Resistant performance of T10 Rojolele transgenic rice events harboring cry1B::cry1Aa fusion genes against the rice yellow stem borer Scirpophaga incertulas Wlk.

S Nugroho*, D I Sari, F Zahra, S Rachmawati, B S Maulana and A Estiati

Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI), CSC-BG, Jl. Raya Bogor KM 46, Cibinong, West Java, Indonesia

*E-mail: nugroho_satya@yahoo.com

Abstract. Rice yellow stem borer (Scirpophaga incertulas Wlk) (rice YSB) is one of the most important insect pests in rice (Oryza sativa L.). Plant damage caused by the pest attack in all growth stages could significantly reduce yield. Cry toxins from Bacillus thuringiensis are known to be effective against rice YSB, therefore transgenic rice events cv Rojolele harbouring the cry1B::cry1Aa fusion genes driven by the maize ubiquitin promoter have been developed to improve rice resistance. To determine that the resistant traits has been stably inherited and expressed, in-planta and in-vitro bioassays of the T10 generation were performed on 6 independent transgenic rice events using the 1st instar larvae of rice YSB. In-planta bioassay was performed on 5-week-old rice plants grown in pots in a transgenic glasshouse, in 7 replicates. Each plant was invested with 20 1st instar larvae of rice YSB. The in-vitro bioassay was performed using the stems of the transgenic rice events against 10 1st instar larvae of rice YSB, in aerated Petri dishes in a culture room under room temperature, in three replicates. Results showed that the T10 generation of the transgenic rice events stably maintained the transgene integration and their resistance against the rice YSB.

1. Introduction

Rice (Oryza sativa L), is one of the major food crops in the world. In Indonesia, over 90% of all Indonesian consume rice as their staple food. With the increasing number of populations, rice production in Indonesia will also need to be increased. Currently, rice production in Indonesia has met the national demands (http://bulog.co.id/berita/37/7478/10/9/2020/Konsumsi-Nasional-Beras-Aman-Hingga-Akhir-2020.html). However, rice production always faces problems of both biotic and abiotic stresses, which can reduce yield significantly.

One of the most important biotic stress encounters in rice paddy field is damage caused by the rice yellow stem borer/rice YSB (Scirpophaga incertulas Wlk). The impact of rice YSB on rice production is significant, since it can attack the rice plants in both vegetative and generative development stages. Damage symptom caused by this pest during the vegetative stage is called deadheart, while damage symptom during the generative stage is called whitehead. Rice experiencing deadheart might recover, but with reduced yield (up to 30% recovery). On the contrary, rice experiencing a whitehead is less likely to recover and tends to experience total yield loss [1]. It is predicted that 1% of rice YSB infestation correlates with 1% yield loss [2].
In an attempt to overcome rice YSB, the most common approach is by the application of pesticides, which is expensive, unsustainable, and detrimental to human and animal health, and the environment. Therefore, breeding programs to improve rice resistance to obtain promising rice varieties that are resistant to rice YSB are needed. However, the unavailability of germplasm in rice and its close relatives for rice YSB resistance, has hampered the development of resistant rice varieties by conventional breeding [3]. An approach by introducing foreign genes from different species or organism can be seen as an alternative. This approach has been successful in developing insect-resistant Bt transgenic crops of different commodities, such as Bt corn, Bt soybean, Bt cotton and Bt eggplant [4-6]. In rice, many Bt events have been reported. Those include the overexpression of single cry genes, fusion of cry genes and stacking of cry genes with other genes, such as those encoding trypsin inhibitor, herbicide-tolerant and disease-resistant genes. Examples were synthetic cry2Aa gene [7], synthetic cry1Ca [8], synthetic cry1Ab and cry1Ac genes [9] and synthetic cry1Ab [10] for the single gene overexpression. Overexpression of fusion genes in rice have also been reported, such as Cry1Ab-1Ac fusion proteins [11-13], Cry1Ab-Cry1B fusion proteins [14] and stacked events expressing cry1Ac and CpII (cowpea tripolin inhibitor) [15].

LIPI has been developing Bt rice using different cry genes and promoters combinations to target rice YSB, such as cry1Ab fused to the maize ubiquitin promoter and GNA (Galanthus nivalis agglutinin or snowdrop lectin) to target rice brown planthopper [16] fused to the maize ubiquitin promoter [17,18], selectable marker-free cry1Ab gene driven by CaMV35S promoter [19], cry1B driven by the wound-inducible promoter mpi (maize proteinate inhibitor) [20-22] and cry1B-cry1Aa fusion gene controlled by maize ubiquitin promoter [23]. The rice variety backgrounds were indica rice cultivars IR64 and Cisadane and javanica rice cultivar Rojolele. The application of the cry fusion gene and the inducible promoter is an attempt to reduce the risks of plants’ resistant breakdown. At this moment, six transgenic Rojolele events overexpressing the fusion gene cry1B::cry1Aa and four events overexpressing cry1B under mpi inducible promoter were selected to be pursued for further variety released. Previously, the resistance of the Bt rice for the cry1B::cry1Aa fusion genes events against rice YSB developed at LIPI have been reported at early generation [24] and advance generation T7 for the mpi::cry1B events [25] [26]. Here, the efficacy of GM rice events at advance generation (T10) of the Rojolele rice harbouring the cry1B::cry1Aa fusion gene against rice YSB is reported. This efficacy test is important to ensure the stability of expression of the resistance fusion genes is maintained throughout the generations of the rice events and provide information for the stability status of the events as requested by the biosafety committee in Indonesia.

2. Materials and Methods

2.1. Materials
Six homozygous T10 transgenic Rojolele events (X22, W3, U10, P8, Y7 and Q10) harboring the cry1B::cry1Aa fusion genes driven by the maize ubiquitin promoter were obtained from the seed collection of Research Center for Biotechnology-LIPI. Non-transgenic Rojolele variety was used as baseline control. IR64 variety was used as susceptible elite variety control in the in planta efficacy tests. The rice YSB used was obtained from the Indonesian Center for Rice Research in Sukamandi-Subang-West Java. Adult rice YSB were maintained in Cisadane rice plants in pots covered in plastic roll to lay eggs. The hatched 1st instar larvae were used in the in planta and in vitro efficacy studies.

2.2. Plant cultivation and maintenance
Rice plants were grown in the biosafety containment belongs to the Research Center for Biotechnology-LIPI. Seeds of the six individual transgenic events and the non-transgenic control Rojolele and IR64 rice lines were germinated in the soil in a plastic container. Twenty-one-day old homogenous germinating seeds were analyzed for inserts by PCR, and 7 selected transgenic plants from each event were transferred to seven individual pots containing top soil and manure (70:30 v/v). The plants were maintained by routine watering using tap water.
2.3. Confirmation of transgene inserts

The presence of the fusion genes was determined by PCR. Total genomic DNA’s were isolated from the leaves of 11 plants from each of the events, except for Q10 from 12 plants, in Eppendorf tubes using a modified miniprep CTAB protocols based on van Heusden et al [27]. The PCR reactions were conducted in the Biometra Thermocycler (AnalytikJena) using DreamTaq DNA polymerase (Thermo Scientific) following the manufacturer’s suggestion. The primers used were designed to amplify 816 bp fragment covering the junction region between the cry1B and cry1Aa genes (Forward Primer: CryF: GCCCAAGAAGCTGTCAACGC, and reverse primer CryR: CGATGTCGAGAAGCTGAAGGG).

PCR was performed for 30 cycles under the following conditions: denaturation at 95°C for 1 minute, annealing at 62°C for 30 seconds and extension at 72°C for 2 minutes.

2.4. In-planta bioassay

The in-planta bioassay was performed based on Heinrich et al. [28] in the biosafety containment belongs to the Research Center for Biotechnology-LIPI. Seven plants harboring the cry1B::cry1Aa fusion genes inserts based on the PCR assays of each of the six transgenic events were used. Seven non-transgenic Rojolele and IR64 rice plants were used as the controls. The tillers of the five-week-old plants (during the 1-2 tiller stage) were infested with 20 first instar larvae of rice yellow stem borer, placed near the auricles of the youngest leave. The observation was performed at 3, 7, 10, 14 and 21 days after inoculation (DAI). The percentage of deadhearts and the D value from each transgenic rice events and control plants were calculated as described previously [25,26]. The percentage of deadheart was calculated using the following formula:

$$\text{Deadhearts} = \frac{\text{Number of deadhearts tiller} \times 100\%}{\text{Total number of tillers}}$$

The D value was calculated using the following formula:

$$\text{D value} = \frac{\% \text{ Deadhearts of the evaluated event}}{\% \text{ Deadhearts of the susceptible control line}} \times 100\%$$

The susceptible control lines in this case is rice cultivar IR64. The D value is converted into scales (0 – 9); where 0 = 0%, 1 = 1-10%, 3 = 11-20 %, 5 = 21-30 %, 7 = 31-60% and 9 = 61-100% [29]. Plant in the scale of 0 is considered highly resistant, 1 is resistant, 3 is moderately resistant, 5 is moderately susceptible, 7 is susceptible and 9 is highly susceptible [30].

2.5. In-vitro bioassay

The in-vitro bioassay was performed in plastic Petri dishes in the growth room at room temperature. The lids of the Petri dishes were cut 2x2 cm² and covered with cloths to allow air circulation. Stems of each transgenic rice events and the non-transgenic Rojolele rice lines were cut 5 cm long and placed on top of tissue papers in the Petri dishes individually for three replicates. To keep the moisture in each Petri dish, 1 mL sterile dH₂O was applied to the tissue paper. In each Petri dish, 10 first instars of rice yellow stem borer were added to the rice stems. Observations for larvae mortality were performed on day three after the inoculation.

3. Results and Discussion

3.1. Confirmation of inserts

PCR reactions to amplify 816 bp of the cry1B::cry1Aa fusion junction using primers specific to cry1B (CryF) and cry1Aa (CryR) showed that all the plants tested were transgenic harboring the fusion genes (figure 1). The results also indicated that the fusion genes were stably inserted in the genome of all rice events and that the rice events were homozygous for the inserted fusion genes. The fact that the fusion
genes were stably inserted in the genome is important information requested when commercial release is being sought.

![PCR Confirmation of Transgenic Lines](image)

**Figure 1.** Insert confirmation of T10 plants by PCR. M: λ Hind III; P: Plasmid pC-Cry1B-Cry1Aa; K+: Positive control plants; K-: negative control plant; A: Water; 1-11: X22, 12-22: W3, 23-33: U10, 34-44: P8, 45-55: Y7 and 56-67: Q10.

### 3.2. In-planta bioassay

In this experiment, plants at their early stage of development were exposed to extreme biotic stresses by the infection of 20 first instar larvae of rice YSB into each plant. This condition was created to mimic heavy infestation in the field, as well as to give a real challenge of rice YSB infestation to the plants. The results of this experiment showed that the transgenic lines were resistant to rice YSB. As shown in table 1, at the early stage of infestation (3 DAI and 7 DAI) all transgenic rice events showed the percentage of deadheart lower than the percentage of deadheart in control varieties. The percentage of deadheart in Rojolele and IR64 were 100 and 85.71%, respectively. However, there were differences observed in the resistance of the transgenic rice events; transgenic rice event X22 showed no observed deadheart. Meanwhile, rice events P8 and Y7 showed 1 deadheart (14.29%) while in W3 and U10 showed 2 deadhearts (28.57%) and Q10 showed 3 deadhearts (42.86%).

The performances of the transgenic rice events improved by time, as indicated by the lowering of the percentage of deadhearts. At 7 DAI all transgenic rice events experiencing deadheart showed recovery, except for events W3. However, at 10 DAI all transgenic plants of all transgenic events recovered completely. The variations in the percentage of deadhearts, especially at the early stages, could be due to the level of the expressions of the cry1B::cry1Aa fusion genes among the events, which may be due to positional effects or insertion sites. The different performance of transgenic events has also been reported, among many were the transgenic sugar cane resistance to the mosaic virus [31], transgenic potato [32] and transgenic rice overexpressing Waxy gene [33].

Different cases were observed with the non-transgenic controls. Rice cultivar IR64, which was used as the susceptible control, at the early stages of infection (3, 7 and 10 DAI’s) showed 85.71% of deadheart. However, at the later stages (14 and 21 DAI’s) showed 100% deadheart. Meanwhile, the non-transgenic Rojolele cultivar showed 100% deadheart during the early stages. The plants managed to show some recovery, indicated by the improvement in the percentage of deadheart to 85.71%. However, it is still classified as highly susceptible.

The results were consistent with Usyati et al. [24] finding when an earlier generation of the same events were tested under in-planta condition in the glasshouse. Since this is the first transgenic events expressing cry1B::cry1Aa fusion genes, the uses of the fusion genes in other rice or other crops have not been reported previously; therefore, comparison among results cannot be done. However, results of this experiments is similar to other fusion genes applications, such as that reported by Ho et al [14] when applying cry1Ab::cry1B fusion genes in a Vietnamese elite variety.
The rice plant recovery after the infestation of the rice YSB can be expected if the damage does not impact the apical meristem. Once the apical meristem is damaged, no recovery can be expected. The 100% recoveries of the transgenic rice events indicated that although the rice YSB caused damage, they were killed during the process before reaching the apical meristem of the plants. The 1st instar larvae of the rice YSB were placed at the leaf axial of rice plants during the infestation and they would bore into the stem, causing damage along the way, depending on the degree of resistance of the plants. Transgenic rice plants producing toxic crystal Cry1B-Cry1Aa protein fusion killed the larvae, while the non-transgenic rice cultivar with no resistance against the rice YSB were lethally damaged.

International Rice Research Institute (IRRI) established a very stringent scoring system for rice damages including that caused by the rice YSB [29]. This stringent scoring system is aimed to select the released varieties to have the best resistance to the rice YSB. Based on the scoring system, the transgenic rice events tested, although all except for X22 showed deadhearts, at the end of the experiment period could be categorized as high resistance, while both control cultivars were highly susceptible. The resistance to rice YSB of all transgenic rice events tested increased to more than 85% compared to the non-transgenic Rojolele control and 100% compared to IR64, the susceptible elite variety control. The resistance performances of the transgenic rice events and the control cultivars against the rice YSB in general can be seen in figure 2. Figure 2A showed the 5 weeks old rice performance prior to the inoculation with the rice YSB. Figure 2 B-C showed the early infection stage in which some transgenic plants were recovering. Figure 2D-E showed the recovered transgenic rice plants representing the 6 events tested.

| No | Event | Observation time (Day after Infection/DAI) |
|----|-------|------------------------------------------|
|    |       | 3 DAI                                    |
|    |       | PD (%) | D | S | C | PD (%) | D | S | C | PD (%) | D | S | C | PD (%) | D | S | C | PD (%) | D | S | C |
| 1  | Rojolele | 100 | 116.7 | 9 | HS | 85.7 | 100 | 9 | HS | 85.7 | 97.9 | 9 | HS | 85.7 | 9 | HS | 85.7 | 9 | HS |
| 2  | IR64   | 85.7 | 100 | 9 | HS | 85.7 | 100 | 9 | HS | 87.5 | 100 | 9 | HS | 100 | 100 | 9 | HS | 100 | 100 | 9 | HS |
| 3  | X22    | 0 | 0 | 0 | HR | 0 | 0 | 0 | HR | 0 | 0 | 0 | HR | 0 | 0 | 0 | HR |
| 4  | W3     | 28.6 | 33.3 | 7 | S | 28.6 | 33.3 | 7 | S | 0 | 0 | 0 | HR | 0 | 0 | 0 | HR |
| 5  | U10    | 28.6 | 33.3 | 7 | S | 0 | 0 | 0 | HR | 0 | 0 | 0 | HR | 0 | 0 | 0 | HR |
| 6  | P8     | 14.3 | 16.7 | 3 | MR | 0 | 0 | 0 | HR | 0 | 0 | 0 | HR | 0 | 0 | 0 | HR |
| 7  | Y7     | 14.3 | 16.7 | 3 | MR | 0 | 0 | 0 | HR | 0 | 0 | 0 | HR | 0 | 0 | 0 | HR |
| 8  | Q10    | 42.9 | 50 | 7 | S | 14.3 | 16.7 | 3 | MR | 0 | 0 | 0 | HR | 0 | 0 | 0 | HR |

PD: % Deadheart (%), D: D-Value, S: Scale, C: Category of Resistance, HS: Highly Susceptible, S: Susceptible, MR: Moderately Resistance, HR: Highly Resistance.
Figure 2. Performance of the transgenic rice events harboring cry1B::cry1Aa fusion genes and the control plants against the rice YSB. A. During inoculation (5-week-old), B. 7 days after inoculation (DAI), C. 10 DAI, D. 14 DAI and E. 21 DAI. 1. IR64, 2. Non-transgenic Rojolele, 3. U10, 4. Q20, 5. P8, 6. X22, 7. Y7 and 8. W3.

3.3. In-vitro bioassay
The in-planta efficacy test was established to try to imitate the natural infestation in the field. However, the faith of the rice YSB used in the experiments is not completely known as they can travel anywhere, including being consumed by predators. On the contrary, the in-vivo test performed in the lab, try to confine the experiment set up, so that the survival status of the rice YSB treated with the transgenic materials can be observed by counting their mortality or survival rates.

The in-vitro efficacy tests showed similar results to the in-planta tests, as shown in figure 3. The transgenic rice plants of all events tested showed improved resistance against the rice YSB compared to the non-transgenic control plants. All the 1st instar larvae applied were killed (100% mortality) in all events tested except for U10 in which 1 out of 10 1st instar larvae was survived (90% mortality) after 3 days of treatment (figure 3). Compared to the non-transgenic Rojolele, improvements of resistance between 90% (U10) to 100% (W3, Y7, Q20, X22, and P8) were observed. The appearance of the survived and killed instar can be seen in figure 4.
Figure 3. Mortality rates of the first instar rice YSB, 3 days after treatment.

Figure 4. First instar rice YSB after 3 days of in-vitro bioassays. A. A life instar on non-transgenic Rojolele stem, B. Dead instars recovered from plates containing transgenic Rojolele stems. Bar: approximately 2 mm.

4. Conclusions
Based on the in-planta and in-vitro efficacy studies, the six transgenic rice events tested could be categorized as highly resistant to the rice YSB (*S. incertulas* Wlk) compared to the control (highly susceptible). Different responses of resistance among the six events were observed during the in-planta bioassay, in which one event (X22) exhibit no deadheart, while the others showed a different percentage of deadheart during the early stage of infection but recovered completely at the end of the experiment.
The results also showed that the resistance to rice YSB was stably inherited to the T10 generation of the six transgenic events.

References

[1] Wunn J, Kloti A, Burkhardt P, Ghosh Biswas G, Launis K, Iglesias V A and Potrykus I 1996 Biotechnol. 14 171–6
[2] Baehaki S E 2013 Iptek Tanaman Pangan 8 1–14
[3] Bennett J, Cohen M B, Kattiyar S K, Ghareyazie B and Khush G S 1997 Advances in Insect Control: The Role of Transgenic Plants ed N Carrozi and M Koziel (London: Taylor and Francis) pp 75–93
[4] Shelton A M, Hossain M J, Paranjape V, Azad A K, Rahman M L, Khan ASMMR, Prodhun M Z H, Rashid M A, Majumder R, Hossain M A, Hussain S S, Huesing J E and McCoandless L 2018 Front. Bioeng. Biotechnol. 6 106
[5] Perlak F J, Deaton R W, Armstrong T A, Fuchs R L, Sims S R, Greenplate T J and Fischhoff D A 1990 Nat. Biotechnol. 8 939–43
[6] Cohen M B, Chen M, Bentur J S, Heong K L and Ye G Y 2008 Integration of Insect-resistant Genetically Modified Crops with IPM Systems ed J Romeis, A M Shelton and G G Kennedy (Dordrecht: Springer) pp 223–48
[7] Chen H, Xu C G, Tang W and Li X H 2005 Theor. Appl. Genet. 111 1330–7
[8] Tang W, Chen H, Xu C G, Li X H, Lin Y J and Zhang Q F 2006 Mol. Breed. 18 1–10.
[9] Cheng XY, Sardana R, Datta S 2006 Mol. Breed. 18 1–10.
[10] Ho N H, Baisak N, Oliva N, Datta K, Frutos R and Datta S 2006 Crop Sci. 46 781–9
[11] Zhuang Y, Zhuang Y, Zhao K, Peng Y and Guo Y 2004 J. Agric. Biotech. 12 76–9
[12] Shu Q Y, Ye Q Y, Cui H R, Cheng X Y, Xiang Y B, Wu D X, Gao M W, Xia Y W, Hu C, Sardana R and Altosaar I 2000 Mol. Breed. 6 433–9
[13] Wang Y, Su K, Li Y, Han L, Liu Y, Hua H and Peng Y 2016 Insect Sci. 23 78–87
[14] Tu J, Zhang G, Datta K, Xu C, He Y, Zhang Q, Kush G S and Datta S K 2000 Nat. Biotechnol. 18 1101–4
[15] Tu J, Datta K, Alam M F, Fan Y L, Kush G S and Datta S K 1998 Plant Biotechnol. 15 195–203
[16] Ho N H, Baisak N, Oliva N, Datta K, Frutos R and Datta S 2006 Crop Sci. 46 781–9
[17] Zhao H, Zhang Y, Zhao K, Peng Y and Guo Y 2004 J. Agric. Biotech. 12 76–9
[18] Rao K V, Rathore K S, Hodges T K, Fu X, Stoger E, Sudhakar D, Williams S, Christou P, Bharathi M, Bown DP, Powell KS, Spence J, Gatehouse A M R and Gatehouse J A 1998 Plant J. 15 549–57
[19] Slamet-Loedin I H, Novalina, Satoto, Damayanti D, Sutrisno, Mulyaningsih E S, Christou P and Aswinoor H 2003 Advances in Rice Genetics ed G S Khush, D S Brar and B Hardy (Manila: International Rice Research Institute) pp 565–6
[20] Satoto, Sulistyowati Y, Hartana A and Slamet-Loedin I H 2008 Indones. J. Agric. Sci. 9 35–43
[21] Sulistyowati Y, Rachmat A, Zahra F, Rahmawati S and Nugroho S 2011 Ann. Bogor. 15 27–32
[22] Breitler J C, Marfà V, Royer M, Meynard D, Vassal J M, Verschaffelt B, Frutos R, Messegner J, Gabarra R and Guiderdoni E 2000 Plant Cell Rep. 19 1195–202
[23] Breitler J C, Vassal J M, Catala M C, Meynard D, Marfa V, Melé E, Royer M, Murillo I, San Segundo B, Guiderdoni E and Messegner J 2004 Plant Biotechnol. J. 2 417–30
[24] Estiati A, Rahmawati S and Slamet-Loedin I H 2007 Ann. Bogor. 11 1–5
[25] Rahmawati S and Slamet-Loedin I H 2006 Hayati 13 19–25
[26] Estiati A, A, Astuti D and Slamet-Loedin I H 2009 J. Entomologi Indonesia 6 30–41
[27] Estiati A, Nrurhasanah A and Nugroho S 2013 Ann. Bogor. 17 17–26
[28] Estiati A, Astuti D, Nrurhasanah A and Nugroho S 2020 Proc. IOP Conf. Ser.: Earth Environ. Sci. (IOP Publishing) 439 012054
[27] Van Heusden A W, Van Ooijen J W, Vrielink-Van Ginkel R, Verbeek W H J, Wietsma W A and Kik C 2000 Theor. Appl. Genet. 100 118–26
[28] Heinrich E A, Medrano F G and Rapusas H R 1985 Genetic Evaluation for Insect in Rice (Manila: International Rice Research Institute) p 356
[29] International Rice Research Institute (IRRI) 2013 Standard Evaluation System for Rice (Manila: International Rice Research Institute) p 65
[30] Devasena N, Soundararajan R P, Reuolin S J, Jeyaprakash P and Robin S 2018 J. Entomol. Zool. Stud. 6 874-8
[31] Yao W, Ruan M, Qin L, Yang C, Chen R, Chen B and Zhang M 2017 Front. Plant Sci. 8 104.
[32] Dale P J and McPartlan H C 1992 Theor. Appl. Genet. 84 585-91
[33] Yu H, Liu Q, Xu L, Lu M, Cai X, Gong Z, Yi C, Wang Z and Gu M 2009 Acta Agron. Sin. 35 967–73