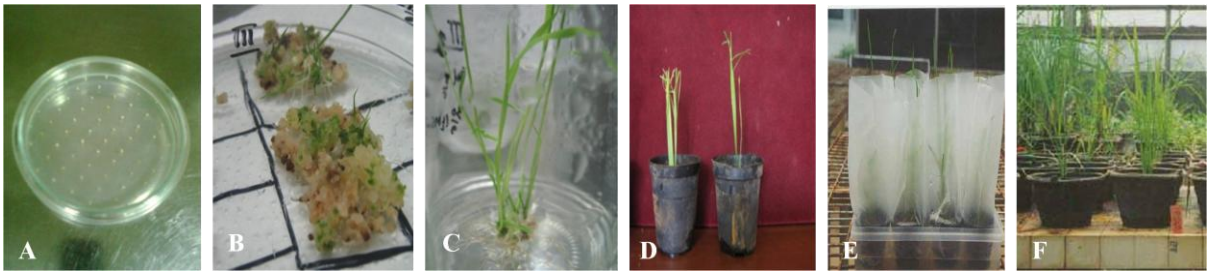

Figure 2. Part of transformation process of indica rice cv Ciherang. A. Immature embryos of Ciherang on cocultivation medium; B. Regenerated plants from hygromycin resistant calli; C. Planlet; D & E. Aclimatization; and F. Transgenic plants grown in greenhouse.

Table 1. Regeneration and transformation efficiency of rice cv Ciherang transformed with p2TDNAcryIAb vector.

Number of experiments	Total immature embryos inoculated (A)	Number of *hygromisin resistance* calli (B)	Number of regene rated calli (C)	Regenera tion efficiency (%) (C/B*100%)	Number of PCR positif indepen dent plants* (D)	Transfor mation efficiency (%) (D/A*100%)
1	150	96	9	9.37	9	6
2	100	33	4	12.12	4	4
3	100	8	1	12.50	1	1
Total	**350**	**137**	**14**	**10.22**	**11**	**4**

*Independent plants: plants coming from different calli

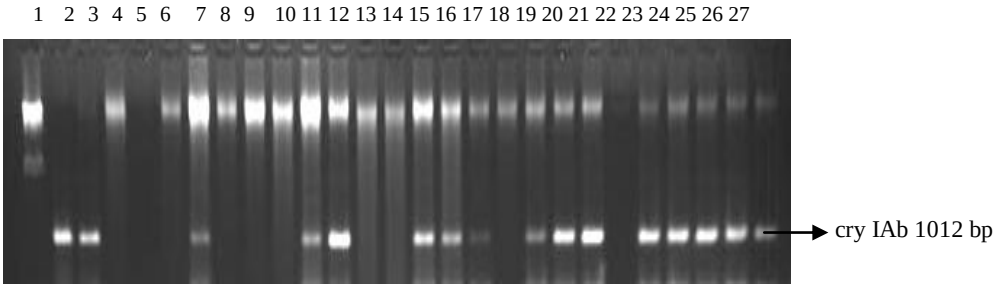

Figure 3. PCR analysis with cry 1Ab primer. Lane 1= Marker, lane 2= fragmen cry 1Ab, lane 3= positive plant control, lane 4= negative plant control, lane 5 = water control, lane 6-27= samples.

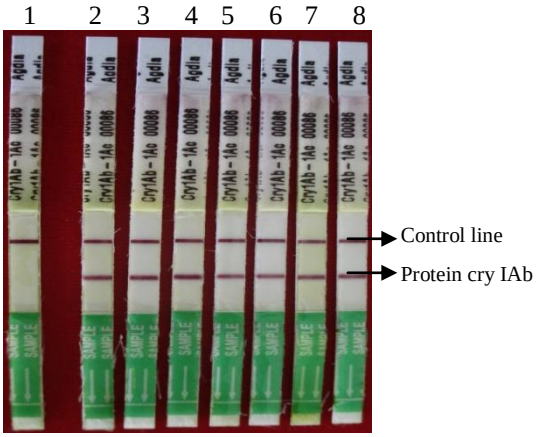

Figure 4. Immunostrip assay Lane 1= negative control plant (non transgenic), Lane 2-8 = sample of transgenic plants.

References

Agdia. 2003. Bt-cryIA(b)/lA(c) *ImmunoStrip* Tests. Strip tests for the detection Bt- cryIA(b)/lA(c) protein. Agdia Incorporated. Elkhart, Indiana, USA. p1-3.

Biro Pusat Statistik. 1996. Survey Pertanian. Luas dan intensitas serangan organisme pengganggu tanaman dan bencana alam padi, palawija dan sayuran di Jawa. Biro Pusat Statistik, Jakarta.

Conner, A. J. 1997. Genetically Engineered Crops. Environmental and Food Safety Issues. The Royal Society of New Zealand. Miscellaneous Series 39.

Ge, X. J., Z. H. Chu, Y. J. Lin & S. P. Wang. 2006. A tissue culture system for different germplasms of *indica* rice. Plant-Cell-Reports, 25(5): 392-402.

Hare, P. D., & N. M Chua. 2002. Excision of selectable marker genes from transgenic plant. Nature Biotechnology, 20: 575-580.

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

Ho, N. H , N. Baisakh, N. Oliva, K. Datta, R. Frutos, & S. K. Datta. 2006. Translational Fusion Hybrid *Bt* Genes Confer Resistance against Yellow Stem Borer in Transgenic Elite Vietnamese Rice (*Oryza sativa* L.) Cultivars. Crop. Sci., 46:781-789.

Lu, H. J., X. R. Zhou, Z. X. Gong, & N. M. Upadhyaya. 2001. Generation of selectable marker-free transgenic rice using double right-border (DRB) bibary vectors. Austr. J. Plant Physiol., 28: 241-248.

Matthew, P. R., M. B. Wang, P. M. Waterhouse, S. Thornton, S. J. Fieg, F. Gubler, & J. V. Jacobsen. 2001. Marker gene elimination from transgenic barley, using co-transformation with adjacent "twin T-DNAs'" on a standard *Agrobacterium* transformatiom vector. Mol. Breed., 7: 195- 202.

Opabode, J. T. 2006. *Agrobacterium*-mediated transformation of plants: emerging factors that influence efficiency. Biotech. Mol. Biol. Rev., 1: 12-20.

Purnamaningsih, R. 2006. Induksi kalus dan optimasi regenerasi empat varietas padi melalui kultur *in vitro*. J. Agrobiogen., 2: 74-80.

Rachmat, A. 2006. Konstruksi vektor ekspresi gen untuk mengeliminasi gen penyeleksi antibiotik pada tanaman padi (*Oryza sativa* L.) transgenik. Thesis. Post Graduate Program. Bogor Agricultural University (in Indonesia with English abstract).

Rahmawati, S., D. A. Jefferson, D. Sopandi, Suharsono & I. H. Slamet-Loedin. 2010. Comparative analysis of rice transformation using *Agrobacterium tumefaciens* and *Rhyzobium leguminosarum*. Indonesian Journal of Biotechnology, 15 (1): 37-45.

Rao, P. S. P. & G. Padhi. Improved sources of plant resistance to yellow stem borer (YSB) *Scirpophaga incertulas* walker in rice. Intl. Rice Res. Newsl., 13:5.

Roy, M., R. J. Jain, J. S. Rohila, & R. Wu. 2000. Production of agronomically superior transgenic rice plant using Agrobacterium transformation method: present status and future prospectives. Curr. Sci. ,79: 954-960.

Rowell, C. 2008. They are Small, but Terrible: Yellow Stem Borer. SEARCA RDD News Release. www.searca.org/web/news/2008/aug/web/14.html.

Van Heusden, W. A., J. W. van Ooijen, R. Vrielinkvan Ginkel, W. H. J. Verbeek, W. A. Wietsma, & C. Kik. 2000. A genetic map of an interspecific cross in *Allium* based on amplified fragment length polymophism (AFLP) markers. Theor. Appl. Gen., 100: 118-126.

Wang, J., M. E. Lewis, J. H. Whallon & K. C. Sink. 1995. Chromosomal mapping of T-DNA inserts in transgene *Petunia* by in situ hybridization. Transgenic Res., 4: 241-246.

Xing, A., Z. Y. Zhang, S. Sato, P. Staswik, & T. Clemente. 2000. The use of the two T-DNA binary systemto derive marker-free transgenic soybeans. In Vitro Cell Dev. Biol. Plant., 36: 456-463.

Zhou, H. Y., S. B. Chen, X. G. Li, G. F. Xiao, X. L. Wei, & Z. Zhu. 2003. Generating marker-free transgenic tobacco plants by *Agrobacterium* mediated transformation with double T-DNA binary vector. Acta Botanica Sinica, 45 (9): 1103 -1108.

Zuo, J. R., Q. W. Niu, S. G. Moller, & N. H. Chua. 2001. Chemical-regulated, site-specific DNA excision in transgenic plants. Nature Biotechnology, 19: 157-161.