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Midgut metabolomic profiling of fall armyworm (*Spodoptera frugiperda*) with field-evolved resistance to Cry1F corn

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ABSTRACT

Populations of the fall armyworm (*Spodoptera frugiperda*) have developed resistance to transgenic corn producing the Cry1F insecticidal protein from the bacterium *Bacillus thuringiensis* (Bt). Resistance in *S. frugiperda* from Puerto Rico is genetically linked to a mutation in an ATP Binding Cassette subfamily C2 gene (*SfABCC2*) that results in a truncated, non-functional Cry1F toxin receptor protein. Since ABCC2 proteins are involved in active export of xenobiotics and other metabolites from the cell, we hypothesized that Cry1F-resistant fall armyworm with a non-functional SfABCC2 protein would display altered gut metabolome composition when compared to susceptible insects. Mass spectrometry and multivariate statistical analyses identified 126 unique metabolites from larval guts, of which 7 were found to display statistically significant altered levels between midguts from susceptible and Cry1F-resistant *S. frugiperda* larvae when feeding on meridic diet. Among these 7 differentially present metabolites, 6 were found to significantly accumulate (1.3–3.5-fold) in midguts from Cry1F-resistant larvae, including nucleosides, asparagine, and carbohydrates such as trehalose 6-phosphate and sedoheptulose 1/7-phosphate. In contrast, metabolomic comparisons of larvae fed on non-transgenic corn identified 5 metabolites with statistically significant altered levels and only 2 of them, 2-isopropylmalate and 3-phosphoserine, that significantly accumulated (2.3- and 3.5-fold, respectively) in midguts from Cry1F-resistant compared to susceptible larvae. These results identify a short list of candidate metabolites that may be transported by SfABCC2 and that may have the potential to be used as resistance markers.

1. Introduction

The fall armyworm (*Spodoptera frugiperda*) is an important polyphagous pest native to tropical regions in the Americas with a long migratory range (Nagoshi et al., 2017). Larvae of this insect have also been reported as a devastating invasive pest of corn in Africa (Day et al., 2017; Goergen et al., 2016). Options for effective control of *S. frugiperda* larvae were greatly improved in 2003 by commercialization of transgenic corn producing the Cry1F insecticidal protein from the bacterium *Bacillus thuringiensis* (Bt) (Siebert et al., 2008). As other Cry toxins, the Cry1F protein recognizes receptors in the midgut of *S. frugiperda* larvae as a critical step to kill enterocytes and ultimately disrupt the midgut epithelium and facilitate deadly septicemia (Adang et al., 2014). Field-evolved resistance to Cry1F corn has been reported in *S. frugiperda* populations from Puerto Rico (Storer et al., 2010), Florida and North Carolina (Huang et al., 2014), Brazil (Farias et al., 2014; Monnerat et al., 2015), and Argentina (Chandrasena et al., 2018).

In a strain of *S. frugiperda* from Puerto Rico, resistance to Cry1F was linked to a mutation in an ATP Binding Cassette subfamily C2 (*SfABCC2*) gene that resulted in a truncated protein and reduced Cry1F toxin binding (Banerjee et al., 2017). This observation mirrored similar cases of resistance to Cry toxins linked to mutations in ABCC2 genes in *Heliothis virescens* (Gahan et al., 2010), *Plutella xylostella* (Baxter et al., 2011), *Helicoverpa armigera* (Xiao et al., 2014), *Bombyx mori* (Atsumi et al., 2012) and *Spodoptera exigua* (Park et al., 2014). Members of the ABCC2 protein family play a critical role in detoxification and chemoprotection by controlling the efflux of both exogenous and endogenous compounds across the cell membrane (Jemnitz et al., 2010). These proteins have broad specificity and act upon many different compounds, including representatives of most classes of insecticides (Dermauw and Van Leeuwen, 2014). However, ABC transporters are not always involved in resistance to insecticides (Porretta et al., 2016),

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and functional interactions need to be tested experimentally. Despite diversity in susbtrate specificity and binding, ABC proteins commonly perform active export of xenobiotics from cells to reduce their intracellular concentration and thus reducing noxious effects (Locher, 2016). Consequently, we hypothesized that altered ABCC2 in Cry1-resistant insects would lead to accumulation of certain metabolites in gut cells when compared to susceptible larvae. In this work, we test this hypothesis by performing the first metabolomic analysis of the midgut epithelium from an insect and quantitatively comparing metabolite patterns between susceptible and Cry1F-resistant *S. frugiperda* larvae. Identification of metabolome alterations in the midgut of resistant larvae suggests candidate metabolites that may be transported by SfABCC2 as potential targets in the development of novel technologies to monitor for resistance to Cry toxins and Bt crops linked to alterations in ABCC2 genes. However, further studies are necessary to demonstrate the link between the significantly differentially expressed metabolites and the mutated ABCC2 gene.

2. Materials and methods

2.1. Insect strains

Eggs of a Cry1F-susceptible strain of *S. frugiperda* (Ben) were purchased from Benzon Research (Carlisle, PA). The Cry1F-resistant *S. frugiperda* strain 456LS3 was originated from field collections in Puerto Rico and its resistance phenotype is described elsewhere (Jakka et al., 2015). The 465LS3 strain was crossed with Ben and re-selected at the F2 generation on corn event TC1507 (producing Cry1F toxin) for 7 days, and this process repeated thrice to generate a similar genomic background between the strains, except for the resistance allele present in 456LS3 (Banerjee et al., 2017). For the purpose of this work, heterozygous strains were generated by mating 25 female/male moths from the Ben and 456LS3 strains to generate strains 456F or 456M according to the sex of the resistant adult in the parental cross (F for female and M for male). All insect rearing and treatments were performed in incubators at 26 ± 2 °C, 44% relative humidity, and an 18-h light/6-h dark photoperiod.

2.2. Protoxin preparation

The Cry1F protoxin was produced in a recombinant *Bacillus thuringiensis* (Bt) HD-73 strain generously provided by Dr. Jie Zhang (Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China) and described elsewhere (Zhao et al., 2015). The bacteria were grown on 1/3 tryptic soy broth (TSB) agar containing erythromycin (5 μg/mL) for three days, and then a single colony was suspended in 1 mL of autoclaved water and heated for 45 min at 70 °C to kill vegetative cells. An aliquot (500 μL) of the suspension was used to inoculate 500 mL of 1/3 TSB medium containing erythromycin. The culture was incubated for 4 days at 28 °C with agitation (160 rpm) until sporulation was confirmed by microscopic observation. Cultures were then collected by centrifugation (14,981 *g*, 4 °C for 10 min) and pellets washed three times with 1 M NaCl containing 0.1% Triton X-100 with brief (5 min) sonication before centrifugation. Washes were repeated for another 2 cycles as before using distilled water. The final pellet was re-suspended in 50 mL of solubilizing solution (50 mM Na_2CO_3, 0.1% β-mercaptoethanol, 0.1 M NaCl, pH 10.5), sonicated as above and then incubated overnight at 30 °C with agitation (180 rpm). After centrifugation at 27,167 *g* for 30 min, the supernatant containing solubilized Cry1F protoxin was purified using anion exchange chromatography as described elsewhere (Gouffon et al., 2011). One major absorbance peak containing Cry1F protoxin (based on band size in SDS-10%PAGE) was detected during the elution, and fractions in that peak were pooled, aliquoted and keep at −80 °C until used.

2.3. Bioassays

Eggs of susceptible, resistant and heterozygous strains were collected, and diet contamination bioassays were carried out under standard laboratory-controlled environmental conditions (26 ± 2 °C temperature, 44% RH, 16L: 8D photoperiod) using meridic diet (Beet Armyworm diet, product #F9219B, Frontier Scientific Services, Newark, DE). Diet was poured on individual wells of 128-cell polystyrene bioassay trays (Bio-BA-128) and after setting a solution of Cry1F protoxin was applied on the surface. A range of 11 different toxin concentrations (0.0035, 0.007, 0.014, 0.028, 0.056, 0.112, 0.224, 0.445, 0.896, 1.792 and 3.585 μg/cm^2) prepared in sterile MilliQ water and a water control were tested with Ben and heterozygous neonates. Four different Cry1F protoxin concentrations (4.75, 14.4, 28.8 and 36.1 μg/cm^2) were tested for 456LS3 neonates. Sixteen neonates per concentration were tested and bioassays were replicated twice. Mortality was determined after 7 days of incubation and used to determine the Cry1F protoxin concentration killing 50% of the larvae (LC_{50}) for each strain by Probit analysis using the PoloPlus software (LeOra, 1987). Differences between LC50s were considered significant if there was no overlap between the corresponding 95% confidence intervals.

2.4. Larval treatments

Neonates from the Ben, 456LS3, 456M, and 456F populations described above were placed either on meridic diet or V3—V4 stage leaves from corn event TC1507 producing Cry1F toxin or the non-transgenic isoline (2T777). Leaf sections (2.5 × 1.5 cm) were placed in individual plastic cups containing 1% agar to prevent desiccation. A single *S. frugiperda* neonate was placed in each cup, with 60 larvae used per strain. Leaf sections were replaced after the first 6 days of larval growth, and then every day as needed. Larvae were kept on corn leaves or meridic diet until they reached 4th instar (11–14 days), and then weighed. Weight measurements were rank transferred before statistical analysis using two-way analysis of variance (ANOVA) testing for the effect of the different insect strains/treatments on the average weight measurements with $p < 0.01$.

2.5. Tissue sample preparation and metabolite extraction

Polar metabolites were extracted from pools of five dissected 4th instar guts to create a single biological replicate of approximately 100 mg, and a total of four biological replicates were prepared for each strain/treatment. Pooled guts were homogenized to fine pieces on ice using disposable micro tissue homogenizers (Kimble™), snap frozen in liquid nitrogen and stored at −80 °C until further use.

Sample preparation and extraction was done at 4 °C unless otherwise specified. Extraction and detection methods were modified from procedures published elsewhere (Rabinowitz and Kimball, 2007; Tague et al., 2018). Briefly, each biological replicate was extracted with 1.3 mL of extraction solvent (40:40:20 HPLC grade methanol: acetonitrile: water with formic acid at a final concentration of 0.1 M) pre-chilled to 4 °C, and allowed to proceed for 20 min at −20 °C. The supernatant was collected after 5 min of centrifugation at 13,300 × *g* and the pellets were re-extracted with 200 μL of fresh extraction solvent and allowed to sit for 20 min at −20 °C before being centrifuged at the same conditions as mentioned earlier. Both supernatant fractions were combined, and a stream of dry nitrogen was used to evaporate the solvent. Sterile water (300 μL) was used to for suspend the metabolites prior to being placed in the instrument's autosampler.

2.6. Ultra performance liquid chorography- high resolution mass spectrometry (UPLC–HRMS) metabolomic analysis

Samples extracted above were separated on a Dionex Ultimate 3000

UPLC (Thermo scientific, Waltham, MA) by injecting a 10 µL sample on to a Synergi 2.5 µm reverse phase Hydro-RP 100 Å, 100 mm × 2.00 mm LC column (Phenomenex, Torrance, CA) kept at 25 °C. All solvents used for mobile phases were HPLC grade and purchased from Fisher Scientific. Solvent A consisted of 97:3 water: methanol, 10 mM tributylamine, and 15 mM acetic acid and solvent B was 100% methanol. The gradient steps included from 0 to 5 min 0% B, from 5 to 13 min 20% B, from 13 to 15.5 min 55% B, from 15.5 to 19 min 95% B, and from 19 to 25 min 0% B, with a flow rate of 200 µL/min. The eluent was introduced into the mass spectrometer via an electrospray ionization source conjoined to an Exactive™ Plus Orbitrap Mass Spectrometer (Thermo Scientific, Waltham, MA) (Lu et al., 2010). Data was collected in negative mode from 85 to 1000 *m/z* with a resolution of 140,000 and an automatic gain control of 3×10^6 ions.

Raw data was analyzed using MAVEN (Clasquin et al., 2012; Melamud et al., 2010) to align and annotate peaks within a 5 ppm window. Area under the chromatographic curve was integrated based upon an in-house verified list of metabolites using exact *m/z* and known retention times. All metabolite values were normalized based on the exact mass of the sample extracted prior to all statistical calculations. Heat maps were made by Cluster 3.0 (de Hoon et al., 2004) and Javatreeview 1.1 (Saldanha, 2004), using $\log_2$ transformed data. P-values were calculated using Student's T-test. Partial Least Squares Discriminant Analysis (PLSDA) and Variable Importance in Projection (VIP) scores were constructed using the statistical package DiscriMiner in R version 3.1.1. (Sanchez, 2013). These VIP scores provide a metric for determining how much influence a metabolite has on the group separation seen in the PLSDA plots. A value > 1 indicates that the metabolite contributes to the group differentiation, and this was considered as a significant VIP score (Farrés et al., 2015; Li et al., 2017; Xia and Wishart, 2011). Global pathway maps were created by imputing KEGG IDs for each metabolite detected into iPath 2.0 (Yamada et al., 2011).

3. Results

3.1. Cry1F protoxin bioassays

As expected from resistance in 456LS3 being recessive and autosomal (Jakka et al., 2015), bioassays with purified Cry1F protoxin showed no significant difference (based on overlapping 95% confidence intervals) in toxicity against Ben, 456F, and 456M larvae (Table 1). In contrast, only low mortality (12.5%) was observed when larvae from the 456LS3 strain were exposed to the highest Cry1F protoxin concentration tested (36.1 µg/cm^2). Based on this observation, we estimated that larvae from 456LS3 were > 75 fold resistant to Cry1F compared to larvae from the Ben, 456F or 456M strains. In agreement with these observations, exposure of neonates from the Ben, 456M or 456F strains to Cry1F corn leaf tissue resulted in 100% mortality after seven days, while no mortality was observed for larvae of the 456LS3 strain (data not shown).

No significant difference was detected when comparing weights of 4th instar larvae from all strains fed on control diet ($p = 1$). When comparing artificial diet to corn leaf tissue, the mean larval weights from all the strains was significantly higher (Tukey- Kramer, $p < 0.05$) on meridic diet compared to non-Bt corn leaves (Fig. 1). No significant differences were detected when comparing all four strains fed on meridic diet ($p = 1$), yet a significant difference in mean larval weight was detected between parental (456LS3 and Ben) strains compared to heterozygous (456F and 456M) strains when feeding on non-Bt corn leaf tissue ($p < 0.05$). Weights of larvae from the 456LS3 strain were not significantly different when fed on non-Bt or Cry1F corn leaves ($p = 0.9994$).

Table 1
Susceptibility of *S. frugiperda* lines to Cry1F protoxin in diet incorporation bioassays.

Strain	LC50 (µg/cm^2)	95% CI[a] (lower- upper)	Slope ± SE
Ben	0.52	0.39–0.71	1.90 ± 0.20
456LS3[b]	NA	NA	0.02 ± 0.00
456M	0.46	0.38–0.56	2.20 ± 0.30
456F	0.62	0.52–0.75	1.64 ± 0.18

[a] CI = confidence intervals.
[b] Mortality for 456LS3 was < 50% at the highest Cry1F protoxin concentration tested.

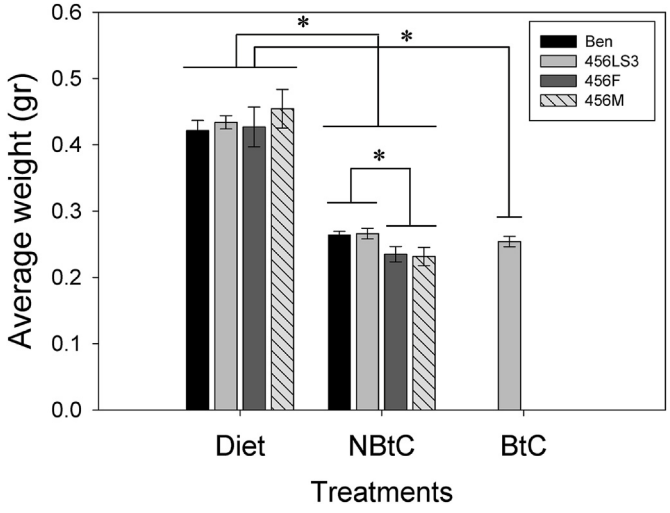

Fig. 1. Average weight of *Spodoptera frugiperda* neonates fed on diet (Diet), corn event TC1507 producing Cry1F (BtC) or the non-transgenic isoline 2T777 (NBtC) until reaching 4th instar. Shown are the average larval weight and the corresponding standard error from 4 biological replicates with 5 larvae each per treatment (n = 20). Data was analyzed using ANOVA, significant differences in comparisons connected with lines ($p < 0.05$) are denoted with an asterisk.

3.2. Metabolomics and multivariate data analysis

Untargeted metabolomic profiling analyses identified a large number of endogenous metabolites in larval guts, but only 126 were selected for further analysis based on annotation via known exact *m/z* and retention time. These identified metabolites were classified into different categories including amino acids, carbohydrates, organic acids, nucleotides, nucleosides, fatty acids, vitamins and other metabolites. The cellular pathways related to these metabolites are represented in Supplementary Fig. 1. As expected from an analysis that was focused on polar metabolites and that preferentially annotates small hydrophilic compounds, pathways with metabolites detected included metabolism of nucleotides, energy, carbohydrates, cofactors and vitamins as well as biosynthesis of secondary metabolites and degradation of xenobiotics. No metabolites related to lipid, glycan or terpenoid metabolism were identified, due to their hydrophobic nature.

In general, the majority of detected metabolite levels were higher in 456LS3 compared to the other strains (data not shown). The metabolite profiles of all *S. frugiperda* strains fed on either meridic diet (shorten as diet) or non-Bt corn (shorten as NBtC) were evaluated using multivariate analysis (Fig. 2). For each strain, the metabolite profiles were represented using a Partial Least Squares Discriminate Analysis (PLSDA) scores plot (Fig. 2A and B). In these PLSDA plots, the 456LS3 and Ben strains clearly displayed unique metabolite profiles from one another, while the heterozygous strains (456F and 456M) did not separate in the PLSDA and formed a third group distinct from Ben and 456LS3. These differences appeared clearer in metabolites from insects fed on meridic diet (Fig. 2A) than when fed on non-transgenic corn (Fig. 2B).

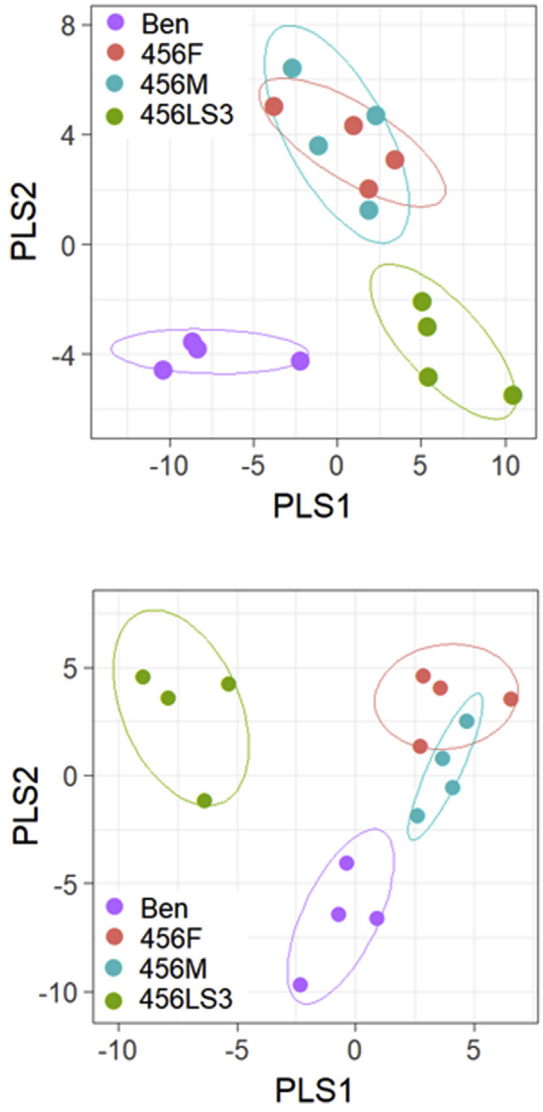

Fig. 2. Partial Least Squares Discriminate Analysis (PLSDA) scores plots displaying different clusters when comparing metabolite profiles of midguts from *S. frugiperda* larvae from different strains fed on meridic diet (Upper panel) or non-Bt corn leaf tissue (Lower panel).

In order to determine which metabolites drove the separation among the groups, the metabolites with Variable Importance in Projection (VIP) scores > 1 were further examined as this VIP score indicates that the metabolite contributes to the group differentiation. When fed the meridic diet, 51 metabolites had significant VIP scores and resulted in a clear separation of the three unique groups: 456LS3, 456F/456M, and Ben (Supplementary Table 1). The same grouping of strains was observed using metabolites detected when larvae were fed on the non-Bt corn leaf tissue, with 63 metabolites with significant VIP scores (Supplementary Table 1). Twenty of the metabolites with significant VIP scores were common to the meridic diet and non-Bt corn (Table 2), although the VIP scores varied for the same metabolite depending on treatment. Considering the total number of metabolites with significant VIP scores for each treatment, 43% of the important metabolites for the strain separation on artificial diet were shared with the non-Bt leaf treatment, while 35% of the significant metabolites for the non-Bt leaf treatment were shared with the artificial diet.

Table 2
Metabolites with VIP[a] score > 1 that support differentiation of Ben, 456M/456F and 456LS3 samples in the PLSDA multivariate space regardless of the food source. Shown are VIP scores for samples from larvae fed on diet (Diet) and non-Bt corn leaf (NBtC).

Metabolite	Diet	NBtC
Aconitate	1.13	1.27
Allantoate	1.16	1.11
Cysteine	1.43	1.01
D.Gluconate	1.69	1.06
dAMP	1.21	1.09
dCMP	1.24	1.18
dTMP	1.15	1.10
Gluconolactone	1.20	1.00
Hydroxyisocaproic.acid	1.01	1.34
Hydroxyphenylacetate	1.43	1.30
Inosine	1.21	1.28
N-Acetylglutamine	1.39	1.23
Pantothenate	1.31	1.13
S-Ribosyl L-homocysteine	1.19	1.28
Sedoheptulose 1/7-phosphate	1.38	1.07
Trehalose.6.phosphate	1.34	1.26
UDP N-acetylglucosamine	1.02	1.02
Uracil	1.30	1.03
Uric acid	1.18	1.23
Uridine	1.01	1.02

[a] VIP, Variable Importance in Projections scores were obtained from PLSDA using the DiscriMiner software package.

3.3. Comparative metabolite profiling

Comparison of the relative levels for all detected metabolites in the Ben, 456M, and 456F compared to the 456LS3 gut metabolome for larvae fed on meridic diet (Fig. 3) or non-Bt corn leaf tissue (Fig. 4) identified metabolites with increased and reduced levels associated to the 456LS3 sample. Given that metabolome differences may result from differential metabolic states in larvae, and that we detected small but significant weight differences between parental and F1 lines when feeding on corn leaf tissue (Fig. 1), we focused metabolite profiling on all four strains fed on meridic diet, on Ben and 456LS3 fed on non-Bt corn, and on 456LS3 when fed on Cry1F corn compared to non-Bt corn.

Out of the total 126 metabolites identified, only 7 metabolites were found to display statistically significant ($p < 0.05$) altered levels (Table 3) when comparing metabolite patterns between susceptible (Ben, 456M and 456F) and Cry1F-resistant (456LS3) larvae fed on meridic diet. Altered metabolites included organic acids (hydroxyphenylacetate), amino acids (asparagine), nucleosides (dCMP, dTMP, and dAMP), and carbohydrates (trehalose 6-phosphate and sedoheptulose 1/7-phosphate). When comparing abundance, only hydroxyphenylacetate appeared to be at lower levels in the guts of 456LS3 larvae, while the other 6 metabolites significantly accumulated in 456LS3 compared to the other strains.

In the case of the gut metabolome samples fed on non-Bt corn leaf tissue (Fig. 4 and Table 4), we observed five metabolites with significantly different levels ($p < 0.05$). Three of these metabolites, two fatty acids (3-hydroxyisovaleric acid and hydroxyisocaproic acid), and an organic compound (phenyllactic acid) were found at significant lower levels in the 456LS3 compared to all other larval guts, while amino acid derivative 3-phosphoserine and fatty acid 2-isopropylmalate accumulated in the guts of resistant compared to larvae of the Ben, 456M and 456F strains (Table 4).

Metabolomic profiling of larval guts from the 456LS3 strain fed on Cry1F-producing corn and non-Bt corn leaf tissue identified 7 metabolites with distinct abundance between the two treatments. All these metabolites significantly accumulated in the guts when the larvae fed on the non-Bt isoline, and included metabolites from diverse metabolic pathways (Table 5).

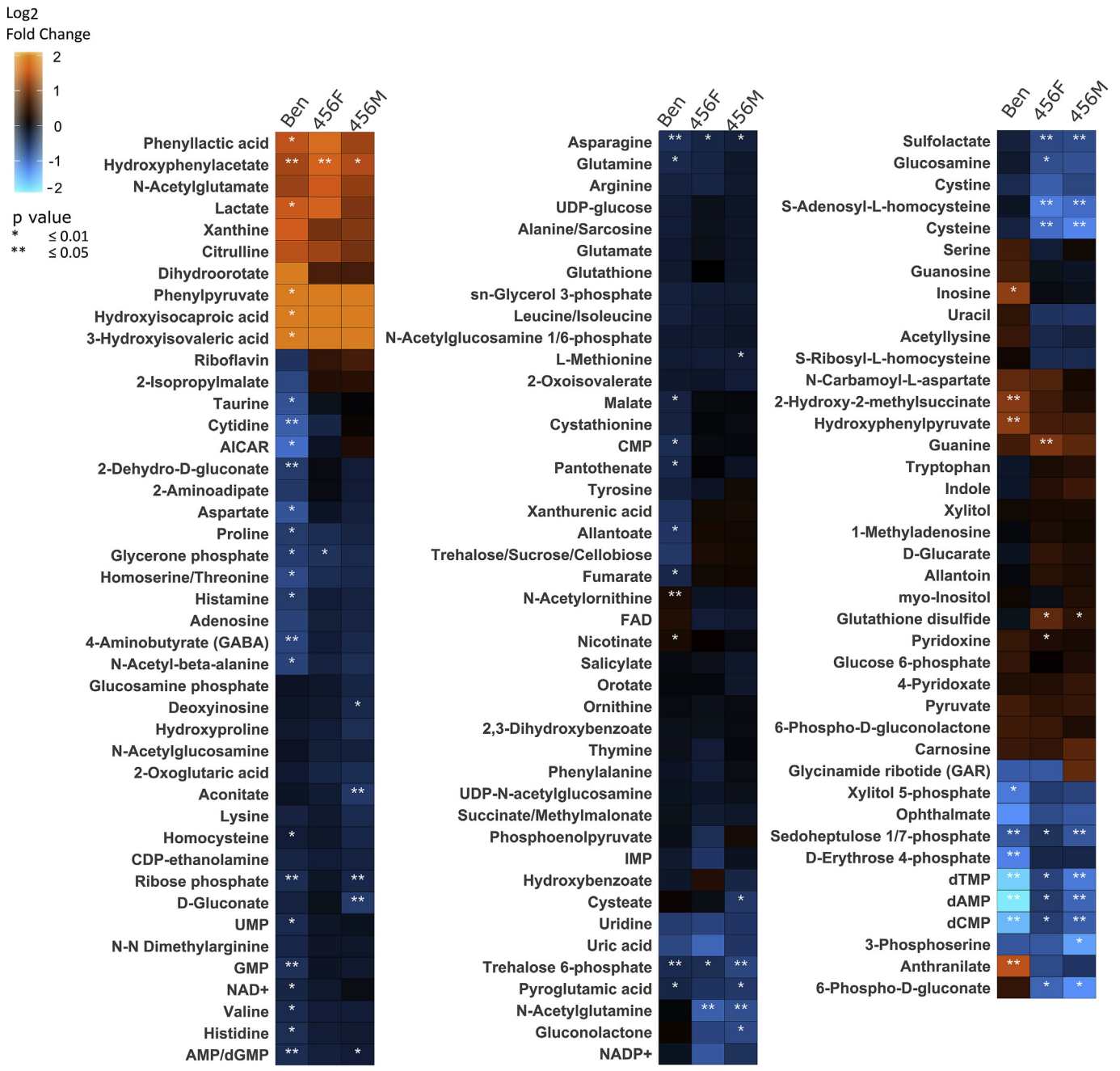

Fig. 3. Heat maps of relative levels of metabolites detected in guts of *S. frugiperda* larvae fed on meridic diet. The specific larvae compared in each column are shown at the top. Data are $\log_2$ ratios of metabolites identified when comparing metabolomes from Ben, 456F, or 456M to metabolomes in guts of larvae from the 456*LS*3 strain. The relative fold levels for each metabolite in each comparison are shown by the key, orange indicates induction > 0, blue indicates repression < 0, with maximums at > 2 and < −2. Asterisks represent level of significance (*$p < 0.05$, **$p < 0.01$), as noted in the key. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

Members of the ABC transporter gene family are involved in cellular defense against xenobiotics, including insecticides (Porretta et al., 2016). Despite their functional diversity (Locher, 2016), generally ABC proteins actively export xenobiotics from cells to reduce their intracellular concentration. Selected ABC transporter genes have been reported as functional receptors for Cry toxins in insects (Banerjee et al., 2017; Bretschneider et al., 2016; Endo et al., 2017; Tanaka et al., 2013), and mutations in these genes or alterations in their expression are linked to resistance against diverse Cry toxins (Banerjee et al., 2017; Baxter et al., 2011; Fabrick et al., 2014; Xiao et al., 2014). Given their ATP-driven transport activity, our hypothesis was that mutations affecting functionality of ABC transporter genes would result in alterations in the intracellular metabolome. In support of this hypothesis, previous reports demonstrated that a truncated ABCC2 protein in Cry1Ac-resistant *Helicoverpa armigera* larvae resulted in accumulation and altered susceptibility to selected synthetic pesticides (Xiao et al., 2016). In testing our hypothesis, we performed a comparative metabolite analysis between the midgut epithelium from a Cry1F-resistant strain of *S. frugiperda* homozygous for a mutation in an ABC subfamily C2 gene (*SfABCC2mut*) predicted to result in a truncated non-functional ABCC2 protein (Banerjee et al., 2017), and larvae with the wild type (*SfABCC2*) genotype or heterozygous for the *SfABCC2mut* allele.

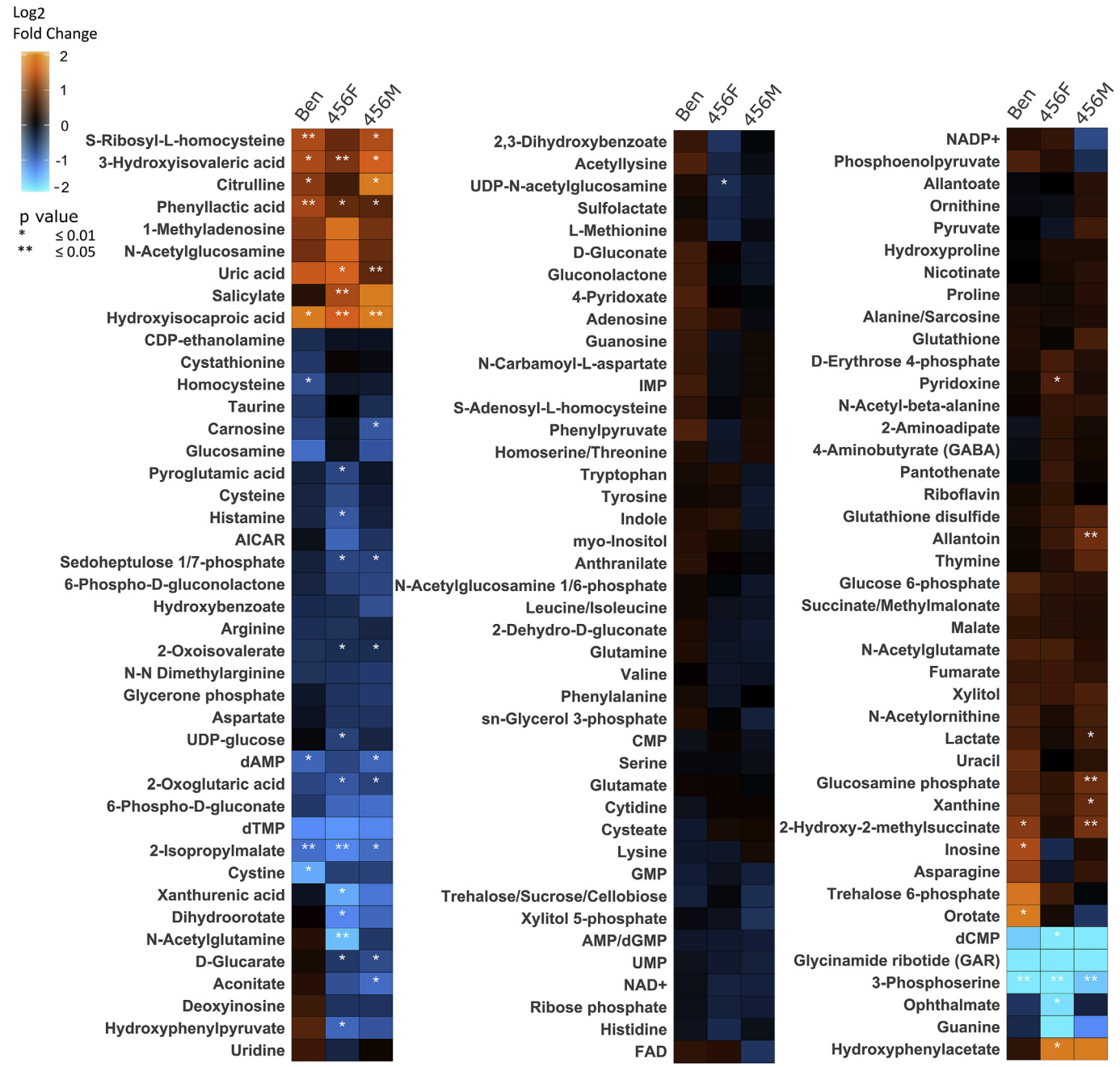

Fig. 4. Heat maps of relative levels of metabolites detected in guts of *S. frugiperda* larvae fed on non-Bt corn leaf tissue. The specific larvae compared in each column are shown at the top. Data are $\log_2$ ratios of metabolites identified when comparing metabolomes from Ben, 456F, or 456M to metabolomes in guts of larvae from the 456LS3 strain. The relative fold levels for each metabolite in each comparison are shown by the key, orange indicates induction > 0, blue indicates repression < 0, with maximums at > 2 and < -2. Asterisks represent level of significance ($^*p < 0.05$, $^{**}p < 0.01$), as noted in the key. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Results from bioassays with Cry1F protoxin were in agreement with previous reports (Jakka et al., 2015) and supported high levels of resistance in the 456LS3 strain, and that resistance is transmitted as a recessive trait. Reduced larval weights in larvae fed corn versus meridic diet, independently of the *S. frugiperda* strain, are in agreement with optimized nutritional value and adaptation of the strains to meridic diet. Lack of significant effects of Cry1F-corn compared to non-transgenic corn on 456LS3 larvae support no fitness costs associated with the *SfABCC2mut* allele, as previously reported (Jakka et al., 2014). Given the significant differences between meridic diet and non-transgenic corn treatments, we considered metabolome comparisons between the strains for both diets independently. Moreover, since parental and F1 strains displayed significantly different weight when grown on non-Bt corn tissue, we focus metabolic profiling to Ben-456LS3 comparisons for that treatment. It's interesting to note that we found a clear separation of the Ben, 456F/M and 456LS3 groups in the multivariate analysis (PLSDA), but there were only seven metabolites categorized as significant in the comparative analysis. This could point to multiple small changes in a suite of metabolites being related to the resistant phenotype; indicating that a measurement of a set of metabolites would be necessary to differentiate between the phenotypes.

While there is a lack of information on substrate specificity for ABCC2 proteins in insects, much is known in similar proteins in mammals. In BLASTp searches of the mammalian subset of proteins in

Table 3
Metabolites with significantly altered levels ($p < 0.05$) in Ben, 456M and 456F lines compared to larvae from the 456LS3 strain fed on meridic diet. Shown are the fold change (FC) and corresponding p-value and VIP scores. Row shaded in black with white font represents lower metabolite levels in guts of larvae from the 456LS3 compared to other strains. All the other metabolites accumulated in guts from larvae of the 456LS3 compared to other strains tested.

	Ben/456LS3		456F/456LS3		456M/456LS3	
Metabolite	**FC* (p-value)**	**VIP**	**FC (p-value)**	**VIP**	**FC (p-value)**	**VIP**
Organic acids						
Hydroxyphenylacetate	2.51 (0.007)	1.40	3.23 (0.002)	1.82	2.62 (0.044)	1.45
Amino acids						
Asparagine	0.73 (0.002)	1.31	0.79 (0.029)	1.43	0.82 (0.015)	1.35
Nucleosides						
dCMP	0.34 (0.001)	1.43	0.65 (0.016)	1.48	0.53 (0.002)	1.60
dTMP	0.31 (0.004)	1.36	0.63 (0.047)	1.31	0.46 (0.009)	1.50
dAMP	0.28 (0.001)	1.40	0.67 (0.027)	1.42	0.51 (0.006)	1.55
Carbohydrates / conjugates						
Trehalose 6-phosphate	0.76 (0.005)	1.26	0.74 (0.007)	1.42	0.62 (0.0003)	1.61
Sedoheptulose 1/7 phosphate	0.55 (0.0002)	1.45	0.72 (0.023)	1.50	0.56 (0.0001)	1.66

*Fold changes (FC) were calculated by dividing the value of metabolites in larvae receiving meridic diet of Ben, 456M and 456F lines by 456LS3 line.

NCBI, the most similar proteins to SfABCC2 are members of the multidrug resistance-associated protein 4 (MRP4) group (maximum 41% identity, 0.0 Evalue), which are unidirectional transporters localized in diverse tissues and involved in resistance to xenobiotics (Belinsky et al., 2007). Substrates of MRP4 include nucleotide analogues, glucuronide and sulphate conjugates, prostaglandins, methotrexate, and bile acids (Saidijam et al., 2018). There is also evidence for specificity towards anionic conjugates, including N-acetyl-(2,4-dinitrophenyl)-cysteine (van Aubel et al., 2002). In agreement with these observations, half of the metabolites accumulating in 456LS3 compared to Ben or F1 larvae when fed meridic diet were nucleosides (dCMP, dTMP, dAMP). Although preliminary, these observations support that lack of a functional SfABCC2 may result in accumulation of metabolites (nucleosides and cysteine derivatives) that may be otherwise transported out of the cell by this protein. In mammals transport of nucleosides is a major feature of MRP4 proteins (Saidijam et al., 2018), and this may also be the case for the orthologous SfABCC2. Other metabolites accumulated in 456LS3 larvae lacking a functional SfABCC2 were carbohydrates (trehalose 6-phosphate and sedoheptulose 1/7-phosphate) and an amino acid (asparagine), which have not been described as substrates for MRP4. Interestingly, different metabolites were found to accumulate in 456LS3 compared to wild type larvae fed meridic diet and non-Bt corn leaf tissue. This observation suggests important nutritional and metabolite differences based on diet, which is reflected in the significant differences detected in larval weight between the diet treatments. Differences in metabolite composition between meridic diet and corn leaf tissue may have prevented detection of common significant differences between susceptible and resistant larvae when fed on these two diverse diets.

Interestingly, we detected 7 metabolites that were at lower levels in guts of larvae from the 456LS3 strain when they fed on Cry1F-producing corn compared to the non-Bt isoline. In this case, metabolic alterations detected would reflect interactions between Cry1F and the midgut epithelium that in 456LS3 are not conducive to toxicity. In fact, while Cry1F binding to the SfABCC2mut protein from 456LS3 larvae is not detected (Banerjee et al., 2017), the toxin still displays (albeit reduced) binding to midgut brush border membrane vesicles (BBMV) from 456LS3 compared to Ben (Jakka et al., 2015), suggesting the potential for Cry1F interacting with midgut proteins. While it is complex to discern the physiological relevance of the detected metabolome changes in that case, it is interesting to note the reduced levels of components of the TCA cycle (2-oxoglutaric acid) and glutathione metabolism (cystine and pyroglutamic acid), which may suggest alterations on pathways related to energy conservation and reduced

Table 5
Metabolites with significantly altered levels ($p < 0.05$) in guts from 456LS3 larvae fed leaf tissue from Cry1F-producing or the non-Bt corn isoline. Shown are the fold change (FC) and corresponding p-value and VIP scores.

Metabolite	FC (p-value)	VIP
Amino acid metabolism		
3-Phosphoserine	0.30 (0.007)	1.69
Cystine	0.41 (0.026)	1.68
Purine metabolism		
AICA ribonucleotide	0.63 (0.041)	1.39
TCA cycle		
2-Oxoglutaric acid	0.50 (0.009)	1.66
Glutathione metabolism		
Pyroglutamic acid	0.61 (0.018)	1.61
N-Acetyl-beta-alanine metabolism		
N-Acetyl-beta-alanine	0.61 (0.031)	1.51
Valine biosynthesis		
2-Oxoisovalerate	0.68 (0.047)	1.39

*Fold changes (FC) were calculated by dividing the value of metabolites of 456LS3 larvae receiving Bt-corn by 456LS3 line fed on non-Bt corn.

Table 4
Metabolites with significantly altered levels ($p < 0.05$) in Ben, 456F and 456M when compared to 456LS3 larvae fed non-Bt corn leaf tissue. Shown are the fold change (FC) and corresponding p-value and VIP scores. Rows shaded in black with white font represents lower metabolite levels in guts of larvae from the 456LS3 compared to other strains. All the other metabolites accumulated in guts from larvae of the 456LS3 compared to other strains tested.3

	Ben/456LS3		456F/456LS3		456M/456LS3	
Metabolite	**FC (p-value)**	**VIP**	**FC (p-value)**	**VIP**	**FC (p-value)**	**VIP**
Fats and fatty acids						
3-Hydroxyisovaleric acid	2.54 (0.020)	1.67	2.03 (0.009)	1.60	3.05 (0.014)	1.67
Hydroxyisocaproic acid	6.75 (0.026)	1.76	6.06 (0.000)	1.76	6.20 (0.004)	1.79
Organic acids and derivatives						
Phenyllactic acid	2.43 (0.003)	1.75	1.80 (0.014)	1.54	1.69 (0.044)	1.37
2-Isopropylmalate	0.44 (0.008)	1.72	0.39 (0.007)	1.60	0.45 (0.039)	1.33
Amino acids and derivatives						
3-Phosphoserine	0.24 (0.007)	1.79	0.11 (0.006)	1.80	0.29 (0.006)	1.59

oxidative stress when feeding on Cry1F corn. Although speculative, reduced levels of AICA ribonucleotide, which in hepatocytes has been shown to participate in activation of AMP-activated protein kinase and inhibition of autophagy (Meijer and Codogno, 2006), may suggest increased autophagy in 456LS3 when feeding on Cry1F-corn compared to the non-transgenic isoline. Further work to identify the pathways involved in these metabolome differences would be needed to identify physiological effects of feeding on Bt versus non-transgenic corn by Bt-resistant larvae.

In summary, we used comparative metabolomics on the midgut epithelium of susceptible and Cry1F-resistant *S. frugiperda* larvae in an attempt to identify candidate metabolites transported by SfABCC2, a transporter putatively involved in xenobiotic transport outside of the cell. Importantly, as far as we know this work represents the first metabolomics analysis of an insect midgut epithelium, and provides a list of candidate metabolites that accumulate in Cry1F-resistant *S. frugiperda* lacking a functional SfABCC2 protein. Further work is needed to determine if any of these metabolites is a substrate for SfABCC2 transport. Specifically, nucleosides are good candidates as they accumulate in Cry1F-resistant larvae and are metabolites selectively and uniquely transported by homologous proteins to SfABCC2 in mammals. It is important to note that we found diverse metabolomic results depending on the diet used (meridic versus corn leaf) to rear the larvae, which suggests that diet has an important impact on the gut metabolome. Further functional assays are needed to confirm substrate specificity for SfABCC2 and to identify metabolites that may serve as markers for resistance to Cry toxins when linked to mutations altering ABC transporter function.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2019.01.002.

References

Adang, M., Crickmore, N., Jurat-Fuentes, J.L., 2014. Diversity of *Bacillus thuringiensis* crystal toxins and mechanism of action. In: In: Dhadialla, T.S., Gill, S. (Eds.), Advances in Insect Physiology, vol. 47. Academic Press, San Diego, CA, pp. 39–87 Insect Midgut and Insecticidal Proteins.

Atsumi, S., Miyamoto, K., Yamamoto, K., Narukawa, J., Kawai, S., Sezutsu, H., Kobayashi, I., Uchino, K., Tamura, T., Mita, K., Kadono-Okuda, K., Wada, S., Kanda, K., Goldsmith, M.R., Noda, H., 2012. Single amino acid mutation in an ATP-binding cassette transporter gene causes resistance to Bt toxin Cry1Ab in the silkworm, *Bombyx mori*. Proc. Natl. Acad. Sci. U.S.A. 109, E1591–E1598.

Banerjee, R., Hasler, J., Meagher, R., Nagoshi, R., Hietala, L., Huang, F., Narva, K., Jurat-Fuentes, J.L., 2017. Mechanism and DNA-based detection of field-evolved resistance to transgenic Bt corn in fall armyworm (*Spodoptera frugiperda*). Sci. Rep. 7, 10877.

Baxter, S.W., Badenes-Perez, F.R., Morrison, A., Vogel, H., Crickmore, N., Kain, W., Wang, P., Heckel, D.G., Jiggins, C.D., 2011. Parallel evolution of *Bacillus thuringiensis* toxin resistance in Lepidoptera. Genetics 189, 675–679.

Belinsky, M.G., Guo, P., Lee, K., Zhou, F., Kotova, E., Grinberg, A., Westphal, H., Shchaveleva, I., Klein-Szanto, A., Gallo, J.M., Kruh, G.D., 2007. Multidrug resistance protein 4 protects bone marrow, thymus, spleen, and intestine from nucleotide analogue-induced damage. Cancer Res. 67, 262–268.

Bretschneider, A., Heckel, D.G., Pauchet, Y., 2016. Three toxins, two receptors, one mechanism: mode of action of Cry1A toxins from *Bacillus thuringiensis* in *Heliothis virescens*. Insect Biochem. Mol. Biol. 76, 109–117.

Chandrasena, D.I., Signorini, A.M., Abratti, G., Storer, N.P., Olaciregui, M.L., Alves, A.P., Pilcher, C.D., 2018. Characterization of field-evolved resistance to *Bacillus thuringiensis*-derived Cry1F delta-endotoxin in *Spodoptera frugiperda* populations from Argentina. Pest Manag. Sci. 74 (3), 746–754.

Clasquin, M.F., Melamud, E., Rabinowitz, J.D., 2012. LC-MS data processing with MAVEN: a metabolomic analysis and visualization engine. Curr. Protoc. Bioinformatics Chapter 14 Unit14 11.

Day, R., Abrahams, P., Bateman, M., Beale, T., Clottey, V., Cock, M., Colmenarez, Y., Corniani, N., Early, R., Godwin, J., Gomez, J., Moreno, P.G., Murphy, S.T., Oppong-Mensah, B., Phiri, N., Pratt, C., Silvestri, S., Witt, A., 2017. Fall armyworm: impacts and implications for Africa. Out. Pest Manag. 28, 196–201.

Dermauw, W., Van Leeuwen, T., 2014. The ABC gene family in arthropods: comparative genomics and role in insecticide transport and resistance. Insect Biochem. Molec 45, 89–110.

de Hoon, M.J., Imoto, S., Nolan, J., Miyano, S., 2004. Open source clustering software. Bioinformatics 20, 1453–1454.

Endo, H., Tanaka, S., Imamura, K., Adegawa, S., Kikuta, S., Sato, R., 2017. Cry toxin specificities of insect ABCC transporters closely related to lepidopteran ABCC2 transporters. Peptides 98, 86–92.

Fabrick, J.A., Ponnuraj, J., Singh, A., Tanwar, R.K., Unnithan, G.C., Yelich, A.J., Li, X., Carriere, Y., Tabashnik, B.E., 2014. Alternative splicing and highly variable cadherin transcripts associated with field-evolved resistance of pink bollworm to Bt cotton in India. PLoS One 9, e97900.

Farias, J.R., Andow, D.A., Horikoshi, R.J., Sorgatto, R.J., Fresia, P., dos Santos, A.C., Omoto, C., 2014. Field-evolved resistance to Cry1F maize by *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Brazil. Crop Protect. 64, 150–158.

Farrés, M., Platikanov, S., Stefan, Tsakovski, Tauler, R., 2015. Comparison of the variable importance in projection (VIP) and of the selectivity ratio (SR) methods for variable selection and interpretation. J. Chemometr. 29, 528–536.

Gahan, L.J., Pauchet, Y., Vogel, H., Heckel, D.G., 2010. An ABC transporter mutation is correlated with insect resistance to *Bacillus thuringiensis* Cry1Ac toxin. PLoS Genet. 6, e1001248.

Goergen, G., Kumar, P.L., Sankung, S.B., Togola, A., Tamò, M., 2016. First report of outbreaks of the fall armyworm *Spodoptera frugiperda* (J E Smith) (Lepidoptera, Noctuidae), a new alien invasive pest in West and Central Africa. PLoS One 11 (10), e0165632.

Gouffon, C., Van Vliet, A., Van Rie, J., Jansens, S., Jurat-Fuentes, J.L., 2011. Binding sites for *Bacillus thuringiensis* Cry2Ae toxin on heliothine brush border membrane vesicles are not shared with Cry1A, Cry1F, or Vip3A toxin. Appl. Environ. Microbiol. 77, 3182–3188.

Huang, F., Qureshi, J.A., Meagher Jr., R.L., Reisig, D.D., Head, G.P., Andow, D.A., Ni, X., Kerns, D., Buntin, G.D., Niu, Y., Yang, F., Dangal, V., 2014. Cry1F resistance in fall armyworm *Spodoptera frugiperda*: single gene versus pyramided Bt maize. PLoS One 9, e112958.

Jakka, S.R., Gong, L., Hasler, J., Banerjee, R., Sheets, J.J., Narva, K., Blanco, C.A., Jurat-Fuentes, J.L., 2015. Field-evolved Mode 1 resistance of the fall armyworm to transgenic Cry1Fa-expressing corn associated with reduced Cry1Fa toxin binding and midgut alkaline phosphatase expression. Appl. Environ. Microbiol. 82, 1023–1034.

Jakka, S.R., Knight, V.R., Jurat-Fuentes, J.L., 2014. Fitness costs associated with field-evolved resistance to Bt maize in *Spodoptera frugiperda* (Lepidoptera: Noctuidae). J. Econ. Entomol. 107, 342–351.

Jemnitz, K., Heredi-Szabo, K., Janossy, J., Ioja, E., Vereczkey, L., Krajcsi, P., 2010. ABCC2/Abcc2: a multispecific transporter with dominant excretory functions. Drug Metab. Rev. 42, 402–436.

LeOra, S., 1987. Polo-plus, POLO for Windows. LeOra Software, Petaluma, CA.

Li, X.Z., Yang, X.Y., Wang, Y., Zhang, S.N., Zou, W., Wang, Y., Li, X.N., Wang, L.S., Zhang, Z.G., Xie, L.Z., 2017. Urine metabolic profiling for the pathogenesis research of erosive oral lichen planus. Arch. Oral Biol. 73, 206–213.

Locher, K.P., 2016. Mechanistic diversity in ATP-binding cassette (ABC) transporters. Nat. Struct. Mol. Biol. 23, 487–493.

Lu, W., Clasquin, M.F., Melamud, E., Amador-Noguez, D., Caudy, A.A., Rabinowitz, J.D., 2010. Metabolomic analysis via reversed-phase ion-pairing liquid chromatography coupled to a stand alone orbitrap mass spectrometer. Anal. Chem. 82, 3212–3221.

Meijer, A.J., Codogno, P., 2006. Signalling and autophagy regulation in health, aging and disease. Mol. Aspect. Med. 27, 411–425.

Melamud, E., Vastag, L., Rabinowitz, J.D., 2010. Metabolomic analysis and visualization engine for LC-MS data. Anal. Chem. 82, 9818–9826.

Monnerat, R., Martins, E., Macedo, C., Queiroz, P., Praça, L., Soares, C.M., Moreira, H., Grisi, I., Silva, J., Soberon, M., Bravo, A., 2015. Evidence of field-evolved resistance of *Spodoptera frugiperda* to Bt corn expressing Cry1F in Brazil that is still sensitive to modified Bt toxins. PLoS One 10, e0119544.

Nagoshi, R.N., Fleischer, S., Meagher, R.L., Hay-Roe, M., Khan, A., Murua, M.G., Silvie, P., Vergara, C., Westbrook, J., 2017. Fall armyworm migration across the Lesser Antilles and the potential for genetic exchanges between North and South American populations. PLoS One 12, e0171743.

Park, Y., Gonzalez-Martinez, R.M., Navarro-Cerrillo, G., Chakroun, M., Kim, Y., Ziarsolo, P., Blanca, J., Canizares, J., Ferre, J., Herrero, S., 2014. ABCC transporters mediate insect resistance to multiple Bt toxins revealed by bulk segregant analysis. BMC Biol. 12, 46.

Porretta, D., Epis, S., Mastrantonio, V., Ferrari, M., Bellini, R., Favia, G., Urbanelli, S., 2016. How heterogeneous is the involvement of ABC transporters against insecticides? Acta Trop. 157, 131–135.

Rabinowitz, J.D., Kimball, E., 2007. Acidic acetonitrile for cellular metabolome extraction from *Escherichia coli*. Anal. Chem. 79, 6167–6173.

Saidijam, M., Karimi Dermani, F., Sohrabi, S., Patching, S.G., 2018. Efflux proteins at the blood–brain barrier: review and bioinformatics analysis. Xenobiotica 48, 506–532.

Saldanha, A.J., 2004. Java Treeview–extensible visualization of microarray data. Bioinformatics 20, 3246–3248.

Sanchez, G., 2013. Package 'DiscriMiner'. https://CRAN.R-project.org/package=DiscriMiner.

Siebert, M.W., Babock, J.M., Nolting, S.P., Santos, A.C., Adamczyk, J.J., Neese, P.A., King,

J.E., jenkins, J.N., McCarty, J., Lorenz, G.M., Fromme, D.D., Lassiter, R.B., 2008. Efficacy of Cry1F insecticidal protein in maize and cotton for control of fall armyworm (Lepidoptera: Noctuidae). Fla. Entomol. 91, 555–565.

Storer, N.P., Babcock, J.M., Schlenz, M., Meade, T., Thompson, G.D., Bing, J.W., Huckaba, R.M., 2010. Discovery and characterization of field resistance to Bt maize: *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Puerto Rico. J. Econ. Entomol. 103, 1031–1038.

Tague, E.D., Bourdon, A.K., MacDonald, A., Lookadoo, M.S., Kim, E.D., White, W.M., Terry, P.D., Campagna, S.R., Voy, B.H., Whelan, J., 2018. Metabolomics approach in the study of the well-defined polyherbal preparation Zyflamend. J. Med. Food 21, 306–316.

Tanaka, S., Miyamoto, K., Noda, H., Jurat-Fuentes, J.L., Yoshizawa, Y., Endo, H., Sato, R., 2013. The ATP-binding cassette transporter subfamily C member 2 in *Bombyx mori* larvae is a functional receptor for Cry toxins from *Bacillus thuringiensis*. FEBS J. 280, 1782–1794.

van Aubel, R.A.M.H., Smeets, P.H.E., Peters, J.G.P., Bindels, R.J.M., Russel, F.G.M., 2002. The MRP4/ABCC4 gene encodes a novel apical organic anion transporter in human kidney proximal tubules: putative efflux pump for urinary cAMP and cGMP. J. Am. Soc. Nephrol. 13, 595–603.

Xia, J., Wishart, D.S., 2011. Web-based inference of biological patterns, functions and pathways from metabolomic data using MetaboAnalyst. Nat. Protoc. 6, 743–760.

Xiao, Y., Zhang, T., Liu, C., Heckel, D.G., Li, X., Tabashnik, B.E., Wu, K., 2014. Mis-splicing of the ABCC2 gene linked with Bt toxin resistance in *Helicoverpa armigera*. Sci. Rep. 4, 6184.

Xiao, Y., Liu, K., Zhang, D., Gong, L., He, F., Soberón, M., Bravo, A., Tabashnik, B.E., Wu, K., 2016. Resistance toBacillus thuringiensis mediated by an ABC Transporter mutation increases susceptibility to toxins from other bacteria in an invasive insect. PLoS pathog 12 (2) e1005450.

Yamada, T., Letunic, I., Okuda, S., Kanehisa, M., Bork, P., 2011. iPath2.0: interactive pathway explorer. Nucleic Acids Res. 39, W412–W415.

Zhao, C., Jurat-Fuentes, J.L., Abdelgaffar, H.M., Pan, H., Song, F., Zhang, J., 2015. Identification of a new cry1I-type gene as a candidate for gene pyramiding in corn to control *Ostrinia* species larvae. Appl. Environ. Microbiol. 81, 3699–3705.