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Full Length Research Paper

Detection of unintended effects in genetically modified herbicide-tolerant (GMHT) rice in comparison with non-target phenotypic characteristics

Xianbin Jiang [1, 2, 3] and Guoying Xiao [1*]

[1]Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha 410125, Hunan, China
[2]Rice Research Institute, Guangxi Academy of Agricultural Sciences, Nanning 530007, Guangxi, China.
[3]Graduate University of Chinese Academy of Sciences, Beijing 100049, China.

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In the process of safety assessments of genetically modified (GM) crops, particular attention should be paid to their unintended effects. This study was focused on detection of unintended effects in genetically modified herbicide-tolerant (GMHT) rice (*Oryza sativa* L. ssp. *indica*) Bar68 -1 in comparison with non-target phenotypic characteristics between Bar68 -1 and its unmodified counterpart, D68. The results showed that there were no significant differences between Bar68 -1 and D68 in most of the morphological or agronomic traits, including main culm length, main culm thickness, flag leaf length, tillering dynamics, seed setting, 1000-grain weight, grain length, grain width, length-width ratio of grain, brown rice length, brown rice width, length-width ratio of brown rice, grain yield, biomass of aerial part, yield, rate of seed germination and ratooning. There were statically significant differences between Bar68-1 and D68 in the parameters such as flag leaf width, spikelets per panicle, panicle length and harvest index, but the values of the changed traits are within rice normal ranges and in the same scales according to GB/T 19557.7 - 2004 from Chinese standard and SES from IRRI. Therefore, these differences were not considered biologically relevant. Results indicated that the unintended effects were not detected in Bar68-1 in this study.

Key words: Unintended effects, safety assessment, biosafety, GM crops, herbicide tolerance, transgenic plant, rice (*Oryza sativa* L.), bar gene.

INTRODUCTION

The main objective of safety assessment of GM plants and their products is to make it clear that whether GM foods are as safe as their traditional counterparts and whether they will introduce any added or new risks to the health of consumers (Cellini et al., 2004). In the process of these safety assessments, particular attention should be paid to unintended effects of GM foods, especially to their long-term and potential impacts on human health (Deng et al., 2008). The "unintended effects" in GM crops and their products have been defined as "in achieving the objective of conferring a specific target trait (intended effect) to the host organism by the insertion of defined DNA sequences, additional traits could, theoretically, be acquired or existing traits lost (unintended effects)" (FAO/WHO, 2000). Unintended effects may be caused by the process of genetics engineering for GM plants (Haslberger, 2003). If the gene introduced has pleiotropic effects concerning with functional gene coding region or regulatory elements, unintended effects may happen in that case, and bar gene has pleiotropic effects (Miki et al., 2009).

Targeted compositional analysis has been applied to detect unintended effects and the research results showed that differences between some GM potatoes and controls were statistically significant, while these differences appeared to be random and not associated with any specific construct, which revealed no consistent

*Corresponding author. E-mail: xiaoguoying@isa.ac.cn. Tel: +86-731-84619770. Fax: +86-731-84612685.

Abbreviations: GM, Genetically modified; **GMHT,** genetically modified herbicide-tolerant; **SES,** standard evaluation system for rice; **IRRI,** international rice research institute.

Table 1. Arrangement of field experiments (2006 - 2008).

Year	Sowing date	Transplant date	Harvest date	Paddy field area (m^2)	Plot numbers per variety	Plot area (m^2)	Hill space (cm)
2006FH	Mar 25	Apr 20	July 10	600	3	40.0	16.67 × 20
2006SH	July 5	July 25	Oct 13	480	2	31.6	16.67 × 20
2007	May 18	June 5	Aug 29	2145	4	120.0	20 × 20
2008	May 25	June 20	Sept 5	2145	4	120.0	20 × 20

Notes: 2006FH means first harvest season in 2006; 2006SH means second harvest season in 2006.

differences between GM and non-GM potato (Shepherd et al., 2006). As for this current method of targeted compositional analysis, concerns have been raised for it is biased and does not consider the possibility of unintended effects which could result from the genetic modification (Cellini et al., 2004). The analysis of non-target traits can increase the chances of detecting unintended effects; for it investigates the physiology of the GM plants as comprehensively as possible without statistical bias (Rischer and Oksman-Caldentey, 2006). The unintended effects of genetically modified herbicide-tolerant (GMHT) rice Bar68 -1 with bar gene were analyzed in comparisons with non-target phenotypic characteristics in this paper. It will provide useful information for safety assessment of this GMHT crop and reduce the uncertainties. And it will provide a new approach to detect the unintended effects.

MATERIALS AND METHODS

Materials

GMHT rice Bar68 -1, an *indica* rice variety with *bar* gene resistant to glufosinate, was developed from eight transgenic lines transformed by particle bombardment (Xiao et al., 2007). An *indica* rice cultivar D68, donor plant of Bar68-1, was served as the control (CK).

Experimental design and crop management

Experiments were carried out in Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha, Hunan, China (28.20N, 113.08E) in 2006, 2007 and 2008 with approval documents of No. 2005-095 and No. 2006-060. A regular field management practice was applied as described earlier (Xiao et al., 2006). Field experimental arrangement was listed in Table 1. Every field is composed of two parts, one for planting GMHT rice and the other for the non-transgenic control.

Sampling and measurement

Observation of the traits followed national standards of China GB/T 19557.7 - 2004 (Chinese Standard, 2004) and the Standard Evaluation System for Rice (SES) from International Rice Research Institute (IRRI, 2002). In addition, tillering dynamics were investigated by sampling 60 hills and then calculated the average of tillers per plant; ratoon ability was expressed by ratooning rate (R). R = (tillers per plant in ratoon season)/ (tillers per plant in seedling season).

Statistical analysis

By using SPSS 15.0 statistical program (SPSS Inc., Chicago, IL, USA), two-tailed t-test with α = 5% for each comparison was performed to compare means between GMHT and non-GM rice groups. Differences between means were considered significant for $p<0.05$. Before analysis, the data of seed setting rate and seed germination percentage were normalized by arcsine transformation. Variability around the mean was represented as ± standard error of sample mean (SE).

RESULTS

The data of economic and morphological characteristics of Bar68 - 1 and D68 were provided in Table 2. No statistically significant differences were found between Bar68 -1 and D68 in non-target traits, which include plant height, main culm length, main culm thickness, flag leaf length, panicles per plant, seed setting, 1000-grain weight, grain length, grain width, grain length-width ratio, brown rice length, brown rice width, brown rice length-width ratio, grain yield, biomass of aerial part and yield. The flag leaf width, spikelets per panicle, panicle length and harvest index had shown statistically significant variations between Bar68-1 and D68, while they were within the same scales according to GB/T 19557.7-2004 (Chinese Standard, 2004) and SES (IRRI, 2002). Comparisons between GMHT rice Bar68-1 and the control in different years or different growing seasons showed that non-target phenotypic characteristics were not significantly different except for panicle length in the first harvest of 2006 (2006FH) and the second harvest of 2006 (2006SH), panicles per plant in 2006SH, and spikelets per panicle in 2006FH.

Table 2. Comparisons of non-target traits of Bar68-1 and that of D68 (2006-2008).

Traits	df	Mean ± S. E.		Mean difference	T	*P*-value [a]	95% CI	SES scale [b]		GB scale [c]	
		Bar68-1	D68 (CK)					Bar68-1	D68	Bar68-1	D68
Plant height (cm)	241	84.5±0.6	86.1±0.7	-1.6	-1.747	0.082	-3.4 to 0.2	1	1	-	-
Main culm length(cm)	196	60.8±0.5	60.8±0.6	0.0	-0.056	0.955	-1.6 to 1.5	-	-	3	3
Main culm thickness (mm)	38	4.9±0.1	4.8±0.1	0.1	0.097	0.924	-0.2 to 0.3	-	-	5	5
Flag leaf length(cm)	187	26.5±0.5	27.7±0.6	-1.2	-1.608	0.110	-2.6 to 0.3	-	-	5	5
Flag leaf width(cm)	38	1.08±0.02	1.15±0.02	-0.07	-2.082	0.044*	-0.13 to -0.002	-	-	5	5
Panicles per plant	331	13.5±0.3	12.8±0.3	0.7	1.503	0.134	-0.2 to 1.6	5	5	5	5
Spikelets per panicle	241	118.0±2.3	126.0±2.4	-8.0	-2.431	0.016*	-14.5 to -1.5	-	-	5	5
Panicle length (cm)	241	23.9±0.2	24.6±0.2	-0.7	-2.638	0.009**	-1.3 to -0.2	-	-	5	5
Seed setting (%)	241	80.03±1.07	81.62±1.01	-1.59	-1.077	0.283	-4.51 to 1.32	3	3	7	7
1000-grain weight (g)	96	18.700±0.404	18.967±0.225	-0.267	-0.578	0.565	-1.185 to 0.650	-	-	1	1
Grain length(mm)	116	9.59±0.06	9.55±0.07	0.04	0.417	0.678	-0.15 to 0.23	-	-	7	7
Grain width (mm)	116	2.74±0.05	2.73±0.05	0.01	0.210	0.834	-0.12 to 0.15	-	-	3	3
Grain length-width ratio	116	3.56±0.07	3.57±0.07	-0.01	-0.131	0.896	-0.21 to 0.19	-	-	4	4
Brown rice length(mm)	116	6.68±0.08	6.77±0.06	-0.09	-0.959	0.340	-0.29 to 0.10	3	3	7	7
Brown rice width(mm)	116	2.26±0.04	2.28±0.04	-0.02	-0.391	0.697	-0.13 to 0.19	-	-	3	3
Brown rice length-width ratio	116	3.00±0.07	3.03±0.07	-0.03	-0.259	0.796	-0.22 to 0.17	1	1	-	-
Grain yield (g $plant^{-1}$)	156	22.8±0.9	21.5±0.8	1.3	1.054	0.293	-1.1 to 3.7	-	-	-	-
Biomass of aerial part (g $plant^{-1}$)	156	36.9±1.4	35.5±1.2	1.4	0.756	0.451	-2.2 to 5.0	-	-	-	-
Harvest index	156	0.62±0.00	0.60±0.01	0.02	2.015	0.046*	0.0004 to 0.04	-	-	-	-
Yield (kg/hm^2)	45	5536.8±183.0	5473.5±244.8	63.3	0.210	0.835	-543.4 to 670.0	-	-	-	-

CI: Confidence interval
* 5% significant level
** 1% significant level
[a] *P*-values from independent samples t-test (two-tailed)
[b] SES scale means scale values determined by Standard Evaluation System for Rice (SES) (IRRI, 2002)
[c] GB scale means scale values determined by guidelines for the conduct of tests for distinctness, uniformity and stability — Rice (*Oryza sativa* L.) (Chinese Standard, 2004).

These characteristics include plant height, panicle length, panicles per plant, spikelets per panicle, yield and seed germination (Figure 1). The mean values of panicle length for Bar68 - 1 and D68 were 22.0 and 23.0 in the first harvest of 2006 (2006FH) respectively and 22.5 (Bar68 -1), 23.7

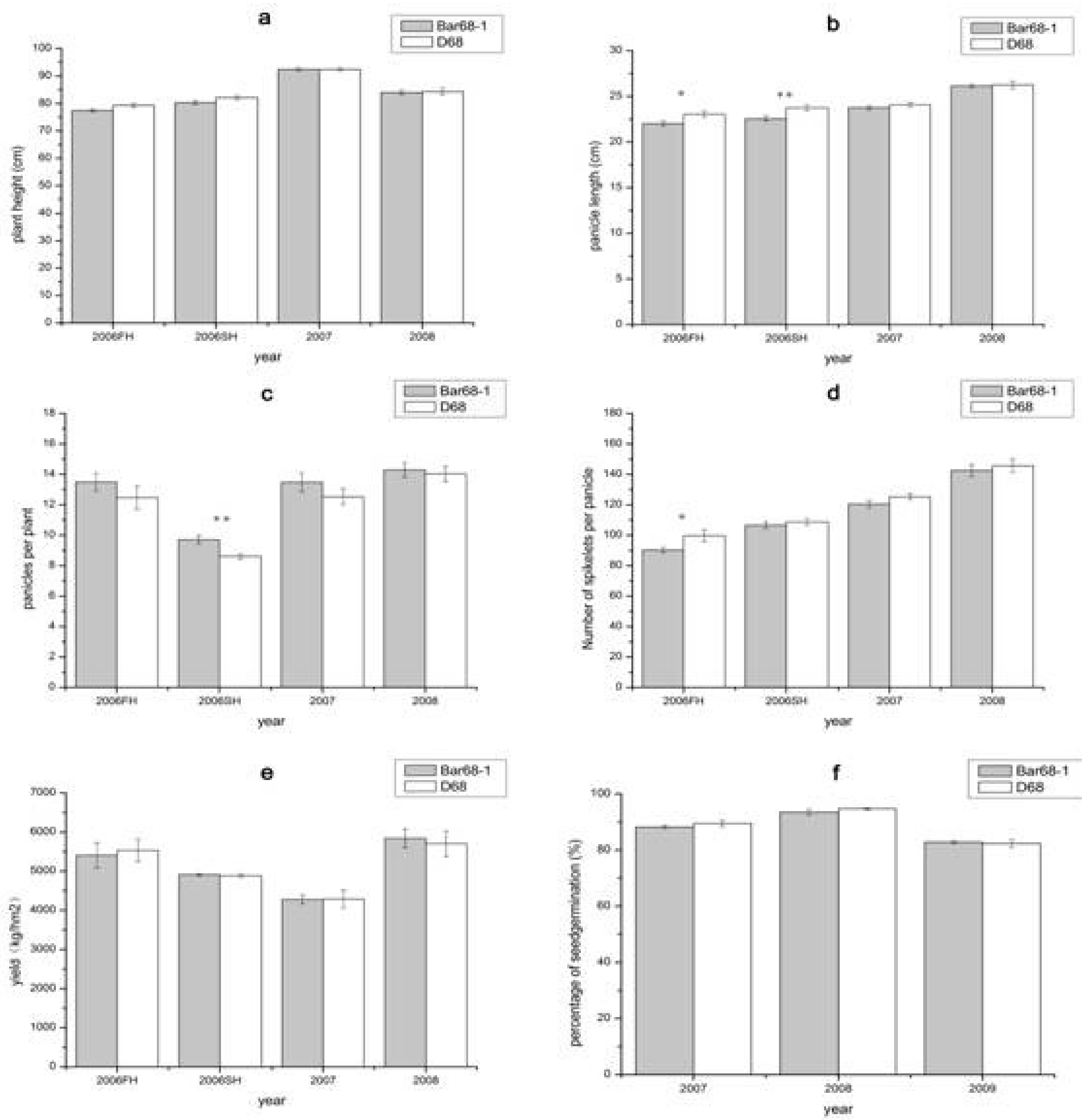

Figure 1. Comparisons of non-target phenotypic characteristic between GMHT rice Bar68 -1 and non-GM rice D68
a-f represent phenotypic characteristics of rice in the order of plant height, panicle length, panicles per plant, spikelets per panicle, yield, seed germination. * or ** means significant differences at 5% or 1% level.

(D68) in the second harvest of 2006 (2006SH), GB scale for each of them was 5; panicles per plant in 2006SH were 9.7 (Bar68 - 1) and 8.6 (D68), and GB scales for them were 3 (Bar68 - 1), 3 (D68), GB scales were 7 (Bar68 - 1), 7 (D68); spikelets per panicle in 2006FH were 90.3 (Bar68 - 1) and 99.7 (D68), and GB scales were3 (Bar68 -1), 3 (D68). SES scale means scale values determined by Standard Evaluation System for Rice (SES) (IRRI, 2002); GB scale means scale values determined by guidelines for the conduct of tests for distinctness, uniformity and stability - rice (*Oryza sativa* L.) (Chinese Standard, 2004). Differences of ratooning rate between Bar68 -1 and D68 were not significant at every sample time in 2007 and 2008 (Figure 2), and so was the tillering dynamics (Figure 3).

DISCUSSION

Most key nutrients and anti-nutrients in GMHT rice Bar68 -1 have the same value as those in non-GM counterpart D68 except iron and vitamin E, and the values of iron and vitamin E in Bar68 -1 rice were within established reference ranges for rice grains (Li et al., 2008). And

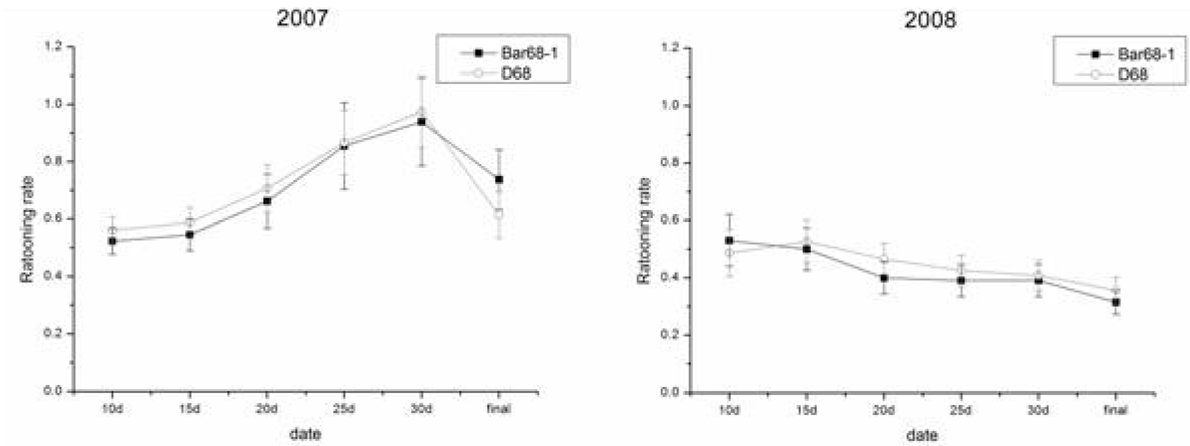

Figure 2. Ratooning dynamics of Bar68-1 and D68.

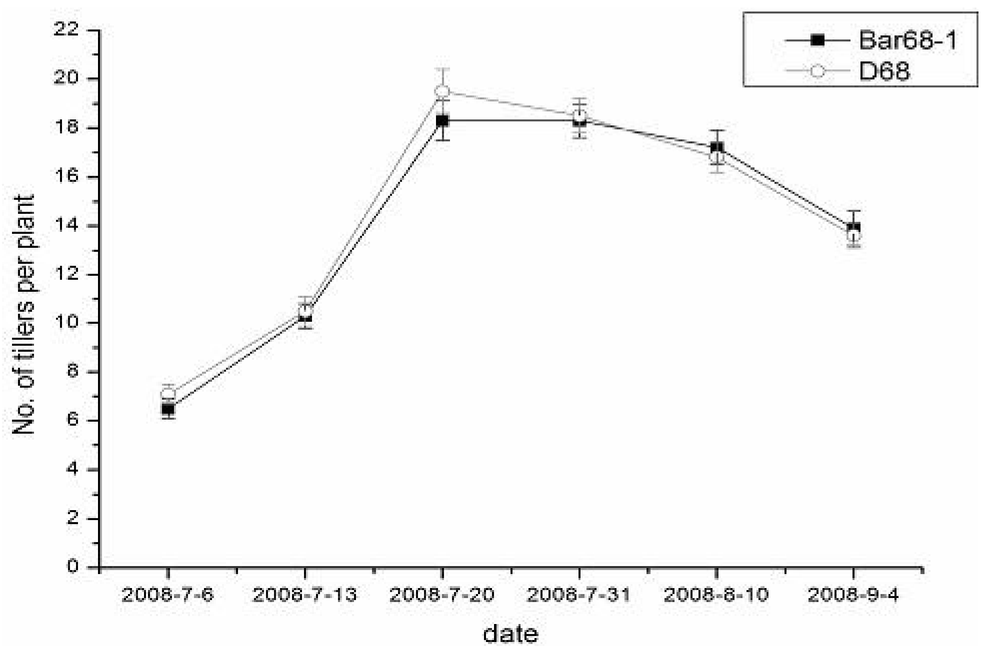

Figure 3. Tillering dynamics of Bar68-1 and D68 (2008).

nutritional components of GMHT rice LLRICE62 were equivalence to its control too (Oberdoerfer et al., 2005). If only important nutrients and specific anti-nutritional compounds or intermediates analyzed, unintended effects of GM crops would not be detected (Jelenic, 2005; Li et al., 2005). Therefore, more ways are needed to detect the unintended effects of GM crops. The data in this paper showed that there were no significant differences in most of the traits between Bar68 -1 and D68. There were statistically significant variations in the traits of Bar68 - 1 when compared with those of D68, but mean values of the changed traits were within rice normal ranges and within the same scales determined by GB/T 19557.7 - 2004 (Chinese Standard, 2004) and SES (IRRI, 2002). Therefore, these differences were not considered biologically relevant. In other words, unintended effects in GMHT rice Bar68 -1were not detected in this study. These results were similar to experiment of photo-synthetic characteristics of insect-resistant transgenic cotton (*Gossypium hirsutum* L.), which showed that there were no obvious differences in the shapes of stomatal conductance between GM cotton and non-GM cotton, and significant difference in the shapes of net photo-synthetic rate did occur but it could not be detected in all the growing seasons (Sun et al., 2009).

In the cultivation of GM crops, phenotypic or agronomic traits of the transgenic line are evaluated through selection, which will result in elimination of most of the candidate varieties (NRC, 2004). More than 99% of the specimens produced in process of plant breeding are discarded (Chassy, 2009). This selection process takes many years and removes major unintended effects (Cellini et al., 2004). In this study, unintended effects were not detected in GMHT rice Bar68 -1. There would be three reasons for that: 1) unintended effects did not occur in this event of transformation; 2) it occurred, but were removed in subsequent breeding process; 3) it occurred, but more methods are needed to increase the effectiveness of unintended effects detection. Incidentally, conclusion would be more objective and comprehensive if it's drawn from years of experimental results than just from one year data. Some inappropriate conclusions may be drawn from one year data for the traits such as spikelets per panicle, panicles per plant, or panicle length in our study. Profiling techniques such as genomics, proteomics, and metabolomics might increase the chances of detecting unintended effects (Noteborn et al., 2002). Most of transcriptome profiles of transgenic plant MON810 were the same as those in near-isogenic varieties compared by microarray when plants are grown in the field, but there were differences when plants are cultured *in vitro* (Coll et al., 2009). A proteomics approach was applied to detect the unintended effects of GM plants and 102 significantly altered protein spots were detected (Ren et al., 2009). Proteomic profiling of 550 - 600 proteins tested in two-dimensional gels to detect unintended effects in GM potato, and most of them were found at similar levels except for 21 proteins (Khalf et al., 2010). Metabolic profiles of transgenic rice with *Cry*1Ac and *SCK* genes were compared to wild type to detect unintended effects, and significant differences were found in sucrose, mannitol and glutamic acid (Zhou et al., 2009). While, there was an imperfection for the "omics" technologies, interpretation of the data obtained is rather difficult, so that their power in the detection of unintended effects is currently limited (Cellini et al., 2004). Detection of variation in non-target phenotypic characteristics, the method used in this article has some advantages: (1) it is simple and easy to use; (2) it is clear and easy to read the test results; (3) it can be processed during the work of GM crops breeding, no additional work is needed.

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