Segregation Analysis of Transgenic Rice Plants cv Rojolele Harboring *cry1B* Gene and Plant Selection for Potential Resistant to Yellow Stem Borer

Amy Estiati*, Yuli Sulistyowati, Fatimah Zahra, Ade Nena Nurhasanah, Syamsidah Rahmawati and Inez H.S-Loedin

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

Abstract

Transgenic rice plants harboring resistant gene to yellow stem borer, *cry1B* gene had been obtained. However, the *cry1B* gene in second generation of transgenic rice lines still segregating following Mendelian ratio 3:1. For further use in the breeding programs, it is important to ensure that the gene is dominant gene and the plants are homozygous for *cry1B* gene. Selection of homozygous transgenic rice lines containing *cry1B* gene at third, fourth and fifth generation had been conducted by PCR. The presence of *cry1B* gene was determined by showing the PCR product of 1.9 kb. Segregation analysis proved that six transgenic rice lines i.e. 3R7.8.15.1, 3R7.8.15.9, 3R7.8.15.10, 3R7.8.15.15, 3R7.8.15.21, 3R7.8.15.29 are homozygous lines for *cry1B* gene. Moreover, bioassay studies at vegetative stage on six homozygous transgenic rice lines showed that these transgenic rice lines are potential resistant to yellow stem borer comparing with non-transgenic plants, with the score of 0 (indicated no symptom) for six transgenic lines and score of9 (more than 60% damaged tillers) for non-transgenic plants. However, to confirm the efficacy of *cry 1B* gene to yellow stem borer in natural condition, confined field trial in endemic area of yellow stem borer should be conducted.

Keywords: bioassay, *cry1B* gene, transgenic rice, yellow stem borer

---

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

Introduction

Rice production in Indonesia meet the challenges such as abiotic and biotic stresses. Yellow stem borer (*Scirpophaga incertulas* Walker) which is one of the important pests in Indonesia is belong to biotic stresses. Yellow stem borer attack at vegetative and generative stage of plant development. During the first infestation while the plants are at vegetative stage, the larvae damage the growing tillers and provoke symptom called deadhearts. While, the damage produced by growing larvae at reproductive stage (generative) blocked nutrients transport from the stem to the grain, resulting in whitehead symptom. At this stage, the panicles are formed but with the grains empty (Marfä *et al*., 2002). In Indonesia, the average damage by yellow stem borer in the last 10 years was 84.952 ha (Direktorat Perlindungan Tanaman, 2007). In many place in Java, yellow stem borer is a dominant insect and its population reached up to 90% of total population of stem borer (Hendarsih *et al*., 2007). Therefore, the development of rice varieties that resistant to yellow stem borer is being crucial.

The development of resistant rice plant to control yellow stem borer is desirable and environmental friendly. Unfortunately, until now no rice resistant plants have been found and no genes for host resistance have been mapped in plant genome (Bennett *et al*., 1997). *Cry* gene that isolated from soil bacteria *Bacillus thuringiensis* can express crystal protein which is highly toxic to lepidopteran, dipteran, and or coleopteran insects, among which are economically important pests of rice such as yellow stem borer (Breitler *et al*., 2000; Cohen *et al*., 2000; Marfä *et al*., 2002). Unfortunately, gene transformation from bacteria to plant by conventional breeding is not possible. Modern biotechnology can facilitate gene transformation between unrelated species, thus *cry* gene from bacteria could be inserted into

commercially rice plant to develop rice resistant to yellow stem borer. *Agrobacterium*-mediated transformation of *cry1B* gene under wound inducible gene promoter *mpi* (maize proteinase inhibitor) into rice plant cv. Rojolele had been conducted (Estiati *et al*., 2007). This is the first research working with *cry1B* gene in Indonesia. Rojolele had been chosen because this variety is a popular variety in Indonesia. It has aromatic fragrance and good in grain quality and taste (Keputusan Mentan, 2003; Lestari *et al*., 2011).Furthermore, gene transformation technique on Rojolele variety has been established in our laboratory.

The *mpi* promoter controlling the expression of maize proteinase inhibitor protein is induced by wounding and insect feeding (Tamayo *et al*., 2000). Since the gene expression under control of the *mpi* promoter is induced by mechanical wounding, the *cry1B* gene that fused with *mpi* promoter should be expressed only when the plant is damaged by yellow stem borer. Furthermore, the used of *mpi* promoter is one of the strategies to prolonged the resistance breakdown by yellow stem borer.

Four putative transgenic rice lines cv Rojolele harboring *cry1B* gene had been obtained (3R2, 3R5, 3R7 and 3R9). In this article we focused on one putative transgenic rice line i.e. 3R7. The existence of *cry1B* gene in transgenic rice plants at first (3R7) and second generation (3R7.8) generation had been proved by Polymerase Chain Reaction (PCR). At second generation of transgenic plants, the *cry1B* gene segregated following Mendelian ratio as 3:1. This showed that the *cry1B* gene is a single dominant gene (Estiati *et al*., 2007).

Although the *cry1B* gene was proved to be exist in transgenic rice, the existence of the *cry1B* gene should be stable through the generation. The prerequisites for gene stability in plants are the gene should be homozygous, thus segregation analysis for obtaining homozygous plants for *cry1B* gene should be carried out. In this article, the segregation analysis for selecting homozygous plants for *cry1B* gene using PCR at third (T2), fourth (T_3) and fifth (T4) generation of transgenic rice plants population were studied and bioassay to select transgenic rice lines potential resistance to yellow stem borer were also carried out.

Materials and Methods

Plant materials. Rojolele trangenic rice harbouring *cry1B* gene under wound inducible gene promoter (maize proteinase inhibitor, *mpi*) at third (T_2), fourth (T_3) and fifth (T_4) generations and control plants (Rojolele non-transgenic, IR64 and IR74). Transgenic rice lines harboring *cry1B* gene was developed in our laboratory. The seeds of Rojolele non-transgenic, IR64 and IR 74 had been obtained from Indonesian Center for Rice Research-Sukamandi. Each plant was grown in polybag containing soil and manure with ratio 2:1. All plants were grown in biosafety containment.

Segregation analysis for selecting homozygous plants. Selection of homozygous rice line was conducted based on the presence of *cry1B* gene with showing the PCR product of 1.9 kb. The segregation pattern of *cry1B* gene was analyzed using a Chi Square test. Sixteen seeds at third generation (3R7.8) line were planted and the DNA were isolated. Fresh leaf material from transgenic rice lines and control plants were collected from the greenhouse and the genomic DNA was extracted from leaves following methods by Zheng *et al* (2000). PCR was conducted using a pair of specific primers for *cry1B* gene with sequences are 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 mixture 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: 1 min denaturation at 95°C, 1 min annealing at 62°C, 1 min 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. When all plants tested from 3R7.8 line amplified the *cry1B* gene, this proved the homozygousity status of the parents of lines. In the contrary, if some of individual plants tested of 3R7.8 line showed unexpected phenotype, the plant population must have originated from heterozygous parent plant. For obtaining homozygous line in succession generations, the individual plant from population that segregated following

Mendelian ratio 3:1 were planted. The segregation pattern of *cry1B* gene was analyzed using a Chi Square test. DNA was isolated from leaves and PCR was carrid out. This procedure was repeated until all of the plants tested showed the expected bands.

Bioassay. Bioassay to identify and selected transgenic rice line potential resistant to yellow stem borer had been conducted in biosafety containment. Plants to be tested were put in a plastic cylinder to avoid any cross-larval contamination.Individual plants from each of Rojolele transgenic rice lines and control plants (Rojolele non-transgenic, IR64 and IR74) were subjected to infestation with two yellow stem borer larvae per tiller in each pot at 21days after plantation (DAP) (Figure 1). Observation at vegetative stage had been conducted at 2 and 4 weeks after infestation (WAI). Score was attributed to the plants following the Standard Evaluation System. The number of total and damaged tillers per plant at vegetative were recorded to evaluate the percentage of damage plants produced by yellow stem borer larvae following formula as follows:

At vegetative stage:

$$\frac{\Sigma \text{ deadhearts of line tested}}{\Sigma \text{ number of tillers from the same line}} \times 100\%$$

Percentage of damaged plants was converted to D value with formula:

$$D = \frac{\% \text{ damaged plants of line tested}}{\% \text{ damaged plants of control susceptible plants}}$$

Note: D value was converted to scale 0-9. Score 0 indicated no symptom; 1 indicated 1-10% damaged tillers; 3 indicated 11-20% damaged tillers; 5 indicated 21-30% damaged tillers; 7 indicated 31-60% damaged tillers and 9 indicated more than 60% damaged tillers. The plants was categorized as resistant if the D value in scale of 0,1 and 3 meanwhile the plants was categorized as susceptible if the D value in scale of 5, 7, and 9 (Heindrich *et al*., 1985).

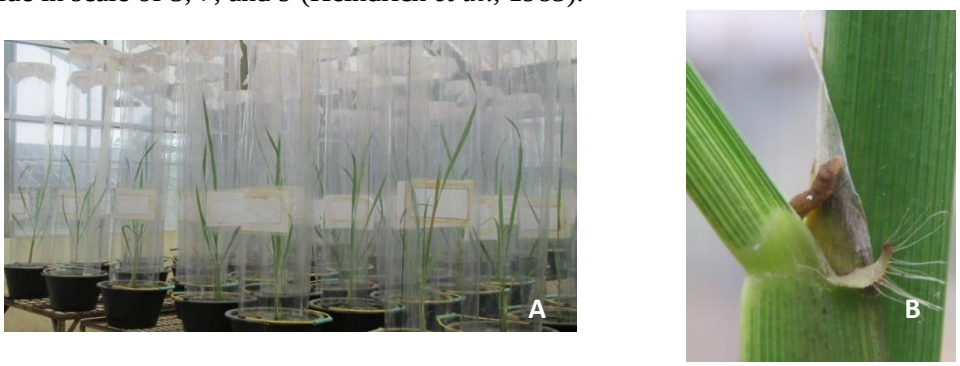

Figure 1. Bioassay for selecting transgenic rice plants potentially resistant to yellow stem borer in biosafety containment. (A) Plants to be tested were put in a plastic cylinder to avoid any cross-larval contamination; (B) infestation with two yellow stem borer larvae per tiller in each pot at 21days after plantation

Results and Discussion

Segregation analysis

For segregation analysis, the minimum number of seeds needed is 11. Sedcole (1977) explained that statistically 11 is the minimum number of plants that must be tested to show at least one unexpected phenotype appeared under 95% probability for a character controlled by a recesive allele. If from the 11 individual plants 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. In other words, 11 sample number is the minimum set of number to ensure the homozigosity at the level of confidence 95%.

PCR analysis from sixteen plants at third generation (3R7.8) showed that the *cry1B* gene were present in some of plants tested and the *cry1B* gene was inherited following Mendelian segregation as 3:1. This explained that the 3R7.8 was heterozygous line and the *cry1B* gene is a single dominant gene (Table 1). Further selection for obtaining the homozygous rice line is the single plant of heterozygous transgenic rice line 3R7.8 that positively contained *cry1B* gene were selfing to produce progeny seeds of fourth generation lines (3R7.8.15). Twenty six seeds from 3R7.8.15 were grown and analyzed for *cry1B* gene inheritance. The results showed that from 26 plants tested, 20 plants showed the expected bands and the *cry1B* gene was segregated following Mendelian ratio 3:1.Since from twenty plants positively containing *cry1B* gene, four plants produce less than 11 seeds, then for further experiment these plants did not included.

The 16 single plant of transgenic rice line 3R7.8.15 that positively contained *cry1B* gene were selfing to produce progeny seeds of fourth generation lines (3R7.8.15.1, 3R7.8.15.4, 3R7.8.15.5, 3R7.8.15.8, 3R7.8.15.9, 3R7.8.15.10, 3R7.8.15.11, 3R7.8.15.15, 3R7.8.15.16, 3R7.8.15.19, 3R7.8.15.20, 3R7.8.15.21, 3R7.8.15.23, 3R7.8.15.25, 3R7.8.15.27 and 3R7.8.15.29).Segregation analysis showed that the unamplified *cry1*B gene were still present in individual plants of some rice lines tested (3R7.8.15.4, 3R7.8.15.5, 3R7.8.15.8, 3R7.8.15.11, 3R7.8.15.16, 3R7.8.15.19, 3R7.8.15.20, 3R7.8.15.23, 3R7.8.15.25 and 3R7.8.15.27 lines). This indicated that those lines are still heterozygous plant.

Meanwhile, the segregation analysis from 3R7.8.15.1, 3R7.8.15.9, 3R7.8.15.10, 3R7.8.15.15, 3R7.8.15.21 and 3R7.8.15.29 lines indicated that all individual plants tested from each of those lines showing the PCR product of 1.9 kb. This proved that the status of these six transgenic rice lines is homozygous plants for *cry1B* gene. In this experiment, only homozygous transgenic rice lines were selected for bioassay experiment. Seven transgenic rice lines that segregated following Mendelian segregation 3:1 will be kept as a candidate for future experiment for selecting homozygous lines, while the rest of three transgenic rice lines that did not segregated as 3:1 would not be used. The segregation analysis data of *cry1B* gene at third (T_2), fourth (T_3) and fifth (T_4) generations of rice lines are shown in Tables 1-3.Meanwhile, the amplification of *cry1B* gene from some transgenic rice lines are shown in Figures 2 and 3.

Bioassay

Six homozygous transgenic rice lines harboring *cry1B* gene were selected. Five to ten individual transgenic plants from each of selected homozygous lines (3R7.8.15.1, 3R7.8.15.9, 3R7.8.15.10, 3R7.8.15.15, 3R7.8.15.21, 3R7.8.15.29) and control plants (Rojolele non-transgenic, IR74 and IR64) were used. To evaluate the damaged plants by yellow stem borer larvae, the number of damaged tillers on each pot was observed.

The percentage of damaged tillers on six transgenic rice lines at vegetative stage at 2 weeks after infestation (WAI) and 4 WAI were 0%, while the percentage of damaged tillers on three non-transgenic rice plants (Rojolele non-transgenic, IR74 and IR64) at 2 WAI were 85.35%, 100%, 99.17%, respectively. The percentage of damaged tillers on six transgenic lines at vegetative stage at 4 WAI were 0%, while the percentage of damaged tillers in non-transgenic plants (Rojolele non-transgenic, IR74 and IR64) were 73.32%, 100% and 64.43%, respectively (Figure 4). These percentage numbers was converted following the Standard Evaluation System to determine the score of infested plants. This conversion showed that the score of six transgenic rice lines at 2 WAI and 4 WAI were 0 while the score of three non-transgenic plants were 9 (Table 4). The observation at generative will be carried out.

According to Heindrich *et al*. (1985), the plants was categorized as resistant if the D value in scale of 0,1 and 3 meanwhile the plants was categorized as susceptible if the D value in scala of 5, 7 and 9. Thus, from these these data showed that 3R7.8.15.1, 3R7.8.15.9, 3R7.8.15.10, 3R7.8.15.15, 3R7.8.15.21 and 3R7.8.15.29 lines are potential lines that resistant to yellow stem borer at biosafety containment level.

To evaluate the resistant transgenic rice plants to yellow stem borer attack in natural condition, confined field trial in endemic area of yellow stem borer will be conducted.

Discussion

Selection of homozygous rice lines containing *cry1B* gene had been conducted. Selection based on the segregation pattern of *cry1B* gene through generation using a Chi Square test. Since all individual plants tested from each of transgenic rice lines 3R7.8.15.1, 3R7.8.15.9, 3R7.8.15.10, 3R7.8.15.15, 3R7.8.15.21, 3R7.8.15.29 at fifth generation showing the *cry1B* gene, this proved that these six transgenic rice lines are homozygous plants for *cry1B* gene

The efficacy study to identify the transgenic rice lines that potentially resistant to yellow stem borer in biosafety containment had been carried out. Observation of damaged tillers on six transgenic rice lines at vegetative stage showed that the transgenic rice lines 3R7.8.15.1, 3R7.8.15.9, 3R7.8.15.10, 3R7.8.15.15, 3R7.8.15.21, 3R7.8.15.29 are potentially resistant to yellow stem borer comparing with control plants.

Since only transgenic rice lines harboring single copy number of *cry1B* gene insertion that will be selected for further experiment, thus Southern hybridization to determine the copy number of *cry1B* gene integrated in transgenic rice genome should be carried out. To identify the expression of *cry1B* gene in selected transgenic rice plants, ELISA or Western blotting will be conducted. Assessment of homozygous transgenic rice lineharboringa single copy of *cry1B* gene under *Ubi* promoter against striped stem borer (*Chilo suppressalis*) in the greenhouse, had been conducted. The results showed that the transgenic plants appeared to be fully protected against stripped stem borer comparing with non-transgenic riceand the expression of *cry1B* gene could be demonstrated as 66 Kda (Breitler *et al.*, 2000; Marfä *et al.*, 2002). Moreover, to confirm the efficacy of *cry 1B* gene to yellow stem borer in natural condition, confined field trial in endemic area of yellow stem borer will also be conducted.

Table 1. Segregation analysis for selecting homozygous plants at third generation of line 3R7.8

Total plants tested	Segregation analysis		X^2	Df:1; α;0.05; X^2:3.84 Segregation Ratio
	+*cry1B* gene	-*cry1B* gene		
16	14	2	1.333	3:1

Table 2. Segregation analysis for selecting homozygous plants at fourth generation of line 3R7.8.15

Total plants tested	Segregation analysis		X^2	Df:1; α;0.05; X^2:3.84 Segregation Ratio
	+*cry1B* gene	-*cry1B* gene		
26	20	6	0.051	3:1

Table 3.Segregation analysis of 16 transgenic rice lines selecting for homozygous lines

Line	Total plants tested	Segregation analysis		X^2	Df:1; α;0.05; X^2:3.84 Segregation Ratio
		+*cry1B* gene	-*cry1B* gene		
3R7.8.15.1	16	16	0		Homozygot
3R7.8.15.4	12	9	4	0.231	3:1
3R7.8.15.5	13	12	1	2.077	3:1
3R7.8.15.8	11	7	4	0.758	3:1
3R7.8.15.9	16	16	0		Homozygot
3R7.8.15.10	14	14	0		Homozygot
3R7.8.15.11	36	32	4	3.074	3:1
3R7.8.15.15	28	28	0		Homozygot
3R7.8.15.16	24	20	4	0.889	3:1
3R7.8.15.19	30	29	1	7.511	
3R7.8.15.20	25	24	1	5.880	
3R7.8.15.21	30	30	0		Homozygot
3R7.8.15.23	27	26	1	6.531	
3R7.8.15.25	22	20	2	2.970	3:1
3R7.8.15.27	14	11	3	0.095	3:1
3R7.8.15.29	37	37	0		Homozygot

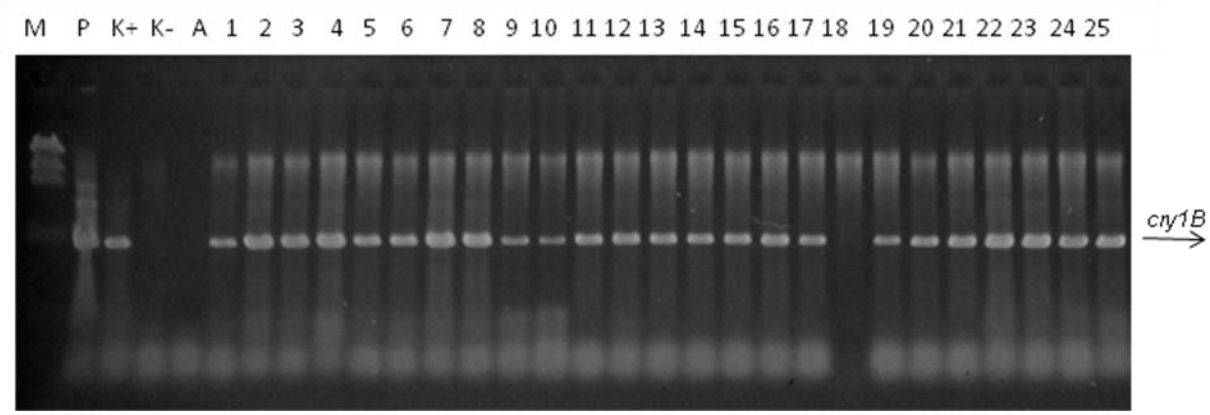

Figure 2. PCR analysis from individual plants of 3R7.8.15.20line.Lane M: λ *Hin*dIII;lane P: pCAMBIA 1301;lane K+: positive control plant; lane K-: untransformed plant as a negative control; lane A: dH_2O as a replacement for DNA; lane 1-25: individual transgenic plants

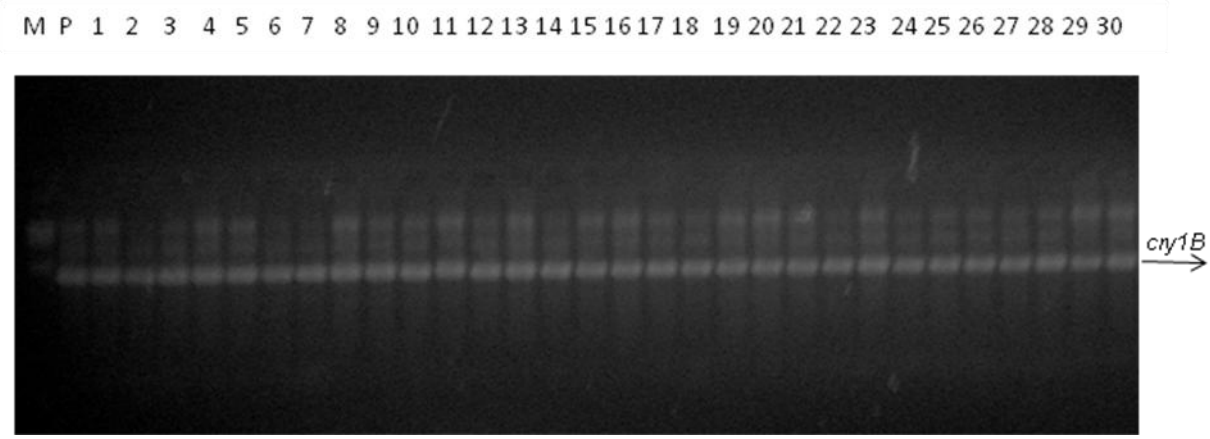

Figure 3. PCR analysis from individual plants of 3R7.8.15.21 line.Lane M: λ *Hin*dIII;lane P: pCAMBIA 1301; lane 1-30: individual transgenic plants

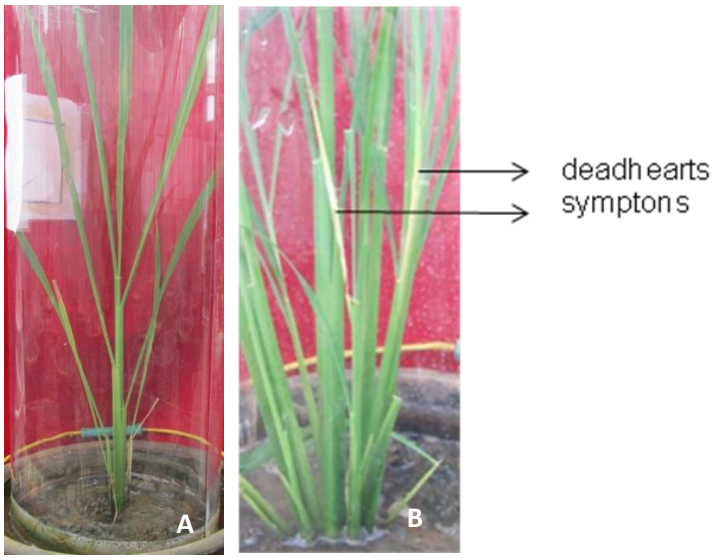

Figure 4.Comparison between transgenic rice plants (A) and non- transgenic rice plants (B) infestedby yellow stem borer.

Table 4. Analysis of six transgenic rice lines infested with yellow stem borer larvae at vegetative stage

Lines	D-value		Scale	Resistant/Susceptible
	2 weeks after infestation	4 weeks after infesation		
3R7.8.15.1	0	0	0	Resistant
3R7.8.15.9	0	0	0	Resistant
3R7.8.15.10	0	0	0	Resistant
3R7.8.15.15	0	0	0	Resistant
3R7.8.15.21	0	0	0	Resistant
3R7.8.15.29	0	0	0	Resistant
Rojolele non-transgenic	85.35	73.32	9	Susceptible
IR64	100	100	9	Susceptible
IR74	99.17	64.43	9	Susceptible

Acknowledgements

This work is financially supported by DIPA 2011. Lidya Sasmawati's technical assistance in PCR experment was appreciated.

References

Bennett, J., M. B. Cohen, S. K. Katiyar, B. Ghareyazie, & G. S. Khush. 1997. Enhancing insect resistance in rice through biotechnology. In: Carrozi, N., M. Koziel (eds.) Advances in insecxt control: the role of transgenic plants. Taylor and Francis, London, pp. 75-93.

Breitler, J. C., V. Marfä, M. Royer, D. Meynard, J. M. Vassal, B. Vercambre, R. Frutos, J. Messeguer, R. Gabarra, & E. Guiderdoni. 2000. Expression of a *Bacillus thuringiensis cry1B* synthetic gene protects Mediterranean rice against the striped stem borer. Plant Cell Reports, 19: 1195-1202

Cohen MB. 2000. Bt rice: practical steps to sustainable use. International Rice Research Notes 25(2):4-10

Direktorat Jenderal Tanaman Pangan.2007. Informasi Perkembangan Serangan OPT Padi Tahun 2006, Tahun 2005 dan Rerata 5 Tahun (2000-2004). Jakarta: Direktorat Jenderal Tanaman Pangan, Direktorat Perlindungan Tanaman.

Estiati, A., S. Rahmawati, D. Astuti, & I. H. S. Loedin. 2007. *Agrobacterium*-mediated transformation of javanica rice plants with a *cry1B* gene under the control of wound-inducible gene promoter. Annales Bogorienses 11(1): 1-7 (ISSN: 0517-8452).

Hendarsih S., D. Kertoseputro & N. Kurniawati. 2007.Penyebaran penggerek batang padi di Pulau Jawa.Laporan DIPA 2007. BB Padi.

Heindrich, E. A., F. G. Medrano, & H. R. Papuas. 1985. Genetic evaluation for insect resistance in rice. Los Banos: International Rice Research Institute.

Keputusan Menteri Pertanian Nomor: 126 Kpts/TP.240/2/2003 Tentang Pelepasan Galur Padi Sawah Lokal Rojolele Sebagai Varietas Unggul Dengan Nama Rojolele.

Lestari, A.P., B. Abdullah, A. Junaedi, H. Aswidinnoor. 2011. Performance of grain quality and aroma of aromatic new plant type promising rice lines.Indonesian Journal of Agricultural Science 12(2): 84-93.

Marfä, V., E. Melé, R. Gabarra, J. M. Vassal, E. Guiderdoni, & J. Messeguer. 2002. Influence of the developmental stage of transgenic rice plants (cv. Senia) expressing the *cry1B* gene on the level of protection against the stripped stem borer (*Chilo suppressalis*). Plant Cell Reports ,20: 1167-1172.

Sedcole, J. R. 1977. Number of plants necessary to recover a trait. Crop Sci., 17: 667-668.

Tamayo, M. C., M. Rufat, M. J. Baravo, & B. S. Segundo. 2000. Accumulation of a maize proteinase inhibitor in response to wounding and insect feeding, and characterization of its activity toward digestive proteinases of *Spodoptera liioralis* larvae. Planta, 211: 62-71.

Zheng, S. J., L. I. Khrustaleva, B. Henken, E. Sofiari, E. Jacobsen, C. Kik, & F. A. Krens. 2000. *Agrobacterium tumefaciens*- mediated transformation of *Allium cepa* L.: the production of transgenic onions and shallots. In Towards onions and shallots (*Allium cepa* L.) resistant to beet armyworm (*Spodoptera exigua* Hübner) by transgenesis and conventional breeding. Thesis. pp. 73-96.