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ORIGINAL ARTICLE

Different response of an elite *Bt* restorer line of hybrid rice (*Oryza sativa* L.) in adaptation to nitrogen deficiency

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Abstract Transgenic *Bacillus thuringiensis* (*Bt*) rice have been reported to acquire effective resistance against the target pests; however, the insertion and expression of alien *Bt* genes may have some unintended effects on the growth characteristics of rice. A screen-house experiment was conducted and repeated twice to investigate the growth characteristics and Bt protein expressions in two *Bt* rice lines [MH63 (*Cry2A**) and MH63 (*Cry1Ab/Ac*)], which had different Bt protein expression levels in leaves, under zero nitrogen (N0) and recommended nitrogen (NR) fertilizer applications. Compared to the counterpart MH63, MH63 (*Cry2A**) under N0 experienced accelerated leaf senescence and a lower internal N use efficiency (IE_N), resulting in a 23.2% decrease in grain yield and a lower accumulated biomass. These variations were revealed to be correlated to the higher ratio of the Bt protein content to the soluble protein content (BTC/SPC) with a maximum value of 4.3‰ in MH63 (*Cry2A**) leaves in the late growth stage. Under NR, no differences in growth characteristics between MH63 (*Cry2A**) and MH63 were found. The growth characteristics of MH63 (*Cry1Ab/Ac*), with a lower BTC/SPC in the late growth stage compared to MH63 (*Cry2A**), were identical to those of MH63 under the two N applications. Results show that the transgenic *Bt* rice MH63 (*Cry2A**), with a relatively higher Bt protein expression in the late growth stage, had an inferior adaptation to nitrogen deficiency compared to its non-*Bt* counterpart. And this inferior adaptation was found to be correlated with the higher BTC/SPC in MH63 (*Cry2A**) leaves in the late growth stage.

Keywords *Bt* rice · Grain yield · N use efficiency · Leaf senescence · Bt protein · Bio-burden

Abbreviations

AE_N	N agronomic efficiency
Bt	*Bacillus thuringiensis*
BTC/SPC	Ratio of the Bt protein content to the soluble protein content
BTC	Bt protein content
C	Carbon
CMS	Cytoplasm male sterile
FL	Flowering stage
FS	Filling stage
IE_N	Internal N use efficiency
K	Potassium
LSD	Least significant difference
MT	Mid-tillering stage
N0	Zero nitrogen
NPR	Net photosynthetic rate
NR	Recommended nitrogen
NUE	N use efficiency
P	Phosphorus
PE_N	N physiological efficiency
PFP_N	N partial factor productivity

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PI	Panicle initiation stage
PM	Plant maturity stage
RE_N	N recovery efficiency
SD	Standard deviation
SPC	Soluble protein content

Introduction

Lepidopteran has caused great yield loss of rice (Pandi et al. 2009) and thus scientists have been working on development of lepidopteran-resistant transgenic *Bt* rice for decades (Fujimoto et al. 1993; Tu et al. 2000a, b; Lu et al. 2001; Chen et al. 2005; Tang et al. 2006). This transgenic *Bt* rice was the result of effective genes selection and genetic manipulation. Specifically, *Cry1Ab/Ac* and *Cry2A** were selected among the screened *Bt* genes and inserted into MH63, a indica cytoplasm male sterile (CMS) restorer line which is widely used for rice breeding in Asia (Tu et al. 2000b; Chen et al. 2005). Biosafety certificate of commercial production in China was granted by the Chinese Ministry of Agriculture for MH63 (*Cry1Ab/Ac*) in 2009 (Chen et al. 2011).

The performance of transgenic crops in different environments must be thoroughly evaluated prior to commercial production to ensure stability and sustainability. Studies on *Bt* rice have mainly concentrated on breeding (Tu et al. 2000b; Tang et al. 2006), evaluations for resistance (Yu et al. 2011; Jiang et al. 2014), food and environment safety (Clark et al. 2005; Yuan et al. 2013; Li et al. 2015b). Some transgenic *Bt* rice were reported to have acquired effective resistance against the target pests (Tu et al. 2000a; Chen et al. 2005; Tang et al. 2006). These rice thus have a distinct yield advantage over their non-*Bt* counterparts in field when no pesticide was applied. Meanwhile, some authors found certain unintended effects of *Bt* transgene on rice traits such as lower setting rates (Tu et al. 2000a; Shu et al. 2002; Xia et al. 2010; Wang et al. 2012b), fewer grains (Kim et al. 2008) and decreased plant height and root length (Shu et al. 2002), that commonly lead to a reduced grain yield. The distinct advantage of *Bt* rice in pests resistance does not take away the fact that the unintended effects are the usual precursors would lead to lower yield (Jiang et al. 2014). It is also possible that its advantage is only limited to environments where no pesticide was applied. Therefore, it is important to investigate the untended effects of transgenes by comparing the growth and physiology characteristics of *Bt* rice with their non-*Bt* counterparts in strict pest control environment.

Nitrogen plays an important role in growth and physiology metabolism of crops (Huber 1985; Hocking and Meyer 1991; Kropff et al. 1993; Lawlor 2002). As a newly imparted anabolic process, Bt protein synthesis will consume extra N in transgenic *Bt* crops; therefore, scientists have devoted considerable attention to determine whether transgenic *Bt* crops change relative to N utilization and metabolism. In cotton, Chen et al. (2004) showed that *Bt* cotton had more vigorous N metabolism in the reproductive stage than its non-*Bt* counterpart. Specifically, in *Bt* cotton, higher total leaf N concentration, higher soluble protein content, more free amino acids, and higher nitrate reductase and glutamic-pyruvic transaminase activity were observed in the reproductive stage. In maize, Ma and Subedi (2005) discovered that several *Bt* hybrid maize strains had different N distributions in the plants compared to their non-*Bt* counterparts. Moreover, it was reported that a *Bt* hybrid maize (Pioneer 38W36*Bt*) had more N accumulation in comparison to its non-*Bt* counterpart (Pioneer 38W36), although there were no differences between them in leaf N concentrations and chlorophyll contents at the silking and maturity stages (Subedi and Ma 2007). To date, few studies have been done to examine the N utilization of *Bt* rice. Investigating the responses of newly bred *Bt* rice lines to both adequate and inadequate N applications is a feasible and quick way to study their N utilization characteristics.

Bt protein expression in transgenic crops are regulated by both the genes and plant nutriture. Genetically, a relatively higher Bt protein expression should be guaranteed to prolong effectiveness of *Bt* crops (Cohen et al. 2000). Physiologically, the Bt protein content was found to be significantly affected by N and soluble protein concentrations in leaves. Promoted plant N nutriture would increase the Bt protein expression in *Bt* crops (Bruns and Abel 2003; Dong and Li 2007; Wang et al. 2012a). Moreover, there exist temporal and spatial differences in Bt protein expression in *Bt* crops. As the photosynthetic and easily affected part in plant, leaves usually had the highest Bt protein expression (Fearing et al. 1997; Adamczyk et al. 2001; Kranthi et al. 2005; Siebert et al. 2009). The Bt protein expression in plant organs generally climbed up in growing term at first and then decreased in decrepitude term (Greenplate 1999; Olsen et al. 2005). To put this in perspective, one concern is whether the Bt protein expression will in turn affect related mechanisms of *Bt* crops.

As effective insect-resistant *Bt* genes, *Cry1Ab/Ac* and *Cry2A** would be widely used in transgenic breeding in the future. Meanwhile, MH63 is an important CMS restorer line for rice breeding in Asia. Therefore, possible effects of the two *Bt* genes on yield formation and physiology of *Bt* MH63 should be fully evaluated before its application. This study aims to (1) investigate responses in yield formation of *Bt*-MH63 to different N applications (2) and to explore the physiological mechanisms of the differences in the responses.

Materials and methods

Plant materials

Two *Bt* rice lines and their non-*Bt* counterpart were used in the study. They were MH63 (*Cry2A**), MH63 (*Cry1Ab/Ac*) and MH63, respectively. The *Cry2A** gene was synthesized by modifying the wild-type *Cry2Aa* genes of *Bt* (Chen et al. 2005) while the *Cry1Ab/Ac* gene is a hybrid *Bt* gene derived from *Cry1Ab* and *Cry1Ac* (Tu et al. 1998). The *Bt* rice lines were provided by the National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan, China.

Experimental design

A pot experiment was conducted in a screen-house from June to October of 2012 and 2013 at Huazhong Agricultural University, Wuhan City (29°58′N 113°53′E), Hubei Province, China (Fig. S2). The air temperature and radiation during the growing season in the screen-house were shown in Table S1. The treatments were laid out in a randomized block design with two fertilizer applications [N0 (0 g N pot^{-1}) and NR (4 g N pot^{-1})] as the blocking factor and the three varieties, replicated three times. Four ten-day-old seedlings were transplanted at one seedling per hill in each pot (40 cm in diameter and 30 cm in height) containing 12 kg of sieved, air-dried paddy soil. The chemical properties of the paddy soil were as follows: pH, 6.32; organic carbon (C), 12.78 g kg^{-1}; total N, 0.92 g kg^{-1}; NO_3^-–N, 5.14 mg kg^{-1}; NH_4^+–N, 2.11 mg kg^{-1}; available phosphorus (P), 6.48 mg kg^{-1}; and available K, 102 mg kg^{-1}. For NR, N fertilizer [$CO(NH_2)_2$] was applied at a rate of 4 g N pot^{-1} with 50, 20 and 30% at basal stage, mid-tillering stage (MT) and panicle initiation stage (PI), respectively. For N0 and NR, potassium (K) fertilizer (KCl) was added at a rate of 4 g K_2O pot^{-1} with 50% at the basal stage and 50% at the PI. P fertilizer (NaH_2PO_4) was applied at a rate of 2 g P_2O_5 pot^{-1} at the basal stage. A two-cm water depth was maintained in pots until harvest. Chemicals were applied during the growth season to control pests and diseases to avoid yield losses.

Sampling and data collection

Leaf senescence

The SPAD values of four topmost fully expanded leaves per pot were measured using a chlorophyll meter [SPAD-502, Soil–Plant Analysis Development Section, Minolta Camera Co., Osaka, Japan] at the MT, PI, flowering stage (FL), filling stage (FS, 15 days after the FL), and plant maturity stage (PM). The net photosynthetic rates (NPR) of the four topmost fully expanded leaves were measured using a Li-Cor 6400XT portable photosynthesis system (Li-Cor, Lincoln, NE, USA) at the FS.

BTC and SPC analysis

The four topmost fully expanded leaves per pot were sampled and stored at −80°C for BTC and SPC analysis. About 20 mg of the fresh leaves was grinded in the extraction buffer. The extracting solution was then diluted for the enzyme-linking reaction. The enzyme-linking reaction was conducted using the kits provided by the Enviro-Logix (Portland, Me.) and Envirologix (Envirologix, USA) manufacturers. The results of the enzyme-linking reaction was read using a microplate reader (Multiskan MK3, Labsystem, P.R. China). The *Cry2A** or *Cry1Ab/Ac* content was calculated according to the readings. About 0.5 g of the fresh leaves was grinded in the extraction buffer and then centrifuged. The extracting solution was pooled for Coomassie blue dye-binding assay (Bradford 1976). The SPC was calculated according to the absorbance reading. Thereafter, BTC/SPC ratio was calculated.

NUE and yield

The aboveground plants in the sampling pots were taken at the MT, PI, FL, FS and PM. Sampled plants were dissected and oven-dried at 70°C to determine the total biomass. The N concentrations of the plant tissues at PM were determined using the method of micro-Kjeldahl digestion, distillation, and titration (Bremner and Mulvaney 1982). Finally, the total N uptake was computed as the sum of products of the biomass and N concentration.

The number of panicles per pot was counted at the PM. The panicles were threshed and then oven-dried at 70°C. Filled and unfilled spikelets were counted respectively. The setting rate was equal to the ratio of the filled spikelets to the total spikelets (filled spikelets + unfilled spikelets). The filled spikelets were weighed to determine the grain yield and the 1000-grain weight. N use efficiency (NUE) was calculated according to the methods of Novoa and Loomis (1981) and Peng et al. (2006):

$$\text{N recovery efficiency } (RE_N, \%) = 100 \times (TN_N - TN_{N0})/FN,$$

$$\text{N physiological efficiency } (PE_N, g\ g^{-1}) = (GY_N - GY_{N0})/(TN_N - TN_{N0}),$$

$$\text{N agronomic efficiency } (AE_N, g\ g^{-1}) = (GY_N - GY_{N0})/FN,$$

$$\text{N partial factor productivity } (PFP_N, g\ g^{-1}) = GY_N/FN,$$

$$\text{Internal N use efficiency } (IE_N, g\ g^{-1}) = GY/TN,$$

where TN, TN_N, TN_{N0}, FN, GY, GY_N and GY_{N0} is the total N uptake of the plants in the pot, total N uptake of the plants in the pot with N fertilizer, total N uptake of the plants in the pot without N fertilizer, amount of N fertilizer applied, grain yield, grain yield from the pot with N fertilizer, and grain yield from the pot without N fertilizer, respectively.

Statistical analysis

Means were compared and grouped using analysis of variance by SAS 9.1 (SAS Institute, Inc., Cary, NC, USA), and the least significant difference (LSD) post hoc test, respectively. Data are presented as the mean ± standard deviation (SD, $n = 3$). Differences of the means were statistically significant at $\alpha = 0.05$. CORR procedure in SAS was used to analyze correlations among the growth characteristics of *Bt* rice.

Results

Phenology

The mean monthly air temperature in 2012 and 2013 inside the screen-house for the rice growing season ranged from approximately 25–32 °C and from approximately 25–33 °C, respectively (Table S1). The mean daily radiations in 2012 and 2013 ranged from approximately 13 MJ m^{-2} $days^{-1}$ to 18 MJ m^{-2} $days^{-1}$ and from approximately 12 MJ m^{-2} $days^{-1}$ to 19 MJ m^{-2} $days^{-1}$, respectively. The mean air temperature of the rice growing season in 2013 was about 1 °C higher than that in 2012.

Grain yield and yield components

No significant difference was observed between the grain yields of MH63 (*Cry2A**) and its non-*Bt* counterpart MH63 under NR (Table 1). However, grain yields of MH63 (*Cry2A**) were 26.0% and 20.4% lower than those of MH63 in 2012 and 2013 under N0, respectively. Lower setting rate relative to MH63 was the primary reason for the reduction in MH63 (*Cry2A**) grain yield under N0. No differences in grain yield and yield components between MH63 (*Cry1Ab/Ac*) and MH63 were observed. MH63 (*Cry2A**) and MH63 showed different responses in grain yield to N application. The grain yields of MH63(*Cry2A**) were 68.7 and 69.4% lower under N0 than under NR in 2012 and 2013, whereas those of MH63 were 59.0 and 63.1% lower under N0 than under NR in 2012 and 2013, respectively. The grain yield and yield components except the 1000-grain weight were significantly affected by the N application. Both the genotype and N application × genotype exerted strong influences on the grain yield and the setting rate.

Biomass accumulation

No difference in biomass accumulation was found between *Bt*-MH63 and MH63 under NR in 2012 and 2013 (Fig. 1). However, under N0, MH63 (*Cry2A**) was found to have significantly lower total biomass accumulation compared to MH63 (*Cry1Ab/Ac*) and MH63 after the FL in 2012 and 2013. N application significantly affected the biomass accumulations of the three varieties in 2012 and 2013.

Nitrogen utilization

The total N uptake level was much lower under N0 than that under NR in 2012 and 2013 (Fig. S1). There were no differences in the total N uptakes between *Bt*-MH63 and MH63 under both N applications. However, there were some differences in the N recovery efficiency (RE_N), N physiological efficiency (PE_N) and IE_N of the three varieties (Table 2). Under N0, MH63 (*Cry2A**) had lower IE_N than the other varieties in 2012 and 2013. Under NR, MH63 (*Cry2A**) had higher PE_N and RE_N than those of MH63 under NR in 2012 and 2013, respectively. Moreover, a lower PE_N was found in MH63 (*Cry1Ab/Ac*) relative to MH63 under NR in 2013. The N application, genotype and N application × genotype all showed significant effects on IE_N. The genotype significantly affected the PE_N.

Leaf senescence

Between the N application treatments, leaf SPAD values were found to be consistent and significantly higher in all growth stages when N is applied in 2012 and 2013 (Table 3). Across varieties under NR, no differences in leaf SPAD and NPR were detected. However, under N0, MH63 (*Cry2A**) had lower leaf SPAD throughout the late growth stages (FS and PM) along with lower NPR at FS than MH63. No significant differences in the SPAD values and NPRs were found between MH63 (*Cry1Ab/Ac*) and MH63. N applications exerted strong influence on the SPAD value at each growth stage. The SPAD and NPR in the late stages were significantly affected by both the genotype and its interaction with N application.

Bt protein and soluble protein

N applications significantly increased the soluble protein contents (SPCs) and Bt protein contents (BTCs) in leaves of *Bt*-MH63 at MT, PI, FL, FS and PM in 2012 and 2013 (Fig. 2). The BTCs were higher in MH63 (*Cry2A**) leaves than those in MH63 (*Cry1Ab/Ac*) under both N0 and NR. The

Table 1 Grain yields and components of *Bt*-MH63 and MH63 under different N applications in 2012 and 2013

N application	Genotype	Panicles (no. pot^{-1})	Spikelets (no. panicle^{-1})	Setting rate (%)	1000-grain weight (g)	Grain yield (g pot^{-1})
2012						
N0	MH63 (*Cry2A**)	26.0 ± 1.0a	84.4 ± 4.5a	67.7 ± 3.1b	26.8 ± 0.3a	39.9 ± 3.3b
	MH63 (*Cry1Ab/Ac*)	26.3 ± 1.5a	85.2 ± 4.0a	82.4 ± 3.8a	27.1 ± 1.0a	50.4 ± 3.7a
	MH63	27.3 ± 0.6a	88.7 ± 3.0a	83.3 ± 3.8a	26.6 ± 0.9a	53.9 ± 2.8a
	Mean	26.6B	86.1B	77.8A	26.8A	48.0B
NR	MH63 (*Cry2A**)	67.3 ± 3.2a	94.6 ± 3.2a	71.8 ± 5.1a	27.9 ± 1.0a	127.5 ± 10.4a
	MH63 (*Cry1Ab/Ac*)	69.7 ± 3.2a	97.2 ± 4.7a	73.1 ± 3.9a	27.4 ± 0.5a	135.6 ± 11.6a
	MH63	71.3 ± 3.8a	93.8 ± 4.2a	69.5 ± 3.9a	28.1 ± 0.7a	131.5 ± 11.4a
	Mean	69.4A	95.2 A	71.5A	27.8 A	131.5A
Analysis of variance						
N application		**	*	*	NS	**
Genotype		NS	NS	**	NS	*
N application × genotype		NS	NS	*	NS	*
2013						
N0	MH63 (*Cry2A**)	27.7 ± 1.5a	82.3 ± 3.8a	61.7 ± 2.5b	27.2 ± 0.7a	38.2 ± 3.4b
	MH63 (*Cry1Ab/Ac*)	29.0 ± 2.6a	80.8 ± 3.4a	77.1 ± 3.3a	26.3 ± 1.3a	47.6 ± 2.6a
	MH63	28.7 ± 0.6a	78.5 ± 3.3a	77.8 ± 4.0a	27.4 ± 0.8a	48.0 ± 3.1a
	Mean	28.4B	80.5B	72.2A	27.0A	44.6B
NR	MH63 (*Cry2A**)	73.0 ± 3.6a	91.1 ± 5.0a	66.7 ± 3.5a	28.2 ± 1.3a	124.8 ± 7.1a
	MH63 (*Cry1Ab/Ac*)	75.0 ± 2.6a	87.3 ± 5.1a	68.5 ± 3.9a	26.6 ± 0.6a	119.3 ± 11.2a
	MH63	76.7 ± 3.5a	92.3 ± 6.1a	67.1 ± 4.6a	27.3 ± 0.8a	130 ± 18.6a
	Mean	74.9A	90.2A	67.4A	27.4A	124.7A
Analysis of variance						
N application		**	*	*	NS	**
Genotype		NS	NS	*	NS	**
N application × genotype		NS	NS	*	NS	*

Data are presented as the mean ± standard deviation (SD, $n = 3$). Uppercase letters indicate LSD ($\alpha = 0.05$) grouping of means between the two N applications within each year. Lowercase letters indicate LSD ($\alpha = 0.05$) grouping of means across genotypes within an N application for each year. Means with the same letter are not significantly different

NS not significant

* Significant source of variation at $\alpha = 0.05$ while ** at $\alpha = 0.01$

BTCs in the *Bt*-MH63 leaves increased starting at MT, peaked at FL, and then declined. This decline was much slower in MH63 (*Cry2A**) leaves than that in MH63 (*Cry1Ab/Ac*). The SPCs in the leaves of the two varieties had the highest values at PI. Under N0, at FS, MH63 (*Cry2A**) had lower SPCs in leaves than MH63 (*Cry1Ab/Ac*). BTC/SPC from the FL to the PM were consistently at higher levels in the MH63 (*Cry2A**) leaves than those in MH63 (*Cry1Ab/Ac*). It is interesting to note that, MH63 (*Cry2A**) showed the highest BTC/SPC (4.3‰) at FS under N0.

Correlations among the growth characteristics of *Bt* rice

There were significant positive correlations among the grain yield at PM, biomass at PM, SPAD at FS and NPR at FS (Table 4). The SPAD and IE_N showed significant negative and positive correlations with the setting rate, respectively. What is more, the SPAD, grain yield, biomass and IE_N showed significant negative correlations to the BTC/SPC in the *Bt*-MH63 leaves.

Discussion

Our results showed that MH63 (*Cry2A**) had lower grain yield than its counterpart MH63 under N0 (Table 1). This reduction in the yield of MH63 (*Cry2A**) was due to the decreased setting rates observed exclusively in an N deficient environment. Reduction in the setting rate of MH63 (*Cry2A**) had also been found in our previous field experiment (Jiang et al. 2013). This apparent interaction

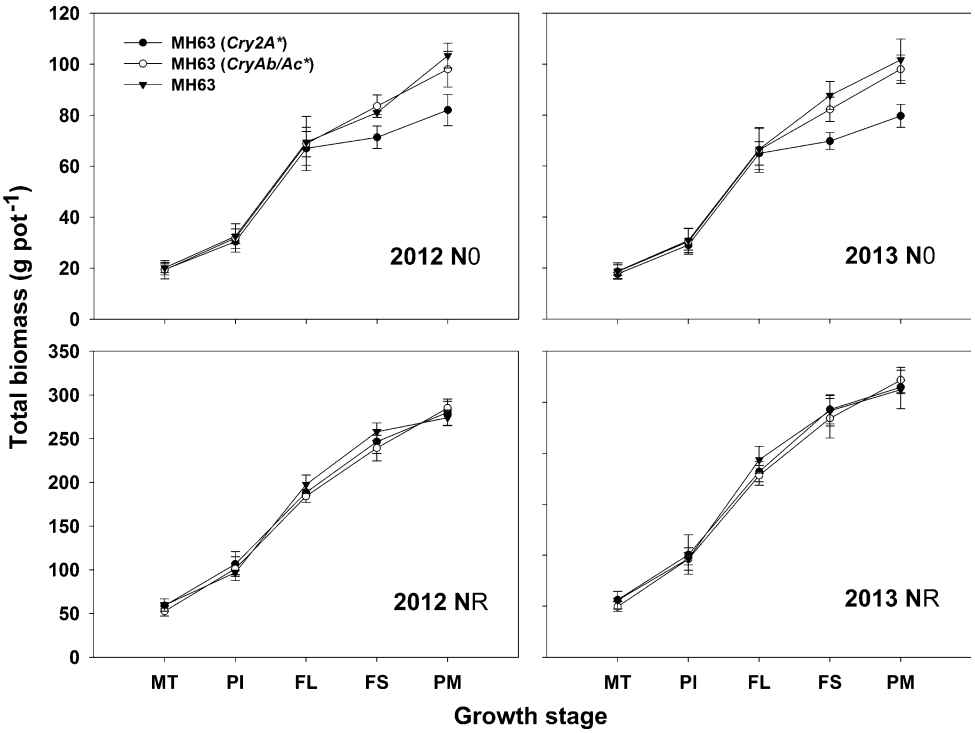

Fig. 1 Changes in the total biomass of *Bt*-MH63 and MH63 under different N applications in 2012 and 2013. *MT* mid-tillering stage, *PI* panicle initiation stage, *FL* flowering stage, *FS* filling stage, *PM* plant maturity stage. The *vertical bars* indicate standard deviations

between genotype and environment suggests that the negative effect of N deficiency on yield formation is more obvious in MH63 (*Cry2A**) than that in MH63. Lower setting rates, as it happens, were also reported in several other *Bt* rice lines with a *Cry1C** (Wang et al. 2012b), *Cry1Ab* (Kim et al. 2008), *Cry1Ac* (Shu et al. 2002), *Xa21* (Tu et al. 2000a) or *Bt/CpTI* (Xia et al. 2010) gene. As a common observation in *Bt* rice, lower setting rate should be considered in *Bt* rice breeding.

Photosynthesis after FL contributed more than 60% of the carbohydrates for yield formation in rice (Mae 1997). Postponed leaf senescence can sustain a high photosynthetic activity in rice, while accelerated leaf senescence especially during the grain filling stage will reduce the rice yield (IRRI 2002). In our study, accelerated leaf senescence, reflected by lower values for SPAD, NPR (Table 3), and SPC (Fig. 2) at the FS, was found in MH63 (*Cry2A**) under N0. The SPAD, NPR, biomass and grain yield (Table 4) were later found to be positively correlated with each other. As such, lower grain yield (Table 1) and less biomass (Fig. 1) were produced during the late growth stage in MH63 (*Cry2A**) under N0.

Leaf senescence and yield formation processes were closely related to the N metabolism in plant (Dalling et al. 1976; Yamaya et al. 2002; Jin et al. 2015). In our study, there was no difference in N uptake between *Bt*-MH63 and MH63 (Fig. S1). And yet, MH63 (*Cry2A**) had the lower IE_N under N0. This suggested that the N absorbed by MH63 (*Cry2A**) under N0 was not efficiently converted into grain. Variations in NUE and leaf senescence indicated that the N utilization and metabolism in MH63 (*Cry2A**) were more or less changed under N deficient environment. Variations in N utilization and metabolism were also observed in some *Bt* cotton lines (Chen et al. 2004; Sun et al. 2007; Poongothai et al. 2010). Dong and Li (2007) and Yukui et al. (2009) indicated that the changes in element utilization and distribution in *Bt* cottons were probably due to the decreased soluble element-binding proteins as influenced by the Bt protein expression in the plant. Coincidentally, lower SPC in *Bt* rice MH63 (*Cry2A**) under N0 at FS was also found in our study (Fig. 2). This indicates that the Bt protein expression might have interfered the expression of other proteins. Further studies are still required to ascertain the reasons for changes in the N metabolism of *Bt* crops.

Transgenes may bring unexpected fitness cost to crops (Marrelli et al. 2006; Xia et al. 2010). The added burden caused by constitutive high or over expression of transgenes was considered to be an important reason for the fitness cost (Gurr and Rushton 2005). However, high or over expression of Bt protein is essential for transgenic plant to ensure the effective insect resistance (Kota et al. 1999; Cohen et al. 2000). Temporal and spatial regulation in the expression of transgenes is an effective way to lessen the added burden of resistance expression (Hammond-Kosack and Parker 2003; Michelmore 2003). Generally, BTC in the plant dramatically declined during the late growth stage due to both plant aging and transcriptional regulation (Kranthi et al. 2005; Olsen et al. 2005; Poongothai et al.

Table 2 Nitrogen use efficiency of *Bt*-MH63 and MH63 under different N applications in 2012 and 2013

N application	Genotype	RE_N (%)	PE_N (g g^{-1})	AE_N (g g^{-1})	PFP_N (g g^{-1})	IE_N (g g^{-1})
2012						
N0	MH63 (*Cry2A**)	–	–	–	–	33.4 ± 0.8b
	MH63 (*Cry1Ab/Ac*)	–	–	–	–	40.5 ± 1.0a
	MH63	–	–	–	–	39.0 ± 0.7a
	Mean	–	–	–	–	37.6A
NR	MH63 (*Cry2A**)	53.2 ± 1.2a	42.7 ± 1.5a	22.8 ± 1.5a	32.7 ± 1.0a	35.3 ± 1.5a
	MH63 (*Cry1Ab/Ac*)	51.7 ± 0.7a	41.4 ± 2.6ab	21.0 ± 1.1a	34.4 ± 1.4a	37.1 ± 0.9a
	MH63	52.3 ± 0.8a	38.3 ± 1.6b	19.1 ± 1.8a	33.2 ± 2.0a	36.3 ± 0.8a
	Mean	52.4	40.8	21.0	33.4	36.2A
Analysis of variance						
N application		–	–	–	–	–*
Genotype		NS	–*	NS	NS	–*
N application × Genotype		–	–	–	–	–**
2013						
N0	MH63 (*Cry2A**)	–	–	–	–	32.3 ± 1.0b
	MH63 (*Cry1Ab/Ac*)	–	–	–	–	39.8 ± 1.2a
	MH63	–	–	–	–	38.4 ± 0.6a
	Mean	–	–	–	–	36.8A
NR	MH63 (*Cry2A**)	57.5 ± 1.9a	38.0 ± 1.3a	21.6 ± 1.1a	30.9 ± 1.7a	34.7 ± 1.3a
	MH63 (*Cry1Ab/Ac*)	52.8 ± 1.4b	33.2 ± 1.0b	18.1 ± 0.8a	29.4 ± 1.0a	35.1 ± 2.8a
	MH63	49.1 ± 0.6b	41.3 ± 1.2a	20.3 ± 0.9a	31.6 ± 0.9a	35.5 ± 1.2a
	Mean	53.1	37.5	20.0	30.6	35.1A
Analysis of variance						
N application		–	–	–	–	–*
Genotype		–*	–*	NS	NS	–*
N application × genotype		–	–	–	–	–*

Data are presented as the mean ± standard deviation (SD, n=3). Uppercase letters indicate LSD ($\alpha = 0.05$) grouping of means between the two N applications within each year. Lowercase letters indicate LSD ($\alpha = 0.05$) grouping of means across genotypes within an N application for each year. Means with the same letter are not significantly different. RE_N is N recovery efficiency; PE_N, N physiological efficiency; AE_N, N agronomic efficiency; PFP_N, N partial factor productivity; IE_N, internal N use efficiency

NS not significant

* Significant source of variation at $\alpha = 0.05$ while ** at $\alpha = 0.01$

2010). In our study, BTC in MH63 (*Cry1Ab/Ac*) leaves was lower than that in MH63 (*Cry2A**), and it dramatically reduced with the plant aging under N0 (Fig. 2). BTC in MH63 (*Cry2A**) leaves, by contrast, was higher, and crucially, the BTC maintained relatively high level in MH63 (*Cry2A**) during the maturation stage under N0 (Fig. 2). Coincidentally, phenotypical differences were found in MH63 (*Cry2A**) but not observed in MH63 (*Cry1Ab/Ac*). The higher BTC and decreased SPC led to a higher BTC/SPC in MH63 (*Cry2A**) than that in MH63 (*Cry1Ab/Ac*) under N0 at the FS (Fig. 2). Defects in morphology and development such as stunted plant and sterility were reportedly caused by a high BTC/SPC in *Bt* rice lines with *Cry1Ac* and *Cry2A Bt* genes (Gahakwa et al. 2000). In our study, the SPAD and NPR were found to be negative correlated to the BTC/SPC in MH63 (*Cry2A**) (Table 4). This implies that the accelerated leaf senescence in MH63 (*Cry2A**) was correlated with the high BTC/SPC in the plant under the N deficient environment. It is worth noting that inferior adaptations to nutrient deficiency and the other environmental stresses were also observed in *Bt* cotton in some studies. Wei-Dong et al. (2007) studied the changes in root exudates of *Bt* cotton and found that *Bt* cotton had less root organic acid exudation than its non-*Bt* counterpart under N deficient environment, and root organic acid exudation has been considered as important regulation substances against environmental stress (Bano et al. 1993; Rose et al. 2011; Tombesi et al. 2015). Zhang et al. (2007) demonstrated that some *Bt* cotton strains had lower biomass than its non-*Bt* counterpart under K deficient

Table 3 Leaf senescence of *Bt*-MH63 and MH63 under different N applications in 2012 and 2013

N application	Genotype	SPAD			FS		PM
		MT	PI	FL	SPAD	NPR (μmol CO_2 m^{-2} s^{-1})	SPAD
2012							
N0	MH63 (*Cry2A**)	27.1 ± 1.2a	31.6 ±1.2a	33.7 ± 2.3a	17.5 ± 1.3b	6.13 ± 0.76b	7.33 ± 0.30b
	MH63 (*Cry1Ab/Ac*)	27.4 ± 1.6a	30.5 ± 2.3a	35 ± 1.7a	24.1 ± 1.0a	8.29 ± 0.62a	8.92 ± 0.39a
	MH63	28.1 ± 0.9a	29.9 ± 2.0a	34.5 ± 2.2a	24.2 ± 1.7a	8.47 ± 0.51a	9.17 ± 0.29a
	Mean	27.5B	30.7B	34.4B	21.9B	7.63B	8.47B
NR	MH63 (*Cry2A**)	34.6 ± 1.6a	37.0 ± 2.0a	41.2 ± 2.6a	31.1 ± 1.0a	12.14 ± 0.28a	11.26 ± 0.25a
	MH63 (*Cry1Ab/Ac*)	33.9 ± 1.2a	36.2 ± 1.1a	40.7 ± 1.6a	33.2 ± 0.7a	11.82 ± 0.71a	12.09 ± 0.34a
	MH63	34.3 ± 1.1a	35.9 ± 1.1a	39.5 ± 1.4a	32.4 ± 1.2a	11.48 ± 0.56a	11.87 ± 0.35a
	Mean	34.3A	36.4A	40.5A	32.2A	11.81A	11.7 4A
Analysis of variance							
N application		–**	–**	–**	–**	–**	–*
Genotype		NS	NS	NS	–*	–*	–*
N application × genotype		NS	NS	NS	–*	–*	–*
2013							
N0	MH63 (*Cry2A**)	26.2 ± 0.7a	28.1 ± 0.8a	32.0 ± 1.0a	16.1 ± 0.4b	6.84 ± 0.12b	6.75 ± 0.27b
	MH63 (*Cry1Ab/Ac*)	23.8 ± 0.8a	28.9 ± 1.3a	32.3 ± 2.0a	23.2 ± 0.9a	9.08 ± 0.26a	8.29 ± 0.17a
	MH63	26.4 ± 1.1a	28.5 ± 1.5a	33.7 ± 1.2a	24.3 ± 0.7a	8.76 ± 0.33a	8.58 ± 0.37a
	Mean	25.5B	28.5B	32.7B	21.2B	8.23B	7.87B
NR	MH63 (*Cry2A**)	33.5 ± 1.4a	38.1 ± 0.9a	39.0 ± 1.2a	28.7 ± 1.1a	11.38 ± 0.45a	10.26 ± 0.43a
	MH63 (*Cry1Ab/Ac*)	35.4 ± 1.1a	36.2 ± 0.7a	39.8 ± 1.3a	31.4 ± 0.9a	12.24 ± 0.77a	10.85 ± 0.35a
	MH63	32.9 ± 1.6a	36.8 ± 0.8a	39.3 ± 0.9a	29.2 ± 1.0a	12.04 ± 1.18a	9.91 ± 0.15a
	Mean	33.9A	37.0A	39.4A	29.8A	11.89A	10.34A
Analysis of variance							
N application		–**	–**	–**	–**	–**	–**
Genotype		NS	NS	NS	–*	–*	–*
N application × genotype		NS	NS	NS	–*	–**	–*

Data are presented as the mean ± standard deviation (SD, $n = 3$). Uppercase letters indicate LSD ($\alpha = 0.05$) grouping of means between the two N applications within each year. Lowercase letters indicate LSD ($\alpha = 0.05$) grouping of means across genotypes within an N application for each year. Means with the same letter are not significantly different. Leaf senescence was monitored at mid-tillering stage (MT), panicle initiation stage (PI), flowering stage (FL), filling stage (FS), and plant maturity stage (PM). *NPR* net photosynthetic rate

* Significant source of variation at $\alpha = 0.05$ while ** at $\alpha = 0.01$. *NS* means not significant

environment. Besides, Li et al. (2015a) and Ma et al. (2015) reported greater influences of pathogens and CeO_2 nanoparticles on *Bt* cottons, respectively. Plant stress responses are associated with increased demands for energy and redistributions of elements (Gargallo-Garriga et al. 2014). Under the nutrient deficient environment, the nutrient elements and energy were insufficient to support the normal physiology metabolisms in the plant. The additional nutrient and energy consumptions caused by the Bt protein synthesis would more easily influence the metabolic balance when the plant was in a poor nutriture. We infer that the fitness cost and bio-burden would be more severe for varieties with higher Bt protein expressions under N deficient environments, and BTC/SPC is proposed to be an indicator for assessment of the bio-burden.

In conclusion, different response of the elite *Bt* restorer line MH63 (*Cry2A**) such as declining yield and lower biomass were found under the condition of N deficiency. The declining yield in MH63 (*Cry2A**) was caused by the lower setting rate, which was a common variation observed in *Bt* rice lines. Further reasons for the above variations in MH63 (*Cry2A**) were accelerated leaf senescence, which means a weaker photosynthesis, and a lower internal IE_N, which means a poorer N conversion efficiency to yield. Moreover, these variations were revealed to be correlated to the relatively higher BTC in MH63 (*Cry2A**) in the late growth stage. Higher BTC and lower SPC in MH63

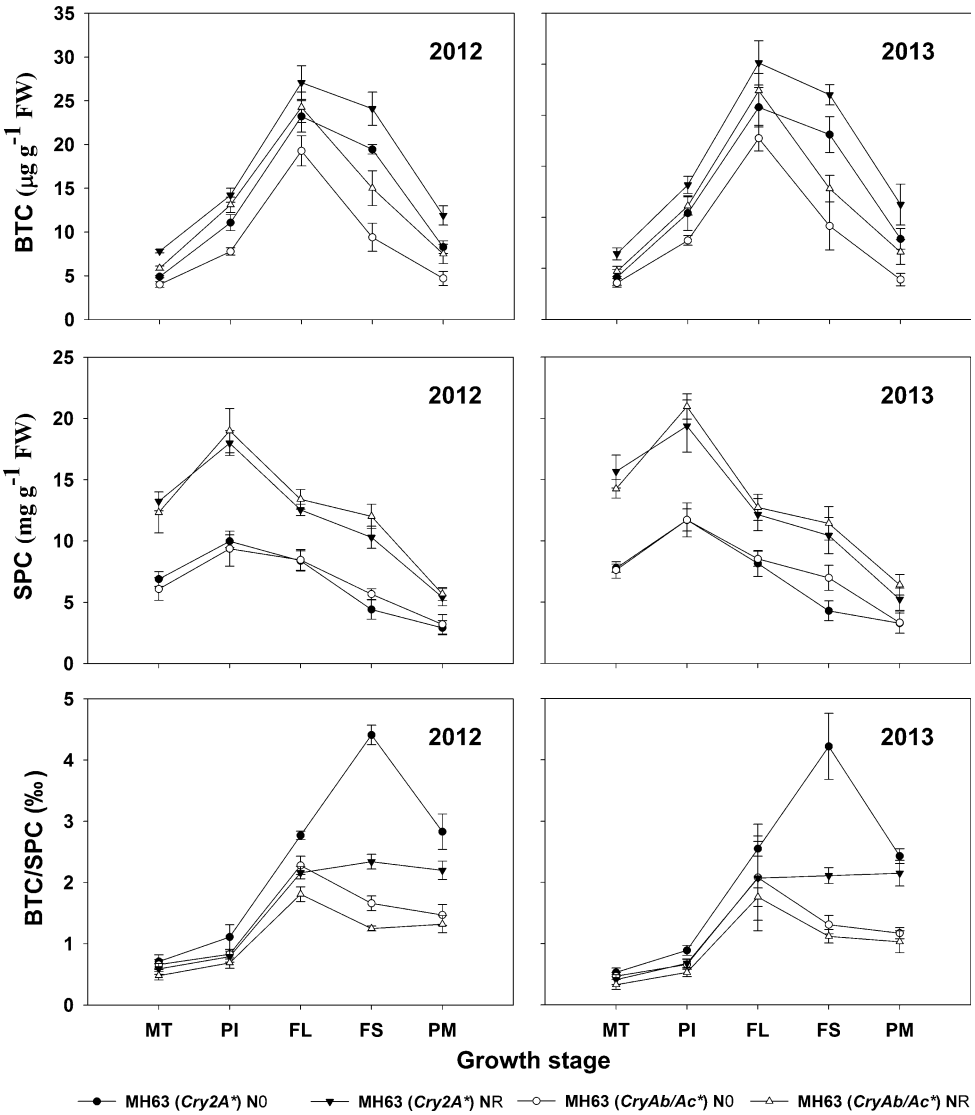

Fig. 2 Changes in the BTC, SPC and BTC/PC of *Bt*-MH63 leaves under different N applications in 2012 and 2013. *BTC* Bt protein content, *FW* fresh weight, *SPC* soluble protein content, *BTC/SPC* ratio of BTC to SPC, *MT* mid-tillering stage, *PI* panicle initiation stage, *FL* flowering stage, *FS* filling stage, *PM* plant maturity stage. The *vertical bars* indicate standard deviations

Table 4 Correlation among growth characteristics of *Bt*-MH63 under different N applications in 2012 and 2013

	BTC/SPC	SPAD	NPR	Setting rate	Grain yield	Biomass
SPAD	−0.43*					
NPR	−0.26	0.62**				
Setting rate	0.14	−0.33*	−0.22			
Grain yield	−0.41*	0.42*	0.39*	0.12		
Biomass	−0.44*	0.46*	0.53*	−0.16	0.67**	
IE_N	−0.32*	−0.26	−0.11	0.37*	0.23	0.14

BTC/SPC is the ratio of the Bt protein content to the soluble protein content; NPR, the net photosynthetic rate; IEN, the internal N use efficiency. The BTC/SPC, SPAD and NPR were values collected at the filling stage (FS). The setting rate, grain yield, biomass and IE_N were values collected at the plant maturity stage (PM)

* Significant correlation at $\alpha = 0.05$ while ** at $\alpha = 0.01$

(*Cry2A**) gave rise to the high BTC/SPC when the N supply was inadequate. The BTC/SPC in MH63 (*Cry1Ab/Ac*) was much lower in the late growth stage, and no unintended variations were observed in MH63 (*Cry1Ab/Ac*). The BTC/SPC is proposed to be an indicator for assessment of the bio-burden of Bt protein expression.

From this perspective, temporal and spatial regulations in the expression of Bt protein to maintain the desired BTC/SPC can be done to lessen the bio-burden of Bt protein expression. What's more, considerations must be given to both stable adaptation and effective resistance for the newly developed *Bt* rice lines.

Author contribution statement YJ and CGC conceived the research and designed the experiments; YJ, LL, LLZ, MLC, CFL, MZ and JPW performed the experiments and data analysis; YJ wrote the manuscript; AD revised the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The Authors declare that they have no conflict of interest.

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