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The influence of Bt-transgenic maize pollen on the bacterial diversity in the midgut of *Apis mellifera ligustica*

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Abstract – Honeybees are subjected to direct contact with transgenic maize pollen due to their feeding activities on pollen. The potential side effects of transgenic *cry1Ab*-maize pollen on the midgut bacteria of the larvae and worker bees of *Apis mellifera ligustica* were investigated through denaturing gradient gel electrophoresis under both laboratory and field conditions. Newly emerged bees were fed transgenic *cry1Ab*-maize pollen, normal maize pollen, linear *cry1Ab* gene DNA, supercoiled plasmid DNA, and sugar syrup under the laboratory conditions. The results showed that there were no significant differences in the midgut bacterial community composition among the five treatments. No significant effects were observed in the midgut communities between larvae and adult honeybees fed transgenic *cry1Ab*-maize pollen and non-transgenic maize pollen in the field trials.

transgenic maize / *Apis mellifera* / intestinal community / denaturing gradient gel electrophoresis

1. INTRODUCTION

Accumulated hectareage of genetically modified crops planted globally from 1996 to 2011 exceeds 1.6 billion ha (Clive 2012). Planting *Bacillus thuringiensis* (Bt) crops effectively reduces the pesticide use by 443 million kg of active ingredient and has brought \$34.2 billion in economic benefits to date. In 2011, biotech maize covered 51 million ha and 32 % of the global biotech area, and it was the second largest

planted crop after cotton. However, the worldwide planting of Bt maize has triggered concerns about their potential impacts on the environment and human health. The main concern is the harmful effects that these crops may exert on nontarget organisms. Many studies demonstrate that transgenic maize with *cry1Ab*, *cry1F*, or *cry3Bb* gene do not exert harmful effects on nontarget arthropod (Gathmann et al. 2006; Higgins et al. 2009; Al-Deeb et al. 2003) and rhizosphere soil bacterial communities (Baumgarte and Tebbe 2005; Blackwood and Buyer 2004). The endophytic bacterial communities of maize were not significantly affected by transforming with *cry3Bb1*, *cry1A105*, or *cry1Ab2* gene (Prischl et al. 2012). Meanwhile, no obvious changes have been observed in the decomposition of *cry1Ab*- or

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cry1F-maize residue (Daudu et al. 2009; Zwahlen et al. 2007; Gruber et al. 2012; Shan et al. 2008). Risk assessments and ecological effects of these commercialized Bt crops have been thoroughly investigated. But relatively few investigations focus on newly developed Bt crops which have the potential to be commercialized in the future.

Honeybees are economically valuable pollinators that are required for the seed production of many crops and wild plants. Because of their ecological and economic importance, they were widely considered as an important insect in the biosafety assessment of transgenic crops (Duan et al. 2008; Romeis et al. 2008). Gut bacteria coevolves with honeybees and plays an important role in honeybee health and growth. The effects of Bt maize on honeybees and gut bacterial structures of honeybees have been investigated. It was reported that syrups containing Cry1Ab protoxin (1,000 µg/kg) did not affect syrup consumption and learning abilities of honeybees (Ramirez-Romero et al. 2005), whereas, pure Cry1Ac protein are unlikely to influence the development of honeybee larvae (Lima et al. 2011). Maize transformed with stacked Bt genes (*cry1A.105*, *cry2Ab2*, and *cry3Bb1*) or single gene (*cry1Ab*) showed no effects on the weight and survival of honeybee (Hendriksma et al. 2011; Rose et al. 2007). Exposure and feeding on *cry1Ab*-maize pollen did not harm the hypopharyngeal gland development of honeybees (Malone et al. 2004), and no significant effects on bacterial communities were observed (Babendreier et al. 2007).

Toxicity of Bt-toxin Cry1Ah to *Ostrinia furnacalis* is higher than that of the Cry1Ac, Cry1Ab, and Cry1Ie proteins in our previous studies (Xue et al. 2008). Cry1Ah shared amino acid residue identity of 82 % with *cry1Ab* and 67 % with *cry1F* which were widely used in commercialized Bt crops. Meanwhile, domain II of Cry1Ah was very similar to that of Cry1Ab and Cry1Ac, and domain III was similar to that of Cry1Ac. Transgenic maize harboring the *cry1Ah* gene performs well in controlling *O. furnacalis* larvae under both laboratory and field conditions (Wang et al. 2008) and has the potential to be commercialized. Their effects on honeybee development have been analyzed (Dai et al. 2012a, b). But there

are no reports focused on the midgut bacterial structure fed with Bt-*cry1Ah* maize. In this study, the effects of transgenic Bt-*cry1Ah* maize pollen and high-dose foreign DNA (linear and superhelical plasmids) on the midgut bacterial community of worker bees (*Apis mellifera ligustica*) were first investigated under laboratory conditions; then, the influences of transgenic maize pollen on the midgut bacterial community of larvae and worker bees were studied in the field trials.

2. MATERIALS AND METHODS

2.1. Insect, plant material, and DNA preparation

A. mellifera ligustica was kindly provided by the Institute of Apicultural Research, Academy of Agricultural Sciences, Beijing, China. The transgenic maize (expressing the Bt-*cry1Ah* gene) and non-transgenic maize (X090) were provided by Biotechnology Research Institute, Chinese Academy of Agriculture Sciences (Beijing, China). The quantity of Cry1Ah protein was 65.21 ng/g of maize pollen (Wang 2010).

Plasmid DNA (HF366) containing the *cry1Ah* gene was extracted using the *AxyPre* Plasmid Miniprep Kit (Axygen, USA) according to the manufacturer's protocol. The linear *cry1Ah* gene was extracted from the plasmid by digestion with *Bam*HI.

2.2. Laboratory feeding of honeybees

Newly emerged bees (less than 12 h old) were assigned randomly to wooden cages (22×16×6 cm) with mesh on two sides. For each treatment, a total of 70 bees were used. All the cages were kept in a dark incubator at 29±1 °C and 65±5 % relative humidity. Bees were fed sugar syrup (60 % (w/v) sucrose solution) from a gravity feeder fitted on each cage (Dai et al. 2012a). Five treatments were designed to examine the effects of Bt-transgenic maize pollen on the intestinal bacterial community of the honeybees. Pure sugar syrup (60 % (w/v) sucrose solution) served as a negative control. The other four treatments were 160 mg/mL of transgenic pollen, 160 mg/mL of non-transgenic pollen, 900 ng/mL of plasmid DNA, and 900 ng/mL of linear *cry1Ah* which

were all mixed thoroughly in to sugar syrup (60 % (w/v) sucrose solution). For each cage, 4 mL of the mixture was supplied every day. Bees were collected for further analysis after 15 days.

2.3. Field feeding of honeybees

Transgenic and non-transgenic maize were planted in two separated fields (8×50 m, 50 m apart) at the Langfang Experimental Station, Ministry of Agriculture China (Hebei Province, China) in July and August of 2010. Approximately 1,200 plantlets were planted per field plot in 12 rows with 0.5 m spacing between the rows. At the beginning of flowering, each plot was divided into three mesh cages (8×16 m; height in the center, 3.5 m and mesh size, 1 mm). Bee hives were set up in the center of mesh cages as described by Dai et al. (2012b). Three colonies of *A. mellifera ligustica* (10,000 honeybees per colony) were introduced per field plot. So the hives contained predominantly open and capped brood and eggs less than 48 h old that had been laid by the queen from the experimental hive. Larvae hatched shortly from these combs, and they were fed by worker bees which collected maize pollen in the field cages. The larvae were sampled after 0, 3, 6, and 9 days, respectively. Adult bees were marked with enamel paint after emergence and collected 0, 15, and 30 days later, respectively.

2.4. Honeybee dissection and DNA extraction

The bees were placed at −20 °C for an hour before dissection. The midguts of the honeybees were isolated in sterile conditions, and ten samples were collected for each treatment, kept in 2.0-mL eppendorf tubes, and stored at −70 °C. DNA extraction was performed according to the QIAamp DNA Stool Mini Kit protocol (QIAGEN, Germany).

2.5. Amplification of the 16S rDNA gene V3 region and DGGE analysis

The V3 region of the 16S rDNA gene was amplified using primers 343f (5'-ACTCCTA

CGGGAGGCAGCAG-3') and 534r (5'-ATTACCGCGGCTGCTGG-3') (Nakatsu et al. 2000). A "GC-clamp" (5'-CGCCCGCCGCGCGCGGCGGGCGGGG CGGGGGCCCCGGGG-3') was added to the 5' end of the forward primer. PCR reactions were performed in a volume of 50 µL containing 1.0 µL DNA template, 1.0 µL of each primer, and 25 µL 2× Prime STAR Mix (TaKaRa). To reduce spurious PCR products, the touchdown procedure was used. We started with an annealing temperature of 65 °C for 1 cycle. The temperature was subsequently decreased by 1 °C every cycle until we reached a touchdown at 55 °C, where a further 20 cycles was carried out in an Eppendorf Mastercycler Gradient thermocycler (Brinkman Instruments, Westbury, NY). The PCR products (about 230 bp) were separated by electrophoresis in agarose gels (1 %, w/v) and recovered with a DNA Gel Extraction Kit (AxyGEN, USA).

Denaturing gradient gel electrophoresis (DGGE) was performed using the D-code system (Bio Rad) with 8 % (m/v) acrylamide (acrylamide/bis-acrylamide, 37.5:1 m/m). The gel contained a gradient of denaturant ranging from 35 to 50 % (100 % denaturant is 7 mol/L urea and 40 % deionized formamide). Approximately 500 ng of PCR products were mixed with 2× DGGE loading buffer, and DGGE was run at 200 V for 4 h at 60 °C. After electrophoresis, the gel was stained with SYBR Green I (AMRESCO, USA) and visualized with UV illumination (Bio-Rad, USA). DGGE profiles were analyzed using Quantity One version software (BioRad). The phylogenetic clustering tree was constructed using the complete linkage method.

All the bands marked on the figure were collected, mashed, and then dipped in 100 µL dd H₂O overnight at 4 °C. The solution (5 µL) was taken as template and amplified with primers 343f and 534r using the above protocol. The PCR products (about 230 bp) were recovered, ligated into a pMDT-19 vector, and sequenced by Shanghai Sangon Biotechnology Co. (Shanghai, China). The tree shown was calculated by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method implemented in the MEGA package.

2.6. Detection of horizontal gene transfer of full-length *cryIAh* gene

PCR was used to determine whether the full-length *cryIAh* gene (2,040 bp) was transferred to the gut bacteria with the transgene-specific primers *cryIAh-F* (5'-GGGATCCATCCTATTTAC-3') and *cryIAh-R* (5'-GGTACCTTATCAAAGCTCGTC-3'). PCR conditions included an initial denaturing step for 4 min at 94 °C followed by 30 cycles of denaturing at 94 °C for 0.5 min, annealing at 54 °C for 0.5 min, and extension at 72 °C for 1.5 min. The final elongation time was 10 min at 72 °C.

3. RESULTS

3.1. DGGE patterns of intestinal bacterial communities of adult honeybees under laboratory conditions

The DGGE profiles of the bacterial 16S rDNA V3 region was generated from the midgut microbial community of honeybees fed on transgenic and non-transgenic maize pollen (Figure 1). The five feeding treatments were *cryIAh*-maize pollen, non-transgenic maize pollen, pure sugar syrup, and sugar syrup with a *cryIAh* plasmid or the linear

cryIAh gene. The DGGE profiles of midgut microbial communities showed high diversity with large numbers of bands, and consistent bands specific to the five treatments were not observed. The dendrogram generated by this analysis contained three major clusters, separated at a minimum similarity level of approximately 56 % (Figure 1). The transgenic treatment group II and non-transgenic treatment group II formed one separate cluster with approximately 71 % of internal similarity (Figure 1). Almost all similarity coefficients between replicates were higher than that between treatments (Table 1), except for the similarity between plasmid and linear DNA treatments (Cs=59.4), and the similarity between non-transgenic pollen and pure sugar syrup treatments (Cs=54.1). The likeness coefficient revealed that there were no significant differences between the midgut bacterial community structures of *A. mellifera ligustica* fed on transgenic pollen, non-transgenic pollen, pure sugar syrup, or a high concentration of foreign DNA.

For phylogenetic affiliation, the important clone sequences were aligned with closely related reference sequences (Martinson et al. 2011; Cox-Foster et al. 2007). Three clusters were identified, namely Betaproteobacteria,

Figure 1. DGGE image and cluster analysis of gut bacterial community structure from honeybees feeding on laboratory conditions.

Table I. Similarity coefficient (Cs) calculated from the DGGE banding patterns of bacterial communities from honeybees feeding on laboratory conditions.

	Plasmid	Linear	Transgenic	Normal	Sugar
Plasmid	59.4				
Linear	59.4	66.8			
Transgenic	48.4	51.6	55.1		
Normal	51.1	53.1	51.0	54.0	
Sugar	49.8	55.9	48.6	54.1	58.3

Gammaproteobacteria, and Firmicutes (Figure 2), which consistently occurred in bacterial community of honeybees (Martinson et al. 2011; Cox-Foster et al. 2007).

3.2. DGGE patterns of intestinal bacterial communities of larvae fed under field conditions

The DGGE patterns were generated from larvae samples collected 0, 3, 6, or 9 days after fed on Bt-maize and non-transgenic maize pollen in field trials (Figure 3). Consistent bands specific to either Bt-maize pollen or non-transgenic pollen treatments were not observed. The dendrogram generated here indicated that samples collected from the same time tended to cluster, no matter what kind of diet

they had. Moreover, the Bt-maize and non-transgenic pollen of 3 days’ treatment formed two separate clusters with 78 and 74 % similarity, respectively (Figure 3). Almost all the similarity coefficients between replicates were higher than that between treatments (Table II). The result indicated that *cryIAh*-maize pollen did not clearly affect the midgut bacterial community structures of *A. mellifera ligustica*. For phylogenetic affiliation, the 11 clone sequences were aligned with closely related reference sequences. Three clusters were identified, namely Alphaproteobacteria, Gammaproteobacteria, and Firmicutes (Figure 4), which consistently occurred in bacterial community of honeybees. Most DGGE bands (Figure 3, B2, B3, B4, B6, B7, B9, and B11) showed the highest similarity to Gammaproteobacteria.

Figure 2. UPGMA dendrogram based on 16S rRNA gene sequences from midgut bacteria of honeybees feeding on laboratory conditions and closely related reference sequences.

Figure 3. DGGE image and cluster analysis of gut bacterial community structure from larvae feeding under field conditions.

3.3. DGGE patterns of intestinal bacterial communities of adult honeybees fed under field conditions

The DGGE profiles were generated from adult samples collected 0, 15, or 30 days after fed on Bt-maize and non-transgenic maize pollen in field trials (Figure 5). The dendrogram generated by this treatment contained three major clusters, and samples collected from the same time tended to cluster. Similar to the result of larva treatment, sampling time had a much more clear effect than Bt-maize pollen. The samples collected after 30 days' treatment on Bt-maize and non-transgenic pollen formed one separate cluster with

70 % similarity (Figure 5). All similarity coefficients between replicates were higher than that between treatments (Table III). Furthermore, similarity between the same time treatments was higher than that between different time points. The result indicated that no obvious effect was observed in the midgut bacterial community structures of adult honeybees fed on transgenic pollen and non-transgenic pollen in field trials. For phylogenetic affiliation, the 15 clone sequences formed three clusters, namely Betaproteobacteria, Gammaproteobacteria, and Firmicutes (Figure 6). The main cluster was Gammaproteobacteria which was the same as results in larva groups, whereas Alphaproteobacteria phylotype did not appear.

Table II. Similarity coefficient (Cs) calculated from the DGGE banding patterns of bacterial communities from larvae feeding on field conditions.

	0d No	0d Bt	3d No	3d Bt	6d No	6d Bt	9d No	9d Bt
0d No	68.8							
0d Bt	47.8	69.1						
3d No	68.7	68.7	72					
3d Bt	63.9	73.8	73.8	71.5				
6d No	51.8	59.9	57.4	57.4	41.7			
6d Bt	41.1	47.6	48.9	46.8	46.8	64.7		
9d No	37.8	48.4	48.0	44.6	50.9	50.9	79.5	
9d Bt	36.9	42.2	40.1	40.9	38.9	65.2	65.2	91.2

Figure 4. UPGMA dendrogram based on 16S rRNA gene sequences from midgut bacteria of larvae feeding under field conditions and closely related reference sequences.

3.4. PCR detection of the full-length *cryIAh* gene in bacteria of the honeybee midgut

Using *cryIAh*-specific primers, horizontal transfer of the full-length *cryIAh* gene to indigenous bacteria of *A. mellifera ligustica* was investigated. When genomic DNA from the gut bacteria was used as template, the whole *cryIAh* gene (~2.0 kb) was detected from the

samples in the high-dose exogenous DNA (plasmid or linear DNA) treatment groups (Figure 7, lanes 5 and 7). Meanwhile, the *cryIAh* band did not appear when samples digested with DNase I (TaKaRa) before bacterial genomic DNA extraction (Figure 7, lanes 6 and 8). No positive results were observed in the treatments of sugar syrup, non-transgenic pollen, and transgenic pollen under laboratory conditions (Figure 7, lanes 2 to 4). No bands

Figure 5. DGGE image and cluster analysis of gut bacterial community structure from adult honeybees feeding under field conditions.

Table III. Similarity coefficient (Cs) calculated from the DGGE banding patterns of bacterial communities from adult feeding on field conditions.

	0d No	0d Bt	15d No	15d Bt	30d No	30d Bt
0d No	69.4					
0d Bt	53.2	56.0				
15d No	38.3	43.8	55.4			
15d Bt	45.2	47.7	49.5	60.5		
30d No	38.2	43.1	41.0	58.8	82.5	
30d Bt	36.3	46.2	36.6	50.6	73.1	74.3

appeared both in non-transgenic pollen and transgenic pollen treatment of larvae and adult honeybees in field trials (Figure 7, lanes 9 to 12). Thus, we concluded that the full-length *cry1Ah* gene from transgenic pollen or high-dose treatments did not transfer to indigenous bacteria in the midgut of *A. mellifera ligustica*.

4. DISCUSSION

Only one study evaluated the potential risk of purified Bt-protein and Bt-maize pollen on gut bacterial communities of honeybees, and no significant effects were observed (Babendreier et al. 2007). However, the effects of Bt-

expressing maize on midgut bacterial communities of *A. mellifera ligustica* under field conditions have not been investigated yet. In this study, the effects of transgenic maize pollen containing Bt-Cry1Ah toxin on midgut bacterial communities of both larvae and adult of honeybees (*A. mellifera ligustica*) were investigated in the field trails, and no obvious differences were observed. Furthermore, no significant differences were found in midgut bacterial structures among newly emerged honeybees fed on transgenic *cry1Ah*-maize pollen, normal maize pollen, linear *cry1Ah* gene DNA, supercoiled plasmid DNA,

Figure 6. UPGMA dendrogram based on 16S rRNA gene sequences from midgut bacteria of adult bees feeding under field conditions and closely related reference sequences.

Figure 7. Detection of horizontal transfer of full-length *cryIAh* gene by PCR.

and sugar syrup under the laboratory conditions. These results were consistent with previous explorations (Babendreier et al. 2007). Moreover, larva and adult honeybee samples collected at the same time, whether fed with transgenic maize pollen or normal pollen, tended to cluster in the field trails according to DGGE profiles. The effect of different sampling time periods was greater than that of diet treatments. Additionally, the number of midgut bacteria of adult honeybees was higher than that of larvae, which might be influenced by different gut structure.

The dominant midgut bacterial population of *A. mellifera ligustica*, which were α -, β -, γ -Proteobacteria and Firmicutes, were the same as previously reported for *Apis cerana* (Yoshiyama and Kimura 2009) and *A. mellifera* (Mohr and Tebbe 2006; Martinson et al. 2011; Cox-Foster et al. 2007). Furthermore, in this study, members of the Gammaproteobacteria were found to be the most common group of bacteria in honeybee midguts as in intestines (Babendreier et al. 2007). But the diversity found was relatively low as compared with these studies.

The full-length *cryIAh* gene from transgenic pollen or high-dose DNA treatments were not transferred to indigenous bacteria in the midgut of *A. mellifera ligustica*, which was consistent with the results of bees fed with herbicide-resistant (transformed with *pat* gene) oilseed rape pollen (Mohr and Tebbe 2007). This demonstrated that neither a full-length *cryIAh* gene nor a partial *pat* gene (386 of 800 bp) integrated into the genome of gut bacteria. Thus, it cannot be excluded that horizontal gene transfer took place at a very low rates. If more pairs of primer were used, the results should be more convincing.

In the environmental risk assessment of biotech crops, honeybee is a key species. The results here indicated that there were no significant direct adverse effects of transgenic *cryIAh* maize pollen on the midgut bacterial community composition of *A. mellifera ligustica* in both laboratory and field conditions.

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L'influence du pollen de maïs-Bt transgénique sur la diversité bactérienne de l'intestin moyen d'*Apis mellifera ligustica*

Maïs transgénique / *Apis mellifera* / flore intestinale / électrophorèse sur gel à gradient dénaturant

Der Einfluss von Bt-transgenem Maispollen auf die bakterielle Diversität im Mitteldarm von *Apis mellifera ligustica*

transgener Mais / *Apis mellifera* / Darmflora / denaturierende Gradientengelelektrophorese

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