Optimization of the *Cry2Aa* Gene and Development of Insect-resistant and Herbicide-tolerant Photoperiod-sensitive Genic Male Sterile Rice

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Abstract

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In this study, an optimized *Cry2Aa* gene was obtained after codon optimization based on the preferred codons in rice. The novel fusion gene *Cry2Aa#* was designed by adding the sequence coding the signal peptide of PR1a at the 5' end and the endoplasmic reticulum retention signal peptide KDEL at the 3' end of the optimized *Cry2Aa* gene, respectively. The *Cry2Aa#* and *Bar* genes were transformed into 4008S, a photoperiod-sensitive genic male sterile (PGMS) line in rice, using the *Agrobacterium* transfer method. A total of 65 regenerated plantlets confirmed by PCR were produced, in which eight transgenic lines had single-copy insertions as confirmed by Southern blot analysis. The high variability of *Cry2Aa#* gene expression was observed among independent transgenic lines with single-copy insertion, and the spatiotemporal difference of Cry2Aa protein expression was discovered in each transgenic line. The results showed that the transgenic lines were highly resistant to glufosinate, rice leaf roller and striped stem borer, which not only confirmed the effective optimization of the *Cry2Aa* gene but also produced a useful germplasm for breeding insect-resistant and herbicide-tolerant hybrid rice varieties.

Keywords: *Bar* gene; *Cry2Aa#* gene; herbicide tolerance; insect resistance; PGMS line; rice

The rice leaf rollers (*Cnaphalocrocis medinalis*), striped stem borer (*Chilo suppressalis*), yellow stem borer (*Tryporyza incertulas*) and plant hopper are the major insects endangering rice production. The stem borers are responsible for steady annual damage of 5–10% of the rice crop in Asia (Pathak & Khan 1994). The germplasm resources for breeding rice varieties resistant to stem borers have not been found yet, so the use of *Bt* gene is the only effective way to breed the rice varieties resistant to rice stem borers (Chen *et al.* 2009). *Bt* rice offers the potential to generate benefits of around US$ 4 billion annually from an average yield increase of up to 8%, and an 80% decrease in the use of insecticides (James 2009).

The main source of the insecticidal toxins produced in commercial transgenic crops is from the soil bacterium *Bacillus thuringiensis*. At an early stage, the introduction of unmodified *Bt* genes into tobacco (Barton *et al.* 1987; Vacek *et al.* 1987), tomato (Fischhoff *et al.* 1987) and cotton (Perlak *et al.* 1990) resulted in low levels of protein expression. Murray *et al.* (1991) indicated that the AT-rich regions and the codons rarely used in plants resulted in low levels of protein expressions in plant cells. Perlak *et al.* (1991) reported that the expressions of insecticidal proteins in tomato and tobacco plants were increased by using the partially modified and totally synthesized *Cry1A(b)* and *Cry1A(c)* genes; the majority of plants transformed with the partially modified gene had a 10-fold higher level of insecticidal proteins and the plants with the synthesized gene had a 100-fold higher level of insecticidal proteins compared with the wild-type gene. So far, there are many reports on transformed rice with modified *Bt* gene, but most used *Cry1A* gene (Fujimoto *et al.* 1993; Nayak *et al.* 1997; Lee *et al.* 2009; Qi *et al.* 2012).

The studies show that the recombinant proteins can be targeted to specific subcellular sites such as chloroplast and vacuole by signal sequences, and the localization of foreign proteins can enhance the expression levels in transgenic plants (Wong *et al*. 1992; Kim *et al*. 2009). Wandelt *et al*. (1992) and Schouten *et al*. (1996) indicated that the accumulation levels of foreign proteins in the cells were markedly increased by adding a signal peptide sequence at the 5' end and the retention signal peptide sequence KDEL at the 3' end of foreign gene in the transgenic plant cells, respectively.

A number of inbred varieties are usually used as the receptors for developing insect-resistant and/or herbicide-tolerant rice varieties, and there are also some reports on using the restorer line and maintainer line of three-line hybrid rice for transformation (Alam *et al*. 1999; Ramesh *et al*. 2004; Chen *et al*. 2005; Wang *et al*. 2010). But there are few reports on using male sterile lines as transformation materials (Xiao 2009). In this study, the original *Cry2Aa* gene was optimized, the modified *Cry2Aa#* gene along with *Bar* gene as a selection maker was transferred into 4008S via *Agrobacterium*-mediated method. Finally, the function of the modified *Cry2Aa#* gene was verified.

MATERIAL AND METHODS

Optimization and synthesis of *Cry2Aa#* gene. The optimization of wild-type *Cry2Aa* gene (Genebank No. AY496458) was carried out on the premise of keeping the amino acid sequence of the Cry2Aa protein constant. First, the codons of *Cry2Aa* gene were optimized based on the preferred codons in rice, and the AT-rich sequences and potential poly(A) addition signal sequences were eliminated at the same time. Next, four potential intron-exon boundary sequences were eliminated by the replacement of synonymous codon, and the frequencies of codon ACG, TCG, GCG and CCG were maximally decreased to prevent DNA methylation. Then, the secondary structure of mRNA was analysed and nine stable stem-loop structures were eliminated. In order to target the insecticidal protein to the endoplasmic reticulum (ER), the fusion gene *Cry2Aa#* was designed by adding the sequence coding the signal peptide of the tobacco pathogenesis-related proteins PR1a at the 5' end and ER retention signal peptide KDEL at the 3' end of the optimized *Cry2Aa* gene, respectively. The *Cry2Aa#* gene was synthesized after adding the restriction site of *Sma*I (cccggg) at the 5' end and the restriction site of *Sac*I (gagctc) at the 3' end of the optimized *Cry2Aa#* gene, respectively.

Construction of the plant expression vector. The plant expression vector pC3300-Cry2Aa# containing *Cry2Aa#* and *Bar* gene was generated using the backbone of pCAMBIA3300, which is shown in Figure 1.

Transformation of rice. The variety 4008S, a wide compatible *japonica* PGMS line provided by Prof. P.J. Zhang from Rice Research Institute of Anhui Province, was transformed by *Agrobacterium*-mediated method (Hiei *et al.* 1994). Dehulled rice seeds were sterilized and placed on NB medium containing 2 mg/l 2,4-D, 3% sucrose and 0.8% agar for 6–8 days to induce callus at 32°C in the dark, then subcultured in the same conditions for 4 days. The calli were co-cultured for 3 days at 24°C with the *Agrobacterium* EHA105 (OD_{600} = 0.02) that carried the expression vector, then directly transferred to the selection medium (MS basal medium supplemented with 500 mg/l cefotaxime, 400 mg/l carbenicillin, 2.0 mg/l 2,4-D, 500 mg/l proline, 0.8% agar and 6 mg/l glufosinate) for 15 days at 32°C. Subsequently, the surviving calli were transferred to the selection medium containing 8 mg/l glufosinate for another 15 days at 32°C. Afterwards, the surviving calli were regenerated on the MS medium supplemented with 2 mg/l 6-BA, 0.2 mg/l NAA, 3% sucrose, 500 mg/l proline, 500 mg/l glutamine and 0.8% agar. Finally, the regenerated plantlets were transferred to 1/2 MS medium containing 6 mg/l glufosinate for culturing strong plantlets.

PCR and Southern blot. Rice genomic DNA was isolated by the CTAB method (Murray & Thompson 1980). The pair of PCR primers for the *Cry2Aa#* gene was Cry2Aa-F (5'-CAGACAACCCTCAGCCTCAGATG-3') and Cry2Aa-R (5'-GACCGTTGATAGT-

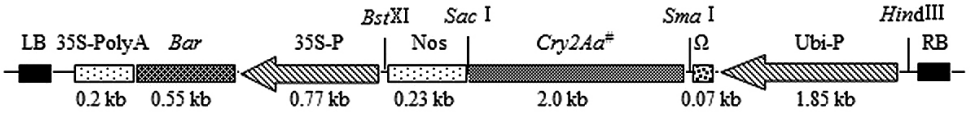

Figure 1. The T-DNA region of plasmid pC3300-Cry2Aa#; the *Cry2Aa#* gene was driven by a maize ubiquitin promoter with Ω enhancer and terminated by nopaline synthase (Nos) terminator, the *Bar* gene used as a selectable marker was driven by CaMV 35S promoter and terminated by CaMV 35S polyA; LB – left border of T-DNA region; RB – right border of T-DNA region

GACCCGAATG-3') that amplified a 496-bp fragment. The pair of PCR primers for the *Bar* gene was Bar-F (5'-CACCATCGTCAACCACTACATCG-3') and Bar-R (5'-TAAATCTCGGTGACGGGCAGGAC-3') that amplified a 485-bp fragment. The PCR reaction for *Cry2Aa#* and *Bar* was run in the same programme: initial denaturation at 95°C for 5 min; then 30 cycles of 94°C for 1 min, 58°C for 45 s and 72°C for 45 s; and final extension at 72°C for 10 min. A 10 µl aliquot from each reaction was used for electrophoresis on 1.0% agarose gel.

Genomic DNA (30 µg per sample) was digested by *Hin*dIII, an enzyme with a single restriction site in T-DNA region, separated on a 1.0% agarose gel and transferred onto the nylon membrane by capillary action. The DIG DNA Labelling and Detection Kit DIGD-110 (LabKit, Shenzhen, China) was used for probe labelling and band detection by Southern blot. The primers for probe labelling were the same as mentioned above.

RT-PCR. Total RNA was isolated from transgenic lines A-2, B-4, G-5 and F-1 of T_1 generation and nontransgenic plants by using Trizol LS reagent (Invitrogen, Carlsbad, USA). The cDNA was synthesized using the Fermentas First Strand cDNA Synthesis Kit (Fermentas, Ottawa, Canada). The primers used for RT-PCR analysis were RCry2Aa-F (5'-ACCGCTCGTTACACGCTTAG-3') and RCry2Aa-R (5'-GTTGACGCCATCGTTGTTTG-3') that amplified a 153-bp fragment. The RT-PCR was performed in the following programme: denaturation at 95°C for 5 min; then 35 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 40 s; and final extension at 72°C for 10 min. A 10 µl aliquot from each reaction was used for electrophoresis on the 1.0% agarose gel.

Quantification of the Cry2Aa protein. Approximately 20 mg fresh leaves, 20 mg stem cuts and 10 mature grains of T_1 transgenic plants were collected separately. The expression of *Cry2Aa#* gene in transgenic lines was quantified by an enzyme-linked immunosorbent assay (ELISA) using the Envirologix Kit APP005 (Envirologix, Portland, USA).

Assay of herbicide tolerance. The marked leaves of T_1 transgenic plants at tillering stage were smeared with 750 mg/l glufosinate to validate the expression of *Bar* gene; the glufosinate resistances of T_2 transgenic plants were determined by spraying the plants with 0.375 g/M^2 of glufosinate at the four-leaf stage. The results were observed and photographed after 7 days.

Bioassay of insect resistance. The adult rice leaf rollers (*Cnaphalocrocis medinalis*) were caught from the rice field of China National Rice Research Institute to lay eggs and hatch larvae in the laboratory. The fresh rice leaves of T_1 transgenic line G-5 and nontransgenic control were harvested at booting stage and cut into 6–8 cm segments. Four cuts of fresh leaves, with both ends wrapped in moistened filter paper and inoculated with 12 first-instar larvae of the rice leaf roller, were placed into a test tube (20 × 2.5 cm) and cultured in a growth chamber at 28°C, 85% relative humidity and 12 h illumination per day. The bioassay of the leaf roller resistance was divided into three groups fed for 2 days, 4 days and 5 days, respectively. Each group included three replicates.

The first-instar larvae of the striped stem borer (*Chilo suppressalis*) were obtained from artificial rearing. The bioassay of the striped stem borer resistance was conducted with three duplicates according to leaf-section bioassay method (Ye *et al*. 2000). The damage to leaf tissues and the larval mortality were observed and photographed after feeding for 7 days.

RESULTS

Optimization of *Cry2Aa#* gene. The design of the optimized *Cry2Aa#* gene minimized the rare codons of rice and the codons susceptible to methylation; eliminated all AT-rich sequences, poly(A) addition signal sequences, intron-exon boundary sequences and stable stem-loop structure in mRNA; realized the orientation and retention of the product by signal peptide. As a result, the GC content of the optimized gene was increased to 57.47% while that of the wild type was 34.81%; 25.87% (492/1902) of nucleotides and 67.51% (428/634) of codons were replaced in the optimized *Cry2Aa* gene.

Molecular identification of transformants. A total of 65 regenerated plantlets confirmed by PCR with *Cry2Aa#* and *Bar* genes were obtained at a transformation frequency of 4.3% by *Agrobacterium*-mediated method. Eight transgenic lines (A-2, B-4, G-5, E-1, F-1, K-1, L-1 and M-2) with single-copy insertion were selected from the 65 transformants of T_1 generation. The Southern blot results of six transgenic lines for the *Cry2Aa#* gene are shown in Figure 2A. The intact transcription of *Cry2Aa#* gene in transgenic lines (A-2, B-4, G-5, K-1 and F-1) was validated by RT-PCR (Figure 2B).

Quantification of Cry2Aa protein. Four transgenic lines (A-2, B-4, G-5 and F-1) with single-copy insertion were selected to analyse the expression levels of Cry2Aa protein. The results showed that the Cry2Aa protein concentrations in the fresh leaves of T_1 transgenic plants at tillering stage (MacLean *et*

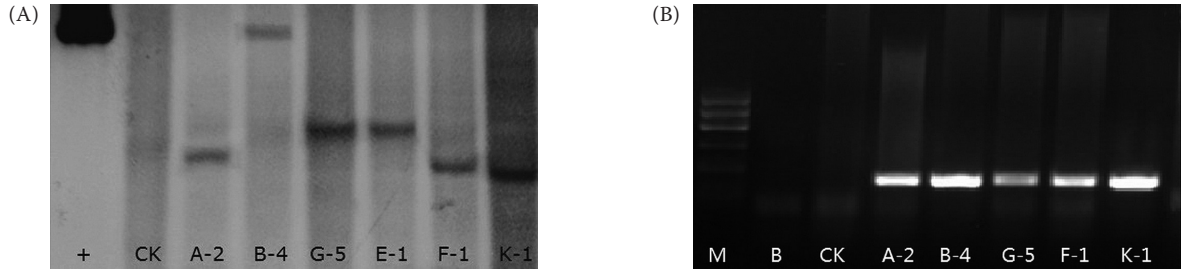

Figure 2. The Southern blot (A) and RT-PCR (B) analysis on *Cry2Aa*# gene in T_1 transgenic rice; + – pC3300-Cry2Aa#; CK – nontransgenic rice plants; M – DNA marker; B – blank control; A-2~K-1 – transgenic line A-2~K-1

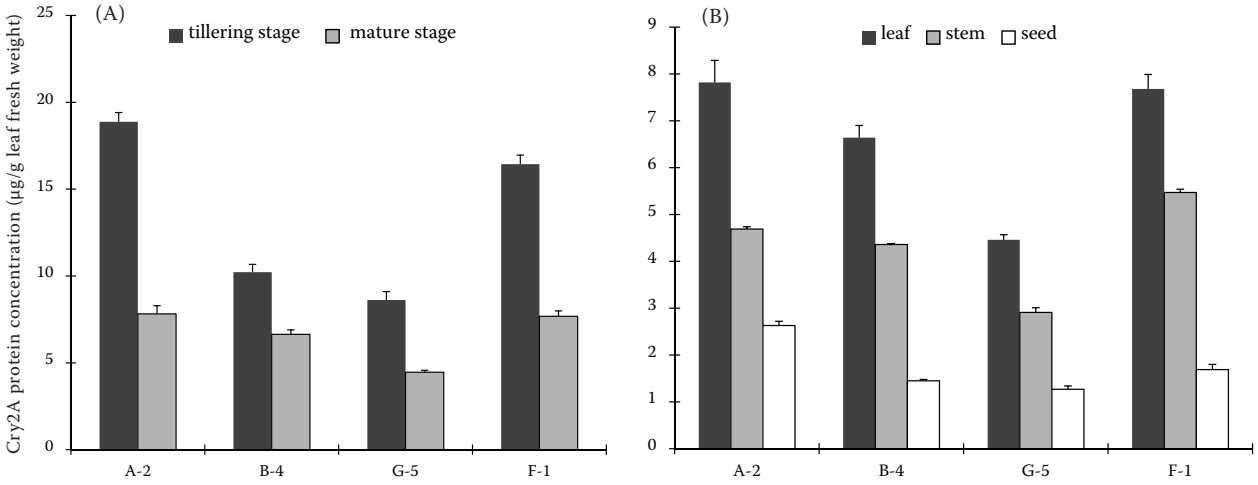

Figure 3. The spatiotemporal expression of Cry2Aa protein in T_1 transgenic rice: (A) the Cry2Aa protein concentration in fresh leaf of T_1 transgenic plants at tillering and mature stage, (B) the Cry2Aa protein concentration among leaf, stem and seed of T_1 transgenic plants at mature stage; the bars represent mean ± SD

al. 2002) ranged from 8.62 to 18.88 μg/g, while they ranged from 4.46 to 7.82 μg/g at mature stage; for each transgenic line, the Cry2Aa protein concentration in the leaves of T_1 transgenic plants at tillering stage was significantly higher than that at mature stage (Figure 3A). Furthermore, the contents of Cry2Aa protein

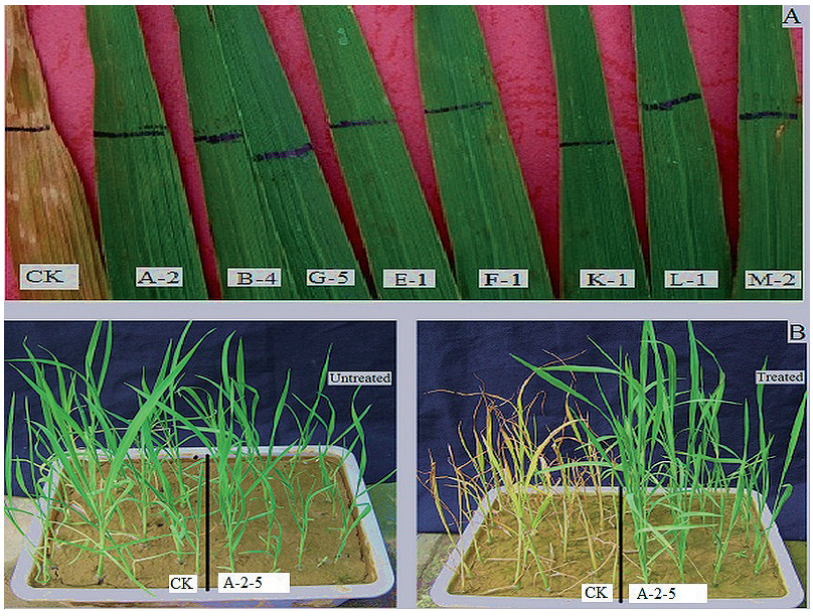

Figure 4. The assessment of glufosinate resistance in transgenic rice: (A) analysis of glufosinate resistance in T_1 generation; CK – negative control; A-2~M-2 – transgenic line A-2~M-2; (B) analysis of glufosinate resistance of T_2 transgenic line A-2; CK – control plants; B2A4008S – transgenic line A-2 of T_2 generation

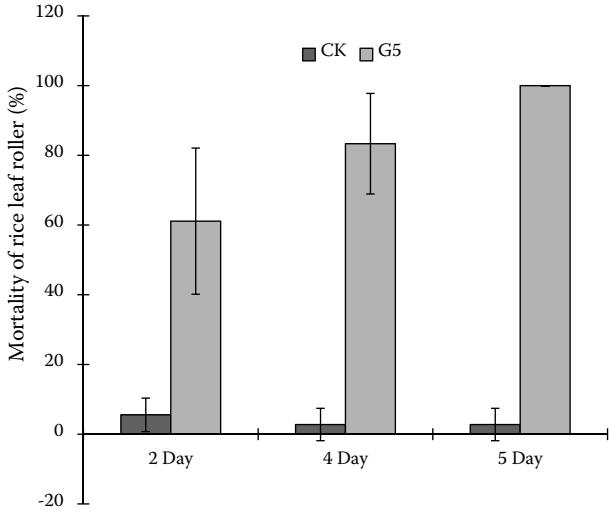

Figure 5. The mortality of rice leaf roller fed on the leaf of transgenic rice for different days; CK – nontransgenic plant; G5 – transgenic line G-5; the bars represent mean ± SD

varied significantly in different tissues at mature stage (MacLean *et al.* 2002) for each line. The contents of Cry2Aa protein were the highest in leaves, followed by those in the stems and seeds (Figure 3B).

Glufosinate resistance. After 7 days, the leaves of the control were bleached seriously while the leaves of all transgenic lines maintained their normal green colour (Figure 4A). The transgenic plants of T_2 generation could still grow normally while the non-transgenic rice plants died (Figure 4B).

Insect resistance. Among the four transgenic lines (A-2, B-4, G-5 and F-1) mentioned above, the Cry2Aa protein content of line G-5 was the lowest, but the plant was still highly resistant to rice leaf roller and striped stem borer (Figures 5 and 6).

After feeding on leaves of transgenic line G-5 for 5 days, all of the rice leaf roller larvae were dead; after feeding for 7 days, all of the striped stem borer larvae were killed.

DISCUSSION

Since Perlak *et al.* (1991) reported that the modification of the coding sequence enhanced the plant expression of *Bt* gene, there have been similar or other approaches reported by other scholars to improve the expression levels of exogenous genes in transgenic plants. Fujimoto *et al.* (1993) reported that the *Cry1A(b)* gene was extensively modified based on the codon usage of rice genes to highly express the Cry1A(b) protein, and the highest expression level of this modified *Cry1A(b)* gene was up to 0.05% of total soluble leaf protein. Chen *et al.* (2005) reported that a novel *Cry2A** gene was optimized based on the plant codon usage and the Cry2A protein concentrations in the fresh leaves of transgenic rice plants ranged from 9.65 to 12.11 μg/g at tillering stage. Tang *et al.* (2006) reported that the *Cry1C* gene was optimized based on the preferred codons in plants, the highest Cry1C protein content in the fresh leaves of transgenic *indica* rice was up to 1.38 μg/g at heading stage. In this study, five approaches of optimization were used to enhance protein expression: (1) minimizing the codons rarely used in rice by preferred codon substitution; (2) maximally decreasing the frequencies of the codons ACG, TCG, GCG and CCG to avoid DNA methylation; (3) eliminating poly(A) addition signal sequences and intron-exon boundary sequences, (4) optimizing the secondary structure of mRNA for improving the efficiency of protein translation; (5) adding orientation and ER retention signal peptide sequences. The results indicated that the Cry2Aa protein concentrations in the fresh leaves of T_1 transgenic plants at tillering stage ranged from 8.62 to 18.88 μg/g; the mean and maximum of Cry2Aa protein concentration were both higher than those of other reports, which indicated that the approaches for the optimization of the *Cry2Aa#* gene were practical and successful.

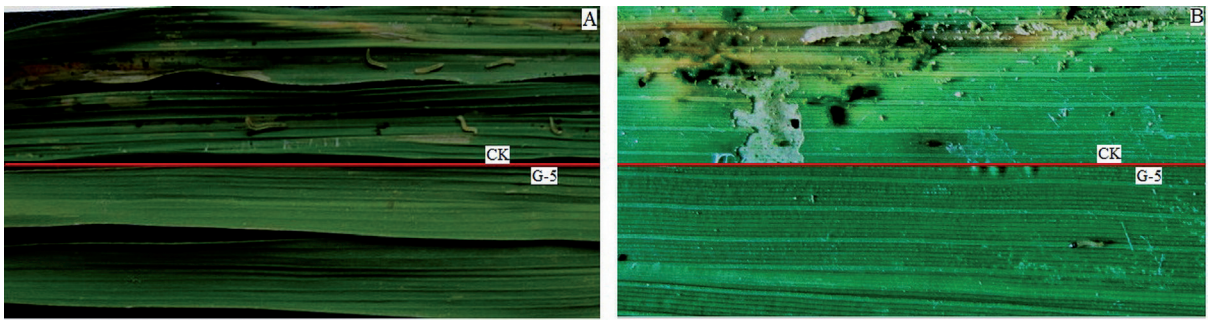

Figure 6. The bioassay of leaf roller (A) and striped stem borer (B) fed on the leaf of transgenic line G-5; CK – nontransgenic plant; G-5 – transgenic line G-5

Greenplate *et al*. (1998) found that the larvae of cotton bollworm (*Helicoverpa armigera*) could survive and resist Bt toxin due to a reduction of Bt toxin expression at latter stages and the spatiotemporal diversity of Bt toxin expression in Bollgard cotton. In this study, the Cry2Aa protein concentration in leaves at tillering stage was significantly higher than that at mature stage for the same transgenic line, and a declining trend of the Cry2Aa protein concentration was found from vegetative to reproductive stage; the Cry2Aa protein concentration was the highest in the leaves, followed by those in the stems and seeds. So the rice pests with a high lethal dose of Bt toxin such as pink stem borer (*Sesamia inferens*) possibly survived in stems where the Bt protein expression was lower at later stages, and finally finished the alternation of generations. Moreover, the grain as the edible part neither needs a high level of Bt protein expression nor suffers damage from the main target pests such as rice leaf roller, striped stem borer and yellow stem borer. Therefore, it is necessary to use a tissue-specific promoter in the future to realize the highly tissue-specific expression of Bt protein in transgenic rice varieties in order to confer defensive ability for crucial tissues and organs where the pests infest, such as leaf and stem.

In this study, the synthetic *Cry2Aa#* gene along with *Bar* gene was transferred into 4008S, resulting in the development of insect-resistant and herbicide-tolerant male sterile lines. Because of the dominant effect of the introduced foreign genes, the hybrid rice produced by this transgenic PGMS line can resist both pests and the herbicide, which makes the cultivation convenient, less costly and environmentally friendly. In addition, the mechanical harvest of F_1 hybrid seeds can be realized by spraying the herbicide to kill the restorer line after pollination when the herbicide-tolerant PGMS line and herbicide-sensitive restorer line are mixedly sown in the field, which brings benefits to the companies to reduce the cost in hybrid seed production (Xiao 1997).

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