Insect Bioassay in Biosafety Containment to Select Transgenic Rice (*Oryza sativa* L.) Harboring *Cry1B* Gene Resistant to Yellow Stem Borer (*Scrirpophaga incertulas* Walk.)

Amy Estiati*, Ade Nena, and Satya Nugroho

Research Center for Biotechnology, Indonesian Institutes of Sciences (LIPI), Indonesia

Abstract

Development of rice varieties resistant to yellow stem borer (YSB) is very crucial. *Agrobacterium*-mediated transformation of *Cry1B* gene under wound inducible gene promoter *mpi* (maize proteinase inhibitor) into a local rice variety Rojolele had been conducted. PCR analysis proved that *Cry1B* gene had been integrated into plant genome of 3R25 and 3R5 rice lines. Segregation analysis using PCR for *Cry1B* gene of the two putative transgenic rice lines at third (T_2), fourth (T_3), fifth (T_4) and sixth (T_5) generations of 3R25 and 3R5 lines proved that 3R25.7.27, 3R25.7.13.8.2, 3R25.7.13.8.6, 3R25.7.13.8.8, 3R5.26.2 and 3R5.26.5 are homozygous lines for *Cry1B*. Insect bioassay on three randomly picked homozygous transgenic rice lines to study the efficacy of *Cry1B* gene toward YSB was conducted in biosafety containment by infestingYSB larvae at first instar into 3R25.7.27, 3R25.7.13.8.6 and 3R5.26.5 transgenic rice lines, using non-transgenic Rojolele, IR64 and IR74 as susceptible controls. The results showed that the precentages of deadhearts symptoms of 3R25.7.27, 3R5.26.2 and 3R25.7.13.8.6 rice lines were lower than those of the susceptible control lines with scores of 0,1 and 0, respectively. While the scores of all three susceptible control plants were 9. The results proved that lines 3R25.7.27, 3R25.7.13.8.6 and 3R5.26.2 were categorized as resistant lines while the non-transgenic Rojolele, IR64 and IR74 were categorized as susceptible lines. The results also showed that the *Cry1B* gene was expressed and produced insecticidal protein CRY1B which were active against YSB to protect rice plant toward YSB infestation.

Keywords: transgenic rice, *Cry1B* gene, insect bioassay, Rojolele, yellow stem borer

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*Corresponding author:
Cibinong Science Center, Jl. Raya Bogor Km. 46 Cibinong 16911, Indonesia
Tel. +62-21-8754587, Fax. +62-21-8754588
E-mail. nugroho_amy@yahoo.com

Introduction

Yellow stem borer (*Scirpophaga incertulas* Walker) is a destructive pest of rice and has been a serious problem in Indonesia. This insect attacks rice plant at vegetative and reproductive stages of plant development. At vegetative stage, the larvae damage the growing tillers, in which the young tillers and leaves of the tillers turn brown and die, which will provoke a symptom called deadhearts. However, deadhearts at the early vegetative stage may not affected grain yield, because plants injured by stem borers at vegetative stage can partially recover by producing more tillers. The critical stage of yellow stem borer's attack is during reproductive stage. During this stage, injury to tillers can destroy the panicles resulting in whiteheads symptoms. Tillers that are lost at the reproductive stage are not replaceable causing yield loss (Bandong & Litsinger, 2005; Lv *et al*., 2008 in Texas Rice, 2011). Thus, the damage at the reproductive stage can contribute in reduced filled grains. Every 1% whitehead sympoms is correlated with 1% rice production loss (Balai Besar Penelitian Padi, 2010; 2011).

In Indonesia, the average damage by yellow stem borer in the last 10 years was 84.952 ha (Direktorat Perlindungan Tanaman, 2007). In many places in Java, yellow stem borer is the dominant insect and its population may reach up to 90% of total population of stem borer (Hendarsih *et al*., 2007). To control stem borer, the preventive approach by using high-dose pesticides continuosly may result in a negative impact to environmental including effect on non-target organisms, insect resistance breakthrough and human health.

Therefore, the development of rice varieties resistant to yellow stem borer (YSB) is crucial.

However, extensive screening for resistance to YSB done at the International Rice Research Institute (IRRI) resulting in no resistance rice germplasms identified. Bioassay toward YSB on a number of local rice germplasms had also been conducted at Indonesian Center For Rice Research – Ministry of Agriculture by infesting yellow stem borer larvae into rice plants but unfortunately, no resistant donor in the rice germplasms had been found (Usyati, 2012 personal communication). Furthermore, no resistant gene has been mapped in plant genome (Bennett *et al*., 1997 in Breitler *et al*., 2000; Hoa & Nhu, 2011).

Cry gene that was isolated from soil bacteria *Bacillus thuringiensis* (*Bt*) produced insecticidal crystal proteins called δ-endotoxins which is specifically toxic to Lepidoptera, Coleoptera, Hymenoptera and Diptera among which are economically important pests of rice such as the yellow stem borer (Breitler *et al*., 2000; Cohen *et al*., 2000; Marfä *et al*., 2002; Bravo *et al*., 2007).

There are several successful reports on applying transgenic rice expressing *Cry* gene to combat rice stem borer. Basmati transgenic rice lines harboring two different *Bt* genes, *Cry1Ac* and *Cry2A* were evaluated against YSB under field conditions and showed that the transgenic rice lines showed up to 100 and 96% resistance against YSB at vegetative and generative stages, respectively (Rahman *et al*., 2007). Kumar *et al.* (2010) reported that insect bioassay revealed complete protection of transgenic rice expressing translational fusion of two *Cry* genes namely *Cry1B*::*Cry1Aa* from YSB infestation. Insect bioassay test for resistance to YSB in two indica rice varieties IR64 and Mot Bui harboring *Cry1Ab* or *Cry1Ac* in the laboratory and green house revealed that a large number of transgenic rice lines were highly resistant compared to those of the non-transgenic rice lines (Hoa & Nhu, 2011).

Transformation of *Cry1B* gene under wound inducible gene promoter *mpi* (*maize proteinase inhibitor*) into rice plant cv. Rojolele had been done succesfully resulting in four putative transgenic lines (Estiati *et al*., 2007). The *mpi* promoter is able to control the *Cry1B* gene expression, where the gene is expressed only if the plant is wounded both by mechanical injury or insect infestation, so that the expression of the gene is inducible (Tamayo *et al*., 2000). Previously, we have reported that one of the lines, namely 3R7, which was homozygous for *Cry1B*, showed improved resistant to YSB compared to the control plants (Estiati *et al*., 2012).

In this paper, segregation analysis of two putative transgenic rice lines harboring the *Cry1B* gene driven by the *mpi* promoter (3R25 and 3R5) at third (T_2), fourth (T_3), fifth (T_4) and sixth (T_5) generations done to obtain homozygous lines followed by the efficacy studies done against YSB in the biosafety containment, are reported. Efficacy studies in the biosafety containment need to be conducted before similar studies under natural conditions in the field are performed.

Materials and Methods

Plant Materials. Two transgenic rice lines (*Oryza sativa* L.) i.e. 3R25 and 3R5 harboring *Cry1B* gene under wound inducible gene promoter (*mpi*) at second generation (T_1) and control non-transgenic plants (Rojolele non-transgenic, IR64 and IR74 varieties) were grown in the biosafety containment at the Research Center for Biotechology, Indonesian Institute of Sciences (LIPI). Non-transgenic Rojolele was chosen as the isogenic negative control line, while IR64 and IR4 varieties were included as the susceptible control lines. Each plant was grown in polybag containing soil and manure in 2:1 ratio. The rice cultivation were conducted in the biosafety containment facility.

Segregation Analysis for Selecting Homozygous Transgenic Rice Lines. The materials used in this experiment were two transgenic rice lines (*O. sativa* L.) i.e. 3R25 and 3R5 harboring *Cry1B* gene under wound inducible gene promoter (*mpi*) at second generation (T_1). For analyzing the segregation pattern of *Cry1B* gene, a population of each line should be build. The self- pollinated of T_2 seeds of the T_1 transgenic plants were harvested and planted. The segregation pattern of *Cry*1B gene in transgenic rice was studied based on the PCR positive result on T_2 population. The T_3 seeds resulted from the T_2 showing positive *Cry1B* were planted and analysed their gene inheritance. The same

procedure was follow until homozygous population were obtained. Segregation analysis was done using the Chi Square ($\chi 2$) test in which observed values were compared to theoretical values corresponding to the integration of one or more copies of the transgene. Transgenes are inherited sexually as a dominant trait with inheritance conforming to a 3:1 Mendelian ratio. Non-Mendelian segregation wouldn't be used due to either unstable transmission of the transgene or poor expression (Tizaoui & Kchouk *et al*., 2012). Thus, in our experiment to select homozygous transgenic rice lines, only population following Mendelian ratio 3:1 was used for the next segregation experiment. A population of one line identified as homozygous, if all individual plants tested in PCR analysis showing the presence of *Cry1B* gene with the length of 1.9 kb. Meanwhile, a population identified as heterozygous if there is one individual plant showing no *Cry1B* gene. Lines proved as a homozygous for *Cry1B* gene were used for insect bioassay. As a negative control for PCR, Rojolele non-transgenic was included in this experiment.

Young leaf material from individual transgenic rice and control plants were collected from the greenhouse and the genomic DNA was extracted from leaves following methods by Zheng *et al.* (2000). PCR analysis was conducted using a pair of specific primers for *Cry1B* gene with sequences as follow: *Cry1B* forward: 5'- GCT GTG TCC AAC CAC TCC GC -3' and *Cry1B* reverse 5'- GTA CCG AAT TGG GCT GCA GG –3'. The reactions were carried out in a Biometra Thermocycler. PCR reaction containing DreamTaq Green PCR Master Mix (2×)(Fermentas), 0.5 µM of each primer and about 100 ng plant genomic DNA. PCR analysis was carried out as follow: 3 min pre-denaturation at 95°C, 1 min denaturation at 95°C, 1 min annealing at 62°C, 1 min extension at 72°C, 10 min final extension at 72°C for 40 cycles. The existence of *Cry1B* gene was detected by loading the DNA on a 1.2% agarose gel with 0.5×TBE at 100 volt, for running time about 30 min. The *Cry1B* fragment should be amplified in transgenic plants, and no *Cry1 B* gene would be detected from control plants.

Insect Bioassay. Ten individual homozygous transgenic rice plants from each line and control susceptible plants were assayed for resistance toward yellow stem borer. For control susceptible plant, Rojolele non transgenic, IR4 and IR74 were used. All individual plant tested were put in each pot covered with plastic cylinder to avoid any cross-larval contamination. Seven yellow stem borer larvae at first instar were infested into a tiller of each plant at 21 days after plantation (DAP). The efficacy test was conducted in biosafety containment-Research Center for Biotechnology, Indonesian Institute of Sciences. Observation was carried out at 2 and 4 weeks after infestation (WAI). The percentage of deadhearts and D value from each transgenic rice lines and control plants were calculated as described by Estiati *et al.* (2012).

Results

Segregation Analysis

In order to select homozygous lines, PCR analysis on progeny of 3R25 and 3R5 transgenic rice lines had to be carried out. Eleven plants is the minimum plants in a population that must be tested to show at least one unexpected phenotype appeared under 95% probability for a character controlled by a recessive allele. If from the 11 individual plants tested, there is one phenotype from unexpected recessive allele, the plant population must have originated from heterozygous parent plant. On the contrary, if all of the 11 individual plants showed phenotype from expected dominant allele, it means that the population was originated from homozygous parent plant for the respective gene (Sedcole, 1977).

Thirty seeds of each 3R25.7 and 3R5.26 rice lines were harvested and planted. DNA from young leaves of transgenic rice plants and Rojolele non-transgenic were isolated and PCR analysis using primer pairs specific for *Cry1B* gene was conducted. PCR analysis on 30 individual plants tested of each 3R25.7 and 3R5.26 rice lines showed that the *Cry1*B gene were present in 27 and 23 plants, respectively. Meanwhile, no bands of *Cry1B* gene was present in Rojolele non-transgenic plant. $\chi 2$ test on each population of 3R25.7 and 3R5.26 rice lines showed that the segregation pattern

of *Cry1B* gene in 3R25.7 and 3R5.26 rice lines are following Mendelian ratio as 3:1. From this results could be concluded that 3R25.7 and 3R5.26 lines had been proved as heterozygous lines. Furthermore, from this segregation it is explained that the *Cry1B* gene is a single dominant gene and inserted in one locus (Table 1). Electrophoresis to prove the existence of *Cry1B* gene on population of each lines are showed at Figure 1-2. *Cry1B* gene was not present at lane 6, 8 and 28 (Figure 1) and at lane 6, 7, 23, 27, 28, 33 and 35 (Figure 2). The presence of *Cry1B* gene was determined by showing the PCR product of 1.9 kb.

Table 1. Segregation pattern of *Cry1B* gene in T_2 3R25.7 and 3R5.26 rice lines

Lines	Total plants tested	Segregation analysis		Chi Square value	Df:1; α;0.05 χ^2:3.84
		+*Cry1B* gene	-*Cry1B* gene		
3R25.7	30	27	3	3.60	Ratio 3:1
3R5.26	30	23	7	0.04	Ratio 3:1

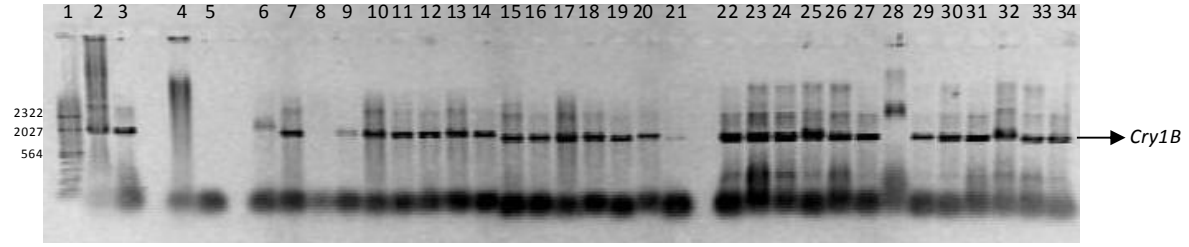

Figure 1. PCR analysis from individual plants of 3R25.7 line. Lane 1: λ *Hin*dIII; lane 2: pCAMBIA 1301 harboring *Cry1B* gene as a positive control; lane 3: transgenic plant as a positive control; lane 4: untransformed plant as a negative control; lane 5: dH_2O as a replacement for DNA; lane 6-34: individual plant tested

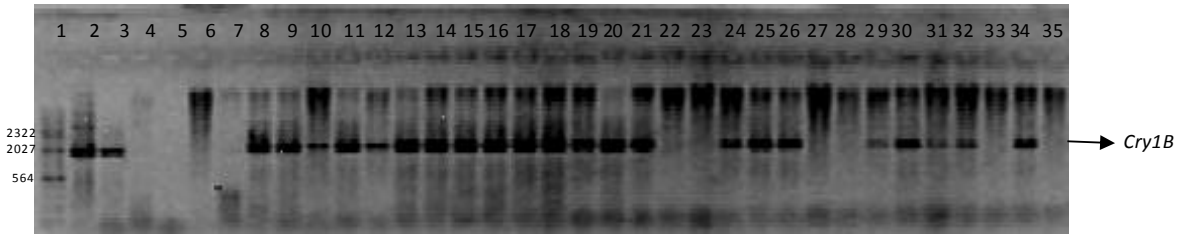

Figure 2. PCR analysis from individual plants of 3R5.26 line. Lane 1: λ *Hin*dIII; lane 2: pCAMBIA 1301 harboring *Cry1B* gene as a positive control; lane 3: transgenic plant as a positive control; lane 4: untransformed plant as a negative control; lane 5: dH_2O as a replacement for DNA; lane 6-35: individual plant tested

Since 3R25.7 and 3R5.26 lines were still heterozygous, the segregation analysis on succesive generation had to be conducted. The seeds from selected individual progeny plants of each 3R25.7 and 3R5.26 lines namely 3R25.7.27, 3R25.7.13, 3R5.26.1, 3R5.26.2 and 3R5.26.5 positively harboring *Cry1B* gene were maintained to produce seeds and the seeds were harvested. Thirty seeds of each 3R25.7.27, 3R5.26.1, 3R5.26.2, 3R5.26.5 lines and 28 seeds of 3R25.7.13 line were grown.

PCR analysis on 30 individual plant of each 3R25.7.27, 3R5.26.2 and 3R5.26.5 lines showed that all individual plant tested amplified the PCR product of *Cry1*B gene with the size of 1.9 Kb (Figure 3-4). This proved that 3R25.7.27, 3R5.26.2 and 3R5.26.5 are homozygous lines and had been chosen for insect bioassay. Meanwhile, PCR analysis on 30 individual plant tested of 3R5.26.1 line and 28 individual plants tested of 3R25.7.13 line showed that the *Cry1*B gene were present in only 29 and 18 plants, respectively. At Figure 5, no band of *Cry1B* gene could be seen at lane 16. Segregation analysis using χ2 test showed that the segregation pattern of *Cry1B* gene in 3R25.7.13 line following Mendelian ratio as 3:1, thus, it had been proved as heterozygous

line (Table 2), meanwhile segregation analysis in 3R5.26.1 did not follow Mendelian ratio as 3:1. Thus, 3R5.26.1.line would not be included for further experiment. According to Tizaoui and Kchouk (2012), non-Mendelian segregation would not be used due to either unstable transmission of the transgene or poor expression.

Table 2. Segregation pattern of *Cry1B* gene in T_3 3R25.7.27, 3R25.7.13, 3R5.26.1, 3R5.26.2 and 3R5.26.5 rice lines

Lines	Total plants tested	Segregation analysis		Chi square value	Df:1; α;0.05 χ^2:3.84
		+*Cry1B* gene	-*Cry1B* gene		
3R25.7.27	30	30	0		Ratio 1:0 (Homozygot)
3R25.7.13	28	18	10	1.71	Ratio 3:1
3R5.26.1	30	29	1	7.50	
3R5.26.2	30	30	0		Ratio 1:0 (Homozygot)
3R5.26.5	30	30	0		Ratio 1:0 (Homozygot)

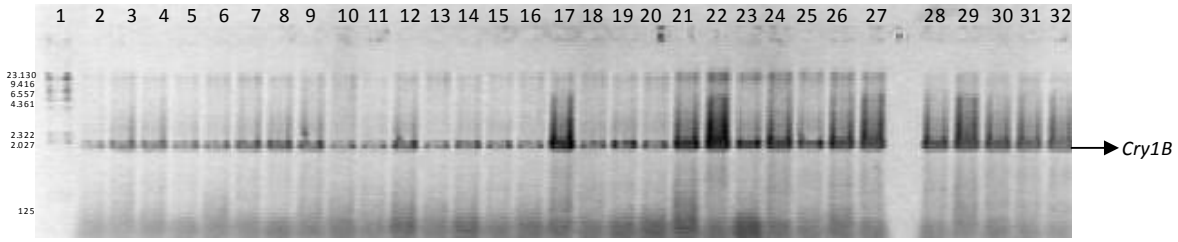

Figure 3. PCR analysis from individual plants of 3R25.7.27 line. Lane 1: λ *Hin*dIII; lane 2: pCAMBIA 1301 harboring *Cry1B* gene as a positive control; lane 3-32: individual plant tested

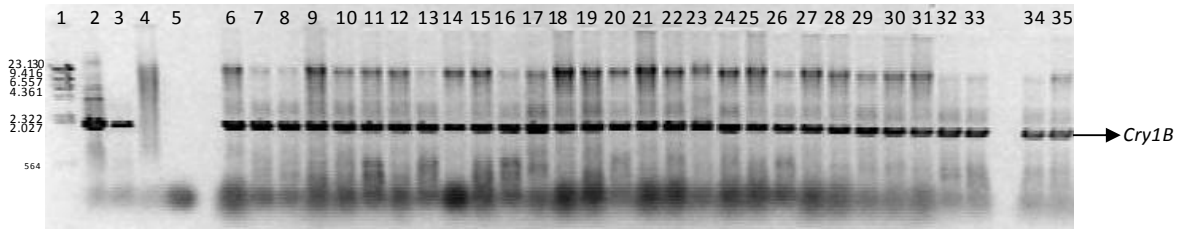

Figure 4. PCR analysis from individual plants of 3R5.26.2 line. Lane 1: λ *Hin*dIII; lane 2: pCAMBIA 1301 harboring *Cry1B* gene as a positive control; lane 3: transgenic plant as a positive control; lane 4: untransformed plant as a negative control; lane 5: dH_2O as a replacement for DNA; lane 6-35: individual plant tested

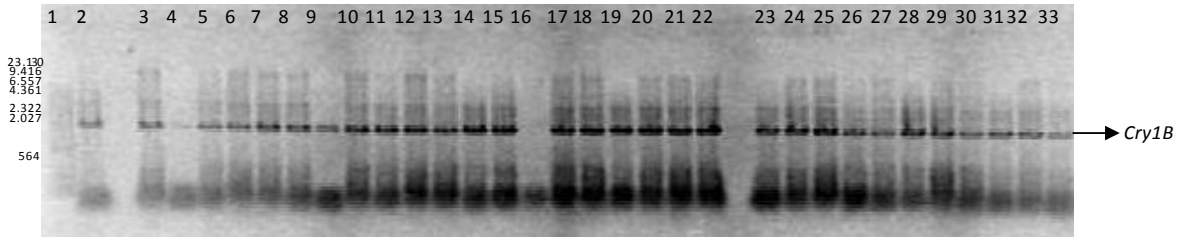

Figure 5. PCR analysis from individual plants of 3R5.26.1 line. Lane 1: λ *Hin*dIII; lane 2: pCAMBIA 1301 harboring *Cry1B* gene as a positive control; lane 3: transgenic plant as a positive control; lane 4-33: individual plant tested

Since 3R25.7.13 line was still heterozygous line, segregation analysis on progeny of 3R25.7.13 had been carried out. One selected individual progeny plants namely 3R25.7.13.8 positively harboring *Cry1B* gene were maintained and thirty seeds were grown. DNA from 30 individual plant were isolated and PCR analysis was carried out. PCR analysis on 30 individual plants showed that the *Cry1B* gene were present in 25 plants and absent in 5 plants. No amplicon of *Cry1B* gene were present in lane number 2, 6, 11, 12 and 17 (Figure 6). Segregation analysis using χ2 test showed that the segregation pattern of *Cry1B*

gene in 3R25.7.13.8 line following Mendelian ratio as 3:1, thus proved that 3R25.7.13.8 was heterozygous lines (Table 3). Segregation analysis was further done in T_6. The seeds from selected individual progeny plants of 3R25.7.13.8 line i.e. 3R25.7.13.8.2, 3R25.7.13.8.6, 3R25.7.13.8.8 and 3R25.7.13.8.25 positively harboring *Cry1B* gene were grown. PCR analysis on 15 individual plants of each 3R25.7.13.8.2 and 3R25.7.13.8.8 lines and 30 individual plants of 3R25.7.13.8.6 line were exposed to PCR. The result showed that all individual plants tested resulted in PCR product of the *Cry1*B gene with the size of 1.9 Kb (Figure 7-8). This proved that 3R25.7.13.8.2, 3R25.7.13.8.8 and 3R25.7.13.8.6 are homozygous lines and had been chosen for insect bioassay. Meanwhile, PCR analysis on 16 individual plants of 3R25.7.13.8.25 line showed that the *Cry1*B gene were present in only 14 plants and the segregation pattern following Mendelian ratio 3:1 (Table 3). Thus, the 3R25.7.13.8.25 line would not be used in insect bioassay.

Insect Bioassay

Three randomly picked homozygous transgenic rice lines were used in insect bioassay i.e. 3R25.7.27, 3R25.7.13.8.6.and 3R5.26.2. Ten individual plants of each 3R25.7.27, 3R25.7.13.8.6. and 3R5.26.2 and control non-transgenic plants (Rojolele, IR64 and IR74 varieties) were infested with seven yellow stem borer larvae at first instar per tiller in each pot at 21 days after plantation (DAP). The observation of damaged tillers were conducted at 2 and 4 weeks after infestation (WAI). The percentage of deadhearts symptoms from 10 individual transgenic plants of each 3R25.7.27, 3R25.7.13.8.6 and 3R5.26.2 lines at 2 weeks after infestation (WAI) were 0%, 0% and 4%, respectivelly. Meanwhile, the percentage of deadhearts symptoms from 10 individual plants of each non-transgenic rice varieties i.e. Rojolele non-transgenic, IR64 and IR74 at 2 WAI were 47.82%, 66.66% and 65.82%, respectivelly. From this data showed that the precentage of damaged tillers in non-transgenic rice plants were higher than transgenic rice plants, although no deadhearts symptom were observed in 3R25.7.27 and 3R25.7.13.8.6 rice lines. To determine the score number of each lines, the percentage of deadhearts was converted to D value which is IR64 had been used as a control susceptible variety. The D value of 3R25.7.27, 3R25.7.13.8.6 and 3R5.26.2 lines were 0%, 0% and 6%, respectively, meanwhile the D value for Rojolele, IR64 and IR74 varieties were 71.73%, 100% and 98.73%, respectively. Finally the D value was converted to determine the score. According to Heinrichs *et al* (1985), the score of 3R25.7.27, 3R5.26.2 and 3R25.7.13.8.6 rice lines were 0, 1 and 0 respectively, meanwhile the D value for Rojolele, IR64 and IR74 varieties were 7, 9 and 9, respectively. The plants was categorized as resistant if the score was 0, 1, 3 or 5, meanwhile the plants was categorized as susceptible if the score was 7 or 9. According to scoring number, 3R25.7.27, 3R5.26.2 and 3R25.7.13.8.6 transgenic rice lines were categorized as resistant lines, meanwhile Rojolele, IR64 and IR74 varieties were categorized as susceptible varieties (Table 4).

The second observation of the deadhearts sympoms was conducted at 4WAI. The percentage of deadhearts symptoms from 10 individual plants of each 3R25.7.27, 3R25.7.13.8.6, 3R5.26.2 rice lines and Rojolele, IR64 and IR74 varieties were 0%, 0%, 5.40%, 65.50%, 54.54% and 86.50%, respectively. From this data showed that the precentage of damaged tillers at 4WAI in non-transgenic rice plants were still significantly higher than transgenic rice plants. The D value of 3R25.7.27, 3R25.7.13.8.6 and 3R5.26.2 lines were 0%, 0%, 9.90%, respectively with the score number were 0, 0 and 1 respectively. Meanwhile the D value for Rojolele, IR64 and IR74 varieties were more than 100%, with the score number was 9. From this score number showed that 3R25.7.27, 3R25.7.13.8.6, and 3R5.26.2 lines were categorized as resistant lines, meanwhile Rojolele, IR64 and IR74 varieties were categorized as susceptible varieties (Table 4). The deadhearts symptoms at 2 and 4 WAI was shown at Figure 9.

Table 3. Segregation pattern of *Cry1B* gene in T_4 and T_5 3R25.7.13.8, 3R25.7.13.8.2, 3R25.7.13.8. 6, 3R25.7.13.8.8 and 3R25.7.13.8.25 rice lines

Lines	Total plants tested	Segregation analysis		Chi-Square value	Df:1; α;0.05 χ^2:3.84
		+*Cry1B* gene	-*Cry1B* gene		
T4 3R25.7.13.8	30	25	5	1.11	Ratio 3:1
T5 3R25.7.13.8.2	15	15	0		Ratio1:0(Homozygot)
T5 3R25.7.13.8.8	15	15	0		Ratio1:0 (Homozygot)
T5 3R25.7.13.8.6	30	30	0		Ratio 1:0(Homozygot)
T5 3R25.7.13.8.25	16	14	2	1.33	Ratio 3:1

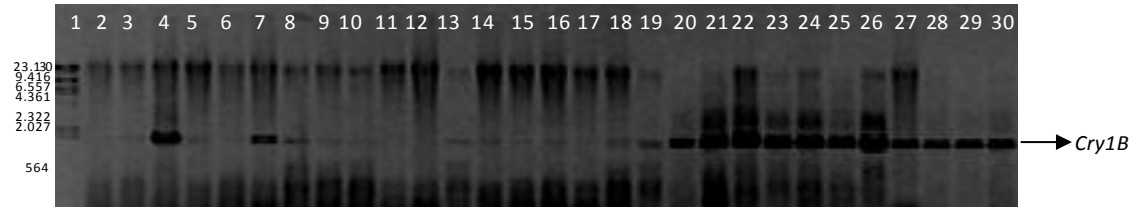

Figure 6. PCR analysis from individual plants of 3R25.7.13.8 line. Lane 1: λ *Hin*dIII; lane 2-30: individual plant tested

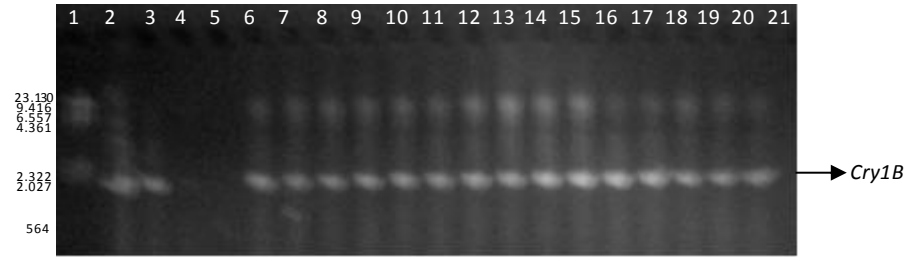

Figure 7. PCR analysis from individual plants of 3R25.7.13.8.2 line. Lane 1: λ *Hin*dIII; lane 2: pCAMBIA 1301; lane 3: transgenic plant as a positive control; lane 4: untransformed plant as a negative control; lane 5: dH_2O as a replacement for DNA; lane 6-21: individual plant tested

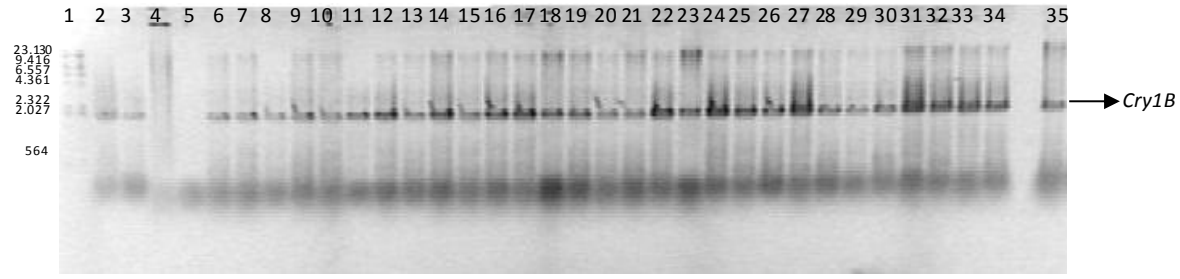

Figure 8. PCR analysis from individual plants of 3R25.7.13.8.6 line. Lane 1: λ *Hin*dIII; lane 2: pCAMBIA 1301; lane 3: transgenic plant as a positive control; lane 4: untransformed plant as a negative control; lane 5: dH_2O as a replacement for DNA; lane 6 -35: individual plant tested

Table 4. Resistance/susceptible determination of 3R25.7.27, 3R5.26.2 and 3R25.7.13.8.6 transgenic rice lines and control plants (Rojolele, IR64 and IR74 varieties) infested with yellow stem borer larvae

Lines	D- value (%)		Score		Resistant/Susceptible
	2WAI	4WAI	2WAI	4WAI	
3R25.7.27	0	0	0	0	Resistant
3R5.26.2	6	9.90	1	1	Resistant
3R25.7.13.8.6	0	0	0	0	Resistant
Rojolele	71.73	120	7	9	Susceptible
IR64	100	100	9	9	Susceptible
IR74	98.73	158	9	9	Susceptible

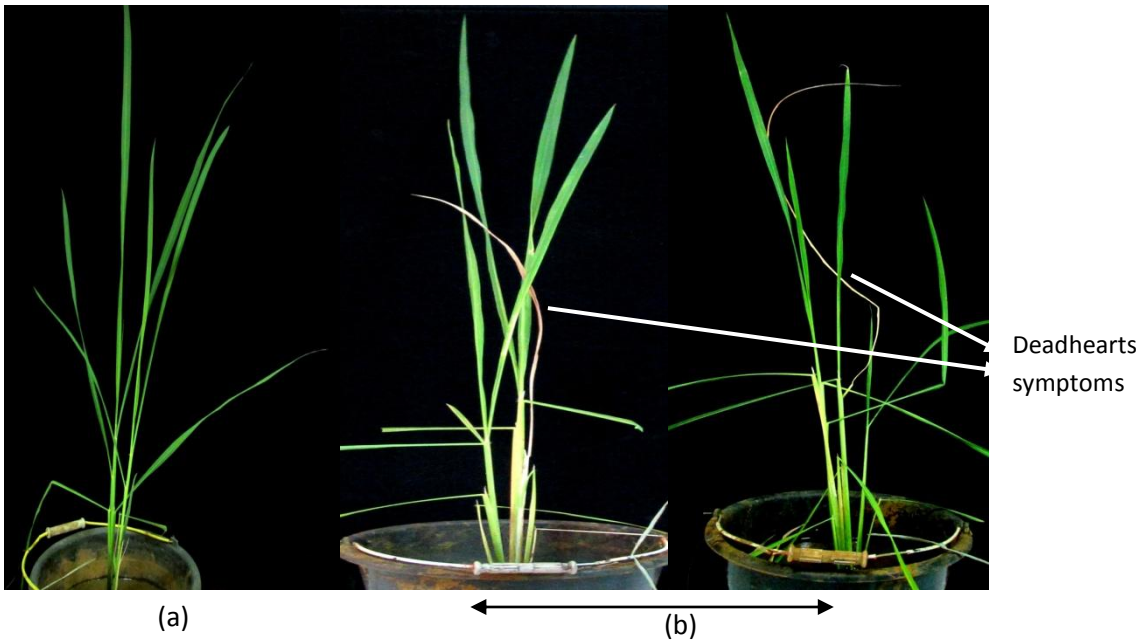

Figure 9. The efficacy studies of *Cry1B* gene in transgenic rice plants and non- transgenic rice plants infested by yellow stem borer larvae. (a) No deadhearts symptoms detected in Rojolele transgenic rice line 3R5.26.2.4 ; (b) deadhearts symptom in Rojolele non-transgenic rice varieties.

Discussion

Yellow stem borer is one of the limited factors to increase national rice productivity. Until now no rice germplams had been identified as resistant plants toward YSB. Bioassay study by infesting yellow stem borer larvae into some of rice germplasms which is conducted at International Rice Research Institute (IRRI) and Indonesian Center For Rice Research Sukamandi–Departement of Agriculture, resulting in no resistance rice germplasms identified. Genomic and Crop Improvement Laboratory-Research Center for Biotechnology, Indonesian Institute of Sciences had developed transgenic rice harboring *Cry1B* gene.

Segregation analysis to study the inheritance of *Cry1B* gene in successive generation of 3R25 and 3R5 transgenic rice lines, showed that the *Cry1B* gene is dominant gene and inserted in one locus. PCR analysis which is facilitated segregation analysis showed that 3R25.7.27, 3R25.7.13.8.2, 3R25.7.13.8.6, 3R25.7.13.8.8, 3R5.26.2 and 3R5.26.5 are homozygous lines. There must be no segregation of *Cry1B* gene in successive generation since the *Cry1B* gene had been stable integrated into plant genome.

Insect bioassay in biosafety containment to verify the efficacy of *Cry1B* gene in three homozygous transgenic rice lines showed that 3R25.7.27, 3R5.26.2, and 3R25.7.13.8.6 lines are more resistant comparing with non-transgenic rice plants (Rojolele, IR64, and IR74 varieties). This results were similar to the once obtain from the previous experiment on another different stem borer resistant line (Estiati *et al*., 2012). This result proved that the *Cry1B* gene in transgenic rice plants expressed insecticidal activity toward yellow stem borer. Breitler *et al*. (2000) reported that stable accumulation of CRY1B which is representing 0.4% of the total soluble protein in transgenic rice plants was able to fully protected plants from attacks of third and fourth instar striped stem borer (*Chilo suppressalis* Walker. Marfà *et al.* (2002), reported that transgenic rice plants cv. Senia expressing *Cry1B* gene was protected against SSB at vegetative and generative stages of plant development under green house condition. The same result came from feed bioassay under laboratory condition showed that indica transgenic rice harboring *Cry1B* gene can control the damage caused by *Spodoptera frugiperda* (Pinto *et al*., 2012). Both *C. supressalis* and *S. frugiperda* which is insect pests in rice are belong to the same ordo with yellow stem borer i.e. Lepidoptera.

From these results we can concluded that transgenic rice lines cv. Rojolele harboring *Cry1B* gene that we had been developed appears to be a good strategy to protect plants from rice insect pests belong to Lepidopteran especially toward yellow stem borer which is an important pest in Indonesia.

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