Differential responses of leaf water-use efficiency and photosynthetic nitrogen-use efficiency to fertilization in Bt-introduced and conventional rice lines

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Abstract

Leaf stomatal conductance (g_s), transpiration rate (E), and light-saturated net photosynthetic rate (P_{Nmax}) at three developmental stages (tillering stage, jointing-booting stage, and milking stage) and leaf total nitrogen concentration (LTNC) and δ^{13}C value at milking stage were measured for a conventional rice line (Minghui 63) and its corresponding *Bacillus thuringiensis* (Bt)-gene [*cry1A* (*b* and *c*)] introduced line (Bt line) under three fertilizer levels. Compared to conventional line, Bt line showed lower g_s, which was associated with lower P_{Nmax} and E, but instantaneous water-use efficiency (WUE), measured as the ratio of P_{Nmax} to E, was higher in the Bt line than in the conventional line, particularly in the jointing-booting stage. However, δ^{13}C values were not significantly different across treatments, suggesting that intrinsic water-use efficiency (WUE$_{in}$) might be indistinguishable between Bt and conventional lines. LTNC was higher but P_{Nmax} was lower in Bt line compared to conventional line, resulting in significantly lower photosynthetic nitrogen-use efficiency (PNUE). This might result from the additional cost of producing Bt protein in the Bt line due to the effect of competing nitrogen with photosynthetic machinery. Bt-gene introduction and expression does not significantly change WUE$_{in}$ but may significantly decrease leaf PNUE. Thus we suggest that Bt rice should be carefully examined in relation to environmental risks (*e.g.* water-body pollution) before planting commercially.

Additional key words: *Bacillus thuringiensis* rice, δ^{13}C, instantaneous water-use efficiency, intrinsic water-use efficiency, photosynthetic nitrogen-use efficiency, stomatal conductance.

Introduction

In 1996, genetically modified plants (GMPs) were commercially employed for the first time. By 2009, 14 million farmers from 25 countries accepted and cultivated GMPs with total planted area of 134 million ha (James 2010). GMPs have shown their substantial agricultural, economic, and environmental benefits with enhanced crop production, farmer earnings and decreased pesticide usage (James 2010). However, the development and planting of GMPs, especially those lines that express alien toxins such as Bt (*Bacillus thuringiensis*) protein, have led to broad concerns about the associated ecological and environmental risks.

Although the control of Bt toxin over target insect populations and the positive effect on crop production (Pray *et al.* 2002, Qaim and Zilberman 2003) have been well documented in previous studies (Felke *et al.* 2002, Carriere *et al.* 2003, Marvier *et al.* 2007), recent research indicates that outbreaks of nontarget insect species may dramatically decrease crop production (Lu *et al.* 2010). Transgenic Bt crops have also extensively been demonstrated to affect soil biota (Sims and Martin 1997, Watrud and Seidler 1998, Saxena and Stotzky 2000, Motavalli *et al.* 2004, Li *et al.* 2009) and gene flow between species or populations (Quist and Chapela 2001, Hanson *et al.* 2005, Ortiz *et al.* 2005, Pineyro *et al.* 2009), with substantially adverse consequences to ecosystem functioning.

Nevertheless, it is not clear whether Bt-introduction

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Abbreviations: Bt – *Bacillus thuringiensis*; E – transpiration rate; g_s – stomatal conductance; LTNC – leaf total nitrogen concentration; P_{Nmax} – light-saturated net photosynthetic rate; PAR – photosynthetic active radiation; PNUE – photosynthetic nitrogen-use efficiency; RH – air relative humidity; WUE – instantaneous water-use efficiency; WUE$_{in}$ – intrinsic water-use efficiency; δ^{13}C – the difference in ^{13}C/^{12}C ratio between sample material and standard material.

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affects the efficiency of water and nutrient use in crops, despite this issue being of great relevance to many countries. For example, in China, rice constitutes 24.02% of total arable land area (29.24 million ha) and represents 36.30% of the total food yield in 2008 (State Statistical Bureau 2009). Moreover, China is facing moderate water shortages (Jiang 2010); agricultural water use accounts for 62% of national total annual water consumption and experiencing continued and severe shortage. Out of a nationwide 121.72 million ha of the arable land, only 58.47 million ha can be effectively irrigated (State Statistical Bureau 2009, Jiang 2010). Therefore, if WUE_{in} of rice is negatively affected by the introduction of the Bt gene, water-deficiency is likely to worsen. Similarly, if nutrient-use efficiency declines due to the introduction of Bt gene, more chemical fertilizers may have to be used to maintain present rice yield, which would, in turn, potentially intensify environmental pollution around farmlands. In today's China, the issue of water quality has become unprecedentedly serious. This is to be attributed primarily to eutrophication of waterbodies, especially within major grain-producing areas such as the Yangtze and the Zhujiang Deltas (*e.g.* Xie *et al.* 2008).

China has vowed to release Bt-rice for commercial use, despite the fact that Bt-gene introduction might affect resource-use efficiency. The expression of the Bt protein consumes additional resources that could be used by crops for plant growth and reproduction and thus tends to lower efficiencies relative to conventional crops. In addition, Bt might change the interactions between crop plants and microbes, including ectomycorrhizal and endomycorrhizal fungi, which may further alter the plants' water/nutrient absorptive ability (Giovannetti *et al.* 2005, Liu and Du 2008, Liu 2010). Last, the Bt gene might unpredictably affect the expression of the genes responsible for plant water and nutrient-use efficiency by changing gene expression levels, expression timing and/or enzymatic activity (*sensu* Ren *et al.* 2004, Yang *et al.* 2006, Wu *et al.* 2008). Unfortunately, empirical work addressing the effects of Bt-introduction on crop water-/ nutrient-use efficiency is still lacking.

WUE_{in} and nitrogen-use efficiency can be examined at the leaf level in transgenic rice. Leaves constitute the plant organ completing the processes of both water transpiration and CO_2 sequestration. The ratio of photosynthetic rate to E is often used to characterize WUE. Similarly, leaf PNUE can be characterized as the ratio of photosynthetic rate to leaf nutrient concentration. However, since crop lifetime efficiency cannot be derived from WUE calculations, WUE_{in} should be considered. Carbon 13 isotope composition ($\delta^{13}C$) has been widely used to characterize the WUE_{in} because it is associated with the ratio of CO_2 partial pressure inside (P_i) and outside the leaves (P_{amb}). Previous studies have extensively demonstrated a positive correlation between the WUE_{in} and $\delta^{13}C$ (Farquhar *et al.* 1982, 1989).

The primary aim of this study is to test whether Bt-gene introduction affects WUE_{in} and nitrogen-use efficiency in the Bt-transferred rice line (Bt Minghui 63) recently allowed for commercial use. We achieve these goals by measuring leaf P_{Nmax}, E, LTNC, and $\delta^{13}C$ and deriving estimates of WUE, PNUE, and WUE_{in}.

Materials and methods

Rice plantation: Experiments with rice plantation and field measurements were conducted at the Gulou Campus (118°46′E, 32°3′N), Nanjing University, Eastern China, in 2009. The study site is within the Northern subtropical monsoon climate zone. Mean annual temperature is 16.1°C, with monthly means in January and July reaching 1.5°C and 29.0°C, respectively. Mean annual precipitation is 975 mm, more than 70% of which occurs in summer, between June and August.

The experiment was started at the beginning of May and ended in late October. The conventional line, Minghui 63, and its corresponding Bt-transferred line, Bt Minghui 63, were used as the study material. Bt Minghui 63 possesses a Bt fusion gene consisting of both *cry1Ab* and *cry1Ac* gene sequences (Tu *et al.* 2000). We planted these crops following the regular timing and techniques employed by local peasants. In early May, seeds of these two lines were placed on the soil surface of two large incubators (50 cm × 30 cm × 30 cm depth) in a greenhouse. The soil was carefully removed from the cross section of a nearby barren mountain (total N concentration = 0.34 g kg^{-1}). The low soil fertility allowed for a nutrient gradient in the fertilization experiment. Water was added during the first two weeks to keep seedlings alive. On the 15th day after germination, seedlings were transplanted and cultured in the open air.

Sixty seedlings (thirty for each line) were planted in sixty pots. Each pot (25 cm in diameter and 30 cm in depth) was filled with 10 kg of basal soil (same as that used for seed germination). For each line, three fertilizer levels were set with 1.5, 3.0, and 4.5 g urea per pot, respectively, applied at jointing-booting stage of rice growing as basal dressing, each level having 10 replicates. The level of 3.0 g urea per pot is the approximated amount applied by local peasants. Thus, the factorial experiment had two factors (*i.e.* line and fertilizer) with two and three levels, respectively, forming six treatments in total.

After transplantation, natural rainfall was the only water source. No apparent insect damage was observed for the planted rice, and less than 1% leaf area loss was observed during the experiment. All the plants were subjected to inspection every day and weeds were immediately removed after emergence. No pesticide or herbicide was applied during the experiment.

Physiological measurements: *Li-Cor 6400XT* portable photosynthesis system (*Li-Cor, Lincoln*, NE, USA) was used to measure and record photosynthetic parameters under saturated light conditions [Murata 1961 photosynthetic active radiation (PAR) was set as 1,250 µmol m^{-2} s^{-1}] at three stages (tillering stage, jointing-booting stage, milking stage) of the planted rice. CO_2 concentration was set at 370 ppm and the air relative humidity (RH) ranged between 50% and 70%. During measurements, flow rate was set constant at 500 µmol s^{-1} and leaves were maintained at 30°C, conditions assumed to be optimal for rice growth and most frequently observed for the rice growing season in the study area. One flag leaf of each of three individuals from each treatment was selected for the measurements. WUE was calculated as P_{Nmax} /E ratio.

LTNC was measured for four or five individuals randomly selected from each treatment. Leaves were sampled from individuals at milking stage immediately after the last measurement of photosynthetic parameters. All sampled leaves were dried to constant mass at 70°C, ground into powder and sieved through 0.147-mm mesh. Samples were then digested in $H_2SO_4/HClO_4$ solution. Leaf total nitrogen mass was measured with the Kjeldahl method (Miller and Miller 1948) for each sample, and LTNC was determined as the leaf total nitrogen mass divided by the sample mass. We calculated PNUE as P_{Nmax}/LTNC ratio for the leaves measured at milking stage.

Stable carbon isotope: Leaves were collected immediately after rice ripening in mid-October, then dried and sieved, as for LTNC assessment. The sampling timing made it possible to accurately evaluate lifetime WUE_{in} but not the efficiency relative to specific developmental stages. The carbon isotope composition of leaves was assessed at the Stable Isotope Lab of the Chinese Academy of Sciences, Beijing, China, using an isotope ratio mass spectrometer (*DELTA ^{plus} XP*, with 0.1‰ analytical precision). Results were expressed as $\delta^{13}C$ values, using the formula proposed by Farquhar (1982):

$$\delta^{13}C = (R_{sam} - R_{std})/R_{std} \times 1{,}000‰ \tag{1}$$

where sam and std refer to sample and standard material, respectively. $\delta^{13}C$ values were used to characterize leaf WUE_{in}.

Statistical analysis: To evaluate the effects of line, fertilizer level, and development stage on the measured WUE, PNUE, and $\delta^{13}C$ values, as well as additional traits, three-way and two-way *ANOVA*s (for some traits, without the effect of stages) were conducted, and post-hoc *Fisher*-LSD tests were used to determine the significance level of the difference between treatments. All statistical analyses were performed with *STATISTICA for Windows* (*StatSoft Inc.*, 2001).

Results

WUE: Three-way *ANOVA* revealed significant effects of line and development stage on P_{Nmax}, *E*, WUE, and g_s (Table 1). Conventional rice generally had higher P_{Nmax} than Bt rice (Fig. 1*A*). For both lines, P_{Nmax} were substantially higher at tillering stage than at jointing-booting stage and milking stage (Fig. 1*A*, Table 2). Moreover, fertilization enhanced P_{Nmax} although the trend was not evident for the Bt rice, especially at the latter two stages (Table 2). Conventional rice had higher *E* than Bt rice (Table 2). *E* remained almost constant across stages in conventional rice, but it was substantially lower in the jointing-booting stage than the other stages in the Bt-rice (Table 2).

WUE was not significantly altered by nitrogen addition but stage and line had significant effects on WUE (Tables 1, 2), as indicated by the significant difference between two lines at jointing-booting stage (but not for the other two stages). Conventional rice had

Table 1. Results of three-way analysis of variance showing the effects of nitrogen (fertilization), line (Bt *vs*. conventional line), and stage (tillering stage, jointing-booting stage, milking stage) on stomatal conductance (g_s), light-saturated net photosynthetic rate (P_{Nmax}), transpiration rate (*E*) and instantaneous water-use efficiency (WUE). df – degrees of freedom.

Index	g_s			P_{Nmax}			*E*			WUE		
Factor	df	F	*p*	df	F	*p*	df	F	*p*	df	F	*p*
Stage	2	76.901	<0.001	2	214.632	<0.001	2	11.146	<0.001	2	104.960	<0.001
Nitrogen	2	2.291	0.111	2	5.006	<0.05	2	0.165	0.849	2	1.709	0.191
Line	1	70.664	<0.001	1	122.922	<0.001	1	164.819	<0.001	1	14.901	<0.001
Stage × nitrogen	4	1.812	0.140	4	1.081	0.375	4	1.630	0.180	4	0.605	0.660
Stage × line	2	1.560	0.220	2	4.085	<0.05	2	16.515	<0.001	2	15.234	<0.001
Nitrogen × line	2	0.075	0.928	2	0.123	0.884	2	0.012	0.988	2	0.174	0.841
Stage × nitrogen × line	4	2.490	0.054	4	0.451	0.771	4	0.467	0.760	4	0.441	0.778
Error	53			53			53			53		

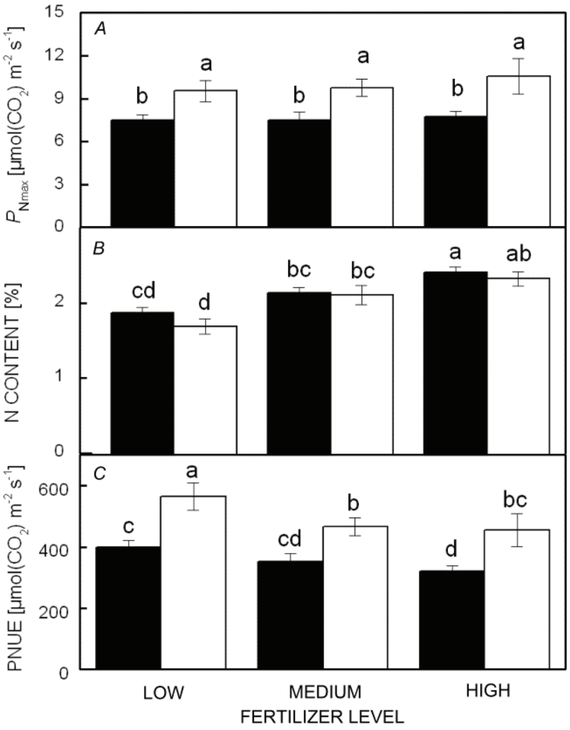

Fig. 1. *A*: Light-saturated photosynthetic rates (P_{Nmax}), *B*: leaf total N concentrations, and *C*: photosynthetic nitrogen-use efficiencies (PNUE) of Bt and conventional rice lines under three fertilizer levels at the milking stage. Bt line: *shaded columns*; conventional line: *blank columns*. The error bars denote 1 SE. $n = 3$ for each treatment. The same *lowercase letters* above columns indicate no significant difference between values for the two treatments ($p<0.05$).

generally higher g_s than Bt rice (Table 2). For both lines, g_s was lower at tillering stage (Table 2). Besides, g_s was influenced by nitrogen levels at jointing-booting stage in Bt and conventional lines and at milking stage in Bt line (Table 2). Additionally, P_{Nmax}, *E*, and WUE were all affected by the interaction between stage and line, but g_s was not influenced by this interaction (Table 1).

δ^{13}C: There were no significant differences in δ^{13}C across treatments (Table 3). Line, nitrogen level and the interaction between these had no significant effect on rice leaf δ^{13}C value (Fig. 2). *Fisher*-LSD test showed that δ^{13}C values remained highly constant across different treatments and no significant difference was observed between any two treatments (all $p>0.05$).

PNUE: At milking stage, *i.e.* the only stage in which PNUE was examined, the introduction of Bt gene negatively affected P_{Nmax} but nitrogen addition did not affect P_{Nmax} (Table 3). However, nitrogen addition positively influenced LTNC and there was no significant increase in LTNC as a result of Bt-gene introduction (Fig. 1*B*). This led to both nitrogen addition and Bt gene introduction having negative effects on leaf PNUE, although the interactive effect of the two factors was not significant (Table 3). PNUE decreased slowly from the low to medium fertilizer level in the Bt-line; it declined dramatically from the low to medium fertilizer level but remained relatively constant from the medium to high fertilizer level in the conventional line (Fig. 1*C*).

Discussion

WUE can be influenced by both variation in P_{Nmax} or *E* or both. We attempted to examine the sources of variation in WUE by inspecting differences in P_{Nmax} and *E* between the Bt and the conventional lines. The introduction of the Bt gene significantly decreased both P_{Nmax} and *E* in all three stages. The relative stability of WUE within and between lines at tillering stage lies in the nearly identical proportion of change for both rates, which were generally higher than the other two stages possibly because of younger leaves (Table 2). The data from the milking stage showed a similar trend. Nitrogen addition generally showed no effect on WUE presumably because of the relative stability of both P_{Nmax} and *E* within lines and the proportional changes of P_{Nmax} and *E* between lines (Table 2). However, at jointing-booting stage, P_{Nmax} of Bt rice was 64%‑68% of that of conventional rice depending on the different nitrogen levels, but the *E* for the Bt rice was only 42%‑46% of that of conventional rice, inevitably leading to the increase of WUE of Bt rice (Table 2).

Since both P_{Nmax} and *E* are often positively correlated to leaf g_s (Raschke 1979, Cowan 1982), we further inspected variation in g_s among treatments. g_s of the Bt line was obviously smaller than that of the conventional line, which is consistent with the lower P_{Nmax} and *E* in the Bt line (Table 2). Furthermore, relative to H_2O, CO_2 in the leaves has an additional diffusive resistance (Nobel 1983), suggesting that the decrease of g_s might lead to less decrease of P_{Nmax} than of *E* (Raschke 1979, Cowan 1982). This could at least partly explain the asynchronous changes of P_{Nmax} and *E* at jointing-booting stage. In addition, differences in g_s across different fertilizer levels within the same line were much smaller than differences between lines (Table 2). The reasons for the relative stability of *E* within line across nitrogen levels at the same stage become self-evident. However, it is not clear why g_s was significantly decreased by the introduction of the Bt gene in rice.

δ^{13}C: Leaf δ^{13}C is a common and useful index of seasonally integrated WUE_{in}. Generally, δ^{13}C is positively correlated to WUE_{in} (Farquhar *et al.* 1982, 1989). In our study, high stability of δ^{13}C values among six treatments revealed no difference in WUE_{in}, following

Table 2. Leaf instantaneous water-use efficiency (WUE), light-saturated net photosynthetic rate (P_{Nmax}), transpiration rate (E) and stomatal conductance (g_s), of Bt *vs*. conventional rice lines under three fertilizer levels (low, medium, and high) at three development stages, means ± SE, n = 5.

Fertilizer level	Parameter	Bt line			Conventional line		
		Low	Medium	High	Low	Medium	High
Tillering stage	WUE [μmol(CO_2) $mmol^{-1}$ (H_2O)]	4.00 ± 0.27	4.34 ± 0.28	4.29 ± 0.18	4.14 ± 0.31	4.05 ± 0.42	4.03 ± 0.10
	P_{Nmax} [μmol(CO_2) m^{-2} s^{-1}]	11.45 ± 1.08	12.97 ± 1.26	14.20 ± 0.42	16.88 ± 0.17	16.95 ± 0.63	18.65 ± 0.85
	E [mmol(H_2O) m^{-2} s^{-1}]	2.85 ± 0.17	2.98 ± 0.20	3.33 ± 0.24	4.15 ± 0.33	4.27 ± 0.26	4.64 ± 0.27
	g_s [mol(H_2O) m^{-2} s^{-1}]	0.0607 ± 0.0037	0.0650 ± 0.0055	0.0642 ± 0.0049	0.0892 ± 0.0080	0.0924 ± 0.0075	0.0991 ± 0.0066
Jointing-booting stage	WUE [μmol(CO_2) $mmol^{-1}$ (H_2O)]	3.33 ± 0.14	3.40 ± 0.29	3.63 ± 0.09	2.06 ± 0.04	2.31 ± 0.07	2.66 ± 0.15
	P_{Nmax} [μmol(CO_2) m^{-2} s^{-1}]	6.24 ± 0.36	6.45 ± 0.60	6.59 ± 0.14	9.17 ± 0.32	10.07 ± 0.21	10.31 ± 0.29
	E [mmol(H_2O) m^{-2} s^{-1}]	1.87 ± 0.08	1.96 ± 0.37	1.82 ± 0.07	4.46 ± 0.12	4.40 ± 0.20	3.93 ± 0.24
	g_s [mol(H_2O) m^{-2} s^{-1}]	0.1128 ± 0.0043	0.1356 ± 0.0157	0.1122 ± 0.0026	0.1255 ± 0.0008	0.1586 ± 0.0088	0.1644 ± 0.0083
Milking stage	WUE [μmol(CO_2) $mmol^{-1}$ (H_2O)]	2.31 ± 0.11	2.44 ± 0.21	2.52 ± 0.10	2.55 ± 0.11	2.52 ± 0.10	2.59 ± 0.13
	P_{Nmax} [μmol(CO_2) m^{-2} s^{-1}]	7.47 ± 0.41	7.49 ± 0.57	7.71 ± 0.41	9.54 ± 0.74	9.77 ± 0.61	10.54 ± 1.25
	E [mmol(H_2O) m^{-2} s^{-1}]	3.24 ± 0.16	3.10 ± 0.27	3.06 ± 0.14	3.78 ± 0.46	3.90 ± 0.27	4.06 ± 0.38
	g_s [mol(H_2O) m^{-2} s^{-1}]	0.0995 ± 0.0026	0.0974 ± 0.0074	0.1225 ± 0.0074	0.1594 ± 0.0222	0.1503 ± 0.0119	0.1474 ± 0.0153

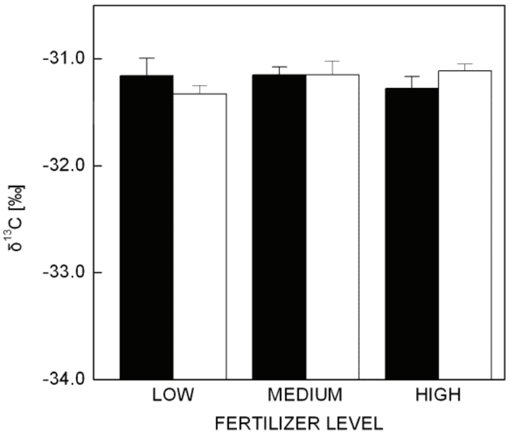

Fig. 2. Leaf $\delta^{13}C$ of Bt and conventional rice lines under three fertilizer levels. Bt line: *shaded columns*; conventional line: *blank columns*. The error bars denote 1 SE. $n = 5$ for each treatment.

introduction of Bt gene or different nitrogen levels. Considering Bt rice had a higher WUE at jointing-booting stage (Table 2), there seems to be a paradox between the results for WUE and WUE_{in}. The paradox derives from the surprisingly high WUE of Bt rice, which was probably caused by the unusually low g_s at jointing-booting stage (Table 2), as observed earlier. Thus, we tend to attribute the inconsistency between WUE_{in} and WUE to the different responses of WUE to Bt-gene introduction among different developmental stages (Table 2), *i.e.* the significant difference in WUE at jointing-booting stage might be offset by the variation in WUE at other developmental stages.

The relatively constant WUE_{in} between the two lines (Table 3, Fig. 1) seems to imply there could be no hidden risk for water supply and the large-scale commercialization of Bt rice might not hinder extant rice plantations. More importantly, because of its higher WUE at jointing-booting stage which is the most water-dependent stage in rice life history, Bt rice may have potential advantages in the context of agricultural expansion.

Table 3. Results of two-way analysis of variance showing the effects of nitrogen (fertilizer addition) and line (Bt and conventional line) on $\delta^{13}C$, light-saturated net photosynthetic rate (P_{Nmax}), leaf total nitrogen concentration (LTNC) and photosynthetic nitrogen-use efficiency (PNUE) at the milking stage. df – degrees of freedom.

Index	$\delta^{13}C$			P_{Nmax}			LTNC			PNUE		
Factor	df	F	*p*	df	F	*p*	df	F	*p*	df	F	*p*
Nitrogen	2	0.334	0.710	2	0.452	0.645	2	23.578	<0.001	2	4.361	<0.05
Line	1	0.001	0.981	1	17.858	0.001	1	2.755	0.112	1	26.099	<0.001
Nitrogen × line	2	1.195	0.322	2	0.160	0.854	2	0.198	0.822	2	0.298	0.747
Error	22			14			21			14		

PNUE: Within lines, PNUE declined when leaf total nitrogen increased, which could be attributed to the huge difference in LTNC that resulted in merely tiny amplitude in P_{Nmax} (Fig. 2*A*,*B*). This shows that both Bt and conventional rices might be using nitrogen more efficiently when the nitrogen supply is relatively insufficient. The pattern is consistent with many previous studies (*e.g.* Cordell *et al.* 1998).

Conventional rice, characterized by higher P_{Nmax} but lower LTNC, showed higher PNUE than Bt rice. This is in contrast to the well-known tendency of P_{Nmax} to be generally positively correlated to mass-based leaf N content (*e.g.* Reich *et al.* 1999). This unusual result might originate from the following mechanism. The photosynthetic nitrogen in Bt rice might be relatively less despite having higher LTNC relative to the conventional line. Photosynthesis is an enzyme-mediated process that largely depends on ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Rubisco, as the key enzyme that accounts for up to 30% of total leaf nitrogen, is often positively correlated to nitrogen availability and might directly influence photosynthetic capacity (Sims *et al.* 1998). However, Bt rice, which should allocate part of its nitrogen resource to construct Bt protein absent in conventional rice, might have less nitrogen available to synthetize the Rubisco enzyme and other nitrogen-demanding compounds involved in photosynthesis. Consequently, the low PNUE of Bt rice might result from nitrogen competition between Bt protein synthesis and photosynthesis. This explanation is in line with results of Field and Mooney (1986) who proposed that the expression of N-containing secondary compounds for defense against predation could lead to the decline of PNUE. Noticeably, the maintenance of WUE_{in} is often achieved by decreasing g_s. The reduction of g_s is expected to limit *E* and CO_2 supply in leaves. As a consequence, P_{Nmax} is also decreased, which in turn leads to the decrease in PNUE. In other words, maintaining WUE_{in} may incur a PNUE cost, as suggested by several previous studies (Field *et al.* 1983, Evan 1991, Gutierrez and Meinzer 1994). However, we did not find the negative relationship between WUE_{in} and PNUE in the present study. Rice is not a drought-tolerant plant. In order to maintain normal growth, water should be sufficiently supplied. Therefore, the abundant rainfall during our experiment might have obscured the differences in WUE_{in} between Bt and conventional rice.

To summarize, our results show that rice suffers a significant loss in g_s after Bt gene introduction. The decreased g_s maintained WUE_{in} values stable for the transgenic line as a consequence of the synchronous decrease of P_{Nmax} and E. However, PNUE decreased in the transgenic line possibly because of competition for leaf nitrogen between Bt-gene and photosynthetic processes. Given that there is little evidence for herbivore damage physiologically affecting leaf resource use efficiencies, it is possible that more fertilizer will be applied to Bt rice to maintain high yield in large field. We suggest that extreme prudence should be applied in the commercialization of Bt rice, given its relatively low PNUE and the potential heavy risk of significant pollution.

References

Carriere, Y., Ellers-Kirk, C., Sisterson, M., Antilla, L., Whitlow, M., Dennehy, T.J., Tabashnik. B.E.: Long-term regional suppression of pink bollworm by *Bacillus thuringiensis* cotton. – P. Natl. Acad. Sci. **100**: 1519-1523, 2003.

Cordell, S., Goldstein, G., Mueller-Dombois, D., Webb, D., Vitousek, P.M.: Physiological and morphological variation in *Metrosideros polymorpha*, a dominant Hawaiian tree species, along an altitudinal gradient: the role of phenotypic plasticity. – Oecologia **113**: 188-196, 1998.

Cowan, I.R.: Regulation of water use in relation to carbon gain in higher plants. – Encyclopedia Plant Physiol. **12B**: 489-613, 1982.

Evan, H.D., William, H.S.: Resource-use efficiency and drought tolerance in adjacent great basin and sierran plants. – Ecology **72**: 51-58, 1991.

Farquhar, G.D., Ehleringer, J.R., Hubick, K.T.: Carbon isotope discrimination and photosynthesis. – Annu. Rev. Plant Phys. **40**: 503-537, 1989.

Farquhar, G.D., O'Leary, M.H., Berry, J.A.: On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. – Aust. J. Plant Physiol. **9**: 121-137, 1982.

Felke, M., Lorenz, N., Langenbruch, G.-A.: Laboratory studies on the effects of pollen from Bt-maize on larvae of some butterfly species. – J. Appl. Entomol. **126**: 320-325, 2002.

Field, C., Merino, J., Mooney, H.A.: Compromises between water-use efficiency and nitrogen-use efficiency in five species of California evergreen. – Oecologia **60**: 384-389, 1983.

Field, C., Mooney, H.A.: The photosynthesis-nitrogen relationships in wild plants. – In: Givnish, T.J. (ed.): On the economy of plant form and function. Pp. 25-55. Cambridge Univ. Press, Cambridge 1986.

Giovannetti, M., Sbrana, C., Turrini, A.: The impact of genetically modified crops on soil microbial communities. – Riv. Biol. – Biol. Forum. **98**: 393-417, 2005.

Gutierrez, M.V., Meinzer, F.C.: Carbon isotope discrimination and photosynthetic gas-exchange in coffee hedgerows during canopy development. – Aust. J. Plant Physiol. **21**: 207-219, 1994.

Hanson, B.D., Mallory-Smith, C.A., Price, W.J., Shafii, B., Thill, D.C., Zemetra, R.S.: Interspecific hybridization: potential for movement of herbicide resistance from wheat to jointed goatgrass (*Aegilops cylindrica*). – Weed Technol. **19**: 674-682, 2005.

James, C.: Global status of commercialized biotech/GM Crops: 2010. ISAAA Brief No. 42. ISAAA South Asia Office, New Delhi 2010.

Jiang, W.L.: Strategic research on how to solve the water problem in China. – Impact Sci. Soc. **7**: 24-29, 2010.

Li, X.G., Liu, B., Heia, S., Liu, D.D., Han, Z.M., Zhou, K.X., Zheng, Y.P.: The effect of root exudates from two transgenic insect-resistant cotton lines on the growth of *Fusarium oxysporum*. – Transgenic Res. **18**: 757-767, 2009.

Liu, W.K.: Do genetically modified plants impact arbuscular mycorrhizal fungi? – Ecotoxicology **19**: 229-238, 2010.

Liu, W.K., Du, L.F.: Interactions between Bt transgenic crops and arbuscular mycorrhizal fungi: a new urgent issue of soil ecology in agroecosystems. – Acta. Agr. Scand. B-S. P. **58**: 187-192, 2008.

Lu, Y.H., Wu, K.M., Jiang, Y.Y., Xia, B., Li, P., Feng, H.Q., Wyckhuys, K., Guo, Y.Y.: Mirid bug outbreak in multiple crops correlated with wide-scale adoption of Bt cotton in China. – Science **328**: 1151-1154, 2010.

Marvier, M., McCreedy C., Regetz, J., Kareiva1 P.: A meta-analysis of effects of Bt cotton and maize on nontarget invertebrates. – Science **316**: 1475-1477, 2007.

Miller, G. L., Miller, E.E.: Determination of Nitrogen in Biological Materials. – Anal. Chem. **20**: 481-488, 1948.

Motavalli, P.P., Kremer, R.J., Fang, M., Means, N.E.: Impact of genetically modified crops and their management on soil microbially mediated plant nutrient transformations. – J. Environ. Qual. **33**: 816-824, 2004.

Murata, Y.: [Studies on the photosynthesis of rice plants and cultural significance.] – Bull. Natl. Inst. Agric. Sci. Jap. Ser. D **9**: 1-169, 1961. [In Jap.]

Nobel, P.S.: Biophysical Plant Physiology and Ecology. – W. H. Freeman, New York 1983.

Ortiz-Garcia, S., Ezcurra, E., Schoel, B., Acevedo, F., Soberon, J., Snow, A.A.: Absence of detectable transgenes in local landraces of maize in Oaxaca, Mexico (2003-2004). – P. Natl. Acad. Sci. **102**: 12338-12343, 2005.

Pineyro-Nelson, A., van Heerwaarden, J., Perales, H.R., Serratos-hernandez, J.A., Rangel, A., Hufford, M.B., Gepts, P., Garay-Arroyo, A., Rivera-Bustamante, R., Alvarez-Buylla, E.R.: transgenes in Mexican maize: molecular evidence and methodological considerations for GMO detection in landrace populations. – Mol. Ecol. **18**: 750-761, 2009.

Pray, C.E., Huang, J., Hu, R.F., Rozelle, S.: Five years of Bt cotton in China - the benefits continue. – Plant J. **31**: 423-430, 2002.

Qaim. M., Zilberman, D.: Yield effects of genetically modified crops in developing countries. – Science **299**: 900-902, 2003.

Quist, D., Chapela, I.H.: Transgenic DNA introgressed into traditional maize landraces in Oaxaca, Mexico. – Nature **414**: 541-543, 2001.

Raschke, K.: Movements of stomata. – Encyclopedia Plant Physiol. N.S. **7**: 383-441, 1979.

Reich, P.B., Ellsworth, D.S., Walters, M.B., Vose, J.M., Gresham, C., Volin, J.C., Bowman, W.D.: Generality of leaf trait relationships: A test across six biomes. – Ecology **80**: 1955-1969, 1999.

Ren, M.Z., Chen, Q.J., Zhang, R., Guo, S.D.: Structural characteristic and genetic expression of nondulin-like gene and its promoter in cotton. – Acta Bot Sin. **46**: 1424-1423, 2004.

Saxena, D., Stotzky, G.: Insecticidal toxin from *Bacillus thuringiensis* is released from roots of transgenic *Bt* corn in vitro and in situ. – FEMS Microbiol. Ecol. **33**: 35-39, 2000.

Sims, S.R., Martin, J.W.: Effects of the *Bacillus thuringiensis* insecticidal proteins CryIA(b), CryIA(c), CryIIA, and CryIIIA on *Folsomia candida* and *Xenylla grisea* (Insecta: Collembola). – Pedobiologia **41**: 412-416, 1997.

Sims, D.A., Seemann, J.R., Luo, Y.: The significance of differences in the mechanisms of photosynthetic acclimation to light, nitrogen and CO_2 for return on investment in leaves. – Funct. Ecol. **12**: 185-194, 1998.

State Statistical Bureau: China Statistical yearbook, 2009. http://www.stats.gov.cn/tjsj/ndsj/2009/indexch.htm

Tu, J., Zhang, G., Datta, K., Xu, C., He, Y., Zhang, Q., Khush, G.S., Datta, S.K.: Field performance of transgenic elite commercial hybrid rice expressing *Bacillus thuringiensis* δ-endotoxin. – Nat. Biotechnol. **18**: 1101-1104. 2000.

Xie, Y.X., Xiong, Z.Q., Xing, G.X., Yan, X.Y., Shi, S.L., Sun, G.Q., Zhu, Z.L.: Source of nitrogen in wet deposition to a rice agroecosystem at Tai lake region. – Atmos. Environ. **42**: 5182-5192, 2008.

Watrud, L.S., Seidler, R.J.: Non-target ecological effects of plants, microbial, and chemical introductions to terrestrial system. – In: Huang, P.M., Adriano, D.C., Logan, T.J., Checkai, R.T. (ed.): Soil Chemistry and Ecosystem Health **52** (Sp.): 313-340. Soil Science Society of America, Madison 1998.

Wu, J.H., Luo, X.L., Wang, Z., Tian, Y.C., Liang, A.H., Sun, Y.: Transgenic cotton expressing synthesized scorpion insect toxin AaHIT gene confers enhanced resistance to cotton bollworm (*Heliothis armigera*) larvae. – Biotechnol. Lett. **30**: 547-554, 2008.

Yang, X.J., Yang, Z.M., Mao, C.H., Du, J.: Dependence of dielectric properties on Bt particle in EP/BT composites. – Rare Metals **25** (Sp.): 250-254, 2006.