Status of DIMBOA and phenolic acids in transgenic Bt corn

C. R. Niea,b,c, S. M. Luoa, C. X. Linb, R. S. Zenga, J. H. Huanga, and J. W. Wanga

aInstitute of Tropical and Subtropical Ecology, South China Agricultural University, Guangzhou, 510642, P. R. China.
bCentre for Ecological and Environmental Technologies, South China Agricultural University, Guangzhou, 510642, P. R. China.
cInstitute of Crop Genetics and Breeding, Foshan University, Foshan, 528231, P. R. China.

Abstract. Experiments were conducted to investigate the status of DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoazin-3-one) and phenolic acids in leaves of some transgenic Bt corn hybrids. Comparison between Bt corn hybrids and their corresponding non-transgenic near-isogenic lines suggested that the introduction of the Bt gene has adverse effects on the biosynthesis and accumulation of DIMBOA and some phenolic acids, such as ferulic acid, in the corn plants. Under conditions of either water or nitrogen stress, the accumulation of DIMBOA in the leaves of the Bt corns could be enhanced. The results of this study suggest that caution must be taken when considering the introduction of Bt corns into China because Bt corns may have a weaker capacity, relative to traditional Chinese corn hybrids, to synthesise DIMBOA and some forms of phenolic acids in the leaves and, therefore, reduce the plant's resistance to pathogens, diseases, and pests other than the targeted corn borer.

Additional keywords: Bt protein, water stress, nitrogen stress.

Introduction

Transgenic Bt corn provides a tool for control of infestations of the European corn borer, which has resulted in the commercialisation of Bt corn in North America (Obrycki et al. 2001; James 2004). The potential benefits of Bt corn have also attracted corn producers in other countries and regions, including China (Wang et al. 1995; Liu and Wang 2003). Although many people believe that Bt proteins are not harmful to life other than the corn borer, others argue that the ecological effects of growing Bt corn remain uncertain (Hilbeck et al. 1999; Losey et al. 1999; Stotzky 2000; Obrycki et al. 2001; Quist and Chapela 2001; Saxena and Stotzky 2001).

Cyclic hydroxamic and phenolic acids are among the common secondary metabolites of gramineous plants, such as corn, wheat, and rye (Niemyer 1988; Classen et al. 1990; Anwar et al. 1995). These materials and their derivatives have implications for the resistance of plants to herbivores, pests, and pathogens (Bennett and Wallsgrove 1994). Phenolic compounds constitute a large group of secondary plant products and have been the focus of many studies in chemical ecology. Phenolic acids have been reported to have a major role in many areas of plant-animal, plant-pathogen, and plant-plant interactions (Waterman and Mole 1994). If the introduction of the Bt gene adversely affects the production and accumulation of these endogenous toxins in corn plants, the resistance of Bt corn to pathogens, diseases, and pests other than corn borers may be weakened.

The objectives of this study were (a) to analyse the spatial and temporal pattern of DIMBOA (one of the most important cyclic hydroxamic acids) in the leaves of a variety of Bt corns, (b) to examine the effects of the expression of Bt protein on the accumulation of DIMBOA and phenolic acids in Bt corns in comparison with their corresponding near-isogenic non-Bt lines, and (c) to assess the suitability of replacing traditional Chinese corn hybrids with Bt corns in terms of difference in levels of DIMBOA between the Bt corns and a corn hybrid widely grown in China.

Materials and methods

Materials

Soil

The soil (0–20 cm) used in all experiments was collected from a paddy field in Guangzhou, China. It was a clayey loam with a pH of 6.1, 1.81% organic matter, 0.09% total N, 0.16% total P, and 0.56% total K.

Plant materials

Four transgenic Bt corn hybrids were used in the experiments. These included 3 hybrids of American origin (34B24, P67, G30) and one hybrid of Chinese origin (Nongda61). To examine the effects of the insertion of the Bt gene on the accumulation of DIMBOA and phenolic acids in the Bt corn plants, 2 non-transgenic corn hybrids, 34B23 (the near-isogenic non-Bt line of 34B24) and G26 (the near-isogenic non-Bt line of G30), were also used. In addition, a corn hybrid (Nongda3138) that has been widely grown in China was selected for the assessment of the suitability of replacing the dominant Chinese corn hybrid by Bt corn hybrids.
Chemical materials
Pure protocatechuric acid, β-hydroxybenzoic acid, vanillin acid, caffeic acid, syringic acid, p-hydroxybenzoin acid, and ferulic acid were purchased from ACROS ORGANICS (New Jersey, USA).

Experimental design

Pot experiment

Three separate experiments were conducted. The first experiment compared the concentrations of DIMBOA and phenolic acids in the leaves as a whole (mixture of all leaves in each plant) among all the 7 Bt corn hybrids, as well as in individual leaves occurring at different locations in each plant. For each hybrid, 4 seeds were sown in each pot (in 4 replicates). Each pot was filled with 5 kg of the soil and the pots were placed randomly in a greenhouse-like plant growth chamber that had a glass covering and was enclosed by a metal screen. After germination, 2 selected seedlings were retained in each pot, and the rest of the seedlings were removed. At the 11th day after sowing, one of the two plants in each pot was harvested, all the leaves in the plant were mixed, and the mixed sample was analysed for DIMBOA and phenolic acids. The remaining plant in each pot was harvested on the 20th day after sowing when the plant had 6–7 leaves. The 1st, 3rd, and 5th upper leaves of each plant were collected and analysed separately for DIMBOA and Bt protein.

The second experiment monitored the change in concentration of DIMBOA in the leaves of the Chinese Bt corn hybrid, Nongda61. Fifty seeds were sown in each pot (in 4 replicates). Each pot was filled with 5 kg of the soil and the pots were placed randomly in the same greenhouse as in Exp 1. After germination, 20 selected seedlings were retained in each pot, and the rest were removed. The plants in each pot were harvested at 6, 9, 12, and 15 days after sowing; 5 plants were removed from a pot each time, and leaves were mixed to form a pooled sample for determination of DIMBOA.

The third experiment examined the effects of stress conditions on the accumulation of DIMBOA in Hybrid 34B24. There were 2 factors (water and nitrogen) and 2 levels (normal and stress) for each factor. Under stress conditions, no nitrogen was added to the soils, and water was provided only when the plants showed signs of wilting. Under normal stress conditions, no nitrogen was added to the soils, and water was allowed to stand at room temperature for 15 min, adjusted to pH 3 with 0.1 M H3PO4, centrifuged at 12200 rpm for 20 min, and partitioned 3 times against n-butyl ether (equal volumes). The ether extract was evaporated to dryness under vacuum, and the residue was dissolved in 2 ml of methanol. Samples were passed through a 0.45-mm filter membrane and stored at −20°C before being analysed.

DIMBOA and various forms of phenolic acids in the extracts were determined by high performance liquid chromatography (HPLC) (column, Hypercil ODS C18 column (250 mm × 4 mm), eluent, 25% (v/v) methanol containing 0.75% (v/v) acetic acid, flow rate, 1 ml/min; detection, 280 nm) with DAD detector by using external standard curves for authentic compounds. The HPLC method was validated with regard to accuracy, linearity, and precision.

Concentrations of DIMBOA in the leaf as a whole of different corn hybrids

The concentration of DIMBOA was highly variable among the 4 Bt corn hybrids (Table 1). The mean concentration of DIMBOA was in the following decreasing order: P67 > Nongda61 > G30 > 34B24. Two pairs of transgenic and non-transgenic hybrids, Hybrids 34B24 and G30, of transgenic Bt corns had a relatively lower level of DIMBOA than their corresponding near-isogenic lines of non-transgenic corns, i.e. 34B23 and G26, respectively. The non-transgenic hybrid, Nongda5138, which is grown in a large area in North China, had the highest level of DIMBOA among the corn hybrids.

Concentrations of phenolic acids in the leaves as a whole of different corn hybrids

Variations in the concentrations of various forms of phenolic acids existed among the investigated corn hybrids (Table 2).
Table 1. Concentrations of DIMBOA (mg/kg, fresh wt. basis) in the mixed leaves of 11-day-old seedlings of corn
The HPLC injections (subsamples) were conducted for each corn extraction. The mean of the subsamples was used in the statistical analysis. Within the column, means with the same letter were not significantly different \((P > 0.05)\) using REGWQ multiple test.

<table>
<thead>
<tr>
<th>Corn hybrid</th>
<th>DIMBOA</th>
<th>Bt corn</th>
</tr>
</thead>
<tbody>
<tr>
<td>34B23</td>
<td>57.63±0.76c</td>
<td>–</td>
</tr>
<tr>
<td>34B24</td>
<td>29.88±0.74c</td>
<td>+</td>
</tr>
<tr>
<td>G26</td>
<td>51.36±0.58bc</td>
<td>–</td>
</tr>
<tr>
<td>G30</td>
<td>58.90±0.82d</td>
<td>+</td>
</tr>
<tr>
<td>P67</td>
<td>74.25±0.98b</td>
<td>+</td>
</tr>
<tr>
<td>Nongda3138</td>
<td>216.53±4.13a</td>
<td>–</td>
</tr>
<tr>
<td>Nongda61</td>
<td>69.60±0.58bc</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 2. Concentrations of phenolic acids (mg/kg, fresh wt. basis) in the leaves of seedlings of Bt and non-Bt corn
Within the column, means with the same letter were not significantly different \((P > 0.05)\) using REGWQ multiple test.

<table>
<thead>
<tr>
<th>Corn hybrid</th>
<th>Vanillic acid</th>
<th>Syringic acid</th>
<th>Ferulic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>34B23</td>
<td>16.67±2.76c</td>
<td>5.06±0.63d</td>
<td>49.00±12.25a</td>
</tr>
<tr>
<td>34B24(Bt)</td>
<td>13.80±3.85c</td>
<td>4.81±0.69d</td>
<td>24.65±3.87c</td>
</tr>
<tr>
<td>G26</td>
<td>21.36±4.36b</td>
<td>8.32±1.21e</td>
<td>53.82±11.31a</td>
</tr>
<tr>
<td>G30(Bt)</td>
<td>23.18±3.25b</td>
<td>8.69±0.66c</td>
<td>34.17±5.15b</td>
</tr>
<tr>
<td>P67(Bt)</td>
<td>74.25±11.28c</td>
<td>11.67±0.96d</td>
<td>28.6±2.37bc</td>
</tr>
<tr>
<td>Nongda3138</td>
<td>81.94±10.81a</td>
<td>13.84±2.38b</td>
<td>7.57±1.28d</td>
</tr>
<tr>
<td>Nongda61(Bt)</td>
<td>77.30±11.83a</td>
<td>19.24±4.36a</td>
<td>4.65±0.79b</td>
</tr>
</tbody>
</table>

Fig. 1. Change in DIMBOA concentration in leaves of a Bt corn hybrid (Nongda61) during the experiment.

Comparison of leaf DIMBOA concentration at different growth stages between Bt and non-Bt near-isogenic lines
The DIMBOA concentrations of the 1st and 3rd leaves of 34B24 and its non-transgenic near-isogenic line (34B23) at different growth stages can be seen in Table 3. At all 3 growth stages (39, 53, and 67 days after sowing), Bt corn (Hybrid 34B24) had a significantly lower DIMBOA concentration than its corresponding non-transgenic corn (Hybrid 34B23) in both the 1st and the 3rd upper leaf.

Relationship between Bt protein and DIMBOA in Bt corn
The concentration of DIMBOA was negatively related to Bt protein \((r^2 = 0.755b)\) in the 1st, 3rd, and 5th upper leaves of Bt corn seedlings 20 days after sowing when the plants had 6–7 leaves (Fig. 2).

Leaf DIMBOA concentration of Bt corns under stress conditions
At all stages of growth and for all leaves, the concentrations of DIMBOA were significantly higher under conditions of water stress, nitrogen stress, or a combination of both than under normal conditions of growth (Fig. 3). The concentration of DIMBOA at the same location decreased with increasing period of growth. The DIMBOA concentration of each plant

In particular, corn hybrids of American origin had a higher concentration of ferulic acid than hybrids of Chinese origin, whereas corn hybrids of Chinese origin had higher concentrations of vanillic acid and syringic acid than the hybrids of American origin. There was no significant difference in the concentrations of vanillic acid and syringic acid between Bt transgenic and non-transgenic near-isogenic lines of American origin, whereas corn hybrids of Chinese origin had higher concentration of ferulic acid than hybrids of Chinese origin.
Table 4. Concentrations of DIMBOA (mg/kg, fresh wt. basis) at different growth stages (days after sowing) of corn

<table>
<thead>
<tr>
<th>Corn hybrid</th>
<th>1st upper leaf</th>
<th>3rd upper leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>39-days</td>
<td>53-days</td>
</tr>
<tr>
<td>34B23</td>
<td>123.71 ± 10.26a</td>
<td>71.20 ± 8.51a</td>
</tr>
<tr>
<td>34B23(Bt)</td>
<td>77.98 ± 6.76b</td>
<td>13.24 ± 5.15b</td>
</tr>
</tbody>
</table>

Within the column, means with the same letter were not significantly different (P > 0.05) using REGWQ multiple test.

Discussion

The high variation in the concentration of DIMBOA in leaves as a whole among the investigated corn hybrids was in agreement with previous findings (e.g. Argandoña and Luis 1985). However, the lower concentration of DIMBOA in transgenic Bt corn hybrids, relative to their corresponding non-transgenic near-isogenic lines, suggests that the introduction of Bt toxin may have adverse effects on the accumulation of DIMBOA in corn plants. A possible explanation is that gene expression of Bt protein consumes some energy and resources of the plant that may result in inhibition in the biosynthetic pathways leading to DIMBOA and this could cause a reduction in biosynthesis of DIMBOA. This possibility was supported by the fact that there was a significantly negative relationship between the concentrations of DIMBOA and Bt protein in most Bt corn hybrids. A similar phenomenon was reported in Bt cotton, which had significantly lower levels of total hydroxybenzene and tannin than their near-isogenic non-Bt counterparts (Wu et al. 2000).

In addition to DIMBOA, phenolic acids also have an important role in counteracting plant diseases and pests (Classen et al. 1990). Although there was no significant difference in the concentration of vanillic acid and syringic acid between Bt corns and their corresponding non-transgenic lines, Bt corns had a significantly lower concentration of ferulic acid than their corresponding non-transgenic counterparts, suggesting that the introduction of the Bt gene also inhibits or changes some biosynthetic pathways in the secondary metabolism leading to the reduction of some secondary metabolites, such as ferulic acid.

The concentration of DIMBOA in Bt corn plants under either water or nitrogen stress was higher than under normal conditions. It has been reported that stress conditions enhance the accumulation of DIMBOA in non-Bt corn plants (Morse...
et al. 1991; Massardo et al. 1994; Bergvinson et al. 1995; Larsen and Christensen 2000). The results of this study suggest that enhancement of DIMBOA accumulation also applies to transgenic Bt corn under stress conditions. The generally lower concentration of DIMBOA in the older (lower leaf) than that in the younger leaf (upper leaf) of the same plant reflects the stronger capacity of younger than the older leaves to synthesise hydroxamic acids. This appears to be a self-defence mechanism through which younger leaves are protected from attack by pests (Cambier et al. 2000). The investigated Bt corns had higher Bt protein in the older than in the young leaves, indicating that the expression of the Bt gene has a more important role in protecting older leaves from attack by corn borers.

Conclusions
It is likely that the introduction of the Bt gene adversely affects the biosynthesis and accumulation of DIMBOA and some phenolic acids, such as ferulic acid, in corn plants. This suggests that ecological risk may exist when introducing Bt corn to a new area, because the Bt corn may be subject to attacks from pathogens and pests that are not sensitive to Bt toxin due to the reduced amounts of DIMBOA and some forms of phenolic acids contained in the leaves of Bt corn. The non-Bt corn hybrid Nongda3138, which is widely grown in China, was superior to all the investigated Bt corn hybrids in terms of DIMBOA level. Therefore, caution must be exercised when considering the replacement of the traditional Chinese corn hybrid by Bt corn hybrids.

Acknowledgments
We thank Dr Cindy Nakatus at the Purdue University (USA) and Professor Jingrui Dai at the China Agricultural University for kindly providing the transgenic Bt corn hybrids used in this study. This work was supported by the National Natural Science Foundation of China (Projects No. 30270270 and No. 30270230) and the Guangdong Provincial Natural Science Foundation (Projects No. 021043, No. 000569, and No. 039254).

References
Niemeyer HM (1985) Hydroxamic acids (4-Hydroxy-1,4-benzoxazin-3-ones), defence chemicals in the gramineae. Phytochemistry 24, 353–361.

Manuscript received 19 December 2004, accepted 23 June 2005

http://www.publish.csiro.au/journals/ajar