Bt rice does not disrupt the host-searching behavior of the parasitoid Cotesia chilonis

Qingsong Liu1, Jörg Romeis1,2, Huilin Yu1, Yongjun Zhang1, Yunhe Li1 & Yufa Peng1

We determined whether plant volatiles help explain why Cotesia chilonis (a parasitoid of the target pest Chilo suppressalis) is less abundant in Bt than in non-Bt rice fields. Olfactometer studies revealed that C. chilonis females responded similarly to undamaged Bt and non-Bt rice plants. Parasitoids preferred rice plants damaged by 3rd-instar larvae of C. suppressalis, but did not differentiate between caterpillar-infested Bt and non-Bt plants. According to GC-MS analyses of rice plant volatiles, undamaged Bt and non-Bt rice plants emitted the same number of volatile compounds and there were no significant differences in the quantity of each volatile compound between the treatments. When plants were infested with and damaged by C. suppressalis larvae, both Bt and non-Bt rice plants emitted higher numbers and larger amounts of volatile compounds than undamaged plants, but there were no significant differences between Bt and non-Bt plants. These results demonstrate that the volatile-mediated interactions of rice plants with the parasitoid C. chilonis were not disrupted by the genetic engineering of the plants. We infer that parasitoid numbers are lower in Bt than in non-Bt fields because damage and volatile induction by C. suppressalis larvae are greatly reduced in Bt fields.

Genetically engineered (GE) crops have been widely adopted, with 181 million hectares of GE crops grown globally in 20141. Nearly half of these crops were modified by inserting cry genes from Bacillus thuringiensis (Bt). These genes encode insecticidal Cry proteins that can suppress lepidopteran and coleopteran pests. Production of insect-resistant GE (IRGE) plants has increased farmer incomes and decreased the use of broad-spectrum chemical insecticides that endanger human and ecosystem health1–4.

Despite these benefits, the potential environmental risks associated with the planting of IRGE crops have received substantial attention, with a particular focus on the potential effects on valued nontarget organisms. Among nontarget organisms, natural enemies of pest arthropods are of particular interest because they help regulate herbivore populations and thus contribute to sustainable agro-ecosystems5. Many field studies have assessed the potential effects of Bt crops on the abundance of nontarget arthropods, and meta-analyses indicate that nontarget species are generally more abundant in Bt cotton and Bt maize fields than in non-transgenic fields managed with insecticides6–9. When Bt fields are compared with insecticide-free control fields, however, nontarget taxa – such as specialist parasitoids of target pests – are less abundant in Bt fields6–9. The reduced abundance of parasitoid wasp species of the target pests in Bt crop fields is associated with the lack of their hosts and does not appear to be caused by toxicity of the Bt Cry proteins6–9.

In searching for their hosts, parasitoids often rely on the chemical communication between the parasitoids, their hosts, and/or the host plant. More specifically, parasitoids can use herbivore-induced plant volatiles (HIPVs) to locate their hosts10–13. The lower abundance of parasitoid species in Bt than in non-Bt crops might result from differences in plant volatiles as a consequence of two potential mechanisms: i) the insertion of foreign insecticidal genes may change the production of plant volatiles due to pleiotropic effects, epistasis, or insertional mutagenesis; and ii) compared to Bt plants, non-Bt plants

1State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China. 2Agroscope, Institute for Sustainability Sciences ISS, Zurich, Switzerland.

Received: 25 March 2015
Accepted: 22 September 2015
Published: 15 October 2015

Correspondence and requests for materials should be addressed to Y.L. (email: yunhe.li@hotmail.com)

DOI: 10.1038/srep15295
www.nature.com/scientificreports
will be more heavily damaged by target pests and thus release greater amounts of plant volatiles or some new volatiles, making them more attractive to the parasitoid wasps. A few studies have investigated the potential effects of Bt plants on parasitoid behavior as mediated by plant volatiles. Most of these studies have reported that undamaged Bt plants and the corresponding undamaged non-Bt control plants emitted similar types and quantities of volatiles and were similarly attractive to parasitoid species; when infested with and damaged by caterpillars, however, the non-Bt plants receive more damage than the Bt plants and consequently release qualitatively and quantitatively different volatile profiles, resulting in higher attractiveness to parasitoid wasps\textsuperscript{14–19}. Although Yan et al.\textsuperscript{20} reported that the expression of a foreign gene in Bt cotton plants affected their volatile profiles, the authors did not determine whether this difference affected insect behavior.

For controlling lepidopteran rice pests, such as the striped stem borer, *Chilo suppressalis* (Lepidoptera: Crambidae), the yellow stem borer, *Scirpophaga incertulas* (Lepidoptera: Pyralidae), and the rice leaf roller, *Cnaphalocrocis medinalis* (Lepidoptera: Pyralidae), various IRGE rice cultivars have been developed in China; these IRGE rice cultivars express the Bt genes *cry1Ab*, *cry1Ac*, *cry1Aa*, *cry1Ab/Ac*, or *cry1C*\textsuperscript{21,22}. The IRGE rice lines exhibit high levels of resistance against the target pests, and laboratory and glasshouse studies have verified that the Cry proteins produced by Bt rice plants have no negative effects on nontarget species outside the order of Lepidoptera\textsuperscript{22–28}. Field studies confirmed that the planting of Bt rice did not significantly affect populations of non-target arthropods, with the exception of parasitoid wasps that attack the target pests\textsuperscript{29–31}. No data have been reported regarding the potential effect of Bt rice plants on the host-searching behavior of parasitoid wasps.

The transgenic rice line T1C-19 expresses a synthesized *cry1C* gene and has a high level of resistance to lepidopteran insect pests including stem borers and leaf rollers\textsuperscript{3,32}. T1C-19 thus has the potential to be commercialized in China, and was used in the current study to investigate the effects of Bt genetic engineering on the host-searching behavior of parasitoid wasps. Our study system consisted of the Bt rice line T1C-19, its non-transformed parental rice line, the target pest *C. suppressalis*, and its parasitoid *Cotesia chilonis* (Hymenoptera: Braconidae). We performed a series of olfactory experiments to evaluate the attractiveness of volatiles from undamaged or caterpillar-damaged plants of both rice lines to the parasitoid. In addition, the volatiles from undamaged and damaged plants were characterized in order to determine whether there are qualitative or quantitative differences in the volatile bouquet produced by the Bt and non-Bt plants.

**Results**

**Response of *C. chilonis* to rice plants damaged by 3\textsuperscript{rd}-instar *C. suppressalis* larvae.** The mean body weight increase of 3\textsuperscript{rd}-instar *C. suppressalis* larvae was 0.91 ± 0.14 mg when fed non-Bt rice stems, and was 0.75 ± 0.12 mg when fed Bt rice stems for 24h. However, the difference detected between the two treatments was not significant (*t* = −0.851, df = 64, P = 0.398).

Females of the parasitoid *C. chilonis* were more strongly attracted to the odors of undamaged (UD) Bt or non-Bt rice plants than to clean air (non-Bt UD vs. Air, *χ*² = 10.60, *P* = 0.001; Bt UD vs. Air, *χ*² = 7.2, *P* = 0.007) (Fig. 1). The attraction of the parasitoid to undamaged Bt vs. undamaged non-Bt rice plants did not differ significantly (Bt UD vs. non-Bt UD, *χ*² = 0.007, *P* = 0.933) (Fig. 1).

*C. chilonis* females were more attracted to rice plants damaged by 3\textsuperscript{rd}-instar *C. suppressalis* larvae (HD) than to undamaged plants regardless of plant genetics (either Bt-transgenic or non-transformed).

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**Figure 1. Responses of *Cotesia chilonis* to pairs of odor sources in dual-choice tests using a Y-tube olfactometer.** Odor sources: Bt UD: undamaged Bt rice plants, non-Bt UD: undamaged non-Bt rice plants, Air: clean air. Asterisks indicate a significant difference within a choice test: **P < 0.01; n.s. indicates a non-significant difference (*P > 0.05*) (χ² test). The bars indicate the percentages of parasitoids that selected either odor source. Gray shading indicates herbivore-undamaged plants. The numbers indicate the number of parasitoids that made a choice, and the numbers in parentheses indicate the total number of parasitoids tested.
Response of *C. chilonis* to rice plants damaged by 1st- or 3rd-instar *C. suppressalis* larvae. *C. chilonis* females did not exhibit a significantly different preference for Bt or non-Bt rice plants that were damaged by 30 1st-instar *C. suppressalis* larvae (1st non-Bt HD vs. 1st Bt HD; χ² = 0.057, P = 0.811). In contrast, non-Bt plants infested with 3rd-instar larvae were significantly more attractive to *C. chilonis* females than were Bt rice plants infested with 1st-instar *C. suppressalis* larvae (3rd non-Bt HD vs. 1st Bt HD; χ² = 7.451, P = 0.006) (Fig. 3).

Volatile analyses. There were great differences in the composition and quantities of volatiles released by the herbivore-damaged and undamaged Bt and non-Bt rice plants (one-way ANOVA: F = 21.88, d.f. = 3, P < 0.001 for volatile quantity) (Table 1 and Fig. 4). Pair-wise comparisons showed that undamaged Bt and undamaged non-Bt rice plants emitted the same numbers and similar amounts of volatiles (P = 0.374) (Table 1). When damaged by caterpillars, both Bt and non-Bt rice plants released significantly higher amounts of volatiles and several new compounds (unknown 1, unknown 2, unknown 4,
2-nonanone, methyl salicylate, α-bergamotene, germacrene D, 2-tridecanone, and α-cedrene. The total amounts of volatiles released by herbivore-damaged rice plants were 4.6- and 4.8-times higher for Bt rice plants (Bt UD, HD vs. non-Bt UD, HD) than for undamaged rice plants (all P < 0.001) (Table 1). The total amount of volatiles emitted did not significantly differ between damaged Bt plants and damaged non-Bt plants (P = 0.954); the mean quantities of individual volatiles also did not significantly differ between damaged Bt plants and damaged non-Bt plants (all P > 0.05) (Table 1).

Discussion
The results from our olfactometer tests showed that female C. chilomis parasitoids, when given a choice between clear air and odor emitted by undamaged rice plants, strongly responded to the undamaged rice plants whether they were Bt or non-Bt. This finding is consistent with our GC-MS analyses: undamaged Bt and non-Bt rice plants released similar types of volatile compounds at similar concentrations. The bouquet that we identified is comparable to that reported in previous studies for other conventional rice lines.33-35. In contrast, Y an et al. reported that the egg parasitoid Trichogramma pretiosum (Hymenoptera: Trichogrammatidae) showed an equal preference for undamaged Bt and non-Bt cotton plants. Similarly, the larval parasitoids Microplitis mediator (Hymenoptera: Braconidae)29 were equally attracted to undamaged Bt and non-Bt maize. In addition, the adult stages of some phytophagous insects, which also use plant volatiles to locate their host plants, did not show any preference for Bt or non-Bt plant lines34,37-46. In contrast, Yan et al.20 reported that higher concentrations of α-pinene, β-pinene, and an unknown minor compound were emitted by Bt cotton

| Peak no. | Plant volatile     | Retention time (min) | Mean quantities of volatiles* |
|---------|-------------------|----------------------|------------------------------|
|         |                   |                      | Bt UD | Non-Bt UD | Bt HD | Non-Bt HD |
| 1       | 2-heptanone       | 5.14                 | 28 ± 6 a | 11 ± 4 a | 331 ± 117 b | 163 ± 43 b |
| 2       | 2-heptanol        | 5.34                 | 14 ± 4('a) | 10 ± 3 a | 232 ± 57 b | 107 ± 23 b |
| 3       | unknown 1         | 5.84                 | – | – | 8 ± 1 a | 6 ± 1 a |
| 4       | α-pinene          | 5.98                 | 8 ± 2 a | 10 ± 3 a | 9 ± 1 a | 16 ± 4 a |
| 5       | unknown 2         | 8.23                 | – | – | 6 ± 1 a | 5 ± 1 a |
| 6       | D-limonene        | 8.31                 | 33 ± 4 a | 26 ± 4 a | 238 ± 30 b | 204 ± 40 b |
| 7       | unknown 3         | 9.09                 | 4 ± 0 a | 4 ± 0 a | 8 ± 1 a | 6 ± 1 a |
| 8       | unknown 4         | 9.38                 | – | – | 5 ± 1 a | 6 ± 1 a |
| 9       | 2-nonanone        | 9.97                 | – | – | 89 ± 23 a | 40 ± 5 a |
| 10      | linalool          | 10.21                | 163 ± 38 a | 104 ± 22 a | 180 ± 52 a | 144 ± 36 a |
| 11      | methyl salicylate | 12.41                | – | – | 51 ± 38 a | 36 ± 18 a |
| 12      | copaene           | 16.44                | 4 ± 1 a | 5 ± 1 a | 18 ± 2 b | 23 ± 5 b |
| 13      | β-caryophyllene   | 17.41                | 6 ± 1 a | 8 ± 2 a | 11 ± 3 a | 13 ± 3 a |
| 14      | α-bergamotene     | 17.74                | – | – | 5 ± 2 a | 5 ± 1 a |
| 15      | germacrene D      | 18.67                | – | – | 9 ± 2 a | 12 ± 2 a |
| 16      | 2-tridecanone     | 19.07                | – | – | 64 ± 21 a | 25 ± 7 a |
| 17      | β-selinene        | 19.54                | 4 ± 1 a | 6 ± 2 a | 27 ± 5 b | 31 ± 8 b |
| 18      | α-cedrene         | 19.70                | – | – | 16 ± 8 a | 26 ± 6 a |
|         | Total             |                      | 251 ± 44 a | 167 ± 29 a | 1145 ± 256 b | 805 ± 93 b |

Table 1. Volatiles collected from the headspace of undamaged Bt rice plants (Bt UD), undamaged non-Bt rice plants (non-Bt UD), herbivore C. suppressalis-damaged Bt rice plants (Bt HD), and herbivore-damaged non-Bt plants (non-Bt HD). “−” indicates that the concentration of the volatile was below the detection level. *Values are mean normalized quantities of volatiles (%).
than by non-Bt cotton. However, Yan et al.\textsuperscript{20} did not determine whether this difference was caused by the insertion of the cry gene or whether this difference affected the searching behavior of phytophagous insects or parasitoid wasps.

It is well known that plant volatiles induced by herbivore damage can serve as host-searching cues for natural enemies\textsuperscript{8,10,11,13,17,19}. More specifically, a previous study confirmed that \textit{C. chilonis} females strongly preferred rice plants damaged by \textit{C. suppressalis} larvae to undamaged rice plants\textsuperscript{47}. Our results also demonstrate that \textit{C. chilonis} females were more attracted to rice plants damaged by 3\textsuperscript{rd}-instar \textit{C. suppressalis} larvae than to healthy rice plants. The GC-MS analyses confirmed that damaged Bt and non-Bt rice plants emitted significantly larger amounts of volatiles than healthy rice plants. In addition, the damaged plants released several new chemicals, including 2-nonanone, methyl salicylate, \(\alpha\)-bergamotene, germacrene D, 2-tridecanone, \(\alpha\)-cedrene, and two unknown compounds.

Because \textit{C. suppressalis} is a target pest of Bt rice, and because its larvae are expected to cause less damage to Bt plants than to non-Bt plants, it is reasonable to hypothesize differences in the volatile bouquet and in parasitoid behavior between caterpillar-infested Bt and non-Bt rice plants. These differences, however, were not evident in the current study. This may be explained by the fact that both Bt and non-Bt rice plants can be infested with 3\textsuperscript{rd}-instar \textit{C. suppressalis} and that such larvae cause comparable damage to Bt and non-Bt rice plants during a 24-hour duration of feeding. This interpretation was confirmed by our observation that the mean weight increase of 3\textsuperscript{rd}-instar \textit{C. suppressalis} larvae fed non-Bt rice plants was only a little higher than those fed Bt rice plants, a non-significant difference. These results suggest that the planting of Bt rice plants will not affect the host-searching behavior of the larval parasitoid \textit{C. chilonis}. Nevertheless, higher densities of parasitoids (including \textit{C. chilonis}) have been recorded in non-Bt rice fields\textsuperscript{29,48,49}. This could be explained by the fact that Bt rice plants are much less damaged in the field than in our study\textsuperscript{24}. In contrast to our experiments, where three rice plants were infested with six 3\textsuperscript{rd}-instar \textit{C. suppressalis} plants in the field would be infested by neonates that would be effectively killed by the \textit{Bt} toxin before they cause significant damage. Consequently, parasitoids in the field are likely to be more attracted to or arrested by non-Bt rice plants than by Bt-rice plants, because the non-Bt plants would support much higher densities of lepidopteran larvae as hosts and will probably also release higher amounts of volatiles in response to herbivore damage. Our olfactometer tests with 1\textsuperscript{st}- and 3\textsuperscript{rd}-instar larvae of \textit{C. suppressalis} could better reflect the field condition. The results showed that the parasitoids did not distinguish between Bt and non-Bt rice plants that were damaged by 1\textsuperscript{st}-instar \textit{C. suppressalis}, although it was expected that the neonates might cause higher damage on non-Bt rice plants.

Figure 4. Chromatographic profiles of volatiles obtained from headspace collections from rice plants. Undamaged Bt rice plants (Bt UD), undamaged non-Bt rice plants (non-Bt UD), herbivore \textit{C. suppressalis}-damaged Bt rice plants (Bt HD), and herbivore-damaged non-Bt line (non-Bt HD). Peaks: 1, 2-heptanone; 2, 2-heptanol; 3, unknown 1; 4, \(\alpha\)-piene; 5, unknown 2; 6, D-limonene; 7, unknown 3; 8, unknown 4; 9, 2-nonanone; 10, linalool; 11, methyl salicylate; 12, copaene; 13, \(\beta\)-caryophyllene; 14, \(\alpha\)-bergamotene; 15, germacrene D; 16, 2-tridecanone; 17, \(\delta\)-selinene; 18, \(\alpha\)-cedrene. The added reference compound was 1-tridinene (IS).
compared to Bt plants. These results may suggest that the relatively little damage caused by neonates of C. suppressalis within 24h on either Bt or non-Bt plants is not enough to affect the host-searching behavior of the parasitoid C. chilonis. However, female C. chilonis were significantly more attracted to the odors emitted by non-Bt rice plants damaged by 3rd-instar C. suppressalis than to Bt rice plants damaged by 1st-instar C. suppressalis, a scenario that is likely to occur under field conditions. These results further explained the ecological phenomenon that higher densities of C. chilonis are observed in non-Bt rice fields than in Bt rice fields.

Our results agree with those of earlier studies with Bt cotton. Moraes et al.\textsuperscript{15} reported that 2nd-instar Spodoptera frugiperda (Lepidoptera: Noctuidae) larvae caused damage to both Bt and non-Bt cotton, and that damaged plants emitted a higher quantity of volatiles than undamaged plants. Although the parasitoid T. pretiosum was significantly attracted to damaged cotton plants, it did not distinguish between Bt and non-Bt plants. Several studies have documented that the greater damage caused by the target pests on non-Bt plants than on Bt plants caused a larger induction of volatiles or plant defense compounds in the non-Bt plants; this difference has been reported for cotton\textsuperscript{20,21}, maize\textsuperscript{16}, and oilseed rape\textsuperscript{14,18,19,52}. It was also reported that herbivore-damaged non-Bt oilseed rape plants were more attractive to the parasitoids Cotesia plutellae (Hymenoptera: Braconidae)\textsuperscript{18,19} and C. vestalis (Hymenoptera: Braconidae)\textsuperscript{52} than were Bt-transgenic oilseed rape plants. As expected, when larvae of a Bt-resistant Plutella xylostella (Lepidoptera: Plutellidae) strain were used in such studies, they caused equal feeding damage on Bt and non-Bt oilseed rape, and the parasitoid of P. xylostella was equally attracted to damaged Bt and non-Bt oilseed rape plants\textsuperscript{18,19}. In some studies, although herbivore-damaged non-Bt plants released significantly higher amounts of volatiles than the less-damaged Bt plants, parasitoid wasps did not differentiate between Bt and non-Bt plants\textsuperscript{16}. This phenomenon was explained by the fact that the ratio of different functional chemical compounds emitted by Bt and non-Bt plants was not altered.

Our study is the first to elaborate the mechanism by which the populations of parasitoid wasps of the target pest are affected by volatiles released from Bt and non-Bt rice. The results suggest that the volatile-mediated interactions between rice plants and a parasitoid of a lepidopteran rice pest are not disrupted by the genetic engineering of the rice plant.

Materials and Methods

Plants. The transgenic Bt rice line, T1C-19, and its corresponding non-transformed near-isoline, Minghu63 (MH63), were used in this study. T1C-19 plants express a synthesized cry1C\textsuperscript{*} gene driven by the maize ubiquitin promoter; the gene encodes the Cry1C protein that targets lepidopteran rice pests\textsuperscript{32}. MH63 is an elite indica restorer line for cytoplasmic male sterility in China. The rice seeds were provided by Prof. Yongjun Lin (Huazhong Agricultural University, Wuhan, China).

Pre-germinated seeds of both lines were sown simultaneously in a greenhouse, and the seedlings were transplanted after three weeks into individual plastic pots (630 cm\textsuperscript{3}) containing a mixture of peat and vermiculite (3:1). Plants were watered daily and supplied with nitrogenous fertilizer every 3 d. All plants were maintained in a glasshouse at 27 ± 3°C with 65 ± 10% RH and a 16 L : 8 D photoperiod. Four weeks after transplanting, the plants were at the tillering stage and were used for the experiments. A previous study had revealed that at this growth stage, the target pest C. suppressalis is almost 100% controlled, i.e., Bt rice plants at this growth stage experience no or extremely low damage from C. suppressalis feeding\textsuperscript{24}.

Insects. The C. suppressalis specimens used in this study were obtained from a laboratory colony that had been reared on an artificial diet\textsuperscript{23}. The C. chilonis wasps used in this study were provided by Prof. Gongyin Ye (Zhejiang University, Hangzhou, China) and were obtained from a laboratory colony that had been reared on the larvae of C. suppressalis. The parasitoid colony has been kept in the laboratory for nearly 20 generations, while field-collected parasitoids were introduced yearly to the colony to intro-
duce fresh genetic material. Each C. suppressalis larva (4th–5th instar) was exposed to a mated female wasp in a glass tube (2.4 cm diameter, 7 cm height) for 24h to ensure that the larva had been parasitized. Parasitized larvae then were provided with sufficient artificial diet until they yielded parasitoid cocoons or pupated. The cocoons were collected and transferred to clean Petri dishes (9 cm diameter) until adult emergence. If not used immediately, the cocoons were stored at 15 ± 1°C and 70 ± 5% RH for up to 2 weeks. Newly emerged adult wasps were supplied with a honey : water solution (20%, v-v) as a food source and were allowed to mate freely for >24h. Mated female wasps (2 to 3 days old) were used in the bioassays. Female wasps were naïve, i.e., they had not previously contacted rice plants or hosts. Both C. suppressalis and C. chilonis were kept in environmental chambers (RXZ, 380 L, Ningbo Jiangnan Instrument Factory, Zhejiang, China) at 27 ± 1°C with 70 ± 5% RH and a 16 L:8 D photoperiod.

Chemicals. Super Q (80–100 mesh) was purchased from Alltech (Deerfield, IL, USA). Methylene chloride (≥99.8% purity) and 2-nonanone (99% purity), were purchased from Aladdin Reagent Co., Ltd. (Shanghai, China). Linalool (97% purity), 2-heptanol (98% purity), 2-heptanone (99% purity), methyl salicylate (≥99% purity), β-caryophyllene (≥98.5% purity), α-pinene (≥99% purity), D-limonene (≥99% purity), and 1-tridecene (96% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA).
Response of *C. chilonis* to rice plants damaged by 3rd-instar *C. suppressalis* larvae. Plant treatments. The potted rice plants were washed with running water to remove soil and silt. Three plants were placed in 50-ml conical flasks filled with distilled water, and the plants and flasks were wrapped with aluminum foil. Two plant treatments were compared for both *Bt* and non-*Bt* rice plants: (i) undamaged plants (UD), and (ii) herbivore-damaged plants (HD). The HD plants had been infested with six early 3rd-instar *C. suppressalis* larvae for 24 h. The larvae had previously been starved for 2 h. Visual observations indicated that 3rd-instar larvae of *C. suppressalis* could cause similar damage on *Bt* and non-*Bt* rice plants within 24 h. Subsequently, undamaged or herbivore-damaged plants were enclosed in a frosted glass bottle (3142 cm³); a port at the bottom was used for the inlet of air, and a port at the top was used for the outlet of air. Plants in these bottles were used for the olfactometer bioassays and for the collection of volatiles as described in the following sections. During the olfactometer bioassay and the collection of volatiles, *C. suppressalis* larvae were left on plants. All experiments were conducted at 27 ± 2°C and 40% RH. The lighting level in the frosted glass bottle was 3000 ± 150 Lux.

**Damage on rice plants by 3rd-instar larvae of *C. suppressalis***. To quantitatively measure the degree of damage to *Bt* and non-*Bt* rice plants by 3rd-instar larvae of *C. suppressalis*, a bioassay was conducted in which *Bt* and non-*Bt* rice stems were provided to the larvae for 24 h. A thin, moist layer of absorbent cotton was laid on the bottom of a Petri dish (6 cm diameter) and a moistened filter paper (5.5 cm diameter) was placed on the cotton layer. Subsequently two 4–5-cm segments that were cut from the middle part of the main rice plant stems were placed onto the filter paper. One end of the stem segment was covered with saturated cotton wool to keep the rice stems fresh. After being weighed on an electronic balance (Mettler Toledo ME204, Zurich, Switzerland; 220 g full scale, d = 0.0001 g), the 3rd-instar larvae of *C. suppressalis* were individually transferred to each Petri dish. The Petri dish then was sealed with Parafilm. A total of 29 and 37 larvae were tested for *Bt* and non-*Bt* rice, respectively. The insects were kept in an environmental chamber (RXZ, 380 L, Ningbo Jiangnan Instrument Factory, Zhejiang, China) under a 16 L:8 D photoperiod at 27 ± 1°C, 70 ± 5% RH. After 24 h feeding, *C. suppressalis* larvae were weighed again, and the weight increase of each insect was calculated.

**Olfactometer bioassays**. The rice plants enclosed in glass bottles as described in the previous section were used as odor sources. Dual-choice (Y-tube) olfactometers (16-cm stem; 16-cm arms at 75° angle; 2 cm internal diameter) were used to investigate the behavioral responses of naïve *C. chilonis* females to one odor source vs. a blank (clean air) or pairs of different odor sources. The experiments were conducted in a chamber (110 × 80 × 70 cm) formed with iron sheets and equipped with four 16-W cool white fluorescent lights at the top, and the light was filtered by an acrylic plate to ensure an even distribution of light (1650 ± 10 Lux). In addition, a light meter (Shenzhen Huachang Technology Co., Ltd., China) was used to monitor the light distribution. An airstream was generated by a membrane pump (Beijing Institute of Labour Instruments, Beijing, China). Air was filtered through activated charcoal, humidified by passage through a jar containing distilled water, and passed through the odor source before entering the olfactometer. The air-flow through the two olfactometer arms was monitored by two separate airflow meters (Changzhou Kede Thermo-technical Instrument Co., Ltd., Changzhou, China) at 250 ml/min. All parts of the system were connected by 0.6-cm-diameter Teflon tubes.

Individual *C. chilonis* females were introduced into the Y-tube at the base of the stem, and the position of the wasp was observed for 5 min. A ‘first choice’ was recorded when the wasps moved over a threshold line at the middle of either arm for at least 5 s. Wasps that had not made a choice during the observation time were recorded as ‘no choice’, and their data were not included in the statistical analyses. Each wasp was tested only once. A total of 80–160 females were tested for each treatment. All tests were conducted between 9:00 and 18:00 h in a climate-controlled laboratory room (27 ± 2°C, 40% RH). The position of the arms of the Y-tube were reversed after two tests (replicates) to avoid position bias, and the Y-tube was replaced with a clean one after it was used to test four wasps. To compensate for any unforeseen asymmetry in the bioassays, the odour sources were swapped after 10 individuals were tested and were replaced by a new set of plants after they were used to test 20 wasps. All glassware and connected Teflon tubes were washed with detergent (5%, v/v) followed by alcohol (90%, v/v) and then were rinsed with distilled water. The female parasitoids were exposed to the following eight pairs of odor sources: (i) *Bt* UD vs. clean air, (ii) non-*Bt* UD vs. clean air, (iii) *Bt* UD vs. non-*Bt* UD, (iv) *Bt* HD vs. *Bt* UD, (v) *Bt* HD vs. non-*Bt* UD, (vi) non-*Bt* HD vs. non-*Bt* UD, (vii) non-*Bt* HD vs. *Bt* UD, and (viii) *Bt* HD vs. non-*Bt* HD. Comparisons (i) and (ii) were conducted to test whether the volatiles released by undamaged *Bt* or non-*Bt* rice plants were attractive to the parasitoid wasps, while comparison (iii) tested whether the wasps could distinguish between undamaged *Bt* and non-*Bt* rice plants, i.e., whether the *Bt* transformation had caused a change in the volatile emission of the rice plants. Comparisons (iv), (v), (vi), and (vii) tested whether the damage to rice plants caused by 3rd-instar *C. suppressalis* larvae would affect the volatile emission profile of either non-*Bt* or *Bt* rice plants and affect the host-searching behavior of the parasitoid *C. chilonis*. Comparison (viii) tested whether the parasitoid wasps could differentiate between the volatiles emitted by damaged *Bt* and non-*Bt* rice plants. In particular, the comparison between herbivore-damaged non-*Bt* rice plants and undamaged *Bt* rice plants (vii) reflected the field situation where lepidopteran larvae cause negligible or no damage to the *Bt* plants34. With these comparisons, two research
hypotheses could be tested: i) the insertion of Bt genes will not change the production of rice plant volatiles; and ii) the non-Bt rice plants are more damaged by C. suppressalis than the Bt plants and consequently release qualitatively and quantitatively different volatile bouquets, resulting in higher attractiveness to parasitoid wasps.

**Response of C. chilonis to rice plants damaged by 1st- or 3rd-instar C. suppressalis larvae.** An additional olfactometer bioassay was conducted to compare the responses of C. chilonis females to Bt rice plants that had been infested with 1st-instar caterpillars, which might occur in the fields, and non-Bt rice plants that had been infested with 1st or the 3rd-instar larvae of C. suppressalis. The material and methods used in this experiment were the same as described above. The female parasitoids were exposed to two pairs of odor sources: (i) non-Bt rice plants infested with 1st-instar C. suppressalis larvae vs. Bt rice plants infested with 1st-instar C. suppressalis larvae (1st non-Bt HD vs. 1st Bt HD); and (ii) non-Bt rice plants infested with 3rd-instar C. suppressalis larvae vs. Bt rice plants infested with 1st-instar C. suppressalis larvae (3rd non-Bt HD vs. 1st Bt HD); both comparisons reflected the field situation. Each plant was infested with 30 newly hatched 1st-instar C. suppressalis larvae (<2 h) or 10 3rd-instar larvae. After 24 h feeding, the C. suppressalis damaged plants were used for the olfactometer bioassays using the method as described above.

**Collection of headspace volatiles.** Volatiles were collected as described by Sun et al.54 and Zhao et al.54 with minor modifications. Volatiles were collected from both uninfested and C. suppressalis-infested Bt and non-Bt rice plants that were used in the bioassays. Air was filtered through activated charcoal, molecular sieves (5 Å, beads, 8–12 mesh, Sigma-Aldrich), and silica gel Rubin (cobalt-free drying agent, Sigma-Aldrich) before entering the glass bottles. Air was blown in through the base collection ports of the bottles and drawn out at the top ports of the bottles through Super Q traps (80/100 mesh, Alltech Associates, Deerfield, IL, USA). Before volatiles were collected, the system was purged for 20 min at 600 ml/min. Subsequently, the volatiles emitted from the top of the sample bottles were trapped on 100 mg Super Q in a glass tube (5 mm diameter, 8 cm height) plugged with glass wool at a flow of 400 ml/min for 8 h. The insects were left on the plants during volatile collection. For each treatment, 7 to 10 collections (from 7 to 10 replicate bottles) were made simultaneously. As a negative control, volatiles also were collected from a bottle containing an aluminum foil-wrapped flask that was filled with distilled water only. Volatile compounds that were detected in the control were considered to be air contaminants and were excluded from the plant samples.

Volatiles collected by Super Q traps were extracted with 600 μl of methylene chloride, and 2000 ng of 1-tridecene in 10 μl of methylene chloride was added to the samples as an internal standard (IS). All extracts were stored at −30°C.

**Identification and quantification of volatiles.** An Agilent gas chromatography (GC) (6890N) coupled with a mass spectrometry (MS) system (5973 MSD; Agilent Technologies, Inc., Palo Alto, CA, USA) was used for separation and detection of plant volatiles. A 1-μl volume of each sample was injected into a DB5-MS column (30 m × 0.25 mm ID ¥ 0.25 μm film thickness; Agilent Technologies). The inlet was operated in a split-less injection mode, and the injector, ion source, and mass quadrupole were maintained at 250°C, 230°C, and 150°C, respectively. Helium was used as the carrier gas with a flow of 1.0 ml min⁻¹ in constant flow mode. The GC-MS was operated in scan mode with a mass range of 33–300 amu at 5.24 s⁻¹ and was in an electron impact ionization mode (EI) at 70 eV. The GC oven temperature was initially kept at 40°C for 2 min and then increased to 250°C at 6°C min⁻¹ and kept for 2 min at 250°C. Collected volatiles were identified by comparing their retention time and mass spectra with authentic standard compounds. If standards were unavailable, tentative identifications were made based on referenced mass-spectra available from the Nist 05 library (Scientific Instrument Services, Inc., Ringoes, NJ, USA) or previous studies. The total ion chromatogram peak area of the volatile compounds was calculated by Enhanced Data Analysis software D.02.00.275 MSD Chemstation (Agilent Technologies, Inc., Palo Alto, CA, USA).

**Statistical analyses.** Student’s t-tests were used to compare the body-weight increase of the C. suppressalis larvae that had fed on Bt or non-Bt rice stems. Heterogeneity among different plant pairs for each pairwise comparison was tested by the method of replicated G-tests of goodness-of-fit.55 As different plant pairs were homogeneous in each comparison (all P > 0.05), data were pooled. The behavioral responses of the female wasps to pairs of odors were analyzed using Chi-square (χ²) tests, with an expected response of 50% for either olfactometer arm. Data for individuals that made no choice within 5 min were excluded from the analyses. The datasets of the ratios of recorded peak area of each volatile or the total volatile relative to the internal standard were log10(x + 1) transformed before the analyses to satisfy the assumption of homogeneity of variance. Consequently they were analyzed using one-way ANOVA, and Tukey honestly significant difference (HSD) tests were conducted to separate the means between the treatments (Bt UD, non-Bt UD, Bt HD, and non-Bt HD). All statistical analysis were conducted with SPSS 22.0 (IBM SPSS, Somers, NY, USA).
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Acknowledgements
We thank Prof. Yongjun Lin (Huazhong Agricultural University) for providing transgenic rice seeds and Prof. Gongyin Ye (Zhejiang University) for providing the C. chilonis. We thank Dr. Fengqi Li for technical assistance with the GC-MS analysis. This work was supported by the National Natural Science Foundation of China (Grant No. 31272041).

Author Contributions
Y.L. and Q.L. designed the study. Q.L. performed all the experiments. Y.L., J.R., Y.P. and Q.L. analyzed data and wrote the manuscript. H.Y., Y.Z. and Y.P. provided experimental materials. All authors have read and approved the manuscript for publication.

Additional Information
Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Liu, Q. et al. Bt rice does not disrupt the host-searching behavior of the parasitoid Cotesia chilonis. Sci. Rep. 5, 15295; doi: 10.1038/srep15295 (2015).

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