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Effects of a Corn Root Defense Substance on Western Corn Rootworm *Diabrotica virgifera virgifera* LeConte Larvae

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By

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The objectives of this research were to evaluate the effects of hydroxamic acids, a group of corn root defense substances on western corn rootworm (*Diabrotica virgifera virgifera* LeConte) larvae and identify putative molecular mechanisms for hydroxamic acid detoxification. Corn line 428G carries a homozygous mutant *bx1* gene, which makes it unable to synthesis hydroxamic acids. In contrast, H88 is the wild type parental line of 428G and is able to synthesize hydroxamic acids. Larvae were fed on 428G and H88 roots for 7 days for comparison. No significant difference was observed in growth, development, or survival rate. A RNA-Sequencing study was conducted to identify the differentially expressed genes that responded to dietary hydroxamic acids. A larval transcriptome sequence was assembled *de novo* from RNA-Sequencing data. Nine gene sequences were declared significantly differentially expressed. A putative cytochrome P450 gene was up regulated in the larvae feeding on hydroxamic acid-containing corn

roots (H88). The results of this research suggested that hydroxamic acids do not have a significant inhibitory effect on western corn rootworm larvae. An inducible mechanism, mediated by cytochrome P450s, may be involved in hydroxamic acid detoxification. The results of this research advanced current understanding of western corn rootworm-corn interactions, and also provided a foundation for further study of the molecular mechanisms of hydroxamic acid detoxification by western corn rootworm.

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CHAPTER 1

Literature Review

WESTERN CORN ROOTWORM AND CORN

Origin and Importance

The western corn rootworm (*Diabrotica virgifera virgifera* LeConte) is a native North American insect species. It was first recorded in Kansas in 1867 and was only found in Nebraska, east Colorado, west Kansas, and a small portion of South Dakota and Iowa before 1955 (Chiang, 1973; Smith & Lawrence, 1967). After the 1950s, the eastward expansion of western corn rootworm was facilitated by continuous corn cultivation and improving irrigation systems in the Corn Belt (Meinke et al., 2009). By the year 2006, the western corn rootworm was distributed all over the North American Corn Belt, northeast to New England (Boucher, 2006) and southeast to Northern Georgia (Hudson & All, 1996) and Alabama (Flanders, 2006). A European introduction was first reported near Belgrade airport in 1992 (Ciosi et al., 2008; Gray et al., 2009; Kiss et al., 2005). Western corn rootworm successfully established in more than 11 European countries within two decades (Ciosi et al., 2008; Miller et al., 2005).

Western corn rootworm is one of the most significant pest species of corn (*Zea mays* L.) in North America. Larvae feed on corn root tissues, which reduces water uptake (Hou et al., 1997; Riedell, 1990) and impairs the mechanical support. Crown roots are excised by intense rootworm consumption, which increases the susceptibility to lodging

(Levine & Oloumi-Sadeghi, 1991). The 0-3 node-injury scale is currently used to evaluate corn rootworm pressure in the field (Oleson et al., 2005). Adults feed on corn silks, pollen and floral tissue and may interfere with pollination, which can reduce the yield (Meinke et al., 2009).

The western corn rootworm has a remarkable economic importance. It is estimated that *Diabrotica* species can cost $1.7 billion in control and losses annually in the US (Sappington et al., 2006). Soil treatment by chemical insecticides (Mayo & Peters, 1978; Meinke et al., 1998), Crop rotation with non-host plants (Chandler, 2003; Chandler et al., 2000), and the introduction of Bt transgenic corn (Vaughn et al., 2005), are three current major approaches to controlling western corn rootworm in North America. Insecticide baits were previously used for western rootworm control (Chandler, 2003). Corn lines expressing double strand interfering RNA (dsRNA) has also been discussed as potential approach (Baum et al., 2007).

Biology and Life History in the Corn Belt

Western corn rootworm is a univoltine insect that overwinters in the egg stage (Ball, 1957). In temperate regions, the eggs are laid in the soil during July to September and followed by obligate egg diapause (Krysan, 1978; Meinke et al., 2009). The diapause duration period is genetically controlled (Meinke et al., 2009). The diapause may serve as a strategy against drought conditions in winter (Meinke et al., 2009). In nature, the post-diapause eggs continue dormancy until the temperature is higher than the threshold

temperature of 11 ˚C (Wilde, 1971). The post-diapause development is determined by soil temperature. The optimal temperature is 28 ˚C (Schaafsma et al., 1991). Soil water is also necessary for western corn rootworm eggs to hatch (Krysan, 1978; Meinke et al., 2009).

The egg hatch initiates during late May to early June (Meinke et al., 2009). The larvae develop through three instars (Hammack et al., 2003). After eclosion from eggs, the neonate larvae search for fine corn roots (Chiang, 1973). They locate corn roots by semiochemicals released from corn roots, including carbon dioxide (Strnad et al., 1986), 6-methoxy-2-benzoxazolinone (MBOA) (Hibbard & Bjostad, 1988), and free fatty acids (Hibbard et al., 1994). When the larvae grow older, they move inward and consume the root core (Chiang, 1973). Third instar larvae pupate in the soil. Females take 45 to 20.7 days to complete larval development and metamorphosis at the temperature range between 18 and 30 ˚C respectively. The males develop a little faster than females (Meinke et al., 2009). The beetles emerge during late June to early July and peak in July (Nowatzki et al., 2002). The total development duration is affected by many factors, including the phenology of corn (Bergman & Turpin, 1986; Meinke, 1995; Musick et al., 1980), soil insecticide use (Boetel et al., 2003), and the adoption of transgenic crops (Becker, 2006; Nowatzki et al., 2008).

Although the primary diet of western corn rootworm is corn, their life cycle can be supported by western wheatgrass (*Pascopyrum smithii* (Rydb.)), pubescent wheatgrass

(*Elytrigia intermedia* (Host)), side-oats grama (*Bouteloua curtipendula* Michx.) as well as several prairie grass species (Oyediran et al., 2004). Female adults may also consume soybean leaves in soybean-corn rotation fields, but they provide little nutrition to them (Gray et al., 2009; O'Neal et al., 2002).

Control and Adaptation

Chemical control has been used for western corn rootworm since the 1940s when the damage by rootworm first became significant (Ball & Weekman, 1962). Crop rotation and application of Bt transgenic corn are alternatives to chemical control. However, the physiological plasticity and adaptability of western corn rootworm have resulted in adaptations to current management approaches (Gassmann et al., 2011; Gray et al., 2009; Meinke et al., 2009).

Soil insecticides are applied in the cornfield at planting to control larvae (Gray et al., 1992; Mayo & Peters, 1978; Meinke et al., 1998). In the Midwest US, 50-60% of corn fields are treated with soil insecticide for rootworm management (Levine & Oloumi-Sadeghi, 1991). Foliar sprays are used for controlling beetles to prevent corn silk loss. They also significantly suppress egg density in the next generation (Meinke et al., 1998).

Organochlorine pesticides were first applied as soil treatments in 1949 in Nebraska (Ball & Weekman, 1962), and were replaced by carbamate and organophosphate in 1970s. Both types of pesticide are also used in foliar sprays for adult control. The intensive use

of pesticides, as well as continuous corn cultivation kept a strong selective pressure on western corn rootworm populations. Insufficient control by organochlorine pesticides was reported in the field in 1959 (Ball & Weekman, 1962; Meinke et al., 1998; Roselle et al., 1959). Three years later, low sensitivity to organochlorine pesticides was verified in adults (Ball & Weekman, 1962). After carbamate and organophasphate pesticides were adopted, significant resistance in adults was reported in Nebraska in the late 1990s. Foliar applications of oganophasphate and carbamate insecticides also led to resistance evolution in western corn rootworm (Meinke et al., 1998). The data from Nebraska demonstrated foliar sprays provide a higher selective pressure than soil treatments (Meinke et al., 1998). The evidence of soil insecticide resistance was also reported in 3rd instars (Wright et al., 2000).

The physiological mechanisms of pesticide resistance have been studied in western corn rootworm. Both oxidative (P450-based) and hydrolytic (esterase-based) pathways are involved in pesticide detoxification (Miota et al., 1998). Constitutively elevated expression of esterase was found in organophosphate insecticide resistant populations (Wright et al., 2000; Zhou et al., 2003). Cytochrome P450s are also involved in carbaryl and organophosphate resistance in western corn rootworm, which indicates that western corn rootworm adopts N-demethylation to deactivate some insecticides (Scharf et al., 2001; Scharf et al., 2000).

Transgenic corn expressing Bt endotoxin has been developed to control western

corn rootworm (Moellenbeck et al., 2001). Bt corn expressing Cry3Bb1 (Environmental Protection Agency, 2003; Vaughn et al., 2005), mCry3A (Environmental Protection Agency, 2010), and Cry34/35Ab1 (Environmental Protection Agency, 2010) are available in the United States. Selection experiments in the greenhouses have produced colonies with resistance to Cry3Bb1 (Meihls et al., 2008) and Cry34/35Ab1 (Lefko et al., 2008). The resistant colonies show significantly higher survival than control larvae feeding on non-Bt isolines. Field populations evolved resistance were first identified in Iowa, where Cry3Bb1 corn was continuously grown for several seasons (Gassmann et al., 2011). A possible reason for resistance is that Cry3Bb1 corn offers a low-to-moderate dose of toxin to western corn rootworm, which doesn't eliminate heterozygous individuals that pass the resistant allele to offspring (Gassmann et al., 2011; Meihls et al., 2008; Siegfried et al., 2005). Insufficient refuge may have contributed to the increasing level of resistance in those populations (Gassmann et al., 2011).

Gene silencing by double stranded RNA (dsRNA) was developed as a genomic tool in research (Fire et al., 1998). It is also considered as a potential approach to control pests by silencing essential genes (Price & Gatehouse, 2008). In western corn rootworm, silencing of vacuolar ATPase (v-ATPase) by feeding dsRNA causes significant mortality and morbidity in both larvae (Baum et al., 2007) and adults (Rangasamy & Siegfried, 2012). Genetic engineering enable the expression dsRNA in plant cells (Huang et al., 2006), which suggests that corn lines expressing dsRNA may be available in the future to

control western corn rootworm.

Crop rotation is an effective cultural method for managing many specialist pests, including western corn rootworm. Corn-soybean rotation is used to manage corn rootworms in many farms in the Midwest of the US. However, the efficacy of crop rotation has been reduced by the appearance of rotation resistant western corn rootworm populations (Levine et al., 2002). Severe damage caused by rotation resistant western corn rootworm was first reported in central Illinois. Since then, the problem has spread throughout part of the Corn Belt (Gray et al., 2009). The females of rotation resistant populations have lost their strict fidelity to cornfield for ovipositing eggs. Instead, they lay a significant number of eggs in the non-host soybean field that will be planted with corn the next year (Gray et al., 2009). The adaptation to crop rotation is a genetic trait, although the molecular mechanisms are not clear (Gray et al., 2009).

INSECT-PLANT INTERACTIONS

Plants and insect herbivores interact in complex ways. It is estimated that over 50% of total insect species are herbivores (Janz et al., 2006). Plants provide food resources but also stresses for insect herbivores, through low food quality, physical barriers, toxic defense chemicals and antifeedant compounds. A successful herbivore must evolve strategies to overcome these stresses.

Plants evolved a sessile life, and their ability to avoid grazers and herbivores is limited (Milchunas & Noy-Meir, 2002). Some studies have also suggested plants can

compensate their loss by increasing photosynthesis in the remaining tissues (Strauss & Agrawal, 1999; Trumble et al., 1993). Tough structures of plants can reduce susceptibility to insects as physical barriers (Howe & Jander, 2008).

Plant chemistry is a major factor in insect-plant interactions. Plants have evolved a large number of chemicals for defending themselves. In response, herbivores adopt strategies to avoid or deactivate defense chemicals. The arms race between plants and insects is a form of co-evolution (Bernays & Graham, 1988; Ehrlich & Raven, 1964). Although several researchers challenged the idea of co-evolution, (Bernays & Graham, 1988), studies in many plant species revealed chemical defense systems with delicate signal pathways. Secondary metabolites and insecticidal proteins are two major end products of defense pathways and interact directly with herbivores.

Plant Secondary Metabolites

The term of secondary metabolites was first defined by Kossel as plant chemicals that are not directly involved in growth and reproduction (primary metabolism) (Bourgaud et al., 2001). In the 1950s, researchers started to understand their physical and ecological significance (Fraenkel, 1959). However, due to the diversity in structure and function of secondary metabolites across plant species, only a few of them have been well studied. The classification of plant secondary metabolites was considerably difficult. Most plants contain secondary metabolites from the following families: phenolic compounds, terpenes, sulphur-containing compounds, and alkaloids (Bourgaud et al.,

2001; Crozier, 2006). Other secondary metabolites compounds have been also identified.

Plant secondary metabolites have important roles in plant-insect, plant-microbe, and plant-plant interactions (Wink, 1999). Humans have a long history of using plant secondary metabolites as dyes, flavors, medicines and insecticides (Balandrin et al., 1985). Plant secondary metabolites are responsible for some resistance traits in many plant-breeding programs (Wink, 1988).

The functions of secondary metabolites in plant-insect interactions can be categorized into three types: toxins (antibiosis), feeding deterrents, and multi-trophic interactions (Wink, 1988). An individual compound may have different types of functions. For example hydroxamic acids, the major secondary metabolites in Poaceae, demonstrate antibiosis to European corn borer (*Ostrinia nubilalis*) (Reid et al., 1990) and greenbug (*Schizaphis graminum*) (Argandona et al., 1981; Givovich & Niemeyer, 1995), and antifeedant effects on Asian corn borer (*Ostrinia furnacalis*) (Yan et al., 1999) and several other insect herbivore species (Givovich & Niemeyer, 1995; Reid et al., 1990). The multi-trophic interactions mediated by secondary metabolites are referred as indirect defenses. Plants use secondary metabolites as signals to recruit natural enemies. For example, corn seedlings release terpenoid volatiles in response to lepidopteran insect feeding. Those compounds can attract parasitoid wasps, the natural enemies of herbivores (Turlings et al., 1990). Mechanical damage and oral secretions are both critical to trigger the release of the volatiles (Turlings et al., 1990). The inducible volatiles were identified

as belonging to the terpene family (Dicke et al., 2009). This phenomenon has been observed in more than 15 plant species (Degenhardt et al., 2003) and also underground, where corn root released volatiles can help entomopathogenic nematodes locate western corn rootworm larvae (Rasmann et al., 2005).

There are three strategies that have been adopted by plants to produce defensive secondary metabolites (Hartmann, 1985; 1996). The first strategy is constitutive expression, in which the secondary metabolites are always present in plant tissues, such as the accumulation of nicotine in tobacco plants (*Nicotiana* ssp.). The second strategy is inducible expression, in which expression only occurs when the plant is attacked by herbivores. A third strategy is to produce defense chemicals constitutively, but as inactive precursors. The release of active compounds is triggered by herbivore attack. For example, hydroxamic acids are converted to inactive beta-glucosides immediately after synthesis. The free hydroxamic acids are released a short period after the plant is challenged by a herbivore (Niemeyer, 1988).

The synthesis, activation and release of plant secondary metabolites are controlled by sophisticated and intricate signal pathways mediated by Jasmonic acid (JA) and ethylene. Both compounds are plant hormones and are up regulated when the plants are challenged by insect herbivory (Gundlach et al., 1992). An increased level of secondary metabolites is observed with up regulated JA and ethylene (Gundlach et al., 1992).

Insecticidal Proteins

Plant proteins with antibiosis effects also contribute to plant defenses. Toxins from *Bacillus thuringiensis* and *Galanthus nivalis* agglutinin (GNA) transgenic plants belong to this category. However plants also express endogenous insecticidal proteins to interfere directly with herbivores.

Protease inhibitors consist of a large group of insecticidal proteins that have been identified in many plant species. Plant protease inhibitors interfere with the digestive enzymes in the insect midgut (Green & Ryan, 1972). Studies in many plant species have demonstrated that the expression of protease inhibitors is inducible and regulated by plant signaling pathways (Koiwa et al., 1997). In corn, maize protease inhibitor (MPI) is produced in response to mechanical wounding (Cordero et al., 1994) and fall armyworm (*Spodoptera littoralis*) feeding (Tamayo et al., 2000). It strongly inhibits serine proteases, elastase, and chymotrypsin, which are the three most significant proteases in lepidopteran larvae (Cordero et al., 1994).

Plants also use proteases as a weapon against insects by proteolysis of the insect's peritrophic matrix. Papaya (*Carica papaya*) and fig (*Ficus virgata*) contain a high concentration of papain or papain-like enzymes. They both demonstrate inhibitory effects on the growth and development of lepidopteran larvae (Konno et al., 2003). A cysteine protease, named maize insect resistance 1-cysteine protease (Mir1-CP), was identified in corn line MP708 (Lopez et al., 2007; Pechan et al., 2000). This protein is induced by lepidopteran larval feeding. It is also regulated by the collaboration of Jasmonic acid and

ethylene pathways (Ankala et al., 2009).

Chitinase is an inducible plant defense protein that responds to pathogen infection in many plant species. An antibiosis function against insects was identified in corn seeds (Lin et al., 1992). Up regulated chitinase level in response to insect herbivory, and mechanical wounding has been demonstrated in sorghum (Krishnaveni et al., 1999). Chitinase is also a candidate gene in transgenic crop breeding for pest control (Ding et al., 1998).

HYDROXAMIC ACIDS

As a successful corn pest, western corn rootworm encounters antibiosis secondary metabolites, including hydroxamic acids. Hydroxamic acids are a series of compounds containing 1,4-benzoxazin-3-one groups (Cambier et al., 2000). They naturally occur in corn and other Poaceae plants as several different forms. The major compounds are: DIMBOA (1,4-ben-zoxanzin-3(4H)-ones), DIBOA (2,4-dihydroxy-2H-1,4-benzoxazin-3(4H)-one) and HMBOA (2-hydroxy-7-methoxy-1,4-benzoxazin-3(4H)-one) (Tipton et al., 1967). In corn, DIMBOA is the most abundant. Hydroxamic acids are stored as inactive forms 2-β-*O*-D-glucosides (Niemeyer, 1988) in the plant. The active hydroxamic acids are released when a plant is challenged by insect herbivores or pathogens (Niemeyer, 1988; Oikawa et al., 2001).

Biosynthesis

The *de novo* DIMBOA biosynthesis pathway in maize has been reviewed by Frey et al. (1997b; 2009) (Figure 1.1). It starts with Indole-3-glycerol-phosphate, an intermediate compound in the tryptophan synthesis pathway. A series of *bx* genes located on the short arm of chromosome 4 encode the enzymes for DIMBOA synthesis (Frey et al., 1997a; Frey et al., 2009; Gierl & Frey, 2001). Gene *bx1* encodes a branch enzyme that converts indole-3-glycerol-phosphate into indole. The *bx2* to *bx5* encode redox enzymes, which convert indole to DIBOA in microsomes. Cytochrome P450s are involved in those steps. DIBOA is a highly active compound and it is quickly transferred into cytosol and glucosylated by *BX8* and *BX9*. A methoxy group is added to C-7 by *BX6* and *BX7* in the cytosol to synthesize the final product DIMBOA-Glc (Figure 1). The DIMBOA-Glc is stored in the vacuole. When the plant is attacked by insects, the DIMBOA-Glc is quickly hydrolyzed to frees DIMBOA. In corn, two methoxy groups are added to the DIBOA, forming DIM_2BOA (8-methoxy-DIMBOA-Glc), an alternative defense compound (Xie et al., 1991).

Hydroxamic acids are constitutively synthesized in corn plants. They are not present in the seeds (Argandona et al., 1981; Niemeyer, 1988). Biosynthesis starts after germination. The concentration of hydroxamic acid reaches its maximum at several days after germination, then declines rapidly (Cambier et al., 2000; Niemeyer, 1988). Young corn leaves tend to have higher concentration than the older tissues (Niemeyer, 1988). Plant varieties, nutrients and environmental conditions affect hydroxamic acid levels

(Niemeyer, 1988).

Functions

Hydroxamic acids are associated with plant resistance to insect herbivores, pathogens and in some cases, herbicides (Niemeyer, 1988). After DIMBOA is released, it decomposes to give the downstream product MBOA. The MBOA is very active and interacts with nucleophilic biological macromolecules (Niemeyer, 1988). Hydroxamic acids have demonstrated antibiosis effects on European corn borer (*Ostrinia nubilalis*) (Reid et al., 1990) and several aphid species including greenbug (*Schizaphis graminum*) (Argandona et al., 1981; Givovich & Niemeyer, 1995). Hydroxamic acids have been shown to interfere with insect detoxification systems by inhibition of esterase and moderately inhibition of glutathione-S-transferases in *Rhopalosiphum padi* (Mukanganyama et al., 2003).

DIMBOA also has an antifeedant effect on many corn pest species, including aphids (Givovich & Niemeyer, 1995) and Asian corn borer (*Ostrinia furnacalis*) (Yan et al., 1999). Conflicting results have been reported for European corn borer and western corn rootworm. Robinson et al (1978) reported an antifeedant effect of DIMBOA on European corn borer, while Campos et al. (1989) found no that effect was not significant. Similarly, Xie et al. (1992) declared an antifeedant effect of DIMBOA on western corn rootworm larvae, while the result from Robert et al. (2012) showed no significant difference. Other functions of hydroxamic acids have been reviewed, including

allelopathy to other plants, triggering of reproduction of the mammal montane vole (*Microtus montanus*), affecting mineral uptake in plants, modification of auxin receptors, plant detoxification and mutagenesis (Niemeyer, 1988).

Other hydroxamic acids are also distributed in corn plants. HDMBOA has a higher concentration in old and very young leaves (Cambier et al., 2000). In corn roots, HDMBOA is the dominant hydroxamic acid at 6 days after germination (Cambier et al., 2000). MBOA is also present in corn, it may serve as a rapid response against herbivores (Oikawa et al., 2001).

Applications

The concentration of hydroxamic acid is an important trait for plant breeding. Resistance to European corn borer is related to DIMBOA content (Reid et al., 1990). The inherence of DIMBOA is quantitative and controlled by a series of loci (Dunn et al., 1981; Klun et al., 1970).

DNA SEQUENCING TECHNOLOGY

DNA sequencing has become a powerful tool in western corn rootworm research. Genome and transcriptome sequencing projects will benefit understanding of genetic variation, invasion mechanisms, gene identification, and expression analysis (Miller et al., 2010). DNA sequencing technologies have evolved in two major generations.

First generation sequencing technology

First generation sequencing refers to the techniques of chemical degradation

(Maxam & Gilbert, 1977) and chain termination (Sanger et al., 1977b) . The chemical degradation method uses chemical reagents to cleave DNA at given positions (Maxam & Gilbert, 1977). The chain termination method involves DNA polymerase I mediated DNA synthesis and radioactively labeled dideoxynucleotides to stop extension (Sanger et al., 1977b). Both methods use polyacrylamide gel electrophoresis (Maniatis et al., 1975) and autoradiophotography to display sequences on polyacrylamide gels. The chain termination method (Sanger sequencing) was improved by fluorescent-labeled dideoxynucleotides (Ansorge et al., 1986), capillary electrophoresis and laser detection (França et al., 2002) and semiautomated workflows. Commercial facilities for semi-automated Sanger Sequencing are available, generating 700-800 bp high quality reads for researchers (França et al., 2002).

Before 2004, first generation sequencing was the only technique for DNA sequencing. The first organism (Phage ΦX174) (Sanger et al., 1977a), first free-living organism (*Haemophilus influenzae*) (Fleischmann et al., 1995), first eukaryote (*Saccharomyces cerevisiae*) (Goffeau et al., 1996), first animal (*Caenorhabditis elegans*) (The C. elegans Sequencing Consortium, 1998), the first plant (*Arabidopsis thaliana*) (Arabidopsis Genome Initiative, 2000), and human (*Homo sapiens*) were sequenced using Sanger sequencing. The workflow of genome (or transcriptome) sequencing was described by Sulston et al. (1992). First the genome (or cDNA from transcriptome) is sheared into fragments. A sequence library is constructed by cloning fragments into

sequencing vectors. Vector carrying fragments are randomly selected for Sanger sequencing. In order to eliminate gaps, the read coverage (sequencing depth) is several times the size of the genome.

Second generation sequencing technology

Parallel sequencing technology that emerged in the early 2000s was named "Second Generation Sequencing". The first second generation sequencing method was developed by Roche/454 in 2004 (commercialized by 454 Life Sciences) (Margulies et al., 2005; Metzker, 2010). Later, two alternative parallel sequencing platforms Solexa (commercialized by Solexa, now part of Illumina) (Bentley, 2006) and SOLiD (Sequencing by Oligonucleotide Ligation and Detection, commercialized by Applied Biosystems, Inc., now part of Life Technologies) became available (Mardis, 2008). These new sequencing methods feature *in vitro* template preparation. All of them are able to generate a large number of reads in one sequencing run.

The workflow of second-generation sequencing consists of library construction, sequencing reactions, and data analysis. Instead of in vivo library construction using vectors, the sequencing templates for second-generation sequencing are clusters prepared by emulsion PCR (454 and SOLiD) (Williams et al., 2006) or bridge PCR (Solexa) (Adessi et al., 2000; Fedurco et al., 2006; Shendure & Ji, 2008). These clusters are fixed on the 2-D surface where the sequencing reactions take place. A CCD (Charge-coupled Device) is used to capture light signals emitted from templates during sequence reactions,

by which the sequence data is obtained (Metzker, 2010).

The sequencing reactions fundamentally differentiate three second-generation technologies. Roche/454 and Solexa adopted synthesis-based reactions, in which nucleotides are added in a fixed order (Metzker, 2010). Roche/454 uses Pyrosequencing. When one nucleotide is added to the primer, pyrophosphate is released. The free pyrophosphate is catalyzed by sulfurylase and luciferase, causing energy to be released as light and captured by the CCD (Ahmadian et al., 2000). Solexa adopted a cyclic reversible termination reaction (Metzker, 2010). The added nucleotide is conjugated with a 3'-blocker connected to a nucleotide-specific fluorophore for identification. Then the 3'-blocker is removed for next sequencing extension. The 3'-blocker nucleotide prevents multiple incorporations in tandem, a phenomenon that reduces the accuracy in Roche/454 method. The SOLiD platform uses a ligation-based approach. A series of 8-nucleotide probes are added, each of them is bound to a fluorescent dye and has two barcode bases at 3' end to anneal to the template. One dye represents four different two-base combinations, thus, four colors will be present in sequencing reactions. After two barcode bases are incorporated to the next position of the primer, the rest of the probe is cleaved, causing the signal to be released from the dye. In order to get the sequence information, at least 5 to 7 rounds of sequence reactions are necessary, each starts with a primer with one base longer at its 5' end (Metzker, 2010).

There are several new features of next generation sequencing technology: first, the

read lengths generated by second generation sequencing platforms are between 25 and 500 base pairs, shorter than ~800 basepair reads from Sanger sequencing. Second, the coverage (sequence depth) per run is much higher. Third, the cost per base pair is much lower (Shendure & Ji, 2008). The corresponding data analysis is also required to be capable of handling massive amounts of data.

GENE EXPRESSION ANALYSIS METHODS

In order to understand biological processes of western corn rootworm, such as growth, development, and adaptation to insecticides, plant defense substances, transgenic crops, and crop rotation, it is necessary to analyze the functions and abundance of genes expressed from the genome. Methods developed for gene expression analysis, can be categorized into three groups based on the methodologies they rely on: (1) hybridization based, (2) sequencing based, and (3) quantitative PCR.

Hybridization based methods

Northern blot was the first method to be developed for gene expression analysis. The electrophoresed sample RNA is hybridized with a specific P^{32} labeled DNA probe on a piece of aminobenzyloxymethyl treated paper. After washing off non-hybridized probes from the paper, the mRNA with complementary sequence with the probe is visible by autoradiography (Alwine et al., 1977). Northern blotting was also used for quantifying target genes (Barbu & Dautry, 1989).

DNA microarrays were derived from northern blot and allow higher accuracy and

throughput in quantification. Probes are fixed on the surface of a high-density array, each corresponding to a target gene. Sample RNA is converted to cDNA by reverse transcription and labeled with a fluorescent dye. The cDNA pool is hybridized to the array. After washing, the cDNAs with complementary sequences of probes are retained on the array. The position and intensity of fluorescent signal from labeled cDNA provides both qualitative and quantitative measures of gene expression (van Hal et al., 2000). Differential gene expression analysis, such as comparing expression profiles between two treatments, can be performed by labeling two RNA samples with distinct fluorescent dyes (van Hal et al., 2000).

Microarrays enable the researcher to screen high-throughput gene expression at genome level in one test (Schena et al., 1995). However, one prerequisite is that the subject organism has an available genome sequence, or at least that most of the transcriptome sequence is known. Microarrays are used intensively with model organisms including yeast (*Saccharomyces cerevisiae*) (Lashkari et al., 1997), *Arabidopsis thaliana* (Schena et al., 1995), and human (Schena et al., 1996). For model organisms, commercialized platforms are available, providing standard protocols from experimental design to data analysis (Holloway et al., 2002; Tan et al., 2003).

Sequencing based methods

Since the first generation sequencing era, methods have been developed for transcriptome profiling, including large-scale cDNA sequencing (Okubo et al., 1992),

serial analysis of gene expression (SAGE) (Velculescu et al., 1995), cap analysis of gene expression (CAGE) (Shiraki et al., 2003), and massively parallel signature sequencing (MPSS) (Brenner et al., 2000). The high cost and limited sequencing depth restrict the use of these methods. RNA-Sequencing (RNA-Seq) (Mortazavi et al., 2008) methods become popular for qualitative and quantitative analysis of transcriptome when second generation sequencing technology became available.

The main procedures of RNA-Seq are described below. RNA is sheared into fragments and converted to cDNA by random priming (Mortazavi et al., 2008). The cDNA library is sequenced using high-throughput sequencing platforms, which generate millions of reads ranging in length from 25 (Solexa) to 500 (Roche/454) nucleotides. The next step is to align the read sequences to a reference genome sequence (Mortazavi et al., 2008), transcriptome sequence or transcriptome sequence *de novo* assembled from the reads (Wang et al., 2009). RNA-Seq is capable of profiling expression by searching reads aligned to genes in reference databases. The expression levels are determined by counting number of reads aligned to each gene (Wang et al., 2009).

RNA-Seq is a highly reproducible method comparable to microarrays (Marioni et al., 2008). It also has advantages over microarrays. First, RNA-Seq can be used on organisms with an unknown genome or transcriptome sequence by aligning reads to a de novo assembly from the reads themselves (Dunning et al., 2013). Second, the quantification is more accurate than microarrays with a higher dynamic range (Wang et

al., 2009; Wilhelm & Landry, 2009). Third, the background noise of RNA-Seq is almost negligible (Wang et al., 2009). RNA-Seq also has extra benefits to researchers for finding low expressed genes and defining exon boundaries (Wang et al., 2009).

Quantitative real-time PCR

Real-time PCR is a method to quantify a target sequence in an initial template. It was derived from traditional PCR and uses two distinctive methods to detect the copies of PCR products in real time: dual fluorogenic probes and SYBR green. The dual fluorogenic system applies a set of dual fluorogenic labels in PCR probes. The fluorescent signal is released when the daughter strand is extended (Heid et al., 1996). SYBR green is a fluorescent dye that binds to the minor groove of double stranded DNA (Morrison et al., 1998). PCR product is quantified by detecting fluorescence intensity of SYBR green during amplification (Morrison et al., 1998; Pfaffl, 2001).

Target genes are quantified in two ways: absolute quantification and relative quantification. Absolute quantification procedure measures the initial concentration of the target gene in sample mRNA. An internal or external standard curve is necessary to calibrate the system (Morrison et al., 1998; Pfaffl, 2001). Relative quantification measures the expression level changes among different samples (Pfaffl, 2001). A reference gene (usually a house keeping gene) that is expressed equally in all samples is necessary as an endogenous standard (Pfaffl, 2001). Because real-time PCR is a convenient, accurate and reproducible method, it is widely used to verify genes identified

by other methods (Pfaffl, 2001).

JUSTIFICATION AND OBJECTIVES

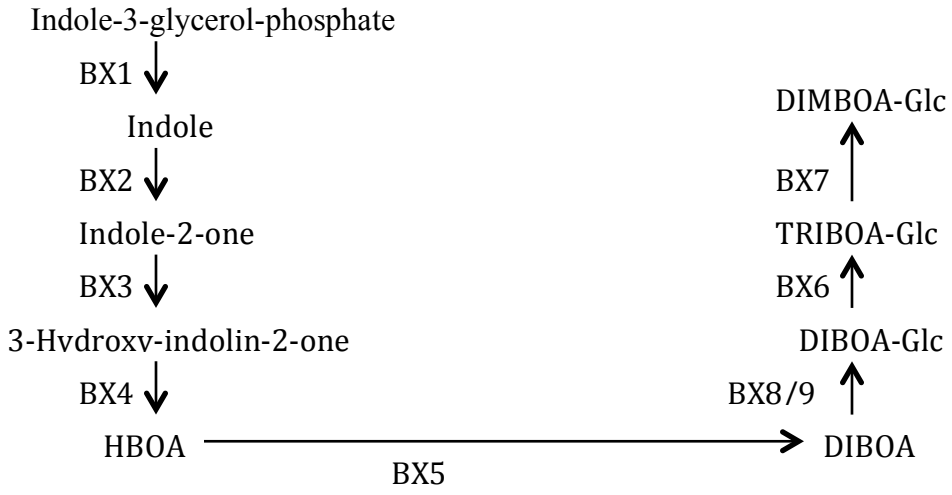
Many studies have been done on corn defense chemicals, including chemistry, physiological functions, synthesis, distribution and applications. However, the effects of corn defense chemicals on western corn rootworm are not very clear. The physical and biochemical interactions between western corn rootworm larvae and hydroxamic acids, the most significant secondary metabolites in corn plants, have not been well studied. The overall goal of this study is to understand the effect of hydroxamic acids to western corn rootworm larval growth, development and gene expression. This goal is addressed under three objectives

(1) Compare the larval growth, development and survival rate on mutant corn plants that do not produce hydroxamic acids, and on wild type parental lines that do produce hydroxamic acids. Larval dry weight, instar distribution, and survival rate were used to evaluate the effects of hydroxamic acid.

(2) Sequence the transcriptome from western corn rootworm larvae from both corn lines using next generation sequencing technology. Assemble a transcriptome to obtain a set of reference sequences for expression analysis.

(3) Analyze the RNA sequencing data to obtain gene expression profiles. Identify differentially expressed genes to understand the physiological response of western corn rootworm larvae.

Figure 1.1: *De novo* biosynthesis of hydroxamic acids in corn (Frey et al., 2009)



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CHAPTER 2

The Effect of Hydroxamic Acids on Western Corn Rootworm *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) Growth and Development

Introduction

The western corn rootworm (*Diabrotica virgifera virgifera* LeConte) is one of the most destructive corn pests in North America. Larvae live underground, feeding on root tissue and adults feed on corn silk, pollen, leaves and floral tissue. The western corn rootworm causes great damage to corn production. It is estimated that *Diabrotica* species cost $1.7 billion annually in the United States (Sappington et al., 2006). Western corn rootworm is native to North America, but its distribution expanded rapidly during the last 60 years (Chiang, 1973; Gray et al., 2009; Meinke et al., 2009; Smith & Lawrence, 1967). The invasion of Europe by this species was reported in 1992 (Ciosi et al., 2008; Kiss et al., 2005). Now the species has expanded to over 11 European countries (Ciosi et al., 2008; Miller et al., 2005).

The western corn rootworm is a very adaptable insect. Although many management strategies have been used for western corn rootworm including crop rotation, chemical insecticides, and transgenic Bt corn (Meinke et al., 2009), its resistance to soil insecticides (Meinke et al., 2009), transgenic Bt corn (Gassmann et al., 2011), and crop rotation (Gray et al., 2009) has been reported. Given the adaptability to environmental stresses, it is a good model to study insect-host interactions.

Western corn rootworm is a specialist herbivore, feeding almost exclusively on corn (*Zea mays* L.) (Branson & Krysan, 1981; Krysan & Smith, 1987; Meinke et al., 2009). It presumably has adapted to stresses imposed by corn, including secondary metabolites, which are significant components of plant chemical defense. The most abundant secondary metabolite in corn is DIMBOA, a hydroxamic acid. DIMBOA is a toxic chemical that is constitutively synthesized and stored as an inactive 2-β-*O*-D-glucoside in plant cells (Niemeyer, 1988). When the plant is attacked by herbivores, the free DIMBOA is quickly released (Niemeyer, 1988; Oikawa et al., 2001). Previous studies on the effects of hydroxamic acids on insect herbivore presented mixed results. Early reports indicated hydroxamic acids have a significant behavior-modifying and feeding-deterrent effect on western corn rootworm larvae (Assabgui et al., 1995; Xie et al., 1992b). However recent research on native corn lines demonstrated that levels of hydroxamic acid are not related to larval survivorship and development (Davis et al., 2000). Fresh weight differences were insignificant whether larvae fed on DIMBOA-containing roots or non-DIMBOA-containing roots for 24 hours (Robert et al., 2012). MBOA, a bioactive downstream compound of DIMBOA, is also found to have no significant toxicity to western corn rootworm larvae (Abou-Fakhr et al., 1994). Choice studies in the some research have indicated that DIMBOA might be one of chemical cues for larvae to locate the most nutritious root tissue (Hibbard & Bjostad, 1988; Robert et al., 2012).

The mechanisms by which western corn rootworm copes with hydroxamic acids are not clear. The objective of the studies in this chapter is to test the sublethal effect of hydroxamic acids on western corn rootworm larvae. Since hydroxamic acids are present in all natural and commercial corn lines, the sublethal effect will be only measured when western corn rootworm larvae are feeding on hydroxamic acid free corn. The objective of this study is to compare the larval growth, development and survival rate on hydroxamic acid free corn and its wild type counterpart.

Materials and methods

Hydroxamic acid free mutant and wild type corn lines

Mutant corn line 428G cannot synthesize hydroxamic acids because it carries a homozygous *bx1* mutation (Frey et al., 2009). H88 is the wild type parental line of 428G that has a functional hydroxamic acid synthesis pathway. A limited number of 428G and H88 seeds were received from the Maize Genetics Cooperation Stock Center at the University of Illinois at Urbana-Champaign and Purdue University respectively. In order to obtain enough seeds for experiments, both lines were propagated by self-pollination in two separate greenhouses at University of Nebraska-Lincoln. Each individual plant was grown in a #16 pot filled with soil. Necessary fertilization and pest management were provided. After the shoots appeared, they were covered with a shoot bag to avoid any possible unwanted cross breeding. Hand pollination was implemented on the day when the tassels were completely open and starting to shed pollen. At that day, corn stalks were

bent and the tassels were slid into tassel bags carefully. Then the silks were cut with clean scissors at 1.5 inch above the silk base. On the next morning between 9 and 10 am, the tassel bags were gently shook to collect pollen, then dumped the pollen on the silk. After finished hand pollination, the tassel bags were left on the pollinated shoot. After the seeds matured, the ears and seeds were harvested and stored at 4 ˚C.

In order to confirm the hydroxamic acid traits in the two lines, a colorimetric hydroxamic acid assay was carried out (Long et al., 1974). Ten seeds from each line were randomly sampled and placed in 15 ml centrifuge tube filled with moist vermiculate for seven days. The root tissue was crushed on filter paper soaked with 0.1M $FeCl_3$. Seedlings from H88 produced a purple color, caused by the coordinate complex formed by hydroxamic acids and ferric ion. In contrast, 428G seedlings did not produce a color change

Washing and surface sterilization of western corn rootworm

Non-diapause western corn rootworm eggs were received from USDA-ARS Northern Grain Insects Research Laboratory at Brookings, SD (Branson, 1976). The eggs were deposited between two layers of wet soil in a petri dish. The eggs were shipped and incubated in at room temperature.

The eggs were washed and surface sterilized to minimize the contamination by microorganisms. The soil provided a moist condition and protection for eggs. The optimal time for washing is right before mass hatching, which was indicated by the

emergence of first neonate larva on the top of soil. Washing consisted of 7 steps. First eggs mass were placed in a sieve and washed by tap water to remove the soil, then transferred the egg mass into 50 ml centrifuge tube, rinsed the egg mass to remove the debris. Forty milliliter of 58.3 g/L MgSO4 solution was added into the tube. At this concentration, the eggs are floating and heavier soil residue remains at the bottom of tube. After this step, the eggs were rinsed in the sieve by tap water and recovered into 50 ml tube. Then the eggs were surface sterilized by 40 ml of Lysol Soap for 3 minutes, 10% formaldehyde for 2 minutes, and 1% bleaches for 1.5 minutes. The eggs were completely rinsed in the sieve by tap water and recovered into 50 ml tube after each chemical treatment. Finally the eggs were rinsed twice by distill water. The clean eggs were deposited on moist filter paper placed in petri dish in room temperature.

Survival rate analysis of western corn rootworm feeding on H88 and 428G lines

A bioassay was conducted in a growth chamber with settings of 27 ˚C, 14: 10 hour light: dark cycle. In order to reduce the interference of fungus, the seeds were coated with 0.1 grams of thiabendazole fungicide for every 100g seeds. The seeds, fungicides and water were mixed completely in a Ziploc bag. Coated seeds were placed separately in 15-ml centrifuge tubes with 13 ml sterile vermiculite moistened with 7 ml of water. One milliliter of vermiculite covered the top of the seed. One milliliter of water was added into each tube every three days to maintain the moisture. Two 6x6 15-ml centrifuge tube racks were set in the chamber on different days, each rack represents a block. Eighteen

H88 and 18 428G plants were placed randomly in each block. A randomized block design was used to minimize chamber environment variation.

Infestation occurred at day 7 after planting. Each seedling was carefully pulled out of vermiculite. Three neonate larvae were picked up using a paintbrush (size: 18/0) under a VistaVision stereo zoom microscope and transferred to the exposed corn root base. The seedlings were placed into the same tube and replenished with autoclaved vermiculite. At day 14 after planting, seedlings and vermiculite were put into a petri dish and examined under the microscope. The surviving larvae were recovered from roots. The number of survival larvae from each tube was recorded.

The survival rate between larvae feeding on H88 and 428G plants was analyzed by SAS 9.2 using Glimmix procedure for multinomial data (SAS Institute Inc., 2008). The block was set as a random variable.

Growth analysis of western corn rootworm feeding on H88 and 428G lines

Dry weight was used to estimate the assimilation rate for nutrients by larvae. The larvae collected from first rack of survival study were used for dry weight analysis. The experimental setting was described above. One 6x6 rack housing 18 H88 and 18 428G plants were set. Seven days after planting, seedlings were infested by western corn rootworm neonate larvae at the density of 3 individuals per seedling. Fourteen days after planting, the surviving larvae were recovered from seedlings and lyophilized by FreeZone 2.5 liter Benchtop Freeze Dry System at -50 ˚C, 0.012 MPa overnight. The dry

weight of each individual was measured by Sartorius R190P balance. The data were analyzed by SAS 9.2 (SAS Institute Inc., 2008) using a one-way analysis of variance (ANOVA) procedure with Tukey's test (5% level of significance).

Development analysis of western corn rootworm feeding on H88 and 428G lines

The instar distribution is a critical parameter to evaluate the development rate of a population. The instar of western corn rootworm can be determined by measuring head capsule width: 1st instar: 0-260 μm, 2nd instar: 260-420 μm and 3rd instar: 420-560 μm (Hammack et al., 2003).

Larvae recovered from the second rack from survival analysis were used for development analysis. The experimental setup was described above. Eighteen H88 and 18 428G plants were completely randomized placed in a 6x6 rack. Seedlings were infested by western corn rootworm neonates at the density of 3 larvae per plant on day 7 after planting. The surviving larvae at day 14 were recovered and kept in 70% ethanol solution. To determine the development stage of recovered larvae, the head capsule width was measured under stereo zoom microscope. The head capsule measurements were transformed to instar as described as above (Hammack et al., 2003). A two-factor chi-square test was applied to instar structure using SAS 9.2 (SAS Institute Inc., 2008).

Results

Survival rate analysis of western corn rootworm feeding on H88 and 428G lines

Western corn rootworm larvae were recovered from all 428G plants and H88 plants

in the test. For 428G, 13 plants had 1 surviving larvae, 18 plants had 2 surviving larvae, and 5 plants had 3 surviving larvae. For H88, 9 plants had 1 survivor, 16 plants had 2 survivors, and 11 plants had 3 survivors (Figure 2.1). The SAS GLIMMIX model using multinomial correction did not indicate a significant difference in survival rate between two plant types ($F=2.51$; $P=0.1178$).

Growth analysis of western corn rootworm feeding on H88 and 428G lines

A total of 32 western corn rootworm larvae were recovered from 428G seedlings. The average body dry weight was 0.45 g. There were 33 larvae recovered from H88 seedlings; the average dry weight was 0.50 g (Figure 2.2). There was no significant difference between the two groups ($F=1.05$; $P=0.3122$).

Development analysis of western corn rootworm feeding on H88 and 428G lines

A total of 73 larvae were recovered from all test plants, all of which are in either 2nd or 3rd instar. For 428G, 32 larvae were recovered, including 4 3rd instar larvae and 28 2nd instar larvae. Five 3rd instar and 36 2nd larvae were recovered from H88 plants (Figure 2.3). The chi-square test did not show a significant difference ($\chi^2=0.0015$; $P=0.9686$) in instar distribution (Table 2.1).

Discussion

In this chapter, the experiments demonstrated that a natural level of hydroxamic acids does not have significant effects on western corn rootworm larvae growth, development or survival. The difference in instar distribution was not significant,

indicating that the development of western corn rootworm was not delayed or promoted by hydroxamic acids. These results are comparable with the results from Davis et al. (2000) and Robert et al. (2012).

Although hydroxamic acids show a significant antibiosis effect on some lepidopteran and hemipteran species (Givovich & Niemeyer, 1995; Reid et al., 1990), the same effect on western corn rootworm was not significant, which indicates that the overall cost to western corn rootworm of coping with hydroxamic acids is small. The mechanism by which western corn rootworms cope with hydroxamic acids is unknown. There are three hypotheses that may explain the results of this study.

The first hypothesis is that western corn rootworms are generally insensitive to hydroxamic acids, which allows larval survival and growth at high hydroxamic acid levels. Tobacco hornworm (*Manduca sexta* L.) is a specialist feeding on tobacco (*Nicotiana attenuata*), which contains a high concentration of nicotine. Nicotine has a detrimental effect on insects by interacting with acetylcholine receptors in the central nervous system (Gepner et al., 1978). The central nervous system of tobacco hornworm is insensitive to nicotine (Morris, 1984). However, the interaction between protein and hydroxamic acids is less specific than ligand-receptor interactions such as nicotine and acetylcholine receptors. Hydroxamic acids can interact with many macromolecules (Niemeyer, 1988). It is less possible for western corn rootworm to evolve a large number of macromolecules adapted to high hydroxamic acids level.

The second hypothesis is that western corn rootworm has an inducible, but not energetic costly detoxification system to deactivate hydroxamic acids. Although larvae feeding on hydroxamic acid-containing corn invest extra energy in detoxification, the cost is tiny and undetectable over a 7-day period. Xie et al. (1992a) suggested that a detrimental effect on western corn rootworm larvae was observed when the corn roots were treated with high concentration (250-1000 ppm) of DIMBOA or MBOA. Native corn lines contain 80-1480 ppm of hydroxamic acids (Long et al., 1977). The development experiment also indicated that most of recovered larvae were in their 2nd instar. The root consumption of 2nd instar larvae is less than 3rd instar ones, which is considered as the most destructive stage. The negative effect of endogenous hydroxamic acids could be too small to identify at this stage.

The third hypothesis is that western corn rootworm adopted a costly deactivation system, but it is constitutively expressed whether hydroxamic acids present or not. Since western corn rootworm is a specialist feeding on corn and several grass species that contain hydroxamic acids, the stress is constant during evolution. A constitutively expressed mechanism eliminates the complex signal pathways, which would provide a faster response than inducible detoxification. Although the detoxification metabolism for hydroxamic acids may be costly, the larvae feeding on 428G roots cannot turn off the redundant enzymes and have to pay equal amount of energy cost, according to this hypothesis, which explains the non-significant difference in larval growth, development

and survival between hydroxamic acids and non-hydroxamic acid feeding groups.

To verify these hypotheses, comparison on survival, growth and development between hydroxamic acids non-hydroxamic acid feeding larvae should be extended to 3rd instar or adult stage. It is also necessary to identify whether hydroxamic acid detoxification mechanisms are constitutive, or inducible. Chapter 3 and 4 in this thesis will investigate the differential expression analysis using RNA-Seq.

Firgue 2.1: The distribution of survival rate of western corn rootworm larvae feeding on 428G and H88 plants

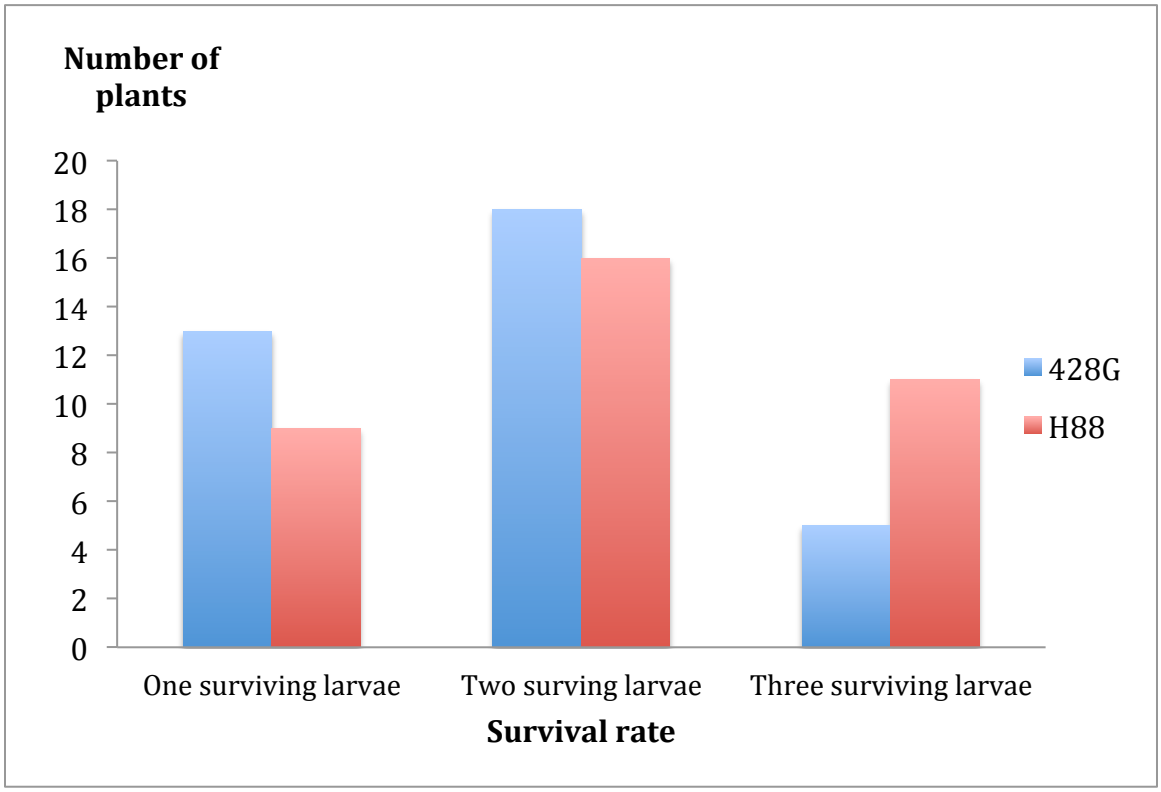

Figure 2.2: Average dry weight of western corn rootworm larvae recovered from 428G and H88 plants

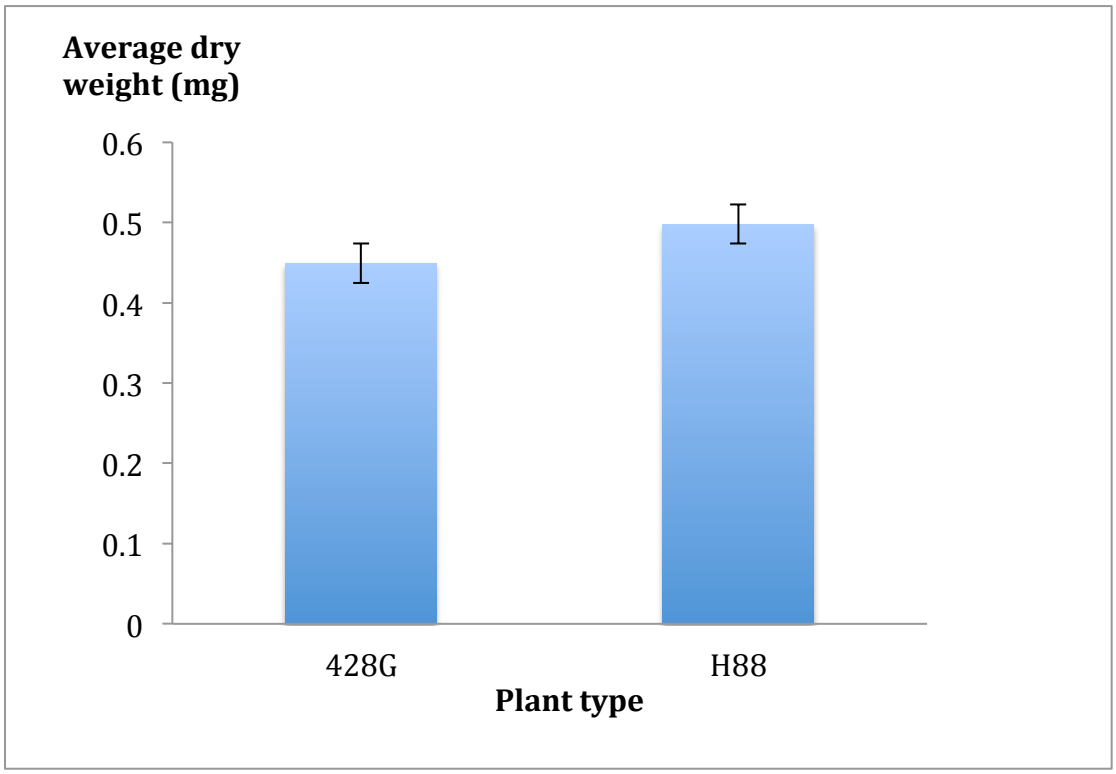

Figure 2.3: Instar structure of recovered larvae from 428G and H88 plants

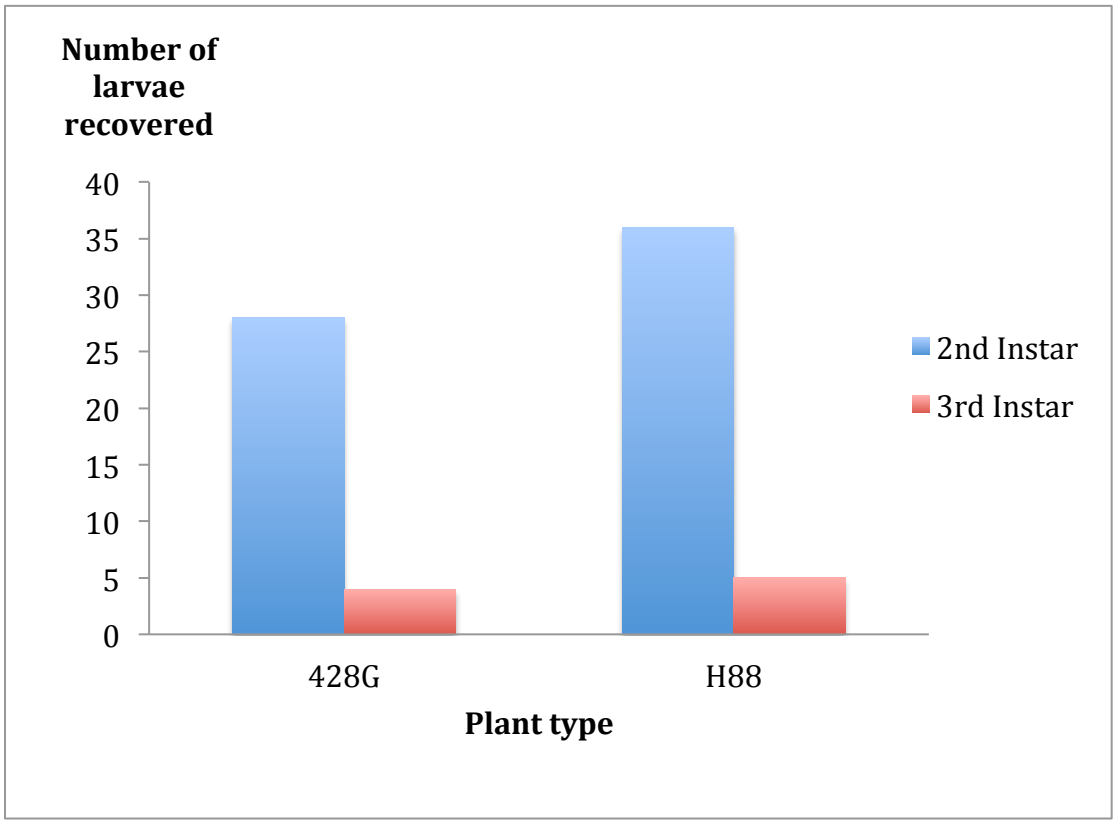

Table 2.1: Instar structure table of larvae recovered from 428G and H88 plants

Frequency Percent Row % Col %	H88	428G	Total
Second instar	36 49.32 56.25 87.8	28 28.36	64 87.67
Third instar	5 6.85 55.56 12.2	4 5.48 44.44 12.5	9 12.33
Total	41 56.16	32 43.84	73 100

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CHAPTER 3

RNA Sequencing and *de novo* Transcriptome Assembly of Western Corn Rootworm *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae)

Introduction

A transcriptome is the complete set of messenger RNA (mRNA) and noncoding RNA (ncRNA) copied from a genome. The mRNA delivers genetic information encoding protein sequences while ncRNA displays regulatory functions in cell processes such as translation and gene splicing (Mattick & Makunin, 2006). A transcriptome is also a tool for genetics studies, especially for non-model species without available genome sequences. Before second generation sequencing technologies became available, the cost of sequencing a transcriptome was very high. Studies of transcriptomes were focused on Expressed Sequence Tags (EST), the collection of short sub-sequences of cDNA, to discover new genes, annotate sequences, and study their functions (Parchman et al., 2010).

Parallel Sequencing, known as "second generation sequencing technology" or "next-generation sequencing technology" emerged during the early 2000s. Three platforms are commonly used: Roche/454, Illumina and Applied Biosystem SOLiD. These platforms feature higher throughput and lower costs than conventional Sanger sequencing. Although primarily developed for genome sequencing projects, transcriptome studies also gain from benefits of these platforms. Full-length sequences of

the RNA of a cell, tissue or organism can be obtained by transcriptome sequencing using second generation sequencing technologies.

Because the read length is shorter than Sanger sequencing (25 to 500 bp per read, shorter than 800 bp from Sanger sequencing) (Shendure & Ji, 2008), assembling short reads into an intact, full-coverage genomes or transcriptomes is especially important. For organisms with sequenced genomes, the strategy is to align the reads to a reference genome sequence followed by determination of transcript sequences. Tools such as Scripture (Guttman et al., 2010) and Cufflinks (Trapnell et al., 2010) were developed for this strategy. For most organisms without an available genome, or cases where the transcriptomes are highly variable such as cancer cells, a *de novo* assembly is necessary (Grabherr et al., 2011).

In the first generation sequencing era, the *de novo* assembly algorithms were based on finding overlaps between reads, which are "read-centric" methods. These methods do not fit next generation sequencing data because of the shorter read length and increased sequence depth. The computations become unfeasible when dealing with the huge numbers of overlaps using heuristic algorithms (Flicek & Birney, 2009). For longer reads generated from Roche/454, some read-centric assemblers are still available (Kumar & Blaxter, 2010). For shorter reads from Illumina and SOLiD, new assemblers using "graph-centric" algorithms have been adopted using *de Bruijn* graphs (Flicek & Birney, 2009; Grabherr et al., 2011; Zerbino & Birney, 2008).

Some published short read assemblers, like ABySS (Simpson et al., 2009), Velvet (Zerbino & Birney, 2008) and ALLPATHS (Butler et al., 2008) were designed for genome sequencing. However, these assemblers did not fit transcriptome sequences very well. First, the genome sequencing reads are expected to distribute evenly across the whole genome, whereas in transcriptomes, expression levels (quantity of mRNA) changes among genes. Second, alternative splicing is common in transcriptomes. Third, in some organisms, the transcripts encoded by adjacent loci may overlap to form a chimeric transcript in these assemblers (Grabherr et al., 2011; Haas & Zody, 2010). To overcome these difficulties, assemblers such as SOAPdenovo (Li et al., 2010), and Oases (Schulz et al., 2012) were developed for transcriptomes by modifying genome assemblers (Zhao et al., 2011). Trinity (Grabher et al., 2011) was designed specifically for transcriptome assembly.

The western corn rootworm (*Diabrotica virgifera virgifera* LeConte) is the most significant pest of corn in North America. Although originally from a limited geographical distribution in Southwest and Great Plains in the United States, it spread over much of the US in last 60 years (Meinke et al., 2009) and recently invaded Europe (Miller et al., 2005). The western corn rootworm is a very adaptable species that evolved pesticide resistance (Meinke et al., 1998), *Bt* transgenic crop resistance (Gassmann et al., 2011), and resistance to crop rotation (Gray et al., 2009).

Although the physiology and mechanisms of adaptation have been studied, the

whole genome sequence of the western corn rootworm is not available (Miller et al., 2010). Preliminary studies suggested that the western corn rootworm has a large genome of 2.58 Gbp (Coates et al., 2012). EST sequencing has been done for western corn rootworm midgut (Siegfried et al., 2005). However no transcriptome sequence for the whole larvae has been reported. In this chapter Illumina sequencing, assembly and characterization of whole western corn rootworm larval is described.

Materials and Methods

Insect

Eggs of the western corn rootworm non-diapause inbred strain PED-12 were received from USDA-ARS Northern Grain Insects Research Laboratory at Brookings, SD. The eggs were washed and surface sterilized before hatching. The details of washing and surface sterilization were described at Chapter 2.

An ideal transcriptome sequence should contain as many full-length sequences as possible. In this study, total RNA from two groups of larvae was pooled together to assemble the transcriptome sequence. One group of larvae fed on corn line 428G, which carries a mutant *bx1* gene that blocks the hydroxamic acid synthesis pathway; another group fed on wild type line H88 with natural levels of hydroxamic acids. The experimental setup was described in Chapter 2. Eighteen H88 and 428G plants were grown individually in 15 ml centrifuge tubes filled with vermiculite. The plants were completely randomized in a 6X6 centrifuge tube rack. Seven days after planting, three

neonate larvae were placed to the root of each plant. After 7 days infestation on corn roots, all the larvae were recovered and flash frozen in liquid nitrogen. The frozen larvae were kept in an -80 ˚C freezer to prevent the degradation of RNA.

RNA extraction and purification

Six larvae were randomly selected from the 428G and H88 feeding groups. Total RNA was extracted from each individual using an RNeasy Mini Kit (Qiagen) and treated with DNase I using RNase-Free DNase Set (Qiagen) during extraction. The integrity and concentrations of samples were examined using a Bioanalyzer (Agilent).

Sequencing

RNA samples were subjected to RNA-Seq at the Institute for Genomic Biology at University of Illinois at Urbana-Champaign using an Illumina HiSeq 2000 system. Twelve paired-end cDNA libraries of 200 base pairs inserts were constructed, each represented the transcriptome of an individual larva. The libraries were sequenced in two separate lanes to increase sequencing depth. The raw data were analyzed with Casava1.8.2 (pipeline 1.8) to remove linker sequence. The results were received in fastq files, which contain read sequences and associated quality scores.

Assembly

The assembly and data analysis procedures were performed on a server equipped with 16 processors and 128 GB RAM running the Centos Linux operating system (version 6).

Prior to assembly, all the reads were cleaned and trimmed using Sickle (UC Davis Bioinformatics Core) with default settings. The Phred quality score threshold was 20 for trimming. Only trimmed pair-end reads were used in assembly.

Trinity (Grabherr et al., 2011) was chosen for transcriptome assembly. The assembly process was run using a PERL pipeline that controlled execution of the inchworm, chrysalis and butterfly programs. Assembly parameters were set to default value, except 16 CPUs were assigned to the program. A fasta file containing the transcriptome sequence was generated when the program finished.

To test the effect of sequencing coverage, data from first sequencing lane was used to make an assembly. A second assembly was made using pooled data from both lanes. To identify the putative gene functions, the two-lane transcriptome assembly was annotated using Blast2GO (Conesa et al., 2005). Blast2GO is a program developed for automatic sequence annotation (Götz et al., 2008) using Gene Ontology (GO), a method of annotation using homology searching (Camon et al., 2003). Blast2Go performs two steps. First, each sequence is used as a query in a BLAST search. Next, GO terms are obtained for each BLAST hit sequence from a database maintained by the Blast2GO developers. Finally, reliable GO terms are assigned to sequences, provided putative functions of the genes (Götz et al., 2008).

Results

Illumina sequencing

Illumina sequencing generated 968,689,944 paired-end reads (484,344,972 pairs), of which 461,731,476 reads (230,865,738 pairs) were generated in the first sequence lane and 506,958,468 reads were generated from the second lane. Each read a uniform length of 100 bp after removing adapter sequences.

Trinity assembly characterization

Trinity generated 117,027 contigs from one-lane sequencing data. The contigs were categorized into 63,372 components. Contigs ranged in length from 201 bp to 33,009 bp (Figure 3.1). Each component in the assembly represents a gene. For the assembly from two-lanes of sequencing data, 126,230 contigs in 71,690 components were generated, ranging from 201 bp to 33,443 bp (Figure 3.2).

Annotation

Blast2GO was applied to the two-lane assembly containing 126,230 contigs. However, 75,815 contigs failed to return BLAST results due to connectively limitations of the software. For the 50,415 contigs with BLAST returns, 16,989 (33.7%) contigs had hits. Among all BLAST hits, 31,051 hits were to *Tribolium castaneum* sequences, 11,324 hits were to pea aphid *Acyrthosiphon pisum* sequences, and 10,173 hits were to *Drosophila melanogaster* sequences (Figure 3.3). When considering the top hit of each contig, 9,092 (53.5%) contigs had top hits to *Tribolium castaneum* sequences, 838 (4.93%) contigs had top hits to silkworm *Bombyx mori* and 835 (4.91%) contigs had top hits to *Acyrthosiphon pisum* (Figure 3.4).

Mapping and annotation resulted in 13,948 contigs being assigned at least one ontological category: cellular component (C), molecular function (F), or biological process (P). The distribution of all GO levels demonstrates that level 6 contains the largest number of GO terms of biological process, while level 3 contains the largest number of GO terms of molecular function, and level 5 contains the largest number of GO terms of cellular component (Figure 3.5).

The BLAST results and GO annotation provided information of putative function of sequences. As an example of searching candidate genes for pesticide detoxification, 128 contigs were found with cytochrome P450 activity. Also, 110 contigs were identified with esterase activity. Blast2GO also identified 565 contigs of transposase and 16,990 contigs of reverse transcriptase, which may be involved in transposable elements (TE) insertion in genome sequence.

Discussion

Second generation sequencing technology provides a powerful tool for analyzing transcriptome of both model and non-model species. However without a preliminary genome sequence, the *de novo* assembly of a transcriptome is still difficult (Schuster, 2008). Many studies have used the Roche/454 platform, which generates longer reads (Dunning et al., 2013; Meyer et al., 2009; Parchman et al., 2010). The Illumina platform became popular when *de Bruijn* graph based assemblers became available (Garg et al., 2011). We used Trinity to assemble western corn rootworm transcriptome sequences

from Illumina data. The number of reads for the two-lane assembly was 109.8% greater than for the one-lane assembly. However the number of contigs was only increased by 7.86%. The contig length distribution was similar. For comparison, 101,915 contigs were generated by Trinity using western corn rootworm larvae midgut, eggs and midgut EST library (Siegfried et al, unpublished data), and 56,305 components were declared (Table 3.1). The assemblies have 14.8% (one-lane assembly) and 23.9% (two-lane assembly) more contigs than midgut transcriptome. The results indicated that there are additional genes in larval transcriptome that are expressed in organs other than midgut.

The assemblies from Trinity were highly fragmented, containing a lot of subcomponents and multiple sequences for many genes. One possibility is that the fragmentation is caused by insertion of transposable elements (TE) to exon regions. Transposable elements are segments of DNA that can move and replicate within genomes (Kidwell & Lisch, 1997). TEs have been identified in many insect species including *Drosophila* (Rubin & Spradling, 1982). The insertion of TEs to exon regions will interrupt coding sequence and cause null mutations in some individuals (Kidwell & Lisch, 1997). However, TE mutations are expected to be rare because the TE-induced mutation are expected to be deleterious to insects (Kidwell & Lisch, 1997). Many TEs have been found on western corn rootworm through BAC sequencing in intron or intergenic regions (Coates et al., 2012), which indicated that TEs are unlikely to be a major factor causing fragmentation. Another possibility is alternative splicing, when multiple mRNA are

generated from the same pre-mRNA by different combinations of exons (Graveley, 2001). It will result in a group of contigs sharing the same component in transcriptome assembly.

The third possible reason for fragmentation is limitations of the assembler used in this study. The *de Bruijn* graph algorithm requires the user to assign a k-mer length, which represents the length of string used as nodes in the *de Bruijn* graph (Grabherr et al., 2011; Zerbino & Birney, 2008). Shorter k-mers increase sensitivity for detecting rarely expressed genes, but also result in many highly fragmented transcripts, whereas longer k-mers result in a more contiguous assembly, but fail to discover low expressed genes (Surget-Groba & Montoya-Burgos, 2010; Zerbino & Birney, 2008). We used a default k-mer length of 25 in order to increase the sensitivity. New assemblers that adopt a multiple k-mer strategy may increase the integrity while retaining the rare transcripts in assembly (Surget-Groba & Montoya-Burgos, 2010).

BLAST-based annotation allows the researchers to transfer knowledge from model organisms to non-model species. Among the genes with BLAST hits, most of them had homologs in the coleopteran *Tribolium castaneum*, the only beetle with a sequenced genome (Richards et al., 2008). GO annotation in this research revealed the functions of assembled sequences. In this study many cytochrome P450s and esterases were identified, which will be useful to further understand the mechanisms of the interaction between western corn rootworm and corn. Also, a large number of transposases and reverse

transcriptase were discovered in transcriptome, which explained the high TE content in genome discovered by BAC sequencing.

Figure 3.1: The size distribution of contig length resulting from one-lane sequencing data and Trinity assembly.

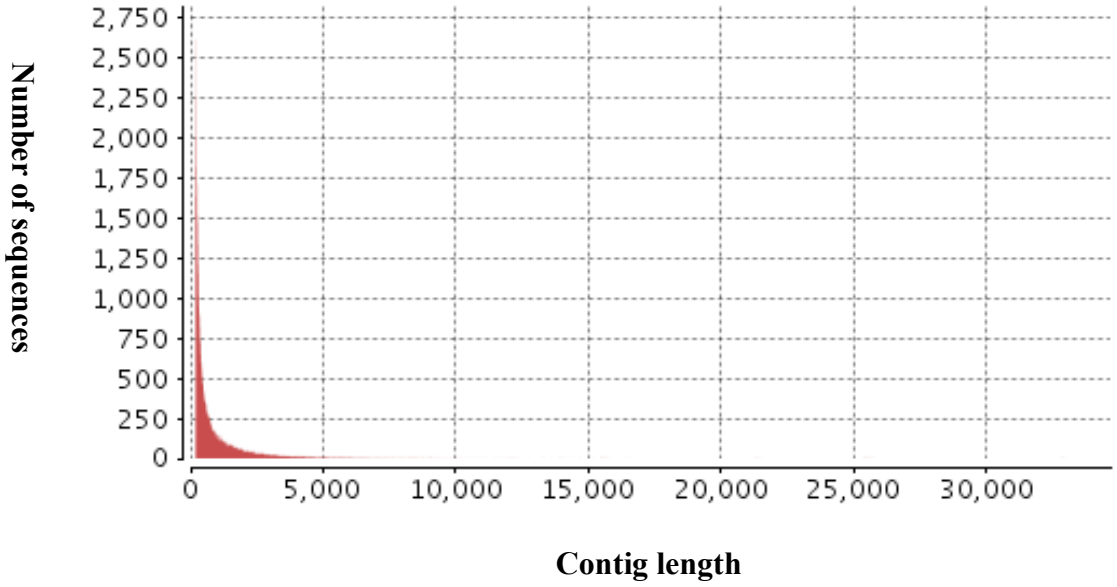

Figure 3.2: The size distribution of contig length resulting from two-lane sequencing data and Trinity assembly.

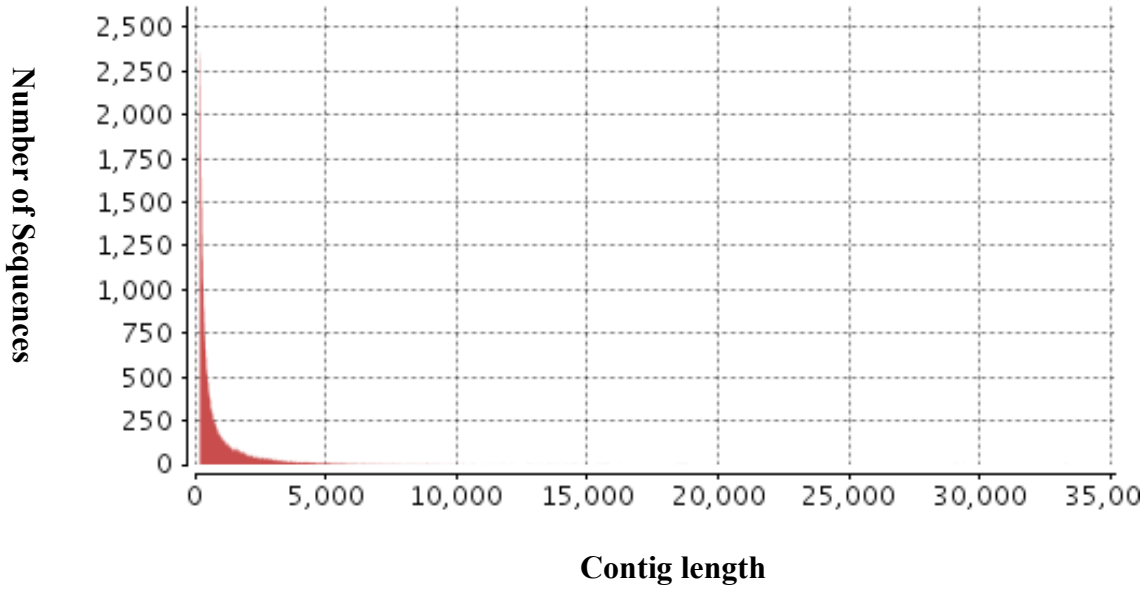

Figure 3.3 Species distribution of BLAST hits of two-lane transcriptome assembly

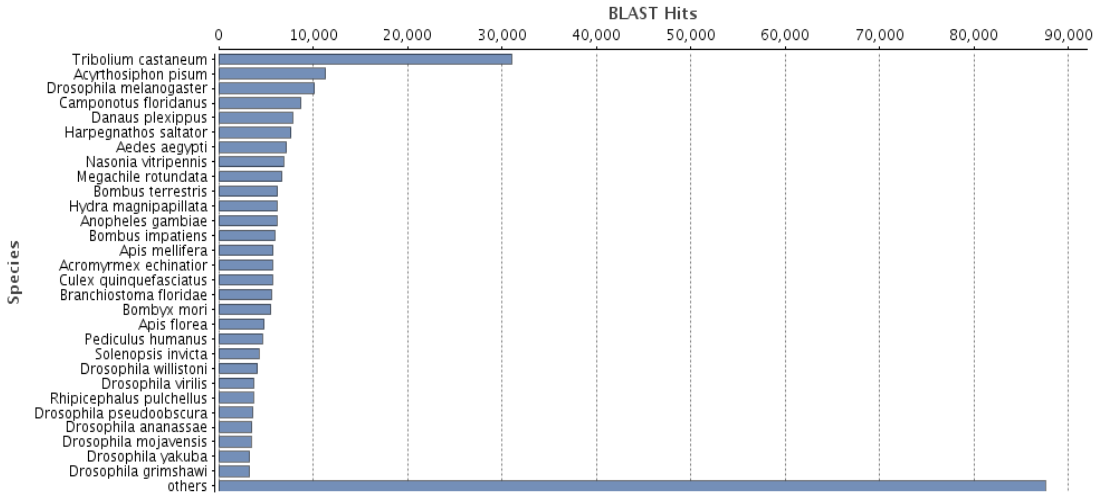

Figure 3.4 Top hits distribution of two-lane transcriptome assembly

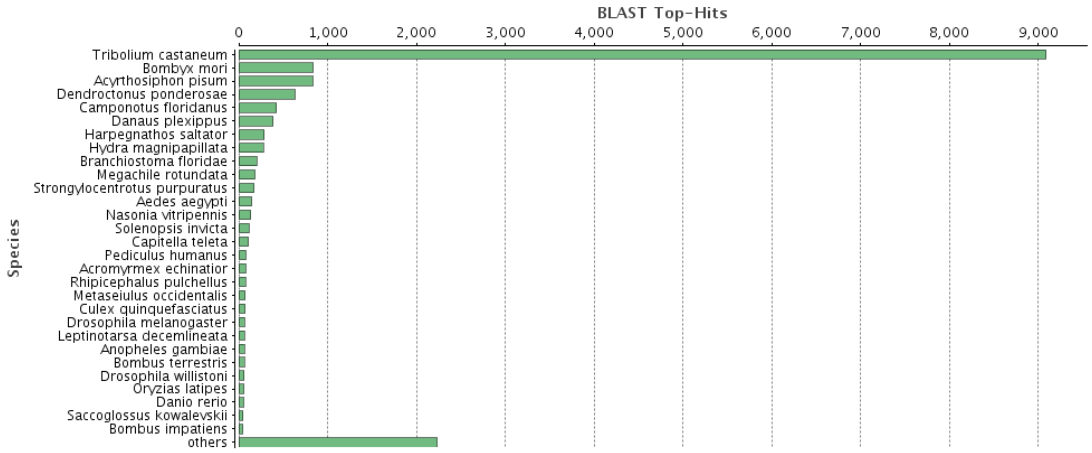

Figure 3.5 GO-level distributions of annotated contigs of two-lane transcriptome assembly

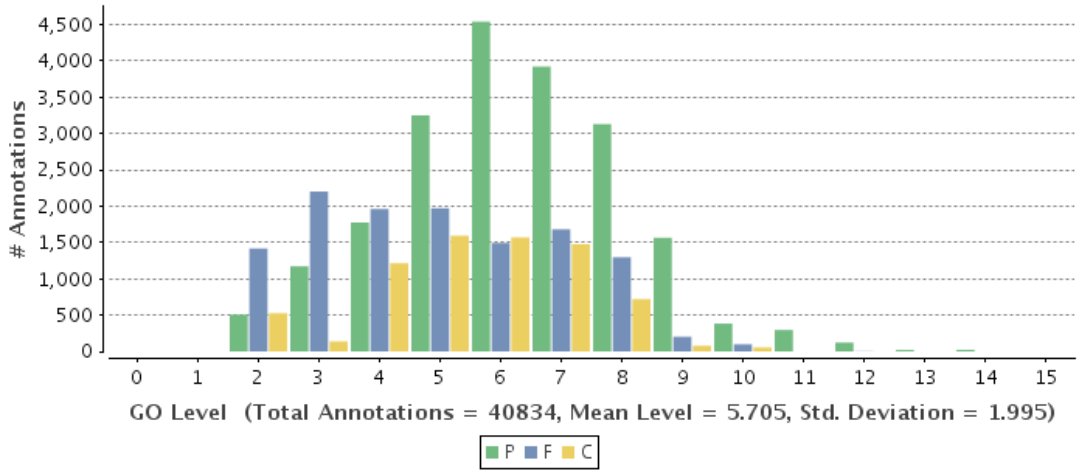

Table 3.1: The total number of contigs and components of assembly using one lane, two lane and midgut data. The size of sequencing dataset is presented.

	Dataset size	Total contigs	Components (Genes)
One-lane assembly	461,731,476	117,027	63,372
Two-lane assembly	968,689,944	126,230	71,690
Midgut assembly	664,431 reads from Roche/454; 229,720,904 reads from Illumina (75 bp paired-end); 17,782 ESTs from NCBI (Coates et al., 2012) (From Siegfried et al. unpublished data)	101,915	56,305

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CHAPTER 4

Gene Expression Analysis of Western Corn Rootworm (*Diabrotica virgifera virgifera* LeConte) in Response to Hydroxamic Acids Using RNA-Sequencing Technology

Introduction

Plant secondary metabolites are of great importance in plant-insect interactions. Plants produce an array of secondary metabolites, which are involved in insect toxicity, herbivore repellence, and indirect defenses (Wink, 1988). The diversity of plant secondary metabolites also has ecological and evolutionary effects on herbivores' host specificity by attracting some species while repelling others (Fraenkel, 1959). In order to successfully survive on and colonize a given plant species, insects must evolve mechanisms to overcome the chemical stresses provided by secondary metabolites.

The western corn rootworm (*Diabrotica virgifera virgifera* LeConte) is a significant corn pest. It is also considered a specialist herbivore. The larvae and adults feed on corn (*Zea mays* L.) and a few other Poaceae species (Oyediran et al., 2004). The original distribution of the western corn rootworm in the US was restricted to a small region of the Midwest and Southern Great Plain of the United States. This species expanded rapidly during the last 60 years due to continuous corn cultivation in the Corn Belt (Meinke et al., 2009). Introduction of this species to Europe was first reported around Belgrade airport in 1992 (Ciosi et al., 2008; Kiss et al., 2005). After two decades western corn rootworm has been founded in more than 11 European countries (Ciosi et al., 2008;

Miller et al., 2005).

The western corn rootworm has demonstrated strong plasticity and adaptability to control strategies in pest management. Strategies including soil insecticide treatment, foliar insecticide sprays, crop rotation with non-host crops, and Bt transgenic crops targeting western corn rootworm have been adopted (Meinke et al., 2009). Resistance against major soil and leaf insecticides arose rapidly (Meinke et al., 2009). Populations with resistance to Bt transgenic corn were identified recently (Gassmann et al., 2011). Some populations have adapted their oviposition behavior to corn-soybean rotation by ovipositing eggs in nearby soybean fields as well as cornfields (Gray et al., 2009). Due to its broad distribution and adaptability, western corn rootworm causes significant losses in corn production. It is estimated that *Diabrotica* costs $1.7 annually in the United States, including yield losses and control costs (Sappington et al., 2006).

Given the host specificity, adaptability and the economic importance, western corn rootworm is a potential model for studies of plant-insect interactions. Hydroxamic acids are the most significant secondary metabolites in corn. The major form is DIMBOA, while other forms, such as MBOA, DIBOA and DIM_2BOA are also occur within the plant (Niemeyer, 1988). Hydroxamic acids are constitutively synthesized in corn plants (except seeds) and stored as inactive 2-β-*O*-D-glucosides. After challenge by insects, the free hydroxamic acids are released (Niemeyer, 1988; Oikawa et al., 2001). The toxicity, inhibitory, and antifeedant effects of hydroxamic acids on many corn pests have been

discussed (Argandona et al., 1981; Givovich & Niemeyer, 1995; Mukanganyama et al., 2003). However, mixed results were reported for western corn rootworm. Xie et al (1992a; 1992b) fed larvae with hydroxamic acid treated corn roots. Significant feeding deterrent effects were observed. Other studies also showed that larval survival rate and corn root damage both have negative relationships with hydroxamic acid concentration in roots (Assabgui et al., 1995; Xie et al., 1992b). A recent study presented a completely different result that hydroxamic acid are not related to larval survivorship and development (Davis et al., 2000). Neonates feeding on roots containing hydroxamic acids, and their non-hydroxamic acid counterparts for 24 hours did not differ in fresh weight (Robert et al., 2012). MBOA, another hydroxamic acid, did not show toxicity to western corn rootworm larvae (Abou-Fakhr et al., 1994). Some studies have demonstrated that hydroxamic acids serve as attractants for host location, rather than repellent, to western corn rootworm neonates (Hibbard & Bjostad, 1988; Robert et al., 2012).

In Chapter 2, no significant effects of hydroxamic acids were found on larval growth and development over a one-week period. However the mechanism by which western corn rootworm copes with hydroxamic acids has not been studied. Two hypotheses were proposed. The first hypothesis is that western corn rootworm larvae express enzymes for hydroxamic acid detoxification constitutively. An alternative is that hydroxamic acid detoxification pathways are inducible, but energetically inexpensive. RNA-Sequencing (RNA-Seq) is a powerful tool for both qualitative and quantitative analysis of

transcriptomes using second generation sequencing technology (Wang et al., 2009). In Chapter 3, an assembled transcriptome sequence was produced. In order to identify whether western corn rootworm has inducible detoxification mechanisms, an RNA-Seq experiment was conducted to analyze the gene expression changes in response to plant defenses.

Materials and Methods

Insects and Plants

Eggs of western corn rootworm non-diapause inbred strain PED-12 were received from USDA-ARS Northern Grain Insects Research Laboratory at Brookings, SD. The mutant corn line 428G was received from the Maize Genetics Cooperation Stock Center at University of Illinois at Urbana-Champaign. The wild-type parental corn line H88 was received from Purdue University. The 428G plants carry a homozygous *bx1* mutation, which makes them unable to synthesis hydroxamic acids (Frey et al., 2009). The details of seed propagation, incubation and surface sterilization of western corn rootworm eggs were described in Chapter 2.

Feeding treatments

Larvae from the treatment group were fed on H88 roots, while control larvae were fed on 428G roots with no hydroxamic acids. The experimental setup was as described in Chapter 2 and 3. Eighteen H88 and 428G plants were grown individually in 15 ml centrifuge tubes filled with sterile vermiculite. The tubes holding plants were positioned

in a 6X6 centrifuge tube rack in a completely randomized design. Seven milliliters of water were given to each tube when the seeds were deposited. One milliliter of water was added to each tube every three days.

Seven days after planting, the seedlings were pulled carefully out of tubes. Three neonate larvae were positioned on the surface of the roots of each plant. Then the seeds were returned into the same tubes and the roots were covered with sterile vermiculite. At day 14, all the surviving larvae were recovered and flash frozen in liquid nitrogen. The frozen larvae were stored in an -80 ˚C freezer to prevent any possible degradation.

RNA extraction, purification, sequencing and assembly

Six larvae were randomly selected from the treatment group (fed on H88) and control group (fed on 428G). Total RNA was extracted from each larva using an RNeasy Mini Kit (Qiagen) and treated with RNase-Free DNase (Qiagen) to remove DNA contamination. Before submitting samples for sequencing, the integrity and concentrations were examined using a Bioanalyer (Agilent) to make sure the minimum requirements were met.

Total RNA samples were subject to RNA-Seq at the Institute for Genomic Biology at the University of Illinois at Urbana-Champaign using an Illumina HiSeq 2000 system. Six samples from treatment larvae and six samples from the control group were submitted. Twelve paired-end cDNA libraries of 200 bp fragements were constructed, each representing the transcriptome of an individual. The libraries were sequenced in two

separate lanes to increase sequence depth. After sequencing, raw data were analyzed with Casava 1.8.2 (pipeline 1.8) to remove linker sequence. The results were provided in fastq files, which contain read sequences and associated quality scores.

Transcriptome sequences assembled *de novo* from RNA-Seq reads (two-lane assembly in Chapter 3) were used as reference transcriptome in this study. The details of assembly were described in Chapter 3. Western corn rootworm egg and midgut transcriptome sequence (Siegfried et al., unpublished data) were also used as references for comparison purpose.

Sequence mapping and differential expression

The quantification of RNA-Seq relies on counts of sequencing reads that map to each gene from reference sequence. The first step of data analysis is to align reads to the reference transcriptome. Reads were aligned to reference sequences using bowtie, an alignment tool based on Burrows Wheeler transform methods, designed for second-generation sequencing data alignment (Flicek & Birney, 2009; Langmead et al., 2009). First, a bowtie index was built for each transcriptome assembly using the bowtie-build command. Second, the reads were aligned to the indexed reference using paired-end mode. Only one mismatch for each read was allowed. The bowtie outputs (created in SAM format) were converted into BAM format. Because some sequences may be artificially duplicated by PCR during library construction, which could cause a significant bias in statistical analysis, SAMtools (Li et al., 2009) was implemented on

bowtie outputs to eliminate artificial duplicates.

The R package DESeq (1.10.1) (Anders & Huber, 2010) was used to analyze RNA-Seq data. DESeq implements a test, based on the negative binomial distribution, for differential gene expression in RNA-Seq data. The bowtie outputs were loaded into R and a table of the number of reads mapped to each gene from each sample was generated. The row counts were normalized for library size and the negative binomial test was implemented. A false discovery rate (FDR) of 0.1 was used to account for multiple tests. Genes (contigs of transcriptome reference) with an adjusted p value less than 0.1 were declared significantly different. A BLASTX search against the NCBI non-redundant protein database was performed for differentially expressed contigs.

Results

A total of 968,689,944 reads from two-lane sequencing were unambiguously aligned to larval and midgut transcriptome. The Bowtie alignment was performed using the larval transcriptome (described as two-lane assembly in Chapter 3) and midgut transcriptome. Among all the samples, 56.5% to 62.7% of reads aligned to the larval transcriptome (mean=59.8%), significantly higher than 38.6% to 47.0% of reads aligned to the midgut transcriptome (mean=42.1%). The percentage of aligned reads to larval transcriptome among samples fed on H88 was between 57.3% and 59.6% (mean=58.4%), while 56.5% to 60.6% reads from the samples fed on 428G were aligned to the same reference (mean=59.2%) (Table 4.1).

The results of DESeq analysis are presented in Table 4.2. Nine contigs were significantly differentially expressed between H88 and 428G feeding larvae. Five of them were up regulated in H88 feeding larvae, while 4 of them were declared down regulated (Figure 4.1). The expression fold change was calculated using the mean of normalized counts (base mean) of H88 feeding larvae divided by the base mean value of 428G feeding larvae, which may be reported as 0 if no transcription is detected in H88 feeding larvae, or infinite if no transcription is detected in the 428G feeding larvae. A MAplot was produced visually represent the expression difference of all genes in transcriptome. Most genes did not show significant differences in expression (marked in black), while only a few significant genes were present (Figure 4.2).

BLASTx search demonstrated that among 9 differential expressed contigs, 2 of them have top-hits. The other 7 contig did not present any BLAST results. A contig from the larval transcriptome (comp148366_c0_seq6, padj=0.0299) had a top hit to *Tribolium castaneum* cytochrome P450 9Z4 (CYP9Z4, accession number: NP_001164248). The maximum identity is 67% (69% of query coverage). Although declared significant by DESeq, the expression levels of comp148366_c0_seq6 was not consistent among the larvae feeding on H88 (Table 4.3). Another significant contig is comp41282_c0_seq1 (padj= 0.070). It had top hit to *Diabrotica virgifera virgifera* cathepsin L-like cysteine proteinase CAL1 (DvCAL1) (accession number: AAG17127.1; 91% of query coverage, 73% of identity). The expression of DvCAL1 is significantly higher in H88 feeding

larvae.

Discussion

This study demonstrated the gene expression changes in western corn rootworm larvae caused by dietary hydroxamic acids. Hydroxamic acids are major defense substances in corn. The mechanisms of how western corn rootworm copes with hydroxamic acids are not clear. By using RNA-Seq, it is possible to search transcriptome-wide for differentially expressed genes without an available genomic sequence. In this study nine contigs were identified as differentially expressed, which indicated that hydroxamic acids have effects on gene expression. However of 9 differentially expressed contigs, only 2 had BLAST annotations. The remaining 7 contigs lack BLAST homologous sequences in the database. It is possible that those contigs are western corn rootworm specific genes that have not been studied before. Another possibility is that these contigs are fragments of variable untranslated regions that interact with hydroxamic acids that are not included in non-redundant protein databases.

This study identified a differentially expressed cytochrome P450 in western corn rootworm. This gene is a candidate for hydroxamic acid detoxification due to the up regulation in H88 feeding larvae. However the up regulation of this contig varies among treatment samples. Four of 6 individuals were strongly up regulated, while 2 of them remained low. This phenomenon could be due to an uneven distribution of hydroxamic acids in the root system.

Cytochrome P450 monooxygenases (P450s) are universal proteins that occur in plants, animals, and microbes. They function as oxidases by adding oxygen into substrates. As of 2009, a total of 11,294 P450s were identified and categorized into 977 families (Nelson, 2009). In insects, 1675 P450s have been identified, which belong to 59 families from four clades (Feyereisen, 2006; Nelson, 2009). P450s are involved in many physiological functions as well as xenobiotic metabolism, including insecticide resistance (Ffrench-Constant et al., 2004). Insect P450s also demonstrated important roles in plant-insect interactions (Schuler, 2011). In western corn rootworm, P450s are involved in detoxification of many pesticides including organophosphates (Miota et al., 1998) and carbamates (Scharf et al., 1999). Constitutively enhanced expressed P450s are implicated in methyl parathion and carbaryl resistance in western corn rootworm (Scharf et al., 2001).

The CYP 9 family has not been studied in western corn rootworm. However the CYP9 members have been studied in *Heliothis virescens* (Rose et al., 1997), *Manduca sexta* (Stevens et al., 2000) and *Depressaria pastinacella* (Li et al., 2004). In *Manduca sexta*, CYP9s are induced by many allelochemicals in their diets (Stevens et al., 2000). CYP9A1 is also involved in pesticide resistance in *Heliothis virescens* by constitutive up regulation in thiodicarb resistant colonies. This study found that the expression level of a CYP9-like contig is significantly elevated by hydroxamic acids, indicating that this contig may be involved in hydroxamic acid detoxification.

Physiological studies of European corn borer (*Ostrinia nubilalis*) provided clues regarding possible hydroxamic acid detoxification mechanisms. In European corn borer, cytochrome b5, NADPH-cytochrome c reductase, NADH-cytochrome c reductase, NADH oxidase, and O-demethylase are up regulated by DIMBOA. MBOA can up regulate cytochrome b5, NADH-cytochrome c reductase, and glutathione S-transferase activities as well (Feng et al. 1992). An explanation of these up-regulated oxidoreductases is that hydroxamic acids are degraded in the insect body through oxidation-reduction reactions, which can be mediated by cytochrome P450s.

RNA-Seq also identified that a cathepsin L-like cysteine proteinase (DvCAL1) was differentially expressed. DvCAL1 is a digestive enzyme that belongs to the family of cathepsin-like proteins that are produced in the western corn rootworm midgut (Siegfried et al., 2005). DvCAL1 is strongly inhibited by soybean protease inhibitor soyacystatin (Koiwa et al., 2000). However, in this study, the DvCAL1 was up regulated by dietary hydroxamic acids. It conforms that hydroxamic acids may function as host plant locating cues, which stimulate the midgut cell to express protease.

In conclusion, this study demonstrated an inducible response to hydroxamic acids in western corn rootworm larvae. A putative CYP9 family of cytochrome P450 monooxygenase was up regulated and involved detoxification of hydroxamic acids. A DvCAL1 gene encoding cathepsin-like protease was also upregulated, indicating that hydroxamic acids may have feeding stimulant effects on western corn rootworm midgut.

However, only a few contigs were found to be differentially expressed in this study. It is possible that only a few genes are respond to hydroxamic acids. In order to further identify the impact of hydroxamic acids on western corn rootworm at molecular level, an increase in replication and sequencing depth might enhance the sensitivity in detecting differentially expressed genes. RT-PCR studies should be used to validate the differentially expressed genes and further studies on gene function are also necessary.

Figure 4.1: The base mean chart of differentially expressed gene declared by DESeq (adj.p<0.1).

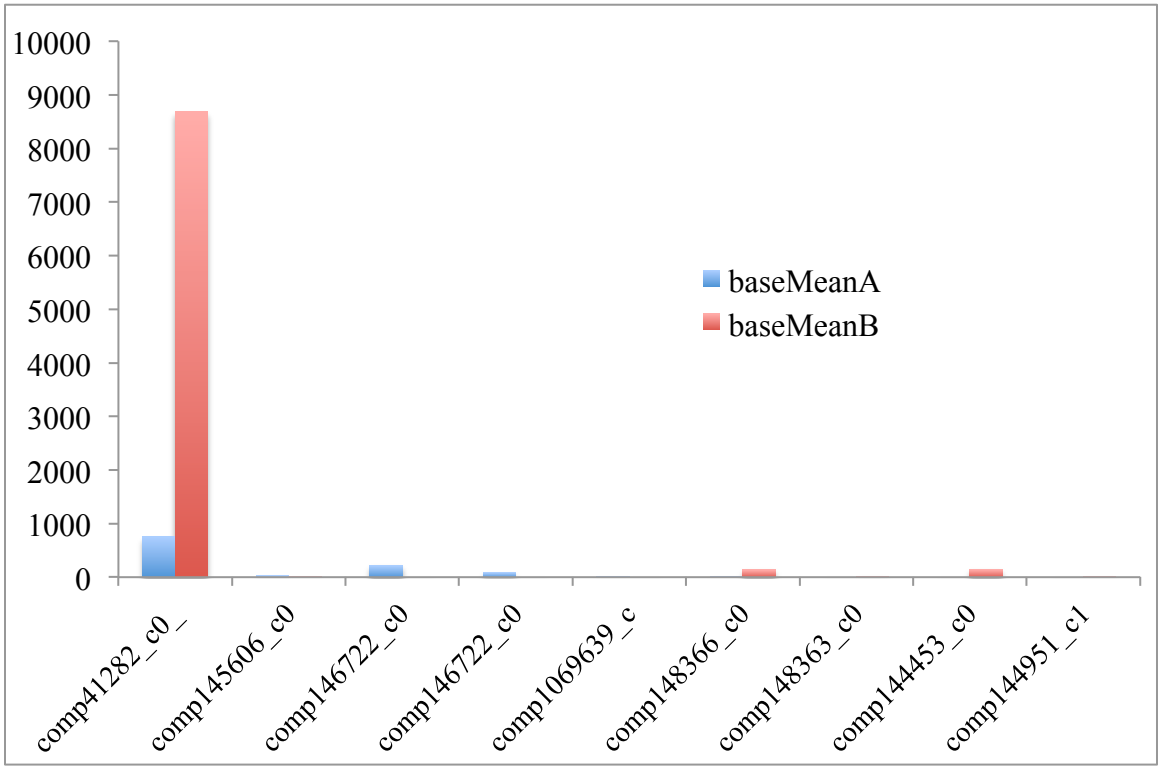

Figure 4.2 MAplot of expressed contigs in DESeq analysis. The non-significant contigs are marked in black while significantly differentially expressed contigs are marked in red.

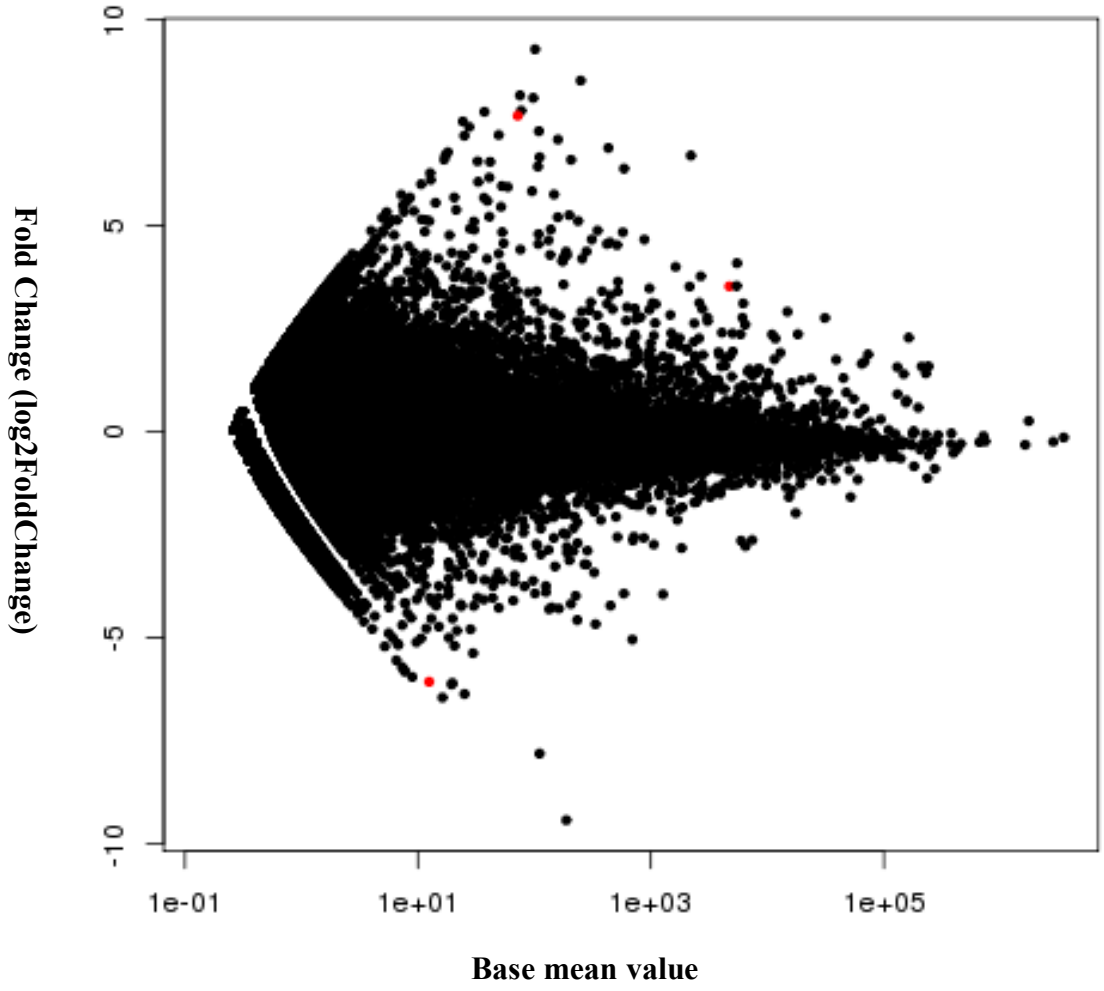

Table 4.1: The total number of reads, aligned reads to larval transcriptome, and the percentage of aligned reads. For comparison, the percentage of reads aligned to a midgut transcriptome is presented.

Sample	Sequence reads	Aligned reads	Percentage of reads aligned to assembly	Percentage of reads aligned to midgut transcriptome
H88-1	71,802,934	20,817,068	57.98%	43.65%
H88-2	77,978,306	22,547,857	57.83%	40.72%
H88-3	83,260,692	23,837,990	57.26%	38.60%
H88-4	79,967,266	23,833,038	59.61%	39.82%
H88-5	80,653,942	23,416,818	58.07%	44.39%
H88-6	96,284,188	28,620,500	59.45%	41.17%
428G-1	81,709,070	24,771,041	60.63%	42.60%
428G-2	93,056,636	29,185,730	62.73%	47.00%
428G-3	69,482478	21,581,938	62.12%	43.60%
428G-4	78,495,460	22,161,416	56.47%	43.29%
428G-5	81,010,336	22,901,145	56.54%	40.30%
428G-6	74,988,636	21,304,626	56.82%	39.86%

Table 4.2: Significantly differentially expressed contigs declared by DESeq (cut-off p.adj=0.1) and the BLASTX top hits of each contig.

Contig name	foldChange	p.adj	BLASTx top-hit
comp41282_c0_seq1	11.547	0.0700	*Diabrotica virgifera virgifera* cathepsin L-like cysteine proteinase CAL1 (CAL1) mRNA, partial cds
comp145606_c0_seq1	0.015	0.00242	N/A
comp146722_c0_seq4	0	0.0162	N/A
comp146722_c0_seq7	0	0.0700	N/A
comp1069639_c0_seq1	0	0.0162	N/A
comp148366_c0_seq6	204.421	0.0299	*Tribolium castaneum* cytochrome P450 9Z4 (CYP9Z4), mRNA
comp148363_c0_seq2	Inf	0.0427	N/A
comp144453_c0_seq2	Inf	0.00242	N/A
comp144951_c1_seq10	Inf	0.0427	N/A

Table 4.3: Number of reads from each sample that aligned to sequence comp148366_c0_seq6, a putative CYP9 gene.

Sample	Number of reads
H88-1	0
H88-2	212
H88-3	166
H88-4	192
H88-5	322
H88-6	0
428G-1	0
428G-2	0
428G-3	2
428G-4	2
428G-5	0
428G-6	0

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