Theor Appl Genet (2009) 119:635–644
DOI 10.1007/s00122-009-1075-5

ORIGINAL PAPER

Normal expression of insect-resistant transgene in progeny of common wild rice crossed with genetically modified rice: its implication in ecological biosafety assessment

Hui Xia · Bao-Rong Lu · Jun Su · Rui Chen · Jun Rong · Zhiping Song · Feng Wang

Received: 7 June 2008 / Accepted: 15 May 2009 / Published online: 6 June 2009

Abstract Transgene outflow from genetically modified (GM) rice to its wild relatives may cause undesirable ecological consequences. Understanding the level of transgene expression in wild rice following gene flow is important for assessing such consequences, providing that transgene escape from GM rice cannot be prevented. To determine the expression of a transgene in common wild rice (*Oryza rufipogon*), we analyzed the content of Cry1Ac protein in three GM rice lines containing a *Bt* transgene, their F_1 hybrids with common wild rice and F_2 progeny at different growth stages, using the sandwich enzyme-linked immunosorbent assay. The average content of Cry1Ac protein in leaf samples of the wild rice lines ranged between 0.016 and 0.069% during the entire growth period, whereas that in stems varied between 0.12 and 0.39%. A great variation in Cry1Ac protein content was detected among individuals of F_1 hybrids and F_2 progeny, with some wild individuals showing higher level of *Bt* toxin than the cultivated GM rice. The results suggest that the *Bt* transgene can express normally in the interspecific hybrids between insect-resistant GM rice and common wild rice, and may have similar effects on the target insects as in GM rice.

Communicated by M. Bohn.

H. Xia · B.-R. Lu (✉) · J. Rong · Z. Song
The Ministry of Education Key Laboratory for Biodiversity Science and Ecological Engineering, Institute of Biodiversity Science, Fudan University, 200433 Shanghai, China
e-mail: brlu@fudan.edu.cn

J. Su · R. Chen · F. Wang (✉)
Fujian Province Key Laboratory of Genetic Engineering for Agriculture, Fujian Academy of Agricultural Sciences, 350003 Fuzhou, China
e-mail: wf@fjage.org

Introduction

The extensive environmental release and commercial production of genetically modified (GM) crops with novel traits have increasingly aroused ecological biosafety concerns and debates worldwide (Snow 2002; Lu and Snow 2005; Hails and Morley 2005). One of the major ecological biosafety concerns is the escape of genetically engineered genes (transgenes) through cross-pollination into wild or weedy relatives of GM crops. Transgenes with evolutionary selective advantage dispersed into weedy or wild populations may result in fitness changes of individuals that have picked up the transgenes, causing unwanted ecological consequences (Snow and Palma 1997; Riches and Valverde 2002). These include the promotion of weed populations in agro-ecosystems (Darmency 1994; Halfhill et al. 2005), influences on genetic integrity of wild gene pool (Gepts and Papa 2003), and non-target effects (Poppy 2000; Hellmich et al. 2001). To understand the potential ecological consequences caused by transgene escape, questions concerning three successive steps should be addressed: (1) At what frequency can a transgene disperse from GM crops into their wild relative populations through gene flow? (2) At what level will a transgene express and inherit after being incorporated by wild relatives? (3) How will a transgene change the fitness of wild individuals that have incorporated the transgene, and the surviving/competitive ability of wild populations? Knowledge about each of these questions will provide the scientific bases for biosafety assessment of potential ecological consequences caused by transgene outflow to wild relatives (Lu and Snow 2005).

Rice (*Oryza sativa* L.) is an important world cereal crop that provides staple food for nearly one-half of the global population (Lu and Snow 2005). Biotechnology research and development have long been applied in rice and great progress has been made in the past decades. Many countries (e.g., China, India, Vietnam, and Iran) have invested enormously in research and development of GM rice, and consequently, a large number of GM rice lines have been developed (Lu and Snow 2005; Wang and Johnston 2007). In 2005, Iran became the first country in the world that commercialized *Bt* transgenic rice (James 2006). Recently, the USA has approved the non-regulated cultivation of two herbicide-tolerant rice varieties, "Liberty Link® Rice" (LLRICE06 and LLRICE62), containing a *bar* gene (see: US Regulatory Agencies Unified Biotechnology Website, http://usbiotechreg.nbii.gov/database_pub.asp). As the largest rice producing and consuming country, China is also actively exploring transgenic biotechnology for the improvement of rice varieties (Wang and Johnston 2007). In fact, a large number of GM rice lines have been developed in China, and some are under preparation (including biosafety assessment) for commercialization (Xiong 2004). However, due to the strict biosafety assessment protocols, commercial production of GM rice has been significantly delayed in China. As one of the centers of origin and diversity for cultivated rice and the northernmost distribution region of wild *Oryza* species, China has taken a precautious biosafety approach to assess potential ecological consequences caused by transgene outflow. Given that gene flow from cultivated rice to wild or weedy rice is unavoidable under normal cultivation practices where wild rice exists (Ellstrand et al. 1999; Song et al. 2003; Chen et al. 2004), it is important to determine subsequent levels of transgene expression in wild rice and thereafter the fitness change in wild rice populations that contain a transgene, as a part of the biosafety assessment for ecological consequences.

The insect-resistance *Bt* (*Bacillus thuringiesis*) gene has been widely used in crop improvement through genetic engineering. Insect-resistant GM crops include *Bt* cotton (Hilder and Boulter 1999; Pray et al. 2002), *Bt* maize (Alcalde and Camara 2007; Andersen et al. 2007), *Bt* cabbage (Bhattacharya et al. 2002), and *Bt* rice (Nayak et al. 1997; Tu et al. 2000; High et al. 2004). One type of *Bt* transgenes that encodes the Cry1Ac toxin is effective to kill the larvae of a number of rice lepidopteran insects, such as rice stem borers (*Sesamia nonagrioides*, *Scirpophaga incertulas*, and *Chilo suppressalis*) and rice leaffolders (*Cnaphalocrocis medinalis*). These target rice herbivores occur frequently in cultivated rice and they are also found in some populations of common wild rice (Cohen et al. 2008). These pests mainly attack stems (by rice stem borers) and leaves (by leaffolders) of rice plants after the jointing stage (when plants stop major tilling and start to elongate rapidly), causing yield losses up to ~20% for cultivated rice (Muralidharan and Pasalu 2006). Studies have demonstrated that transgenic *Bt* rice can effectively reduce damage from the target insect pests (Khanna and Raina 2002; Huang et al. 2005; Han et al. 2007). If the *Bt* transgene disperses from GM rice into wild rice individuals and expressed normally in wild rice plants, it may provide a selective advantage to the wild rice populations exposed to lepidopteran insects, resulting in unwanted ecological consequences (Cohen et al. 2008). In addition, from the germplasm point of view, wild rice is the reservoir of many beneficial genes for cultivated rice improvement. If the transgene spreads into wild rice populations, providing resistance against these insects, other useful genes, for example those giving resistance to the insects naturally, may be lost over time in the wild rice populations.

Evidence has shown that *Bt* transgenes can express stably in wild *Brassica* hybrids after the transgenes were incorporated into wild relatives (Halfhill et al. 2002; Zhu et al. 2004; Ammitzbøll et al. 2005). Whether the *Bt* transgene that introgresses into individuals of common wild rice (*Oryza rufipogon*) will express normally is still unknown, and this knowledge gap will hinder the appropriate prediction of potential ecological consequences caused by *Bt* transgene escape to wild rice relatives. Common wild rice is the putative ancestor of cultivated rice, and widely distributed in most tropical regions in Asia where cultivated rice is grown. It is also found in northern Australia, Latin America, and Africa as an introduced species (Naredo et al. 1997; Lu and Snow 2005; Vaughan et al. 2005a). In some rice growing countries such as India, Vietnam, Cambodia, and Malaysia, common wild rice occurs abundantly and is a noxious weed infesting rice fields (Vaughan et al. 2005b).

In this study, we applied the sandwich enzyme-linked immunosorbent assay (ELISA) method to measure the content of Cry1Ac protein in F_1 hybrids of common wild rice crossed with *Bt* GM rice lines, and their F_2 progeny produced in the course of our GM rice biosafety research. The sandwich ELISA has been proven to be an effective method for quantifying the *Bt* Cry1Ac protein (Sims and Berberich 1996; Takahashi et al. 1998) and estimating *Bt* transgene expression in vivo organism, such as cotton (Adamczyk and Sumerford 2001) and rice (Qin et al. 2003; Bashirn et al. 2005). The objectives of this study were to determine the level and variation pattern between tissues and growth stages of *Bt Cry1Ac* transgene expression in hybrid lines of common wild rice crossed with cultivated GM rice, compared to the GM rice parents. This knowledge will facilitate our decision making on further ecological risk assessment of transgene outflow from GM rice to its wild relatives.

Materials and methods

Plant materials

Three double-inserted insect-resistant GM rice lines (KeFeng6, Ba23, and Ba28) were used in the experiment to test the same target transgenes in three different genetic backgrounds (Table 1). Two insect-resistance transgenes, i.e., *Bacillus thuringiesis* (*Bt*) and cowpea trypsin inhibitor (*CpTI*), were transferred into the three GM rice lines and tightly linked with the hygromycin resistance gene (*hpt*), used as the selectable marker. *Bt* was under the control of the maize ubiquitin (*Ubi*) promoter and encodes the Cry1Ac protein (toxin) that kills lepidopteran larvae. KeFeng6 was produced from Minghui-86, a traditional rice variety, using *Agrobacterium*-mediated transformation technology and selfing to the T7 generation until individuals of the line with stable insect resistance and genetic inheritance (Chen et al. 2006). Ba23 was bred from the backcrossed lines of KeFeng6 × F96110 (a commercial rice variety), and Ba28 was selected from the backcrossed lines of KeFeng6 with a complex rice variety having three parents (Duoxi1/W311/Minghui-69). These two transgenic lines (Ba23 and Ba28) were homozygous for *Bt*, which showed stable inheritance and agronomic performance.

Four genotypes of common wild rice (*O. rufipogon*), coded as R1, R2, R3, and R4, and collected from different localities (Table 1), were used for producing F_1 hybrids and F_2 progeny with the GM rice lines. The four genotypes of common wild rice were unrelated, and therefore represented different genetic backgrounds based on the analysis of simple sequence repeat (SSR) molecular markers (unpublished data). One GM parent and its hybrid descendents developed from the same wild rice genotypes were defined as a family (e.g., KF-6, F_1-K/R1, and F_2-K/R1). A total of 13 lines (one type of GM parent or F_1 hybrid or F_2 progeny) belonged to five families including GM rice parents, F_1 hybrids, and F_2 progeny were obtained for the transgene expression experiment (Table 1). To guarantee all the hybrids and F_2 progeny included in the experiment contained the *Bt* transgene, the experimental materials were subjected to PCR analyses using the specific primer pairs for amplifying the *Bt* transgene, according to Rong et al. (2005).

Experimental design

A total of 14 experimental lines (treatments), each with three replicates, were included in the experiment: three GM rice parents, five F_1 hybrids, five F_2 progeny, and one

Table 1 Genetically modified (GM) insect-resistant rice lines (containing *Bt*/*CpTI* transgenes) and interspecific hybrids and F_2 progeny with common wild rice (*Oryza rufipogon*) from different localities in the ELISA (enzyme-linked immunosorbent assay) experiment for transgene (*Bt*) expression

Parent and hybrid progeny	Code	Notes
Parents		
KeFeng-6	KF-6	This material is T7 generation, containing tightly linked double-inserted homozygous *Bt*/*CpTI* genes
Ba-23	Ba-23	Bred from KF-6, homozygous for *Bt*/*CpTI*
Ba-28	Ba-28	Bred from KF-6, homozygous for *Bt*/*CpTI*
Minghui-86	M-86	Non-transgenic cultivated rice, used as blank control
Oryza rufipogon	R1, R2, R3, R4	Four plant individuals with different genotypes are included. They were coded as R1, R2, R3 and R4
F_1 hybrid (♂ × ♀)		
KF-6 × R1	F_1-K/R1	R1 was collected from Suixi, Guangdong Province, China, hemizygous for *Bt*/*CpTI*
KF-6 × R2	F_1-K/R2	R2 was collected from Huilai, Guangdong Province, China, hemizygous for *Bt*/*CpTI*
KF-6 × R3	F_1-K/R3	R3 was collected from Dongxiang, Jiangxi Province, China, hemizygous for *Bt*/*CpTI*
Ba-23 × R1	F_1-Ba23/R4	Hemizygous of *Bt*/*CpTI*
Ba-28 × R4	F_1-Ba28/R1	R4 was collected from Suixi, Guangdong Province, China, hemizygous for *Bt*/*CpTI*
F_2 progeny		
KF-6 × R1	F_2-K/R1	Mixed with homozygous and hemizygous individuals
KF-6 × R2	F_2-K/R2	
KF-6 × R3	F_2-K/R3	
Ba-23 × R1	F_2-Ba23/R4	
Ba-28 × R4	F_2-Ba28/R1	

A non-GM rice parental variety (Minghui-86) was used as blank control

non-GM rice control. Each replicate of all the experimental lines included nine individuals planted in an 80 × 80 cm plot. A total of 42 plots were arranged within a paddy field of 11 × 9 m^2 and the plots were separated by a space of 50 cm. A complete randomized one-factor design was adopted in the field layout for this experiment. Plants of GM rice parents, non-GM control, and F_2 progeny were generated from germinated seeds, whereas those of the F_1 hybrids were developed from the ratooned tillers. The experiment was conducted in the authorized and confined Biosafety Experimental Field at Wufeng Village of Fuzhou City, Fujian Province in China. Management of the experimental materials and paddy field followed the usual procedures by local farmers. No pesticides were applied to the experimental field.

Sample collection

About one gram of leaf samples from the uppermost leaves of the main tillers were collected from each individual for transgene expression analyses at five growth stages: tillering (individuals start to produce most tillers), jointing (tillers begin to elongate), booting (panicles initiate and elongate), flowering, and seed-maturing. In addition, about the same amount of samples from the uppermost internodes (stem) of a main tiller at the jointing stage was also collected for the analyses (Table 2), to see if the *Bt* transgene expresses differently at different growth stages and in different organs of a plant (Scott et al. 1998). Six individuals per plot were randomly sampled with about 0.2 g leaf or stem tissue from each replicate. The collected samples were cleaned with distilled water and immediately subjected to protein extraction.

Measurement of *Bt* transgene expression

Total soluble protein extraction and measurement

PBS buffer (1.2 ml) (0.2 g KCl, 3.58 g $Na_2PO_4{\cdot}12H_2O$, 0.24 g KH_2PO_4 and 8 g NaCl dissolved in 1,000 ml double-distilled water; pH ~7.4) was added to the total soluble protein extracted from the samples following grinding and allowed to set for about 1 min on ice. The mixture was centrifuged at 10,000 rpm for 10 min at 4°C and the collected supernatant fluid was maintained at 4°C. A part of the supernatant fluid was diluted 100 times before the total soluble protein was measured by the modified Bradford method (Stoscheck 1990). Diluted supernatant (100 µl) was added to 1,000 µl Bradford reagent (1,000 ml double-distilled water solution with 100 mg Commassi Blue, 50 ml 95% ethanol, and 100 ml 85% (w/v) H_3PO_4). After 5 min incubation at 25°C, the amount of total soluble protein was obtained by measuring the absorbance at 595 nm on a Beckman model DV^R 7400 UV-spectrum. Bovine serum albumin (Roche) was used for establishing standard curves.

Sandwich ELISA for Cry1Ac test

The ELISA experiment was conducted in the Gene Engineering Laboratory of Fujian Academe of Agriculture Sciences. In the testing system, the polyclonal antibody of Cry1Ae from rabbit was used to determine the expression level of Cry1Ac, because the cryIAe and Cry1Ac have nearly the same amino-acid sequences and the cryIAe antibody will detect Cry1Ac toxin (Qin et al. 2003). The average coefficient of variance of protein component within each test was <5%, indicating that the system for the Cry1Ac expression test was reliable. Lyophilized standard Cry1Ac prepared previously by the Chinese Academy of Agriculture Science was used to establish the standard curve.

A 96-well polyvinylchloride plate was pretreated under UV for about 2 h before 135 µl carbonate-bicarbonate solution (CBS) with 1 µg/ml polyclonal antibody was added to each well. The plate with CBS was incubated at 4°C over night. 10 µl supernatant samples and 90 µl PBS were added to the wells after 235 µl 2% BSA (dissolved in PBS) was used to block the unoccupied sites at 37°C for 1.5 h. The plate was incubated at 37°C for 1.5 h before 100 µl alkaline phosphatase (AP) labeled polyclonal antibody (1 µg/ml)

Table 2 Date and detail information of materials sampled at various stages for enzyme-linked immunosorbent assay (ELISA)

Growth stage	Sampling date	Remarks
Tillering	June 9	12 days after transplanting, individuals having 2–3 tillers
Jointing[a]	July 16	Individuals of GM rice were nearly at the jointing stage
Booting	July 26	Individuals of both GM rice and wild hybrids/ progeny were in booting
Flowering	August 6	Only GM rice individuals were at the flowering stage
	August 29	Only F_1 hybrids and F_2 progeny were at the flowering stage
Maturing	September 29	GM rice was at mature, while wild hybrids and progeny were close to maturity

[a] Leaf and stem samples were collected at this stage

was added to each well, and incubated at 37°C for another 1.5 h. 100 µl *p*-nitrophenyl phosphate (PNPP, PIERCE) was added to each well, and the plate was then scored on a microplate reader (Bio-Rad model 680) after 1.5 h reaction. The plate was washed three times (3 min each) with 235 µl PBST (1 l PBS with 1 ml Tween 20) after each of the above steps.

Data analysis

The level of *Bt* transgene expression was calculated as: the amount of Cry1Ac protein/the amount of total soluble protein. An average value of the Cry1Ac protein content was obtained from samples represented by at least six individual samples in each plot (replicate). Consequently, three average values representing each treatment were obtained from three plots (replicates). Statistical analysis for the content of Cry1Ac protein was based on the average values of various treatments.

The comparison of the Cry1Ac protein content was carried out among different treatments within the same families, including a GM rice parent, its F_1 hybrids, and F_2 progeny derived from the same wild rice genotypes. A One-way ANOVA (Student-Newman-Keuls, SNK) test was performed to determine statistical differences in Cry1Ac protein content among treatments using the SAS software ver. 8.02 (SAS Institute Inc., Cary, NC, USA). The *P* value of <0.05 was taken as the level for significance. The general variation pattern of *Bt* transgene expression at different growth stages was estimated using the average values of all GM rice, F_1 hybrids, and F_2 progeny collected from different experimental lines. The linear regression analysis was performed to investigate the variation trends of *Bt* transgene expression among growth stages in different generations and lines, using the SAS software ver. 8.02 (SAS Institute Inc., Cary, NC, USA).

Results

Expression level of *Bt* transgene in GM rice parents, F_1 hybrids, and F_2 progeny

Before the transgene expression analysis was performed, all the experimental materials were validated for the presence of the *Bt* transgene by PCR analyses using the specific primer pairs of the target transgene (Rong et al. 2005). As a result, the *Bt* transgene was detected in all experimental materials of the three GM rice parents (Kefeng6, Ba23, and Ba28), F_1 hybrids, and F_2 progeny. In contrast, the *Bt* transgene was not detected in the parental wild rice and the blank control (Minhui-86).

Results from this experiment demonstrated in general that the *Bt* transgene (*Cry1Ac*) can express normally in F_1 hybrids and F_2 progeny derived from crosses between common wild rice and GM rice lines (Table 3). The average content of Cry1Ac protein in leaf samples of all the 13 lines that contained the *Bt* transgene varied between 0.016 and 0.069% compared to the total content of soluble protein at the five growth stages, whereas that in stem samples at the jointing stage ranged between 0.12 and 0.39% (Table 3). During the tillering, jointing, and booting stages, the level of Cry1Ac protein showed no significant difference in GM rice parents, F_1 hybrids, and F_2 progeny in most cases. At the flowering stage, the level of Cry1Ac protein in the F_2 progeny was significantly higher than that in GM rice parents in the four comparable families (Table 3). On the contrary, the level of Cry1Ac protein in F_2 progeny was significantly lower than that in GM rice parents at the seed maturing stage in the three comparable families (Table 3). Noticeably, the *Bt* transgene derived from the same GM parental line (e.g., KeFeng6) did not show significant influences by different genotypes (genetic backgrounds) of the wild rice parents. This was not the case for the Cry1Ac protein, where levels in leaf and stem samples were significantly different in the lines of F_1-K/R1, F_1-K/R2, and F_1-K/R3, [which had the same GM rice male parent but different wild rice accession genotypes as female parents, based on the Student–Newman–Keuls (SNK) test (data not shown)]. Similar results were observed when compared the transgene expression level in the F_2 progeny.

Furthermore, three out of 27 examined individuals from the F_2 progeny (F_2-Ba28/R1) and two out of 25 examined individuals from the F_2 progeny (F_2-Ba23/R4) did not show expression of the Cry1Ac protein as tested by ELISA, although all these individuals were proven containing the *Bt* transgene by PCR analyses. This phenomenon was more likely caused by the silencing of the *Bt* transgene in these F_2 progeny.

Expression pattern of *Bt* transgene in various rice lines at different stages

The content of the Cry1Ac protein in leaf samples varied dramatically among individuals of the F_1 hybrids (0.0087–0.16%) and F_2 progeny (0.0075–0.22%), compared with that in the GM rice parents (0.015–0.099%). The similar level of variation was observed in stem samples (F_1 hybrids 0.059–0.69%, F_2 progeny 0.054–0.96% and GM rice 0.039–0.63%).

There was an obvious variation at different growth stages in the average content of Cry1Ac protein (Fig. 1) in GM rice parents (based on three lines), F_1 hybrids (five lines), and F_2 progeny (five lines). The similar variation pattern in the content of Cry1Ac protein was also observed within

Table 3 Comparison of *Bt* transgene expression in leaf and stem samples of GM rice parents, F_1 hybrids with common wild rice, and their F_2 progeny at different growth stages detected by enzyme-linked immunosorbent assay (ELISA)

GM rice line and its derivatives	Average expression level of Cry1Ac protein					
	Leaf					Culm (jointing stage)
	Tillering	Jointing	Booting	Flowering	Seed maturing	
KF-6	0.0422 (0.0017) a	0.0375 (0.0039) a	0.0369 (0.0028) a	0.0387 (0.0017) a	0.0342 (0.0018) b	0.2280 (0.0569) a
F1-K/R1	0.0441 (0.0072) a	0.0296 (0.0028) a	0.0313 (0.0007) ab	0.0408 (0.0035) a	0.0160 (0.0003) a	0.2690 (0.0121) a
F2-K/R1	0.0355 (0.0079) a	0.0386 (0.0084) a	0.0269 (0.0021) b	0.0314 (0.0068) a	0.0242 (0.0041) a	0.2430 (0.0509) a
KF-6	0.0422 (0.0017) a	0.0375 (0.0039) a	0.0369 (0.0028) a	0.0387 (0.0017) a	0.0342 (0.0018) b	0.2280 (0.0569) a
F1-K/R2	0.0450 (0.0069) a	0.0296 (0.0026) a	0.0309 (0.0020) a	0.0421 (0.0017) a	0.0162 (0.0025) a	0.2660 (0.0456) a
F2-K/R2	0.0584 (0.0 60) a	0.0483 (0.0084) a	0.0347 (0.0017) a	0.0631 (0.0033) b	0.0252 (0.0025) a	0.2250 (0.0143) a
KF-6	0.0422 (0.0017) a	0.0375 (0.0039) a	0.0369 (0.0028) a	0.0387 (0.0017) a	0.0342 (0.0018) b	0.2280 (0.0569) a
F1-K/R3	0.0535 (0.0011) a	0.0289 (0.0051) a	0.0307 (0.0028) a	0.0555 (0.0053) ab	0.0185 (0.0011) a	0.1940 (0.0625) a
F2-K/R3	0.0767 (0.0102) b	0.0292 (0.0028) a	0.0384 (0.0048) a	0.0681 (0.0066) b	0.0266 (0.0034) ab	0.2240 (0.0051) a
Ba-23	0.0499 (0.0 064) a	0.0317 (0.0014) a	0.0293 (0.0013) a	0.0269 (0.0028) a	0.0250 (0.0029) a	0.1700 (0.0243) a
F_1-Ba23/R4	0.0475 (0.0 72) a	0.0396 (0.0067) a	0.0371 (0.0035) a	0.0550 (0.0039) b	0.0284 (0.0038) a	0.1990 (0.0260) a
F2-Ba23/R4	0.0530 (0.0133) a	0.0290 (0.0058) a	0.0307 (0.0027) a	0.0455 (0.0089) ab	0.0264 (0.0036) a	0.2080 (0.0099) a
Ba-28	0.0537 (0.0043) a	0.0447 (0.0082) a	0.0372 (0.0019) a	0.0471 (0.0018) a	0.0348 (0.0064) a	0.1750 (0.0221) a
F_1-Ba28/R1	0.0476 (0.0031) a	0.0367 (0.0106) a	0.0358 (0.0017) a	0.0567 (0.0094) a	0.0212 (0.0021) a	0.2800 (0.0368) a
F2-Ba28/R1	0.0499 (0.0068) a	0.0397 (0.0020) a	0.0373 (0.0018) a	0.0780 (0.0039) b	0.0318 (0.0014) a	0.1920 (0.0105) a

Samples from 18 individuals representing each of the GM rice varieties, F_1 hybrids, and F_2 progeny were analyzed. The expression was calculated as the average amount of cry1Ac protein against that of total soluble protein (%). Numbers in parentheses indicate SEs. Different letters following the average values denote their significant differences ($P < 0.05$) under statistical analysis using the Student–Newman–Keuls (SNK) test. Statistical comparison was only conducted among materials derived from the same GM rice lines and wild rice genotypes

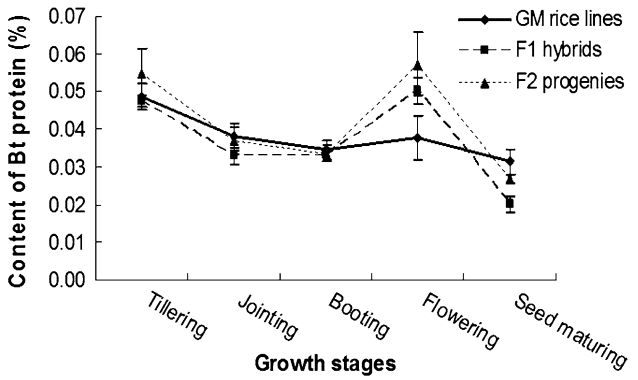

Fig. 1 Variation patterns of Bt Cry1Ac protein content (%) at different growth stages in leaf samples of GM rice parents (*three lines*), F_1 hybrids (*five lines*) and F_2 progeny (*five lines*) based on the average values. The expression of *Bt* transgene showed a gradual decline but with an evident increase at the flowering stage for F_1 hybrids and F_2 progeny. The *vertical bar* represents SEs

different lines. The linear regression analysis involving the average values of all GM rice parents, F_1 hybrids, and F_2 progeny showed negative slopes of the *Bt* protein content (the *y* axis as shown Fig. 1) against the different growth stages (the *x* axis as shown Fig. 1). This analysis demonstrated generally that the content of Cry1Ac protein decreased gradually from the tillering stage until seed maturation, although there was an evident increase in the content at the flowering stage for F_1 hybrids and F_2 progeny (Fig. 1). Further analysis of the cohesiveness of the tendencies among the 13 lines showed a negative slope, with the exception of line F2-Ba28/R1, which had a positive slope. This may have been caused by the exceptionally high value of *Bt* protein content at the flowing stage: the slope became negative when we manually excluded the high values of *Bt* protein content at the flowering stage.

In general, results from this experiment showed that the GM rice parents, F_1 hybrids, and F_2 progeny possessed the same variation pattern of *Bt* transgene expression: a gradual decrease in *Bt* protein content from the tillering stage to seed maturation but with an evident increase at the flowering stage for F_1 hybrids and F_2 progeny.

Discussion

Expression of *Bt Cry1Ac* transgene in interspecific hybrids with GM rice

Results from this study indicate that the *Bt Cry1Ac* transgene can normally express in the F_1 hybrids of common wild rice (*O. rufipogon*) crossed with cultivated GM rice, and in their F_2 progeny. Compared with the level of expression of the *Bt* transgene in the cultivated GM rice parents, the amount of Cry1Ac protein detected in leaf and stem samples was generally high, although considerable

variation (0.0075–0.22% in leaves and 0.039–0.96% in stems) was observed among individuals. The similar amount of Cry1Ac protein detected in the interspecific hybrids with GM rice is more likely attributed to the GM promoter that can maintain its normal function stably in the genetic background of common wild rice. This indicates that a *Bt* transgene incorporated into common wild rice populations will express the same as in its GM rice parents. Given that common wild rice and cultivated rice are closely related (Lu et al. 2002), normal function of the introgressed transgene into wild rice background is plausible. The level of Cry1Ac protein in the experimental materials was determined based on the analysis of more than 300 individuals during their entire growth period, which should be highly reliable for addressing the relevant questions of *Bt* transgene expression in wild rice.

The expression level of the *Bt* transgene did not show significant differences among cultivated GM rice and the hybrid progeny in general, and the significantly higher content of Cry1Ac protein detected in a few hybrid lines at the flowering stage was probably caused by the change of flowering habit in the hybrid lines influenced by wild rice (cultivated GM rice was sampled on 6 August and hybrid lines on 29 August). The significantly lower Cry1Ac protein content in hybrid lines detected at seed maturity was possibly caused by sampling time, as wild materials were collected at very late growth period because the hybrid lines matured much later than the cultivated GM rice lines. There is no clear indication that individuals with homozygous transgenes (i.e., *BtBt*, in GM parents) had significantly higher expression than individuals with hemizygous transgene (i.e., *Bt_*, in F_1 hybrids). This result is very similar to those observed in *Brassica napus* and its wild relatives *Brassica rapa* (Halfhill et al. 2002; Zhu et al. 2004), in which individuals with homozygous or hemizygous *Bt* transgene had the same expression level of Bt Cry1Ac toxin. This finding indicates that a single version of *Bt* transgene will be sufficient to affect the lepidopteran population in wild rice.

ELISA analyses from this experiment clearly showed that Cry1Ac toxin levels in interspecific hybrids were comparable with those in the GM rice lines. This suggests that the target lepidopteran insects might be strongly affected in wild rice populations that have picked up the *Bt* transgene. Interestingly, the content of the Cry1Ac toxin was much higher in stems than in leaves at the comparable stages, indicating potentially a much more powerful effect of the Cry1Ac toxin to the targeted rice stem borers that commonly feed on wild rice (Cohen et al. 2008). Previous studies have indicated that an equivalent amount of 0.01% Cry1Ac toxin of the total soluble protein is sufficient to cause significant mortality on target lepidopteran insects (rice yellow stem borers, *S. incertulas*) (Alam et al. 1998). Another study also showed that Bt Cry1Ac toxin could cause 80–90% mortality of the first-instar larvae of rice yellow stem borers when the toxin level reached 0.013–0.024% in a cut-stem feeding assay (Nayak et al. 1997). However, a higher amount of Cry1Ac toxin (~0.1%) was necessary to be a serious hindrance to the growth and survival of rice striped stem borers (*Chilo suppressalis*) and leaffolders (*Cnaphalocrocis medinalis*), based on the study of field investigations and feeding assays with leaf and stem tissues (Cheng et al. 1998; Maqbool et al. 2001; Han et al. 2006). These results suggest the effective concentration of Cry1Ac protein to kill most target lepidopteran larvae, but the lethal concentration is largely variable (0.01–0.1%) for various insects from different experiments. Based on the results, we can predict that the average amount of Cry1Ac toxin detected in the interspecific hybrids of GM rice from this study should be sufficient to cause the significant mortality of most, if not all, target lepidopteran insects exposed to the wild rice individuals containing the *Bt* transgene. In other words, if target lepidopteran insects occur in wild rice populations, the amount of *Bt* toxin brought by the recurrent transgene flow from GM rice would make considerable impacts on the target lepidopteran insects in natural habitats. As a consequence, *Bt* toxin triggering the reduction in target lepidopteran populations might promote a rapid growth of wild rice populations that are regulated by the pests (Vacher et al. 2004), resulting in further ecological consequences. However, it is worth pointing out that some larvae that are less sensitive to the Cry1Ac toxin at the concentration may survive and develop into resistant populations (Maqbool et al. 2001).

Furthermore, the evident increase of Cry1Ac protein at flowering stage in interspecific hybrids of GM rice wild rice with may have its significance for wild rice because the increased Cry1Ac toxin level in wild rice plants after the booting stage is beneficial to the production of viable seeds in wild rice populations, resulting in more seed-generated individuals in the next generations. The most serious insect damage by rice lepidopteran pests are usually observed at the later stages of rice growth in the field, due to the major occurrence of lepidopteran insects and the interaction between rice and insects (Shen et al. 2002; Bandong and Litsinger 2005; Muralidharan and Pasalu 2006). This analysis emphasizes the importance of higher toxicity for wild rice to prevent attack by lepidopteran larvae at later growth stages.

Potential accumulation of wild individuals with high level of *Bt* toxin under insect pressure

It is important to point out that the content of Cry1Ac protein showed a great variation at different stages in F_1 hybrids and F_2 progeny, with some individuals showing much higher levels of *Bt* toxin than those in cultivated GM

rice at comparable stages, potentially caused by genetic background effects. The ability of some wild individuals to express high levels of *Bt* transgenes may have its critical roles in causing potential ecological consequences, providing that: (1) *Bt* transgene flow from GM rice varieties occurs recurrently, which will maintain a reasonably high frequency of transgene(s) in a wild rice population, and (2) the *Bt* transgene can bring fitness benefit to wild rice individuals/populations. This is owing to the reason that hypothetically the wild rice individuals maintaining the high dosage of Cry1Ac toxin can uphold selective advantage in the populations. Studies have shown that lepidopteran insects may develop resistance to *Bt* toxin at a moderate dosage (Tabashnik et al. 2003; Liang et al. 2008; Gujar et al. 2004). That is why combining a high-dosage of Bt toxin in GM crops with nearby refuges of non-*Bt* crops is commonly adopted as an effective strategy to delay or stop the development of resistance to *Bt* toxin in target insects (Gould 1998). This indicates that high amount of Cry1Ac protein can kill lepidopteran larvae more effectively, even those who are less sensitive to the toxin (Stewart et al. 1996; Maqbool et al. 2001). In fact, it is difficult for target insect to develop resistance to Bt toxin in the wild populations because wild rice individuals that do not contain the transgene will serve as natural refuges. Wild rice individuals with a greater level of *Bt* toxin or stronger ability to kill lepidopteran larvae may enhance the surviving and competitive abilities of the wild rice individuals that have picked up the *Bt* transgene, thus increasing in frequency in wild populations over time. Experimental studies have shown a considerable amount of gene flow (1–18%) from cultivated rice to common wild rice only in one generation (Song et al. 2003; Wang et al. 2006). A science-based biosafety assessment of *Bt* transgene for its influence on fitness of wild rice individuals and impacts on population dynamics of wild rice will provide useful information for the decision making and deployment of transgenic *Bt* rice to minimize the undesirable ecological risks in rice agro-ecosystems and beyond.

In addition, *Bt* transgene silencing was observed in a few F_2 lines from this experiment. The same phenomenon was also reported in the transgenic hybrids of aspen (*Populus tremuloides*) (Kumar and Fladung 2001), melon (*Cucumis melo*) (Hokanson et al. 1997), and transgenic doubled-haploid tobacco (Liu et al. 2003). There are a number of reasons for transgene silencing in wild recipient plant species, including changes at the gene level and transcription level, and epigenetic reasons (Matzke and Matzke 1995). Although we are not able to relate any of these explanations to the *Bt* transgene silencing found in our experiment, we believe that this phenomenon may not be so important for the overall changes of wild rice populations, as wild individuals that contain a *Bt* transgene without its normal function will not behave differently from normal wild rice.

In conclusion, the ELISA analyses from this experiment demonstrated that the *Bt* transgene (*Cry1Ac*) can normally express in interspecific hybrids between GM rice and common wild rice, with comparable levels of *Bt* toxin in the GM parents, which can create common wild rice individuals with the same level, if not higher, of resistance to the target lepidopteran insects as in cultivated GM rice. Some hybrid individuals showed much higher levels of *Bt* toxin, which may provide an opportunity for these individuals to increase their frequencies in a wild population over time because of the selective advantage of these individuals under a strong insect pressure. In addition, the possible development of resistance to the *Bt* toxin by the target lepidopteran insects under a moderate dosage is a concern. This should draw our attention to the potential ecological impacts caused by transgene flow from GM rice to its wild relatives. However, transgene expression should not be viewed in isolation from the complex environment. Therefore, further studies such as the inheritability of the *Bt* transgene in a wild rice population, the change of fitness due to the presence of a *Bt* transgene in wild rice individuals, and the dynamics of a natural wild rice population that have picked-up the *Bt* transgene, should be conducted with solid scientific methodologies to improve our understanding of transgene escape and its relevant ecological consequences for biosafety assessment.

Acknowledgments This research was supported by the Ministry of Science and Technology (Grant no. 2007CB109202), Natural Science Foundation of China (Grant nos. 2007ZD001 and 30570341) and Ministry of Agriculture (Grant no. 2008ZX08011-006).

References

Adamczyk JJ, Sumerford DV (2001) Increased tolerance of fall armyworms (Lepidoptera: Noctuidae) to Cry1Ac delta-endotoxin when fed transgenic *Bacillus thuringiensis* cotton: impact on the development of subsequent generations. Fla Entomol 84:1–6

Alam MF, Datta K, Abrigo E, Vasquez A, Senadhira D, Datta SK (1998) Production of transgenic deepwater indica rice plants expressing a synthetic *Bacillus thuringiensis cryIA(b)* gene with enhanced resistance to yellow stem borer. Plant Sci 135:25–30

Alcalde E, Camara M (2007) 10 years of *Bt* maize—the case of Spain. J Biotechnol 131:21–22

Ammitzbøll H, Mikkelsen TN, Jøergensen RB (2005) Transgene expression and fitness of hybrids between GM oilseed rape and *Brassica rapa*. Environ Biosafety Res 4:3–12

Andersen MN, Sausse C, Lacroix B, Caul S, Messean A (2007) Agricultural studies of GM maize and the field experimental infrastructure of ECOGEN. Pedobiologia 51:175–184

Bandong JP, Litsinger JA (2005) Rice crop stage susceptibility to the rice yellow stem borer *Scirpophaga incertulas* (Walker) (Lepidoptera: Pyralidae). Int J Pest Manage 51:37–43

Bashirn K, Husnain T, Fatima T, Riaz N, Makhdoom R, Riazuddin S (2005) Novel indica basmati line (B-370) expressing two

unrelated genes of *Bacillus thuringiensis* is highly resistant to two lepidopteran insects in the field. Crop Prot 24:870–879

Bhattacharya RC, Viswakarma N, Bhat SR, Kirti PB, Chopra VL (2002) Development of insect-resistant transgenic cabbage plants expressing a synthetic *cryIA(b)* gene from *Bacillus thuringiensis*. Curr Sci India 83:146–150

Chen LJ, Lee DS, Song ZP, Suh HS, Lu B-R (2004) Gene flow from cultivated rice (*Oryza sativa*) to its weedy and wild relatives. Ann Bot Lond 93:67–73

Chen LY, Snow AA, Wang F, Lu BR (2006) Effects of insect-resistance transgenes on fecundity in rice (*Oryza sativa*, *Poaceae*): a test for underlying costs. Am J Bot 93:94–101

Cheng XY, Sardana R, Kaplan H, Altosaar I (1998) Agrobacterium-transformed rice plants expressing synthetic *cryIA(b)* and *cryIA(c)* genes are highly toxic to striped stem borer and yellow stem borer. Proc Natl Acad Sci USA 95:2767–2772

Cohen MB, Arpaia S, Lan LP, Chau LM, Snow AA (2008) Shared flowering phenology, insect pests, and pathogens among wild, weedy, and cultivated rice in the Mekong Delta, Vietnam: implications for transgenic rice. Environ Biosafety Res 7:73–85

Darmency H (1994) The impact of hybrids between genetically-modified crop plants and their related species—introgression and weediness. Mol Ecol 3:37–40

Ellstrand NC, Prentice HC, Hancock JF (1999) Gene flow and introgression from domesticated plants into their wild relatives. Ann Rev Ecol System 30:539–563

Gepts P, Papa R (2003) Possible effects of (trans)gene flow from crops on the genetic diversity from landraces and wild relatives. Environ Biosafety Res 2:89–103

Gould F (1998) Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology. Annu Rev Entomol 43:701–726

Gujar GT, Nair R, Singh BP, Kumari A, Kalia V (2004) Toxicity to the cotton bollworm, *Helicoverpa armigera*, of some Cry1Ac toxins expressed in cotton in India. Crop Prot 27:537–544

Hails RS, Morley K (2005) Genes invading new populations: a risk assessment perspective. Trends Ecol Evol 20:245–252

Halfhill MD, Millwood RJ, Raymer PL, Stewart NC (2002) *Bt*-transgenic oilseed rape hybridization with its weedy relative, *Brassica rapa*. Environ Biosafety Res 1:19–28

Halfhill MD, Sutherland JP, Moon HS, Poppy GM, Warwick SI, Weissinger AK, Rufty TW, Raymer PL, Stewart CN (2005) Growth, productivity, and competitiveness of introgressed weedy *Brassica rapa* hybrids selected for the presence of *Bt Cry1Ac* and *gfp* transgenes. Mol Ecol 14:3177–3189

Han LZ, Wu KM, Peng YF, Wang F, Guo YY (2006) Evaluation of transgenic rice expressing *Cry1Ac* and *CpTI* against Chilo suppressalis and intrapopulation variation in susceptibility to *Cry1Ac*. Environ Entomol 5:1453–1459

Han LZ, Wu KM, Peng YF, Wang F, Guo YY (2007) Efficacy of transgenic rice expressing *Cry1Ac* and *CpTI* against the rice leaffolder, *Cnaphalocrocis medinalis* (Guenee). J Invertebr Pathol 96:71–79

Hellmich RL, Siegfried BD, Sears MK, Stanley-Horn DE, Daniels MJ, Mattila HR, Spencer T, Bidne KG, Lewis LC (2001) Monarch larvae sensitivity to *Bacillus thuringiensis*-purified proteins and pollen. Proc Natl Acad Sci USA 98:11925–11930

High SM, Cohen MB, Shu QY, Altosaar I (2004) Achieving successful deployment of *Bt* rice. Trends Plant Sci 9:286–292

Hilder VA, Boulter D (1999) Genetic engineering of crop plants for insect resistance—a critical review. Crop Prot 18:177–191

Hokanson SC, Hancock JF, Grumet R (1997) Direct comparison of pollen-mediated movement of native and engineered genes. Euphytica 96:397–403

Huang JK, Hu RF, Rozelle S, Pray C (2005) Insect-resistant GM rice in farmers' fields: assessing productivity and health effects in China. Science 308:688–690

James C (2006) Global status of commercialized biotech/GM crops: 2006. ISAAA Brief No 35. ISAAA, Ithaca

Khanna HK, Raina SK (2002) Elite Indica transgenic rice plants expressing modified Cry1Ac endotoxin of Bacillus thuringiensis show enhanced resistance to yellow stem borer (*Scirpophaga incertulas*). Transgenic Res 11:411–423

Kumar S, Fladung M (2001) Gene stability in transgenic aspen (*Populus*). II. Molecular characterization of variable expression of transgene in wild and hybrid aspen. Planta 213:731–740

Liang GM, Wu KM, Yu HK, Li KK, Feng X, Guo YY (2008) Changes of inheritance mode and fitness in *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) along with its resistance evolution to Cry1Ac toxin. J Invertebr Pathol 97:142–149

Liu YS, Levin JS, Murray JS, Wernsman EA, Weissinger AK (2003) Multi-generation analysis of the stability of transgenic virus resistance in doubled-haploid tobacco lines. Mol Breeding 12:145–156

Lu B-R, Snow AA (2005) Gene flow from genetically modified rice and its environmental consequences. BioScience 55:669–678

Lu B-R, Zheng KL, Qian HR, Zhuang JY (2002) Genetic differentiation of wild relatives of rice as referred by the RFLP analysis. Theor Appl Genet 106:101–106

Maqbool SB, Riazuddin S, Thi-Loc N, Gatehouse AMR, Gatehouse JA, Christou P (2001) Expression of multiple insecticidal genes confers broad resistance against a range of different rice pests. Mol Breeding 7:85–93

Matzke MA, Matzke AJM (1995) How and why do plants inactivate homologous (Trans) genes? Plant Physiol 107:679–685

Muralidharan K, Pasalu IC (2006) Assessments of crop losses in rice ecosystems due to stem borer damage (Lepidoptera: Pyralidae). Crop Prot 25:409–417

Naredo MEB, Juliano AB, Lu B-R, Jackson MT (1997) Hybridization of AA genome rice species from Asia and Australia.I. Crosses and development of hybrids. Genet Resour Crop Evol 44:17–23

Nayak P, Basu D, Das S, Basu A, Ghosh D, Ramakrishnan NA, Ghosh M, Sen SK (1997) Transgenic elite indica rice plants expressing Cry1Ac delta-endotoxin of *Bacillus thuringiensis* are resistant against yellow stem borer (*Scirpophaga incertulas*). Proc Natl Acad Sci USA 94:2111–21167

Poppy G (2000) GM crops: environmental risks and non-target effects. Trends Plant Sci 5:4–6

Pray C, Huang J, Hu R, Rozelle S (2002) Five years of Bt cotton in China—the benefits continue. Plant J 31:423–430

Qin CT, Chen ZJ, Su J, Wang F (2003) Comparison among three ELISA methods for quantification of Cry1Ac in transgenic rice. Fujian J Agric Sci 18:258–263 (in Chinese)

Riches CR, Valverde BE (2002) Agricultural and biological diversity in Latin America: implications for development, testing, and commercialization of herbicide-resistant crops. Weed Technol 16:200–214

Rong J, Song ZP, Su J, Xia H, Wang F, Lu B-R (2005) Low frequency of transgene flow between Bt/CpTI rice lines and non-transgenic controls planted at close spacing. New Phytol 168:559–566

Scott A, Woodfield D, White DWR (1998) Allelic composition and genetic background effects on transgene expression and inheritance in white clover. Mol Breeding 4:479–490

Shen JX, Zhang HQ, Zhang SM, Dong GK (2002) Damage and occurrence analysis of *Chilo suppressalis* in late rice field. Entomol Knowl 39:110–113 (in Chinese)

Sims SR, Berberich SA (1996) *Bacillus thuringiensis* CryIA protein levels in raw and processed seed of transgenic cotton: determination using insect bioassay and ELISA. J Econ Entomol 89:247–251

Snow AA (2002) Transgenic crops—why gene flow matters. Nat Biotechnol 20:542

Snow AA, Palma PM (1997) Commercialization of transgenic plants: potential ecological risks. BioScience 47:86–96

Song ZP, Lu B-R, Zhu YG, Chen JK (2003) Gene flow from cultivated rice to the wild species *Oryza rufipogon* under experimental field conditions. New Phytol 157:657–665

Stewart CN, Adang MJ, All JN, Raymer PL, Ramachandran S, Parrott WA (1996) Insect control and dosage effects in transgenic canola containing a synthetic *Bacillus thuringiensis crylAc* gene. Plant Physiol 112:115–120

Stoscheck CM (1990) Quantitation of protein. Methods Enzymol 182:50–68

Tabashnik BE, Carriere Y, Dennehy TJ, Morin S, Sisterson MS, Roush RT, Shelton AM, Zhao JZ (2003) Insect resistance to transgenic *Bt* crops: lessons from the laboratory and field. J Econ Entomol 96:1031–1038

Takahashi Y, Hori H, Furuno H, Kawano T, Takahashi M, Wada Y (1998) Enzyme-linked immunosorbent assays for rapid and quantitative detection of insecticidal crystal proteins of Bt pesticides. J Pest Sci 23:386–391

Tu JM, Zhang GA, Datta K, Xu CG, He YQ, Zhang QF, Khush GS, Datta SK (2000) Field performance of transgenic elite commercial hybrid rice expressing *Bacillus thuringiensis* delta-endotoxin. Nat Biotechnol 18:1101–1104

Vacher C, Weis AE, Hermann D, Kossler T, Young C, Hochberg ME (2004) Impact of ecological factors on the initial invasion of *Bt* transgenes into wild populations of birdseed rape (*Brassica rapa*). Theor Appl Genet 109:806–814

Vaughan DA, Kadowaki K, Kaga A, Tomooka N (2005a) On the phylogeny and biogeography of the genus *Oryza*. Breed Sci 55:113–122

Vaughan DA, Sanchez PL, Ushiki J, Kaga A, Tomooka N (2005b) Asian rice and weedy rice—evolutionary perspectives. In: Gressel J (ed) Crop ferality and volunteerism. CRC Press, Boca Raton, pp 257–277

Wang YQ, Johnston S (2007) The status of GM rice R and D in China. Nat Biotechnol 25:717–718

Wang F, Yuan QH, Shi L, Qian Q, Liu WG, Kuang BG, Zeng DL, Liao YL, Cao B, Jia SR (2006) A large-scale field study of transgene flow from cultivated rice (*Oryza sativa*) to common wild rice (*O*-rufipogon) and barnyard grass (*Echinochloa crusgalli*). Plant Biotechnol J 4:667–676

Xiong L (2004) China could be first nation to approve sale of GM rice. Science 306:1458–1459

Zhu B, Lawrence JR, Warwick SI, Mason P, Braun L, Halfhill MD, Stewart CN (2004) Stable *Bacillus thuringiensis* (*Bt*) toxin content in interspecific F-1 and backcross populations of wild *Brassica rapa* after *Bt* gene transfer. Mol Ecol 13:237–241