Elevated CO₂ alters transgene methylation not only in promoter region but also in coding region 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 (CO₂) 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 CO₂, in future. DNA methylation not only in promoter region but also in coding region 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 CO₂. In this study, the effects of elevated CO₂ (vs. ambient CO₂) on exogenous Bt toxins and transgene expression in promoter region and coding region 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 CO₂ significantly increased belowground biomass, total soluble protein content, transgene methylation levels in promoter region (P1), and in total of promoter region (P1) and coding region (P2 + P3) (i.e., P1 + P2 + P3) at 1 N level, and it also increased transgene methylation levels in coding region (P2), and in total of promoter region and coding region (P1 + P2 + P3) at 2 N level. In addition, elevated CO₂ decreased foliar Bt protein content at 1 N level.

The transgene methylation levels in promoter region and coding region 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 CO₂ or N-fertilizer level. The correlation of transgene methylation in promoter region with transgene expression is even stronger than that in coding region. These data indicate that N fertilization supply will increase the Bt toxin content in transgenic Bt rice, especially under elevated CO₂.

Global atmospheric carbon dioxide (CO₂) concentration has increased from 280 ppm in pre-industrial to 404 ppm currently. It has been projected that it will grow up to 700 ppm at the end of this century. Elevated CO₂ can increase photosynthetic rate, biomass, and C:N ratio of plants. Plants grown under elevated CO₂ accumulate increased level of nonstructural carbohydrates and afford lower nutritional quality of plant tissues for herbivorous insect pests. Broadly speaking, assimilation and allocation profiles of carbon and nitrogen in plant under elevated CO₂ will change the primary and secondary metabolites of plants, thereby affecting the aboveground and belowground herbivorous insects.

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

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During seedling stage of development, responses to environment stress, transgene expression regulation and silencing. Transgene studies have proven that DNA methylation plays a critical role on many aspects of plant growth, including flower stage, grown under ambient and elevated CO2 with different N-fertilizer levels. Bt-transgene expression and methylation in promoter and coding regions of Bt rice with fused Cry1Ab/Ac 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 CO2 and N-fertilizer levels, and their interaction on the beloground 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 CO2 with different N-fertilizer levels (F and P values).

Results

**Belowground and aboveground biomass of Bt rice.** CO2, 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 CO2, elevated CO2 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 CO2, the foliar content of total soluble proteins of Bt rice grown at 1/4 N level were significantly lower than at 1 N and 2 N levels (− 16.14%) than that at 2 N level (P < 0.05; Fig. 2A). Under elevated CO2, 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 − 18.23% at 1 N level) compared with 2 N level (P < 0.001; Fig. 2A).
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.
15.70%; \( P < 0.05 \), Fig. 2A). Compared with ambient \( \text{CO}_2 \), elevated \( \text{CO}_2 \) 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 \( \text{CO}_2 \) level \( (P < 0.05) \) significantly influenced the foliar \( Bt \) protein content of \( Bt \)-rice (Table 1). Under ambient \( \text{CO}_2 \), the foliar \( Bt \) protein content of \( Bt \)-rice significantly increased with the N fertilizer augmentation \( (P < 0.05) \); Fig. 2B). Under elevated \( \text{CO}_2 \), 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 \( \text{CO}_2 \), elevated \( \text{CO}_2 \) 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 \( \text{CO}_2 \) level \( (P < 0.001) \) significantly affected the \( Bt \)-transgene expression in the leaves of \( Bt \)-rice (Table 1). Under ambient \( \text{CO}_2 \), 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 \( \text{CO}_2 \), elevated \( \text{CO}_2 \) 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 promoter region and coding region of \( Bt \)-transgene in the leaves of \( Bt \)-rice.** Promoter region (P1) of \( Bt \)-transgene. \( \text{CO}_2 \), N-fertilizer levels and their interaction significantly affected the methylation levels in the promoter region (P1) of \( Bt \)-transgene in the leaves of \( Bt \)-rice. 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 \( \text{CO}_2 \), respectively \( (P < 0.05) \); Fig. 4), while it was contrary tendency under elevated \( \text{CO}_2 \). 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 \( \text{CO}_2 \) \( (P < 0.05) \); Fig. 4). In addition, compared with ambient \( \text{CO}_2 \), elevated \( \text{CO}_2 \) 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).

\( \text{CO}_2 \), N-fertilizer levels and \( \text{CO}_2 \times \) 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; \( P < 0.05 \) at various N-fertilizer levels and \( \text{CO}_2 \) conditions.
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 CO2 and CO2 × N interactions (P < 0.05; Table 2). Under ambient CO2, 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 CO2 (Fig. 5).

Moreover, compared with ambient CO2, elevated CO2 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 CO2 or N-fertilizer level (Fig. 5).

**Coding region (P2, P3, P2 + P3) of Bt-transgene.** The interaction between CO2 and N-fertilizer levels significantly affected the methylation levels in the coding region (P2 + P3) of Bt-transgene in the leaves of Bt rice (P < 0.05; Table 1). Compared with ambient CO2, elevated CO2 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). CO2 × N-fertilizer interaction significantly affected the methylation levels of cytosines located at CG site in the P2 + P3 fragments of Bt-transgene in the leaves of Bt rice (P < 0.05; Table 2). Compared with ambient CO2, elevated CO2 significantly enhanced the methylation percentages of cytosines located at CG sites in the P2 + P3 fragments of Bt-transgene in the leaves of Bt rice grown 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 CO2 or N-fertilizer level (Fig. 5).

CO2, Nitrogen-fertilizer levels and their interaction did not significantly affect methylation levels in the coding region (P2) and coding region (P3) of Bt-transgene in the leaves of Bt rice (P > 0.05; Table 1). In the coding region (P2), the methylation percentage at 2 N level under elevated CO2 (16.28%) was significantly higher than that under ambient CO2 (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 coding region (P3), the methylation 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 CO2, 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).** CO2 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 CO2 respectively (− 37.10% and − 15.80%; P < 0.05) (Fig. 7). In addition, compared with ambient CO2, elevated CO2 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).

CO2, 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 CO2, 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 CO2 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 CO2 or N-fertilizer level (Fig. 5). Under ambient CO2, 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 CO2, 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 CO2, elevated CO2 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 promoter region and coding region, and the Bt-transgene expression level.** The Pearson’s analysis showed that the methylation level in the promoter region (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 coding region (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).
Discussion

Previous studies showed that elevated CO$_2$ can stimulate plant growth and increase photosynthetic rate, photosynthate production, biomass and C: N ratios\textsuperscript{36}. Hao et al. reported that the biomass of leaf, stem, pod, and total aboveground biomass of soybean increased with elevated CO$_2$\textsuperscript{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 coding region (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 CO₂ 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 CO₂ with different N-fertilizer level.
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 CO2 diffusion conductance and Rubisco content in Bt rice leaves.38–40 Hence, elevated CO2 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 CO2 levels on Bt crops have attracted increasing attention. Our results show that foliar Bt protein content of Bt rice grown at elevated CO2 were significantly lower than that under ambient CO2 at 1 N level. It may be related to the decreased N allocation to Bt protein caused by elevated CO2.

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

| Transgene region | Cytosine methylation patterns | CO2 level (CO2) | N-fertilizer level (N) | CO2 × 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 |
| Coding region (P2) | CG (%) | 1.61 | 0.23 | 0.05 | 0.95 | 3.02 | 0.086 |
|                   | CHG (%) | – | – | – | – | – | – |
|                   | CHH (%) | – | – | – | – | – | – |
| Coding region (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 |
| Coding region (P2 + P3) | CG (%) | 0.66 | 0.43 | 1.79 | 0.21 | 4.95 | 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 CO2 and N-fertilizer levels, and their interaction on the cytosine methylation percentage in the promoter region (P1) and coding region (P2, P3, P2 + P3) of Bt-transgene (P1 + P2 + P3) in the leaves of Bt rice with fused Cry1Ab/1Ac during tillering stage, grown under ambient and elevated CO2 with different N-fertilizer levels (F and P values).
showed that the methylation level in the P1 + P2 + P3 fragments of rice. In addition, the Pearson’s analysis also indicated 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. 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. 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. 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. 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 coding region has a more complex association with gene expression, whereas DNA methylation in promoter region plays a vital role in transgene silencing. 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. Additionally, environmental factors, such as drought and extreme temperature can potentially influence the methylation status.

In rice, 70% of the drought-induced methylation changing sites were reversed to their original status after water recovery. In this study, our results showed that elevated CO2 significantly enhanced the methylation percentages in the promoter region (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 fragment 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 express in the leaves of Bt rice during tillering stage. In general, the methylation status in coding region of Bt-transgene was slightly negatively correlated with the Cry1Ab/1Ac expression level in the leaves of Bt rice during tillering stage. Jiang et al. found that the PTGS methylation in the coding region of Bt-transgene in the leaves of Bt rice during seedling stage remained at a relatively low level, lower than 5%. The methylation level in the coding region of Bt-transgene shows a weak regulation to the transgene expression. Thus, the methylation level in coding region of Bt-transgene in the leaves of Bt rice has a weak regulation to the transgene expression both in tillering and seedling stage. The methylation levels in the promoter region likely affected transgene expression more than that in the coding region 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 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. 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. Additionally, environmental factors, such as drought and extreme temperature can potentially influence the methylation status.

In conclusion, the methylation level in the promoter region and coding region 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 promoter region likely affected transgene expression more than that in the coding region of Bt-transgene in the leaves of Bt rice. In general, the methylation status in coding region of Bt-transgene was slightly negatively correlated with the Cry1Ab/1Ac expression level in the leaves of Bt rice during tillering stage. Jiang et al. found that the PTGS methylation in the coding region of Bt-transgene in the leaves of Bt rice during seedling stage remained at a relatively low level, lower than 5%. The methylation level in the coding region of Bt-transgene shows a weak regulation to the transgene expression. Thus, the methylation level in coding region of Bt-transgene in the leaves of Bt rice has a weak regulation to the transgene expression both in tillering and seedling stage. The methylation levels in the promoter region likely affected transgene expression more than that in the coding region 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 showed moderate regulation to the transgene expression in the leaves of Bt rice during tillering stage.

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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 M163 as the recipient to harbor the fusion gene Cry1Ab/1Ac from transgenic event TT51-1 (GenBank Accession Number: EU880444.1). Expression of the Cry1Ab/1Ac 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 CO2, Ningbo Southeast Instrument CO., LTD, Ningbo, China) connected with a gas-tank system for maintaining the desired atmospheric CO2 concentration. The experimental 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 CO2 concentrations levels were applied continuously, i.e., elevated CO2 (800 ppm, predicted CO2 concentration
in 2100), and ambient CO₂ (about 400 ppm). With each CO₂ level, the N-fertilizer was set at three levels, 1/4, 1 and 2 N; the 1 N was 1.25 mM NH₄NO₃. Therefore, the experiment was consisted of 2 CO₂ concentrations × 3 N-fertilizer levels (total 6 treatment combinations) deployed in six electronically controlled growth incubators as three replications for CO₂ 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 CO₂. 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: NH₄NO₃, 1.25 mM; KH₂PO₄, 0.3 mM; CaCl₂·2H₂O, 1 mM; MgSO₄·7H₂O, 1 mM; Na₂SiO₃·9H₂O, 0.5 mM. (2) Micronutrient solution: MnCl₂·4H₂O, 9 μM; Na₂MoO₄·2H₂O, 0.39 μM; H₃BO₃, 20 μM; ZnSO₄·7H₂O, 0.77 μM; CuSO₄·5H₂O, 0.32 μM; FeSO₄·7H₂O + Na₂-EDTA, 0.32 μM. 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 CO₂ 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 CO₂ 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 CO₂ 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. 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 CO₂ 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-
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).

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

Table 4. DNA sequences of CpG islands in the promoter region (P1) and coding region (P2 and P3) of Bt-transgene in the leaves of Bt rice during tillering stage, grown under ambient and elevated CO2 with different N-fertilizer levels.

Table 5. Primers for bisulfite sequencing of Bt-transgene in the leaves of Bt rice during tillering stage, grown under ambient and elevated CO2 with different N-fertilizer levels.

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 (ANOVA) were performed to examine the effects of CO2 (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 promoter region (P1) and coding region (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 CO2 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 3.86 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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References

1. Long, S. P. & Ort, D. R. More than taking the heat: Crops and global change. *Curr. Opin. Plant Biol.* 13, 241–248 (2010).

2. IPCC. Impacts, Adaptation and Vulnerability. Working Group II Contribution to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change 1132 (Cambridge University Press, Cambridge, 2014).

3. Ainsworth, E. A. & Rogers, A. The response of photosynthesis and stomatal conductance to rising CO$_2$: Mechanisms and environmental interactions. *Plant Cell Environ.* 30, 258–270 (2007).

4. Jackson, R. B., Cook, C. W., Pippen, J. S. & Palmer, S. M. Increased belowground biomass and soil CO$_2$ fluxes after a decade of carbon dioxide enrichment in a warm-temperate forest. *Ecology* 90, 3352–3366 (2009).

5. Liu, Y., Dang, Z., Parajulee, M. N. & Chen, F. Interactive effects of CO$_2$ and temperature on plant chemistry of transgenic bt rice and population dynamics of a non-target plant hopper, nilaparvata lugens (stal) under different levels of soil nitrogen. *Toxins* 11, 261. https://doi.org/10.3390/toxins11050261 (2019).

6. Zavala, J. A., Nabity, P. D. & DeLucia, E. H. An emerging understanding of mechanisms governing insect herbivory under elevated CO$_2$. *Annu. Rev. Entomol.* 58, 79–97 (2013).

7. Hartley, S. E., Jones, C. G., Couper, G. C. & Jones, T. H. Biosynthesis of plant phenolic compounds in elevated atmospheric CO$_2$. *Glob. Change Biol.* 6, 497–506 (2000).

8. Bidart-Bouzat, M. G., Mithen, R. & Berenbaum, M. R. Elevated CO$_2$ influences herbivory-induced defense responses of *Arabidopsis thaliana*. *Ecological Entomology* 45, 415–424 (2002).

9. Sun, Y., Cao, H., Yin, J., Kang, L. & Ge, F. Elevated CO$_2$ changes the interactions between nematode and tomato genotypes differing in the JA pathway. *Plant Cell Environ.* 33, 729–732 (2010).

10. Xu, H. P., Xie, H. C., Wu, S. Y., Wang, Z. Y. & He, K. L. Effects of elevated CO$_2$ and increased N fertilization on plant secondary metabolites and chewing insect fitness. *Front. Plant Sci.* 10, 739. https://doi.org/10.3389/fpls.2019.00739 (2019).

11. Li, Y., Hallerman, E. M., Liu, Q., Wu & Peng, Y. The development and status of Bt rice in China. *Plant Biotechnol. J.* 14, 839–848 (2016).

12. Chen, F., Wu, G., Ge & Parajulee, M. N. Relationships between exogenous-toxin quantity and increased biomass of transgenic Bt crops under elevated carbon dioxide. *Ecotoxicol. Environ. Saf.* 74, 1074–1080 (2011).

13. Li, Y., Peng, Y., Hallerman, E. M. & Wu, K. Biosafety management and commercial use of genetically modified crops in China. *Plant Cell Rep.* 33, 563–573 (2014).

14. Lu, B. R. Challenges of transgenic crop commercialization in China. *Nat. Plants* 2, 16077. https://doi.org/10.1038/nplants.2016.77 (2016).

15. Wang, Y. N. et al. Comparison of three transgenic Bt rice lines for insecticidal protein expression and resistance against a target pest, *Chilo suppressalis* (Lepidoptera: Crambidae). *Insect Sci.* 23, 78–87 (2016).

16. Coviglia, C. E., Stupanovic, R. D. & Trumble, J. T. Plant allocation to defensive compounds: interactions between elevated CO$_2$ and nitrogen in transgenic cotton plants. *J. Exp. Bot.* 53, 323–331 (2002).

17. Chen, F. J., Wu, G., Ge, F., Parajulee, M. N. & Shrestha, R. B. Effects of elevated CO$_2$ and transgenic Bt cotton on plant chemistry, performance, and feeding of an insect herbivore, the cotton bollworm. *Environ. Exp. Appl.* 115, 341–350 (2005).

18. Chen, S., Shelton, A. & Ye, G. Y. Insect-Resistant genetically modified rice in China: From research to commercialization. *Annu. Rev. Environ. Res.* 56, 81–101 (2011).

19. Jiang, S. et al. Impacts of elevated CO$_2$ on exogenous *Bacillus thuringiensis* toxins and transgene expression in transgenic rice under different levels of nitrogen. *Sci. Rep.* 7, 14716. https://doi.org/10.1038/s41598-017-15321-9 (2017).

20. Himanan, S. J. et al. Interactions of elevated carbon dioxide and temperature with aphid feeding on transgenic oilseed rape: Are *Bacillus thuringiensis* (Bt) plants more susceptible to nontarget herbivores in future climate? *Glob. Change Biol.* 14, 1437–1454 (2008).

21. Tsutsui, K., Konno, M., Miyazawa, S. I. & Miyao, M. Sites of action of elevated CO$_2$ on leaf development in rice: Discrimination between the effects of elevated CO$_2$ and nitrogen deficiency. *Plant Cell Physiol.* 55, 258–268 (2014).

22. Coviglia, C. E. & Trumble, J. T. Effect of elevated atmospheric carbon dioxide on the use of foliar application of Bacillus thuringien-ensis. *Biocontrol* 45, 325–336 (2000).

23. Hu, L. et al. Rice MADS3 regulates ROS homeostasis during late anther development. *Plant cell* 23, 515–533 (2011).

24. Jullien, P. E., Susaki, D., Yelagandula, R., Higashiyama, T. & Berger, F. DNA methylation dynamics during sexual reproduction in *Arabidopsis thaliana*. *Curr. Biol.* 22, 1825–1830 (2012).

25. Ma, Y. et al. Disrupted genome methylation in response to high temperature has distinct affects on microspore abortion and anther indehiscence. *Plant Cell* 30, 1387–1403 (2018).

26. Matzke, M. A. & Mosher, R. A. RNA-directed DNA methylation: An epigenetic pathway of increasing complexity. *Nat. Rev. Genet.* 15, 394–408 (2014).

27. Zhong, S. et al. Single-base resolution methylomes of tomato fruit development reveal epigenome modifications associated with ripening. *Nat. Biotechnol.* 31, 154–159 (2013).

28. Yong-Villalobos, L. et al. Methylyme analysis reveals an important role for epigenetic changes in the regulation of the Arabidopsis response to phosphate starvation. *Proc. Natl. Acad. Sci. U.S.A.* 112, E7293–E7302 (2015).

29. Mette, M. F. et al. Transcriptional silencing and promoter methylation triggered by double-stranded RNA. *Embo J.* 19, 5194–5201 (2000).

30. Matzke, M. et al. Genetic analysis of RNA-mediated transcriptional gene silencing. *Biochem. Biophys. Acta* 1677, 129–141 (2004).

31. Matzke, M., Kanno, T., Huettel, B., Daxinger, L. & Matzke, A. J. Targets of RNA-directed DNA methylation. *Curr. Opin. Plant Biol.* 10, 512–519 (2007).

32. Dalakouras, A., Dadami, E., Zwiebel, M., Krzczal, G. & Wassenegger, M. Transgenerational maintenance of transgene body CG but not CHG and CHH methylation. *Epigenetics* 7, 1071–1078 (2012).

33. Lister, R. et al. Highly integrated single-base resolution maps of the epigenome in Arabidopsis. *Cell* 133, 523–536 (2008).

34. Vermeersch, L. et al. Transitive RNA silencing signals induce cytosine methylation of a transgenic but not an endogenous target. *Plant J.* 74, 867–879 (2013).

35. Li, M. Y. et al. NaCl-induced changes of ion fluxes in roots of transgenic *Bacillus thuringiensis* (Bt) cotton (*Gossypium hirsutum* L.). *J. Integr. Agric.* 12, 436–444 (2013).
36. Drake, B. G., Gonzalez-Meler, M. A. & Long, S. P. More efficient plants: A consequence of rising atmospheric CO2?. *Annu. Rev. Plant Biol.* **48**, 609–639 (1997).

37. Hao, X. Y. et al. Effects of free air CO2 enrichment (FACE) on growth and yield of summer soybean. *Acta Ecol. Sin.* **29**, 4595–4603 (2009) (in Chinese).

38. Yamori, W., Nagai, T. & Makino, A. The rate-limiting step for CO2 assimilation at different temperatures is influenced by the leaf nitrogen content in several C-3 crop species. *Plant Cell Environ.* **34**, 764–777 (2011).

39. Reich, P. B., Hobbs, S. E. & Lee, T. D. Plant growth enhancement by elevated CO2, eliminated by joint water and nitrogen limitation. *Nat. Geosci.* **7**, 920–924 (2014).

40. Ruiz, C., Pla, M., Company, N., Riudavets, J. & Nadal, A. High CO2 concentration as an inductor agent to drive production of recombinant phytotoxic antimicrobial peptides in plant biofactories. *Plant Mol. Biol.* **90**, 329–343 (2016).

41. Bruns, H. A. & Abel, C. A. Nitrogen fertility effects on Bt delta-endotoxin and nitrogen concentrations of maize during early growth. *Agron. J.* **95**, 207–211 (2003).

42. Yang, Y. et al. Impacts of nitrogen fertilizer on major insect pests and their predators in transgenic Bt rice lines T2A–1 and T1C–19. *Entomol. Exp. Appl.* **106**, 281–291 (2016).

43. Wang, F. et al. Effects of N treatments on the yield advantage of Bt-SY63 over SY63 (*Oryza sativa*) and the concentration of Bt protein, *Field Crop. Res.* **129**, 39–45 (2012).

44. Dong, H. Z. & Li, W. J. Variability of endotoxin expression in Bt transgenic cotton. *J. Agron. Crop Sci.* **193**, 21–29 (2007).

45. Weinhold, A., Kallenbach, M. & Baldwin, I. T. Progressive 35S promoter methylation increases rapidly during vegetative development in transgenic *Nicotiana attenuata* plants. *Bmc Plant Biol.* **13**, 39. https://doi.org/10.1186/1471-2229-13-39 (2013).

46. Fan, H. H. et al. DNA methylation alterations of upland cotton (*Gossypium hirsutum*) in response to cold stress. *Acta Physiol. Plant.* **35**, 2445–2453 (2013).

47. Xia, H. et al. Differentially methylated epiloci generated from numerous genotypes of contrasting tolerances are associated with osmotic-tolerance in rice seedlings. *Front. Plant Sci.* **8**, 12. https://doi.org/10.3389/fpls.2017.00111 (2017).

48. Chen, B., Salvetit, M. E. & Beckles, D. M. Chilling-stress modifies DNA methylation level in cucumber (*Cucumis sativus*) L. seedling radicle to regulate elongation rate. *Sci. Hortic.* **252**, 14–19 (2019).

49. Wang, W. et al. Drought-induced site-specific DNA methylation and its association with drought tolerance in rice (*Oryza sativa* L.). *J. Exp. Bot.* **62**, 1951–1960 (2011).

50. Stam, M., Mol, J. N. M. & Kooter, J. M. The silence of genes in transgenic plants. *Ann. Bot.* **79**, 3–12 (1997).

51. Vilperte, V., Agapito-Tenfen, S. Z., Wikmark, O. G. & Nodari, R. O. Levels of DNA methylation and transcript accumulation in leaves of transgenic maize varieties. *Environ. Sci. Eur.* **28**, 29. https://doi.org/10.1186/s12302-016-0097-2 (2016).

52. Trtikova, M., Wikmark, O. G., Zemp, N., Widmer, A. & Hilbeck, A. Transgene expression and bt protein content in transgenic Bt maize (MON810) under optimal and stressful environmental conditions. *PLoS ONE* **10**, e0123011. https://doi.org/10.1371/journal.pone.0123011 (2015).

53. Xia, L. Q. & Guo, S. D. The expression of Bt toxin gene under different thermal treatments. *Sci. Agric. Sin.* **37**, 1733–1737 (2004) (in Chinese).

54. Kumar, A., Silim, S. N., Okamoto, M., Siddiqi, M. Y. & Glass, A. D. M. Differential expression of three members of the AMT1 gene family encoding putative high-affinity NH4+ transporters in roots of *Oryza sativa* subspecies indica. *Plant Cell Environ.* **26**, 907–914 (2003).

55. Livak K. J. & Schmittgen T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. *Methods* **25**, 402–408 (2001).