Consumption of *Bt* rice pollen containing Cry1C or Cry2A does not pose a risk to *Propylea japonica* (Thunberg) (Coleoptera: Coccinellidae)

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As a pollen feeder, *Propylea japonica* would be directly exposed to Cry proteins in *Bacillus thuringiensis* (*Bt*)-transgenic rice fields. The effect of Cry1C- or Cry2A-containing transgenic rice pollen on the fitness of *P. japonica* was assessed using two dietary-exposure experiments in the laboratory. In the first experiment, larval developmental time of *P. japonica* was significantly longer when fed pollen from *Bt* rice lines rather than control pollen but other life table parameters were not significantly affected. In the second experiment, *P. japonica* was not affected when fed a rapeseed pollen-based diet containing purified Cry1C or Cry2A at concentrations that were >10-times higher than in pollen, but *P. japonica* was affected when the diet contained E-64 as a positive control. In both experiments, the stability and bioactivity of the Cry proteins in the food sources and the uptake of the proteins by *P. japonica* were confirmed. The results show that *P. japonica* is not sensitive to Cry1C or Cry2A proteins; the effect observed in the first experiment was likely attributable to unknown differences in the nutritional composition of *Bt* rice pollen. Overall, the data indicate that the growing of Cry1C- or Cry2A-transgenic rice should pose a negligible risk to *P. japonica*.

To avoid potential risks to the environment associated with the planting of genetically engineered (GE) plants, any new GE plant must undergo a rigorous environmental risk assessment before it is approved for commercial cultivation. An important part of this assessment, especially in the case of insect-resistant GE plants1–4, is the evaluation of potential effects on valued non-target organisms (NTOs). The non-target risk assessment follows a tiered framework that typically starts with laboratory studies conducted under controlled, worst-case exposure conditions with the main objective of identifying the potential toxicity of the insecticidal proteins produced by the GE plants on surrogate test species1,2,5.

The use of insect-resistant GE plants is an attractive approach for the control of insect pests. In China, many GE rice lines have been developed that produce Cry proteins derived from the soil bacterium *Bacillus thuringiensis* (*Bt*) and targeting lepidopteran pests, such as the Asiatic rice borer, *Chilo suppressalis* (Lepidoptera: Crambidae)6. Two recently developed *Bt* rice lines expressing cry1C or cry2A genes provided good resistance against stem borers and leaf folders throughout the rice growth period in laboratory and field experiments7–9. They therefore may have potential for commercial use in China. Data regarding their potential effects on valued NTOs, however, are limited. Recently, three laboratory studies indicated that larvae and adults of the green lacewing, *Chrysoperla sinica* (Neuroptera: Chrysopidae), are not sensitive to Cry1C and Cry2A proteins when provided in artificial diets or *Bt* rice pollen; the authors of these studies concluded that the planting of the *Bt* rice lines will pose a negligible risk to this species10–12. In addition, consumption of *Bt* rice pollen containing Cry1C or Cry2A protein was found to pose a low to negligible risk to larvae of the silkworm *Bombyx mori* (Lepidoptera: Bombycidae)13.

Throughout East Asia, the ladybird beetle *Propylea japonica* (Thunberg) (Coleoptera: Coccinellidae) is a common and abundant predator in many cropping systems, including maize, cotton, rice, vegetables, and fruit trees14–17. Both the larvae and adults consume aphids, thrips, spider mites, and eggs and young larvae of Lepidoptera18. In addition, they are known to use plant pollen, including rice pollen, as a complementary food source19–21. Therefore, this species may be exposed to plant-produced insecticidal proteins not only indirectly by consuming prey, but also directly by foraging pollen in *Bt* crops. This feeding ecology, together with the fact that
the species can be easily reared and is amenable for testing in the laboratory, makes P. japonica a suitable surrogate species for evaluating the potential effects of Bt-transgenic plants on predacious Coccinellidae22,23. The species has been previously used to assess the non-target effects of Bt-transgenic cotton18,24,25, maize26, and rice14,27 in China.

In the present study, we investigated potential effects of feeding on Bt rice pollen containing Cry1C or Cry2A protein on P. japonica in the laboratory. In addition to this first experiment, we conducted a second experiment in which the beetles were directly exposed to purified Cry1C and Cry2A proteins mixed in rapeseed pollen-based diet at levels that were significantly higher than those present in Bt rice pollen. The second experiment, which used a dietary exposure system recently developed by our group19, was conducted to reduce the possibility that toxic effects were not missed in the laboratory. The second experiment also enabled us to draw general conclusions about the susceptibility of P. japonica to the two Cry proteins independent of the effects of the Bt rice varieties.

Results

Bt rice pollen experiment. Effects on life table parameters. When fed with rice pollen, over 80% of the P. japonica larvae developed to adults, and the percentage did not significantly differ between the Bt rice pollen treatments and the control pollen treatment (χ²-test; both P > 0.05) (Table 1). Similarly, the 15-day larval survival rates did not significantly differ between each Bt rice pollen treatment and the control (Cry1C: χ² = 2.30, P = 0.13; Cry2A: χ² = 2.27, P = 0.13) (Fig. 1). Larval development time, however, was significantly prolonged with Bt rice pollen (Mann-Whitney U-test; both P < 0.001). Adult weight did not significantly differ between each of the Bt pollen treatments and the control pollen (Dunn test; Cry1C: P = 0.47 for females and P = 0.08 for males; Cry2A: P = 0.18 for females and P = 0.12 for males). Fecundity also did not significantly differ between each of the Bt pollen treatments and the control pollen (Dunn test; Cry1C: P = 0.90; Cry2A: P = 0.08). Although differences in this first experiment were significant for larval duration, pupation and eclosion rates, larval survival, adult fresh weights, and especially fecundity tended to be lower with Bt rice pollen than with control pollen (Table 1 and Fig. 1).

Uptake of Cry proteins by P. japonica. At the end of the Bt rice pollen experiment (30 days after adult emergence), ELISA measurements showed that all P. japonica adults that had been fed Bt rice pollen contained Cry protein. The mean concentrations (±SE) of Cry1C and Cry2A in P. japonica adults were 0.16 ± 0.007 and 1.05 ± 0.40 μg/g DW, respectively. No Cry protein was detected in adults fed control pollen.

Stability and bioactivity of Cry proteins in rice pollen. According to ELISA, the original concentrations (mean ± SE) of Cry1C and Cry2A were 2.25 ± 0.33 μg/g and 37.85 ± 3.74 μg/g DW in T1C-19 and T2A-1 rice pollen, respectively. After a 2-d feeding exposure in the environmental chamber, Bt protein contents in the exposed pollen were not significantly different from those in the fresh pollen (Cry1C: t = 1.87, df = 4, P = 0.14; Cry2A: t = 0.48, df = 4, P = 0.66). No Bt protein was detected in samples of control rice pollen that were tested in parallel.

The sensitive-insect bioassay indicated that the mean weight of C. suppressalis larvae was significantly smaller when fed for 7 days on an artificial diet containing extract from the two Bt rice pollens rather than extract from control rice pollen (P < 0.001) (Fig. 2). Pair-wise comparisons revealed no statistical differences between the weights of C. suppressalis larvae fed extract from each Bt pollen that had been either freshly prepared or exposed to P. japonica larvae for 2 days (P > 0.05) (Fig. 2).

Purified Cry protein experiment. Effects on life table parameters. As noted, all of the life table parameters measured in the first experiment tended to be adversely affected by Bt rice pollen treatments but the effect was statistically significant only for duration of the larval stage. Although these results suggest that Bt rice pollen might cause some toxic effects to P. japonica larvae, they could also be explained by other unknown differences between Bt rice pollen and control rice pollen. To further examine the effects of the Cry proteins on P. japonica larvae, and to do so without variation in the pollen, we used a diet containing rapeseed pollen augmented or not augmented with high concentrations of the Cry proteins; for Cry1C the concentration used was 89-times that measured in T1C-19 pollen, for Cry2A the concentration used was 13-times that measured in T2A-1 pollen. The control used the same rapeseed pollen but without Cry protein.

Pair-wise comparisons revealed that the treatments containing Cry1C or Cry2A protein did not differ significantly from the

| Rice line | Pupation rate (%) | Eclosion rate (%) | Days to pupa (±SE) | Adult fresh weight (mg ± SE) | Total fecundity per pair (eggs ± SE) |
|-----------|------------------|------------------|-------------------|-----------------------------|----------------------------------|
| Minghui 63 | 97.3 (75) | 90.7 (75) | 7.99 ± 0.11 (73) | 6.97 ± 0.13 (30) | 177.2 ± 14.64 (30) |
| T1C-19 (Cry1C) | 89.3 (75) | 84.0 (75) | 8.88 ± 0.12 (67) | 6.69 ± 0.24 (27) | 169.5 ± 15.61 (24) |
| T2A-1 (Cry2A) | 90.7 (75) | 81.3 (75) | 8.62 ± 0.10 (68) | 6.53 ± 0.18 (26) | 137.6 ± 10.40 (25) |

Table 1 | Effect of consumption of pollen from Cry1C- or Cry2A-expressing Bt rice (T1C-19 and T2A-1) or the corresponding non-transformed rice line (Minghui 63) on life table parameters of Propylea japonica. Number of replicates is indicated in parenthesis.

Each Bt rice line was compared to the control. An asterisk denotes a significant difference between a Bt rice line and the control.

*P < 0.025.

Mann-Whitney U test with Bonferroni correction (adjusted α = 0.025).

Dunn test.
un-treated (negative) control for pupation rate ($\chi^2$-test; both $P > 0.10$), eclosion rate (both $P > 0.07$), and larval development time ($U = 1374.0, P = 0.08$ for Cry1C and $U = 1440, P = 0.28$ for Cry2A) (Table 2). Similarly, no significant difference was found between each of the Cry proteins and the negative control treatment for female and male FW (Dunnett’s test; all $P > 0.30$). Although total fecundity was reduced by more than 20% in the two Cry protein treatments relative to the control, the difference was not significant ($P = 0.15$ for Cry1C and $P = 0.10$ for Cry2A). In contrast, $P. japonica$ had significantly decreased pupation ($\chi^2 = 9.25, P = 0.002$) and eclosion rates ($\chi^2 = 23.64, P < 0.001$) and a significantly prolonged larval developmental time ($U = 6.0, P < 0.001$) when fed E-64 (Table 2). Likewise, the mean weight of the emerging adults was significantly reduced by E-64 ($P < 0.01$ for both sexes) as was the total fecundity ($P < 0.001$). The survival rates were not significantly affected when $P. japonica$ larvae were fed a rapeseed pollen-based diet containing Cry1C or Cry2A rather than a diet containing pure rapeseed pollen ($P > 0.40$), while survival was significantly lower in the E-64 treatment than in the control treatment ($\chi^2 = 11.1, P = 0.001$) (Fig. 3).

**Uptake of Cry proteins by $P. japonica$.** At the end of the second experiment (20 days after adult emergence), the mean (±SE) Cry protein concentrations in adults were 2.21 ± 0.37 µg/g DW for Cry1C and 6.83 ± 1.37 µg/g DW for Cry2A. No Cry protein was detected in adults that were fed a pure rapeseed pollen-based diet.

**Stability and bioactivity of Cry proteins in rapeseed pollen.** According to ELISA measurements, the original concentrations of Cry1C and Cry2A in the rapeseed pollen diet were 188.80 ± 8.55 µg/g and 337.98 ± 16.5 µg/g DW, respectively. After the 2-day feeding exposure in the environmental chamber, the Cry protein concentrations in the pollen were not significantly different from those in freshly prepared pollen diet (Cry1C: 132.2 ± 20.5 µg/g, $t = 2.54, df = 2, P = 0.13$; Cry2A: 318.9 ± 8.6 µg/g, $t = 1.02, df = 4, P = 0.36$).

The sensitive-insect bioassay showed that the mean weight of *C. suppressalis* larvae was significantly smaller when the artificial diet had been treated with Cry protein (all $P < 0.05$) (Fig. 4). No statistical difference was detected between the weights of *C. suppressalis* larvae when fed a Cry protein-treated artificial diet that had been freshly prepared or that had been exposed to *P. japonica* for 2 days (Fig. 4).

**Discussion**

Laboratory studies are useful for determining whether *Bt*-transgenic plants pose a risk to non-target species2-8. Such studies allow experimenters to expose test organisms to Cry proteins under “worst-case exposure” conditions, i.e., at much higher levels and/or for significantly longer durations than occur in the field. Consequently, conclusions of “no adverse effects” can be made with high certainty3-9.

The results of our first experiment indicated that consumption of Cry1C- or Cry2A-containing transgenic rice pollen by *P. japonica* did not significantly reduce survival, pupation rate, eclosion rate, adult weight, or fecundity. There was a tendency, however, for all of these life table parameters to be lower with Cry1C- or Cry2A-containing transgenic rice pollen than with control pollen. Moreover, the developmental time (days from neonate to pupa) was significantly prolonged when *P. japonica* larvae were fed either Cry1C- or Cry2A-containing rice pollen rather than control pollen. While these effects

**Table 2 | Impact of purified Cry1C, Cry2A, and E-64 provided in a rapeseed pollen-based diet on life table parameters of *Propylea japonica*. Number of replicates is indicated in parentheses.**

| Treatment                  | Pupation rate (%) | Eclosion rate (%) | Days to pupa (d ± SE) | Adult fresh weight (mg ± SE) | Total fecundity per pair (eggs ± SE) |
|----------------------------|-------------------|-------------------|-----------------------|-----------------------------|-------------------------------------|
|                            | Female            | Male              |                       | Female                      | Male                                |
| Control: pure diet          | 84.3 (70)         | 81.4 (70)         | 8.57 ± 0.12 (59)      | 7.10 ± 0.25 [21]            | 103.9 ± 9.87 [19]                   |
| Cry1C 200 µg/g diet         | 84.3 (70)         | 82.9 (70)         | 8.34 ± 0.09 (59)      | 7.42 ± 0.21 [28]            | 82.1 ± 7.11 [20]                    |
| Cry2A 500 µg/g diet         | 82.9 (70)         | 81.4 (70)         | 8.59 ± 0.20 (58)      | 7.51 ± 0.17 [35]            | 80.1 ± 8.31 [22]                    |
| E-64 400 µg/g diet          | 61.4 (70)*        | 41.4 (70)*        | 12.36 ± 0.14 (43)*    | 5.80 ± 0.15 [18]*           | 7.4 ± 2.54 [10]*                    |

Each toxin treatment was compared to the control. An asterisk denotes a significant difference between a toxin treatment and the control.

$^*$Mann-Whitney U-test with Bonferroni correction (adjusted $P = 0.017$).

$^*$Dunnett test.

$^*$t-test with Bonferroni correction (adjusted $P = 0.017$).
might have been caused by toxicity resulting from consumption of Cry1C or Cry2A proteins, they might also have been caused by other unknown nutritional differences between the Bt-transgenic and control rice pollen, as has been reported for Bt-transgenic and control maize pollen21,22.

Even though the developmental time was significantly prolonged when *P. japonica* larvae were fed pollen from *Bt* rice in experiment 1, the effect was rather small, i.e., developmental time was increased by 8–11%. This increase can be regarded as minor and is unlikely to affect the population dynamics of this species in the field considering that individuals will not exclusively develop on *Bt* rice pollen. For comparison higher tier field studies are triggered in the USA when a 50% increase in mortality is observed under laboratory conditions23; in the European Union a trigger of 20% is recommended24.

To eliminate potential differences in pollen unrelated to Cry protein content and to test the effects of even higher concentrations of Cry proteins, we conducted a second experiment in which rapeseed pollen was augmented (or not) with Cry1C or Cry2A rice pollen. For *P. japonica* larvae that directly fed *Bt* rice pollen 10–12,36. Neither study detected any adverse effects on *P. japonica* when the predator fed on *Bt*-pollen that contained Cry1AAb or on *Nilaparvata lugens* (Homoptera: Delphacidae) that had been reared on Cry1Ab-transgenic rice plants25.

The current study is the first to assess the potential effects of Cry1C- and Cry2A-rice on predatory ladybirds in the laboratory, but the effects of Cry1C- and Cry2A-rice have been reported for three other non-target insects. Larvae and adults of the green lacewing, *C. sinica*, were not affected by feeding on Cry1C or Cry2A proteins or on Cry1C- or Cry2A-containing *Bt* rice pollen16,12,56. The planthopper, *Laodelphax striatellus* (Homoptera: Delphacidae), was not adversely affected when fed an artificial diet containing Cry2A at 300 μg/g diet54. Larvae of the silkworm, *B. mori*, were fed Cry1C- or Cry2A rice pollen11. Although *B. mori* larvae were found to be sensitive to both Cry1C and Cry2A proteins, this was expected because *B. mori* is a lepidopteran, and the toxins of Cry1C and Cry2A proteins is lepidopteran specific. Nevertheless, taking natural exposure levels to *Bt* rice pollen into account, the authors concluded that consumption of *Bt* rice pollen will pose a low to negligible risk to *B. mori*.

In summary, the current study suggests that *P. japonica* is not sensitive to Cry1C and Cry2A proteins at concentrations that are more than 10-times higher than those detected in pollen from *Bt* rice. These results are consistent with those of most previous studies that have reported a lack of direct toxicity of the currently used *Bt* proteins (such as Cry1, Cry2, and Cry3) on ladybird beetles21,34,38,39. The increased developmental time for *P. japonica* larvae that directly fed on *Bt* rice pollen may be due to the altered nutritional composition of the *Bt* rice pollen caused by gene transformation or other unclear factors. We believe that this minor effect is unlikely to affect field populations of *P. japonica*, because the larvae will only deploy rice pollen as a supplementary food and thus consume much less rice pollen in the field than in our laboratory experiment. Therefore, we conclude that growing of Cry1C- and Cry2A-transgenic rice should pose a negligible risk to *P. japonica*.

**Methods**

**Ethics statement.** No specific permits were required for the described field studies.

The rice fields where the *P. japonica* used in this study were originally collected were owned by the author’s institute (Institute of Plant Protection, Chinese Academy of Agricultural Sciences, CAAS). These field studies did not involve endangered or protected species.

**Insects.** Specimens of *P. japonica* were collected in 2012 at the experimental field station of the Institute of Plant Protection, Chinese Academy of Agricultural Sciences (CAAS), near Langfang City, Hebei Province, China (39.5° N, 116.7° E). A colony was subsequently maintained in the laboratory and augmented with field-collected insects in 2013. Insects were reared in the laboratory for at least two generations before being used in the current study. Both larvae and adults of *P. japonica* were reared on soybean seedlings infested with *Aphis glycines* (Homoptera: Aphididae). The aphids were replaced daily, ensuring ad libitum food. Newly hatched (<12 h after emergence) *P. japonica* larvae were used for the experiments.
A Bt-susceptible strain of *Othio suppressalis* (Lepidoptera: Crambidae) was maintained in the laboratory on an artificial diet for over 60 generations.

**Rice pollen collection.** Two transgenic rice varieties, T1C-19 and T2A-1, and their corresponding non-transformed near isoinline, Minghui 63, were used for the experiments. T2A-1 plants express a modified cry2A gene, and T1C-19 plants express a modified cry1C gene; these genes encode for Cry proteins targeting lepidopteran rice pests. Minghui 63 is an elite indica restorer line for cytoplasmic male sterility in China. All rice seeds were kindly provided by Prof. Yongjun Lin (Huazhong Agricultural University, Wuhan).

The rice lines were simultaneously planted in three adjacent plots at the experimental field station of the Institute of Plant Protection, CAAS, near Langfang City (39°5′31′′ N, 116°5′31′′ E). Each plot was approximately 0.02 ha, and plots were separated by a minimum of 1 m. Tassels were sown in a seeding box on 8 April 2013, and the seedlings were transplanted into the experimental plots on 1 June, when the seedlings were at the four-leaf stage. The plants were cultivated according to the common local agricultural practices but without pesticide sprays.

During rice anthesis from 30 August to 15 September 2012, rice pollen was collected by shaking the rice tassels in a plastic bag. The collected pollen was air-dried at 5% relative humidity, and a 16:8 h light:dark cycle.

**Insecticidal compounds and bee-collected pollen.** Insecticidal compounds used in this study included the protease inhibitor E-64 [N-[N-(3-carboxyphenylacryl)-2-carbonyl]-L-leucyl]-g-aminomethyl) and the Bt proteins Cry1C and Cry2A. E-64 was purchased from Sigma-Aldrich (St. Louis, MO, USA), and the Cry proteins were purchased from Envirotast-China (an agent for Envirologix Inc., Portland, Maine, USA; www.envirotest-china.com). The proteins were produced and purified at Case Western Reserve University (USA), where the protoxins from Bacillus thuringiensis had been expressed as single-gene products in *Escherichia coli*. The E. coli-derived protoxin inclusion bodies then were dissolved and trypsinated. Subsequently they were isolated and purified by ion exchange HPLC followed by the desalting and lyophilization of the pure fractions. Purity was about 94–96%.

**Bioactivity of the batches of Bt proteins.** A pollen-based diet was previously developed for *P. japonica* in our laboratory using neonate larvae of *C. suppressalis* that were fed for 7 days with an artificial diet containing a series of Bt protein concentrations. The CC50 (concentration resulting in 50% weight reduction compared to the control) was estimated to be 18.1 for Cry1C and 1310.3 ng/ml for Cry2A.

Bee-collected rapeseed pollen used in the experiments was purchased from China-Bee Science & Technology Development Co., Ltd. (Beijing, China). The pollen granules were ground before being fed to *P. japonica*.

**Experiments.**

**Environmental conditions.** All experiments were conducted in an environmental chamber at 26 ± 1°C, 75 ± 5% relative humidity, and a 16:8 h light:dark cycle.

**Feeding system for *P. japonica*.** A pollen-based diet was previously developed for *P. japonica* in our laboratory. This diet, which uses bee-collected rapeseed pollen, supports normal survival and development of *P. japonica* (see Zhang et al., 2014) for a detailed description. The *P. japonica* larvae were individually confined in Petri dishes (6.9 cm diameter, 1.5 cm height). They were fed with pollen on the first day of each instar and then were provided with a mixture of pollen and soybean aphids (natural food) until development into the next instar. For adults, single pairs of *P. japonica* were confined in the same Petri dish and fed with pollen or with a combination of pollen and soybean aphids every other day. The pollen was directly sprinkled on the bottom of the Petri dish, while the aphids were provided on 2-cm segments of heavily infested soybean seedlings. Pollen was replaced every 2 days, while soybean aphids were replaced daily. In addition, an open 1.5-ml centrifuge tube containing solidified 1% agar solution was added to each Petri dish as a water source. All of the aphids were replaced daily. In addition, an open 1.5-ml centrifuge tube containing solidified 1% agar solution was added to each Petri dish as a water source. All of the aphids were replaced daily.

**Purified Cry protein experiment.** The test system used for the second experiment was the same as described by Zhang et al. (2014). Newely hatchated larvae of *P. japonica* were individually assigned to one of four dietary treatments: (i) rapeseed pollen containing Cry1C protein at 200 μg/g dry weight (DW) of pollen; (ii) rapeseed pollen containing Cry2A protein at 500 μg/g DW of pollen; (iii) rapeseed pollen containing E-64 protein at 400 μg/g DW of pollen (positive control); and (iv) rapeseed pollen (negative control). The Cry1C and Cry2A protein concentrations used here were therefore 89 and 13-times higher than the concentrations in the respective Bt rice pollen (see Results). E-64 was selected as a positive control because it has been shown to be readily accepted and toxic to *P. japonica* at 400 μg/g DW; it a gut-active compound like the Cry proteins tested here; and it is stable for the test duration.

**ELISA measurements.** For both experiments, the concentrations of Cry1C and Cry2A in pollen and insects were measured by ELISA using *Cry1C* and *Cry2A*-sensitive *C. suppressalis* larvae as described below.

To determine the stability and bioactivity of the Bt proteins in the rapeseed pollen-based diet during experiment 2, three subsamples were taken from the pollen-based diet immediately after it was prepared and after it had been exposed to *P. japonica* for 2 days. The Cry protein concentrations in the diet samples were determined by ELISA, and bioactivity was determined by bioassay with *Cry1C* and *Cry2A*-sensitive *C. suppressalis* larvae as described below.

**Sensitive-insect bioassay.** Larvae from a *Bt*-susceptible *C. suppressalis* strain were used to determine the bioactivity of the *Cry1C* and *Cry2A* proteins in *Bt* rice pollen and the rapeseed pollen-based diet before and after exposure to the Bt protein. Supernatants from the extracts used for the ELISA analysis were appropriately diluted and thoroughly incorporated into the artificial diet for *C. suppressalis* larvae, resulting in a final concentration of Cry1C and Cry2A of 100 and 3000 μg/g FW of diet, respectively. Extracts of non-*Bt* rice pollen and control rapeseed pollen-based diet served as controls. The artificial diets were cut into slices and individually placed in Petri dishes (9 cm diameter, 1 cm height) together with a neonate larva of *C. suppressalis*. Subsequently, the Petri dishes were sealed with Parafilm and reinforced with surgical tape. After 7 days, the *C. suppressalis* were weighed. Thirty replicates were tested for each treatment.

**Data analyses.** Pair-wise statistical comparisons were made between *Bt* rice pollen and control rice pollen in the first experiment and between the Cry protein or *E*-64 treatments and the control in the second experiment. The effects of the dietary treatments on *P. japonica* were compared using the Kaplan–Meier procedure and pair-wise log-rank chi-square tests or log-rank tests. *T* tests were used to compare pupation and eclosion rates, and Mann–Whitney *U*-tests were used to compare the larval developmental times because such data did not satisfy the assumptions for parametric analyses (normal distribution of residuals and homogeneity of error variances). Bonferroni corrections were
applied for and three pair-wise comparisons, resulting in an adjusted α of 0.025 and 0.017, respectively. Data on adult weight and total fecundity were compared by Dunnett tests in both experiments.

In addition, Student’s t-tests were conducted to compare Cry protein concentrations in fresh Bt rice pollen and in pollen that had been exposed to P. japonica larvae for 2 days. One-way ANOVA followed by Tukey HSD test was carried out to compare Cry protein concentrations in reared pollen-based diet immediately after it was prepared and after it had been exposed to P. japonica larvae for 2 days, and for comparisons of the 7-day larval weight of C. suppressalis that were fed with artificial diets containing the extracts from the different pollen treatments. All statistical analyses were conducted using the software package SPSS (version 13; SPSS, Inc., Chicago, IL).

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