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Does transgenic Cry1Ac + CpTI cotton pollen affect hypopharyngeal gland development and midgut proteolytic enzyme activity in the honey bee *Apis mellifera* L. (Hymenoptera, Apidae)?

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Abstract The transgenic Cry1Ac (Bt toxin) + CpTI (Cowpea Trypsin Inhibitor) cotton cultivar CCRI41 is increasingly used in China and potential side effects on the honey bee *Apis mellifera* L. have been documented recently. Two studies have assessed potential lethal and sublethal effects in young bees fed with CCRI41 cotton pollen but no effect was observed on learning capacities, although lower feeding activity in exposed honey bees was noted (antifeedant effect). The present study aimed at providing further insights into potential side effects of CCRI41 cotton on honey bees. Emerging honey bees were exposed to different pollen diets using no-choice feeding protocols (chronic exposure) in controlled laboratory conditions and we aimed at documenting potential mechanisms underneath the CCRI41 antifeedant effect previously reported. Activity of midgut proteolytic enzyme of young adult honey bees fed on CCRI41 cotton pollen were not significantly affected, i.e. previously observed antifeedant effect was not linked to disturbed activity of the proteolytic enzymes in bees' midgut. Hypopharyngeal gland development was assessed by quantifying total extractable proteins from the glands. Results suggested that CCRI41 cotton pollen carries no risk to hypopharyngeal gland development of young adult honey bees. In the two bioassays, honey bees exposed to 1 % soybean trypsin inhibitor were used as positive controls for both midgut proteolytic enzymes and hypopharyngeal gland proteins quantification, and bees exposed to 48 ppb (part per billion) (i.e. 48 ng g^{-1}) imidacloprid were used as controls for exposure to a sublethal concentration of toxic product. The results show that the previously reported antifeedant effect of CCRI41 cotton pollen on honey bees is not linked to effects on their midgut proteolytic enzymes or on the development of their hypopharyngeal glands. The results of the study are discussed in the framework of risk assessment of transgenic crops on honey bees.

Keywords Hypopharyngeal gland · Midgut enzymes · Risk assessment · Sublethal effects · Imidacloprid · Transgenic cotton pollen

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Introduction

In China in 2009, the pest-resistant transgenic cotton, mainly the *Bacillus thuringiensis* (Bt) cotton, was grown in 70 % of the cotton hectares (Wu et al. 2008; Clive 2009). Bt cotton cultivars have been effective in suppressing key lepidopteran pests (Wu et al. 2008). To delay the development of pest resistance (Gassmann et al. 2009), transgenic Cry1Ac (Bt insecticidal protein) + CpTI (Cowpea Trypsin Inhibitor) cotton cultivar (CCRI41) producing two insecticidal proteins, have been available commercially

since 2002 throughout China (Cui 2003). Currently, the cotton cultivar CCRI41 is planted at a large scale in the Yellow river cotton area in China (Han et al. 2010a).

The widespread adoption of transgenic cotton cultivars has led to great benefits in China, owing to fewer insecticide inputs and less damage from major pests (Wu et al. 2008; Lu et al. 2012). However, few studies assessing potential negative impacts of Bt cotton on key non-target organisms have been undergone (Carrière et al. 2009; Li et al. 2011; Yu et al. 2011), in contrast for example to Bt maize (Ramirez-Romero et al. 2008a; Bøhn et al. 2010; Desneux et al. 2010; Yu et al. 2011; Dai et al. 2012a, b). However, it has been partially documented for the CCRI41 in the case of pollinators (Chen et al. 2011), notably the honey bee *Apis mellifera* L. (Liu et al. 2009; Han et al. 2010a, b). Beyond the specific case of CCRI41 cotton, multiple studies have been conducted to assess the potential impact of transgenic Bt crops on the honey bee *A. mellifera* and, overall results showed that Bt toxins have no lethal effect on *A. mellifera*. Fewer studies have examined the sublethal effects of Bt toxins on the honey bee, *A. mellifera* (Ramirez-Romero et al. 2005; 2008b; Liu et al. 2009; Han et al. 2010a, b; Dai et al. 2012a). Sublethal effects on pollinators, as those demonstrated in many studies on side effects of pesticides on beneficial arthropods (Arno and Gabarra 2011; Stara et al. 2011; Biondi et al. 2012; He et al. 2012 and see Desneux et al. 2007 for a thorough review), could negatively affect their efficiency as pollinators (Brittain et al. 2010) and lead to colony population decrease (Desneux et al. 2007). This highlights the importance of risk assessment of transgenic crops on pollinators (Romeis et al. 2008; Desneux and Bernal 2010).

Healthy honey bee populations depend on landscapes with ample and nutritious sources of pollen yielding flowers (Decourtye et al. 2010; Wratten et al. 2012). The flowering period of cotton usually lasts from June to late August in China, a season during which honey bees do not have many available floral sources but cotton. Honey bee larvae and young adults (less than 12 days old) mainly feed on pollens (Haydak 1970) and, because of the widespread cultivation of this cotton variety and the scarcity of the alternative pollen sources, honey bees are heavily exposed to transgenic cotton (CCRI41) pollen. Therefore, potential malfunctioning of bee hypopharyngeal glands, owing to the reduced consumption of pollen (the primary source of proteins for honey bees) when feeding on CCRI41 pollen (Han et al. 2010a), may potentially compromise hive sustainability. Pollen consumption is positively correlated with gland development (Hrassnigg and Crailsheim 1998) and the highest rate of protein synthesis in hypopharyngeal glands occurs when bees are 8–16 days old (Knecht and Kaatz 1990). Therefore, insufficient pollen consumption in the adult honey bee's early life could cause poor hypopharyngeal gland development (Haydak 1970), thus without actual direct effect of the Bt toxins on the glands (e.g. Malone et al. 2004; Babendreier et al. 2005).

The midgut enzymes play a key role in the digestive process of pollens ingested by honey bee workers (Michener 1974). The total midgut protease activity, which is directly related to digestive capacity of protein-rich pollens, may be a sensitive indicator for assessing food digestive process (Laskowski and Kato 1980; Sagili et al. 2005). Since the direct causes of antifeedant effect identified by Han et al. (2010a) still need to be identified, we assume that the total midgut proteolytic enzyme activity might be reduced when bees consume CCRI41 pollen. The midgut proteolytic reaches its maximal activity when bees are 8 days old (Moritz and Crailsheim 1987), i.e. at the time the bees may be exposed to CCRI41 cotton pollen in the hive. Moreover, midgut protein digestion is associated with the development of hypopharyngeal glands and the production of extractable proteins occurs in the hypopharyngeal glands when bees are fed on pollen (Sagili et al. 2005; Sagili and Pankiw 2007). Sagili et al. (2005) reported that bees fed on pollen containing 1 % soybean trypsin inhibitor (SBTI) significantly reduced hypopharyngeal gland protein content and midgut proteolytic enzyme activity. Findings by Han et al. (2010a) indicated a potential link to the expression of the protease inhibitor CpTI in cotton pollen as a possible cause for the antifeeding reported. Indeed, in studies comparing Cry1 with transgenic protease inhibitors, it was found that only the latter was causing reduced feeding effects in bees (Malone and Pham-Delègue 2001; Sagili et al. 2005; Babendreier et al. 2005, 2007).

The aims of our study were (1) to measure the total midgut proteolytic activity of caged honey bees feeding on CCRI41 pollen according to the methods reported by Sagili et al. (2005), and (2) to determine the side effects of decreased CCRI41 pollen consumption on the development of hypopharyngeal glands in young honey bees. This study provides the first attempt to identify the physiological mechanism(s) involved in the antifeedant effect observed in honey bees exposed to transgenic (Bt) crop products during previous studies (Ramirez-Romero et al. 2008b; Han et al. 2010a).

Materials and methods

Cotton varieties

Transgenic Cry1Ac + CpTI cotton cultivar CCRI41 and its near-isogenic cultivar CCRI23, hereafter referred to as conventional cotton, were provided by Institute of Cotton Research, Chinese Academy of Agricultural Sciences. Both

cultivars were planted early June 2010 in experimental fields of Huazhong Agricultural University, routine management was carried out and pesticide applications were avoided. Thirty grams samples of each pollens type ($n = 30$ plants) were collected using a multi-point field sampling method on August 6th (cotton mid-stage bloom) and September 4th (cotton late bloom), respectively. Pollen derived from June and July (cotton early bloom) was not available owing to frequent heavy rains in the season (resulting in delayed blooming). Samples were stored at −80 °C until they were used for experiments or analyses. Part of the samples was used to quantify the Cry1Ac and CpTI toxins in cotton pollen gathered on the two collection dates (see below) and the rest was used to conduct the studies whose aim was to assess lethal and sublethal effects of CCRI41 cotton pollen on honey bees.

ELISA quantitative detection for transgenic proteins in pollen

We used Envirologix Qualiplate Kits (EnviroLogix Quantiplate Kit, Portland, ME, USA) to estimate Cry1Ac quantities. The quantitative detection limit of the Cry1Ac kit was 0.1 ng ml^{-1} (data from Shanghai YouLong Biotech Co., Ltd). Six known increasing concentrations of Cry1Ac toxin were used to plot the standard calibration curve for further quantification of the Cry1Ac toxin in our samples (Supplementary Fig. 1). The ELISA polyclonal kits to detect CpTI protein were provided by Center for Crop Chemical Control, China Agricultural University, which validated the method. The quantitative detection limit of the CpTI kit was 20 ng g^{-1}. The CpTI ELISA testing method was carried out according to the literature published (Rui et al. 2004). A BIO-RAD 550 plate reader (BIO-RAD Model 550, USA) was used to obtain the ODs of sample pollens.

Before analyzing the two insecticidal proteins, the samples were homogenized in 2 ml extraction phosphate buffered saline tween-20 (PBST; 8.0 g NaCl, 2.7 g $Na_2HPO_4 \cdot 12H_2O$, 0.4 g $NaH_2PO_4 \cdot 2H_2O$, dissolved in 1000 ml water, pH = 7.4) using liquid nitrogen for complete extraction. Three replicates were performed for each sample type. The homogenized samples were washed with an additional 2 ml PBST and kept in 10 ml centrifuge tubes at 4 °C overnight to extract the insecticidal proteins. The tubes were then centrifuged at 6,900 rpm for 20 min. The supernatants were used to detect targeted proteins using the corresponding ELISA kits described above.

Artificial diets

Five different treatments were used, i.e. conventional cotton pollen, CCRI41 cotton pollen, imidacloprid-contaminated conventional cotton pollens (two concentrations were tested, 48 and 96 ppb) and SBTI-contaminated conventional cotton pollen. The artificial diets were formulated by mixing water, honey and pollen described above at a rate of 1:2:7 (weight). For the CCRI41 pollen treatment, the pollen sampled on September 4th was used because the quantification detection results showed the highest amount of Cry1Ac during ELISA tests (see "Results" section "Cry1Ac–CpTI quantification of CCRI41 pollen"). The serine trypsin endopeptidase inhibitor (SBTI) at 1 % (w/v) was used as a positive control (Babendreier et al. 2005; Sagili et al. 2005). Imidacloprid at 48 ppb (ng g^{-1}) contained in food mixture was used because previous works reported sublethal effects in honey bees when exposed to that concentration of imidacloprid (Decourtye and Pham-Delègue 2002; Ramirez-Romero et al. 2008b; Han et al. 2010b). The 96 ppb imidacloprid concentration was used as positive control (low lethal) during chronic exposure to ensure that our exposure bioassay was reliable, i.e. that honey bees feeding on cotton pollen were effectively exposed to potential toxicants.

Chronic exposure

Emerging honey bees were collected from a bee colony during summer and were kept in glass cages (15 × 10.5 × 20 cm) with a piece of mesh covering the top to ensure effective ventilation in the experimental cages. Honey bees had a 2-day adaptive period to the rearing conditions in cages and were then used for the experiments. We used a no-choice dietary feeding method in which a single food source type was provided to bees. Bees received a mixture of water + honey + pollen (using a chopped 10 ml Eppendorf tube introduced into the cage) and no additional sugar was provided. The cages were kept in the dark in an incubator at a temperature of 33 ± 1 °C and 55 ± 5 % relative humidity. Filter papers were placed and changed daily at the bottom of the cage to ensure a clean and dry environment for bees during the assays. Fifty bees were tested per each replicate and four replicates were undertaken per treatment. The bees were exposed to the different treatments for a 7-day period during which water was offered ad libitum.

The honey bee mortality was recorded daily at 5:00 pm. Honey bees were considered dead when they remained completely immobile and these bees were removed from the cages every day (Ramirez-Romero et al. 2008b; Han et al. 2010a). Thereafter, the treated pollens and water were replenished daily. The proportion of dead honey bees at the end of the oral chronic exposure period among CCRI41 pollen, imidacloprid-contaminated pollen (48 and 96 ppb), SBTI-contaminated pollen and conventional pollen were fitted to a log-linear model (proc genmod, SAS Institute 1999) using "food type" and "replicate" as factors. In addition, mortality rates of bees exposed to the various treatments were compared to those recorded in the control

group using Fisher exact tests (Dunn–Sidak adjustment for multiple comparisons).

Assessment of hypopharyngeal gland development

Total protein content extractable from bees' hypopharyngeal glands was considered a parameter for the gland development status and its protein-producing function in honey bees (Patel et al. 1960; Sagili et al. 2005). After the 7-day chronic exposure to the various treatments, the tested honey bees were 11–12 days old, which is the optimal time for hypopharygeal glands development assessment (Fluri et al. 1982; Babendreier et al. 2005; Sagili et al. 2005). At the end of the chronic exposure period, 10 randomly sampled live honey bees from each cage were cold anaesthetized, dissected, hypopharyngeal glands were removed and 10 pairs of glands were placed in a homogenizer (Youyuan Biotech Co., Ltd, Wuhan, China) with 800 µl Tris–Hcl buffer (0.1 M, pH 7.9). The homogenized samples were washed with additional 200 µl Tris–Hcl buffer, kept in 1.5 ml centrifuge tubes and centrifuged at 10,000 rpm for 5 min and then the supernatants were used to quantify the total hypopharyngeal gland protein content.

In this study, *Bradford* assays were employed to measure the total hypopharyngeal gland protein content (Bradford 1976). The protocols are described in details by Sagili et al. (2005) and the 500-0202 Quick Start Bradford Protein Assay Kit 2 (Bio-Rad Laboratories, Hercules, CA, USA) was used. Dry reagent was prepared by mixing dry reagent concentrate (Coomassie Briliant Blue G-250) and distilled water at a rate of 1:4 (vol/vol). Five microlitre supernatant of each sample was added to 1 ml reagent, vortexed thoroughly and then incubated for 5 min at ambient temperature. Bovine serum albumin (BSA) proteins (contained in the Kit), with 7 gradient concentrations, were used to plot standard curves (see Supplementary Fig. 2). An UV/visible spectrometer (Ultrospec 1100 pro, Amersham Biosciences, Piscataway, USA) was used to obtain the ODs of each sample at 595 nm ($n = 3$). The standard curve was generated by linear regression using the CurveExpert Version 1.38 software (Hyams 1993) then the total proteins extracted from hypopharyngeal glands of 10 bees ($n = 4$) from each replicate were calculated (in µg) according to the standard curve. The values were compared using an ANOVA, followed by Tukey's post hoc test. The Cochran test and the Kolmogorov–Smirnov D were used to test the homogeneity of variance and normality of the datasets, respectively.

Assessment of total midgut proteolytic enzyme activity

The midguts of honey bees were dissected from the same bees which had their hypopharyngeal glands removed. The midguts dissected from 10 honey bees taken from each cage were placed in a homogenizer containing 800 µl Tris–Hcl buffer (0.1 M, pH 7.9). The guts were then homogenized in ice and transferred into 1.5 ml centrifuge tubes, and an additional 200 µl Tris–Hcl buffer was added to wash the homogenizer and placed into the tube. The tubes were then centrifuged at 10,000 rpm for 5 min and the supernatant was analyzed to estimate the midgut proteolytic enzyme activity as reported by Sagili et al. (2005).

Azosasein was used as substrate of the proteolysis reaction. Five microlitre of the supernatant from each gut sample was added to 60 µl of assay buffer (0.1 M Tris–Hcl buffer, pH 7.9), 60 µl of 2 % (w/v) azosasein diluted in assay buffer (respectively) and incubated for 6 h at 37 °C. Three hundred microlitre of 10 % (w/v) TCA was added to each mixture and centrifuged for 5 min at 10,000 rpm to remove the residual azosasein after the reaction. 350 µl of supernatant was then added to 200 µl of 50 % (v/v) ethanol diluted in water, immediately followed by the measurement of the absorption at 440 nm using an UV/visible spectrometer (Ultrospec 1100 pro, Amersham Biosciences, Piscataway, USA). Each sample mixture was measured and replicated three times. The absorption of mixture without incubation (no azosasein for proteolysis) was used as a control (i.e. 0 value). Total midgut proteolytic enzyme activity was represented by the OD_{440} value, and they were compared using an ANOVA (followed by Tukey's post hoc test). The Cochran test and the Kolmogorov–Smirnov D were used to test the homogeneity of variance and normality of the datasets, respectively.

Results

Cry1Ac–CpTI quantification of CCRI41 pollen

Mean contents of Cry1Ac protein were 517.3 ± 7.32 ng g^{-1} and 651.0 ± 9.01 ng g^{-1} for August 6th (mid-stage bloom) and September 4th (late bloom) respectively, which suggested relatively higher contents of Cry1Ac than those recorded in 2009 (Han et al. 2010a). Samples collected at the same dates varied less, i.e. low SEM values. CpTI protein was detectable in CCRI41 cotton pollen but the levels were always lower than the lowest level of quantification (20 ng g^{-1}) of the ELISA polyclonal kits used in the experiment.

Chronic mortality of honey bees

The percentages of corrected mortality in honey bees after 7-day chronic exposure period varied from 3.60 to 20.14 % and differed significantly among the treatments (significant "food type" factor) (Table 1). The "replicate" factor and its interaction with the "food type" factor were not

Table 1 Mean percentage of corrected mortality in honey bees after a 7-day period of chronic exposure to CCRI41 (Cry1Ac + CpTI) pollen, conventional pollen containing imidacloprid at 48 ppb (part per billion) or 96 ppb and conventional pollen containing 1 % soybean trypsin inhibitor (SBTI) and conventional pollen

Treatments	Percentage of corrected mortality[a] (%)
CCRI41 (Cry1Ac + CpTI)	3.60 ± 3.32
Imidacloprid at 48 ppb	5.04 ± 3.19
Imidacloprid at 96 ppb	20.14 ± 3.96
1 % SBTI	4.32 ± 3.81

Statistical results

Source of variation	Degrees of freedom	Chi-square	*P* value
Food type	4	15.14	0.0044
Replicate	3	2.54	0.4674
Food type × replicate	12	10.12	0.6050

Statistics from the log linear model were used to analyze the proportion of dead honey bees at the end of the oral chronic exposure period among treatments (food type factor) and as a function of replicate factor (four replicates [cages] per food type with 50 individual bees per replicate)

[a] Corrected mortality based on formula by Abbott (1925), mortality in control group 13.1 %

significant, and thus suggested that mortality results were consistent among the various replicates for a given food type (Table 1). Mortality recorded in groups of honey bees fed on CCRI41 cotton pollen, on 48 ppb imidacloprid-contaminated cotton and on SBTI-contaminated cotton pollen was not significantly different to those recorded in the control group (all $P > 0.05$). Honey bees fed on cotton pollen contaminated with the low lethal concentration of imidacloprid (96 ppb) showed significantly higher mortality rate than those in the control group ($P = 0.007$), i.e. the chronic exposure bioassay used was successful in exposing honey bees to toxicants when feeding on cotton pollen.

Hypopharyngeal gland development

The quantity of proteins extracted from the hypopharyngeal glands of honey bees fed on different diets is reported in Fig. 1. Significant differences in the quantity of hypopharyngeal gland proteins were observed among treatments ($F_{3,15} = 6.189$, $P = 0.006$). Honey bees fed on 1 % SBTI-contaminated pollen showed significantly fewer hypopharyngeal gland proteins than the control group. Those that were fed on imidacloprid-contaminated pollen (48 ppb) or CCRI41 pollen showed slightly lower hypopharyngeal gland protein content though it was not significantly lower than the control group.

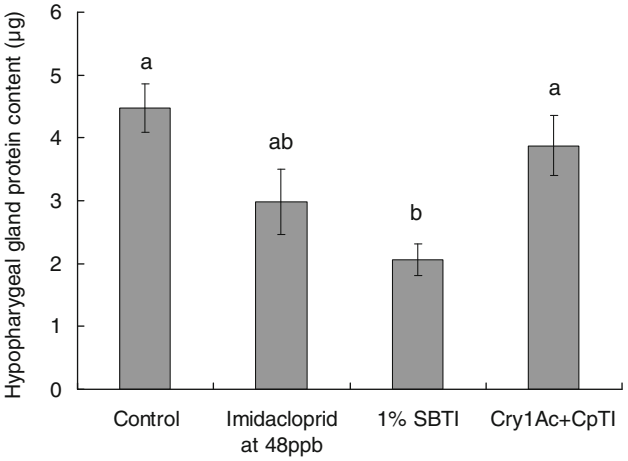

Fig. 1 Mean hypopharyngeal gland protein quantities (± SEM) of bees in groups subjected to a 7-day oral chronic exposure to CCRI41 pollen, conventional pollen containing imidacloprid at 48 ppb (part per billion), and conventional pollen containing 1 % SBTI (soybean trypsin inhibitor) and conventional pollen. Histograms bearing the same letter are not significantly different at the $P > 0.05$ level (ANOVA followed by Tukey's post hoc test)

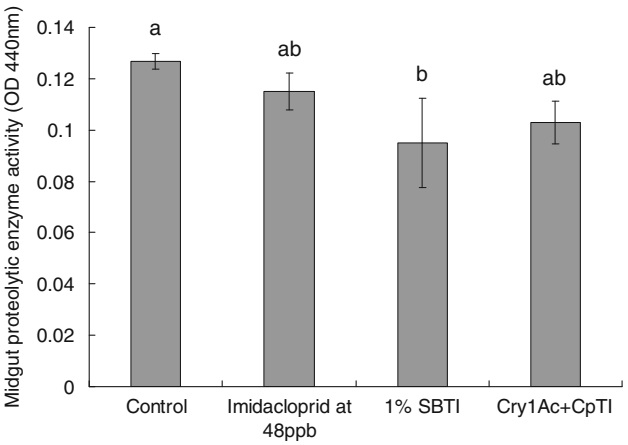

Fig. 2 Mean midgut proteolytic enzyme activities (± SEM) of bees in groups subjected to a 7-day oral chronic exposure to CCRI41 pollen, conventional pollen containing imidacloprid at 48 ppb (part per billion), and conventional pollen containing 1 % SBTI (soybean trypsin inhibitor) and conventional pollen. Histograms bearing the same letter are not significantly different at the $P > 0.05$ level (ANOVA followed by Tukey's post hoc test)

Midgut proteolytic enzyme activity

The values of total midgut proteolytic enzyme activity in honey bees fed on the various diets are reported in Fig. 2. Significant differences in midgut proteolytic enzyme activity were observed among treatments ($F_{3,15} = 3.691$, $P = 0.036$). Honey bees fed on 1 % SBTI cotton pollen diet showed significantly lower midgut proteolytic enzyme activity than the control group. The honey bees that were fed on diet containing imidacloprid at 48 ppb and on the

CCRI41diet showed slightly lower values than the control though not significant ($P > 0.05$).

Discussion

In two companion studies we have demonstrated limited potential side effects of CCRI41 cotton pollen on the honey bee *A. mellifera* (Han et al. 2010a; 2010b) though a decrease in feeding activity in CCRI41-exposed honey bees was observed (Han et al. 2010a). The current study provides further insights in the assessment of potential side effects of CCRI41 cotton on honey bees. Emerging honey bees were exposed to various pollen diets using no-choice feeding protocols in controlled laboratory conditions. We focused on assessing the possible mechanisms underlying the CCRI41 cotton antifeedant effect on honey bees, thus focusing on the potential sublethal effect of CCRI41 pollen on two important physiological traits linked with feeding activity of young adult honey bees. Our results showed no side effects of CCRI41 cotton pollen on both hypopharyngeal gland development and total midgut proteolytic enzyme activity of honey bees when they were subjected to chronic exposure to the transgenic CCRI41 cotton pollen. The negative effects of 1 % SBTI treatment on honey bees (positive control) were consistent with previous reports (Babendreier et al. 2005; Sagili et al. 2005), therefore our results can be considered as fully valid. Furthermore, the experimental setup proved to be efficient in exposing adult honey bees to toxicants through chronic oral exposure, i.e. imidacloprid at 96 ppb induced significant mortality in our assay.

Fluctuation of Cry1Ac expression in transgenic cotton pollen

Regarding temporal dynamics expressed by Cry1Ac insecticidal protein in CCRI41 pollen, a higher level and a different pattern of dynamics were observed compared to the results of quantification carried out on CCRI41 cotton in 2009 (Han et al. 2010a). In the present study, we detected a higher level (twofold higher) of Cry1Ac in cotton pollen and, in particular, a significantly higher level for middle bloom (517.3 ± 7.32 vs 300.0 ± 4.52) and late bloom (651.0 ± 9.01 vs 32.8 ± 3.39), respectively. This indicated temporal dynamics activity of promoter CaMV35S not only at intra-plant level throughout the growing season (Siebert et al. 2009; Bakhsh et al. 2010), but also among generations of the transgenic cotton variety. Since the same cropping practices were used and seeds harvested from the same cultivar from 2009 were used, the potential instability of activity of the promoter CaMV 35S may account for such inter-generation variation in expression of the insecticidal protein. Nevertheless, this variance would require additional data to be fully characterized.

Sublethal effects on honey bees

A decreased midgut proteolytic enzyme activity in honey bees exposed to CCRI41 was thought to be responsible for the observed antifeedant effect in our previous study (Han et al. 2010a). However, there may be some other factors (not studied yet) which could be responsible for the antifeedant effect induced by CCRI41 pollen in honey bees. For example, the bacterial community structure in honey bee intestines may not be a sensitive indicator to identify the effect of Cry toxins on honey bees (Babendreier et al. 2007). The antifeedant effect may be related to altered gut physiologies rather than to direct toxic effects of the Cry toxins on the gut bacterial community. The antifeedant effect of CCRI41 pollen on honey bee may be also linked to its social life style. Social insects like honey bees have an important "cognition" property, i.e. information is processed, stored, retrieved and used in a flexible and adaptive manner in the colony (Giurfa 2003; Srinivasan 2010). In this case, we may assume that the number of individuals showing avoidance to the food source (i.e. to the CCRI41 pollen) may be boosted in the bee population because of frequent communication among individuals and associative learning capacities. Nevertheless, fundamental understanding of feeding mechanisms, digestive functions, and taste receptors of young adult honey bees at molecular level warrant further study in order to fully document the antifeedant effects previously reported on the honey bee (Ramirez-Romero et al. 2008b; Han et al. 2010a).

The development and physiological functions of the hypopharyngeal glands of honey bees were not affected by the antifeedant effect of CCRI41 pollen, even though a decreased consumption of proteins could have reduced the hypopharyngeal gland protein biosynthesis and subsequent gland development (Haydak 1970; Sagili et al. 2005). It was consistent with previous studies which suggested no negative impact of Cry toxins (either through exposure to Cry-contaminated syrups or Bt pollens) on hypopharyngeal gland protein content and gland development on 8–10 day young adult honey bees (Malone and Pham-Delègue 2001; Malone et al. 2004; Babendreier et al. 2005). We did not work on brood (e.g., Sagili et al. 2005; Hendriksma et al. 2011) during our assays and it might lead to an underestimation of the real risk of CCRI41 pollen at the scale of honey bee colony because brood is considered to be a stimulus for hypopharyngeal gland development (Sagili et al. 2005). However, given the complex social organization of honey bee hive (Robinson 1992) the potential risk related to colony maintenance and development still cannot be ruled out (Babendreier et al. 2005).

Implications for risk assessment

Taking into consideration the previous studies as well as the present one on CCRI41 pollen risk assessment on honey bees (Liu et al. 2009; Han et al. 2010a, b), consumption of transgenic pollen would not induce lethal and sublethal effects in the honey *A. mellifera*. Physiological traits such as longevity, midgut proteolytic enzyme activity, the development of hypopharyngeal gland, and associative learning behaviors are not affected by the ingestion of pollen from Bt crops. By contrast, decrease in feeding activity was observed during laboratory bioassays (Ramirez-Romero et al. 2008b; Han et al. 2010a) suggesting that the effects on feeding behavior should not be underestimated nor the potential consequences that this sublethal behavioral effect may have on hive populations (Desneux et al. 2007). Better knowledge about risks associated with CCRI41 pollen on honey bees may also be obtained studying the effects of this food source on young instars development (Hendriksma et al. 2011). All these results were obtained in laboratory conditions (tier 1), which represents a worst-case exposure scenario, therefore the lack of negative effects of CCRI41 on the honey bee may not prompt further semi-field and field assessments experiments in the scheme of the tiered approach (Romeis et al. 2008). However, honey bees represent only one of the few examples of organisms with a highly evolved social structure (Srinivasan 2010) so this could explain that studies on the wide scale side effects may be more complex regarding how the scheme of risk assessment would be implemented.

Overall the risk assessment methods used in current study as well as those in the previous ones (Liu et al. 2009; Han et al. 2010a, b) were found to be simple to carry out, easily-standardized and repeatable. These protocols may be considered useful tools in the context of the tiered approach to accurately assess the risks of either transgenic crops or pesticides on honey bees. Nevertheless, more standardized and validated testing protocols should be developed to meet the needs for precise and robust environmental risk assessment on honey bees.

In conclusion, the two main results: (1) hypopharyngeal glands of young adult honey bees were not significantly affected by the consumption of CCRI41 pollen in laboratory conditions, and (2) we demonstrated that the antifeedant effect induced by CCRI41 pollen in honey bee is not linked to decrease in midgut proteolytic enzyme activity in bees. Further studies are needed to identify the mechanisms underneath the behavioral (antifeedant) effect.

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