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Production of transgenic rice plants resistant to yellow stem borer and herbicide in two Vietnamese varieties via *Agrobacterium tumefaciens*

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ABSTRACT

Embryogenic calli cultured on MS medium for about 30 days of two Vietnamese rice varieties Nang Huong Cho Dao and Mot Bui, were co-cultivated with Agrobacterium tumefaciens LBA 4404 containing hybrid Bt gene [cryIA(b)-cryIB] for insect resistance and selectable marker gene (bar) for herbicide resistance. Subsequently, co-cultivated calli were washed thoroughly to remove bacteria and cultured on medium with phosphinothricin (PPT) 3 mg/l for selection. After 4 rounds of selection (15 days/round), PPT-resistant (PPT^R) calli were cultured on regeneration medium with or without PPT. The regenerated plants were tested initially for PPT resistance by subculture into rooting medium with PPT 3 mg/l. The PPT^R plants were transferred to Yoshida solution and soil pots.

The presence and expression of the Bt and bar genes were confirmed by PCR, quick ELISA, phosphinothricin acetyltransferase (PAT) assay, Southern blot and Western blot analyses. Insect bioassay with neonate larvae showed an enhancement of resistance against the yellow stem borer Scirpophaga incertulas Walker. In greenhouse, spraying transgenic plants expressed PAT enzyme with commercial herbicide BASTA™ (1 % v/v) showed a full resistance. Leaf tip assay, leaf paint assay for PPT resistance were also carried out with positive results.

Key words: Nang Huong Cho Dao, Mot Bui, *Agrobacterium*-mediated transformation, phosphinothricin (PPT), phosphinothricin acetyltransferase (PAT), transgenic plants, yellow stem borer resistance, herbicide resistance.

INTRODUCTION

The most destructive insects for rice are the lepidopteran stem borer and the rice leaf folder which cause annual losses of 10 million tons. Occasional outbreaks can destroy between 60 and 95 % of the crop [Pathak et al. 1994]. Engineering plants with *Bt* gene was one of the early objectives of plant biotechnology. Crops expressing *Bt* genes result in significant saving in terms of cost, time and labor as compared to conventional crop protection utilizing chemicals [Peferoen, 1997]. Herbicide-resistance rice plants are likely to be one of the first practical applications of rice genetic engineering [Datta et al. 1992]. Breeding rice varieties with built-in resistance would be the best approach to manage the insect pest and weed. Conventional resistance breeding has not been successful, as resistant donors for the pest and weed control are not available in the rice gene pool.

Nang Huong Cho Dao (NHCD) and Mot Bui (MB) are two Vietnamese traditional rice varieties widely cultivated in Cuu Long Delta, NHCD is a famous aromatic rice variety in Vietnam. Their good cooking quality and high consuming market in Vietnam have been noticed but the common feature of these varieties is highly susceptible to lepidopteran insects in monsoon season. We obtained the transgenic NHCD containing *hph* and *gusA* genes by biolistic method (Ho et al. 1995). In this report, we present the results on transfer of *Bt* gene along with *bar* gene into these varieties via *Agrobacterium* in order to improve the agronomic traits.

This is the first report on two Vietnamese local rice varieties engineered with hybrid *Bt* gene conferring insect resistance and *bar* gene conferring herbicide resistance.

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MATERIALS AND METHODS

Rice varieties

Experiments were carried out with two widely grown varieties in South Vietnam: Nang Huong Cho Dao and Mot Bui (IRGC 79075) (kindly provided by International Rice Research Insitute's Gene Bank).

Agrobacterium strain

Agrobacterium tumefaciens strain LBA 4404 contains plasmid pBIN-BAR-UBI-IB-AB (Lab for Tissue Culture and Genetic Engineering, International Rice Research Institute, Philippines) harbouring fused insect resistance gene *crylA(b)-crylB* and herbicide resistance gene *bar*.

Establishment and maintenance of embryogenic calli

Mature seed-derived embryos were used for initiation of callus by culture on MS medium (1962) with casein hydrolysate 400 mg/l. After about one month, globular compact calli were formed on the surfaces of scutella. Embryogenic calli were subcultured at 15-20 day intervals on the same MS medium.

Preparation of *Agrobacterium* culture. Infection, co-cultivation with *Agrobacterium* and washing bacteria

Based on the protocol of Datta et al. (1997), first, preculture was done using AAM medium with the appropriate selective agents and shaking. The culture was transferred to AB liquid medium (Chilton et al. 1974) containing the same concentration of antibiotics and shaken vigorously for two days. The bacterial culture was centrifuged and the medium was removed. The bacteria were resuspended in $MgSO_4$ solution, then bacterial suspension was centrifuged and the solution was discarded to remove rid of the antibiotics. The bacteria were resuspended in MS infiltration medium with acetosyringone (200 mM) how to get the final approximate OD ~ 2. The embryogenic calli were submerged in the infiltration medium in a petri dish. The petri dish was placed in a vacuum dessicator for vacuum infiltration. The calli were blotted on a filter paper, transferred to the MS cocultivation medium and kept in the dark at 28°C. After 3 day cocultivation, cocultivated calli were washed several times with sterile water containing cefotaxime 250 mg / l.

Selection

After removing the excess water using the blotting paper, the washed calli were transferred to MS selection medium containing phosphinothricin (PPT) 3 mg/l, cefotaxime 500 mg/l and kept in the dark. Calli were subcultured at 15-day intervals. Calli were passed through selection for 4 selection rounds.

Regeneration of plants

Survived and well-grown calli were transferred to MS regeneration medium containing kinetin 2 mg/l, NAA 1 mg/l, cefotaxime 500 mg/l with or without PPT 3 mg/l (under a 16h photoperiod of 3000 lux intensity at 28°C).

Rooting stage

Regenerated shoots (about 3 cm) were rooted on MS medium containing NAA 0.1 mg/l, PPT 3 mg/l, cefotaxime 250 mg/l.

In vitro leaf assay for PPT resistance

Leaf tips of the in vitro regenerated plants and control were cultured on MS medium with PPT 3 mg/l with the cut surface embedded in solid medium. Noticeable symtomps of either yellowing or bleaching were observed after five days on the medium.

Plant DNA extraction

DNA extraction from rice leaves of PPT-resistant (PPT^R) plants and control for PCR and Southern blot analyses were carried out according to protocol by Datta et al. (1997).

Bacterial plasmid DNA extraction

As described in the kit CONCERT High Purity Plasmid Miniprep System (GIBCO BRL, 2000).

PCR analysis

The presence of the *bar* gene in the PPT^R plants was assessed by PCR analysis. The sequences of the primers are as followed: BAR 1: 5'-GTC TGC ACC ATC GTC AAC C - 3' and BAR 2: 5'- GAA GTC CAG CTG CCA GAA AC - 3'. PCR condition for amplification: 94°C/5', (94°C/30'', 50°C/45'', 72°C/30''): 36 cycles and a 72°C/5' final extension. The expected fragment size is 0.45 kb

Analysis of Phosphinothricin Acetyltransferase (PAT) activity

Based on the method of De Block et al. (1987), the putative transgenic plants and control were analysed for PAT activity by thin layer chromatography in which ^{14}C-labelled acetylated PPT was detected.

Quick ELISA

We used the specific kit (Envirologix, USA) to detect the CryIA(b) and/or CryIA(c) protein: "CryIA(b)/CryIA(c) Lateral Flow Quickstix^{TM} Strip Kit" (Cat # AS 003 BG).

***Ex vitro* leaf assay for PPT resistance**

Based on the method of Wang et al. (1997), leaf tips of the *ex vitro* putative transgenic plants and control were put on MS salts (lack of vitamins and sucrose) plus BA 0.5 mg/l, PPT 3 mg/l with the cut surface embedded in solid medium. Symtomps of either yellowing or bleaching were observed after five days on the medium.

Leaf paint assay for PPT resistance

Leaf blades of the *ex vitro* putative transgenic plants and control were painted with PPT 1% (w/v). Symtomp was observed 5 days after painting.

Southern blot analysis

Total DNA was isolated from leaf tissue of PPT^R plants and control plant. Genomic DNA (10 µg) was digested with *Hind* III (for *crylA(b)-crylB* gene) or *Sma* I (for *bar* gene). Following electrophoresis through a 1% agarose gel, DNA was transferred to Hybond-N^+ Nylon membranes (Southern 1975). Hybridization with the probe was done according to the instruction of the manufacturer (Amersham). The radioactive probes were prepared by the random primer method using (^{32}P)-dCTP. The probe of the *crylA(b)-crylB* gene consisted of the coding region [*Bam* HI-*Bst*E II fragment -*crylA(b)* gene, of plasmid pCIBBt I, 1.8 kb], the expected fragment size: 6.16 kb. The probe of the *bar* gene consisted of the coding region of the *bar* gene (*Sac* I-*Bam* HI fragment of pUBA, 0.8 kb), the expected fragment size: 0.63 kb.

Herbicide application

Plants (PPT^R and control), 6-8 weeks in the transgenic greenhouse, were sprayed with a 1 % (v/v) aqueous solution of the formulated BASTA containing 20 % D,L-PPT (Hoechst) until all leaf surfaces were wet. Data were scored after 7 days.

Western blot analysis

Protein was isolated from fresh leaves of PPT^R and control plants (Datta et al. 1990) and subjected to Western blotting as described by Koziel et al. (1993) and Steward et al. (1996).

Insect bioassay

Plants positive in Southern or Western blot analysis were tested for resistance against the yellow stem borer (YSB) *Scirpophaga incertulas*. A single stem cutting (about 8 cm) with at least one node from a plant at the booting stage was placed on a moistened filter paper in a petri dish (100 x 20 mm). Six neonate larvae of YSB were placed on the stem, and the petri dish was sealed using masking tape. Incubation was performed for 96 h at 25°C. After 96 h, the number of larvae was determined. Mortality rate was expressed as the proportion of dead larvae to applied larvae (%). Missing larvae were grouped within the mortality category. Similar infestation was also carried out for control plant. Each treatment was replicated three times.

RESULTS AND DISCUSSION

Prior to transformation, we examined the effect of PPT on the formation and development of callus of two Vietnamese rice varieties. Callus formation was greatly inhibited when PPT 1-2 mg/l was added into medium and the callus formation was completely inhibited at a concentration of PPT 3-4 mg/l. From these results, we used concentration 3 mg/l for selecting callus and testing the rooting ability of the regenerated plants.

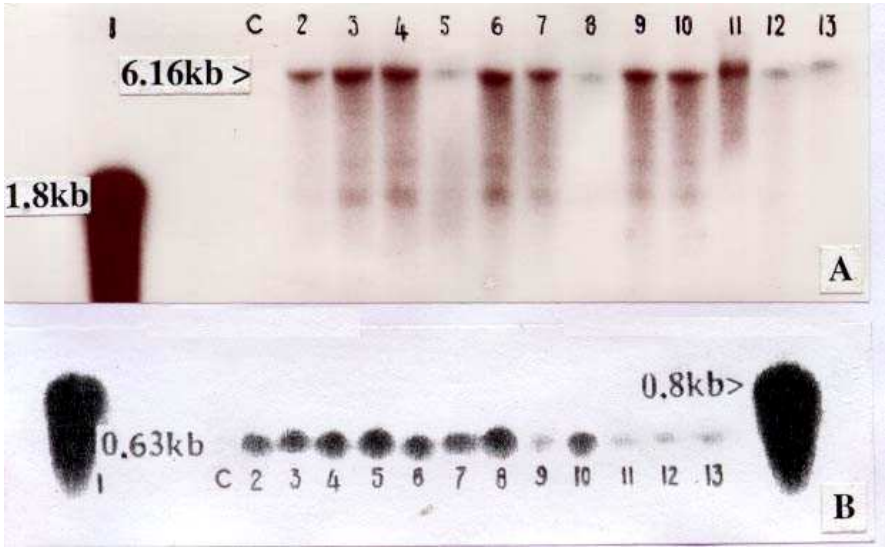
After 3 days of cocultivation, calli were washed off the Agrobacteria thoroughly to prevent further growth of bacteria and cultured on the medium with PPT 3 mg/l. Three-four weeks later, fresh white cell clusters were formed on the cocultivated calli and then they were subcultured onto fresh medium containing the same concentration of PPT for further selection. Calli were subjected to selection for four rounds with about 15 days/round. By this procedure, we obtained 12 putative transgenics NHCD, 5 putative transgenics MB survived on the rooting medium with PPT 3 mg/l. The transformation frequency of NHCD, MB was 2.1 %, ~ 1 %, respectively.

The quick ELISA showed two lines: test line and control line in the case of putative transgenics. These results should be interpreted as positive for *Bt* protein [CryIA(b)]. On the other hand, there was only control line in the control plant.

Southern blot analyses of NHCD and MB showed all transgenics had 6.16 kb -[Promoter-Coding sequence-Terminator] of the hybrid *Bt* gene and 0.63 kb coding sequence of the *bar* gene. In some transgenic lines of NHCD, two additional bands in *Bt* hybridization pattern indicated that recombination and rearrangement of the transgene had occurred. In transgenic MB, there was only a *Bt*-expected band. In each case of NHCD and MB, only *bar* -expected

band was obtained. The expression of hybrid *Bt* gene *crylA(b)-crylB* of transgenic plants was confirmed by Western blot analysis. The result showed the expected 65 kDa protein. In addition to the expected band, the additional band was also obtained (between 46 and 30 kDa). This could be due to post-transcriptional and post-translational changes of the gene. Through insect bioassay, no larvae were found alive on stem cuttings of transgenic plants. It was clear that these transgenics produced a sufficient amount of *Bt* protein to kill larvae. Bioassay result showed the correlation with the Southern and Western blot analyses.

We confirmed also the expression of the *bar* gene by another qualitative analysis. Tests for PPT resistance using leaf tips of the *in vitro* and *ex vitro* plants showed, after 2-3 days on the PPT medium, yellowing of the apical area and the leaf region embedded in the medium of non-transgenic leaf tips. This yellowing proceeded downwards (from leaf tip) and towards (from leaf base) until the whole tissue of the explants was uniformly bleached after 5 days. On the other hand, transgenic leaf sections remained completely healthy and green after 5 days and visibly green for up to 10 days. The assay was very effective at discriminating between *bar*-expressing and non-transgenic plants. There was linked correlation between leaf tip assay and leaf paint assay for PPT resistance. Five days after painting with PPT, there was pronounced distinguishable and widespread symptom of bleaching in non-transgenic plant. Transgenic plants remained green and healthy during that time. These PPT tests have proven very useful for initial screening of primary transgenic plants. It is therefore particularly suitable for screening large segregating populations of transgenic plants. PCR analysis of *bar* gene showed the 0.45 kb band representing the amplified fragment. The experiments on PAT enzyme showed the positive results with detected ^{14}C-labelled acetylated PPT.



A. Southern blot analysis of *crylA(b)-crylB* gene in transgenic rice plants Nang Huong Cho Dao and Mot Bui.

1: Positive control 1.pkb. C: Control plant
2-11: Transgenic plants Nang Huong Cho Dao (expected band: 6.16kb)
12-13: Transgenic plants Mot Bui (expected band: 6.16 kb)

B: Southern blot analysis of *bar* gene in transgenic rice plants Nang Huong Cho Dao and Mot Bui

1: Positive control 0.8kb. C: Control plant
2-11: Transgenic plants Nang Huong Cho Dao (expected band: 0.63 kb)
12-13: Transgenic plants Mot Bui (expected band: 0.63 kb)

CONCLUSION

By using *Agrobacterium tumefaciens* strain LBA 4404 carrying plasmid pBIN-BAR-UBI-IB-AB harbouring hybrid *Bt* gene *cryIA(b)-cryIB* and *bar* gene, we obtained several transgenic rice lines of NHCD and MB. They were highly resistant to yellow stem borer and herbicide. The presence and expression of the *Bt* and *bar* genes were confirmed by qualitative and molecular analyses. The obtained transgenic lines could be useful for rice breeding in Vietnam.

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SUMMARY IN VIETNAMESE

Tạo cây lúa chuyển gen kháng sâu đục thân và kháng thuốc trừ cỏ ở hai giống lúa Việt nam nhờ vi khuẩn *Agrobacterium tumefaciens*.

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Mô sẹo có khả năng sinh phôi, qua nuôi cấy trên môi trường MS khoảng 30 ngày, được nuôi chung với vi khuẩn Argobacterium tumefaciens LBA 4404 mang gen kháng sâu Bt phối hợp cryIA(b)-cryIB và gen kháng thuốc trừ cỏ bar. Tiếp theo, mô sẹo được rửa vi khuẩn và được nuôi cấy trên môi trường chọn lọc có PPT 3 mg/l. Sau 4 chu kỳ chọn lọc (15/chu kỳ), các cụm mô sẹo kháng PPT được đem tái sinh trên môi trường có hoặc không có PPT3 mg/l. Các cây tái sinh được thử tính kháng ban đầu đối với PPT bằng cách nuôi cấy chúng trên môi trường tạo rễ có PPT 3mg/ml. Các cây có sinh trưởng và tạo rễ tốt được đem trồng theo thứ tự ra dung dịch Yoshida và chậu đất.

Sự hiện diện và biểu hiện của gen Bt và gen bar được xác định bằng phân tích PCR, ELISA, thử nghiệm enzym PAT, thí nghiệm lai Southern và Western blot. Thí nghiệm đánh giá tính kháng sâu dùng sâu tuổi 1 cho thấy các cây chuyển gen có khả năng kháng rất cao đối với sâu đục thân Scirpophaga incertulas Walker. Nhận thấy các cây chuyển gen đồng thời cũng kháng thuốc trừ cỏ thương mại BASTA {nồng độ 1% (v/v)]. Ngoài ra, các thí nghiệm dùng đầu lá và quyét dung dịch PPT trên lá để thử tính kháng PPT cũng đã được thực hiện với kết quả dương tính.