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Elevated CO_2 alters transgene methylation not only in promoterregion but also in codingregion of *Bt* rice under different N-fertilizer levels

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The earth has been undergoing climate change, especially in recent years, driven by increasing concentration of atmospheric carbon dioxide (CO_2) and rising earth-surface temperature, which could reduce N allocation to Bt toxin for transgenic *Bt* crops (*Bt* crops), but the N fertilization is considered to be an effective method to enhance the C–N balance in *Bt* crops in the case of elevated CO_2 in future. DNA methylation not only in promoterregion but also in codingregion of transgene plays a critical role in transgene expression regulation and silencing of transgenic crops. Recent research has emphasized the risks of increased transgene silencing of *Bacillus thuringiensis* (*Bt*) rice under elevated CO_2. In this study, the effects of elevated CO_2 (vs. ambient CO_2) on exogenous *Bt* toxins and transgene expression in promoterregion and codingregion of *Bt* rice during tillering stage (cv. HH1 expressing fused *Cry1Ab/Cry1Ac*) were evaluated under three nitrogen (N) fertilizer rate (1/4, 1 and 2 N levels). The aboveground and belowground biomass, and foliar *Bt* protein content of *Bt* rice were all significantly increased with the augmentation of N-fertilizer. And elevated CO_2 significantly increased belowground biomass, total soluble protein content, transgene methylation levels in promoterregion (P1), and in total of promoterregion(P1) and codingregion (P2 + P3) (i.e., P1 + P2 + P3) at 1 N level, and it also increased transgene methylation levels in codingregion (P2), and in total of promoterregion and codingregion (P1 + P2 + P3) at 2 N level. In addition, elevated CO_2 decreased foliar Bt protein content at 1 N level. The transgene methylation levels in promoterregion and codingregion were negatively correlated with *Bt*-transgene expression level. The methylation level of cytosines located at CG sites was higher than those at CHG and CHH sites in P1, P2 and P3 fragments regardless of the CO_2 or N-fertilizer level. The correlation of transgene mehtylation in promoterregion with transgene expression is even stronger than that in codingregion. These data indicate that N fertilization supply will increase the Bt toxin content in transgenic *Bt* rice, especially under elevated CO_2.

Global atmospheric carbon dioxide (CO_2) concentration has increased from 280 ppm in pre-industrial to 404 ppm currently[1]. It has been projected that it will grow up to 700 ppm at the end of this century[2]. Elevated CO_2 can increase photosynthetic rate, biomass, and C:N ratio of plants[3–6]. Plants grown under elevated CO_2 accumulate increased level of nonstructural carbohydrates and afford lower nutritional quality of plant tissues for herbivorous insect pests[7]. Broadly speaking, assimilation and allocation profiles of carbon and nitrogen in plant under elevated CO_2 will change the primary and secondary metabolites of plants, thereby affecting the aboveground and belowground herbivorous insects[8–10].

Rice (*Oryza sativa* L.) is a stable food for more than half of the world's population[11]. Unfortunately, rice yields suffer huge losses by insect pests especially lepidopteran pests[12]. Researchers have developed transgenic rice varieties that produce insecticidal Cry toxins from *Bacillus thuringiensis* (*Bt*) in order to control target lepidopteran

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Parameters	CO_2 level (CO_2)		N-fertilizer level (N)		CO_2 × N	
	F-values	*P*-values	*F*-values	*P*-values	*F*-values	*P*-values
Aboveground biomass (g; fw)	9.30	0.003	193.81	< 0.001	3.29	0.04
Belowground biomass (g; fw)	12.24	< 0.001	244.28	< 0.001	3.25	0.04
Foliar total soluble protein (mg/g; fw)	1.43	0.24	15.73	< 0.001	2.02	0.15
Foliar Bt protein content (μg/g; fw)	0.58	0.46	72.99	< 0.001	3.54	0.045
Bt gene expression	3.84	0.07	4.55	0.03	16.61	< 0.001
Promoterregion methylation of P1 (%)	22.27	< 0.001	4.00	0.047*	23.54	< 0.001
Codingregion methylation of P2 (%)	1.61	0.23	0.05	0.95	3.02	0.086
Codingregion methylation of P3 (%)	0.004	0.95	0.28	0.76	0.46	0.64
Codingregion methylation of P2 + P3 (%)	1.70	0.22	0.13	0.88	3.92	0.049
Transgene methylation of P1 + P2 + P3 (%)	19.82	< 0.001	1.84	0.20	13.34	< 0.001

Table 1. Two-way ANOVAs for the effects of CO_2 and N-fertilizer levels, and their interaction on the belowground and aboveground biomass, foliar contents of total soluble protein and Bt toxin, *Bt*-transgene expression and methylation in promoter and coding regions of *Bt* rice with fused *Cry1Ab/Ac* during tillering stage, grown under ambient and elevated CO_2 with different N-fertilizer levels (*F* and *P* values).

pests[13,14]. Among them, Huahui 1 (HH1), expressing the fused *Cry1Ab/1Ac* gene, has high resistance to the target lepidopteran pests of rice and has been issued bio-safety certificates in China[15].

Because a biologically effective exogenous insect-resistant Bt toxin is expressed in transgenic rice, the stability of Bt toxin expression in plant tissues of *Bt* rice under elevated CO_2 has been of great interests among researchers. Previous studies have investigated the effects of elevated CO_2 on performance of *Bt* crops and stability of the transgenic traits[16–19]. Some studies have suggested that the exogenous gene expression in *Bt* plants transfers certain nutrients from the normal physiological pathways which may change the C-N balance, especially in the case of changed abiotic conditions[16–18,20]. The application of N fertilization can alleviate such nutrient diversion[21]. Coviella et al. found that elevated CO_2 reduced N allocation to Bt toxin, but the reduction was largely diverted by the augmentation of nitrogen[22]. Hence, the N fertilization is considered to be an effective method to enhance the C–N balance in *Bt* plants in the case of elevated CO_2 in the future[16,22].

DNA methylation represents a stable epigenetic mechanism in regulating gene expression[23–26]. Numerous studies have proven that DNA methylation plays a critical role on many aspects of plant growth, including flower development, responses to environment stress, transgene expression regulation and silencing[27–31]. Transgene silencing mainly includes two forms, that is, transcriptional gene silencing (TGS), in which DNA methylation occurs in promoter-region, and posttranscriptional gene silencing (PTGS), in which DNA methylation occurs in coding sequences[32–34]. Li et al. reported that promoter-region methylation repressed gene expression and coding-region methylation usually positively associated with gene expression[35]. During seedling stage of *Bt* rice, the foliar coding-region methylation keeped at low level and showed a moderate regulation of *Bt* gene expression under elevated CO_2 and N augmentation situation[19]. However, how did promoter-region methylation regulate the *Bt*-transgene expression of *Bt* rice under elevated CO_2 was still unclear. Tillering stage is a key period for the construction of rice population. The number of tillers and the quality of growth determine the formation of final yield. So, the higher foliar exogenous-toxin protein content of *Bt* rice grown under elevated CO_2 is important to control target lepidopteran pests and thus get higher yields. Investigating how transgene methylation in promoterregion and codingregion regulate the exogenous transgene expression under elevated CO_2 is vital to ensure higher *Bt*-transgene expression level for *Bt* rice.

In this study, the effects of elevated CO_2 on *Bt*-transgene expression in promoterregion and codingregion of *Bt* rice during tillering stage were investigate under different N-fertilizer levels. The aims of this study were to: (1) explore whether N-fertilizer application under elevated CO_2 condition can alleviate or eliminate the nitrogen limitation in *Bt* rice, (2) investigate how the transgene methylation levels in promoterregion and codingregion regulates *Bt*-transgene expression under elevated CO_2 condition.

Results

Belowground and aboveground biomass of Bt rice. CO_2, N-fertilizer levels and their interaction were significantly affected both the belowground and aboveground biomass of *Bt* rice ($P < 0.05$ or 0.001; Table 1). Both the belowground and aboveground biomass significantly increased with increased N-fertilizer augmentation, respectively ($P < 0.05$; Fig. 1). Compared with ambient CO_2, elevated CO_2 significantly increased the aboveground biomass of *Bt* rice grown at 2 N-fertilizer level (+ 25.74%), and belowground biomass of *Bt* rice grown at 1 N and 2 N-fertilizer levels (+ 27.71% and + 21.19%; $P < 0.05$, Fig. 1).

Foliar contents of total soluble protein and Bt protein of Bt rice. N-fertilizer level significantly affected the foliar content of total soluble protein of *Bt* rice ($P < 0.001$; Table 1). Under ambient CO_2, the foliar content of total soluble proteins of *Bt* rice grown at 1/4 N level were significantly lower (− 16.14%) than that at 2 N level ($P < 0.05$; Fig. 2A). Under elevated CO_2, the foliar content of total soluble proteins of *Bt* rice grown at reduced N-fertilizer level (1/4 N) were significantly lower than that at 1 N and 2 N levels (− 17.27% and −

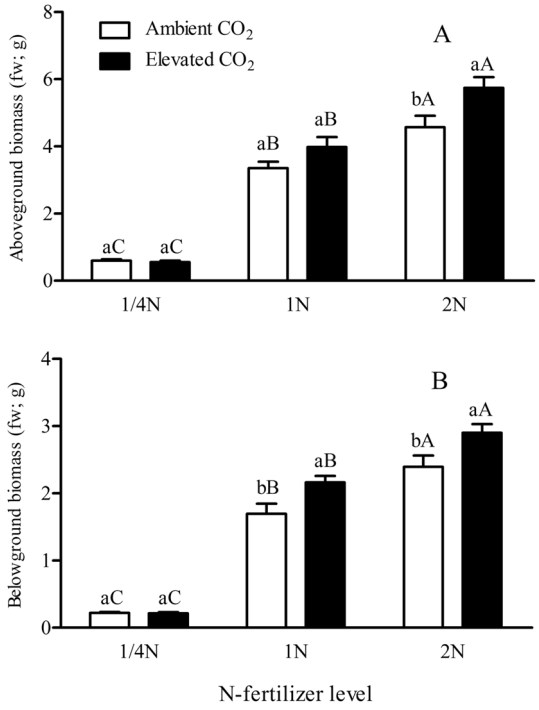

Figure 1. Aboveground (**A**) and belowground (**B**) biomass of *Bt* rice with fused *Cry1Ab/Ac* during tillering stage, grown under ambient and elevated CO_2 with different N-fertilizer levels. (Values are mean ± SE. Values denoted by different lowercase and uppercase letters indicate significant differences between the ambient CO_2 and elevated CO_2 for same N-fertilizer rates, and between the different N-fertilizer rates for same CO_2 level by LSD test at $P < 0.05$. The same in Figs. 2, 3, 4, 5, 6, 7).

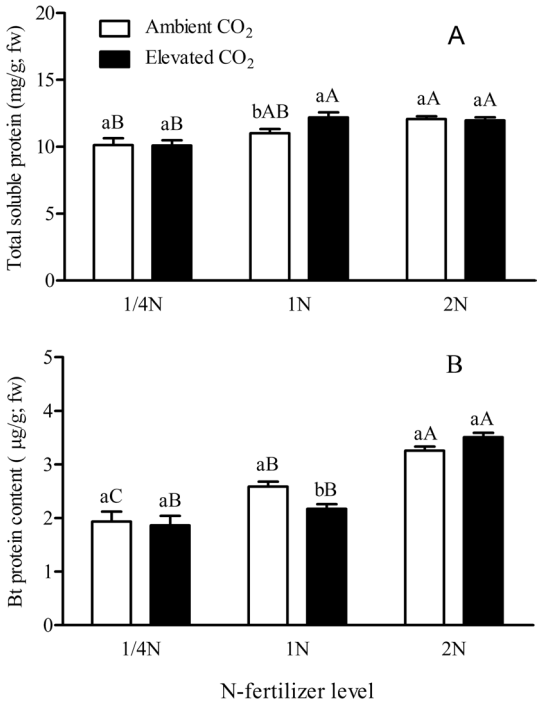

Figure 2. Foliar concentrations of total soluble protein (**A**) and Bt protein (**B**) in *Bt* rice with fused *Cry1Ab/Ac* during tillering stage, grown under ambient and elevated CO_2 with different N-fertilizer level.

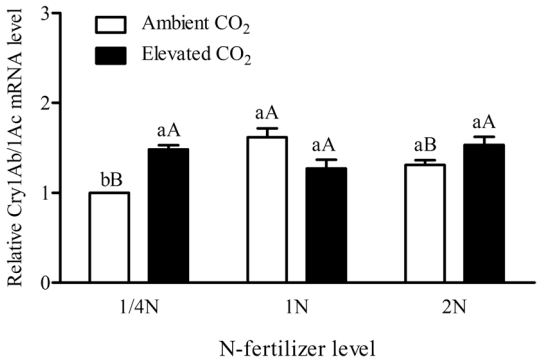

Figure 3. The relative transcript level of *Bt*-transgene in the leaves of *Bt* rice with fused *Cry1Ab/Ac* during tillering stage, grown under ambient and elevated CO_2 with different N-fertilizer level.

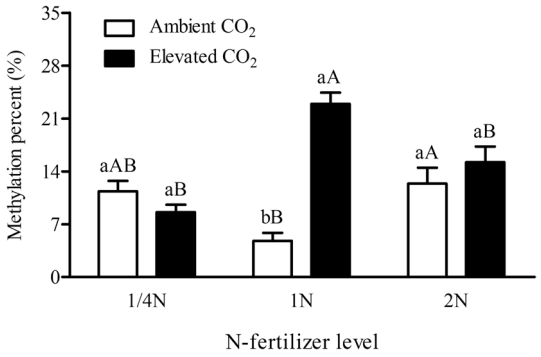

Figure 4. Cytosine methylation levels in the promoterregion (P1) of *Bt*-transgene in the leaves of *Bt* rice with fused *Cry1Ab/Ac* during tillering stage, grown under ambient and elevated CO_2 with different N-fertilizer level.

15.70%; $P < 0.05$, Fig. 2A). Compared with ambient CO_2, elevated CO_2 significantly increased the foliar content of total soluble proteins of *Bt* rice grown at 1 N level (+ 10.75%; $P < 0.05$, Fig. 2A).

N-fertilizer level ($P < 0.001$) and its interaction with CO_2 level ($P < 0.05$) significantly influenced the foliar *Bt* protein content of *Bt* rice (Table 1). Under ambient CO_2, the foliar *Bt* protein content of *Bt* rice significantly increased with the N fertilizer augmentation ($P < 0.05$; Fig. 2B). Under elevated CO_2, the foliar *Bt* protein content of *Bt* rice grown at 2 N level was significantly higher than that at 1/4 and 1 N levels (+ 88.21% and + 61.47%; $P < 0.05$; Fig. 2B). Compared with ambient CO_2, elevated CO_2 significantly decreased the foliar *Bt* protein content of *Bt* rice grown at 1 N level (− 16.04%; $P < 0.05$; Fig. 2B).

Bt transgene expression in the leaves of Bt rice. N-fertilizer level ($P < 0.05$) and its interaction with CO_2 level ($P < 0.001$) significantly affected the *Bt* transgene expression in the leaves of *Bt* rice (Table 1). Under ambient CO_2, the *Bt*-transgene expression level in the leaves of *Bt* rice grown at 1/4 N and 2 N level was significantly down-regulated when compared with that at 1 N level (− 38.16% and − 19.04%; $P < 0.05$; Fig. 3). Compared with ambient CO_2, elevated CO_2 just significantly up-regulated the *Bt*-transgene expression level in the leaves of *Bt* rice grown at 1/4 N level (+ 48.03%; $P < 0.05$; Fig. 3).

Methylation status in the promoterregion and codingregion of Bt-transgene in the leaves of Bt rice. *Promoterregion (P1) of Bt-transgene.* CO_2, N-fertilizer levels and their interaction significantly affected the methylation levels in the promoterregion (P1) of *Bt*-transgene in the leaves of *Bt* rice ($P < 0.05$; Table1). N-fertilizer level differently affected the methylation in the P1 fragment of *Bt*-transgene in the leaves of *Bt* rice. The methylation percentages in the P1 fragment of *Bt*-transgene in the leaves of *Bt* rice grown at 1/4 N level (+ 135.89%) and 2 N level (+ 157.23%) were markedly higher than that at 1 N level under ambient CO_2, respectively ($P < 0.05$; Fig. 4), while it was contrary tendency under elevated CO_2. Significant decreases in the methylation percentages were found in the P1 fragment of *Bt*-transgene in the leaves of *Bt* rice grown at 1/4 N level (− 62.52%) and 2 N level (− 33.75%) in contrast to that at 1 N level under elevated CO_2 ($P < 0.05$; Fig. 4). In addition, compared with ambient CO_2, elevated CO_2 obviously decreased the methylation percentages in the P1 fragment of *Bt*-transgene in the leaves of *Bt* rice grown at reduced N-fertilizer level (1/4 N) (− 24.21%; $P > 0.05$), and markedly enhanced the methylation percentages in the P1 fragment of *Bt*-transgene in the leaves of *Bt* rice grown at recommended normal (1 N: + 376.96%; $P < 0.05$) and increased N-fertilizer level (2 N: + 22.84%; $P > 0.05$, Fig. 4).

CO_2, N-fertilizer levels and CO_2 × N-fertilizer interactions significantly affected the methylation levels of cytosines located at CG and CHH sites in the P1 fragment of *Bt*-transgene in the leaves of *Bt* rice ($P < 0.05$;

Table 2). The methylation levels of cytosines located at CHG site in the P1 fragment of *Bt*-transgene in the leaves of *Bt* rice was just significantly affected by CO_2 and CO_2 × N interactions ($P < 0.05$; Table 2). Under ambient CO_2, the methylation level of cytosines located at CG and CHH sites in the P1 fragment of *Bt*-transgene in the leaves of *Bt* rice grown at 1/4 N level (+ 122.95% and + 140.32%; $P < 0.05$) and 2 N level (+ 112.82% and + 249.95%; $P < 0.05$) were markedly higher than that at 1 N level. In contrast, the methylation level of cytosines located at CG, CHG and CHH sites in the P1 fragment of *Bt*-transgene in the leaves of *Bt* rice grown at 1/4 N level (− 57.77%, − 58.41 and − 72.66%; $P < 0.05$) were significantly lower than that at 1 N level under elevated CO_2 (Fig. 5). Moreover, compared with ambient CO_2, elevated CO_2 markedly enhanced the methylation percentages of cytosines located at CG, CHG and CHH sites in the P1 fragment of *Bt*-transgene in the leaves of *Bt* rice grown at 1 N level (+ 313.79%, + 397.40% and + 511.32%; $P < 0.05$), and CG sites in the P1 fragment of *Bt*-transgene in the leaves of *Bt* rice grown at increased N-fertilizer level (2 N: + 35.67%; $P < 0.05$) (Fig. 5). The methylation level of cytosines located at CG sites was higher than those at CHG and CHH in the P1 fragment of *Bt*-transgene in the leaves of *Bt* rice regardless of the CO_2 or N-fertilizer level (Fig. 5).

Codingregion (P2, P3, P2 + P3) of Bt-transgene. The interaction between CO_2 and N-fertilizer levels significantly affected the methylation levels in the codingregion (P2 + P3) of *Bt*-transgene in the leaves of *Bt* rice ($P < 0.05$; Table 1). Compared with ambient CO_2, elevated CO_2 significantly enhanced the methylation percentages in the P2 + P3 fragments of *Bt*-transgene in the leaves of *Bt* rice grown at increased N-fertilizer level (2 N: + 47.24%; $P < 0.05$) (Fig. 6). CO_2 × N-fertilizer interaction significantly affected the methylation levels of cytosines located at CG site in the P2 + P3 fragments of *Bt*-transgenein the leaves of *Bt* rice ($P < 0.05$; Table 2). Compared with ambient CO_2, elevated CO_2 significantly enhanced the methylation percentages of cytosines located at CG sites in the P2 + P3 fragments of *Bt*-transgene in the leaves of *Bt* ricegrown at 2 N level (+ 67.52%; $P < 0.05$; Fig. 5). The methylation level of cytosines located at CG sites was higher than those at CHG and CHH sites in the P2 + P3 fragments of *Bt*-transgene in the leaves of *Bt* rice regardless of the CO_2 or N-fertilizer level (Fig. 5).

CO_2, Nitrogen-fertilizer levels and their interaction did not significantly affect methylation levels in the codingregion (P2) and codingregion (P3) of *Bt*-transgene in the leaves of *Bt* rice ($P > 0.05$; Table 1). In the codingregion (P2), the methylation percentage at 2 N level under elevated CO_2 (16.28%) was significantly higher than that under ambient CO_2 (9.72%) ($P < 0.05$, Fig. 6). There were no CHG and CHH sites as potential targets in the P2 fragment of *Bt*-transgene (Fig. 5). In the codingregion (P3), the methylated level was very low, not exceeding 5.74% (Fig. 6). The methylation level in the P3 fragment was lower than that in the P2 fragment of *Bt*-transgene in the leaves of *Bt* rice (Fig. 6). N-fertilizer level significantly influenced the methylation level of cytosines located at CG site in the P3 fragment of *Bt*-transgene in the leaves of *Bt* rice ($P < 0.05$; Table 2). Under ambient CO_2, methylation level of cytosines located at CG sites in the P3 fragment of *Bt*-transgene in the leaves of *Bt* rice grown under increased N-fertilizer level (2 N) was significantly lower that at 1 N level (− 70.47%, $P < 0.05$; Fig. 5).

Bt-transgene (P1 + P2 + P3). CO_2 and its interaction with N-fertilizer significantly affected the methylation levels in the *Bt*-transgene (P1 + P2 + P3) in the leaves of *Bt* rice ($P < 0.001$; Table 1). The methylation percentages in the P1 + P2 + P3 fragments of *Bt*-transgene in the leaves of *Bt* rice grown at 1/4 N level were significantly lower than that at 1 N and 2 N level under elevated CO_2 respectively (− 37.10% and − 15.80%; $P < 0.05$, Fig. 7). In addition, compared with ambient CO_2, elevated CO_2 markedly enhanced the methylation percentages in the P1 + P2 + P3 fragments of *Bt*-transgene in the leaves of *Bt* rice grown at recommended normal (1 N: + 87.17%; $P < 0.05$) and increased N-fertilizer level (2 N: + 36.17%; $P > 0.05$) (Fig. 7).

CO_2, N-fertilizer levels and their interactions significantly affected the methylation levels of cytosines located at CHH sites in the P1 + P2 + P3 fragments of *Bt*-transgene in the leaves of *Bt* rice ($P < 0.05$; Table 2). The methylation levels of cytosines located at CG in the P1 + P2 + P3 fragments of *Bt*-transgene in the leaves of *Bt* rice was significantly affected by CO_2 and its interaction with N-fertilizer ($P < 0.05$; Table 2), while the methylation levels of cytosines located at CHG sites in the P1 + P2 + P3 fragments of *Bt*-transgene in the leaves of *Bt* rice was just significantly affected by CO_2 level. The methylation level of cytosines located at CG sites was higher than those at CHG and CHH sites in the P1 + P2 + P3 fragments of *Bt*-transgene in the leaves of *Bt* rice regardless of the CO_2 or N-fertilizer level (Fig. 5). Under ambient CO_2, the methylation level of cytosines located at CHH sites in the P1 + P2 + P3 fragments of *Bt*-transgene in the leaves of *Bt* rice grown at 2 N level were markedly higher than that at 1 N level (+ 128.29%; $P < 0.05$, Fig. 5). Under elevated CO_2, the methylation level of cytosines located at CG and CHH sites in the P1 + P2 + P3 fragments of *Bt*-transgene in the leaves of *Bt* rice grown at 1/4 N level were significantly lower than that at 1 N level respectively (− 33.79% and − 61.01%; $P < 0.05$, Fig. 5). In addition, compared with ambient CO_2, elevated CO_2 markedly enhanced the methylation percentages of cytosines located at CG, CHG and CHH sites in the P1 + P2 + P3 fragments of *Bt*-transgene in the leaves of *Bt* rice grown at 1 N level (+ 62.03%, + 284.85% and + 229.98%; $P < 0.05$) and at CG sites in the P1 + P2 + P3 fragments of *Bt*-transgene in the leaves of *Bt* rice grown at increased N-fertilizer level (2 N: + 53.77%; $P < 0.05$) (Fig. 5).

The correlation between the transgene methylation in promoterregion and codingregion, and the Bt-transgene expression level. The Pearson's analysis showed that the methylation level in the promoterregion (P1) of *Bt*-transgene was negatively correlated with the *Cry1Ab/1Ac* expression level in the leaves of *Bt* rice (Fig. 8). The methylation level in the codingregion (P2 + P3) was slightly negatively correlated with the *Cry1Ab/1Ac* expression level in the leaves of *Bt* rice (Fig. 8). The methylation level in the *Bt*-transgene (P1 + P2 + P3) was negatively correlated with the *Cry1Ab/1Ac* expression level in leaves of *Bt* rice during tillering stage (Fig. 8).

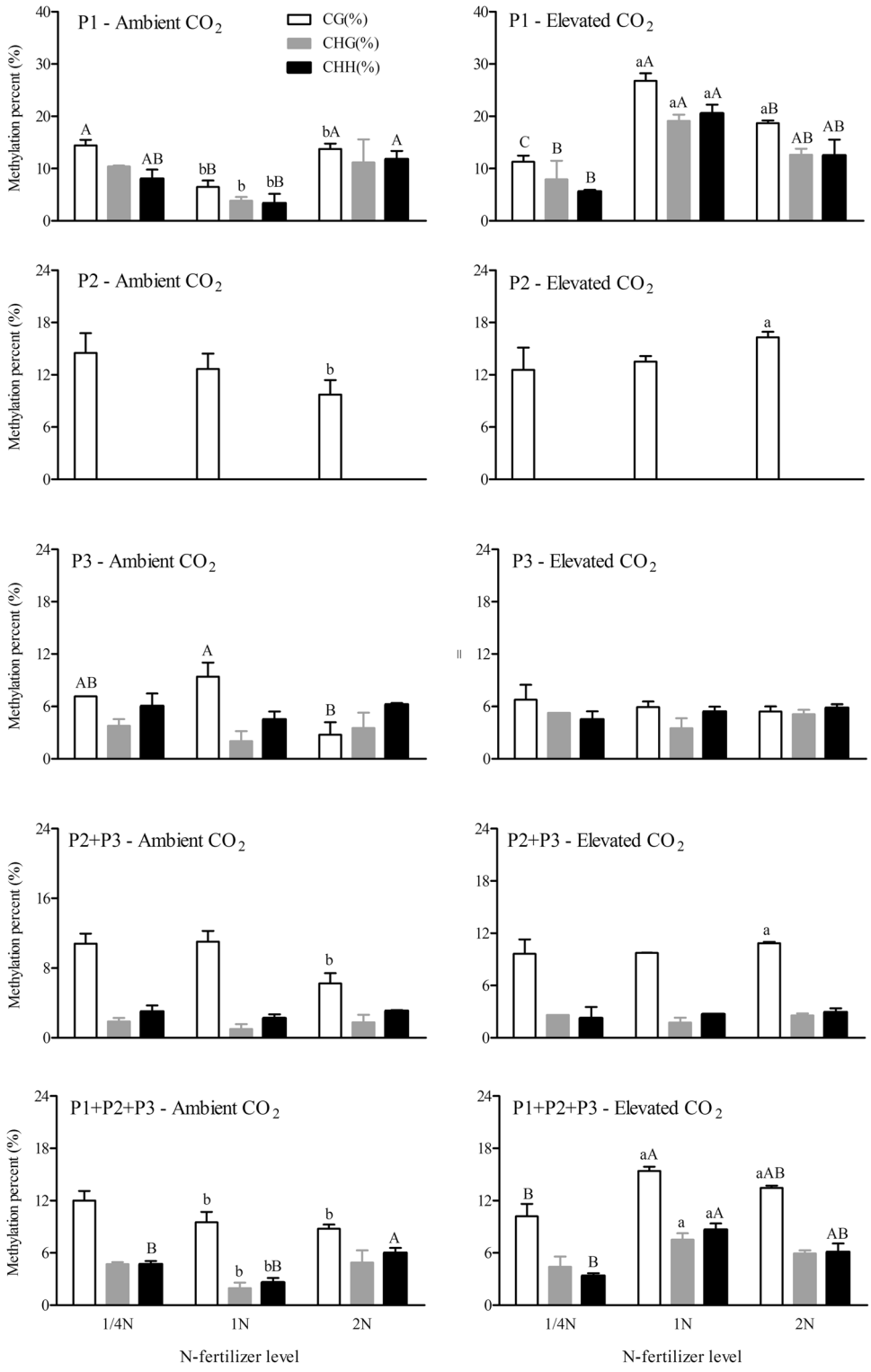

Figure5. Percentage of different methylation patterns (CG, CHG and CHH) in the promoterregion (P1), and codingregion (P2, P3 and P2 + P3) of Bt-transgene (P1 + P2 + P3) in the leaves of *Bt* rice with fused *Cry1Ab/Ac* grown under ambient and elevated CO_2 under three N-fertilizer levels.

Discussion

Previous studies showed that elevated CO_2 can stimulate plant growth and increase photosynthetic rate, photosynthate production, biomass and C: N ratios[36]. Hao et al. reported that the biomass of leaf, stem, pod, and total aboveground biomass of soybean increased with elevated CO_2[37]. Our results indicated that elevated CO_2 and increased N-fertilizer both increased the biomass of *Bt* rice. Also, it appeared that elevated CO_2 showed a positive

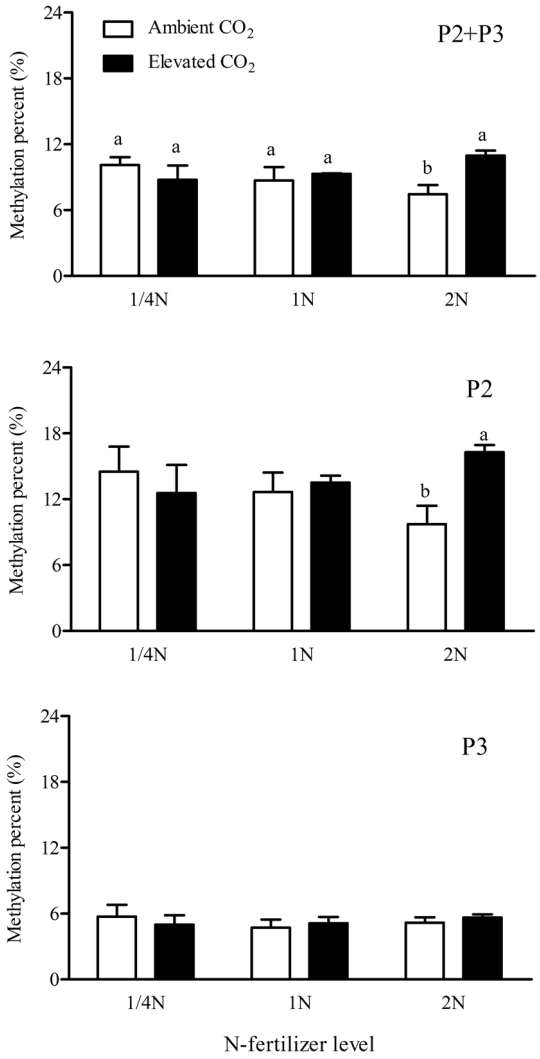

Figure 6. Cytosine methylation levels in the codingregion (P2, P3, P2 + P3) of Bt-transgene in the leaves of *Bt* rice with fused *Cry1Ab/Ac* during tillering stage, grown under ambient and elevated CO_2 with different N-fertilizer level.

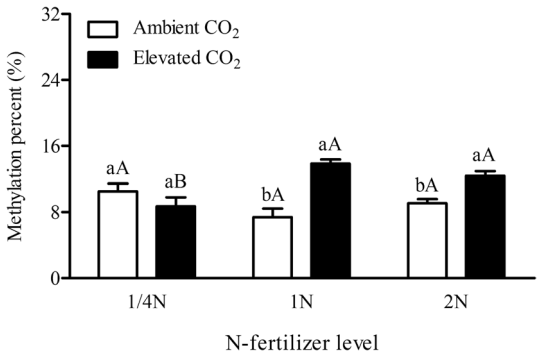

Figure 7. Cytosine methylation levels in the P1 + P2 + P3 of Bt-transgene in the leaves of Bt rice of the transgene promoter and coding-region in the leaves of transgenic *Bt* rice during tillering stage, grown under ambient and elevated CO_2 with different N-fertilizer level.

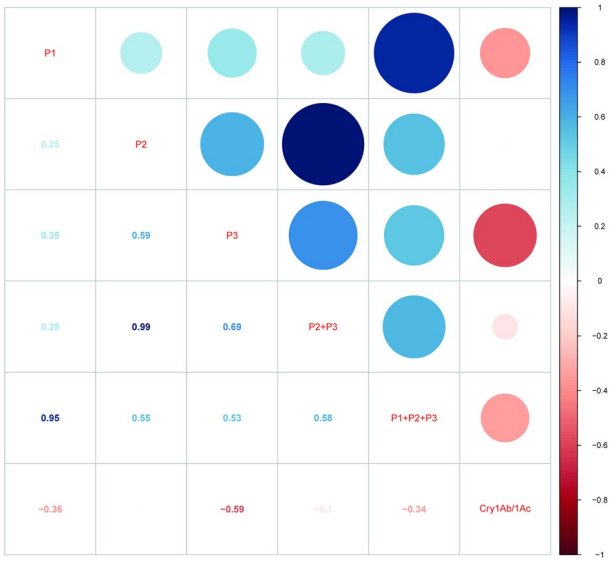

Figure 8. Pearson's analysis on the correlations between the methylation levels in the promoterregion (P1), and codingregion (P2, P3, P2 + P3) of *Bt*-transgene (P1 + P2 + P3) and the *Cry1Ab/1Ac* expression level in the leaves of *Bt* rice leaves during tillering stage, grown under ambient and elevated CO2 with different N-fertilizer level. (P1, CpG island 1 (promoterregion); P2, CpG island 2 (codingregion); P3, CpG island 3 (codingregion); P2 + P3, CpG island 2 + CpG island 3 (codingregion); P1 + P2 + P3, CpG island 1 + CpG island 2 + CpG island 3 (*Bt*-transgene)).

Transgene region	Cytosine methylation patterns	CO_2 level (CO_2)		N-fertilizer level (N)		CO_2 × N	
		F-values	*P*-values	*F*-values	*P*-values	*F*-values	*P*-values
Promoter region (P1)	CG (%)	66.98	< 0.001	7.01	0.0096	58.31	< 0.001
	CHG (%)	5.76	0.034	0.74	0.50	7.33	0.008
	CHH (%)	11.11	0.006	5.02	0.03	15.49	< 0.001
Codingregion (P2)	CG (%)	1.61	0.23	0.05	0.95	3.02	0.086
	CHG (%)	–	–	–	–	–	–
	CHH (%)	–	–	–	–	–	–
Codingregion (P3)	CG (%)	0.18	0.68	5.15	0.02	3.37	0.07
	CHG (%)	3.08	0.11	1.68	0.23	0.002	0.99
	CHH (%)	0.19	0.67	0.83	0.46	0.99	0.40
Codingregion (P2 + P3)	CG (%)	0.66	0.43	1.79	0.21	4.93	0.03
	CHG (%)	3.08	0.11	1.68	0.23	0.002	0.99
	CHH (%)	0.20	0.67	0.83	0.46	0.99	0.40
Transgene (P1 + P2 + P3)	CG (%)	18.64	0.001	2.24	0.15	6.72	0.01
	CHG (%)	18.38	0.001	3.63	0.06	3.57	0.06
	CHH (%)	15.47	0.002	10.41	0.002	12.12	0.001

Table 2. Two-way ANOVAs for the effects of CO_2 and N-fertilizer levels, and their interaction on the cytosine methylation percentage in the promoterregion (P1) and codingregion (P2, P3, P2 + P3) of *Bt*-transgene (P1 + P2 + P3) in the leaves of *Bt* rice with fused *Cry1Ab/Ac* during tillering stage, grown under ambient and elevated CO_2 with different N-fertilizer levels (*F* and *P* values).

effect on the aboveground biomass of *Bt* rice grown under higher N-fertilizer (i.e., 2 N level) and belowground biomass of *Bt* rice grown under 1 N and 2 N-fertilizer. The biomass of *Bt* rice was significantly increased with increased augmentation of N fertilizer. It is expected that the increased nitrogen uptake by the plant would enhance the rate of photosynthesis, resulting in increased biomass accumulation via increased CO_2 diffusion conductance and Rubisco content in *Bt* rice leaves[38–40]. Hence, elevated CO_2 and augmentation of N supply simultaneously increased the rice biomass, likely manifesting synergistically additive effects on biomass accumulation.

In recent years, the potential impacts of future CO_2 levels on *Bt* crops have attracted increasing attention. Our results show that foliar Bt protein content of *Bt* rice grown at elevated CO_2 were significantly lower than that under ambient CO_2 at 1 N level. It may be related to the decreased N allocation to Bt protein caused by elevated

CO_2[16]. Similarly, Coviella et al. found that elevated CO_2 decreased Bt protein content in *Bt* cotton[22]. In this study, the foliar Bt toxin content of *Bt* rice at 2 N level was significantly higher than those at 1 N and 1/4 N level, indicating that the doubling of nitrogen augmentation (i.e., 2 N) resulted in the enhanced foliar Bt protein content level in the leaves of *Bt* rice. Bruns and Abel reported that the Bt protein production of two transgenic *Bt*-transgenic maize lines increased with the augmentation of N fertilizer application[41]. Yang et al. found that the contents of Cry2A and Cry1C in *Bt* rice both increased in the tillering and milking stages with the higher N concentrations applied on rice[42]. Wang et al. documented that the Cry1Ab/1Ac content of *Bt*-SY63 at higher N fertilizer was significantly higher than that without N fertilizer treatment[43]. Moreover, the foliar content of total soluble protein at 1/4 N level was significantly lower than that at 1 N and 2 N level, respectively. The Bt protein content in plant tissues has been shown to significantly correlate with soluble protein and overall nitrogen content[41,44]. Hence, it is plausible to increase the Bt protein content in *Bt* crops by taking appropriate nitrogen management measures.

Epigenetic changes in DNA methylation can affect transgene expression for transgenic crops. DNA methylation occurs in codingregion has a more complex association with gene expression, whereas DNA methylation in promoterregion plays a vital role in transgene silencing[35]. For example, the resistance marker expression of transformed tobacco cultivars was rapidly lost and transgene expression were down-regulated, and hypermethylation within the 35S and NOS-promoters of these cultivars were found[45]. Additionally, environmental factors, such as drought and extreme temperature can potentially influence the methylation status[46–48]. In rice, 70% of the drought-induced methylation changing sites were reversed to their original status after water recovery[49]. In this study, our results showed that elevated CO_2 significantly enhanced the methylation percentages in the promoterregion (P1), and the P1 + P2 + P3 fragments of *Bt*-transgene in the leaves of *Bt* rice during tillering stage grown at 1 N level. In the codingregion, the methylation level in the P2 fragment of *Bt*-transgene, the fagment near the top strand of *Bt*-transgene, was higher than that in the P3 fragment, the fragment amplified from the bottom strand of *Bt*-transgene. Though the methylation level was low in P3 fragment of *Bt*-transgene, it was negatively correlated with the *Cry1Ab/1Ac* expressi in the leaves of *Bt* rice during tillering stage. In general, the methylation status in codingregion in *Bt*-transgene was slightly negatively correlated with the *Cry1Ab/1Ac* expression level in the leaves of *Bt* rice during tillering tage. Jiang et al. found that the PTGS methylation in the codingregion of *Bt*-transgene in the leaves of *Bt* rice during seeding stage remained at a relatively low level, lower than 5%[19]. The methylation level in the codingregion of *Bt*-transgene shows a weak regulation to the transgene expression. Thus, the methylation level in codingregionof *Bt*-transgene in the leaves of *Bt* rice has a weak regulation to the transgene expression both in tillering and seeding stage. The methylation levels in the promoterregion likely affected transgene expression more than that in the codingregion of *Bt*-transgene in the leaves of *Bt* rice. In addition, the Pearson's analysis also showed that the methylation level in the P1 + P2 + P3 fragments of *Bt*-transgene was negatively correlated with the *Cry1Ab/1Ac* expression in the leaves of *Bt* rice. Thus, the methylation level in the P1 + P2 + P3 fragments of *Bt*-transgene was showed moderate regulation to the transgene expression in the leaves of *Bt* rice during tillering stage.

Stable transgene expression and heritability are key factors for the development and application of transgenic crops. Environmental factors, such as soil salinity, water accessibility and temperature all play crucial roles in *Bt* transgene expression[50,51]. Trtikova et al. found that the *Cry1Ab* expression in MON 810 maize under hot/dry stress was significantly lower than that under optimal conditions[52]. Other studies with *Bt* crops have also indicated that environment might influence the levels of transgene expression differently[53]. Our results indicated that the *Bt* transgene expression was significantly up-regulated by elevated CO_2 under 1/4 N level, and *Bt* transgene expression level in the leaves of Bt rice grown at 1/4N and 2N level was significantly down-regulated when compared with that at 1N level under ambient CO_2. Considering the methylation level in promoterregion and codingregion of *Bt*-transgene was negatively correlated with the *Cry1Ab/1Ac* expression level in the leaves of *Bt* rice during tillerage stage, so we speculate that the different transgene expression level among different CO_2 and N treatments was caused by methylation in promoterregion and codingregion of *Bt*-transgene and post-transcriptional regulation in the leaves of *Bt* rice during tillering stage.

In conclusion, the methylation level in the promoterregion and codingregion of *Bt*-transgene were negatively correlated with the *Bt* transgene expression level in the leaves of *Bt* rice during tillering stage. The methylation levels in the promoterregion likely affected transgene expression more than that in the codingregion of *Bt*-transgene in the leaves of *Bt* rice during tillering stage. Elevated CO_2 showed positively effect on the transgene methylation level and negatively effect on the foliar *Bt* toxin content of *Bt* rice grown under 1 N level. The increased N-fertilizer level showed positively effect on the foliar *Bt* toxin content of *Bt* rice during tillering stage. Under elevated CO_2 situation in the future, moderate application of N-fertilizer can increase the foliar Bt toxin content in *Bt* rice. Futhermore, additional studies should be performed to evaluate the efficacy of the transgenic proteins against the target organisms under elevated CO_2, and thus the biological meaning behind it.

Materials and methods

Plant materials. The *Bt* rice cultivar HH1 (Huahui 1) was used in the study. The rice seeds were provided by Prof. Yongjun Lin from Huazhong Agricultural University (Wuhan, China). HH1 was developed by using MH63 as the recipient to harbor the fusion gene *Cry1Ab*/*Ac* from transgenic event TT51-1 (GenBank Accession Number: EU880444.1). Expression of the *Cry1Ab*/*Ac* gene is driven by the rice *actin 1* promoter and the nopaline synthase (NOS) gene terminator (seen in Fig. 9).

Plant growth conditions. This experiment was performed in electronically controlled growth incubator (GDN-400D-4/CO_2; Ningbo Southeast Instrument CO., LTD, Ningbo, China) connected with a gas-tank system for maintaining the desired atmospheric CO_2 concentration. The conditions in the chambers were maintained at 28 °C (day) and 25 °C (night) under a 16: 8 h light/dark photoperiod. The light intensity was 20,000 lx. Two CO_2 concentrations levels were applied continuously, i.e., elevated CO_2 (800 ppm, predicted CO_2 concentration

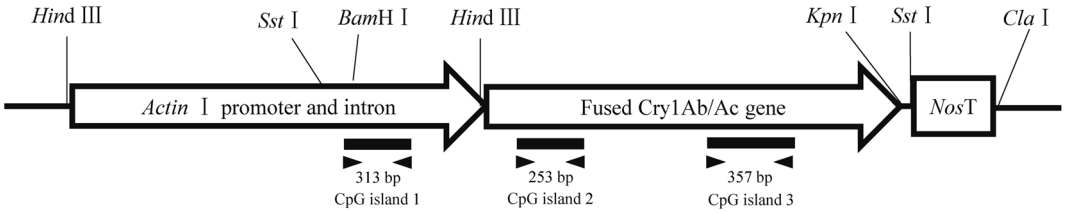

Figure 9. Schematic diagram of the fused *Cry1Ab/Ac* gene and its plasmid.

in 2100), and ambient CO_2 (about 400 ppm). With each CO_2 level, the N-fertilizer was set at three levels, 1/4, 1 and 2 N; the 1 N was 1.25 mM NH_4NO_3. Therefore, the experiment was consisted of 2 CO_2 concentrations × 3 N-fertilizer levels (total 6 treatment combinations) deployed in six electronically controlled growth incubators as three replications for CO_2 main factors.

The rice seeds of *Bt* rice (cv. HH1) were soaked in water for one day, and germinated on a board covered with wet cotton gauze for one day. Then, these seeds were sown into plastic foam covering (0.6 cm thick) on plastic cups (9 cm diameter, 7 cm height) and placed in the electronically controlled growth incubators of ambient and elevated CO_2. In the cup, there were two holes in the plastic foam and one rice seeds into each hole (total two seeds per cup). Thirty cups were placed in each electronically controlled growth chambers with 10 cups per N-fertilizer level. The cups were filled with modified culture solutions[54]; the solution was replaced with fresh solution every day. The composition of modified culture solutions was as follows (per liter): NH_4NO_3, 1.25 mM; KH_2PO_4, 0.3 mM; K_2SO_4, 1 mM; $CaCl_2{\cdot}2H_2O$, 1 mM; $MgSO_4{\cdot}7H_2O$, 1 mM; $Na_2SiO_3{\cdot}9H_2O$, 0.5 mM. (2) Micronutrient solution: $MnCl_2{\cdot}4H_2O$, 9 μM; $Na_2MoO_4{\cdot}2H_2O$, 0.39 μM; H_3BO_3, 20 μM; $ZnSO_4{\cdot}7H_2O$, 0.77 μM; $CuSO_4{\cdot}5H_2O$, 0.32 μM; $FeSO_4{\cdot}7H_2O$ + Na_2-EDTAN[54]. The plastic cups (plants) were re-randomized every two days within the chamber to minimize the positional effect. At tillering stage, the rice plants were collected, labelled, and stored at − 80 °C for various measurements.

Measurement of plant biomass. After sixty-five days for *Bt* rice grown under ambient and elevated CO_2 with different N-fertilizer levels (i.e., tillering stage), ten *Bt* rice plants for each N-fertilizer level were randomly selected from each growth incubator (i.e., 30 rice plants for each fertility-fertilizer level per CO_2 level). The biomass of belowground (root) and aboveground (stem and leaves) plant tissues were individually weighted with an electronic balance (Mettler Toledo AL 104; readability = 0.1 mg, repeatability < ± 0.1 mg).

Measurement of foliar contents of total soluble protein and Bt protein. After the measurement of plant biomass, the foliar contents of total soluble protein and *Bt* protein in the sampled rice plants were measured using the diagnostic kit, A045-2 (Nanjing Jiancheng Bioengineering Institute) and ELISA kits from EnviroLogix (Portland, ME; catalog number AP003), respectively. Three leaves from each sampled plant were taken as a sample unit and weighed. Five samples were measured for each treatment. The samples were individually placed into 2 ml microreaction tubes and homogenized in a Tissue Lyser II (Qiagen) by shaking for 3 min at 30 Hz with two steel balls in each tube. For the determination of foliar total soluble protein content, 0.9% saline was used as an extraction buffer in a proportion of 1:9 (m/v). Then, the measurement was performed by following the kit instructions. Optical density (OD) values were measured using a UV–Vis spectrophotometer (UV-1800PC, Mapada, Shanghai, China) at 595 nm wavelength. For the determination of foliar *Bt* protein content, samples were mixed with extraction buffer PBST (provided with the kit) in a proportion of 1: 10 to 1: 100 (m/v) and then measured the foliar *Bt* protein content in the leaves of *Bt* rice during tillering stage according to the kit instructions. The OD values were measured using a UV–Vis spectrophotometer at 450 nm wavelength.

Bioassay of the transcript expression levels of Bt-transgene. *RNA extraction and reverse transcription.* One leaf per rice plant was excised from 3 plants (total 3 leaves per replication) of each treatment combination of CO_2 and N-fertilizer levels for quantification of transcript expression levels of *Bt*-transgene in the leaves of *Bt* rice during tillering stage. Three samples were measured for each treatment. Total RNA was extracted from leaf tissues using TRIzol reagent following the supplier's protocol (Invitrogen). RNA concentration and integrity were evaluated using the NanoDrop spectrophotometer (Thermo Scientific). First strand cDNA templates were synthesized using Prime Script RT reagent kit (TaKaRa, Japan).

Real-time PCR analysis. Quantitative real-time PCR (qRT-PCR) experiment was carried out using SYBR Premix Ex Taq (TaKaRa, Japan) following the kit instructions. Expression of the target gene (i.e., *Bt*-transgene) was normalized relative to the expression of the housekeeping genes actin1 and ubiquitin. Quantification of the transcript level of *Bt*-transgene in the leaves of *Bt* rice during tillering stage was based on the method of Livak and Schmittgen[55]. Primers used for qRT-PCR are listed in Table 3.

Methylation analysis of Bt-transgene. Genomic DNA were extracted and purified from 30 mg treated leaves of Bt rice from each treatment combination of CO_2 and N-fertilizer levels during tillering stage using DNAsecure Plant Kit (TIANGEN, Beijing, China) following the product instructions. DNA concentration was quantified in the NanoDrop spectrophotometer. Then, 100 ng of isolated DNA was submitted to bisulfite treat-

Primer	Sequence (5′-3′)	GeneBank accession	Description
Cry1Ab/Ac-F	TAGAGTTCGTGTGAGGTA	EU816953	*Bt* protein gene
Cry1Ab/Ac-R	CTGTATTGGAGAAGATGGAT		
Actin1-F	ATGGCAACATTGTGCTCAGTG	*Bt*130427[95]	Rice housekeeping gene
Actin1-R	CCTCCGATCCAGACGCTGTA		
Ubiquitin-F	GCTCCGTGGCGGTATCAT	NC_029258[96]	Rice housekeeping gene
Ubiquitin-R	CGGCAGTTGACAGCCCTAG		

Table 3. Primers used for qRT-PCR in quantifying transcript expression levels of *Bt* transgene.

CpG island	Sequence (5′–3′)
CpG island 1	TTTTTGGTTTTGGTAGTTTGGGTGGGCGAGAGGCGGCTTCGTGCGCGCCCAGATCGGTGCGCGGGAGGGGCG GGATCTCGCGGCTGGGGCTCTCGCCGGCGTGGATCCGGCCCGGATCTCGCGGGGAATGGGGCTCTCGGATGT AGATCTGCGATCCGCCGTTGTTGGGGGAGATGATGGGGGGTTTAAAATTTCCGCCATGCTAAACAAGATCAG GAAGAGGGGAAAAGGGCACTATGGTTTATATTTTTATATATTTCTGCTGCTTCGTCAGGCTTAGATGTGCTAGAT TTTTTTTTTTTTTTTTTGTGGG
CpG island 2	TTGGTGTAAATTGAGTAGTTGATTAATTAGAGGATCGAAGAGTTCGTTAGGAATTAGGTTATTTTTAGGTTGGAA GGATTGAGTAATTTTTATTAAATTTATGTAGAGAGTTTTAGAGAGTGGGAAGTCGATTTTATTAATTAAGTTTTT CGCGAGGAAATGCGTATTTAATTTAACGATATGAATAGCGTTTTGATTATAGTTATTTTATTGTTCGTAGTTTAG AATTATTAAGTTTTTTTTTTGTTCGTGT
CpG island 3	GGAGAGTATTACTGGTCTGGACACCAGATCATGGCCTCTCCAGTTGGATTCAGCGGGCCCGAGTTTACCTTT CCTCTCTATGGAACTATGGGAAACGCCGCTCCACAACAACGTATCGTTGCTCAACTAGGTCAGGGTGTCTAC AGAACCTTGTCTTCCACCTTGTACAGAAGACCCTTCAATATCGGTATCAACAACCAGCAACTTTCCGTTCTTGAC GGAACAGAGTTCGCCTATGGAACCTCTTCTAACTTGCCATCCGCTGTTTACAGAAAGAGCGGAACCGTTGAT TCCTTGGACGAAATCCCACCACAGAACAACAATGTGCCACCCAGGTAAGGATTTTTTTATAGGTTG

Table 4. DNA sequences of CpG islands in the protmoterregion (P1) and coddingregion (P2 and P3) of *Bt*-transgene in the leaves of *Bt* rice during tillering stage, grown under ambient and elevated CO_2 with different N-fefrtilizer levels.

Primer	Sequence (5′–3′)	Description
P1-F	TTTTTGGTTTTGGTAGTTTGG	CpG island 1
P1-R	CCCACAAAAAAAAAAAAAAAAA	
P2-F	TTGGTGTAAATTGAGTAGTTGAT	CpG island 2
P2-R	ACACRAACAAAAAAAAAACTTA	
P3-F	GGAGAGTATTATTGGTTTGGATA	CpG island 3
P3-R	CAACCTATAAAAAAATCCTTACCT	

Table 5. Primers for bisulfite sequencing of *Bt*-transgene in the leaves of *Bt* rice during tillering stage, grown under ambient and elevated CO_2 with different N-fefrtilizer levels.

ment to convert non-methylated cytosines into uracil. The conversion was performed using the DNA Bisulfite Conversion Kit (TIANGEN, Beijing, China). Three types of cytosines -CG, CHG and CHH were analyzed in two regions of transgene: a fragment of the Actin 1 promoter (P1, CpG island 1) and two fragments of *Cry1Ab/1Ac* coding region (P2, CpG island 2 and P3,CpG island 3) (Table 4). The bisulfite sequencing primers were designed using Methyl Primer Express Software (Applied Biosystems) (Table 5).

The target sequences of *Bt*-transgene were amplified from the Bisulfite-treated genomic DNA by PCR with Methylation-specific Kit (TIANGEN, Beijing, China). The PCR conditions consisted of denaturation at 95 °C for 5 min, followed by 35 cycles at 94 °C for 20 s, 60 °C for 30 s, 72 °C for 20 s, and annealing at 72 °C for 5 min. The PCR products were purified using AxyPrep DNA Gel Extraction Kit (Axygen, Union City, USA), cloned into *pEASY*-T3 Cloning Vector and transformed into *Trans* 1-T1 Phage Resistant Chemically Competent Cell (TransGen, Beijing, China). Positive clones were screened with PCR using M13R and M13F primers. Sequencing were done for at least ten independent positive clones from each PCR product was carried out.

Data analysis. All statistical analyses were conducted using SPSS (version 22.0; SPSS Inc., Chicago IL, USA; https://www.ibm.com/products/spss-statistics). DNA methylation levels (%) in CG, CHG and CHH cytosine types were assessed using the kismeth web tool. Two-way analysis of variances (ANOVAs) were performed to examine the effects of CO_2 (Ambient vs. Elevated) and N-fertilizer (1/4, 1 and 2 N), and their interactions on plant biomass, foliar contents of total soluble protein and *Bt* protein, the gene expression levels of *Cry1Ab/Ac*, and the methylation level in the promoterregion (P1) and codingregion (P2, P3, P2 + P3) of *Bt*-transgene (P1 + P2 + P3) in the leaves of *Bt* rice during tillering stage. If there were significant effects of CO_2 level, N-fertilizer

level or their interaction, the least significant difference (LSD) test was used to separate the treatment means at $P < 0.05$. The Pearson's test was performed by R software (version R i386 3.4.2; https://www.r-project.org/) to analyze correlations among methylation level in promoter region and coding region of *Bt*-transgene with the transgene expression level in the leaves of *Bt* rice during tillering stage, grown under ambient and elevated CO_2 with different N-fertilizer levels.

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Author contributions

Y.M.L. and F.J.C. designed the study; Y.M.L., Y.H.W., G.C., and C.X.L. performed the experiments; Y.M.L. wrote the manuscript; Y.M.L., S.L.J, M.N.P. and F.J.C reviewed and polished the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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