# Mechanism of the decrease in exogenous-toxin protein of transgenic Bt rice grown in elevated $CO_2$—Dilution effect hypothesis

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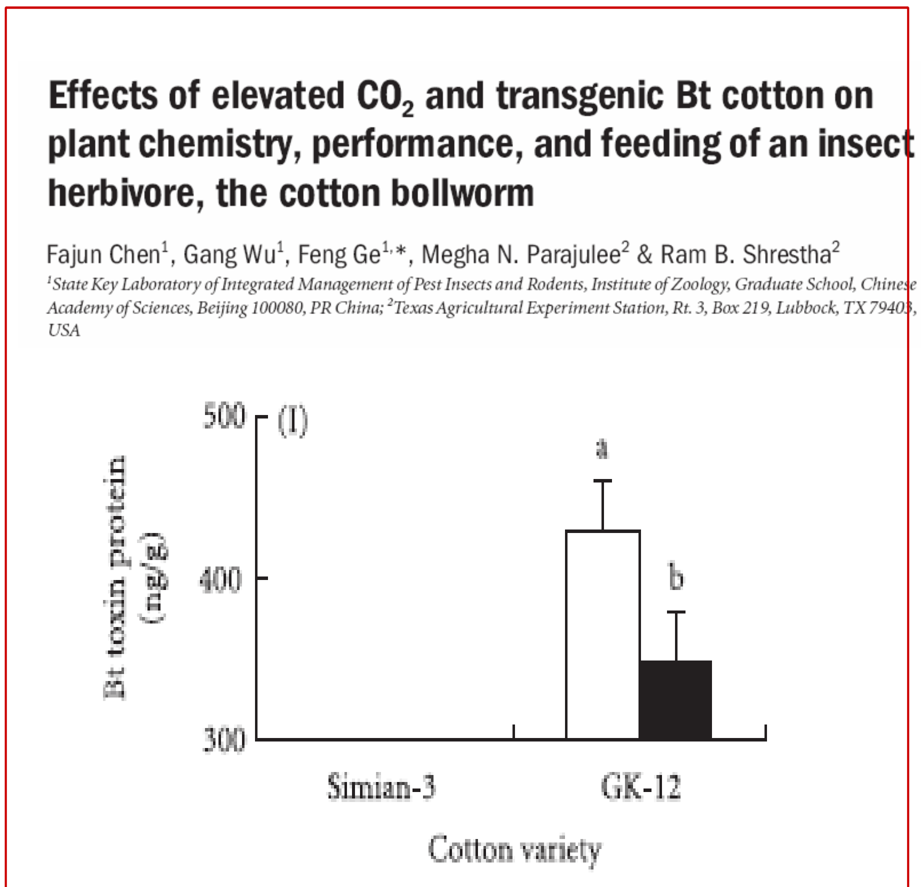
## Introduction

- Biotech crops has been adopted worldwide to control lepidopteran insect pests (James 2005, 2006).
- **In China,**
  - (1) the damage caused by *Helicoverpa armigera* has been greatly alleviated in recent years due, in part, to the adoption of transgenic Bt cotton (Men et al. 2003).
  - (2) some varieties of transgenic Bt rice (e.g., KMD-1 & KMD-2) have been bred, which can been effectively control the occurrence of *Chilo suppressalis* (Wu et al., 2001).
- Elevated $CO_2$ results in marked impacts on plant physiology and secondary metabolism (e.g., Luo et al. 1999).
  - (1) significant increases in photosynthesis, plant biomass and yields and etc.
  - (2) changes in production of secondary metabolites (Wu et al., 2007), e.g., promoting the manufacture of C-based secondary metabolites (e.g., total phenolics, gossypol, condensed tannin) over N-based ones (Bryant et al., 1983; Coviella et al. 2002; Chen et al. 2005; Wu et al. 2007).
- Transgenic Bt cotton appears to be especially responsive to $CO_2$, e.g., significant decrease in exogenous Bt toxin content (Coviella et al. 2002; Chen et al. 2005; Wu et al. 2007).

## Impacts of Elevated $CO_2$ on Transgenic Bt Rice

- **It is presumed that**
  - Elevated $CO_2$ can alter the Bt rice growth (Huang et al., 2004).
  - Hence, ultimately the phenotype allocation to plant chemical components of Bt rice, which may in turn, affect the plant-herbivore interactions (Wu et al. 2007).

## What about the Bt toxin expression of Bt rice grown in elevated $CO_2$? ---Significant decrease in content and amount?



**Effects of elevated $CO_2$ and transgenic Bt cotton on plant chemistry, performance, and feeding of an insect herbivore, the cotton bollworm**

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- GK-12= transgenic Bt cotton cultivar; Simian-3=non-transgenic cultivar [same parental line].
- Different lowercase letters indicate significant differences between $CO_2$ treatments within cotton variety (LSD test; df = 1,4; $P<0.05$).

## Ecological Question?

- How about the mechanism of transgenic Bt cotton grown in elevated $CO_2$ owing to the marked decrease in exogenous Bt toxin content?

## Ecological Risk of Transgenic Bt Rice in Elevated $CO_2$

- Resistance of transgenic Bt rice against target and non-target herbivorous insects in elevated $CO_2$.
- Target resistance may be reduced owing to the subsequent decrease of Bt toxin in elevated $CO_2$?
- Non-target resistance may be enhanced owing to the subsequent increases of secondary defense chemicals in elevated $CO_2$?

## Three Aspects Presumed

(1) **Plant growth - Dilution effects** (Hägele & Martin 1999).

**Do increased carbohydrates of Bt crops, caused by elevated $CO_2$, dilute the Bt toxin content in plant tissues?**

(2) **Bt gene expression may be inhibited** (Xia et al. 2005).

**Methylation of the special promoter (35S) and Bt gene may be enhanced when Bt crops is grown in elevated $CO_2$.**

(3) **Plant physiology - Nitrogen nutrition** (Stitt & Krapp 1999).

**Nitrogen metabolism of transgenic Bt crops may be inhibited under elevated $CO_2$, owing to the N-deficiency in the soil resulted by increased N uptake to compensate the lower leaf-N metabolism.**

## Question 1: Dilution Effect

- **Hypothesis 1:** If the exogenous Bt gene expression isn't affected for transgenic Bt crops grown in elevated $CO_2$
  - Positive correlation should be existing between Bt toxin amount (ng) per plant and biomass (g) per plant
  - Hence, if significant increase in biomass occurs in transgenic Bt crops grown in elevated $CO_2$, total amount of Bt toxin per unit area increases
- **Hypothesis 2:** If the exogenous Bt gene expression is inhibited for transgenic Bt crops grown in elevated $CO_2$
  - Significant correlation should be shown between the increase/ decrease (%) in Bt toxin amount per plant and the increase (%) in tissue biomass per plant for Bt crops grown in elevated $CO_2$

## Question 2: Bt Gene Expression

- The exogenous gene silencing is the key risk causing the reduction in exogenous toxin expression for transgenic crops, owing to the methylation of the special 35S promoter and the Bt gene (Finnegan & McElroy 1994).
- How about the exogenous Bt gene silencing for the Bt crops grown in elevated $CO_2$, whether it results in the significant decrease in Bt toxin in elevated $CO_2$?

## Question 3: Nitrogen Nutrition

- Nitrogen uptake can be enhanced by roots of transgenic Bt crops grown in elevated $CO_2$, which can result in a N-deficiency (Stitt & Krapp 1999).
- The N-deficiency also limits nitrogen metabolism (e.g., assimilation and re-allocation) and Bt gene expression for transgenic Bt crops grown in elevated $CO_2$ (Stitt & Krapp 1999).
- How about the nitrogen metabolism (including some key enzymes, e.g., GS, Fd-GOGAT or NADH-GOGAT, GDH) of transgenic Bt crops grown in elevated $CO_2$?

# In this study, dilution effect hypothesis was tested by measuring Bt-toxin expression (content and total amount) in combination with plant biomass of transgenic Bt rice grown in open-top chambers, under ambient $CO_2$ and elevated $CO_2$

## Materials & Methods

- **OTCs:** The experiment was conducted in six open-top chambers, 2.5 m tall × 4.2 m in diameter (Chen et al., 2005), in Sanhe County, Hebei Province, China (35º57′ N, 116º47′ E). Three OTCs were used for each $CO_2$ treatment, i.e., 375 and 750 µl/l $CO_2$.
- **Rice cultivar:** The transgenic Bt rice cultivar KMD-2 (expressing *Cry1A(b)* genes from *Bacillus thuringiensis* Berliner var. *kurstaki*)(Shu et al., 1998) was used.
- **Growth condition:** The seeds of KMD-2 were planted in white plastic pots (45 cm high × 35 cm in diameter) filled with 8: 3:1 (by volume) loam : cow dung : earthworm frass, with 60 seeds per pot. Thirty pots with 30 stems per pot were placed randomly in each chamber and re-randomized every other day to minimize positional effects. Pure $CO_2$ mixed with ambient air was supplied to the chambers throughout the seedling stage, and enough water was given to every pots.
- **Plant growth:** The index of biomass (g dry weight per stem) was used to indicate plant growth. In this study, total biomass was measured on the 50d and 100d after Bt rice planting (ab. DAP), respectively.
- **Bt toxin protein:** On the 50d and 100d after Bt rice planting, the content of Bt toxin protein was respectively analyzed using ELISA (Chen et al., 1999). Simultaneously, the total amount (ng) Bt toxin protein was gained with the total biomass (g) multiplying the respective Bt toxin protein content (ng/g).
- **Dilution effect test:**

  **(1) Hypothesis 1:** Bt gene expression isn't affected for Bt rice grown in elevated $CO_2$.

  --- Significant increase in Bt toxin amount per stem must be showed in elevated $CO_2$ compared with ambient $CO_2$. And there must be positive correlation between the increase % in Bt toxin amount per stem and the increase % in biomass per stem.

  **(2) Hypothesis 2:** Bt gene expression is inhibited for Bt rice grown in elevated $CO_2$.

  --- No change (even decrease) may be shown in Bt toxin amount per stem when transgenic Bt rice grow in elevated $CO_2$ relative to ambient $CO_2$. And there must be negative correlation between the decrease % in Bt toxin amount per stem and the increase % in the increase % in biomass per stem.
- **Data analysis:** All data were analyzed with a general linear model procedure (SAS Institute, 1996). One-way ANOVA was used to analyze the effects of elevated $CO_2$ on plant biomass, and content and total amount of Bt toxin protein of transgenic Bt rice. And LSD test was used to separate the means between treatments at P<0.05. And Pearson correlations were also used to analyze the relationship between the measured indexes.

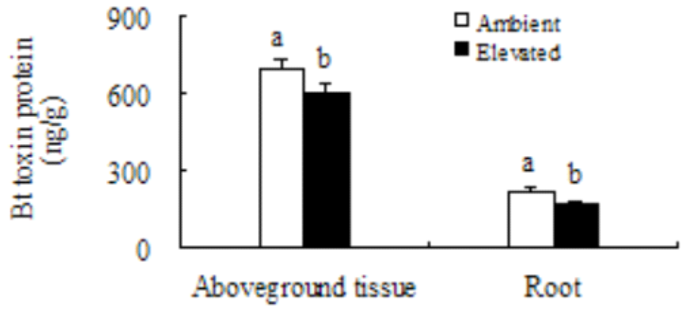

**Figure 1** Bt toxin content of Bt rice grown in ambient and elevated $CO_2$ for 50 days after planting. Different lowercase letters indicate significant differences between $CO_2$ treatments (LSD; df = 1,10; P<0.05).

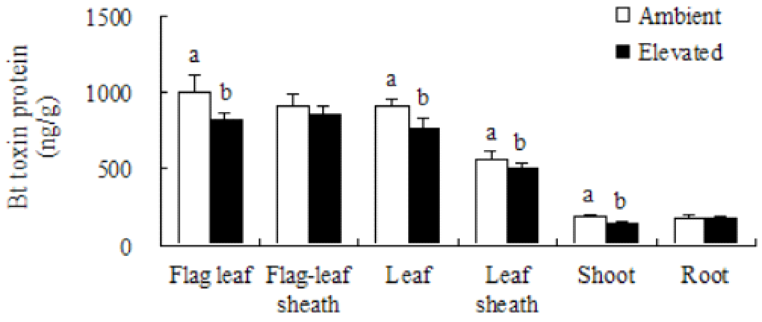

**Figure 2** Bt toxin content of Bt rice grown in ambient and elevated $CO_2$ for 100 days after planting. Different lowercase letters indicate significant differences between $CO_2$ treatments (LSD; df = 1,10; P<0.05).

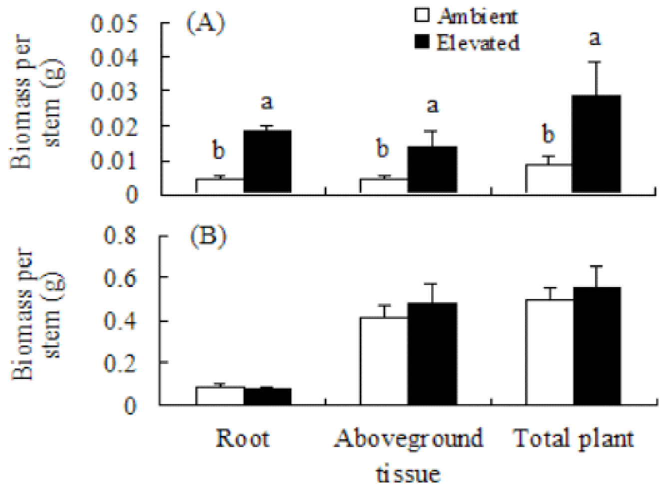

**Figure 3** Biomass per stem of Bt rice grown in ambient and elevated $CO_2$ for 50 days (A) and 100 days (B) after planting. Different lowercase letters indicate significant differences between $CO_2$ treatments (LSD; df = 1,4; P<0.05).

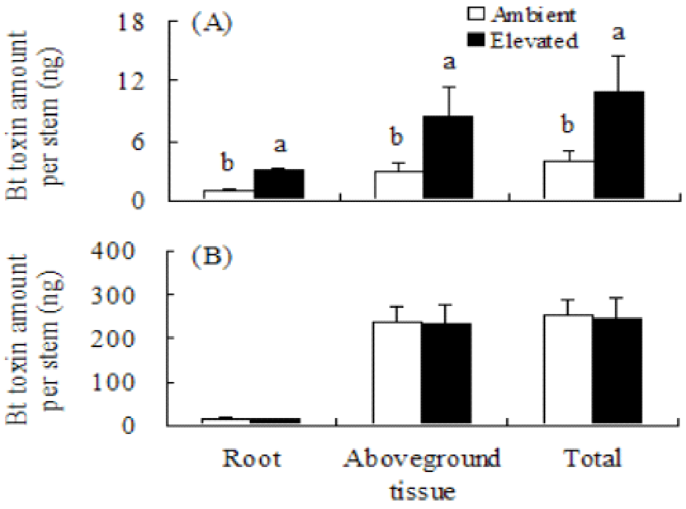

**Figure 4** Bt toxin protein amount per stem of Bt rice grown in ambient and elevated $CO_2$ for 50 days (A) and 100 days (B) after planting. Different lowercase letters indicate significant differences between $CO_2$ treatments (LSD; df = 1,4; P<0.05).

**Table 1** Pearson correlation between the changes (%) of Bt toxin amount per stem and the changes in tissue biomass per stem, when transgenic Bt rice grew in elevated $CO_2$ relative to ambient $CO_2$

| | | Changes in tissue biomass per stem | |
|---|---|---|---|
| | | Root | Aboveground tissues |
| 50 days after planting (DAP) | | | |
| Changes in Bt toxin content | Root | 1.0000 (0.0024)** | |
| | Aboveground tissues | | 1.0000 (0.0013) ** |
| | Total plant | | |
| 100 days after planting (DAP) | | | |
| Changes in Bt toxin content | Root | -0.93 (0.25) | |
| | Aboveground tissues | | -0.9998 (0.013)* |
| | Total plant | | |

Data are the Coefficients (P values) of the Pearson Correlation. * P<0.05, ** P<0.01.

## Results

- **Bt toxin content:** $CO_2$ level significantly affected Bt toxin content of the root ($F_{1,10}$=31.2, P=0.0002) and the aboveground tissues ($F_{1,10}$=16.1, 0.0025) when Bt rice grew for 50 days after planting. And significant decreases in Bt toxin content were shown in elevated $CO_2$ compared with ambient $CO_2$ (P<0.05; Fig.1). Moreover, significant effects of $CO_2$ level were also indicated in the tissues of flag leaf ($F_{1,10}$=12.7, P=0.0052), leaf($F_{1,10}$=24.4, P=0.0006) and leaf sheath ($F_{1,10}$=5.1, P=0.048), and shoot ($F_{1,10}$=74.2, P=0.0001), with significant decreases in Bt toxin content of 100DAP Bt rice grown in elevated $CO_2$ in contrast to ambient $CO_2$ (P<0.05; Fig.2).
- **Biomass:** Elevated $CO_2$ significant increased the root ($F_{1,4}$=126.8, P=0.0001), aboveground tissues ($F_{1,4}$=12.0, P=0.026) and total plant biomass ($F_{1,4}$=12.7, P=0.024) of Bt rice grown for 50 days after planting, but didn't affect the respective biomass of Bt rice grown for 100 days after planting. Significant increases in biomass were found in the root, aboveground tissues and total plant of 50DAP Bt rice grown in elevated $CO_2$ relative to ambient $CO_2$ (P<0.05; Fig.3A).
- **Bt toxin amount:** $CO_2$ level significantly increased Bt toxin amount per stem in the root ($F_{1,4}$=111.4, P=0.0005), aboveground tissues ($F_{1,4}$=9.9, P=0.035) and total plant ($F_{1,4}$=15.7, P=0.017) of 50DAP Bt rice. And significant increases in Bt toxin amount per stem of the above tissues were indicated when Bt rice grew in elevated $CO_2$ compared ambient $CO_2$ (P<0.05; Fig.4A).

  Elevated $CO_2$ didn't increase the Bt toxin amount per stem in the root, aboveground tissues and total plant when Bt rice grew for 100 days after planting. On the contrary, marginal decreases in Bt toxin amount per stem were shown in the above tissues of Bt rice in elevated $CO_2$ compared with ambient $CO_2$ (P>0.05; Fig.4B)..
- **Correlation analysis :** Correlative relationships between the changes in Bt toxin amount per stem and tissues biomass per stem were examined using Pearson's correlation. The increases in Bt toxin amount of the root and aboveground tissues were positively correlated with the increases in root and aboveground tissues' biomass when Bt rice grew for 50 days after planting (Table 1). While, only one significantly negative correlation were found in the aboveground tissues between the decrease in Bt toxin amount and the biomass when Bt rice grew for 100 days after planting (Table 1).

## Conclusion

- The dilution effects were shown in under- (i.e., root) and aboveground tissues of Bt rice grown in elevated $CO_2$ for 50 days after planting. And during this period, the Bt gene expression isn't affected in Bt rice grown in elevated $CO_2$.
- The dilution effects weren't shown in plant tissues of Bt rice grown in elevated CO2 for 100 days after planting. And during this period, the Bt gene expression is inhibited for Bt rice grown in elevated $CO_2$.

## Suggestions

- Some cultural practices could be carried out to improve the Bt-gene expression of transgenic Bt cotton, especially in elevated $CO_2$, e.g.,
  - a) spraying special demethylation reagents (e.g. 5-azaC),
  - b) additional nitrogen-fertilization supply.
- The target receptors (e.g., cadherin-like protein (Hara et al., 2003; Wang et al., 2004)) of the target insect pests should be assayed especially in elevated $CO_2$ to exactly assess the resistant ability of transgenic Bt crops.

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