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Cry1Ab/c in different stages of growth in transgenic rice *Bt*-shanyou63

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1. ABSTRACT

The relationship between the mRNA level and the corresponding protein level of the *cry1Ab/c* gene is not well characterized in transgenic rice (*Bt*-ShanYou63). In this study, we compared *cry1Ab/c* mRNA and its protein expression in leaves at different growth stages in *Bt*-ShanYou63 rice. The results demonstrated that both *cry1Ab/c* mRNA and its protein levels changed at all of the growth stages. The *cry1Ab/c* transcript levels in the leaves were highest during the grain filling stage (3.29, cry/actin) and lowest during the seeding stage (1.06, cry/actin), and the protein levels of Cry1Ab/c was also highest at the grain filling stage (5.71 $\mu g \times g^{-1}$ fresh weight, fw) and lowest during the seeding stage (2.08 $\mu g \times g^{-1}$ fw). There was a significant correlation between *cry1Ab/c* mRNA levels and the protein concentrations ($r=0.742$, $p < 0.01$). However, a linear relationship was not observed between *cry1Ab/c* mRNA levels and the protein levels, and the trend for mRNA expression levels was not consistent with the Cry1Ab/c protein levels in the same growth period in *Bt*-ShanYou63 rice.

2. INTRODUCTION

A number of herbivorous insects, especially lepidopteron pests, cause destructive damage to rice tissue, which results in significant yield losses in many areas of the world (1-2). For a long time, they were mainly controlled by chemical pesticides, which not only increased the production costs but also caused severe environmental pollution. The entomocidal spore-forming soil bacterium *Bacillus thuringiensis* (*Bt*) produce characteristic crystalline insecticidal proteins (Bt proteins or δ-endotoxins), which have been found to be toxic against lepidopterans, coleopterans, dipterans, and nematodes, among other organisms (3).

Since the first crystal protein gene was successfully cloned from *B. thuringiensis* subspecies and expressed in *Escherichia coli* (4), several *Bt* genes have been cloned and transferred into crop plants, including tomato, cotton, maize and rice (5). *B. thuringiensis* protein that was expressed in the transgenic plants provided high protection against certain species of insects (6). A fused *Bt* gene, *cry1Ab/c*, was used to generate transgenic *Bt* rice lines Minghui 63 and Shanyou63, both lines have showed excellent success against both the leaffolder (*Cnaphalocrocis medinalis*) and the yellow stem borer (*Scirpophaga incertulas*) (7-10).

As for transgenic *Bt* crops, a sufficient level of *Bt* transgene expression is necessary for the durable resistance against the target pests (11-12). However, prior work has indicated that the Bt protein content varies with plant age (13-16) and plant structure (17-20), and variations in Bt protein levels may lead to insufficient control of the target pests (21-24). Olsen *et al*. (2005a) (25) suggested

that late-season reductions in efficacy against *Helicoverpa armigera* (Hübner) were mainly attributed to reduction in the Cry1Ac protein content in Bt cotton.

Although the molecular mechanism for variations in Bt protein content in plant tissues has not been fully documented, a number of studies have indicated a close relationship between Bt protein levels and its transcription levels. Adamczyk and Meredith (2004) (26) suggested that a small number of genetic factors control the amount of Cry1Ac protein in *Bt* cotton, and these factors impact the overall *cry1Ac* mRNA transcript levels among different Bollgard lines. The *cry1Ac* mRNA transcript, which differs among Bollgard lines, is correlated with a corresponding Cry1Ac protein expression. The terminal leaves that had higher amounts of *cry1Ac* mRNA transcript had higher amounts of the corresponding Cry1Ac protein (27). Finnegan *et al*. (1998) (28) found that Cry1Ac levels decreased consistently throughout the growing season and attributed part of the decline in Cry1Ac expression to reductions in mRNA levels. Olsen *et al*. (2005b) (29) and Xia *et al*. (2005) (30) reported that the content of Bt protein in the same tissues decreased along with the growth of transgenic cotton plants because of the decrease in full-length *Bt* gene transcripts. These studies illustrate that, as a major step in gene expression, *Bt* exogenous gene transcription has an important influence on Bt protein levels in transgenic cottons.

Rice (*Oryza sativa* L.) is one of the most important food crops in the world. China is the largest rice producer and consumer in the world and has made great efforts to develop transgenic rice. In 2010, *Bt*-Shanyou 63 officially received a certificate for safe production (31-32), which was the first official biosafety production certificate for a genetically modified (GM) cereal crop in China. It is necessary that the potential variation in the content of Bt proteins in the life cycle of transgenic rice be evaluated before it enters into large-scale cultivation. This information would help to elucidate variations of *Bt* rice insect resistance during different growing seasons and control target pests using rational agronomic management strategies. In addition, the relationship between the mRNA level and the corresponding protein level of the *cry1Ab/c* gene in *Bt* rice is still unknown. The objectives of this study are (1) to estimate *cry1Ab/c* mRNA transcripts levels and the Cry1Ab/c protein expression levels in *Bt*-ShanYou63 rice leaves in a greenhouse during the rice growth season, and (2) to determine the relationship between transcription levels and concentrations of the Cry1Ab/c protein.

3. MATERIALS AND METHODS

3.1. Plant materials

The *Bt* rice line, *Bt*-Shanyou63 (*Bt*-SY63), and its non-transgenic rice cultivar Shanyou63 (SY63) were used in the greenhouse experiments. Rice seeds were obtained from the Huazhong Agriculture University, Wuhan, China. *Bt*-SY63, as an indica cytoplasmic male sterile (CMS) line contains a fused *Bt cry1Ab/c* transgene (31), is a hybrid between Huahui No.1 and Zhenshan97A. Laboratory and field tests have confirmed that *Bt*-SY63 rice lines can provide effective control of the lepidopteran pests such as *Chilo suppressalis* (Lepidoptera: Crambidae), *Scirpophaga incertulas* (Lepidoptera: Pyralidae) and *Cnaphalocrocis medinalis* (Lepidoptera: Pyralidae) (7,33-35).

Experiments were conducted between May to October 2012. Rice seeds were sown in petri dishes with wet cotton at 30 °C or one week and then transplanted into a rectangular flower pot (65 cm×45 cm×20 cm) on 7 June 2012. Both *Bt* and non-*Bt* rice plants were grown under natural lighting and ambient temperature in greenhouse.

Leaves were collected from *Bt*-SY63 and its parent cultivar plants throughout the growing season at the 5 stages of development. At the seedling stage (21 June), tillering stage (5 July), jointing stage (30 July), grain filling stage (10 September) and ripening stage (9 October), the uppermost fully elongated leaf blades of the main stems of fifteen rice plants were sampled for quantification analyses. To minimize differences caused by plant growth factors, a single leaf was equally divided longitudinally for both mRNA and Cry1Ab/c protein analyses. Plant samples were frozen in liquid nitrogen, and then stored at −70 °C for future analysis.

3.2. Total RNA Extraction and cDNA synthesis

The RNeasy Plant Mini Kit (Qiagen) was used for isolating total RNA, and an extended DNase treatment was carried out with DNase I (Qiagen) to remove DNA contamination (twice). The concentration of each RNA sample and the 260/280 nm absorbance (A260/A280) ratio were measured using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific); only RNA samples with A260/A280 ratios between 1.8 and 2.0 were used for further analyses. The quality and quantity of RNA were also confirmed by 1.2% agarose gel electrophoresis.

Synthesis of cDNA was carried out by reverse transcription using 1 μg of total RNA from each sample in a reaction volume of 20 μL using random hexamers provided by the PrimeScriptTM RT Reagent Kit (Takara). The RT reaction mixture was incubated at 37 °C for 15 min, heated to 85 °C for 5 sec to denature the reverse transcriptase, and then cooled to 4 °C. In the end, the cDNA synthesis was stored at −20 °C until used in real-time qPCR.

3.3. Real-time quantitative polymerase chain reaction (QPCR)

The house-keeping gene (*actin*) was used as a reference gene to allow for normalization by

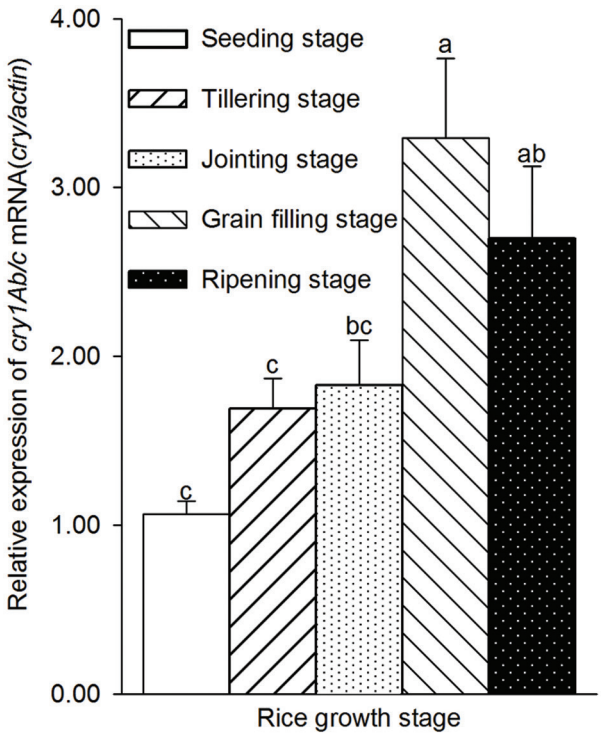

Figure 1. Mean relative expression of *cry1Ab/c* transcripts in leaves at different stages of *Bt*-ShanYou63 rice development. The data in all samples are expressed as mean ± SD, a letter of a or b above bars indicates significant treatment differences in mean (LSD tests, $p < 0.05$).

visual inspection of mRNA levels. For standard curve analysis in real-time PCR assays, plasmid pUC-acitn and pUC-cry1Ab/c were constructed to generate standard curves (*cry1Ab/c* and *actin*, respectively). The serial concentrations of 1.85×10^8, 1.85×10^7, 1.85×10^6, 1.85×10^5, 1.85×10^4, 1.85×10^3 and 1.85×10^2 copies×μL^{-1} were made for plasmid pUC-acitn, and the plasmid pUC-cry1Ab/c were consecutively diluted every tenfold at a range of 1.46×10^8~10^2 copies. Using the quantitative PCR assay, these plots were analyzed repeatedly and reproducibly; each dilution was repeated three times in triplicate.

Real-time PCR was performed in an optical 96-well plate with an ABI step one plus Real Time System (Applied Biosystems, USA). The primer pairs for *cry1Ab/c* were *cry1Ab/c*-F: 5′-CCAGCACCATCACTGTTGTCCTTT-3′ and *cry1Ab/c*-R: 5′-AAGATCAGCTTCAGAATCTCGGGG-3′. The deduced amplification length was 301 bp. The primer pairs for the house-keeping gene *actin* were *actin*-F: 5′-AATGAATTGCGTGTTGCTCCTGAGG-3′ and *actin*-R: 5′-ACCAGTTGTACGACCACTTGCATAG-3′. The primer sequences for the *actin* gene were the same as those used by Liu *et al*. (2008) (36).

The real-time PCR reaction mixture contained 2×SYBR Green Master Mix (Applied Biosystems, USA) 12.5 μL, 0.4 μL of each primer (10 μmol×L^{-1}), 1 μL cDNA or plasmid DNA, and RNase-free H_2O to make a total volume of 25 μL. The mixture was heated initially at 95 °C for 10 min and then followed by 40 cycles with denaturization at 94 °C for 1 min, annealing at 52 °C for 40 s, and extension at 72 °C for 40 s. The fluorescence was measured after each extension at 72 °C. Each assay for unknown samples was performed simultaneously with standard samples and negative control samples. In the end, a melting-curve analysis was performed immediately after the amplification.

3.4. Cry1Ab/c protein quantification

The amount of Cry1Ab/c protein in leaves was determined using the enzyme-linked immunosorbent assay (ELISA) kit by Envirologix (Portland, Maine, USA). To extract Cry1Ab/c protein, 10 mg of each sample was ground to powder under liquid nitrogen, and then suspended in 1 mL of phosphate-buffered saline containing tween20 (PBST buffer, pH 7.4). After centrifugation at 12000 rpm for 10 min at 4 °C, the supernatant was used for quantification of Cry1Ab/c protein according to the protocol provided by the manufacturer. All procedures related to standard curves, dilution factors, positive and negative controls and calculations were conducted as suggested in the kit protocol. Protein quantification was determined by plotting test sample absorbance values on standard curves generated using pure Cry1Ab/c protein. The absorbance was measured at 405 nm.

3.5. Statistical analysis

The mean values and standard deviations (SD) were calculated using Microsoft Excel (Microsoft, USA), and graphs were drawn with SigmaPlot version 10.0 All statistical tests were conducted at the 0.05 level of significance using the Fisher's least significant difference (LSD) method by SPSS17.0 software.

4. RESULTS

4.1. *cry1Ab/c* transcript levels in leaf tissues at different stages of rice development

The transcription of the *cry1Ab/c* and *actin* genes were tested in leaf samples from five rice growth stages. All samples were analyzed using SYBR Green I RT-PCR. The ratio between *cry1Ab/c* and *actin* transcript numbers ranged from 1.06 at the seeding stage to 3.29 at the grain filling stage in leaf of *Bt*-SY63 rice (Figure 1), and *cry1Ab/c* gene transcript were not detectable in SY63 cultivar leaves. As for the *Bt*-SY63 rice samples (Figure 1), the highest transcript level of the *cry1Ab/c* gene was detected at grain filling stage and decreased at ripening stage. The differences in the transcript levels were statistically significant ($p<0.05$).

4.2. Expression of Cry1Ab/c in leaf tissues at different stages of rice development

The amount of Cry1Ab/c protein expressed in different leaf samples were determined by ELISA.

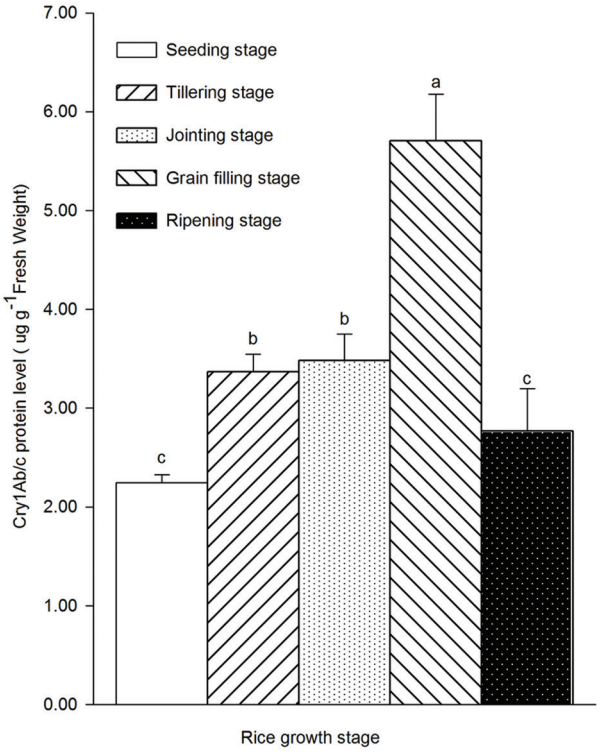

Figure 2. Cry1Ab/c protein level in leaves at different stages of *Bt*-ShanYou63 rice development. The data in all samples are expressed as mean ± SD, bars with a common letter are not significantly different (LSD tests, $p < 0.05$).

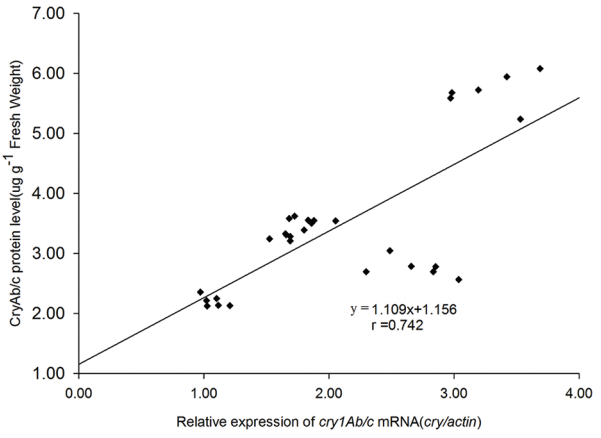

Figure 3. Correlation between *cry1Ab/c* mRNA transcripts and Cry1Ab/c concentrations in leaves of *Bt*-ShanYou63 rice.

The Cry1Ab/c protein was detected in all five stages of *Bt*-SY63. As expected, Cry1Ab/c protein was not detectable in SY63 rice leaves. As shown in Figure 2, mean Cry1Ab/c protein expression levels differed significantly between growth stages. The results clearly showed that Cry1Ab/c expression levels were the highest at the grain filling stage (5.71 $\mu g \times g^{-1}$) and lowest at the seeding stage (2.08 $\mu g \times g^{-1}$). The mean levels of Cry1Ab/c protein measured in the leaves at the tillering stage, jointing stage and ripening stage were 3.37, 3.48, and 2.77 $\mu g \times g^{-1}$, respectively. Cry1Ab/c protein concentration at the grain filling stage was significantly higher than those measured in samples from other stages ($p<0.05$).

4.3. Correlation between transcript levels and expression levels of *cry1Ab/c* gene

The correlation between transcript levels and expression levels for *Bt*-SY63 samples selected as described above are shown in Figure 3. For all of the samples for which a complete data set was generated, there was a significant correlation between transcript levels and Cry1Ab/c protein concentrations (Figure 3). Both transcript levels and Cry1Ab/c protein levels were the highest at the grain filling stage and lowest at the seeding stage. The Pearson product moment correlation coefficient for the whole data was 0.742. It is also worth noting that a linear relationship was not observed. Indeed, the expression trends for mRNA levels were not consistent with the Cry1Ab/c protein levels in the same growth stage. For example, the mRNA level at the ripening stage was 2.70 (cry/actin, significantly higher than other stages except 3.29 at the grain filling stage) while the corresponding Cry1Ab/c protein level was only 2.77 $\mu g \times g^{-1}$.

5. DISCUSSION

Insect-resistant transgenic crops that express *cry* genes from *B. thuringiensis* offer a new opportunity for plant resistance to pests. Large-scale planting of transgenic rice can help to decreased environmental contamination, and significantly reduced the costs of rice production.

As a main component of the insecticidal activity (37), insecticidal protein be expressed in adequate quantities in appropriate plant parts at the requisite time of the season is the key determinant for control targeted pests (38). Thus, Bt protein expression data are essential for assessing and monitoring the biosafety of GM crops (39). *Bt* genes are external genes that differ from internal genes in inheritance and may show some distinct differences to classic genetic laws in plants. Therefore, it is necessary to know the integration and expression of *Bt* genes in GM crops to illuminates the evolution of *Bt* gene.

To explore one gene's function and mechanisms, it is necessary to study it from the points of molecular and gene expression; quantification of mRNA transcript is essential for assessment of the regulatory mechanisms of a gene (40). Real-time RT-PCR results demonstrated that *cry1Ab/c* mRNA changed in the whole *Bt*-SY63 growth stages. Transcript levels were the highest at the grain filling stage and the lowest in the seeding stage

(3.29 and 1.06, cry/actin, respectively). For the tillering, jointing and grain filling stages, the *cy1Ab/c* transcript level was 1.69, 1.83, and 2.70 (cry/actin), respectively. In our study, there was a variation in *cry1Ab/c* expression in *Bt*-SY63 with *actin1* promoters, it may be related to the transgene, the promoter, or a combination in a certain orientation of expression. A more detailed study would be required to understand this phenomenon.

Quantitative estimation of Cry1Ab/c proteins in *Bt*-SY63 was accomplished using ELISA. The expression levels of *cry1Ab/c* changed significantly in the whole *Bt*-SY63 growth period. The results clearly show that the levels of Cry1Ab/c protein in leaves ranged between 2.08 and 5.71 $\mu g \times g^{-1}$ fw. Cry1Ab/c protein levels were the highest in the grain filling stage and the lowest in the seeding stage. Similar findings related to protein expression levels were also reported in other studies of the *Bt* rice lines. Zhang *et al*. (2011) (9) reported that the expression levels of *cry1Ab* (or *cry1Ab/c*) gene were highest in the grain filling stage and lowest in the tillering stage. The heading and ripening stages were relatively stable in the transgenic rice line Huachi B6 (*cry1Ab* gene) and TT51-1 (*cry1Ab/c* gene). Bai *et al*. (2005) (41) indicated that the Cry1Ab concentration was low in leaf samples of KMD1 and KMD2 during the jointing and booting stages, Cry1Ab levels significantly increased in the early grain filling stage and the toxins obviously decreased as plants matured in the maturing stage. However, Kim *et al*. (2013) (39) suggested that expression of mCry1Ac1 was highest in the tillering stage and declined significantly as plants matured from the tillering to the ripening stages (3.1-fold). The different Bt protein levels that were found during the rice developmental stages may have been the result of two factors: (i) The effect of different rice lines and plant ages on the Bt protein level. The insecticidal protein content may be influenced by the plant source, age of the plant samples, methods of protein extraction and adequacy of protein standards (42-43). To minimize experimental variation caused by sampling factors, all samples were taken at the same time from the same part of the plant, and careful manipulation was used to keep variability low in the experimental replicates. (ii) *cry* gene expression was also influenced by environmental factors (44). Sachs *et al*. (1996) (45) demonstrated that *cry1A(b)* gene expression level was larger variation in different cultivated places, even when using the same crop lines at the same age. In the present experiment, Cry1Ab/c proteins in *Bt*-SY63 were examined under greenhouse conditions, with natural lighting, and no fertilizer was applied. Other studies have conducted experiments under field conditions (39). Differences among environmental conditions and cultivation management may have contributed to variations in results.

The relationship between *cry1Ab/c* mRNA and protein expression was examined using the average expression values for all samples. It is evident that *cry1Ab/c* mRNA and Cry1Ab/c protein levels can be greatly influenced by rice growth stages; both *cry1Ab/c* mRNA and Cry1Ab/c proteins changed over time with growth stages. It appears that the level of expressed Cry1Ab/c protein at the seeding and grain filling stages were correlated with mRNA transcript levels. At both stages, there were significantly lower (or higher) amounts of *cry1Ab/c* transcripts and corresponding proteins compared to all other stages (Figure 3), especially in the filling stage, which is a critical period for rice yield and grain quality. Our results showed that both Cry1Ab/c protein levels and *cry1Ab/c* transcript levels were high in the filling stage, which may have led to the accumulation of Bt proteins in the seeds. Qiu *et al*. (2010) (46) reported that the Cry1Ab/c proteins were highly detected in mature seeds of T51-1. In the rice lines *Bt*-SY63, the crop was engineered to express Cry1Ab/c proteins in almost all parts of the plant that are under the control of actin1 promoters (47). A tissue-specific promoter may be used to control gene expression in a tissue-dependent manner. For example, the *cry1Ab/c* gene was only expressed in leaves and stems, but not in seeds or roots in the transgenic rice line L24, which is driven by the maize Phosphoenolpyruvate carboxylase (PEPC) promoter and further dispels the concern for residues of Bt proteins in food (46). mRNA levels at the ripening stage were 2.70 (cry/actin, significantly higher than other stages except 3.29 at the grain filling stage) when corresponding Cry1Ab/c protein had a low expression level, which may have led to insufficient control of targeted pests at the ripping stage. Our results that the *Bt* gene mRNA transcripts levels and Cry1Ab/c protein concentrations did not show a linear relationship in leaves at different stages of *Bt*-SY63 rice have seldom been reported in transgenic food crops so far.

In conclusion, the present study examined the *cry1Ab/c* transcript levels and Cry1Ab/c proteins expression levels in the transgenic rice *Bt*-SY63 and determined the relationship between *cry1Ab/c* transcripts levels and proteins levels. The results demonstrated that both *cry1Ab/c* transcripts and Cry1Ab/c proteins were changed at all growth stages in *Bt*-SY63 and *cry1Ab/c* transcripts levels were not completely relevant to Cry1Ab/c proteins levels. Gene expression levels varied with the nucleotide sequence of the gene, promoter, the insertion point of the gene in the DNA of the transgenic variety, transgene copy number and the internal cell environment (48-49) investigate these results at molecular, genetic, and physiological levels should help in understanding the mechanism for the lack of correlation between mRNA and protein expression in *Bt*-SY63 rice.

6. ACKNOWLEDGMENTS

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