Inheritance and fitness costs of resistance to *Bacillus thuringiensis* toxin Cry2Ad in laboratory strains of the diamondback moth, *Plutella xylostella* (L.)

Jinying Liao1,2,3, Yiqun Xue3, Guangjing Xiao1, Miao Xie1,2,3, Shuting Huang3, Shijun You1,2,3, Kris A. G. Wyckhuys1,2 & Minsheng You1,2,3

The diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae), is one of the main pests of *Brassica* crops worldwide. Management of *P. xylostella* is particularly challenging, as different field populations have readily acquired resistance to a wide range of insecticides, including *Bacillus thuringiensis* (Bt) toxins. In this study, a novel strain of *P. xylostella* (Fuzhou-R2Ad) with 120-fold resistance to Bt Cry2Ad was selected in the laboratory, after screening for 66 generations from the susceptible strain Fuzhou-S. In the absence of Bt Cry2Ad toxin, the Fuzhou-R2Ad had significantly lower fitness as compared to the susceptible strain, which might be related to induced genetic changes to Bt toxins. We used several models to measure the dominance levels of insecticide resistance among different strains and found an incompletely recessive inheritance pattern of the Fuzhou-R2Ad resistance, which might be controlled by multiple genes. This study constitutes the first report of laboratory-acquired resistance to Cry2Ad toxin in *P. xylostella*. Our work presents further insights into the mechanism of Bt resistance and has immediate implications for the integrated pest management of *P. xylostella* globally.

The diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), is one of the world’s most destructive pests of *Brassica* crops, and causes an estimated cost of US$4-5 billion annually in direct damage and pest management globally1,2. Although there are multiple tactics for DBM management, chemically-synthesized insecticides remain the most common and widely-used approach. As insecticide-based management has caused substantial resistance problems in DBM3-7, biological pesticides are increasingly promoted as sustainable and environmentally-friendly alternatives. More specifically, the use of *Bacillus thuringiensis*, a soil-dwelling bacterium, offers durable and effective pest control without negative side effects on humans, vertebrates and most beneficial organisms8,9. This also has led to the development of genetically-modified (GM) crops, using Bt genes that biosynthesize the toxic crystalline (Cry) protein. However, given the ability of DBM to rapidly develop resistance to insecticides, there is significant concern that this pest could equally inherit and sustain resistance to Bt toxins.

A lot of research has been conducted on the genetic basis of insect resistance to Bt toxins10-14. The work has shown that a high level of resistance is primarily conferred through one or several autosomal genes, which are either recessive or incompletely recessive15,16. In contrast, the relatively low resistance is acquired through dominant inheritance mechanisms17,18. Four different models have been defined for insecticide resistance and dominance, based on phenotypic traits. First, a $D_{LC}$ model was applied for insecticide resistance, centred on LC50 values of dose-mortality curves19-21. Next, Roush and McKenzie developed an effective dominance $D_{ML}$ model by assessing mortality at a particular dose of a given insecticide22. Third, the relative fitness of dominance $D_{WT}$ was
calculated based upon the fitness of particular genotypes in insecticide-treated areas\textsuperscript{23,24}. Last, a general formula has been proposed for dominance levels in relation to insecticide resistance\textsuperscript{25}. Overall, dominance levels can be calculated for different traits, including insect fitness in insecticide-treated or untreated areas $D_{\text{WNT}}$. Although the dominance level could be estimated by $D_{\text{LC}}$, $D_{\text{ML}}$, $D_{\text{WT}}$ and $D_{\text{WNT}}$, it may still be varied by environmental influences, genetic information and the selection of an insecticide resistance allele. Using $D_{\text{LC}}$ and $D_{\text{ML}}$ models, it has been shown that the resistance in Cry1Ac-selected strains was incompletely recessive in a field-derived population of DBM\textsuperscript{26}. Pereira et al. has demonstrated a recessive inheritance of Cry1F resistance in European corn borer Ostrinia nubilalis, which was indicated by a dominance level $D_{\text{LC}}$ less than 0.11\textsuperscript{16}. However, to our knowledge, there are no published studies that utilize the various models, especially $D_{\text{WNT}}$, to fully evaluate the degree of the dominance.

In the present study, we evaluate, for the first time, the inheritance properties of a laboratory DBM strain with high resistance to Bt Cry2Ad, by comparing dominance of insecticide resistance between the susceptible strain, the positive and negative cross of the resistant strain and the backcross. Furthermore, we investigate levels of dominance and inheritance of resistance to Cry2Ad toxin in the hybrid, resistant and susceptible strains without selection pressure. Additionally, we estimate whether inheritance of Bt Cry2Ad resistance in P. xylostella is controlled by a single-gene or multiple genes. The results of this research have direct implication for resistance management of DBM to Cry2Ad, and can provide further information to advance the effective control of DBM globally.

**Materials and Methods**

**Cry Toxin.** Cry2Ad toxin was obtained from a Bt strain, BRC-HZP10, which was supplied by the Key Laboratory of Biopesticide and Chemical Biology, Fujian Agriculture and Forestry University (Fuzhou, China). The purity of the extracted Cry2Ad protein reached 88.34\textsuperscript{\%}\textsuperscript{26}. Prior to its use in the experiments, Cry2Ad toxin was prepared in 0.2\% Triton X-100.

**Insect strains.** A susceptible strain of P. xylostella, Fuzhou-S, was collected in 2004 from fields of cabbage (Brassica oleracea var. capitata) in Fuzhou (Fujian, China; 26.08°N, 119.28°E). Whole-genome sequencing was applied to characterize the full genomic mapping\textsuperscript{27}. The Fuzhou-S strain has been kept for over 150 generations under greenhouse conditions without exposure to insecticides, with individuals reared on potted radish seedlings (Raphanus sativus L. var. sativus) under the condition of 25°±1°C, 65 ±5% RH and 16L:8D photoperiod.

A resistant strain was derived from the Fuzhou-S strain, by exposing the 3rd instar larvae of DBM to R. sativus leaves treated with Cry2Ad toxin. Fresh and untreated R. sativus leaves were dipped into the Cry2Ad toxin protein solution at LC\textsubscript{50} concentration for 10s, and excess solution was wiped off with filter paper. After 48 h, the surviving larvae were then selected, allowed to pupate and chosen for production of further progeny\textsuperscript{28}. Similar to the Fuzhou-S strain, the resistant Fuzhou-R2Ad strain has been maintained for about 70 generations in the laboratory without any exposure to insecticides except for Cry2Ad.

**Bioassay.** Following the procedures as outlined above, R. sativus leaves (ca. 10 mm diameter) were treated with five gradient concentrations of Cry2Ad solution. After drying, leaves were fed to the 3rd instar P. xylostella larvae that had previously been starved in clear plastic cups (78 mm (top) and 51 mm (bottom) in diameter, 82 mm height) for 2h\textsuperscript{29–31}. Each concentration was tested for a batch of 12 DBM larvae, and the experiments were independently repeated three times with 10 leaves in each replicate. In a control group, larvae were fed with leaf disks (ca. 10 mm diameter) that had been treated with distilled water containing 0.2\% Triton X-100.

The treated larvae were then transferred to a climate chamber at 25°±1°C, 65 ±5% RH, and a 16 L:8D cycle. After 48 h, fresh untreated R. sativus leaves were added. Mortality of larvae was recorded after 72 h, and a toxicity regression curve was developed to estimate the value of LC\textsubscript{50} with 95\% confidence intervals.

**Hybridization.** After pupation, each pupa was transferred individually into a collection tube for further eclosion. Emerged adults were sexed, and used for production of a F1 generation through reciprocal mass crosses. For one cross, 30 Fuzhou-R2Ad females were allowed to mate with 30 Fuzhou-S males in one laying cage (100 mm diameter and 80 mm height). For a second cross, 30 Fuzhou-S females were paired with 30 Fuzhou-R2Ad males\textsuperscript{32}, larvae from the two parental colonies were defined as F1 (Fuzhou-R2Ad♂×Fuzhou-S♀) and F1’ (Fuzhou-R2Ad♀×Fuzhou-S♂), and subject to the above bioassays. Subsequently, F2 progeny was obtained through single-pair crosses between F1 progeny, and a backcross (BC) was produced by pairing a F1 hybrid with the Fuzhou-S strain (F1×Fuzhou-S). Lastly, 20 susceptible adults (i.e., 10 females and 10 males) were mixed with 20 resistant adults (10:10 sex ratio) for a pooled hybrid (R×S). Dominance of Cry2Ad toxin resistance in F1, F1’ and BC hybrids were determined based on the probit analysis (visualised by slopes of log dose–probit line (LD–P line)), LC\textsubscript{50} value and corresponding 95\% confidence limits.

**Fitness tests.** Newly-hatched larvae from Fuzhou-S, Fuzhou-R2Ad, F1 and F1’ hybrid populations were randomly chosen, and individualized on potted turnip sprouts (ca. 40 mm diameter). On a daily basis, development of P. xylostella was monitored and the relevant biological parameters, including mortality, pupation rate, eclosion rate, and adult sex ratio, were recorded. Single-pair crosses of P. xylostella adults were conducted in 60 mm Petri dishes lined with moist filter paper, and mated females were allowed to lay eggs on the moist filter paper. Mated females were fed with 10\% honey solution, and fecundity of each strain was recorded until all moths died.

Eggs were individually collected and incubated in Petri dishes, and egg eclosion rates were computed. Net population growth rate ($R_n$) was determined, defined as the ratio of new larvae ($N_{n+1}$) to the initial number ($N_n$). The relative fitness of the resistant strain was calculated by:
Relative fitness = \( \frac{R_d(\text{resistant or hybrid strain})}{R_d(\text{susceptible strain})} \)

**Data analysis.** For each bioassay, LD-P line, LC\(_{50}\) value, 95% confidence limits and the relative standard deviation were assessed. Two LC\(_{50}\) values are considered to be significantly different (\( P < 0.05 \)) if their 95% confidence intervals do not overlap\(^3\).

Based on the LC\(_{50}\), the resistance ratio was defined as the ratio between the LC\(_{50}\) value of Fuzhou-R2Ad, F1 or BC and that of the susceptible strain (i.e., Fuzhou-S). Degree of dominance (\( D \)) at LC\(_{50}\) was calculated by:

\[
D = (2\log LC_{RS} - \log LC_{R} - \log LC_{S})/(\log LC_{R} - \log LC_{S})
\]

where LC\(_R\), LC\(_{RS}\) and LC\(_S\) represent lethal concentrations for resistant homozygotes, heterozygotes, and susceptible homozygotes, respectively. The value of \( D \) ranges from -1 to 1, representing a complete recessive towards an absolute dominant. Furthermore, \( D_{LC} \) was calculated by:

\[
D_{LC} = (\log LC_{RS} - \log LC_{S})/(\log LC_{R} - \log LC_{S})
\]

which is equal to \((D + 1)/2\)\(^4\). Hence, the \( D_{LC} \) value varies between 0 (recessive resistance) and 1 (dominant resistance).

We equally applied the \( D_{WT} \) model to evaluate relative fitness of dominance under Bt insecticide selection. \( D_{WT} \) was calculated by:

\[
D_{WT} = (W_{TRS} - W_{TSS})/(W_{TRR} - W_{TSS})
\]

where \( W_{TSS}, W_{TRS} \) and \( W_{TRR} \) represent the relative fitness at a specific insecticide concentration for susceptible homozygotes, heterozygotes, and resistant homozygotes, respectively. If susceptible and resistant strains are considered as homozygous genotypes, \( D_{WT} \) will be taken as \( h\)\(^3\). In a similar fashion as \( D_{LC} \), the \( h \) value ranges from 0 to 1 (i.e., from completely recessive to completely dominant).

Another approach was used to assess dominance. For instance, \( D_{WNT} \) value was calculated by:

\[
D_{WNT} = (W_{NTRS} - W_{NTSS})/(W_{NTRR} - W_{NTSS})
\]

where \( W_{NTSS}, W_{NTRS} \) and \( W_{NTRR} \) represent relative fitness in the absence of insecticide for susceptible homozygotes, heterozygotes, and resistant homozygotes, respectively\(^5\). When the \( D_{WNT} \) value is 0.5, resistance is called co-dominant. \( D_{WNT} \) values ranging from 0 to 0.5 demonstrate partial recessive, while \( D_{WNT} \) values between 0.5 to 1 refer to partial dominance.

To test the genetic mode of inheritance, the expected mortality (\( E \)) of BC and F2 under a certain concentration of insecticide was estimated according to Georgiou’s method\(^6\).

\[
E_{BC} = (W_{1} + W_{2}) \times 0.5
\]

\[
E_{F2} = (W_{1} + W_{2} + W_{3}) \times 0.25
\]

in which \( W_{1}, W_{2}, W_{3} \) represent the actual mortality of Fuzhou-S, Fuzhou-R2Ad, and F1, respectively, for a given dose of insecticide. Chi-square test was employed to compare observed and expected mortality of BC and F2\(^7\).

All of the above analyses, including one-way ANOVA with post-hoc Tukey’s honestly significant difference, were performed by using data processing system (DPS) V9.01, while figures were developed using Prism Graphpad 6.

**Results**

**Cry2Ad resistance ratio.** The resistance to Cry2Ad developed slow, and increased 1.04 times at the 12th generation as compared to the susceptible strain (Table 1). Resistance gradually increased over subsequent generations and by generation 37 a 8.70-fold increase was observed over the susceptible strain. In the 66th generation, the relative resistance ratio was 120.59 (Table 1).

**Biological fitness parameters.** In the Fuzhou-S strain, survival rates (% ± standard error) of the 1\(^{st}\), 2\(^{nd}\), 3\(^{rd}\) and 4\(^{th}\) instar larvae were 90.30 ± 0.50, 57.80 ± 0.77, 93.23 ± 1.63, and 91.00 ± 1.59, respectively (Table 2). For the resistant Fuzhou-R2Ad strain, corresponding survival rates (%) were 67.25 ± 0.59, 58.71 ± 0.19, 100.00 ± 0.12 and 74.90 ± 1.97, respectively. Survival rates of 1\(^{st}\) and 4\(^{th}\) instar larvae of the Fuzhou-R2Ad strain were significantly lower than those of the Fuzhou-S strain, and the relative fitness (\( D_{WT} \)) of the Fuzhou-R2Ad strain was 0.29.

Other fitness parameters, such as egg hatch rate, survival rate of the 2\(^{nd}\)-instar larvae, pupation rate, and female fecundity were significantly higher in F1 hybrid compared to F1’. And the relative fitness values of the positive cross F1 and negative cross F1’ were 0.89 and 0.65, respectively.

**Inheritance properties.** All experimental strains proved susceptible to Cry2Ad, and no significant difference was recorded in LC\(_{50}\) values between F1 and F1’ strains (Table 3). In the pooled hybrid (\( R \times S \)), the LC\(_{50}\) value was significantly lower than that of Fuzhou-R2Ad strain. Also, the overlap in 95% confidence limits of LC\(_{50}\) between F1 and F1’ strains confirmed that Cry2Ad resistance was autosomally inherited, without maternal effects and sex linkage.

**Estimation of dominance.** Upon testing five different Cry2Ad toxin concentrations, LC\(_{50}\) values for F1 and F1’ progenies yielded \( D_{F1} = -0.73 \), \( D_{F1'} = -0.44 \), \( D_{LC:F1} = 0.13 \), \( D_{LC:F1'} = 0.28 \). The effective dominance (\( h \)) varied
Table 1. Resistance ratio of *P. xylostella* to Cry2Ad over multiple generation selection as compared to the susceptible Fuzhou-S strain. *RR* (resistance ratio) is calculated as LC50 (Fuzhou-R2Ad, F1 or BC)/LC50 (Fuzhou-S). LC50 (Fuzhou-S) is expressed as 6.65 ng/mL. Each LC50 value represents the average of 8 independent measurements.

| Generation | number of insects tested | Slope ± SE | LC50 (95% fiducial limits) (ng/mL) | RR* | P (df = 3) |
|------------|--------------------------|------------|-----------------------------------|-----|------------|
| 0          | 216                      | 4.34 ± 0.50| 6.65(5.58–8.28)                   | 1.00| 0.8812     |
| 12         | 216                      | 1.78 ± 0.26| 6.92(4.83–9.11)                   | 1.04| 0.9964     |
| 16         | 216                      | 4.54 ± 0.38| 32.35(26.43–37.92)                | 4.86| 0.6909     |
| 27         | 216                      | 1.68 ± 0.27| 51.53(32.94–70.37)                | 7.76| 0.9999     |
| 37         | 216                      | 2.35 ± 0.32| 57.79(41.96–73.00)                | 8.70| 0.9998     |
| 41         | 216                      | 2.13 ± 0.28| 120.20(96.79–157.82)              | 18.10| 0.9973   |
| 52         | 216                      | 2.35 ± 0.32| 154.45(123.84–206.47)             | 23.26| 0.9058   |
| 66         | 216                      | 1.26 ± 0.31| 800.73(372.94–6142.62)            | 120.59| 0.9633 |

Table 2. Population growth parameters of different *P. xylostella* strains. According to one-way with post-hoc Tukey's honestly significant difference, the same superscript letter following the numbers between rows of a given column indicates no significant difference between the strains at *P* > 0.05. The different upper and lower case letters stand for the significance with *P* < 0.01, and *P* < 0.05, respectively. *Relative fitness of the susceptible Fuzhou-S strain is defined as 1.

| Biological characteristics | Fuzhou-S | Fuzhou-R2Ad | F1 | F1’ | F value | P  |
|---------------------------|----------|-------------|----|-----|---------|----|
| Initial amount of eggs    | 140      | 186         | 118| 87  |         |    |
| Egg hatch (%)             | 80.72 ± 1.22bA | 87.08 ± 0.36bA | 82.26 ± 0.57bAB | 74.57 ± 0.97cC | 36.82| 0.0001|
| Survival rate 1st instar (%) | 90.30 ± 0.5A  | 67.25 ± 0.59cC  | 82.82 ± 0.89bAB | 85.01 ± 1.27bAB | 127.19| 0.0001|
| Survival rate 2nd instar (%) | 57.80 ± 0.77cC | 58.71 ± 0.19cC  | 97.57 ± 1.22A   | 85.74 ± 1.01AB  | 498.22| 0.0001|
| Survival rate 3rd instar (%) | 93.23 ± 1.63bA | 100.00 ± 0.12A  | 96.30 ± 0.15AB  | 100.00 ± 1.03A  | 15.99  | 0.0010|
| Survival rate 4th instar (%) | 91.00 ± 1.59bA | 74.90 ± 1.97cC  | 84.22 ± 0.54bAB | 76.59 ± 1.05bC  | 28.22  | 0.0001|
| Number of pupae           | 17.00 ± 0.67bAB | 16.00 ± 1.00bAB | 21.00 ± 1.00bA  | 12.00 ± 2.08bB  | 8.02     | 0.0085|
| Pupation rate (%)         | 33.42 ± 1.62bC | 25.77 ± 1.02bC  | 53.71 ± 1.47bcA | 83.98 ± 2.46bA  | 227.33  | 0.0001|
| Adult number              | 14 ± 0.67bAB  | 12 ± 0.58bAB   | 18.00 ± 1.00bA  | 10 ± 1.53bB    | 11.49   | 0.0029|
| Emergence rate (%)        | 86.11 ± 3.87bcA | 75.18 ± 2.43bcA | 86.42 ± 0.72bcA | 83.99 ± 2.46bA  | 4.06    | 0.0502|
| Sexual ratio (female: male) | 1.17aA  | 1.00A         | 1.31aA          | 1.50aA        |         |    |
| Fecundity/female          | 102 ± 3.67bAB | 91 ± 5.29bAB  | 129 ± 7.21bA    | 82 ± 8.97bB   | 9.65    | 0.0049|
| Number of offspring eggs  | 1414     | 546          | 1062           | 574           | —       | —    |
| R0                        | 10.10    | 2.93         | 9.00           | 6.60          | —       | —    |
| Relative fitness          | 1.00*    | 0.29         | 0.89           | 0.65          | —       | —    |

between 0.33 up to 0.71, and negatively correlated with the Cry2Ad protein concentration (Table 4). Based on the relative DBM fitness (Table 2), the respective fitness values of F1 and F1’ in insecticide-treated areas *D*<sub>NNW</sub> were 0.15 and 0.49. Hence, *D*, *D*<sub>LC</sub>, and *D*<sub>NNW</sub> parameters indicate that the genes conferring resistance to Cry2Ad in DBM selected strain was incompletely recessive. However, when subject to Cry2Ad at 25.32–202.60 μg/mL, DBM larvae had relatively high *h* values (0.56–0.71), suggesting an incomplete dominant inheritance of the Cry2Ad resistance.

**Genetic mode of inheritance.** LD-P lines and expected values were distinguishable for both BC and F2 crosses (Figs 1 and 2). A plateau was not reached neither after the 50% mortality of BC progeny nor at 25% or 75% mortality levels of F2 hybrids. Chi-square analysis showed that the resistance heredity in experimental DBM strains may be controlled by multiple genes (Tables 3 and 6).

**Discussion**

A thorough understanding of pesticide resistance development in *P. xylostella* is crucial for an effective and sustainable management of this globally-important pest. Past research has shown that the development of Bt resistance depends on the particular Bt strain and the type of Bt toxin. Induced by Bt subspecies *kurstaki*, the resistance ratio of *P. xylostella* strain NO was 30 times. Another *P. xylostella* strain NO-95 selected with high resistance to Bt subspecies *aizawai* has very low resistance to Bt subspecies *kurstaki*. In 2014, a Cry1Ie susceptible *Ostrinia furnacalis* strain of ACB-BS was found to have cross resistance to Cry1Ab, Cry1Ac and Cry1F toxins. Other work has shown that a given Bt toxin produced by the same Bt species may exhibit different impacts on a DBM strains/populations, due to the differential modes of action of the Bt toxins. In this study, we determine that DBM resistance development to the Bt Cry2Ad toxin is possible, after laboratory-based screening for 5 years.
and 66 generations. The resulting Fuzhou-R2Ad resistant strain had 120.59 times higher levels of resistance than the susceptible Fuzhou-S strain.

When unexposed to Bt Cry2Ad toxin, the Fuzhou-R2Ad has significantly lower fitness as compared to the susceptible strain. Similar findings has been made with DBM populations in Hawaii, where Dipel 2X® (a wettable powder formulation of B. thuringiensis subsp. kurstaki strain HD-1) resistant strain NO-QA exhibited reduced survival, egg hatching and mating rates42. Such reduction in fitness is possibly related to induced genetic changes

| Strain or cross | Number of insects tested | Slope ± SE | LC50 (95% fiducial limits) (ng/mL) | RR* | P (df = 3) |
|----------------|----------------------------|------------|---------------------------------|-----|------------|
| Fuzhou-S       | 216                        | 1.44 ± 0.25| 9.84 (6.98–13.61)               | 1.00| 0.8874     |
| Fuzhou-R2Ad    | 216                        | 1.26 ± 0.31| 800.73 (372.94–6142.62)         | 81.37| 0.9633     |
| F1 (Fuzhou-R2Ad♀ × Fuzhou-S♂) | 216 | 1.39 ± 0.22 | 230.27 (155.81–457.35) | 23.40| 0.9737     |
| F1 (Fuzhou-R2Ad♂ × Fuzhou-S♀) | 216 | 1.15 ± 0.25 | 116.91 (77.44–187.60) | 11.88| 0.8206     |
| F1 (F1♀ × S♂)  | 216                        | 1.14 ± 0.25| 77.71 (53.83–107.38)           | 7.90| 0.8943     |
| R × S (pooled) | 432                        | 1.27 ± 0.23| 173.59 (116.62–322.47)         | 17.64| —          |
| S × F1 (F1♀ × S♂) | 216 | 0.83 ± 0.24 | 297.84 (160.45–1591.57) | 30.27| 0.9696     |

Table 3. Susceptibility to Cry2Ad toxin in a susceptible strain (Fuzhou-S), resistant strain (Fuzhou-R2Ad), and different reciprocal crosses of the P. xylostella strains. Resistance ratio is presented by LC50 of a given strain or cross divided by LC50 of the susceptible Fuzhou-S strain.

| Concentration of Cry2Ad (ng/ml) | Strain or cross | Survival (%) | Fitness | h |
|---------------------------------|----------------|--------------|---------|---|
| 25.32                           | Fuzhou-S       | 27.80 ± 1.66 | 0.28    |   |
|                                 | Fuzhou-R2Ad    | 97.22 ± 1.66 | 1.00    |   |
|                                 | F1 (Fuzhou-R2Ad♀ × Fuzhou-S♂) | 77.14 ± 1.19 | 0.79    | 0.71 |
| 50.65                           | Fuzhou-S       | 15.30 ± 1.48 | 0.16    |   |
|                                 | Fuzhou-R2Ad    | 94.44 ± 0.00 | 1.00    |   |
|                                 | F1 (Fuzhou-R2Ad♀ × Fuzhou-S♂) | 65.71 ± 2.83 | 0.69    | 0.64 |
| 101.30                          | Fuzhou-S       | 7.30 ± 1.42  | 0.08    |   |
|                                 | Fuzhou-R2Ad    | 86.11 ± 2.78 | 1.00    |   |
|                                 | F1 (Fuzhou-R2Ad♀ × Fuzhou-S♂) | 51.43 ± 2.89 | 0.60    | 0.56 |
| 202.60                          | Fuzhou-S       | 3.00 ± 1.52  | 0.04    |   |
|                                 | Fuzhou-R2Ad    | 75.00 ± 2.78 | 1.00    |   |
|                                 | F1 (Fuzhou-R2Ad♀ × Fuzhou-S♂) | 45.71 ± 2.03 | 0.61    | 0.59 |
| 405.21                          | Fuzhou-S       | 1.01 ± 0.71  | 0.01    |   |
|                                 | Fuzhou-R2Ad    | 66.67 ± 3.21 | 1.00    |   |
|                                 | F1 (Fuzhou-R2Ad♀ × Fuzhou-S♂) | 22.86 ± 1.28 | 0.34    | 0.33 |

Table 4. Effective dominance (h) of resistance to Cry2Ad in different strains of P. xylostella, as compared to Fuzhou-R2Ad. Mortality (%) is calibrated before fitness calculation, and it is calculated as (WR − WS)/(WR − WS), where WR, WS, and WS represent fitness values at a specific toxin concentration.

Figure 1. The slopes of log dose–probit lines (LD–P lines) for BC and the expected LD–P line of BC progeny (EBC). Expected mortality at concentration x ng/ml is calculated as 0.5 × (mortality of F1 at x ng/ml + mortality of Fuzhou-S at x ng/ml), obtained from regression lines of parental strains.
to Bt toxins, which may remain even in the absence of selection pressure\textsuperscript{24,43}. Hence, it is possible that effective DBM pest control can still be attained for resistant populations by discontinuing Bt Cry2Ad applications.

Inheritance of Bt resistance in the diamondback moth is considered to occur autosomally\textsuperscript{14,28,44}, and similar inheritance models have been recorded for the Asian corn borer \textit{Ostrinia furnacalis}\textsuperscript{38}, the southern house mosquito \textit{Culex quinquefasciatus}\textsuperscript{45}, and the cotton bollworm \textit{Helicoverpa armigera}\textsuperscript{46,47}. As one notable exception, Malaysian populations of \textit{P. xylostella} exhibited maternal effects on Cry1Ac resistance development\textsuperscript{3}. In the current research, we detect susceptibility to Cry2Ad in all experimental strains or crosses, and confirm this to be autosomal resistance to Cry2Ad, without maternal effects or sex linkage (Table 3).

Our work also show that the resistance inheritance to Cry2Ad toxin in DBM strains is incompletely recessive. This is clearly shown by the following parameters: \(D_{F1}\) values of 0.73 and 0.13, \(D_{LC}\) values of 0.44 and 0.28, \(D_{WNT}\) values of 0.15 and 0.49 for F1 and F1' respectively. \(D_{F1}\), \(D_{LC}\) and \(D_{WNT}\) values indicate that resistance to Cry2Ad in the Fuzhou strains of \textit{P. xylostella} is partially recessive. Secondly, the effective dominance is negatively regulated by concentrations of the Bt toxin\textsuperscript{48,49}, namely an incomplete recessivity of resistance at a high Cry2Ad level and an incomplete dominance at low concentrations of Cry2Ad protein. However, when DBM populations are treated with a low dose of toxin, the reduced selection pressure may cause bias because of the increased survival rate in the susceptible strain.

Our work constitutes the first report of Cry2Ad resistance in \textit{P. xylostella}, sheds light upon Bt resistance development, and could guide further pest management interventions against a globally-relevant lepidopteran pest. Caution needs to be taken when extrapolating our findings, as our research is conducted under highly-artificial conditions with laboratory-reared individuals. Hence, one could still encounter an incompletely coincident resistance to Cry2Ad due to variations in DBM field populations\textsuperscript{50}. Further, we postulate that resistant heredity in local

### Table 5.

| Concentration of Cry2Ad (ng/ml) | Observed | Expected | \(\chi^2\) | \(P\) |
|---|---|---|---|---|
| 22.93 | 7 | 29 | 28 | 44 | 3.30 | 0.0692 |
| 45.86 | 8 | 28 | 36 | 36 | 6.56 | 0.0104 |
| 91.72 | 12 | 24 | 43 | 29 | 5.67 | 0.0172 |
| 183.44 | 16 | 20 | 51 | 21 | 6.02 | 0.0141 |
| 366.88 | 19 | 17 | 58 | 14 | 7.74 | 0.0054 |
| \(\sum \chi^2\) |  |  | 29.29 |

### Table 6.

| Concentration of Cry2Ad (ng/ml) | Observed | Expected | \(\chi^2\) | \(P\) |
|---|---|---|---|---|
| 22.93 | 9 | 27 | 16 | 56 | 0.01 | 0.9357 |
| 45.86 | 13 | 23 | 22 | 50 | 0.13 | 0.5663 |
| 91.72 | 19 | 17 | 29 | 35 | 1.05 | 0.2222 |
| 183.44 | 24 | 12 | 37 | 35 | 1.70 | 0.1923 |
| 366.88 | 31 | 5 | 46 | 26 | 4.76 | 0.0131 |
| \(\sum \chi^2\) |  |  | 7.85 |

---

**Figure 2.** LD-P lines for susceptible (Fuzhou-S) and resistant parents (Fuzhou-R2Ad), F1, F2 and expected LD-P line of F2 progeny. Expected mortality at concentration \(x\) ng/ml is calculated as 0.25 \(\times\) (Fuzhou-S mortality + Fuzhou-R2Ad mortality + F1 mortality), obtained from regression lines of parental strains.
diamondback moth populations is conferred by multiple genes (Figs 1 and 2; Table 5). All of the above provide fundamental insights into the mechanism and evolution of Bt resistance, according to the neo-Darwinian theory. Further investigation of Bt resistance genes through molecular biology approaches, including molecular marker selection, would be a great help for the genetic manipulation of the diamondback moth. Moreover, the knowledge obtained from this research could boost the effectiveness of pest management interventions and enable sustainable DBM control globally.

References
1. Zalucki, M. P. et al. Estimating the economic cost of one of the world's major insect pests. Plutella xylostella (Lepidoptera: Plutelidiae): just how long is a piece of string? J Econ Entomol 105(4), 1115–29 (2012).
2. Furlong, M. J., Wright, D. J. & Doss dall, L. M. Diamondback moth ecology and management: problems, progress, and prospects. Annu Rev Entomol 58, 517–41 (2013).
3. Tabashnik, B. E., Cushing, N. L., Finson, N. & Johnson, M. W. Field Development of Resistance to Bacillus thuringiensis in Diamondback Moth (Lepidoptera: Plutelidae). Journal of Economic Entomology 83(5), 1671–6 (1990).
4. Shelton, A. M. et al. Resistance of Diamondback Moth (Lepidoptera: Plutelidae) to Bacillus thuringiensis Subspecies in the Field. Journal of Economic Entomology 86(3), 697–705 (1993).
5. Liu, Y.-B., Tabashnik, B. E. & Pusztai-Carey, M. Field-Evolved Resistance to Bacillus thuringiensis Toxin Cry1C in Diamondback Moth (Lepidoptera: Plutelidae). Journal of Economic Entomology 89(4), 798–804 (1996).
6. Sayed, A. H., Schuler, T. H. & Wright, D. J. Inheritance of resistance to Bt canola in a field-derived population of Plutella xylostella. Pest Manag Sci 59(11), 1197–202 (2003).
7. Shelton, A. M. et al. Assessment of insecticide resistance after the outbreak of diamondback moth (Lepidoptera: Plutelidae) in California in 1997. J Econ Entomol 93(3), 931–6 (2000).
8. Flexner, J. L., Lighthart, B. & Croft, B. A. The effects of microbial pesticides on non-target, beneficial arthropods. Agriculture, Ecosystems & Environment 163(3), 203–34 (1986).
9. ISAAA, Global Status of Commercialized Biotech/GM Crops. Ithaca: The International Service for the Acquisition of Agri-biotech Applications; Report No.: 978-1-892456-66-4 (2016).
10. Council NR, Pesticide Resistance: Strategies and Tactics for Management. Washington, DC: The National Academies Press 484 p (1986).
11. Heckel, D. G. The complex genetic basis of resistance to Bacillus thuringiensis toxin in insects. Biocontrol Science and Technology 4(4), 405–17 (1994).
12. Tabashnik, B. E. et al. Global variation in the genetic and biochemical basis of diamondback moth resistance to Bacillus thuringiensis. Proc Natl Acad Sci USA 94(24), 12780–5 (1997).
13. Ferre, J. & Van Rie, J. Biochemistry and genetics of insect resistance to Bacillus thuringiensis. Annu Rev Entomol 47, 501–33 (2002).
14. Tabashnik, B. E., Finson, N., Schwartz, J. M., Caprio, M. A. & Johnson, M. W. and editors. Diamondback moth resistance to Bacillus thuringiensis in Hawaii. Diamondback moth and other crucifer pests: proceedings of the Second International Workshop, Tainan, Taiwan (1990).
15. Sayed, A. H., Raymond, B., Ibiza-Palacios, M. S., Escrène, B. & Wright, D. J. Genetic and biochemical characterization of field-evolved resistance to Bacillus thuringiensis toxin Cry1Ac in the diamondback moth, Plutella xylostella. Appl Environ Microbiol 70(12), 7010–7 (2004).
16. Pereira, E. J., Storer, N. P. & Siegfried, B. D. Inheritance of Cry1F resistance in laboratory-selected European corn borer and its survival on transgenic corn expressing the Cry1F toxin. Bull Environ Res 98(6), 621–9 (2008).
17. Tang, J. D., Gilboa, S., Roush, R. T. & Shelton, A. M. Inheritance, stability, and lack-of-fitness costs of field-selected resistance to Bacillus thuringiensis in diamondback moth (Lepidoptera: Plutelidae) from Florida. Journal of Economic Entomology 90(3), 732–41 (1997).
18. Gould, E. F. et al. Broad-spectrum resistance to Bacillus thuringiensis toxins in Heliothis virescens. Proc Natl Acad Sci USA 89(17), 7986–90 (1992).
19. Bourguet, D. et al. Variation of dominance of newly arisen adaptive genes. Genetics 147(3), 1225–34 (1997).
20. Bourguet, D., Prout, M. & Raymond, M. Dominance of insecticide resistance presents a plastic response. Genetics 143(1), 407–16 (1996).
21. Bourguet, D. & Raymond, M. The molecular basis of dominance relationships: the case of some recent adaptive genes. Journal of Evolutionary Biology 11(1), 103–22 (1998).
22. Roush, R. T. & McKenzie, J. A. Ecological genetics of insecticide and acaricide resistance. Annu Rev Entomol 32, 361–80 (1987).
23. Mallet, J. & Ray mond, J. A. Preventing insect adaptation to insect-resistant crops: are seed mixtures or refugia the best strategy? Proceedings of the Royal Society of London B: Biological Sciences. 250(1328), 165–9 (1992).
24. Tabashnik, B. E. Evolution of resistance to Bacillus thuringiensis. Annual review of entomology 39(1), 47–79 (1994).
25. Bourguet, D., Genissel, A. & Raymond, M. Insecticide resistance and dominance levels. J Econ Entomol 93(6), 1588–95 (2000).
26. Liao, J. Y., Gao, Y. Q., Wu, Q. Y., Zhu, Y. C. & You, M. S. Purification of the insecticidal Cry2Ad protein from a Bt-isolated BRC-HZP10 strain and toxin assay to the diamondback moth, Plutella xylostella (L.). Genet Mol Res 14(3), 7661–70 (2015).
27. You, M. et al. A heterozygous moth genome provides insights into herbivory and detoxification. Nat Genet 45(2), 220–5 (2013).
28. Liu, Y. & Tabashnik, B. E. Inheritance of Resistance to the Bacillus thuringiensis Toxin Cry1C in the Diamondback Moth. Appl Environ Microbiol 63(6), 2218–23 (1997).
29. Tabashnik, B. E. Plant secondary compounds as oviposition deterrents for cabbage butterfly Pieris rapae (Lepidoptera: Pieridae). J Chem Ecol 13(2), 309–16 (1987).
30. Tabashnik, B. E., Finson, N., Johnson, M. W. & Moar, W. J. Resistance to Toxins from Bacillus thuringiensis subsp. kurstaki Causes Minimal Cross-Resistance to B. thuringiensis subsp. aizawai in the Diamondback Moth (Lepidoptera: Plutelidae). Appl Environ Microbiol 59(5), 1332–5 (1993).
31. Székacs, A., Darvas, B., Ishaaya, I., Palli, S. R. & Horowitz, A. R. Comparative aspects of Cry toxin usage in insect control. Advanced Technologies for Managing Insect Pests. Dordrecht (pp. 195–230). Springer, Netherlands, 2013).
32. Mittal, A., Kalia, V., Singh, D. K. & Gujer, G. T. Inheritance of resistance to Bacillus thuringiensis toxin Cry1A1 in the diamondback moth, Plutella xylostella. Biopestic. Int. 4(2), 110–120 (2008).
33. Sun, J., Liang, P. & Gao, X. Inheritance of resistance to a new non-steroidal edesyne agonist, fufenoizide, in the diamondback moth, Plutella xylostella (Lepidopter: Plutelidae). Pest Manag Sci 66(4), 406–11 (2010).
34. Liu, Y.-B., Tabashnik, B. E., Dennehy, T. J., Patin, A. L. & Bartlett, A. C. Development time and resistance to Bt crops. Nature 400(6744), 519 (1999).
35. Tabashnik, B. E. Delaying insect adaptation to transgenic plants: seed mixtures and refugia reconsidered. P Roy Soc London series B: Biolog Sciences 255(1342), 7–12 (1994).
36. Georgiou, G. P. Genetic studies on insecticide resistance. Adv Pest Control Res 6(1), 171–230 (1965).
37. Sokal, R. & Rohlf, F. J. The principles and practice of statistic in biological research. WH Freman, San Francisco 262–5 (1981).
38. Zhang, T. et al. Inheritance patterns, dominance and cross-resistance of Cry1Ab- and Cry1Ac-selected Ostrinia furnacalis (Guenee). Toxins (Basel) 6(9), 2694–707 (2014).
39. Liang, G. M. et al. Changes of inheritance mode and fitness in Helicoverpa armigera (Hubner) (Lepidoptera: Noctuidae) along with its resistance evolution to Cry1Ac toxin. J Invertebr Pathol 97(2), 142–9 (2008).
40. Wu, Y., Vassal, J. M., Rooy, M. & Pieretti, I. A single linkage group confers dominant resistance to Bacillus thuringiensis 6-endotoxin Cry1Ac in Helicoverpa armigera. J Applied. Entomol 133(5), 375–80 (2009).
41. Groeters, F. R., Tabashnik, B. E., Finson, N. & Johnson, M. W. Resistance to Bacillus thuringiensis Affects Mating Success of the Diamondback Moth (Lepidoptera: Plutellidae). J Econ Entomol 86(4), 1035–9 (1993).
42. Groeters, F. R., Tabashnik, B. E., Finson, N. & Johnson, M. W. Fitness Costs of Resistance to Bacillus thuringiensis in the Diamondback Moth (Plutella xylostella). Evolution 48(1), 197–201 (1994).
43. Alves, A. P., Spencer, T. A., Tabashnik, B. E. & Siegfried, B. D. Inheritance of resistance to the Cry1Ab Bacillus thuringiensis toxin in Ostrinia nubilalis (Lepidoptera: Crambidae). J Econ Entomol 99(2), 494–501 (2006).
44. Hama, H., Suzuki, K. & Tanaka, H. Inheritance and Stability of Resistance to Bacillus thuringiensis Formulations of the Diamondback Moth, Plutella xylostella (LINNAEUS) (Lepidoptera: Yponomeutidae). Applied Entomology and Zoology 27(3), 355–62 (1992).
45. Wirth, M. C., Walton, W. E. & Federici, B. A. Inheritance, stability, and dominance of Cry resistance in Culex quinquefasciatus (Diptera: Culicidae) selected with the three Cry toxins of Bacillus thuringiensis subspp. israelensis. Journal of medical entomology 49(4), 886–94 (2012).
46. Kranthi, K. R. et al. Inheritance of resistance in Indian Helicoverpa armigera (Hübner) to Cry1Ac toxin of Bacillus thuringiensis. Crop Protection 25(2), 119–24 (2006).
47. Mahon, R. I., Olsen, K. M., Saras, K. A. & Young, S. R. Resistance to Bacillus thuringiensis toxin Cry2Ab in a strain of Helicoverpa armigera (Lepidoptera: Noctuidae) in Australia. J Econ Entomol. 100(3), 894–902 (2007).
48. Tan, S. Y. et al. Comparative binding of Cry1Ab and Cry1F Bacillus thuringiensis toxins to brush border membrane proteins from Ostrinia nubilalis, Ostrinia furnacalis and Diatraea saccharalis (Lepidoptera: Crambidae) midgut tissue. J Invertebr Pathol 114(3), 234–40 (2013).
49. Devriendt, M. & Martoret, D. Lack of resistance in the diamondback moth Plutella maculipennis [Lep.:Hyponeutidae] to Bacillus thuringiensis Entomophaga 21(2), 189–199 (1976).
50. Krieg, A. & Langenbruch, G. Susceptibility of arthropod species to Bacillus thuringiensis. In Microbial Control of Pests and Plant Diseases, ed. Burges, H. D., pp. 837–898. New York: Academic. 949 pp (1981).
51. Lande, R. The minimum number of genes contributing to quantitative variation between and within populations. Genetics 99(3–4), 541–53 (1981).

Acknowledgements
This work was supported by the National Natural Science Foundation of China (No. 31230061 and No. 31320103922), Fujian Education and Scientific Research Project for Young and Middle-Aged Teachers (No. JA15176), and Haixia Postdoctoral Exchange Program (No. 201806).

Author Contributions
guantor of integrity of the entire study: Jinying Liao, Shijun You, Minsheng You study concepts: Jinying Liao, Shijun You, Minsheng You study design: Jinying Liao, Minsheng You definition of intellectual content: Jinying Liao, Shijun You, Minsheng You literature research: Jinying Liao experimental studies: Jinying Liao, Yiqun Xue, Guangjing Xiao, Shutong Huang data acquisition: Jinying Liao, Yiqun Xue, Guangjing Xiao, Shutong Huang data analysis: Jinying Liao, Yiqun Xue, Kris A.G. Wyckhuys statistical analysis: Jinying Liao, Yiqun Xue, Kris A.G. Wyckhuys manuscript preparation: Jinying Liao, Yiqun Xue, Shijun You manuscript editing: Jinying Liao, Miao Xie, Kris A.G. Wyckhuys, Shijun You, Minsheng You manuscript review and revision: Jinying Liao, Miao Xie, Kris A.G. Wyckhuys, Shijun You, Minsheng You.

Additional Information

Competing Interests: The authors declare no competing interests.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2019