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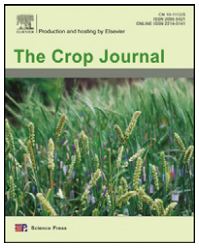

Laboratory testing and molecular analysis of the resistance of wild and cultivated soybeans to cotton bollworm, *Helicoverpa armigera* (Hübner)

*Xiaoyi Wang, Haifeng Chen, Aihua Sha, Rong Zhou, Zhihui Shang, Xiaojuan Zhang, Chanjuan Zhang, Limiao Chen, Qingnan Hao, Zhonglu Yang, Dezhen Qiu, Shuilian Chen, Xinan Zhou**

Department of Soybean, Oil Crops Research Institute, Chinese Academy of Agricultural Sciences, Wuhan 430062, China

ARTICLE INFO

Article history:
Received 11 April 2014
Received in revised form
15 August 2014
Accepted 28 September 2014
Available online 5 October 2014

Keywords:
Soybean
Defoliation
Defoliator resistance
Real-time PCR

ABSTRACT

Identifying a superior soybean variety with high defoliator resistance is important to avoid yield loss. Cotton bollworm (*Helicoverpa armigera* Hübner) is one of the major defoliators of soybean (*Glycine max* [L.] Merr.) worldwide. In this study, we evaluated the effect of *H. armigera* larvae on ED059, a wild soybean (*Glycine soja* Sieb. et Zucc.), and three cultivated soybean varieties: Tianlong 2, PI 535807, and PI 533604, in choice and no-choice assays. The percentage of ED059 leaflets consumed by *H. armigera* was lower than that of the three cultivated soybeans. Larvae that fed on ED059 exhibited low weight gain and high mortality rate. Waldbauer nutritional indices suggested that ED059 reduced the growth, consumption, and frass production of *H. armigera* larvae. Larvae that fed on ED059 showed lower efficiency of conversion of ingested and of digested food than those that fed on Tianlong 2 and PI 533604. However, they showed statistically similar consumption index and approximate digestibility compared with those fed on the three cultivated soybeans. Quantitative real-time PCR analysis revealed that 24 h after insect attack, ED059 had higher transcript levels of *Kunitz trypsin inhibitor 3*, *Cysteine proteinase inhibitor 2*, and *Nerolidol synthase 1* but a lower transcript level of *Pathogenesis-related protein 1* than Tianlong 2. The gene expression results were consistent with the presence of higher levels of jasmonic acid (JA) and transcript levels of the JA biosynthesis enzyme *allene oxide cyclase 3* in ED059 than in Tianlong 2. Our findings indicate that ED059 is a superior soybean line with strong insect resistance that may be mediated via the JA pathway.

1. Introduction

Soybean (*Glycine max* [L.] Merr.) is a primary source of fat and vegetable protein and an important oilseed crop worldwide. Soybean yield is often affected by defoliating insects that reduce leaf area. For example, cotton bollworm (*Helicoverpa armigera* Hübner), which belongs to the Noctuidae family in the order Lepidoptera, is a polyphagous pest that incurs severe loss in economic crops such as soybean worldwide [1,2]. Breeding and the use of soybean varieties with defoliator

* *Corresponding author.*
E-mail address: zhouxinan@caas.cn (X. Zhou).
Peer review under responsibility of Crop Science Society of China and Institute of Crop Science, CAAS.

http://dx.doi.org/10.1016/j.cj.2014.08.004

resistance can reduce crop loss and insecticide use, the latter being important for protecting the environment. Breeders worldwide have focused on identifying soybean cultivars with insect resistance. In the United States, PI 535807 and PI 533604 were cultivated and popularized in the late 1980s because of their resistance to foliar-feeding insects [3,4].

Previous studies have used indices such as the percentage area of leaflets consumed by larvae [5], mass gain and mortality rates of larvae [6], number of bites [7], fecundity [8], and host plant choice [9], to evaluate the resistance level of plants. Many plants naturally develop direct and/or indirect defense mechanisms against insects; for example, certain plants secrete toxic chemical compounds that affect the survival, growth, and reproduction of insects [10]. However, insects counter these defenses by changing their behavior or physiological responses [11,12].

Understanding resistance mechanisms is important for the development of crop varieties that are resistant to herbivorous insects. Antibiosis and antixenosis are the two major types of plant resistance based on the relationship between insects and plants [13,14]. Antibiosis is a resistance mechanism in plants. In this mechanism, the mortality rate of insects increases or larval growth and development decreases after insect feeding. By contrast, antixenosis is a resistance mechanism in which the insects are not attracted to the plant. Defoliator resistance in soybean may rely on one or both of these mechanisms.

Plant defense against defoliators is determined not only by biochemical and morphological features but also by transduction and interaction via signaling molecules [15]. Some defense molecules, such as trichomes, glucosinolates and flavonoids, are expressed constitutively and form the first line of defense. Others are inducible, such as through signal transduction, and involve the expression of certain volatiles and proteinase inhibitors (PIs) [16]. Elucidation of the resistance mechanism of ED059 awaits in-depth biochemical and morphological analyses. Studies have revealed many quantitative trait loci (QTL) associated with response to soybean defoliators, such as common cutworm (*Spodoptera litura*) [17], corn earworm (*Heliothis zea*) [18,19], and Japanese beetle (*Popillia japonica*) [20,21]. For example, candidate genes involved in the flavonoid biosynthesis pathway were found within these QTL regions involved in Japanese beetle resistance [21].

This study aimed to evaluate differences in response to *H. armigera* between the wild soybean line ED059, which has insect resistance, and the susceptible cultivar Tianlong 2 (Fig. S1). Two resistant PI accessions PI 535807 and PI 533604 were also evaluated.

2. Materials and methods

2.1. Plant materials

The cultivated soybean cultivar Tianlong 2 and the wild soybean accession ED059 used in this study were obtained from the Institute of Oil Crops Research, Chinese Academy of Agricultural Sciences, Wuhan, China. PI 535807 and PI 533604 were obtained from the Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Beijing, China.

Seeds were pre-germinated on moistened filter paper in a plant growth chamber at 27 °C, 85% ambient humidity, and 16:8 (light:dark) photoperiod for 3–4 days. The seedlings were then transferred into 18 cm × 18 cm individual plastic pots with nutrition soil (Pindstrup Substrate, Denmark) and vermiculite in a ratio of 2:3 at 27 °C under 16 h of light. All plants used in the experiments had three fully expanded trifoliates.

2.2. Choice test

A choice test of ED059, Tianlong 2, PI 535807, and PI 533604 was conducted in a greenhouse with *H. armigera* in a randomized complete block design. The insects were hatched at 27 °C from a single egg mass supplied by Huazhong Agriculture University, Wuhan, China. For each plant variety, ten plants were used as replicates. At the seedling stage, twenty freshly hatched larvae of *H. armigera* were placed on a leaflet of all plants with three fully expanded trifoliates (20 larvae per plant). All plants and insects were in an open space where the insects could move to their preferred plants, at 27 °C under 16 h of light [22]. The percentage of leaflet area consumed was visually estimated 7 days after infestation.

2.3. No-choice test

A no-choice test of ED059, Tianlong 2, PI 535807, and PI 533604 was conducted in a growth chamber at 27 °C, 85% ambient humidity, and 16:8 (light:dark) photoperiod. Fully expanded leaflets at the seedling stage were collected from plants in the growth chamber. One of the trifoliate leaves was placed in a Petri dish (100 mm × 25 mm) with two moist filter papers at the bottom. The petioles of the detached leaves were inserted into water-soaked cotton to maintain freshness [22]. The no-choice test was divided into two parts. First, each leaf was infested by transferring one freshly hatched larva of *H. armigera* using a brush, and the development of symptoms in ED059, Tianlong 2, PI 535807, and PI 533604 was evaluated after 5 days. Each experiment was conducted in four replicates. Second, one

Table 1 – Defoliation rating as an evaluation index of insect resistance.

Level	Defoliation	Description	Resistance level
1	≤1%	Nearly no damage	Highly resistant
2	1–20%	Needle feeding; holes are not connected	Resistant
3	21–40%	A few holes are connected	Moderately resistant
4	41–60%	Many holes are connected; mesophyll tissue is still present	Moderately susceptible
5	61–80%	Many holes are connected; no mesophyll tissue is present in the damaged area	Susceptible
6	≥80%	Nearly all leaves have been eaten	Highly susceptible

Table 2 – Primer pairs used for real-time PCR.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
KTI3	GGTGCTGGACATGGATGGTAA	ACCGCTTCCTCTTGTAACTGG
CPI2	CACCAATAGGTGGCATTACC	CCCACATACTCCAGATTTGC
NES1	AGACTCTTCAGCATCCGCTTC	TTCAGCCCAAGGTCTTTCCAC
PR-1	CGGAAGCACCGGTAACCTAA	AACCTGAGTGTAGTGCCTGC
LOX3	CCTTGAATCCGATGAGTGTG	CATCGTTCTCGGGTCAAT
actin2/7	TTTGCTGGTGATGATGCTC	ACCTCTTTTTGACTGGGCT
TUA5	ATTTACCCTTCCCCACAGGTTTCA	GCAGATGTCGTAGATGGCTTCGTT
UBQ10	CAAAGCAAAAATCCAGGACAAG	CACCACGAAGACGCAACACAAG
HDC	AGGTCGTTGTTGTCTCAGGTG	CGTGCCGCTTCAGTCTCAG
UKN2	GCCTCTGGATACCTGCTCAAG	ACCTCCTCCTCAAACTCCTCTG
EF1b	GGGTAAGATTCAGTGGCGCT	TAGCAGCCTCCCTTTCCTCT
G6PD	ACCTCGGACACGTTGATGTC	TGGATGCAACGGTGAGTGAA
Fbox	CTCCAAGCCACATGGAGTGT	TTGCGGCCATTCCAGAGATT
60S	GTCGGATCCAACCAGCTTCA	ATCAACCCCACGTTACCGAC
UBC4	TGCAGCTTTAATGATGCGGG	TCTGCCTTACCAGCAACCTG

freshly hatched larva of *H. armigera* was placed in a Petri dish (100 mm × 25 mm). A fresh leaf was added to each dish every 3 days, and the test was stopped after 13 days of infestation. Larval weight, larval mortality rate, and percentage area of leaflets consumed were estimated. The dish experiment was conducted in a randomized complete block design with ten replicates.

Insect resistance level was evaluated as the percentage area of leaflets consumed. Visual defoliation ratings were assessed on a scale of 0 to 6 (Table 1). Level 1 indicates that the defoliation rating was less than 1%, whereas level 6 indicates that the defoliation rating was greater than 80%. The six levels of insect resistance are described as follows: level 1, highly resistant; level 2, resistant; level 3, moderately resistant; level

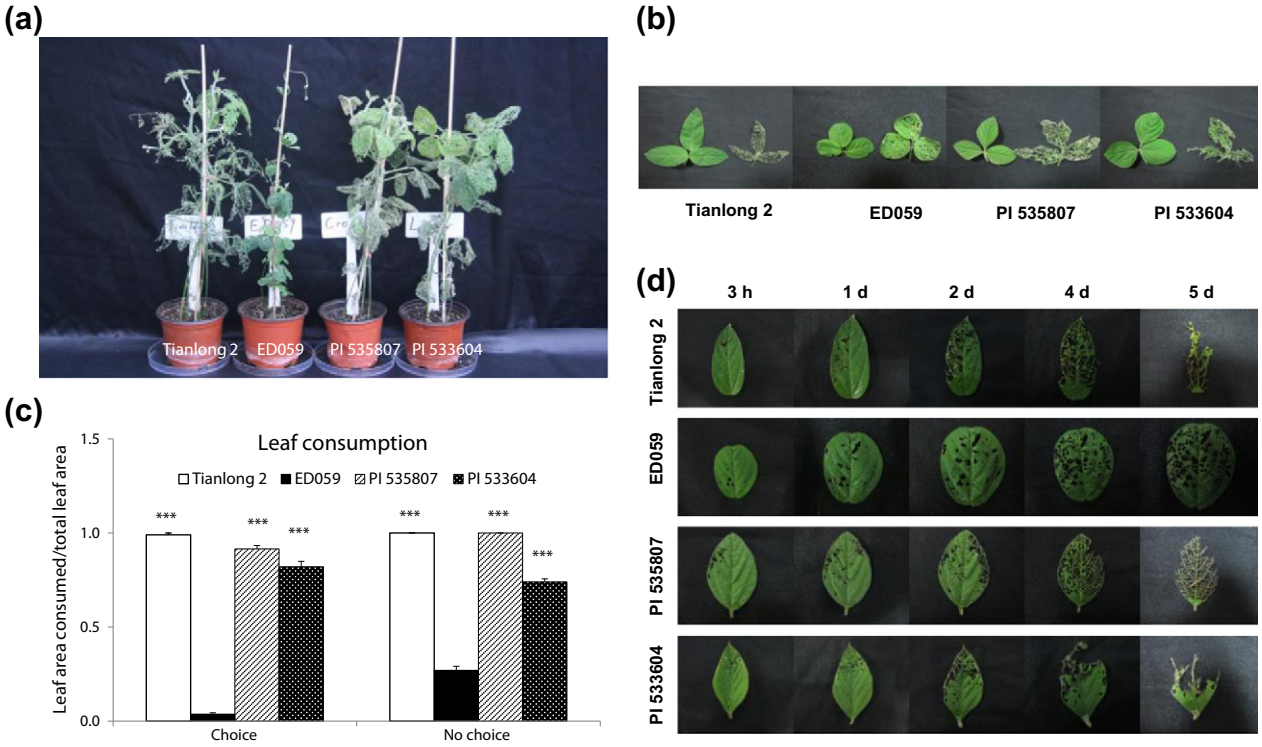

Fig. 1 – Effects of herbivory by *Helicoverpa armigera* on different soybean lines. (a) ED059 exhibited resistance against *H. armigera*. Morphology of ED059, Tianlong 2, PI 535807, and PI 533604 plants at the seedling stage in the choice test after being attacked for 7 days by *H. armigera*. (b) ED059 exhibited resistance against *H. armigera*. Morphology of ED059, Tianlong 2, PI 535807, and PI 533604 leaves in the choice test after being attacked for 7 days by *H. armigera* compared to normal leaves with no insect attack. (c) ED059 exhibited resistance against *H. armigera*. Mean (±SE) leaf area consumed by *H. armigera* after 7 days of feeding in choice test and 5 days of feeding in no-choice test. Asterisks represent significantly different leaf area consumed between ED059 and the three cultivated soybeans [unpaired *t*-test; $^{***}P < 0.001$; $n = 10$ (ten individual plants were used)]. (d) ED059 exhibited resistance against *H. armigera*. Symptom development on ED059, Tianlong 2, PI 535807, and PI 533604 leaves at different time points after infestation with freshly hatched larvae of *H. armigera*.

4, moderately susceptible; level 5, susceptible; and level 6, highly susceptible.

2.4. Analysis of nutritional indices of *H. armigera* on ED059, Tianlong 2, PI 535807, and PI 533604

All plant materials were treated as previously described. A newly hatched *H. armigera* was placed in a dish, and the larva was continuously supplied with fresh leaf material. The experiments on each plant variety were conducted in ten replicates, each with one larva in one dish.

Waldbauer analysis on a dry-weight basis was conducted after 13 days. Dry weight was recorded after drying at 65 °C for 3 days. The mass of leaf consumed (C), body mass gained (G), and mass excreted as frass (F) were measured. The recorded values were used to calculate the consumption index (CI), approximate digestibility (AD), efficiency of conversion of digested food (ECD), and efficiency of conversion of ingested food (ECI) [23]:

$$CI = C / (G \times \text{number of days})$$
$$AD = (C - F) / C$$
$$ECD = G / (C - F)$$
$$ECI = G / C.$$

2.5. Quantitative real-time PCR analysis

At the seedling stage of ED059 and Tianlong 2, five third-instar larvae of *H. armigera* were placed on a leaflet of each plant with three fully expanded trifoliates. After 24 h, the leaves were harvested, flash frozen in liquid nitrogen, and stored at –80 °C until use. Samples from untreated plants were used as controls. Total RNA was extracted with Trizol reagent (Sigma). Exactly 2 μg of total RNA was then reverse transcribed to cDNA using Superscript II reverse transcriptase (Invitrogen). Quantitative real-time PCR analyses were performed with SuperReal PreMix (SYBR Green, Tiangen) on a Rotorgene Q (Qiagen) real-time PCR system. The transcription levels of housekeeping genes can vary considerably in response to changes in experimental conditions and across different types of tissues [24,25]. We accordingly selected 10 housekeeping genes for gene expression evaluation: *TUA5*, *actin2/7*, *HDC*, *UKN2*, *EF1b*, *UBQ10*, *G6PD*, *Fbox*, *60S*, and *UBC4*. The stability of housekeeping gene expression was analyzed with geNorm software (version 3.50) [26]. We determined the value of the stability measure (M) by stepwise exclusion of the least stable housekeeping genes, after which we created a stability ranking and then used the relatively stable housekeeping genes to normalize the expression level of selected genes [26]. The target and housekeeping genes were amplified using gene-specific primers (Table 2). Samples were harvested from three biological replicates for analysis. The relative expression levels of the genes were quantified by the $2^{-\Delta\Delta Ct}$ method. The selected housekeeping genes were used as an internal control to normalize the expression levels of genes.

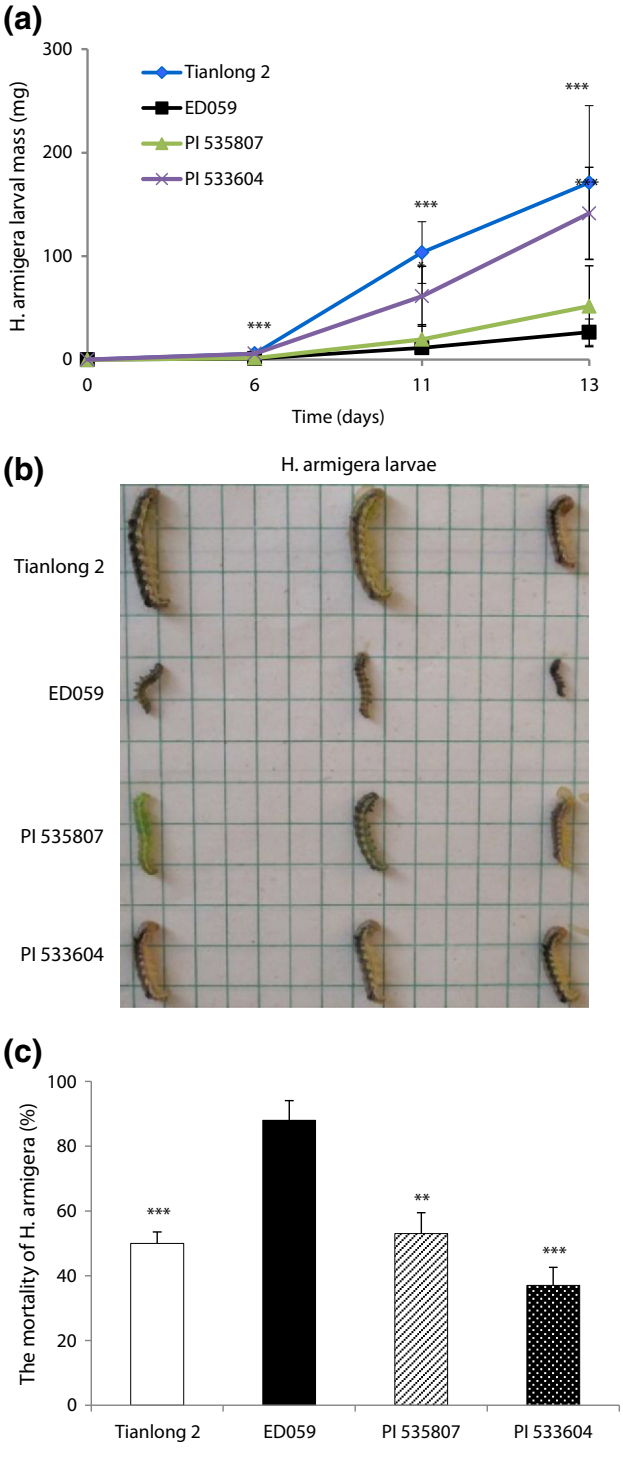

Fig. 2 – Effects of herbivory by *Helicoverpa armigera* on four soybean lines. (a) Mean (±SE) larval mass of *H. armigera* after 6, 11, and 13 days of feeding on ED059, Tianlong 2, PI 535807, and PI 533604. Asterisks indicate significantly different larval mass between larvae feeding on ED059 and the three cultivated soybeans [unpaired *t*-test; $^{*}P < 0.05$; $^{***}P < 0.001$; $n = 10$ (ten individual plants were used)]. (b) Representative *H. armigera* larvae after feeding on ED059, Tianlong 2, PI 535807, and PI 533604 after 13 days. (c) Mean (±SE) mortality rate of *H. armigera* larvae after 13 days of feeding on ED059, Tianlong 2, PI 535807, and PI 533604. Asterisks represent significantly different mortality rate between larval feeding on ED059 and the three cultivated soybeans [unpaired *t*-test; $^{**}P < 0.01$; $^{***}P < 0.001$; $n = 10$ (ten individual plants were used)].

Table 3 – Mean ± SE (standard error) of primary and derived Waldbauer nutritional indices from a 13-day assay on Tianlong 2, ED059, PIs 535807, and 533604.

Waldbauer nutritional indices	Tianlong 2	ED059	PI 535807	PI 533604
Larval mass (mg)	17.61 ± 2.38	2.40 ± 0.66	5.11 ± 1.54	15.02 ± 1.70
Leaf mass consumed (mg)	120.89 ± 14.71	8.03 ± 2.69	58.97 ± 9.03	56.49 ± 7.09
Frass egested (mg)	31.23 ± 4.79	3.06 ± 1.49	12.49 ± 4.06	31.68 ± 2.99
CI[a]	0.54 ± 0.02	0.42 ± 0.10	0.64 ± 0.06	0.30 ± 0.04
AD[b]	0.75 ± 0.01	0.57 ± 0.18	0.76 ± 0.05	0.39 ± 0.06
ECD[c] (%)	0.66 ± 0.07	0.22 ± 0.06	0.20 ± 0.02	0.59 ± 0.10
ECI[d] (%)	0.42 ± 0.06	0.20 ± 0.04	0.21 ± 0.07	0.32 ± 0.01

[a] CI: consumption index.
[b] AD: digestibility.
[c] ECD: efficiency of conversion of digested food.
[d] ECI: efficiency of conversion of ingested food.

2.6. Phytohormone extraction and quantification

Jasmonic acid (JA) was analyzed by high performance liquid chromatography–electrospray ionization–tandem mass spectrometry (HPLC–ESI–MS/MS) as previously described [27]. The plant treatment was the same as that for quantitative real-time PCR analysis. Approximately 0.1 g of the treated leaves was collected at indicated times, frozen in liquid nitrogen, and stored at −80 °C until use.

The treated leaf samples were ground into powder and extracted with 0.5 mL 1-propanol:H_2O:concentrated HCl (2:1:0.002, v/v/v) with internal standards (0.4 μg [$2H_2$]–JA). After centrifugation, the bottom organic phase was transferred to a 10 mL tube and evaporated in a constant nitrogen stream. Each sample was resolubilized in 80% methanol and then extracted using a C18 solid-phase extraction (SPE) cartridge (CNWBOND HC-C18). The obtained eluates were analyzed by HPLC–ESI–MS/MS (Agilent 1200, Agilent Technologies, CA, USA) and then quantified in a hybrid triple–quadruple/linear ion trap mass spectrometer (ABI 4000 Q-Trap, Applied Biosystems, Foster City, CA, USA) using the multiple-reaction monitoring and information-dependent acquisition modes. Standard curves for JA quantification were generated using a series of JA dilutions. All experiments were performed with three biological replicates and each sample was measured three times.

2.7. Data analysis

All data analyses were conducted using Statistica software (SAS Institute Inc., Cary, NC, USA). Differences in the percentage area of leaflets consumed, larval mass, larval mortality rate, and nutritional indices, as well as the relative expression levels of several genes among the soybean lines ED059, Tianlong 2, PI 535807, and PI 533604, were tested using an unpaired *t*-test. A significant difference was declared at $P < 0.05$.

3. Results

3.1. Choice test

In the choice test, a significant difference in *H. armigera* feeding was observed between ED059 and the three cultivated varieties (Tianlong 2, PI 535807, and PI 533604) after 7 days in the greenhouse (Fig. 1-a, b). Only 3.7% of ED059 leaflets were consumed by *H. armigera*, in contrast to 99% of Tianlong 2, 91.5% of PI 535807, and 82% of PI 533604 leaflets ($P < 0.001$, Fig. 1-c). This result indicated that ED059 was the most insect-resistant among the soybean lines tested. When Tianlong 2 was nearly completely consumed, the larvae started to invade ED059. Still, the choice test showed that in the greenhouse, ED059 was resistant to the insect (level 2), whereas Tianlong 2, PI 535807, and PI 533604 were highly susceptible (level 6).

3.2. No-choice test

In the no-choice test, a significant difference in symptom development was observed between ED059 and the three cultivated soybeans after infestation with one freshly hatched larva of *H. armigera* at different time points (Fig. 1-d). The percentage of leaf area consumed was smaller in ED059 than in the three cultivated soybeans. On the fifth day, the extent of defoliation was significantly lower in ED059 (27%) than in Tianlong 2 (100%), PI 535807 (100%), and PI 533604 (74%) ($P < 0.001$, Fig. 1-c). Similarly to the results of the choice test, all leaflets of Tianlong 2, PI 535807, and PI 533604 were completely consumed.

The mass and mortality of newly hatched *H. armigera* larvae placed on ED059, Tianlong 2, PI 535807, and PI 533604 were determined 6, 11, and 13 days after the start of the experiment. The larvae grown on ED059 gained less mass after 13 days of feeding than those grown on the three cultivated soybeans ($P < 0.001$, Fig. 2-a, b). The mortality of *H. armigera* larvae that fed on ED059 (88%) was significantly higher than that of those that fed on Tianlong 2 (50%) ($P < 0.001$, Fig. 2-c), PI 535807 (53%) ($P < 0.01$, Fig. 2-c), and PI 533604 (37%) ($P < 0.001$, Fig. 2-c) after 13 days.

In summary, ED059 not only suffered less damage but was also more toxic to *H. armigera* than were the other soybean lines, as indicated by the poor development and mass gain as well as the increased mortality of larvae that fed on ED059. Thus, our results indicate that ED059 is more insect resistant than Tianlong 2, PI 535807, and PI 533604.

3.3. Nutritional indices of *H. armigera* that fed on ED059, Tianlong 2, PI 535807, and PI 533604

Waldbauer nutritional indices were used to evaluate the response of the larvae to ED059, Tianlong 2, PI 535807, and PI

533604. The averages (±SE) of all nutritional indices are listed in Table 3. *H. armigera* larvae reached the third instar stage after 13 days. A significant difference in leaf mass and dry mass consumed by *H. armigera* was found between ED059 and the three cultivated soybeans ($P < 0.001$, Fig. 3-a). Larvae that fed on ED059 consumed only 6.6% of the leaf mass consumed by those that fed on Tianlong 2. The mass of larvae that fed on ED059 was also significantly different from those of the larvae that fed on the three cultivated soybeans ($P < 0.001$, Fig. 3-b). Interestingly, larvae consumed a large number of leaves of PI 535807, but the mass of the larvae that fed on PI 535807 was not significantly high. This result indicates that an increase in leaf mass consumed may not result in increased larval mass. The dry mass of frass produced by larvae that fed on ED059 (3.1 mg) was significantly lower than those of larvae that fed on Tianlong 2 (31.2 mg) ($P < 0.001$, Fig. 3-c), PI 535807 ($P < 0.01$, Fig. 3-c), and PI 533604 ($P < 0.001$, Fig. 3-c). In addition, CI and AD were both lower in larvae that fed on ED059 than in those that fed on Tianlong 2 ($P < 0.05$) and PI 535807 ($P < 0.01$ or 0.05, Fig. 4-a, b). Furthermore, ECI and ECD were significantly lower in larvae that fed on ED059 than in those that fed on Tianlong 2 and PI 533604 ($P < 0.001$, Fig. 4-c, d).

These results show that ED059 reduced larval growth, frass mass, ECI, and ECD. In addition, significantly less tissue of ED059 than of PI 535807 was consumed by the larvae. CI and AD were also lower in larvae that fed on ED059 than on PI 535807. In sum, the reduction in the mass of the larvae feeding on ED059 can be attributed to the reduced food consumption, frass mass, ECI, and ECD.

3.4. Quantitative real-time PCR analysis of expression of secondary metabolites of insect resistance

We found that ED059 was the most highly insect-resistant and Tianlong 2 the most highly insect-susceptible of the four soybean lines tested. We accordingly chose ED059 and Tianlong 2 to determine whether the selected genes involved in the production of secondary metabolites for insect resistance are differently expressed between the two soybean genotypes. We placed *H. armigera* larvae on both ED059 and Tianlong 2 leaves and collected samples after 24 h. We analyzed the transcripts of *Kunitz trypsin inhibitor 3* (*KTI3*), *Cysteine proteinase inhibitor 2* (*CPI2*), *Nerolidol synthase 1* (*NES1*), and *Pathogenesis-related protein 1* (*PR-1*) in both ED059 and Tianlong 2 by qRT-PCR.

We used the geNorm software to evaluate the stability of housekeeping genes. *HDC* and *EF1b* were ranked as the most stable housekeeping genes in all samples, whereas *UBC4* and *Fbox* consistently ranked low, indicating that they were the most unstable. The optimal numbers of housekeeping genes required for RT-PCR data normalization were determined by geNorm (Table S1). Finally, three housekeeping genes (*HDC*, *EF1b*, and *UKN2*) were selected to normalize the level of gene expression (V3/4 = 0.069 < 0.15) (Table S2).

We measured *KTI3* and *CPI2* transcript levels before and after *H. armigera* attack. *KTI3* and *CPI2* transcript levels were higher in Tianlong 2 than in ED059 before insect attack. However, both *KTI3* and *CPI2* levels increased rapidly 24 h after insect attack in ED059, whereas both *KTI3* and *CPI2* levels decreased in Tianlong 2 (Fig. 5-a, b). In the absence of insect attack, ED059 showed lower expression of *NES1* than Tianlong 2. Insect attack resulted in elevated transcript levels of *NES1* in ED059. In contrast, *NES1* transcript levels in Tianlong 2 declined after insect attack (Fig. 5-c).

The expression level of *PR-1* remained low in ED059 but increased in Tianlong 2 after insect attack (Fig. 5-d). Without

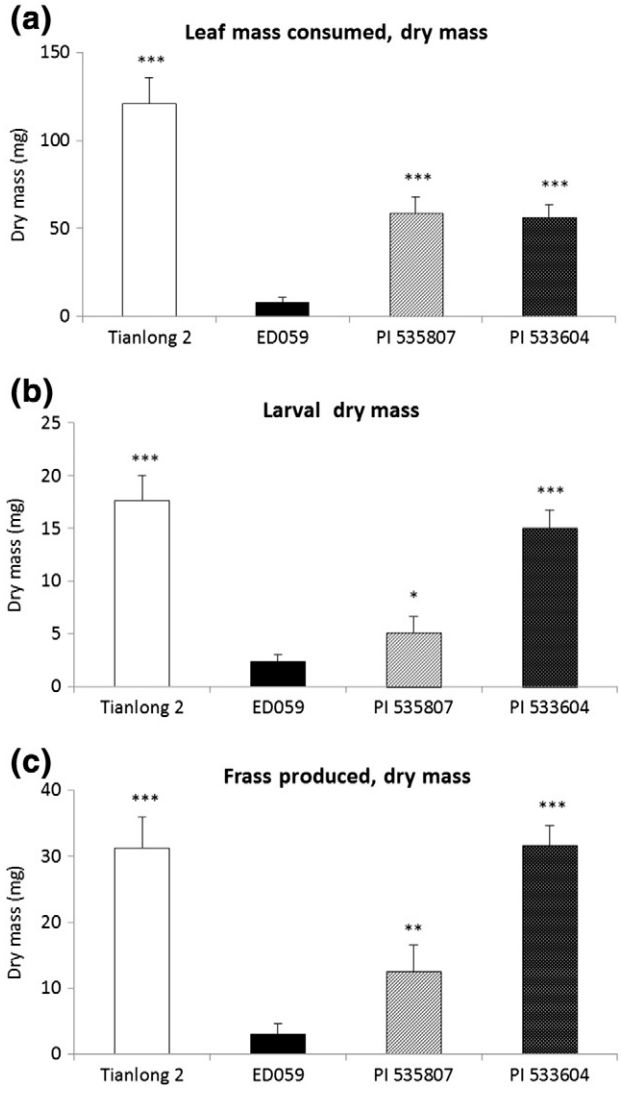

Fig. 3 – Mean (±SE) primary nutritional indices after 13 days of *Helicoverpa armigera* larval feeding on ED059, Tianlong 2, PI 535807, and PI 533604. (a) Total leaf mass consumed. Asterisks indicate significant differences between leaf masses consumed (dry mass) after 13 days of *H. armigera* larval feeding on ED059 and three cultivated soybeans [$^{***}P < 0.001$; $n = 10$ (ten individual plants were used)]. (b) *H. armigera* larval dry mass after feeding on ED059, Tianlong 2, PI 535807, and PI 533604 plants. Asterisks indicate significant differences between larval dry masses after 13 days of *H. armigera* larval feeding on ED059 and three cultivated soybeans [$^{***}P < 0.001$; $n = 10$ (ten individual plants were used)]. (c) Total frass egested. Asterisks indicate significant differences between frass dry masses after 13 days of *H. armigera* larval feeding on ED059 and three cultivated soybeans [$^{**}P < 0.01$; $^{***}P < 0.001$; $n = 10$ (ten individual plants were used)].

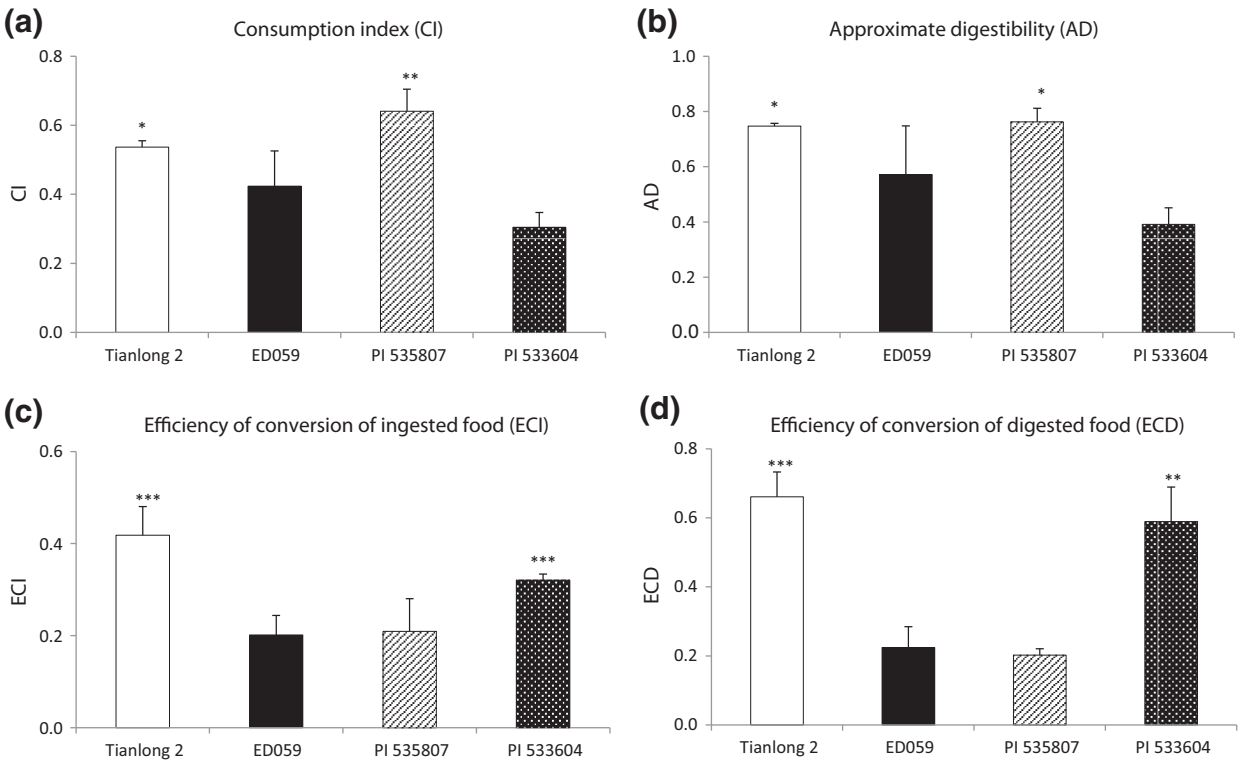

Fig. 4 – Mean (±SE) nutritional indices after 13 days of *Helicoverpa armigera* larval feeding on ED059, Tianlong 2, PI 535807, and PI 533604. (a) Consumption index (CI). (b) Approximate digestibility (AD). (c) Efficiency of conversion of ingested food (ECI). (d) Efficiency of conversion of digested food (ECD). Asterisks represent significantly different CI, AD, ECI, and ECD between larval feeding on ED059 and the three cultivated soybeans [unpaired *t*-test; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $n = 10$ (ten individual plants were used)].

treatment, ED059 showed lower levels of JA than Tianlong 2 (Fig. 6-a). *H. armigera* treatment induced higher levels of JA in ED059 than in Tianlong 2 (Fig. 6-a). We further examined the transcript levels of *allene oxide cyclase 3* (*AOC3*), which is an important enzyme in JA biosynthesis. Without treatment, ED059 and Tianlong 2 had similar transcript levels of *AOC3*. Upon *H. armigera* treatment, the transcript levels of *AOC3* were higher in ED059 than in Tianlong 2 (Fig. 6-b).

4. Discussion

The use of resistant plant varieties is an important component of crop-integrated pest management. This practice not only provides high economic benefits but also offers high social benefits by protecting plants against their natural pests in an environmentally friendly manner. In this study, quantitative assessment of the percentage of leaflet area consumed, as well as the mass and mortality of larvae, showed that *H. armigera* preferred Tianlong 2 and disliked ED059 the most among the soybean varieties examined under choice and no-choice conditions. More feeding on ED059 was initially observed under the no-choice test than under the choice test because of the lack of other food options. However, feeding on ED059 was not observed over longer times.

In this study, we used Waldbauer nutritional indices to evaluate the ingestion capabilities of *H. armigera* larvae that fed on ED059, Tianlong 2, PI 535807, and PI 533604. The capability of an insect to convert consumed food strongly influences its growth and development [28]. Accordingly, plants that were insect-resistant were identified by analysis of the behavior of the insects feeding on them. ED059 significantly reduced larval mass by reducing leaf consumption, ECI, and ECD. Larvae increased their CI and AD to compensate for the reduction in ECI after feeding on ED059. A similar reaction has previously been reported in many other insects subjected to Waldbauer nutritional analysis [29]. The increase in larval mass is associated mainly with the percentage of leaflet area consumed by the larvae. However, in our study, the consumption of leaf mass did not always correlate with the growth of larval mass; the mass of larvae that fed on PI 535807 was low despite the increase in leaf mass consumed. Thus, low leaf intake was not associated with decreases in AD and CI. The observed decrease in the ECD and ECI of larvae feeding on ED059 may be associated with anti-nutritional factors that hinder nutrient absorption and utilization in insects. In summary, ED059 increased the mortality rate but reduced the growth rate of *H. armigera* larvae. Furthermore, larvae were less attracted to ED059 than to other soybean cultivars. Thus, our results indicate that the resistance mechanism of ED059 against defoliators is a combination of antibiosis and antixenosis.

Previous studies have revealed that anti-nutritional factors, such as PIs, lectin, and polyphenol oxidase, reduce larval growth. Other compounds, such as terpenoids, that cause plants to emit a characteristic smell repelling insects have

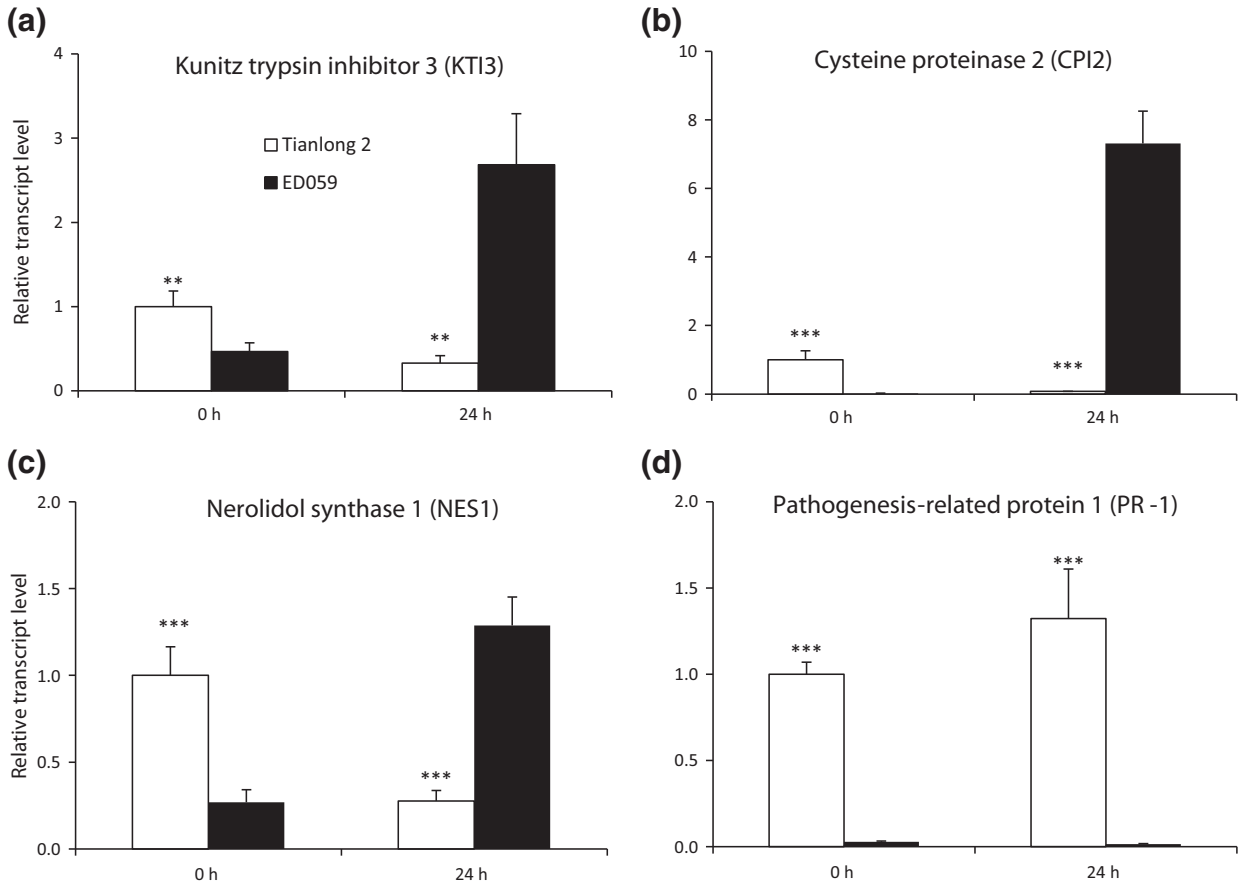

Fig. 5 – Quantitative real-time PCR analysis of transcript levels of *Kunitz trypsin inhibitor 3* (*KTI3*), *Cysteine proteinase inhibitor 2* (*CPI2*), *Nerolidol synthase 1* (*NES1*), and *Pathogenesis-related protein 1* (*PR-1*) in ED059 and Tianlong 2 in response to *Helicoverpa armigera* attack. All values shown are means of three biological replicates. Error bars denote ± SEM. Asterisks represent significantly different transcript levels of *KTI3*, *CPI2*, *NES1*, and *PR-1* between ED059 and Tianlong 2 before and after 24 h *H. armigera* treatment [unpaired *t*-test; $^{**}P < 0.01$; $^{***}P < 0.001$; $n = 3$ (three individual plants were used)].

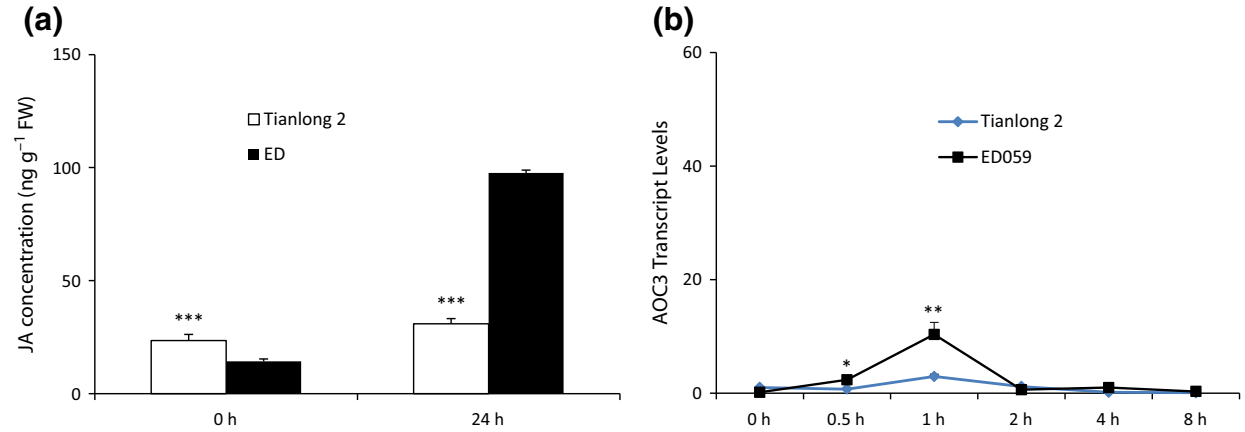

Fig. 6 – Tianlong 2 and ED059 have different levels of *Helicoverpa armigera*-elicited JA. Tianlong 2 and ED059 plants were grown under identical conditions. The second fully expanded leaves were infected with three third-instar larvae of *H. armigera*. Individual leaves from three replicate plants were harvested at the indicated times after treatment. (a) Mean (±SE) JA concentrations in leaves harvested at indicated times were measured with HPLC–MS/MS; (b) The mean (±SE) transcript levels of *allene oxide cyclase 3* (*AOC3*) were measured by qRT-PCR. Asterisks represent significantly different transcript levels between the Tianlong 2 and ED059 at the indicated times [unpaired *t* test; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $n = 3$ (three individual plants were used)].

also been reported [16]. In this study, we measured the differential transcript accumulation of several genes that encode secondary metabolites of insect resistance. In the absence of treatment, differences in transcript levels of *KTI3*, *CPI2*, and *NES1* in ED059 and Tianlong 2 were small. After insect attack, higher levels of *KTI3*, *CPI2*, and *NES1* were observed in ED059 than in Tianlong 2. PIs are important metabolites that contribute to plant responses to herbivores and reduce the growth and survival of insect larvae by inhibiting digestive proteinases in the larval midgut [30,31]. *KTI3* and *CPI2*, which are specific to serine and cysteine proteases, have been proposed to mediate defense against insects [32–34]. ED059 sharply increased *KTI3* and *CPI2* transcripts in response to insect attack, and larvae that fed on ED059 gained markedly less body mass than those that fed on Tianlong 2. *NES1* is associated with the biosynthesis of monoterpene, which is a volatile compound that is toxic to insects [35,36]. Transcript levels of *KTI3*, *CPI2*, and *NES1* increased to the maximum levels in ED059, indicating that PI and monoterpene may both contribute to conferring defense against insects. The biosynthesis of JA is essential for the production of induced defense responses to herbivores in many plants [37], and *AOC3* is an important enzyme that promotes JA biosynthesis [38–41]. We found that *H. armigera* induced higher levels of JA and JA biosynthesis enzyme *AOC3* gene expression in ED059 than in Tianlong 2.

Interestingly, insect attack reduced the transcript level of *PR-1* in ED059 and elevated that in Tianlong 2. *PI* is a reporter for the defense genes induced by the JA pathway, whereas *PR-1* is a reporter for the SA-dependent/independent pathway [42]. Thus, the JA pathway may contribute to the resistance of ED059 against insects. Further investigation of changes in expression levels of other metabolites should be conducted in the future to facilitate understanding of the mechanism of insect resistance in ED059.

Acknowledgments

This work was funded by the China Agriculture Research System (CAAS-04-PS08), the National Transgenic Project of China (2014ZX08004-005), and the Agricultural Science and Technology Innovation Program of China. We thank Dr. Weihua Ma from Huazhong Agriculture University for supplying *H. armigera* eggs.

Supplementary material

Supplementary figure and tables to this article can be found online at http://dx.doi.org/10.1016/j.cj.2014.08.004.

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