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AGROBACTERIUM-MEDIATED TRANSFORMATION OF JAVANICA RICE PLANTS WITH A *CRY1B* GENE UNDER THE CONTROL OF WOUND-INDUCIBLE GENE PROMOTER

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ABSTRACT

A *cry1B* synthetic gene of *Bacillus thuringiensis* has been used for the transformation of the javanica rice plants cv. Rojolele to confer resistance to an important pest, yellow stem borer (*Scirpophaga incertulas*). Embryogenic callus were co-cultivated with the EHA105 strain of *Agrobacterium tumefaciens* harbouring binary vector pCAMBIA 1301 containing *cry1B* gene under the control of wound inducible gene promoter (*mpi*), hygromycin resistance gene (*hpt*) as a selectable marker and intron-containing β-glucuronidase (*gus-intron*) gene as a reporter gene driven by CaMV35S promoter. Previously, our histochemical assay and PCR analysis had proved the integration of *cry1B* gene into the genome of rice plants at first generation. However, the existence of the gene should remain stable throughout generation. In this study, the presence of the *cry1B* transgene in rice transgenic plants at second generation was confirmed by Polymerase Chain Reaction (PCR). Insertion of the *cry1B* gene in the genome of PCR positive plants was verified by Southern blot analysis and showed that integration of *cry1B* into the genomic DNA of javanica rice plants cv. Rojolele. An effective resistance of transgenic plants against stem borer was verified in bioassays.

Keywords: *Agrobacterium*, *cry1B* gene, wound inducible gene promoter, yellow stem borer

INTRODUCTION

Transgenic rice has been developed as a means for controlling disease, weed and insect problems. Until now, more than 30 genes have been transferred to japonica and indica varieties of rice. Within the group of insect resistance genes is *Bt cry*. *Bt* (*Bacillus thuringiensis*) is a gram-positive soil bacterium that express bio-activity against a wide range of insects. The various *Bt* strains produce several toxins, each of which shows a rather narrow host range (Frutos *et al*., 2000). The *Bt* transgenic rice plants transformed with the different *cry* genes are protected against lepidopteran, dipteran, and/or coleopteran insects, among which are economically important pests of rice such as the stripped stem borer (*Chilo suppressalis*), leaffolders (*Cnaphalocrocis medialis* and *Marasmia patnalis*) and the yellow stem borer (*Scirpophaga incertulas*) (Marfä *et al*., 2002).

The yellow stem borer (YSB) is one of the major constraints affecting rice production in Indonesia, which causes yield losses up to 25% (Biro Pusat Statistik, 1996). The plants attacked by yellow stem borer in the field exhibit different symptoms depending on their developmental stage. During vegetative stage, the larvae damage the growing tillers and showing the symptoms called deadhearts, while during reproductive stage, the damage produced by the growing larvae blocks the nutrients transport from the stem to the grain resulting in the grains empty of the panicles as a means of decreasing the rice yields, and this symptoms called whitehead. Conventional rice breeding for yellow stem borer resistance has been proved to be difficult since no gene for host resistance has been found (Bennett *et al*., 1997). An alternative is the development of engineered rice cultivars adapted to local growth conditions and consumer requirements that harbor one or several *endo*-toxin genes from the soil bacterium *Bt* which

encodes insecticidal proteins against stem borer (Breitler *et al.*, 2000). To address this question, the *Bt* transgenic rice plants cv. Rojolele transformed with the *cry1B* gene under the control of wound-inducible maize proteinase inhibitor gene (*mpi*) promoter has been obtained.

The *mpi* gene encodes a maize proteinase inhibitor (MPI) protein whose mRNA accumulates in response to mechanical wounding. Consecutive wounds resulted in higher levels of *mpi* transcripts. The level of accumulation was higher in leaves chewed by larvae than in leaves that had been damaged mechanically (Tamayo *et al.*, 2000). Thus, the use of wound-inducible gene promoter, in combination with the *cry1B* gene could be valuable in regulating the expression of *cry1B* gene, since the expression of this gene is induced by mechanical wounding and expected could prolong the breaking resistance of transgenic plants against stem borer attack.

Histochemical assay at first generation revealed the expression of *gus* gene in leaf tissue of transgenic plants. The presence of the *cry1B* transgene in the putative transformants was confirmed by Polymerase Chain Reaction (PCR). However, the existence of the gene of interest should remain stable throughout generation.

In this paper, we report the stability of the existence *cry1B* gene in the Rojolele transgenic plants at second generation (T1) using PCR and Southern blot hybridization. Bioassays of transgenic plants against yellow stem borer, was also carried out.

Material and Methods

Plant material

Trangenic rice cv. Rojolele harbouring *cry1B* gene under wound inducible gene promoter (*mpi*) at the second generation (T1). The *cry1B* gene under the control of the *mpi* promoter was cloned in binary vector pCAMBIA 1301 containing hygromycin resistance gene (*hpt*) as a selectable marker and intron-containing β-glucuronidase (*gus-intron*) gene as a reporter gene driven by CaMV 35S promoter (Fig. 1). The fusion of promoter *mpi* - *cry1B* gene construct was kindly provided by Dr. Guiderdoni, (CIRAD, France).

Analysis of transgenic plants

PCR

Standard PCR was used to check whether the *cry1B* gene sequence were present in transgenic plants (Zheng *et al.*, 2000). The target for the PCR amplifications is *cry1B* gene with 1.9 kb in length. Fresh leaf materials from putative transgenic rice were collected from the greenhouse. Genomic DNA was extracted from leaves as described by van Heusden *et al.* (2000). Successful PCR was performed using specific primers for *cry1B* gene (forward: 5-GCTGTGTCC AACCACTCCGC-3' and reverse 5- GTACCGAATTGGGCTGCAGG -3). The reactions were carried out in a Perkin Elmer Thermocycler. PCR was performed in a reaction

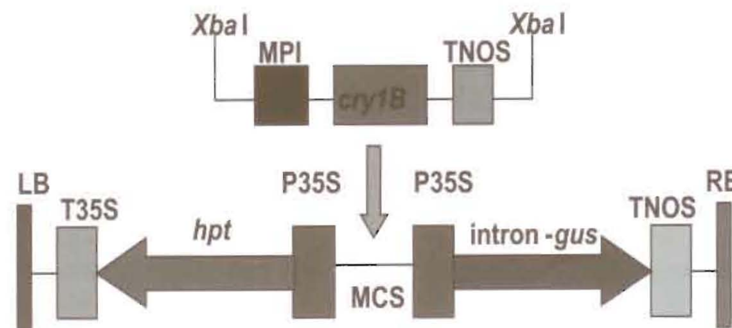

Figure 1. Schematic representation of the T-DNA region of the binary vector pCAMBIA 1301. Abbreviations: RB: right border; LB: left border; MPI: maize proteinase inhibitor gene promoter; P35S and T35S: CaMV35S promoter and terminator;

mixture containing about 40 ng plant genomic DNA, 0.05 mM dNTPs, 2.5 ng/μl of each primer and 0.05 U/l *Taq* polymerase. PCR analysis was carried out under standard condition with 1 min denaturation at 95°C, 1 min annealing at 62°C , 1 min extension at 72°C for 40 cycles. After PCR, the DNA was loaded on a 1.2% agarose gel with 0.5X TBE at 100 volt for running time about 1 hour.

Southern hybridization

Total genomic DNA of transgenic rice was isolated from frozen leaf tissue collected from the greenhouse using a method described by Doyle and Doyle (1989). From each sample, 5 ìg of DNA was digested with *Hin*dIII and DNA fragments were separated on a 0.8% agarose gel at 25 V overnight (Sambrook *et al.*, 1989). DNA was transferred onto nylon membranes (Hybond N^+, Amersham). The 1.9 kb of the *cry1B* coding sequence was used as probe.

Bioassays Bioassays were carried out in the biosafety containment. Four transgenic lines and control plants were subjected to infestation with two yellow stem borer larvae per tiller in each pot. Plants to be tested were put in a plastic cylinder to avoid any cross-larval contamination. Observation were done in 2 and 4 weeks after infestation. Score was attributed to the plants following the Standard Evaluation System. The number of total and damaged tillers per plant was recorded to evaluate the percentage of damage plants produced by larvae following formula below:

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

Percentage of damaged plants was converted to D value using the formula:

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

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%; 7 indicated 31-60% damaged tillers and 9 indicated more than 60% damaged tillers. The plants were categorized as resistant if the D values are in scales of 0, 1, 3, and as susceptible if the D values are in scales of 5, 7 and 9 (Heindrich *et al.*,1985).

Results and Discussion

PCR analysis

Thirty samples from each line (6 lines) were analyzed by PCR to check whether the *cry1B* gene sequences were present. Genomic DNA from transgenic plants at second generation was amplified with the *cry1B* gene primers. The *cry1B* gene was present in all transformed plants from 6 lines at second generation with showing PCR product of 1.9 kb (Fig. 2). However, only 3 lines followed the Mendelian segregation with

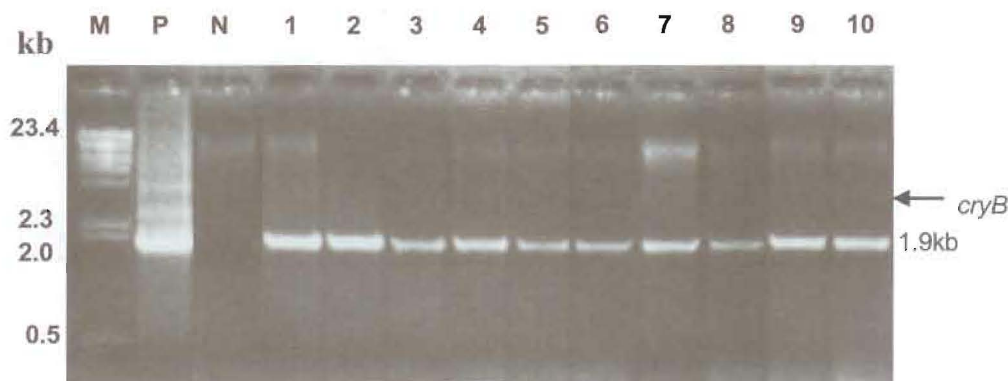

Figure 2. PCR analysis of the transformants for the presence of the *cry1B* transgene. DNA was amplified with *cry1B* primers. Lane M: λ DNA digested with *Hin*dIII; Lane P: plasmid pCAMBIA 1301 as positive control; Lane N: untransformed plant as negative control; Lane 1-10: individual transgenic plants.

3:1 ratio for the presence of *cry1B* gene (not shown results).

Southern hybridization

The successful incorporation of the transgene was further proven by the genomic Southern blot hybridization. To determine the T-DNA integration and the number of T-DNA copy in the plant genome, Southern hybridization was carried out. DNA was extracted from young leaf tissue of individual transgenic plants, digested with *Hin*dIII and hybridized with probe from the PCR product of the *cry1B* gene (Fig. 3) and showed that the integration of *cry1B* into the genomic DNA of aromatic Javanica rice plants cultivar Rojolele had taken place. Three individual transgenic plants isolated from one line (lane 2-4) had different banding patterns. This finding suggested that T-DNA integration could take place in different cells within one callus line.

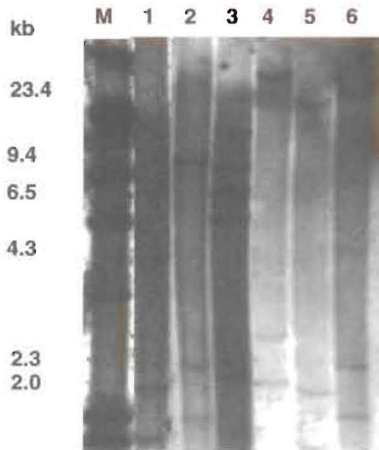

Figure 3. Southern hybridisation of individual transformants. DNA from transformants which were PCR-positive was digested with *Hin*dIII and allowed to hybridize to a probe. DNA from a PCR amplification using *cry1B* primers was used as a probe. Lane M: λ DNA digested with*Hin*dIII; Lane 2-4: plants originated from one line; lane1,5 and 6: plants from three other lines.

Bioassay

To evaluate the damaged plants caused by yellow stem borer larvae, the number of damaged tillers on each pot was observed. The frequency of damaged tillers in four transgenic lines at vegetative stage was 4.99%, 5%, 3% and 5%, while the percentage of damaged tillers in non-transgenic plants was 73.05%. This number was converted following the Standard Evaluation System to determine the score of infested plants. The results showed that the score of the four transgenic and one non-transgenic plants was 1 and 9, respectively. The phenotypes of the transgenic and control plants resulted from bioassay could be seen in Figure 4. At vegetative stage, the young leaves of control plants showed brown color and were very easy to pull out, while

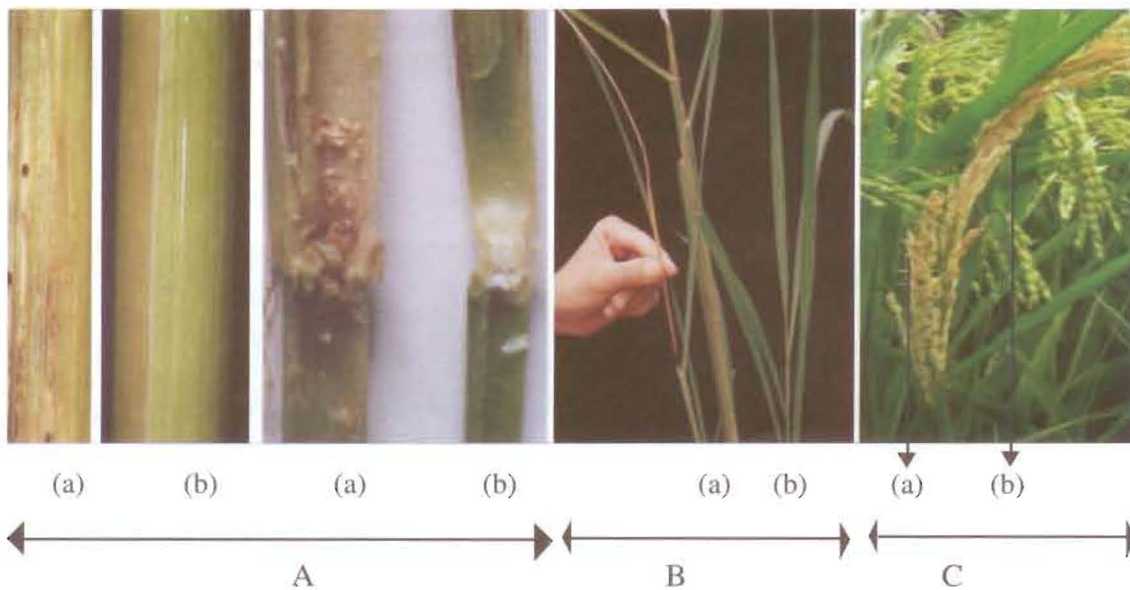

Figure 4. Comparative damage observed on (a) control plants and (b) transgenic plants; (A) on stem, (B) on vegetative stage and (C) on generative stage (C).

in the transgenic plants the young leaves were still green and healthy. At generative stage, the panicles of control plants showed empty no grain inside, while in the transgenic plants the panicles contain grains. In both cases, however, the panicles showed brown color.

Conclusion

The presence of *cry1B* gene under the control of wound- inducible *mpi* gene promoter has proved to be stable and inherited at second generation of transgenic rice cv. Rojolele. Comparative assessment of the resistance against the yellow stem borer of transgenic lines and control plants at vegetative stage showed the percentage of damaged tillers of the transgenic lines was lower compared to the control plants.

On generative stage, the damage resulting in the grains empty of the panicles of non-transgenic plants but not on transgenic plants. The empty panicles can cause decreasing the rice yields.

Acknowledment

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