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Quantitative trait locus mapping and functional genomics of an organophosphate resistance trait in the western corn rootworm, *Diabrotica virgifera virgifera*

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Abstract

The western corn rootworm, *Diabrotica virgifera virgifera*, is an insect pest of corn and population suppression with chemical insecticides is an important management tool. Traits conferring organophosphate insecticide resistance have increased in frequency amongst *D. v. virgifera* populations, resulting in the reduced efficacy in many corn-growing regions of the USA. We used comparative functional genomic and quantitative trait locus (QTL) mapping approaches to investigate the genetic basis of *D. v. virgifera* resistance to the organophosphate methyl-parathion. RNA from adult methyl-parathion resistant and susceptible adults was hybridized to 8331 microarray probes. The results predicted that 11 transcripts were significantly up-regulated in resistant phenotypes, with the most significant (fold increases ≥ 2.43) being an α -esterase-like transcript. Differential expression was validated only for the α -

esterase (ST020027A20C03), with 11- to 13-fold greater expression in methyl-parathion resistant adults ($P < 0.05$). Progeny with a segregating methyl-parathion resistance trait were obtained from a reciprocal backcross design. QTL analyses of high-throughput single nucleotide polymorphism genotype data predicted involvement of a single genome interval. These data suggest that a specific carboxy-esterase may function in field-evolved corn rootworm resistance to organophosphates, even though direct linkage between the QTL and this locus could not be established.

Keywords: pesticide resistance, methyl-parathion resistance, genome mapping, functional genomics.

Introduction

Development of functional resistance to chemical insecticides amongst pest insect populations results in increased survival at field-applied rates, and is a threat to agricultural production owing to the diminished efficacy of control (Knight, 1989; Rouche & Tabashnik, 1990). The endemic range of the western corn rootworm, *Diabrotica virgifera virgifera*, north of Mexico was restricted to the high plains of the USA, including western Kansas and eastern Colorado, where larvae probably fed on native prairie grasses (Branson & Ortman, 1967; Oyediran *et al.*, 2004). Increased planting of farmland in continuous corn during the 1940s was probably a factor contributing to the eastward range expansion of *D. v. virgifera* across the Corn Belt, because it allowed increased population densities, producing greater numbers of migrants and increasing the probability of survival of founder offspring (Gray *et al.*, 2009; Meinke *et al.*, 2009). The invasion front approached the Missouri River by 1960, after which *D. v. virgifera* rapidly expanded across most of the US Corn Belt by 1980, and completed invasion of many East Coast states by the early 1990s (Metcalf, 1983, 1986; Gray *et al.*, 2009; Meinke *et al.*, 2009). Economic losses are inflicted mainly by

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D. v. virgifera larvae feeding on corn roots, which negatively affects plant stability as well as water and nutrient absorption (Chiang, 1973; Kahler *et al.*, 1985; Urias-Lopez & Meinke, 2001). In some circumstances, adults can cause economic damage by feeding on silks and tassels for extended periods, which can reduce pollination success and introduce plant pathogens (Jensen, 1985; Gilbertson *et al.*, 1986).

Management of *D. v. virgifera* is often difficult to achieve because of phenotypic resistance to the most effective control tactics including crop rotation, transgenic toxins expressed by the corn plant, and chemical insecticides (Sappington *et al.*, 2006; Gassmann *et al.*, 2011). Crop rotation takes advantage of the *D. v. virgifera* univoltine life cycle, female preference to lay eggs at the base of corn plants and larval specialization for feeding on grass roots (Krysan *et al.*, 1986). Crop rotations in the Midwest typically incorporate corn and nongrass crops (mainly soybeans) in alternating years, which presents roots that cannot support larval development in years following corn crops. This tactic remains effective in suppressing *D. v. virgifera* populations in most regions, but a behavioural adaptation involving a shift in gut bacterial microbiota community structure (Chu *et al.*, 2013) has evolved in Illinois whereby females oviposit outside of corn, usually in soybean fields, thus circumventing rotation as a management tool (Levine & Oloumi-Sadeghi, 1996; Levine *et al.*, 2002; Miller *et al.*, 2009). The genetic basis for this behavioural trait remains elusive (Knolhoff *et al.*, 2010), but continued geographical spread potentially threatens crop rotation-based control in other parts of the US Corn Belt (Sappington *et al.*, 2006). Similarly, *D. v. virgifera* field populations resistant to commercial corn hybrids expressing the *Bacillus thuringiensis* (Bt) toxins Cry3Bb1 and mCry3A have been documented in several Midwestern states (Gassmann *et al.*, 2011, 2014). Widespread adoption of rootworm-targeting Bt corn by growers since its commercialization in 2003 reflects its initial effectiveness in managing this pest (Rice, 2004), and the rapid evolution of resistance is a disturbing, if not wholly unanticipated, development (Porter *et al.*, 2012; Gassmann *et al.*, 2014; Sappington, 2014).

In cases where growers prefer to grow nonrotated corn, chemical insecticides were the principal management approach prior to the introduction of Bt corn. Now that some Bt toxins are faltering, chemical insecticides are again being recruited for supplemental control even in places where Bt resistance is not yet suspected (Porter *et al.*, 2012; Sappington, 2014). Like any tactic, sustained exposure of insect populations to a chemical insecticide selects for resistance (Georghiou & Lagunes-Tejada, 1991), and rootworms are no exception. For example, cyclodiene insecticides were introduced for control of *D. v. virgifera* in the late 1940s (Hill *et al.*,

1948), and high levels of field-evolved resistance were observed by 1959 (Roselle *et al.*, 1959; Ball & Weekman, 1962). The genetic basis of *D. v. virgifera* resistance to the cyclodiene insecticide aldrin is linked to a conserved nonsynonymous (amino acid changing) resistance to dieldrin (*Rdl*) mutation that disrupts binding at the target site of the gamma-aminobutyric acid receptor (Wang *et al.*, 2013). The *Rdl* mutation confers organochlorine resistance in several other arthropod species, suggesting a common genetic basis amongst species (French-Constant *et al.*, 2000).

Populations of *D. v. virgifera* have also evolved resistance to carbamate and organophosphate (OP) insecticides (Metcalf, 1986; Meinke *et al.*, 1998; Miota *et al.*, 1998; Siegfried *et al.*, 2005; Miller *et al.*, 2009). The mode of action for both of these insecticide chemistries involves the inhibition of acetylcholinesterase during synaptic transmission of nerve impulses. Detoxification of carbamates is the major mechanism of resistance in *D. v. virgifera*, involving up-regulation of cytochrome P450 monooxygenase and general esterase activities (Scharf *et al.*, 1999). Similarly, OP resistance in field populations from Phelps and York Counties, Nebraska, is correlated with increased cytosolic esterase activity (Zhou *et al.*, 2002, 2003), and partly attributed to a single 66-kDa group II esterase purified from resistant individuals (Zhou *et al.*, 2004, 2005). Assaying for mutations that cause resistance represents a novel tool that allows the tracking of changes in resistance allele frequencies in field populations (Riveron *et al.*, 2014).

In the current study we used both quantitative trait locus (QTL) mapping and functional genomic approaches to identify genome regions and differentially expressed genes (DEGs) linked to the OP resistance trait in *D. v. virgifera* populations from Phelps and York County, Nebraska. The results from this study are important for understanding how resistance traits accumulate in pest insect populations, and may be important for developing strategies to slow resistance evolution in other pest insects.

Results

Expression analyses

Transcript levels were compared by cohybridization of differentially labelled cDNAs from OP-resistant and -susceptible *D. v. virgifera* populations to 8331 species-specific microarray probes (Knolhoff *et al.*, 2010). Labelling efficiency was within the optimal range, and any signal bias detected from dye-swap hybridization experiments was used to adjust for differences in signal intensities by the University of Illinois, W. M. Keck Center (data not shown). Analysis of probe hybridization intensities indicated significant differences in expression levels of 81 transcripts between male and female *D. v. virgifera* at cut-offs of $\alpha = 0.0001$ and

fold-difference ≥ 1.5 (data not shown). Owing to previous indications of similar OP susceptibilities between male and female *D. v. virgifera* (Miota *et al.*, 1998), any potential sex bias in estimated transcript levels was not considered.

Comparisons were made amongst replicate pooled samples of 10 *D. v. virgifera* individuals from susceptible Clay Center and resistant Phelps County, Nebraska, locations (Fig. 1A; Table S1), which indicated that 29 DEGs were significant [≥ 4.0 negative logarithm of the P value ($-\log P$); Fig. 1B], and 62 DEGs were identified based on a significance threshold of a ≥ 1.5 fold-difference and P -value ≤ 0.05 (Fig. 1C). Application of both criteria predicted a total of 11 DEGs (Fig. 1D; Table S2), which were used in further analyses. Interestingly, all significant DEGs were over-expressed in the resistant strain compared with the susceptible strain. Sequences of these 11 DEGs were compared against a *D. v. virgifera* transcriptome (Eyun *et al.*, 2014) to obtain more complete sequences. Only

one of the 11 probes was for a gene annotated as a putative detoxification enzyme (probe ST020027A20C03; Table S2; putative α -esterase). Given the involvement of hydrolytic metabolism in resistance to both methyl-parathion and carbaryl (Zhou *et al.*, 2003), this α -esterase gene (*DvvEST*) was considered a potential contributor to observed increases in OP and carbamate insecticide metabolism amongst resistant *D. v. virgifera*. The derived protein sequence from the complete open reading frame (accession number KT210897) showed a catalytic triad associated with α -esterases from other insect species (Fig. 3), and the best 'hit' from GenBank nonredundant (nr) protein database searches was to a putative α -esterase from the Colorado potato beetle (51% amino acid identity; Table S2). Other significant DEGs identified but not associated with insecticide detoxification included probes corresponding to immune response and peptidoglycan recognition proteins, as well as genes involved in

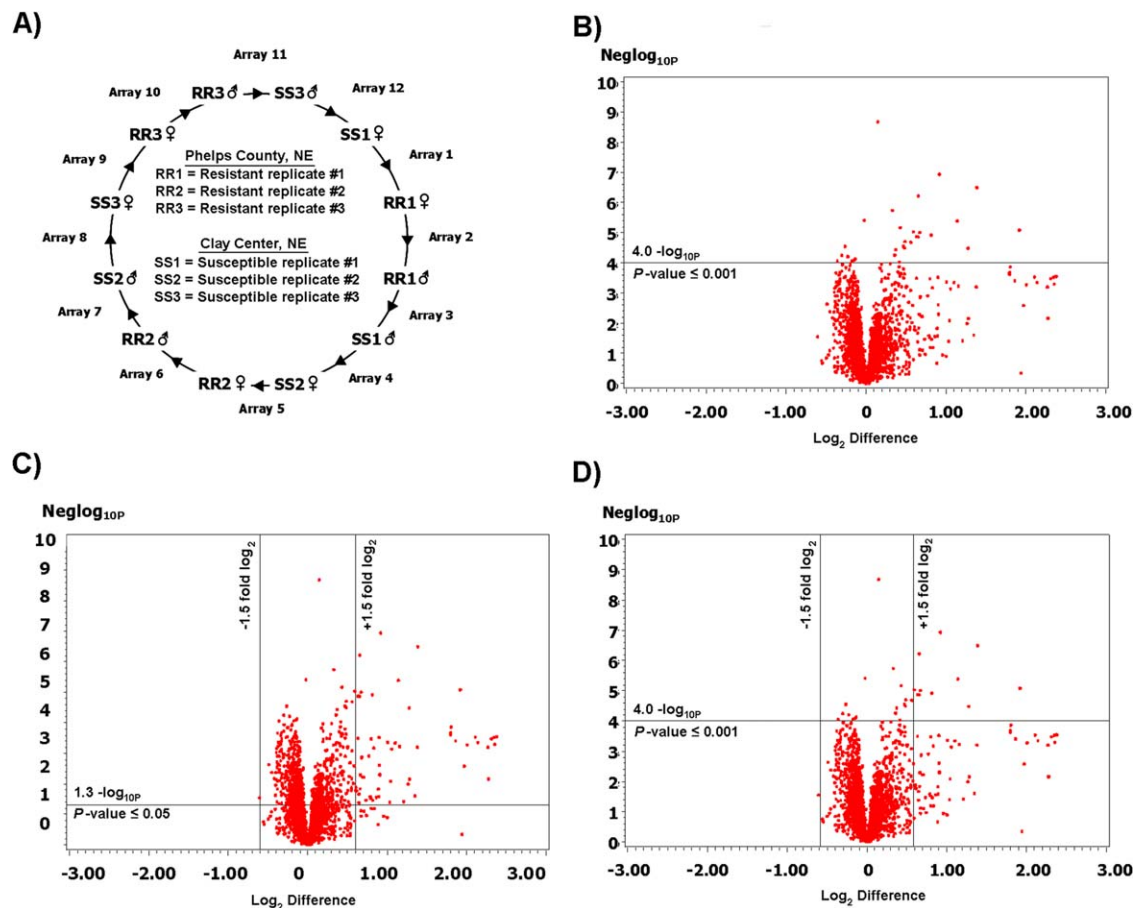


Figure 1. Microarray experimental design and results. (A) diagram showing loop dye-swap design, where arrow heads and tails, respectively, denote Cyanine 5 (Cy5) and Cy3 labelled samples [eg on array 1, Cy5-labelled resistant female (RR1) and Cy3-labelled susceptible female (SS1) are cohybridized]. Population and sex comparisons consisted of six hybridizations each with three biological replicates each (total of 12 array hybridizations). Each biological replicate was split into two Cy3 and Cy5 labelled samples (dye swapped). Also shown are volcano plots representing differences between resistant Phelps County and susceptible Clay Center *Diabrotica virgifera virgifera* populations in transcript levels amongst 8331 genes, respectively, showing genes above significance cut-offs of (B) P -values ≤ 0.001 [above 4.0 negative logarithm of P ($-\log P$)]; (C) fold-differences ≥ 1.5 and P -values ≤ 0.05 (above 1.3 $-\log P$) and (D) both fold-differences ≥ 1.5 and P -values ≤ 0.001 .

catabolic pathways, including cathepsin- and lipase-like enzymes.

Validation of differential esterase expression

Differential abundance of the DvEST α -esterase transcript was validated using quantitative reverse transcriptase PCR (qRT-PCR) with field populations with differing susceptibilities to methyl-parathion. Beetles from resistant populations (Phelps and York Counties in Nebraska) exhibited <10% mortality at a diagnostic methyl-parathion concentration based on the Lethal concentration sufficient to kill 99% of susceptible individuals (LC_{99}) of susceptible populations (Zhou *et al.*, 2003). This was comparable to individuals from the susceptible strain (Saunders County, Nebraska), which showed 100% mortality at this diagnostic dose (Fig. 2A). Corresponding qRT-PCR estimates of relative DvEST α -esterase transcript expression were 11- and 13-fold higher amongst the resistant Phelps and York County field populations, respectively, compared with

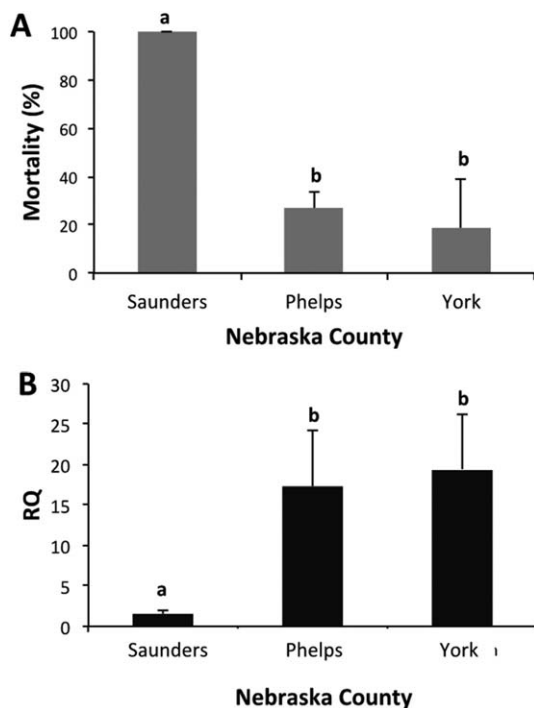


Figure 2. Validation of resistance and differential gene expression between organophosphate-resistant (Phelps and York Counties) and a susceptible population (Saunders County, Nebraska) of *Diabrotica virgifera virgifera* by quantitative reverse transcriptase PCR. (A) Mortality at diagnostic concentration of methyl-parathion; bioassays replicated 10 times with 10 beetles per vial (see Experimental procedures for details of bioassay) and (B) relative quantity (RQ) of *D. v. virgifera* α -esterase (DvEST). Cycle threshold values normalized using the housekeeping gene *beta-actin* and relative comparisons made to the susceptible Saunders Nebraska population (arbitrarily set at 100%); each replicated with five individuals per population. Bars represent mean and SEM and bars with different letters indicate significant differences ($P < 0.05$).

the susceptible Saunders County population (Fig. 3). Furthermore, an analysis of variance (ANOVA) determined that estimates of DvEST α -esterase transcript expression were significantly higher in resistant compared with susceptible individuals ($P < 0.05$).

Backcross pedigrees and phenotypic assays

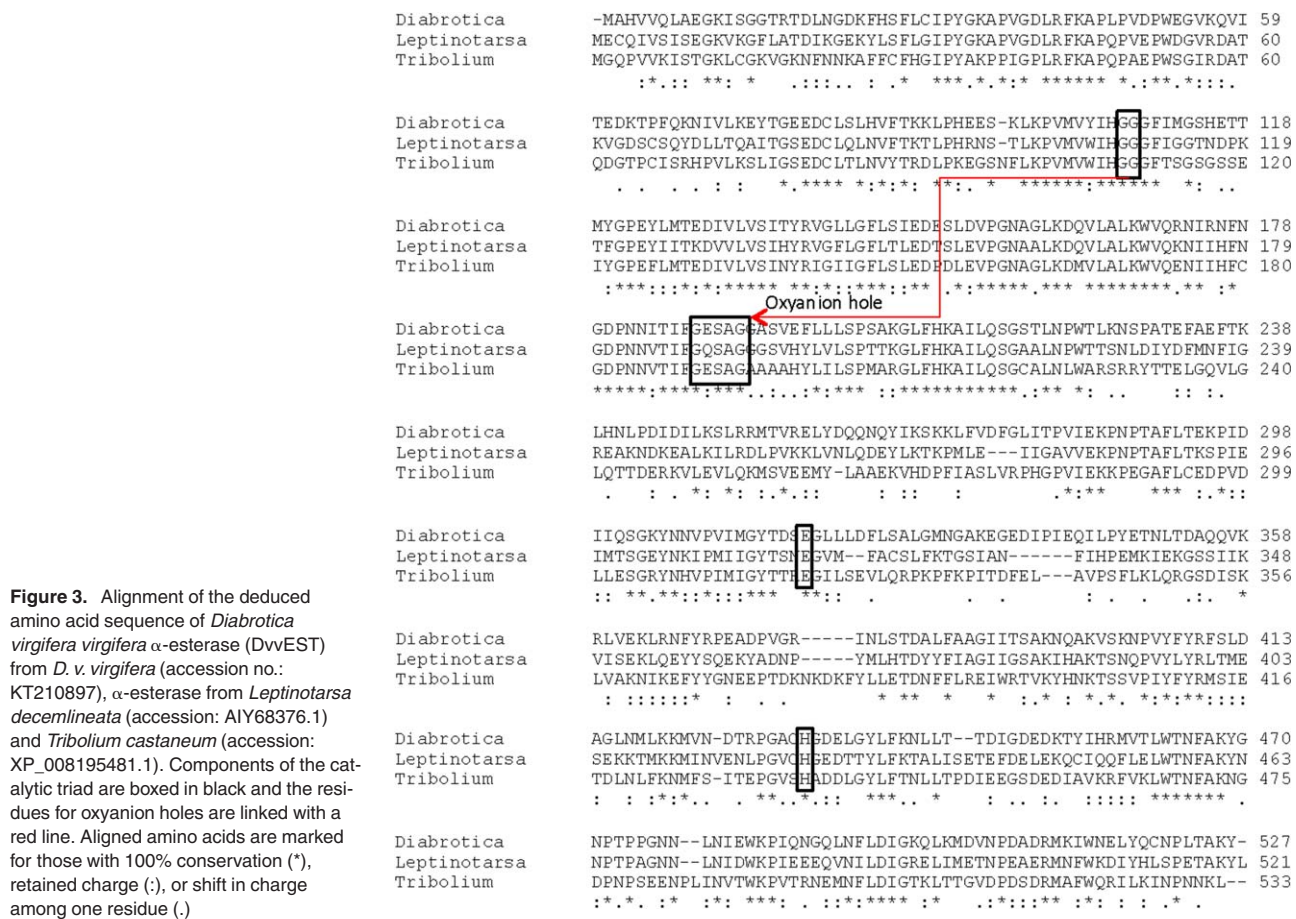
The inheritance of adult *D. v. virgifera* methyl-parathion resistance was evaluated in a reciprocal pedigree design that owing to effective dominance of the trait involved backcross of F_1 progeny to a susceptible individual (Fig. 4), and resulted in the successfully generation of four segregating F_2 populations. Backcrosses produced ≥ 58 F_2 progeny per pedigree from which phenotypes were determined in timed methyl-parathion exposure bioassays (Fig. 5; Table S3). Varying proportions of each population survived the 240-min exposure; 0.259 (35 of 133) and 0.181 (34 of 188) in pedigrees A15 and A09, respectively. A broad distribution of time to death was observed for moribund individuals from families A09 ($n = 153$; mean = 111.8 ± 52.4 min) and A15 ($n = 100$; mean = 114.4 ± 43.7 min). By contrast, no backcross progeny survived beyond 175 min for pedigrees B16 ($n = 99$; mean = 46.9 ± 11.1 min) or B19 ($n = 58$; mean = 87.5 ± 37.1 min).

Genotyping assays

4111 putative single nucleotide polymorphisms [SNPs; 147 from midgut- and 3964 from head-tissue derived expressed sequence tags (ESTs)] were identified in 1467 different contigs (midgut: 62; head: 1405). Using a slightly less stringent quality cut-off score of 95, 5240 SNPs were detected (midgut: 253; head: 4987), distributed over 1715 contigs (midgut: 81; head: 1634). Additional SNPs were detected by digestion of DvEST α -esterase-specific PCR products with *Mlu*CI in pedigrees A09 and A15. Although the mutation was segregating, the susceptible female parent was heterozygous in both pedigrees. Thus, the map position of the DvEST α -esterase gene could not be determined with respect to markers segregating from the resistant male (F_1^{RS}) in subsequent QTL analyses.

Genetic linkage mapping and QTL analyses

Polymorphic SNPs identified from the F_1^{RS} parent in pedigrees were used to determine statistical associations between inheritance of the marker from the original F_0 resistant parent and methyl-parathion survival. In total, 119, 147, 188 and 115 molecular genetic markers were segregating and showed no significant deviation from 1:1 Mendelian expectation in families A09, A15, B16 and B19, respectively. Genetic linkage relationships amongst markers were determined at thresholds of recombination frequency (rf) ≤ 0.3 and logarithm of odds (LOD) score ≥ 3.0 using MAPMAKER software, and resulted in 12 and



16 linkage groups (LGs) per family in pedigrees A09 and A15 respectively with 9.4 ± 3.9 markers per LG (range 3 to 19; Fig. 6). Genetic linkage maps were also constructed for markers segregating in backcross pedigrees B16 (Table S4A) and B19 (Table S4B).

A comparison of the segregation ratios for individual markers from all individuals with resistant individuals (survival times > 240 min) showed significant deviation in the

latter group for 15 and 25 markers in families A09 and A15, respectively (Table 2). Of the markers that showed skewed allelic frequencies amongst methyl-parathion survivors, all were located on LG09 in family A09 and 13 were located on the same LG09 in family A15. The remaining 12 markers in family A15 were all located on LG05 (Table 2). Simple linear regression to test for a one-to-one relationship between marker and phenotype indicated significant associations for 13 of the 15 family A09 markers on LG09 as well as 15 of 17 markers on LG04 (Table 1; P -values ≤ 0.00001 ; Table S5A). Regression similarly indicated that inheritance of 13 markers on LG05 and 12 markers on LG09 in family A15 were associated with OP resistance (P -values ≤ 0.00001 ; Table S5B). All other markers from families A09 and A15 showed no association with the trait. All markers failed to show a one-to-one relationship with increased methyl-parathion survival times amongst *D. v. virgifera* backcross progeny from families B16 and B19 (Table S5C and D).

Permutation tests established threshold LOD scores that corresponded to a P -value of 0.01 in backcross pedigrees A09 (LOD score = 14.81) and A15 (12.49) and were implemented for determining the QTL regions. Interval mapping of the methyl-parathion resistance trait

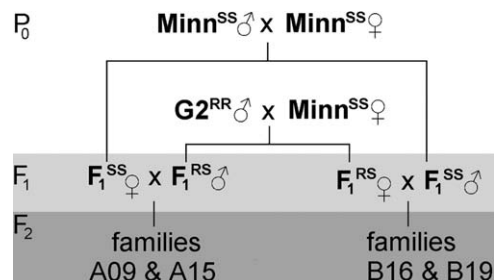


Figure 4. Pedigree design used to generate reciprocal segregating families in the F_2 (backcross) generation carrying a nearly dominant methyl-parathion resistance trait. Backcross of resistant F_1 heterozygotes (F_1^{RS}) to homozygous susceptible backcross parents (F_1^{SS}) resulted in a predicted 1:1 ratio of resistant and susceptible phenotypes in the F_2 generation.

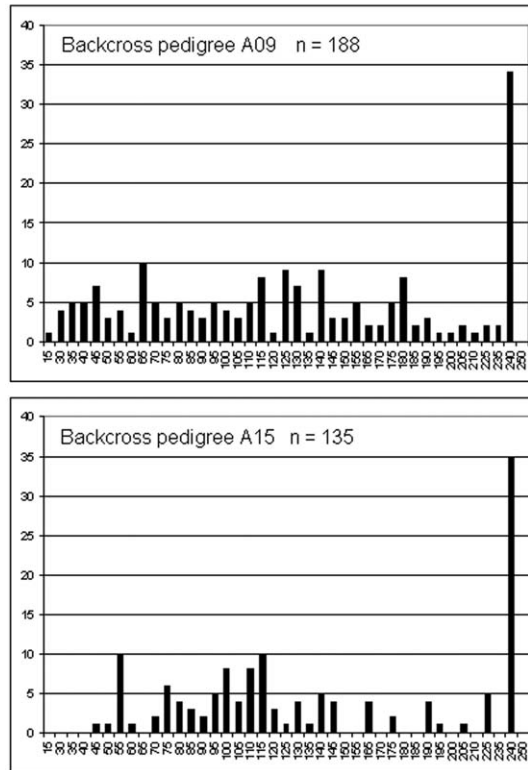
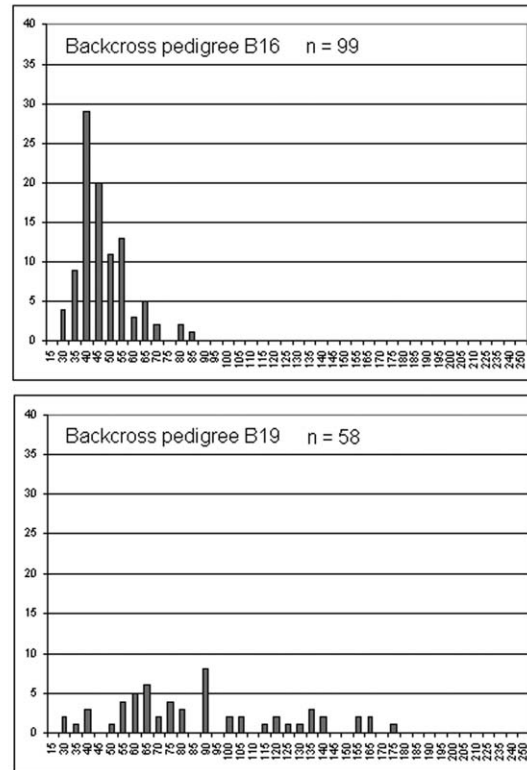
A) Backcross families: male F_1^{RS} parent**B) Backcross families: female F_1^{RS} parent**

Figure 5. Distribution of adult survivorship across time (15 to 240 min) amongst backcross progeny exposed to diagnostic doses of $0.5 \mu\text{g}$ methyl-parathion. Number of individuals in segregating populations with phenotypes defined as survivorship times (min) are shown for F_2 backcross progeny derived from alleles segregating in (A) the male backcross parent (pedigrees A09 and A15) and (B) the female backcross parent (pedigrees B16 and B19).

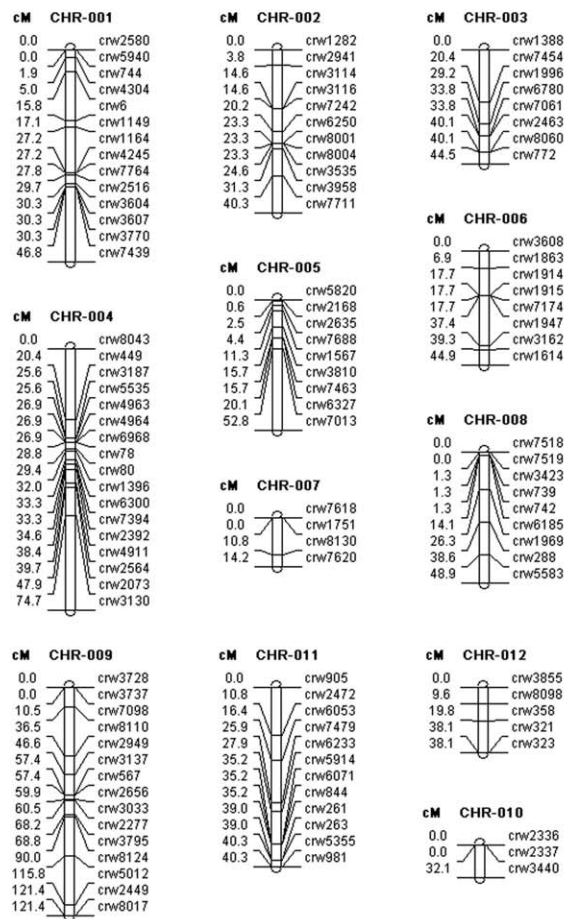
in pedigree A09 resulted in identification of a single genome interval from cM positions 38.8 to 50.0 on LG09 (Fig. 7A), where SNP markers crw8110 and crw567 respectively at LG09 positions 36.5 and 57.4 cM flanked the 21.0 cM QTL interval. Moreover, the marker crw2949 with an estimated LG09 location at 46.5 cM coincided with the QTL interval (LOD score 17.63), and inheritance of the allele from the resistant grandfather accounted for 47.96% of the trait variation and an estimated increase of 82.77 min survival time compared with the pedigree mean. Both LG05 and LG09 had predicted QTL intervals with LOD scores respectively of 18.79 and 19.67 that exceeded the permuted 12.49 cut-off in backcross A15 (Fig. 7B). QTL on LG05 and LG09 in pedigree A15 respectively accounted for 53.92 and 81.67% of the phenotypic variance, and imparted 98.88 and 124.35 min survival advantage to individuals that inherited the locus from the resistant grandparent. SNP markers crw8110, crw2656 and crw3033 on LG05 and LG09 in backcross A15 were positioned on LG09 in backcross A09 (Table 2). Thus, as these markers located on two linkage groups in backcross pedigree A15 were shared with a single LG09 in pedigree A09, marker locations provided logical evidence that LG05

and LG09 from the A15 linkage map may probably be positioned on the same chromosome and flanking markers representing the same QTL.

Discussion

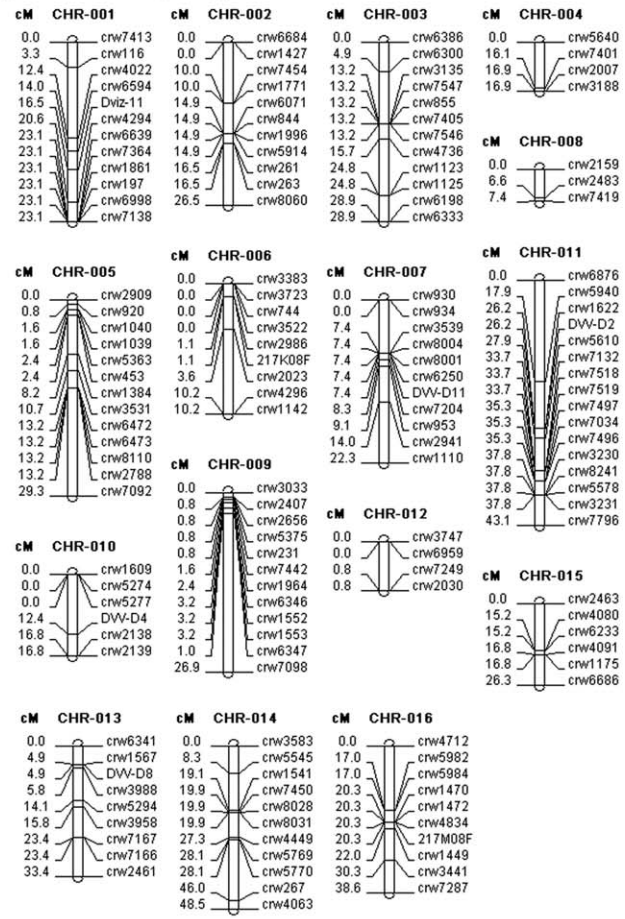
In the western Corn Belt, OP insecticides have been widely applied to reduce adult *D. v. virgifera* populations since the 1970s (Mayo, 1986; Miota *et al.*, 1998). Control failures were first reported during the 1994 and 1995 growing seasons in Phelps and York Counties, Nebraska (Meinke *et al.*, 1998). OP-resistant field populations of insects have been attributed mostly to metabolic mechanisms linked to up-regulation of esterase, glutathione S-transferase or cytochrome P450 monooxygenase detoxifying enzymes as well as target site insensitivity (Oakeshott *et al.*, 2003). Biochemical assessment of general enzyme activity identified esterases as the major factor involved in *D. v. virgifera* resistance to the OP insecticide methyl-parathion. However, these enzymes are encoded by large multigene families (Strode *et al.*, 2008), making it challenging to identify the individual genes directly involved in resistance mechanisms. cDNA microarrays allow examination of many thousands of genes simultaneously (Schena *et al.*, 1995; DeRisi *et al.*, 1997), providing a powerful tool for systematic

A) Backcross pedigree A09



Map distance: 599.1 cM

B) Backcross pedigree A15



Map distance: 405.4 cM

Figure 6. *Diabrotica virgifera virgifera* genetic linkage groups estimated from segregating single nucleotide polymorphism markers in (A) backcross pedigrees A09 and (B) A15.

expression profiling and for characterization of regulatory networks that control gene expression (DeRisi *et al.*, 1997; Cho *et al.*, 1998; Spellman *et al.*, 1998; Winzler *et al.*, 1998). cDNA microarrays have been used to estimate the over-expression of detoxification enzymes in resistant strains and to identify specific genes involved in metabolic resistance in *Drosophila melanogaster* (Daborn *et al.*, 2002; Le Goff *et al.*, 2003; Pedra *et al.*, 2004) and *Anopheles gambiae* (Ranson *et al.*, 2002; David *et al.*, 2005). Microarray experiments are sometimes considered limited in scope because expression can only be estimated for transcripts known *a priori*, which are represented by short oligonucleotide probes. They are often subject to high levels of variation amongst replicate spots representing the same probe, as well as amongst different hybridization experiments (Draghici *et al.*, 2006). Our initial microarray-based identification of 11 candidate DEGs in *D. v. virgifera* included only one gene that is potentially involved with enzymatic detoxification, with the remaining genes

putatively involved in immune response and catabolic pathways. These results agree with a previous findings implicating esterase up-regulation in the resistant phenotype (Zhou *et al.*, 2002, 2003). Our additional qRT-PCR validation experiments demonstrated an estimated 11- to 13-fold increase in this α -esterase transcript amongst methyl-parathion resistant *D. v. virgifera* from field populations. Variation in esterase transcript levels amongst unrelated individuals from field populations, although significantly higher than that of susceptible individuals, demonstrates the effect of genetic background and environment on gene expression. Regardless, this suggests that the trait is at least partially influenced by hydrolytic metabolism. As in the case of more contemporary RNA sequencing (RNA-seq) methods for estimating global changes in transcript levels, this secondary validation of microarray results remains crucial.

QTL mapping procedures use statistical association (linkage disequilibrium) between segregation of a trait and

Table 1. Sequences of oligonucleotide primers used in gene-specific amplification reactions

qRT-PCR				
Gene	Clone/accession no.	Primer name	Primer sequence	T _m (°C)
Beta-actin		DvActin_F	5'-GTTGGATTCTGGTGATGGTG-3'	62.9
		DvActin_R	5'-CTCTTTCTGCTGTGGTGGTG-3'	63.5
P450_6	ST020037B10D05/ EW762827.1	6P450_F	5'-GTAGAAAACGAAGACACCTGTGTTGG-3'	67.4
		6P450_R	5'-CCAAATGCCCTCGTATGCGGG-3'	71.4
P450_9	ST020012A21E08/ EW761497.1	9P450_F	5'-TAGCCTGCAAGAGAAGACAAGAATAAC-3'	65.5
		9P450_R	5'-ACTCATTCCATAAGCACCCG-3'	63.7
P450_24	ST020024B10C10/ EW769439.1	24P450_F	5'-TATCTGTGAAACGTCAATGGGAGTATC-3'	66.6
		24P450_R	5'-TGAGTATCAGCAATCGGCAT-3'	63.2
Esterase3	ST020027A20C03/ EW770686.1	DvE3_F	5'-CCTATCGACATCATCCAGTCAGGGA-3'	70.9
		DvE3_R	5'-CGTTCATTCCAAGTGCCGACAAG-3'	71.4
Esterase20	Contig_1183/ CN498074.1	DvE20_F	5'-CGCGTACTGTGTAGCAATCATCTG-3'	67.5
		DvE20_R	5'-GTGATCTAGCTCCAACCCMCCC-3'	67.8
Esterase29	ST020022A10G08/ EW770370.1	DvE29_F	5'-GTGTTCTGTTCTTTGATAGCAG-3'	60.5
		DvE29_R	5'-GTCCCCATTTCATTTC-3'	61.5
Genotyping				
Gene/ID	Accession no.	Primer name	Primer sequence	T _m (°C)
Esterase3	EW770686.1	Est3-Fa	5'-CCATGGGAAGGGGTAAACA-3'	61.8
		Est3-Ra	5'-CGCCTCAAGCTTTTCAAATGTC-3'	64.3
217K08F	JM105106	217K08F_F	5'-CCTGGCCTATTCTGTGTTT-3'	59.0
		217K08F_R	5'-ATAAAGCGCATTACCCATGC-3'	60.0
217L14F	JM105163.1	217L14F_F	5'-TGCGCAGAGTAGGTATTTTGT-3'	59.0
		217L14F_R	5'-ACGAAAAACGATGGAACGAC-3'	60.0
217M08F	JM105197	217M08F_F	5'-GTCTCTTGTCGCCGTACCAT-3'	60.0
		217M08F_R	5'-TCCAATGCTGGGATAGAGC-3'	60.2

qRT-PCR, quantitative reverse transcriptase PCR; T_m, melting temperature.

molecular genetic markers physically linked to causal genetic factors. QTL mapping can identify genome regions without knowledge of the underlying genes, and thus can be used to scan the entire genome for loci influencing a trait. The density of genetic markers, number of recombinant genotypes sampled and number of recombining generations affect the resolution of a genetic linkage map and the subsequent ability to detect QTL (Yu *et al.*, 2011). High marker densities increase precision by identifying QTL with small effects, and differentiate adjacent QTL that are closely linked (Li *et al.*, 2010; Shi *et al.*, 2011). The genome of *D. v. virgifera* is large (2.56 Gbp; Coates *et al.*, 2012), and although no karyotype data are available, it is probably arranged on nine pairs of autosomes and an unpaired X-chromosome as observed amongst other *Diabrotica* (Stevens, 1908). The 12 to 16 LGs assembled per pedigree had ≥ 119 segregating SNP markers in each. The disparity in LG number between maps and haploid autosomal chromosome number ($n = 10$) suggests that one or more LGs may represent a single chromosome. Individual chromosomes are probably relatively large, and the mean of 9.4 ± 3.9 markers per LG may be too low for recombination rates at the cut-offs we applied ($r_f \leq 0.3$; LOD scores ≥ 3.0). Linkage mapping from an analogously small number of genetic markers resulted in a high number of LGs (Ipek *et al.*,

2005). Thus, additional SNP markers may be required to generate an adequately dense genetic linkage map for *D. v. virgifera*. Methods for simultaneous high-throughput SNP detection and genotyping assays (Elshire *et al.*, 2011) have recently been applied to high-density genetic linkage map construction and QTL isolation in *D. v. virgifera* (Flagel *et al.*, 2014) and other species (Li *et al.*, 2010; Yu *et al.*, 2011; Coates & Siegfried, 2015), and may provide an approach for mapping QTL of additional *D. v. virgifera* resistance traits (see Introduction).

Nevertheless, QTL mapping successfully identified a single 21.0 cM QTL interval in pedigree A09 that influences inheritance of methyl-parathion resistance. The *D. v. virgifera* QTL data detected a single genome region that strongly influences OP resistance, but we were unable to map the position of the DvEST α -esterase gene with respect to the QTL. Thus it remains uncertain if DvEST is physically located in the QTL region, and if *cis*-regulatory mutations or gene duplications (gene amplifications) are the genetic cause of transcriptional up-regulation. Furthermore, low QTL resolution (low marker density) and analysis of backcross (F_2) progeny (low recombination compared to populations) also led to uncertainty as to whether one or more genes in this genome region contribute to metabolic resistance. For instance, two tightly linked QTL 6 cM apart influence a

Table 2. Linkage groups (LGs) associated with quantitative trait loci for organophosphate resistance in *Diabrotica virgifera virgifera* defined by comparison of deviation of genetic markers from Mendelian expected 1:1 ratios amongst methyl-parathion survivors. *P*-values associated with Chi-square (χ^2) tests are shown for genetic markers listed in order of linear arrangement on each LG. Genetic markers shared amongst LGs from different pedigrees are in bold

Backcross pedigree A09				Backcross pedigree A15			
LG09		χ^2 <i>P</i> -value		LG09		χ^2 <i>P</i> -value	
Order	Marker	All F ₂ progeny	F ₂ survivors	Order	Marker	All F ₂ progeny	F ₂ survivors
1	crw3728	0.5788	0.0679	1	crw7092	0.2050	<0.0001
2	crw3737	0.5270	0.0679	2	crw6473	0.1475	<0.0001
3	crw7092	0.8096	0.1141	3	crw6472	0.1475	<0.0001
4	crw8110	0.7518	< 0.0001	4	crw8110	0.1475	<0.0001
5	crw2949	0.7518	< 0.0001	5	crw2788	0.1475	<0.0001
6	crw567	0.8744	0.0010	6	crw3531	0.2050	<0.0001
7	crw3137	0.8744	0.0010	7	crw1384	0.2773	<0.0001
8	crw2656	0.8744	0.0010	8	crw453	0.3653	<0.0001
9	crw3033	0.6917	0.0010	9	crw5363	0.3653	<0.0001
10	crw2277	1.0000	0.0285	10	crw1039	0.4690	<0.0001
11	crw3795	0.7518	0.0285	11	crw1040	0.4690	<0.0001
12	crw8124	0.1139	0.0002	12	crw920	0.5870	<0.0001
13	crw5012	0.1139	0.2733	13	crw2909	0.4690	<0.0001
14	crw2449	0.2684	0.2733				
15	crw8017	0.2684	0.2733				

LG05			
Order	Marker	All F ₂ progeny	F ₂ survivors
1	crw7098	0.2050	<0.0001
2	crw6347	0.3653	<0.0001
3	crw1553	0.3653	<0.0001
4	crw1552	0.3653	<0.0001
5	crw6346	0.3653	<0.0001
6	crw1964	0.4689	<0.0001
7	crw7442	0.5670	<0.0001
8	crw231	0.4689	<0.0001
9	crw5375	0.4689	<0.0001
10	crw2656	0.4689	<0.0001
11	crw2407	0.4689	<0.0001
12	crw3033	0.3174	<0.0001

metabolic resistance trait in *Aedes aegypti* (Saavedra-Rodriguez *et al.*, 2008). Alternatively, regulation of genes and gene networks can be controlled by unlinked genetic loci that act as *trans*-regulators, as in the case of transcriptional up-regulation of two cytochrome P450 monooxygenases involved in *Drosophila melanogaster* dichlorodiphenyltrichloroethane (DDT) resistance (Maitra *et al.*, 2000), or by the regulation of Bt binding receptors in the midgut of Cry1A toxin-resistant lepidopteran larvae (Tiewisiri & Wang, 2011, Coates *et al.*, 2013). Thus, we cannot rule out that mutations in *trans*-regulatory factors in the *D. v. virgifera* OP QTL may directly affect DvEST α -esterase gene expression with metabolic resistance subject to epistatic control, but this requires additional investigation.

Understanding the genetic basis of insecticide resistance may reveal means to better manage resistance evolution in target insect populations. The recent discovery of a one-to-one correlation between a single point mutation in the *Anopheles funestus* glutathione S-transferase epsilon 2 gene and pyrethroid and DDT resistance traits created the opportunity to directly monitor changes in the

frequency of resistance in field populations (Riveron *et al.*, 2014). Incorporating this principle of monitoring direct genotype–phenotype associations into crop pest insect resistance management (IRM) strategies will be valuable in guiding management decisions.

Experimental procedures

Insects and RNA extraction

Laboratory colonies of *D. v. virgifera* used for expression analyses were originally collected from Phelps County ($n = \sim 5000$) and near Clay Center in Clay County, Nebraska ($n = \sim 5000$), in 1995 as adults, and maintained separately at the United States Department of Agriculture Agricultural Research Service (USDA-ARS) North Central Agricultural Research Lab (Brookings, SD, USA). The Phelps colony exhibited high levels of resistance to methyl-parathion, whereas the Clay Center population was determined to be susceptible at the time of collection (Zhou *et al.*, 2002; Parimi *et al.*, 2006). Second instars from both colonies were reared on corn seedling root mats at $24 \pm 1^\circ\text{C}$ and 70% relative humidity. Teneral adults were collected daily, separated by sex (Hammack & French, 2007), flash-frozen in liquid nitrogen, and stored at -80°C . Biological replicates were composed of 10 pooled

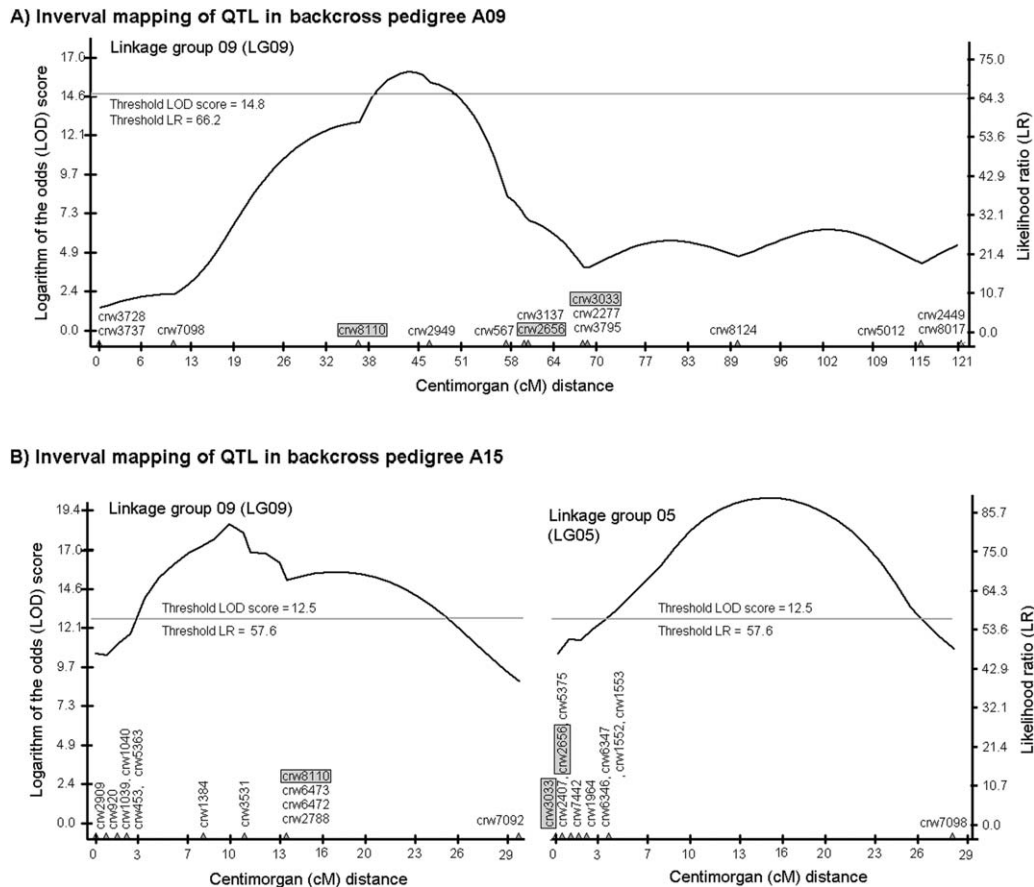


Figure 7. Interval mapping of a quantitative trait locus (QTL) that significantly influences the inheritance of organophosphate insecticide resistance in *Diabrotica virgifera virgifera*. (A) QTL on LG09 (Lod score 17.63) from backcross pedigree A09 showing a single major QTL between 38.8 and 50.0 cM above a significance threshold of 0.01 and accounting for 47.96% for the trait variance. (B) QTL identified in backcross pedigree A15 on LG05 (Lod score 18.79) and LG09 (Lod score 19.67), and accounting for $\geq 53.92\%$ of the trait variance. All QTL designated above a significance threshold of $P \leq 0.01$, and markers shared between genome intervals in both pedigrees are highlighted.

abdomens from tenral adults per sex per population. Three 'sex within population' biological replicates were collected, and total RNA extracted using TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The quantity of extracted RNA was estimated on a NanoDrop1000 (Thermo-Fisher Scientific, Wilmington, DE, USA), and quality evaluated by 1% denaturing agarose gel electrophoresis.

Microarray hybridization and statistical analyses

Microarray slides containing *D. v. virgifera* midgut (Siegfried *et al.*, 2005) and head EST probes were constructed as described by Knolhoff *et al.* (2010). Each slide contained 9120 probes, of which 8331 were *D. v. virgifera* specific; the remaining were blanks, buffer controls and negative controls. First-strand cDNAs incorporating either Cyanine 3 or 5 (Cy3 or Cy5) fluorescent dyes were synthesized and purified as described by Knolhoff *et al.* (2010), quantified by spectrophotometry and dried using a speed-vac.

The microarray experiment was conducted in a loop design, comparing transcription levels between resistant and susceptible strains as well as between sexes within the same strain

(Fig. 1A; Table S1). A dye swap was performed for all array comparisons. Control and calibration experiments were performed with Cy3 and Cy5 only. For each hybridization, 40 μ g PolyA DNA was added to each probe combination and the reaction brought to 40 μ l with sterile water, heated at 95 $^{\circ}$ C for 2–3 min then chilled on ice for 2 min. Hybridization buffer (2X; 40 μ l) was added to each probe combination, and applied in a Corning Hybridization Chamber (Corning Incorporated, Corning, NY, USA) and incubated for 16–20 h (overnight) at 42 $^{\circ}$ C. Slides were washed three times with constant agitation for 4 min; first wash with 1 \times saline sodium citrate (SSC), 0.2% sodium dodecyl sulphate (SDS) at 42 $^{\circ}$ C, and second and third at room temperature with 0.2% SDS and 0.1 \times SSC, respectively. Slides were then spun dry and scanned using a GenePix 4000B fluorescent scanner (Molecular Devices, Sunnyvale, CA, USA) and spot features (ie mean, median and background intensities in the two channels) were extracted from TIFF images using the GENEPIX Pro v. 5 software platform (Molecular Devices). Background-subtracted mean intensities from the two channels in each spot were transformed to intensity log-ratios, $M = (\log_2 \text{red} - \log_2 \text{green})$, and their corresponding geometric means, $A = (\log_2 \text{red} + \log_2 \text{green})/2$. All microarray work was

performed at the Keck Center, University of Illinois at Champaign-Urbana.

Dye-swap data were normalized for each sample using methods described by Lian *et al.* (2007). Normalization was conducted within arrays to minimize technical variation using PROC LOESS (SAS Institute, Cary, NC, USA), enabling recognition of changes caused solely by biological variation amongst samples (Quackenbush, 2002). Normalization between arrays was conducted through scaling, equalizing the means and standard deviations of all distributions (Yang *et al.*, 2002). ANOVA was used to detect significant differences in expression for individual genes, where a dual-mixed model was fitted to the data (Gibson & Wolfinger, 2004) using PROC MIXED of SAS v. 9.1 (SAS Institute). Array, population by sex within array and spot within array were treated as random effects, and population and sex were treated as fixed effects. Least squares mean estimates of transformed fluorescence intensity were calculated and *P*-values assigned for each gene for both comparisons. Criteria for rejecting the null hypothesis were based on fold-change rank ordering associated with lower stringency *P*-values, which are more consistent and provide higher reproducibility of biological significance (Guo *et al.*, 2006). Significant treatment effects were initially based on estimated differences ≥ 1.5 -fold and a *P*-value cut-off of $\alpha \leq 0.05$, but significance thresholds were also increased to $\alpha \leq 0.001$. For each microarray probe that was significantly up-regulated and exhibited highest fold difference between resistant and susceptible populations, the parent EST sequences were used as queries to search the more complete homologous sequences in the previously published *D. v. virgifera* transcriptome (Eyun *et al.*, 2014). Furthermore, homologous sequences in the Eyun *et al.* (2014) transcriptome identified as DEGs from our microarray experiments were used as queries against the nr National Center for Biotechnology Information database using the BLASTx algorithm with an E-value cut-off of 10^{-25} .

Validation of differential gene expression

qRT-PCR was used to validate differential expression of *DvEST* from field populations collected from areas previously identified as being resistant to methyl-parathion (Phelps and York Counties, Nebraska) and one field population with known susceptibility to methyl-parathion (Saunders County, Nebraska). Susceptibility of all three populations was estimated using residual exposure to a diagnostic concentration of methyl-parathion coated on the inside of a 10-ml glass scintillation vial as described by Zhou *et al.* (2003) and Chen *et al.* (2011). For each population, 10 replications of 10 unsexed adults per vial were assayed.

Validation of differential expression of *DvEST* from the same field populations used for methyl parathion bioassays was accomplished by qRT-PCR. Specifically, total RNA was extracted from five adult individuals per field population using TRIzol Reagent [Invitrogen Life Technologies, catalogue no. (Cat.) 15596-026], and five individuals per field population were extracted. The quality and quantity of RNA samples were evaluated on 1% agarose gels and by NanoDrop-2000 spectrophotometry (Thermo-Fisher Scientific), respectively, and then stored at -80°C . The cDNA synthesis was carried out with 500 ng RNA using a QuantiTect Rev Transcription Kit (Qiagen,

Valencia, CA, USA: Cat. 205311) according to the manufacturer's instructions. A web-based primer design program, PRIMER3 (Untergasser *et al.*, 2012), was used to design gene-specific and beta-actin control gene primers (Table 1). The qRT-PCR reaction was conducted with Fast SYBR Green Master Mix (Invitrogen Life Technologies, Cat. 4385612) following the manufacturer's instructions. The synthesized cDNA was diluted 50-fold and 2.0 μl was used as template in each 10- μl qRT-PCR reaction. All reactions were carried out in triplicate on a 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA). The delta delta cycle threshold ($2^{-\Delta\Delta\text{CT}}$) method was used to calculate the relative expression of the target gene (Livak & Schmittgen, 2001). The significance of differences in both mortality at the diagnostic bioassay and in the relative expression of *DvEST* were determined using SAS software v. 9.3 (SAS Institute, 2011). An ANOVA was performed using PROC GLIMMIX with the least-square estimated means procedure to determine differences amongst populations.

Backcross pedigrees and phenotypic assays

OP-resistant and -susceptible *D. v. virgifera* colonies were initiated from field-collected adults from Wabasha County, Minnesota ($n \sim 5000$) and York County, Nebraska ($n \sim 5000$), in 1999 and maintained in the Brookings laboratory as described above as Minn and G2 colonies, respectively. York County, Nebraska, previously showed a high incidence of methyl-parathion resistance (Parimi *et al.*, 2006) that was inherited in a near-complete dominant autosomal fashion amongst F_1 progeny (Parimi *et al.*, 2003).

A reciprocal backcross pedigree design was used to generate F_2 mapping families with a segregating dominant OP resistance trait. Initial crosses were made between two independent parental mate pairs (P_0 generation), with the first cross between a susceptible male and susceptible female from the Minn colony (F_1^{SS}). The second cross was used to establish F_1 progeny with a segregating OP resistance trait (F_1^{RS}) by mating a resistant G2 colony male with a susceptible Minn colony female. Single-pair matings produced segregating backcross (F_2) populations; two unique male $F_1^{\text{RS}} \times$ female F_1^{SS} backcrosses (families A09 and A15), and two unique female $F_1^{\text{RS}} \times$ male F_1^{SS} backcrosses (families B16 and B19) (Fig. 4). All F_2 progeny were assayed for adult survival when exposed to a diagnostic dose of 0.5 μg methyl-parathion per 20-ml glass scintillation vial for 240 min, as described by Zhou *et al.* (2002). Time-to-death phenotypes were recorded by monitoring for a moribund state every 5 min. Beetles remaining unaffected after 240 min. were categorized as 'survivors'. Genomic DNA was extracted from all parental (F_0), backcross parents (F_1^{RS} and F_1^{SS}) and phenotyped F_2 backcross progeny using Qiagen DNeasy Blood and Tissue extraction kits according to the manufacturer's instructions, quantified on a NanoDrop2000 and diluted to 10 ng/ μl with nuclease-free water.

Genotyping assays

All parental (F_0), backcross parents (F_1^{RS} and F_1^{SS}), and phenotyped F_2 backcross progeny were genotyped using the recommended 'core set' of 13 microsatellite markers in *D. v. virgifera* that are apparently free of null alleles (Kim *et al.*, 2008). Primer

pairs were designed from BAC end sequence data (Coates *et al.*, 2012) that PCR-amplified three additional microsatellite loci (Table 1), using methods described by Kim *et al.* (2008). Additionally, a novel set of SNP markers was developed from putative substitution mutations predicted from Sanger read data previously generated for head and midgut ESTs (Siegfried *et al.*, 2005; Knolhoff *et al.*, 2010). Specifically, candidate SNPs were identified at positions in consensus EST contigs by aligning Sanger sequencing trace files using the CONSED computer program (Gordon *et al.*, 1998), and 'forcing' alignments against the consensus sequence of its respective contig. The program POLYPHRED (Nickerson *et al.*, 1997) was then used to detect potential SNPs within each alignment. Sequence quality scores were calculated by POLYPHRED for each read at each nucleotide position (0 to 99), and a quality score cut-off of ≥ 98 was applied, which was expected to provide a true positive rate of 97% (3% false positive rate). GoldenGate assays were designed to detect variation at 1500 SNP loci as described by Whitfield *et al.* (2006), and assays were performed on an Illumina Beadstation (Illumina, San Diego, CA, USA) at the W. M. Keck Center for Comparative and Functional Genomics, University of Illinois at Urbana-Champaign. SNP genotyping data were filtered as described by Whitfield *et al.* (2006). PCR-restriction fragment length polymorphism assays were designed to detect SNPs in the gene that encodes an α -esterase₃ (microarray probe ST020027A20C03; *DvEST* gene). Initial PCR amplification using oligonucleotide primers E3re-F and E3re-R (Table 1) was followed by digestion of amplicons with *Mlu*CI (New England BioLabs, Beverly, MA, USA) as described previously (Coates *et al.*, 2009).

Genetic linkage mapping and QTL analyses

Microsatellite and SNP markers heterozygous in F_1^{RS} parents, ie segregating markers, were used to construct a genetic linkage map separately for each backcross family (A9, A15, B16 and B19) where the F_1^{SS} parent was homozygous for either allele. The F_1^{RS} and F_1^{SS} parental cross generates a segregating 1:1 Mendelian ratio of homozygote to heterozygote F_2 backcross progeny, allowing allele origins to be traced to the initial resistant F_0 parent. Because of the reciprocal backcross design, maps were constructed for markers segregating in the male F_1^{RS} parent from families A09 and A15, and for each female F_1^{RS} parent in families B16 and B19. Predicted Mendelian 1:1 ratios amongst all F_2 progeny were tested using the Chi-square statistic (χ^2), and markers with a P -value ≤ 0.10 were removed from further analyses. Data from Mendelian-inherited markers were used to create an input file for MAPMAKER 3.0 (Lincoln *et al.*, 1992). Markers were assigned to LGs using a Kosambi mapping function, $r \leq 0.3$ and LOD score ≥ 3.0 . LGs with ≤ 2 markers were discarded. The best fit order of markers on each LG was determined using the sum of adjacent recombination frequencies. All potential orders were evaluated by calculating and comparing the maximum likelihood map for each marker sequence.

The putative position of QTL associated with the inheritance of OP resistance in *D. v. virgifera* was initially assigned by evaluating the deviation of genetic markers from Mendelian expectation amongst beetles that survived a 240-min exposure with no

observable affects (survivor group). Specifically, marker alleles inherited at an approximate 1:1 ratio amongst all F_2 progeny from the resistant F_0 parent are expected to be significantly (χ^2 , $P \leq 0.001$) over-represented in the survivor groups when in proximity to a QTL. The LG positions of the markers were determined manually. Similarly, one-to-one associations between genotype and phenotype data were fitted to a simple linear regression model ($y = b_0 + b_1 + e$) using QTL CARTOGRAPHER (Wang *et al.*, 2012), and the null hypothesis that markers are not linked to the QTL ($b_1 = 0$) was assessed using an F -statistic at $\alpha = 0.001$.

Interval mapping assesses the association between a trait's markers using single-factor ANOVA. It estimates QTL position with respect to adjacent linked markers using the LOD score calculated stepwise across LGs. A LOD score is the probability that a QTL is positioned at a given chromosome location, where significance thresholds (α -values) are set according to cumulative distribution functions of the LOD scores. The threshold LOD scores for calling QTL from the current data set were determined by 1000 permutations of the data with a precision level set at 1.0cM using WINQTL CARTOGRAPHER (Wang *et al.*, 2012). Interval mapping was then applied to estimate the position of QTL on LGs from pedigrees A09 and A19 using marker positions imported from MAPMAKER and phenotypic data (time to moribund state when exposed to diagnostic dose of methyl-parathion) using the program WINQTL CARTOGRAPHER with the Kosambi mapping function. The threshold LOD score was thus calculated as 16.81 for $\alpha = 0.01$.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Microarray experimental design.

Table S2. List of differentially expressed genes identified by microarray experiments.

Table S3. Tab-delimited text file of time-to-death phenotypes from pedigrees A09, A15, B16 and B19.

Table S4. Linkage group assignment and estimated genetic distances between single nucleotide polymorphism markers in pedigrees B16 (Table S4A) and B19 (Table S4B).

Table S5. Likelihood ratio test for association between single nucleotide polymorphism markers and time-to-death phenotypes.