Abstract. This research observes and analyses the agronomic characteristics and yield component of six T8 generation of Rojolele transgenic rice lines (P8, Q20, U10, W3, X22, Y7) harboring Cry1B::Cry1Aa gene fusion conferring resistance to yellow stem borer (Scirpophaga incertulas) compared to the nontransgenic Rojolele control plants. The experiment was done in a biosafety containment at the Research Centre for Biotechnology-LIPI in a (4.0 x 3.2) m² pot with 50 cm spacing amongst lines and 25 cm between plants of the same line with 10 replicates each line. PCR analysis using Cry1B::Cry1Aa specific primer showed that all lines tested were transgenic as shown by the presence of 816 bp amplicon. The W3 transgenic line had the most similar agronomic characteristics to the Rojolele control plants in terms of plant height, leaf length, tiller numbers, stem diameter and flowering time. Yield components of the W3 line such as panicle length and total grains were also the closest to the Rojolele control plants compared to other transgenic lines. However, the number of empty grains was the highest. In general, the agronomic characteristics and yield component of all transgenic lines were less than of the Rojolele control plants.

Keywords: Agronomic characteristic, Rojolele transgenic, biosafety

1. Introduction

Yellow stem borer (Scirpophaga incertulas) is one of the most important pests which cause severe damage in lowland rice field in Indonesia. The larvae feed on rice stem and infestation can occur at any rice developmental stage. Infestation at vegetative phase causes the “deadheart” symptom indicated by wilting at the tip of the rice shoots since the larva attacks plant’s growth point. Infestation at generative phase causes “whitehead” which is empty, dried and white-colored panicles because nourishment is cut off as the result of the larva feeding activity [1]. Yellow stem borer infestation causes significant yield loss and has potential of causing crop failure.

Development of rice lines resistant to yellow stem borer cannot be done by conventional plant crossing since naturally-resistant rice parental is not available either from domestic rice varieties or from its wild type. Biotechnology development opens an opportunity to produce rice lines resistant to yellow stem borer using genetic transformation method. By inserting transgene, transgenic rice lines are expected to be able to produce chemical compounds that act as natural insecticide against yellow stem borer.
Many strains of *Bacillus thuringiensis* (Bt) produce crystal protein (cry), called δ-endotoxins which have insecticidal action towards insects from lepidoptera, diptera and coleoptera including yellow stem borer [2]. The toxic property of cry proteins has been used to develop transgenic rice lines by inserting a *Cry1Ab, Cry1Ac, Cry1B* or *Cry1Aa* gene into genome of different rice cultivars [3-6]. Other than single gene transformation, some research were also inserting two *Cry* genes that have different binding site into plant genome, for example transformation of *Cry1Ab::Cry1B, Cry1A::cry1Ac* and *Cry1B::cry1Aa* [7, 8].

The Research Center for Biotechnology has succeeded in transforming *Cry1B::Cry1Aa* gene fusion into a lowland rice variety of Rojolele and producing six T7 generation of transgenic rice lines harboring *Cry1B::Cry1Aa* gene fusion. A glasshouse-scale of efficacy test at vegetative and generative stage showed that all transgenic rice lines were more resistant to yellow stem borer infestation compared to the Rojolele nontransgenic control plants. Segregation analysis using PCR method proved the inheritance stability of the gene until T7 generation [9].

This research observed and analyzed agronomic characteristics and yield component of T8 generation of the six transgenic rice lines harboring *Cry1B::Cry1Aa* gene fusion compared to the Rojolele control plants. The obtained data serves as basic data prior to limited field trial experiment. The research was conducted in a biosafety containment at Research Center for Biotechnology, LIPI during rainy season in 2016. Observation was done on some agronomic characteristics such as plant height, leaf length, leaf width, stem diameter, tiller numbers, productive tiller numbers, flowering time and grain maturity time. The yield components observed were panicle numbers, panicle length, filled grain, empty grain and weight of 1000 grains.

2. Materials and method

2.1. Plant material

Seeds from six transgenic rice lines (T8 generation) harboring *Cry1B::Cry1Aa* gene fusion were used. The six lines were X22 (Rj 04 F2.2 2.4-25-22-12-3-22), U10 (RFZ 3.2.2-1-6-28-1-10), W3 (RFZ 3.3.2A-11-25-12-5-3), Y7 (RFZ 4.2.2-1-27-13-6-7), Q20 (RFZ 4.2.3-28-15-2-8-20) dan P8 (RFZ 4.2.4-21-8-16-7-8) with 10 replicates each line. A nontransgenic Rojolele cultivar was used as control plant (wildtype).

2.2. DNA isolation

Total DNA was isolated from rice leaf at vegetative phase. Leaf (± 5 cm) in a 1.5 mL tube was frozen by soaking in liquid nitrogen and then ground using TissueLyser II (Qiagen). Then 750 μL isolation buffer (lysis buffer [Tris-HCl 0.2 M pH 7.5, EDTA 0.05 M pH 7.5, NaCl 2 M, CTAB 2 % (b/v)], extraction buffer [sorbitol 0.35 M, Tris-HCl 0.1 M pH 7.5, EDTA 5 mM pH 7.5], and sarcosyl 15 % (b/v) by ratio 2.5:2.5:1 was added and mixed. The samples were then incubated for 1 hour in a 65 °C oven. After air-cooling the samples, 750 μL chloroform:isoamylalcohol (24:1) was added into the samples and inverted several times. Samples were then centrifuged at 9000 rpm for 10 minutes at room temperature (RT). The upper layer was then transferred into a 1.5 mL fresh tube then added 400 μL isopropanol and gently inverted until DNA thread was visible. Next, the samples were centrifuged (9000 rpm, RT) for 8 minutes. The supernatant was then discarded and the pellet was added with 400 μL ethanol 70 % and centrifuged (9000 rpm, RT) for 3 minutes. The supernatant was discarded and the pellet was air-dried. The DNA pellet was then diluted in 30 μL TE buffer [Tris-HCl 10 mM pH 7.5, EDTA 1 mM] and stored at -20 °C.

2.3. DNA amplification

For DNA amplification, Dream Taq Green PCR Master Mix (Thermo Scientific) was used. The PCR mixture contained 6.25 μL Dream Taq Green PCR Master Mix, 0.4 μL forward and reverse primer (10 μM), 100 ng DNA sample and nuclease free water to 12.5 μL of total volume. The *Cry1B::Cry1Aa*
specific primers were forward (5’-GCCCAAGAAGCTGTCAACGC-3’) and reverse (5’- CGATGTGAGAACTGTGAGG-3’) resulting in 816 bp DNA fragment. To confirm the PCR process, 100 ng transformation plasmid, 100 ng wild type Rojolele DNA and nontemplate reaction controls were used. The PCR thermal cycle was: 95 °C (10’) 1 cycle, [95 °C (1’), 60 °C (1’), 72 °C (1’)] 35 cycles; dan 72 °C (10’) 1 cycle. The PCR product was separated by agarose gel electrophoresis 0.8 % (b/v).

2.4. Agronomic and yield component observation

Seeds taken from one panicle were sown in seed trays. After 21 days, 10 best grown plants were transferred into a cement container (4 x 3.2 x 1) m³ in biosafety containment with 50 cm space amongst lines and 25 cm between plants of the same line. The plants were fertilized with urea (64 gram), TSP (64 gram) and KCl (51.2 gram) 8 days after transplanting and with urea (128 gram), TSP (64 gram) and KCl (44.8 gram) 33 days after transplanting. At 13 weeks after transplanting the plants were fertilized for the last time with 64 gram of urea. Agronomic characteristics being observed were length and width of leaf taken from the first leaf after the flag leaf, stem diameter, flowering and grain maturity time. On 14 weeks after sowing, measurement was taken on plants height from the ground until the tip of the highest leaf. Tiller number and productive tiller number were also counted. Yield component observed including panicle number, panicle length, filled grains, empty grains, total grains and weight of 1000 grains. Data was analyzed using analysis of variance (ANOVA) and significance was evaluated by Duncan Multiple Range Test (DMRT) at 5 % significance level.

3. Results and discussion

3.1. Confirmation of Cry 1B::Cry 1Aa gene fusion in the T8 generation

Inheritance stability of Cry 1B::Cry 1Aa gene fusion has been proved to be stable until T7 generation [9]. This research used the next generation of the transgenic lines (T8 generation) and PCR analysis was done on all the plants tested using Cry 1B::Cry 1Aa specific primers to confirm the Cry 1B::Cry 1Aa gene fusion in the plants genome. The results showed that all the plants tested were transgenic proved by the presence of 816 bp DNA band which was the resulted size of the Cry 1B::Cry 1Aa PCR amplification (figure 1).

3.2. Agronomic observation

Observation on plants height 14 weeks after sowing showed that all transgenic lines were shorter than the Rojolele control plants. Average height of Rojolele control plants was 175.18 cm while the transgenic lines were no higher than 157 cm. Among the transgenic lines, W3 was the highest with average height of 157 cm and Y7 was the shortest with average height of 141.56 cm (figure 2).

In general, transgenic lines had wider leaf but shorter in length compared to Rojolele control plants. Exception was for U10 which had the same leaf width and W3 which had the same leaf length with Rojolele. Stem diameter of all transgenic lines were also smaller than Rojolele control plants. Among the transgenic lines, W3 had the largest stem diameter.

In terms of tiller numbers, W3 and X22 had the same number as Rojolele (19 tillers) while the other transgenic lines were less in tiller numbers ranging from 13 to 16 tillers per plant. Productive tiller numbers of the transgenic lines were ranging from 8 to 13 productive tillers per plant, less than Rojolele which had 15 productive tillers. However, the Q20 had the most productive tillers among transgenic lines (13 productive tillers) closest to Rojolele. The U10 was recorded as the line with the least in tillers and productive tiller numbers of all the lines tested.

Flowering time was counted from the day of sowing until the day when 50 % of flowers had emerged. The W3 and X22 lines had similarity with Rojolele in flowering time which was around 116 days after sowing (das). Observation on the other transgenic lines showed a variation of flowering time ranging
Figure 1. All samples proved to be transgenic by the presence of 816 bp DNA bands. M: 200 pb marker, P: pC1300::Cry1B::Cry1Aa plasmid, K+: positive plant control, K-: negative plant control, R: nontemplate control.

Figure 2. Transgenic rice plants compared to Rojolele control plants at 14 weeks after sowing. A: Y7, B: P8, C: U10, D: W3, E: Q20, F: X22, G: Rojolele from 120 to 127 days after sowing (das) with the U10 being the line with the longest time to flower (127 das). Grain maturity time was recorded on the day when the first panicle in each plant had fully ripened. Grain maturity time of Rojolele was 154 das while all transgenic lines took longer time which ranging from 157 to 170 das. Among transgenic lines, X22 was the first line to ripe (157 das) while U10 took the longest time which was 170 das (table 1).

Transformation process could change agronomic characteristics of the generated transgenic rice lines such as plant height, tiller numbers, flowering time or grain maturity time as reported in transgenic rice lines harboring OsDREB1A gene conferring drought tolerance [10]. This was also occurred in transgenic rice lines harboring Cry1B::Cry1Aa conferring resistance to yellow stem borer. The growth parameters such as the plant height, leaf length and width, stem diameter, tiller and productive tiller numbers of the six transgenic lines were generally less than the Rojolele control plants. This could happen because the transgene might be inserted in a region of growth regulation gene that affects the plants growth [11].

3.3. Yield component
Panicle numbers and panicle length of the six transgenic rice lines were all less than the Rojolele control plants. The Q20 had the most panicle numbers (13) closest to the Rojolele control plants (15) while
Table 1. Agronomic characteristics of six transgenic lines compared to Rojolele control plants.

| Parameters                  | Line          | P8            | Q20           | U10           | W3            | X22           | Y7            |
|-----------------------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| Plant height (cm)           | 175.18 a      | 143.62 bc     | 146.14 bc     | 150.2 bc      | 157 b         | 144.68 bc     | 141.56 c      |
| Leaf length (cm)            | 73.19 b       | 69.23 bc      | 67.91 c       | 71.82 ab      | 73.93 a       | 67.00 c       | 68.45 c       |
| Leaf width (cm)             | 1.87 b        | 2.02 a        | 2.01 a        | 1.88 b        | 1.96 ab       | 1.98 ab       | 2.027 a       |
| Diameter of stem (cm)       | 2.80 a        | 2.58 ab       | 2.65 ab       | 2.636 ab      | 2.67 ab       | 2.29 c        | 2.55 b        |
| Number of tillers           | 19 a          | 16 ab         | 16 ab         | 13 b          | 18 a          | 18 a          | 13 b          |
| Number of productive tillers| 15 a          | 10 bc         | 13 ab         | 8 c           | 10 bc         | 9 c           | 10 bc         |
| Flowering time (dasb)       | 116 c         | 120 b         | 122 b         | 127 a         | 117 c         | 117 c         | 122 b         |
| Grain maturity time (das)   | 154 e         | 160 cd        | 161 bc        | 170 a         | 158 d         | 157 d         | 163 b         |

Values followed by the same letters in one row are not significantly different at 5% level DMRT. Values are means of 10 replicates.

Table 2. Yield component of six transgenic lines compared to Rojolele control plants.

| Line | Panicle numbers | Panicle length (cm) | Filled grains | Empty grains | Total grains | Weight of 1000 grains (gram) |
|------|-----------------|---------------------|---------------|--------------|--------------|-------------------------------|
| Rojolele | 15 a         | 36.07 a             | 132 a         | 44 c         | 173 a        | 30.49 a                       |
| P8   | 10 bc         | 31.33 cd            | 83 b          | 53 bc        | 136 c        | 27.22 c                       |
| Q20  | 13 ab         | 30.35 d             | 84 b          | 41 c         | 125 c        | 28.94 abc                     |
| U10  | 8 c           | 31.47 cd            | 81 b          | 49 bc        | 130 c        | 30.60 a                       |
| W3   | 10 bc         | 32.36 bc            | 82 b          | 78 a         | 160 ab       | 28.24 abc                     |
| X22  | 9 c           | 33.43 b             | 88 b          | 67 ab        | 155 b        | 28.95 abc                     |
| Y7   | 10 bc         | 30.58 d             | 81 b          | 46 c         | 127 c        | 29.6 ab                       |

Values followed by the same letters in one column are not significantly different at 5% level DMRT. Values are means of 10 replicates.

the U10 had the least panicle numbers (9). However, in terms of panicle length, the Q20 had the shortest panicle while the X22 had the longest panicle of all the transgenic lines. Filled grains of transgenic lines were ranging from 81-88 per plant while the Rojolele control plants had 132 filled grains on average which is significantly higher compared to all the transgenic lines (table 2).

Total grains of the six transgenic lines were all less than the Rojolele control plants (173 grains), ranging from 125 to 160 grains per plant. Despite being the transgenic line with the most panicle
numbers, the Q20 came out to be the least in total grains of all the lines tested (125 grains per plant). This indicates the high number of empty seeds in the Q20 transgenic line. The high number of empty grains can also be seen from the W3 line. Among the transgenic lines, the W3 had the highest number of total grain but almost half of the total grains were empty. As for the weight of 1000 seeds, in general it was similar among all lines tested except for the P8 line which was slightly less in weight (27.22 gram).

4. Conclusion
The transgenic lines W3 had the most similarities to Rojolele control plants followed by X22. Generally, all transgenic lines were less in quantitative characteristics (plant height, leaf width, leaf length, stem diameter) and yield component (panicle numbers, panicle length, filled grains). Delayed flowering and grain maturity time were also recorded in the transgenic lines harboring Cry1B::Cry1Aa gene fusion.

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