Towards the development of soybean resistance to *Dectes texanus* LeConte (Coleoptera: Cerambycidae): Evaluation of conventional soybean resistance in the soybean plant introduction 165673, transcriptomic analyses, and gene silencing by RNA interference

by

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B. Sc., Universidad del Valle, Colombia, 2010

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AN ABSTRACT OF A DISSERTATION

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ABSTRACT

Dectes texanus (Coleoptera:Cerambyciade) larvae devastate soybeans in the U. S. midwestern states by girdling and tunneling inside the stems. Reduction of natural sunflower and ragweed hosts may have promoted the use soybean as a host since the late 1950s. Development of soybean varieties resistant to *D. texanus* is of importance to manage this pest since harvesting as soon as possible is the only option available to farmers to reduce yield losses. The soybean plant introduction (PI) 165673 reduces the number of *D. texanus* larvae, but survivors are still found at 21 d post infestation that may damage and girdle the PI165673 stems at the end of the growing season. Soybean resistance to *D. texanus* can be enhanced with the delivery of double stranded RNA (dsRNA) *in planta* to suppress gene expression by RNA interference (RNAi). DsRNA expressed *in planta* can be designed specifically to target and silence *D. texanus* genes important for development. *D. texanus* genes uniquely or highly expressed when fed soybean compared to those fed their natural hosts or artificial diet can be exploited to impair the development of *D. texanus* with RNAi. Although, dsRNA-based silencing is successful in other cerambycids, its use with *D. texanus* is untested. The objectives of this dissertation are: 1) to evaluate stem girdling and tunneling by *D. texanus* in soybean PI165673 at the end of the 2014 growing season in Kansas; 2) to compare the transcriptomes of *D. texanus* larvae fed soybean, wild sunflower, giant ragweed or artificial diet; and 3) to silence the *Laccase2* (*Lac2*) and *Chitin synthase2* (*CHS2*) genes in *D. texanus* by feeding larvae artificial diet coated with *Lac2* or *CHS2*-dsRNA.

The stems of infested PI165673 had 20% less tunneling by *D. texanus* larvae compared to the susceptible control K07_1544 at the end of the growing season in 2014 in Kansas. However,

this result may be overestimated since the PI16563 plants had not reached full maturity and development compared to K07_1544. Therefore, assessment of stem tunneling (%) and girdling damage should be conducted in Southern States where the PI165637 can finish their development. The PI165673 resistance negatively affected the development and survival of *D. texanus* first instar larvae, but surviving larvae developed until the last instar stage before the end of the growing season in Kansas. These larvae can potentially gridle the PI165673 plants.

A *D. texanus de novo* transcriptome was assembled for differential gene expression analyses. Five and six unigenes were commonly up-regulated and down-regulated in K07_1544-fed larvae compared to those fed wild sunflower, giant ragweed, or artificial diet (FDR < 0.05, fold change cut off ≥ ± 1.5). Unigenes coding for a lipocalin, an ecdysteroid kinase, and a major facilitator transporter were among the commonly up-regulated unigenes in soybean-fed larvae. Unigenes coding for two insect cuticle proteins, a glycosyl hydrolase 45, a transglutaminase, and a chitin binding peritrophin-A domain were among the commonly down-regulated unigenes in soybean fed larvae compared to either native host or artificial diet. Additionally, 41 and 13 unigenes were also up-regulated and down-regulated in larvae fed susceptible K07_1544 plants compared to those fed wild sunflower or giant ragweed, respectively. Cytochrome P450s, carboxylesterases, major facilitator transporters and glycoside hydrolases were the most represented protein families among the up-regulated unigenes in soybean-fed larvae compared to either native host. Up-regulation of unigenes involved in biotransformation of plant allelochemicals, transport of small solutes and hydrophobic molecules, and digestion of plant cell walls by larvae fed soybean may be an important factor in the ability of *D. texanus* to use soybean as a host.

The *Lac2* relative transcript level was at least 6 times lower in dsLac2-fed larvae compared to dsGFP and dsCHS2-fed larvae. However, there were no significant differences in abnormal adult morphology between dsRNA treatments. Differences in *CHS2* transcript levels and larval chitin content were not detected between dsRNA treatments. Lack of treatment differences is most likely related to a small sample size in the experiment.

In conclusion, the PI165673 resistance needs to be accompanied by other sources of resistance to reduce and prevent damage caused by *D. texanus* surviving larvae. Genes within the up-regulated protein families in larvae fed soybean could be used as potential targets for RNAi. However, further gene silencing studies are needed before soybeans expressing *D. texanus* targeted-dsRNA can be available.

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Dedication

To my Mother and first teacher, Marlene Rojas Serna.

A mi Mamá y primera maestra, Marlene Rojas Serna

Chapter 1 - Introduction

Soybean

Soybean, *Glycine max* (L.) Merrill (Fabales: Fabaceae), is a widely cultivated crop around the world. Products derived from soybean seed are important in the food, vegetable oil and livestock industries. Soybean is grown mainly because of its high protein and oil seed content which average 40 and 20%, respectively[1,2]. Processing of the seed to obtain high protein meal and oil has added value to soybean as a crop since oils can be used to make other products, such as biodiesel fuel, cooking oil, meat- and dairy product substitutes, and soyfeed for livestock[3,4]. The demand for biodiesel and food with high protein content has accelerated the growth of soybean production worldwide and the development of high yielding soybean cultivars[5]. Most soybeans are produced in the U. S. A. (35%), Brazil (33%), and Argentina (14%)[6].

Although soybean was introduced to the U. S. A. as a forage crop in the 18th century, soybean yield has increased in this country from 16,900 hg/ha in 1961 to 32,990 hg/ha in 2017[7,8]. This increase in yield is the result of multiple breeding programs that are interested in improving yields, seed composition, pest resistance and tolerance to abiotic stresses[3]. Approximately 26% of the world soybean production was lost due to pests between 2001 and 2003, and ~8.8% was attributed to damage caused by animal pests, including insects[9]. In 2017, ~ 44.5 million bushels of soybeans were lost to insects from ~1.6 billion bushels harvested in 16 U. S. states[10]. The *Dectes* stem borer, *Dectes texanus* LeConte (Coleoptera: Cerambycidae), is included among the insect pests of soybeans, and has been recognized as a potential economic pest since 1968 in Missouri[11].

Dectes stem borer description and life cycle

D. texanus is a long-horned beetle and belongs to the order Coleoptera, family Cerambycidae[12]. It is commonly known as the *Dectes* stem borer, the soybean stem borer, the sunflower stem borer, and the sunflower stem girdler[13]. This insect is a native species of North America and is widely distributed from east of the Rocky Mountains through Northern Mexico[14]. It has been recorded to inhabit plant species from the Asteraceae family, such as common ragweed (*Ambrosia artemisifolia* L.), cocklebur (*Xanthium pennsylvanicum* Vallr.), giant ragweed (*Ambrosia trifida* L.), and wild sunflower (*Helianthus annus* L.)[15,16].

D. texanus has one generation per year, complete metamorphosis[15], and an adult activity period that extends from June to August. However, the beginning and end of this period varies between states. In Missouri, activity occurs from late June until mid-August, in North Carolina from mid-July until mid-August, and in Kansas from late June until late August[15,17,18]. Adult emergence peaks in mid-June in Tennessee, and in early July in Kansas[13,19].

The *D. texanus* adult is dark brown to black with short gray pubescence and has a body with an elongated and narrow shape that ranges from 6-11 mm long and 1.6-4.3 mm wide. There are prominent lateral spines near the base of the pronotum and the elytra have erect black setae projecting above the pubescence. The female has a larger body size and shorter antennae than the male. In the pupal stage, only the female has a pair of genital lobes located on the last abdominal sternite. The sex proportion is about a 1:1 ratio, and adults feed for 2 d before mating[17]. However, mating has been observed 5 d after emergence in Tennessee[19]. Adults mate more than

once in their lifetime, but females mate only with the same partner[19]. The females lay their eggs 3 d after mating, and each female lays an average of 53 eggs in her lifetime. The female places one egg with her ovipositor in the pith after chewing a hole in the petiole. Successful oviposition depends on presence of the pith or if it can be reached with the ovipositor[17,19].

Elongate shaped eggs, averaging 1.5 mm in length, are laid mainly in petioles and soft stems, and are shiny-yellow before hatching[17]. The incubation period in the field lasts from 6 to 10 d in Tennessee[19]. The first instar larva is yellowish white and averages 1.7 mm long. Mature larvae are yellow to dark brown, slender, slightly curved and average 12 – 15 mm long. In the field, the larva completes four instars, a period lasting 9 to 10 mo, but larvae reared in artificial diet undergo six stages[20]. The larvae are legless, but they have strong protuberant dorsoventral ampullae on the first seven abdominal segments[20].

The first instar larva feeds on the pith and the interfascicular parenchyma of the petiole for 14 to 21 d. When the pith is depleted, the larva chews into the main stem. As a result of the feeding damage, the petiole wilts, turns black, drops to the ground, and scar tissue is formed around the entrance hole into the stem[15,17]. The larva bores through the stem toward the lower portion of the plant. When the fourth instar larva reaches the base of the plant, it girdles the stem and overwinters in the stubble below ground[15]. The larva closes the tunnel in the stem with a frass plug as protection from winter and possible enemies[21]. Although, many eggs are laid in the petioles, only one larva survives per stem since *D. texanus* larvae are cannibalistic[17,19]. In mid-June, the overwintered larva becomes active, feeds on woody stubble tissue, cuts an exit hole for adult emergence, and transforms into a pre-pupa[17]. The pupae are yellow-brown and resemble

the size and shape of the adult. The pupal stage lasts 10-15 d followed by an immature adult stage which stays inside the stubble for 1 - 2 d[19]. Adults exit the stubble when the integument hardens[15,17].

Dectes stem borer in sunflower

D. texanus larvae can also damage cultivated sunflowers by causing stalk lodging[22]. A thin stalk seems to exacerbate lodging where *D. texanus* can completely girdle the stem compared to thick stalks[23,24]. Thin-girdled stalks have less strength and brake more easily than thick-girdled stalks[23]. Interestingly, larval boring by *D. texanus* had no effect on percentage of oil seed content and other yield components of the oilseed hybrid, Triumph 665, when compared to un-infested plants[23]. Extensive damage caused by *D. texanus* larvae and abundance of ~1000 larvae per acre on cultivated sunflower were first reported in College Station, TX in 1970[16].

Cultivated sunflower is preferred over wild sunflower by *D. texanus* adults as shown by significantly higher numbers of ovipunctures, eggs, and damage scores on Pioneer 63N82 than on wild sunflower[25]. Release of larger volumes of resinous exudate, lower water content, and tougher epidermis may be related to fewer ovipunctures and eggs in wild sunflower compared to cultivated sunflower[25]. Also, Wild-perennial *Helianthus* species have also been shown to have resistance to *D. texanus* larvae and ovipositing females[26], although females can oviposit successfully in stems of the F1 progeny from hybrid and wild-perennial sunflowers[26]. Although, oilseed sunflower accessions PI386230 and PI650558 display lower percentage of larvae-

infested stalks compared to other accessions[27], they were susceptible to high *D. texanus* population densities in 2002 and 2004 in Colby, KS[27].

Dectes stem borer in sunflower and soybean

D. texanus females preferred to oviposit in sunflower over soybean[28–30]. There is no evidence of specialization for oviposition on either plant[28]. Also, female oviposition preference for sunflower is not affected by plant species from which it emerged [28], and mating success is unaffected by the origin of their mate, either sunflower or soybean[31]. Most likely, host usage by *D. texanus* is related to how close they are to their host. Sunflowers planted in the field border (trap crop) had 57% infested plants compared to 19.2% on soybeans planted in the field's center[29]. Sunflowers planted in half a field (companion crop) had an 81.6% larval infestation rate compared to 30.7% on soybeans[29]. Furthermore, companion-sunflowers made an attraction buffer up to 200 m inside the soybean field, reducing the percent of infestation to ~70% in soybeans closest to sunflower[29].

Using soybean as host is probably costly for *D. texanus*. Sunflower-born females, males, pupae, and larvae are significantly longer and two times heavier than soybean-born individuals[28,30,31]. Also, adults feeding on cultivated sunflower live at least three times longer than those feeding on soybean[30]. Survival of soybean-born adults is higher and significantly positively associated with being heavier during the larval stage[30].

Interestingly, overwintering larvae collected from soybean in 2009 in Missouri had significantly larger head widths than those from sunflower, and no differences existed in larval weights of either soybean- or sunflower-fed larvae[32]. However, sunflower-fed overwintering larva had two times higher lipid content than soybean-fed larva[32], likely due to a three times higher lipid content in sunflower pith than in soybean pith[32]. Conversely, protein and carbohydrate content of sunflower and soybean-fed larvae did not differ significantly, even though, both are significantly higher in soybean than in sunflower pith[32]. Therefore, *D. texanus* larvae may use soybean carbohydrates and proteins to accumulate lipids before overwintering[30,32].

Control of Dectes stem borer

Chemical control. Few insecticides can effectively control *D. texanus* adults or larvae. In North Carolina, diazinon, chlorpyrifos, carbofuran, ethoprop, phorate, and fonofos were evaluated to control overwintering larvae. All were ineffective because they did not penetrate through the stubble, stem or frass plug to reach the larvae[21]. In the same study, spray formulations of carbaryl, malathion, methomyl, and methyl parathion were capable of controlling adults in field cages[21]. However, the authors considered that the use of insecticides in the field would be limited by the lack of knowledge of annual adult emergence and by the requirement for multiple insecticide applications[21].

In Mississippi, soybean plants treated with eight weekly applications of methyl parathion had lower numbers of *D. texanus* adults than untreated plants, but yields were no different

between the two treatments[33]. This lack of difference may be attributed to ensuing larval damage since the insecticide likely did not reach larvae in the stems. In another Mississippi study, yield differences were observed between untreated soybean plants and plants treated with insecticides, but there were no differences in the number of larvae between treatments[34]. It is likely that the use of insecticides to control other soybean pests may help to lower *D. texanus* infestations in Mississippi[35].

In Kansas, *D. texanus* adult mortality was observed 24 h after application of low concentrations of the pyrethroid insecticides lambda-cyhalothrin and permethrin in laboratory experiments[36]. Lambda-cyhalotrhin applied in the field reduced adult *D. texanus* populations. However, in order for insecticide treatments to work, multiple timed applications coinciding with adult emergence will be required to reduce infestations[37,38].

Several studies[39–41] have shown that fipronil, a systemic phenyl pyrazole insecticide applied as a soil- or soybean seed treatment, reduces *D. texanus* infestations up to 100% and that protected plants yield 10% more than untreated control plants. Fipronil also controls larvae that have previously tunneled into and reached the main stem before treatment[40,42]. However, fipronil remains unregistered by the U. S. EPA for use in soybeans[13,40].

Seed of oilseed sunflower hybrids treated with carbofuran had significantly reduced *D. texanus* larval infestations, higher stalk strength, lower lodging and higher oil seed content than untreated and lambda-cyhalothrin treated hybrids in 2005[23]. However, oil seed content was not consistently higher than carbofuran-protected hybrids over the 2 yr experiment[23]. Late-planted

sunflowers (early June) had significant lower numbers of *D. texanus* larvae when treated at the V8 stage with carbofuran than the untreated sunflowers[43]. However, this result was not consistent over 2 yr of study[43].

Cultural practices to reduce yield loss caused by *D. texanus*. Harvesting before lodging occurs has been shown to be more practical and effective than the use of insecticides[15,17]. However, constant field monitoring for *D. texanus* infestations is important for this strategy to be effective, and it is even more important when plants are close to maturity[13].

Burial of soybean stubble, at least 5 cm deep, was also suggested to reduce larval survival, since soil creates a physical barrier for adult emergence[21]. Soil type is a key factor in the success of stubble burial, since hard crust soils and dry conditions reduce adult emergence[21]. The combination of disk-tillage and early to mid-June planting reduced the survival of *D. texanus* larvae in sunflower to 27.4%, on average[26]. However, tillage and stubble burial is incompatible with soil conservation and erosion prevention efforts[13].

The effectiveness of crop rotation for *D. texanus* control remains uncertain because large areas of soybean crops are easily found by *D. texanus*[13,15]. However, there is evidence that second crop soybeans have lower *D. texanus* than full season soybeans[35]. No differences were detected between four irrigation schedules on *D. texanus* larvae abundance in sunflower stalks in Colby, KS[44].

Using cultivated sunflower as a trap crop for *D. texanus* oviposition[29], and increasing plant spacing to grow thicker soybean stalks[24] are other potential cultural control strategies. A higher percentage of *D. texanus*-girdled plants was observed in high density sunflower plots (>30,000 plants/ha) with thin stalks than in low-density plots (<26,000 plants/ha) with thicker stalks[24].

Biological control. Several hymenopteran parasites and one dipteran parasite infest *D. texanus* larvae. Parasitoids collected from giant ragweed included hymenopteran insects from the families Braconidae, Pteromalidae and Ichneumonidae[17]. *Dolichomitus irritator* (F.) (Hymenoptera: Ichneumonidae) and *Zelia tricolor* Coquillett (Diptera: Tachinidae) were found parasitizing larvae in soybean stubble[45,46]. However, there is no information about their efficiency as parasitoids for the development of biological control strategies.

Host plant resistance. The soybean defense system plays an important role in controlling soybean pests, and resistant cultivars would help to minimize yield losses related to *D. texanus*. In North Carolina, 618 soybean genotypes were evaluated for *D. texanus* resistance, but through 3 yr of consecutive screening, there was no consistency in the percent of infestation of putatively resistant plants[47]. However, *D. texanus* infestation and girdling declined in later maturity cultivars (maturity group V to VII) and in plants with higher lignin content[47].

In Mississippi, the cultivar “Tracy” had lower and higher numbers of larvae and adults than plants of the cultivar “Bragg. Therefore, “Tracy” could have an antibiotic effect on *D. texanus* larvae while “Bragg” could have antixenosis resistance to the adult[33]. Screening for *D.*

texanus resistance in Delaware was unsuccessful in identifying sources of resistance, but recent results indicate that the percentage of infested stems was >50% in screened soybean cultivars in maturity groups 4.7-4.8[48].

In Kansas, *D. texanus* larval infestation among commercial soybean cultivars in 2000 ranged from 50- to 68% in irrigated fields and from 17- to 75% in dryland fields[36]. However, there was no consistency in resistance response variables (lodging and girdling) since growing conditions and external factors likely affected the lodging response[36]. Also, there was no consistent resistance response among cultivars between different localities and environmental conditions (irrigated versus dryland fields)[36].

Kansas soybean cultivars and plant introductions were evaluated using the ratio: number of oviposition punctures (OP) / number of live larvae (Lv) to assess *D. texanus* larval resistance[49]. In contrast to previous studies of resistance based on larval stem tunneling and girdling, infestation and plant lodging, the OP/Lv ratio corrects for the fact that different cultivars receive different numbers of oviposition punctures and assesses larval survival[49].

Through four consecutive years of screening, plants of PI (plant introduction) 165673 consistently contained high numbers of oviposition punctures, low numbers of live larvae, and a high OP/Lv ratio compared to the susceptible checks 93M50 and 93M92[49]. The resistance response in PI165673 was similar to the positive antibiosis control, 93M50 plants protected with fipronil systemic insecticide[49]. Therefore, the authors concluded that PI165673 could be used as a resistant parent in the development of *D. texanus* resistant soybean cultivars[49].

In a follow up study, 108 $F_{2:3}$ families from the cross between the susceptible K07-1544 and PI165673 were evaluated for resistance to *D. texanus*. Thirteen $F_{2:3}$ families had higher OP/Lv ratios than K07-1544, and two of these 13 families had a higher ratio than the resistant PI165673 control[50]. Although broad sense heritability among the families (68.2%) indicates that progress in breeding PI165673 resistance into soybean can be achieved by selecting for high OP/Lv ratios[50], environmental variation (σ^2_e) may have been underestimated in this study since it was based mainly on parental variation[50]. Therefore, evaluation of advanced generation K07-1544/PI165673 progeny is needed to confirm both PI165673 resistance phenotypes and potential environmental effects. Finally, frequency distributions of the OP/Lv ratios in these families indicated that *D. texanus* resistance exhibited by PI165673 is polygenic[50] and will require several generations to be incorporated into commercial varieties by conventional breeding.

Interestingly, there were no differences in larval head capsule width, body length and proportion of larvae per instar between larvae collected from PI165673 or K07-1544 plants at 21 d post infestation[50]. This lack of differences in larval growth and development in the resistant and susceptible parents indicates that PI165673 resistance factors that reduce numbers of larvae do not affect larval growth. Therefore, the PI165673 resistance may need to be accompanied by other resistance factor(s) that inhibit or reduce larval development to provide effective control of this pest. Also, data are needed to determine if larvae surviving in PI165673 plants damage stems before harvest and overwinter.

Soybean resistance to *D. texanus* can be complemented by *in planta* delivery of double stranded RNA (dsRNA) to silence genes important for normal larval development[51,52]. DsRNA activates the RNA interference (RNAi) pathway in cells and consequently destroys the targeted messenger RNA (mRNA)[53]. Host plant-induced gene silencing by RNAi has been used in transgenic maize and potato to successfully manage the coleopteran pests, *Diabrotica virgifera virgifera* and *Leptinotarsa decemlineata* (Chrysomelidae) under controlled conditions[54–56].

Information about the success of gene silencing in *D. texanus* is lacking. Therefore, verification of the functionality of the RNAi pathway in *D. texanus* larvae is a vital step in determining if dsRNA can be delivered *in planta* to manage this pest. Gene silencing has been successful with the cerambycids *Monochamus alternatus*[57] and *Anoplophora glabripennis*[58] by injection of dsRNA that target the *Laccase2* (*Lac2*) and inhibitor of apoptosis (*iap*) genes, respectively. Most likely, gene silencing in *D. texanus* will be successful since it has been clearly demonstrated in closely related cerambycids[57–59], and genes involved in RNAi are conserved in coleopteran species[60–62]. Nevertheless, target genes necessary for soybean-induced gene silencing in *D. texanus* by RNAi must be identified.

Genes uniquely or highly expressed in larvae fed soybean are possible candidates for incorporation into soybean plants expressing *D. texanus*-specific dsRNA that reduce larval development. Genomic and transcriptomic resources are scarce for *D. texanus*, and as a result, such genes are unknown. However, *de novo* transcriptome assemblies have been useful to conduct differential expression analyses in other cerambycids[61,63–66].

Therefore, the objectives of my research projects are:

1. Evaluate stem girdling and tunneling by *D. texanus* in soybean PI165673 at the end of the 2014 growing season in Kansas.
2. Compare the transcriptome of *D. texanus* larvae fed soybean, wild sunflower, giant ragweed or artificial diet.
3. Silence the *Lac2* and *Chitin Synthase2* (*CHS2*) genes in *D. texanus* by feeding larvae artificial diet coated with *Lac2* or *CHS2*-dsRNA.

The hypotheses of my research projects are:

1. I observed *D. texanus* larvae surviving in stems of soybean PI165673, 21 d post infestation. Therefore, I hypothesize that larvae can girdle PI165673 plants at the end of the growing season in Kansas.
2. *D. texanus* prefer the native host plants sunflower and giant ragweed over soybean. I hypothesize that *D. texanus* larvae differentially express genes when fed soybean compared to those fed wild sunflower, giant ragweed or artificial diet.
3. Silencing genes by RNA interference is successful in other cerambycids and coleopteran pests. Therefore, I hypothesize that *Lac2* and *CHS2* can be silenced in *D. texanus* by RNA interference.

Chapter 2 - Evaluation of antibiosis resistance to *Dectes texanus* (Coleoptera: Cerambycidae) in the soybean PI 165673 at multiple days during the 2014 growing season in Kansas

Introduction

Host-plant resistance to arthropods is a fundamental component of crop improvement and integrated pest management[67,68]. Soybean varieties resistant to *Dectes texanus* LeConte (Coleoptera: Cerambycidae) are sought because the larval stage causes 15% yield reductions[13], commercial insecticides fail to kill larvae inside stems and require multiple applications to effectively manage adults[37], and cost of *D. texanus* management is included in the seed price[68,69]. Although, commercial soybean varieties resistant to *D. texanus* have not been developed, the soybean PI165673 shows antibiosis resistance to *D. texanus* that reduces the number of larvae compared to the susceptible control 93M50[49]. However, incorporation of this resistance into Kansas commercial varieties will require several generations because it is a quantitative trait, has a 68.2% broad sense heritability among $F_{2:3}$ families[50], and the PI165673 maturity group (VIII) is unsuitable for Kansas growing conditions[70]. Also, 21 d after infestation, the resistance in this plant introduction did not show an effect on larval growth and development of surviving larvae[50]. However, it is unknown if the surviving larvae continues to develop and damage the PI165673 at the end of the growing season. Therefore, the objective of this research is to evaluate stem girdling and tunneling by *D. texanus* in soybean PI165673 at the end of the 2014 growing season in Kansas.

Results

At 7 d post infestation, the larval antibiosis ratio (OL) (# ovipunctures / # larvae) was two times higher in the PI165673 compared to K07_1544 ($F_{1, 8} = 6.51$, $P = 0.03$, Table 2.1). The egg and larval antibiosis ratio (OEL) (# ovipunctures / [# eggs + # larvae]); head capsule width, and percentage of larvae per instar were not significantly different between genotypes ($F_{1, 18} = 2.61$, $P > 0.05$; $F_{1, 18} = 0.05$, $P > 0.05$; $F_{1, 18} = 0.94$, $P > 0.05$; $F_{1, 18} = 0.64$, $P > 0.05$; $F_{1, 8} = 0.02$, $P > 0.05$; Pearson's $\chi^2 = 1.5$, df = 1, $P > 0.05$, respectively; Table 2.1 and 2.2).

At 9 d post infestation, the number of oviposition punctures, eggs, and larvae; the OEL and OL antibiosis ratios; head capsule width, and percentage of larvae per instar were not significantly different between genotypes ($F_{1, 13} = 1.98$, $P > 0.05$; $F_{1, 13} = 0.13$, $P > 0.05$; $F_{1, 13} = 3.59$, $P > 0.05$; $F_{1, 13} = 0.95$, $P > 0.05$; $F_{1, 10} = 1.38$, $P > 0.05$; $F_{1, 10} = 0.01$, $P > 0.05$; Pearson's $\chi^2 = 0.98$, df = 1, $P > 0.05$, respectively; Table 2.3 and 2.4).

Also, at 11 d post infestation, none of the response variables were significantly different ($F_{1, 14} = 0.01$, $P > 0.05$; $F_{1, 14} = 0.03$, $P > 0.05$; $F_{1, 14} = 3.38$, $P > 0.05$; $F_{1, 14} = 0.86$, $P > 0.05$; $F_{1, 11} = 0.01$, $P > 0.05$; $F_{1, 11} = 0.74$, $P > 0.05$; Pearson's $\chi^2 = 0.98$, df = 1, $P > 0.05$, respectively; Table 2.5 and 2.6).

At 13 d post infestation, the OEL antibiosis ratio was two times significantly higher in the PI165673 compared to K07_1544 ($F_{1, 16} = 6.11$, $P = 0.02$, Table 2.7). The number of oviposition punctures, eggs, and larvae; the OL antibiosis ratio; head capsule width, and percentage of larvae

per instar were not significantly different between genotypes ($F_{1, 16} = 0.25$, $P > 0.05$; $F_{1, 16} = 2.61$, $P > 0.05$; $F_{1, 16} = 0.01$, $P > 0.05$; $F_{1, 13} = 2.02$, $P > 0.05$; $F_{1, 13} = 0.21$, $P > 0.05$; Pearson's $\chi^2 = 0.07$, df = 1, $P = 0.07$, respectively; Table 2.7 and 2.8).

At 15 d post infestation, the OEL antibiosis ratio was 0.6 times higher in the PI165673 compared to K07_1544 ($F_{1, 16} = 6.34$, $P = 0.02$, Table 2.9). The number of oviposition punctures, eggs, and larvae; the OL antibiosis ratio, and head capsule width were not significantly different between genotypes ($F_{1, 16} = 3.56$, $P > 0.05$; $F_{1, 16} = 1.93$, $P > 0.05$; $F_{1, 16} = 1.16$, $P > 0.05$; $F_{1, 16} = 2.56$, $P > 0.05$; $F_{1, 12} = 0.12$, $P > 0.05$, respectively; Table 2.9). The percentage of larvae per instar was significantly different between genotypes (Pearson's $\chi^2 = 11.3$, df = 1, $P = 0.03$) where the PI165673 had higher and lower percentage of larvae in first and second instar compared to the K07_1544, respectively (Table 2.10).

At 21 d post infestation, the OL antibiosis ratio and larval head capsule width were significantly different between genotypes (($F_{1, 10} = 5.67$, $P = 0.0385$; $F_{1, 9} = 13.07$, $P = 0.0056$, respectively). The OL antibiosis ratio was three times significantly higher in the PI165673 compared to K07_1544 (Table 2.11). Although, larvae-fed PI165673 had significantly smaller average head capsule widths than larvae-fed K07_1544 (Table 2.11), the percentage of larvae per instar was not significantly different between genotypes (Pearson's $\chi^2 = 4.97$, df = 2, $P = 0.06$, table 2.12). At least 50% of the larvae per genotype were in the first instar stage (Table 2.12). The number of oviposition punctures, eggs and larvae; and the OEL antibiosis ratio were not significantly different between genotypes ($F_{1, 13} = 0.01$, $P > 0.05$; $F_{1, 13} = 1.25$, $P > 0.05$; $F_{1, 13} = 1.07$, $P > 0.05$; $F_{1, 13} = 1.79$, $P > 0.05$, respectively; Table 2.11).

At 120 d post infestation, the OL antibiosis ratio; numbers of oviposition punctures, larvae and stem entrance holes; larval head capsule widths, and percentage of larvae per instar were not significantly different between genotypes ($F_{1, 7.2} = 0.2$, $P > 0.05$; $F_{1, 8.8} = 2.05$, $P > 0.05$; $F_{1, 9} = 2.02$ $P > 0.05$; $F_{1, 8.9} = 4.38$, $P > 0.05$; $F_{1, 9} = 1.14$, $P > 0.05$; Pearson's $\chi^2 = 0.74$, $P > 0.05$, respectively; Table 2.13 and 2.14). The percentage of stem tunneling was significantly lower in the PI16573 compared to K07_1544 ($F_{1, 9} = 20.39$, $P = 0.0015$, Table 2.13). Although all plants in both genotypes were tunneled by *D. texanus*, none of the PI165673 plants had tunnels reaching the base of the plant or were girdled compared to K07_1544 (69.2 and 30.8 %, respectively). The percentage of tunneled and girdled plants were statistically different between genotypes (Pearson's $\chi^2 = 25.9$, df = 1, $P < 0.0001$; Pearson's $\chi^2 = 8.8$, df = 1, $P = 0.0043$, respectively).

Discussion

The high OL antibiosis ratio observed in the PI165673 at 7 d post infestation may be overestimated because 30% of the K07_1544 plants contained larvae whereas 70% of the PI165673 plants had first instar larvae. Therefore, resistance assessment might be more accurate with the OEL antibiosis ratio during the first week of the experiment.

The PI165673 had higher OEL antibiosis ratio at 13 and 15 d post infestation which indicates that the sum of eggs and larvae relative to the number of oviposition punctures is lower in the PI165673 compared to K07_1544. Therefore, the PI165673 contained oviposition

punctures lacking eggs. This behavior was previously described for *D. texanus* on soybean[17] and sunflower[22], but this is the first report on the PI165673.

The high OL antibiosis ratio observed in PI165673 confirm previous published data where this genotype had higher ratio than the susceptible control in choice experiments at 21 d post infestation[49,50]. Also, it confirms that the PI16573 reduces *D. texanus* larval densities relative to oviposition punctures compared to K07_1544. *D. texanus* intraspecific cannibalism[17] may explain the lack of differences in the OL antibiosis ratio between genotypes at 120 d post infestation.

The percentage of second instar larvae was lower in the PI165673 compared to the susceptible control at 15 d post infestation suggesting that first instar larvae feeding in the PI165673 take longer to develop. At 21 d post infestation, larvae fed-PI165673 had smaller head capsule sizes compared to those fed K07_1544 suggesting that the PI165673 could be slowing the development of *D. texanus* larvae. These results are contrary to those observed in 2012 where no differences were detected in the larval head capsule size between genotypes at 21 d post infestation[50]. However, more testing is needed because environmental factors may be influencing the PI16573 quantitative-resistance response since 2014 had a mild summer compared to the hot summer in 2012.

Also, lack of differences in the head capsule size and percentage of larvae per instar between genotypes at 120 d post infestation suggest that the surviving larvae can compensate or overcome the PI165673 resistance factors at some point during their development.

The plant maturity of each genotype may underestimate the percentage of girdled plants observed at 120 post infestation because K07_1544 plants had reached full development whereas PI165673 plants were still green, bushy, and in the pod-filling stage. Additionally, the plant height (data not shown) and plant maturity may bias the percentage of tunnels reaching the plant base and stem tunneling, respectively, since K07_1455 plants were shorter and had reached their maximum growth relative to PI165673. Therefore, assessment of PI165673 girdling by *D. texanus* at the end of the growing season will be best conducted in Southern States where maturity VIII soybeans can complete their development.

In conclusion, PI165673 antibiosis resistance reduces *D. texanus* egg oviposition, larval survivorship and could affect development of first instar larvae. However, surviving larvae can tunnel PI165673 stems, develop until the sixth instar stage, and potentially cause girdling damage. Therefore, the PI165673 resistance needs to be reinforced with other sources of soybean resistance or biotechnology techniques before releasing seed material to farmers in Kansas.

Materials and methods

Field experiment

PI165673 and K07_1544 seeds used in this experiment were provided by the Kansas State University soybean breeding program from the winter nursery in Costa Rica. The

experiment was conducted in summer 2014 in a 12.2 x 12.2 m plot that consisted of 20 rows spaced 61 cm apart, at the Kansas State University Ashland Bottoms Research Station, near Manhattan, KS. Each row had 19 planting spots, spaced 61 cm apart, and a spot was randomly assigned to a genotype. Three seeds per genotype were hand planted in early June, about 2.5 cm deep, due to low seed germination of the resistant genotype in a moist filter paper assay (< 30 %); one seedling per spot was kept after emergence. Only 110 spots had a PI165673 plant. Plot border rows were planted with K07_1544 seed.

Each plant was caged with a galvanized tomato frame (0.6 x 1.4 m, Hummert International) covered with a mosquito mesh (1 x 2 m) 21 d after planting. Cages were hold to the ground using a 2 m rebar and 23 cm tent stakes. The bottom and top of the mesh was buried with soil and tied with a knot, respectively, to prevent the beetles from scaping and other insects from entering. Cages were infested 30 d after planting with four unsexed *D. texanus* beetles per cage that were collected from soybean fields in the research station. *D. texanus* adults have sexual monomorphism.

Plants were sampled at seven dates (7, 9, 11, 13, 15, 21 and 120 d) post infestation; number of plants sampled per date are listed in Table 2.15. Sampling after the first freeze (120 d after infestation) was divided into ten consecutive sampling days (blocks) due to logistic constrains of sampling all plants in the same day. October 29th was the first freeze date in Fall 2014 at the Research Station[71]. Five plants per genotype were evaluated at each sampling day after the first freeze. About 30% of the plants per genotype lacked *D. texanus* oviposition

punctures; most likely because beetles got stuck at the top of the mesh before oviposition (Table 2.15). These plants were not considered in the analyses.

The number of oviposition punctures (OvP), eggs, larvae, and stem entrance holes; plant height; stem and tunnel length; and girdling damage were counted, measured and evaluated for each plant, respectively. The egg and larval antibiosis ratio (OEL, OvP / [#eggs + #larvae]) and larval antibiosis ratio (OL, # OvP / #larvae) were calculated for each sampling date, except for 120 d post infestation when only OL was calculated since no eggs were found inside the plants. Larval head capsules were measured across their widest point using a Leica® MZ APO stereomicroscope. Larval instar was determined based on the head capsule width range described for each *D. texanus* instar by Hatchett et al. 1975[17]. Larvae exceeding the range of sixth instar stage were still considered sixth instar.

Statistical analyses

Data listed in table 2.16 were assessed for normality and homogeneity of variances at each sampling date using Kolmogorov-Smirnov[72], distribution of residuals, Levene[73] and Brown-Forsythe[74] tests. A Poisson or negative binomial distribution was used to analyze data that failed these tests after fitness of both distributions were assessed based on a Pearson's chi-square/DF statistic for conditional distributions (overdispersion test)[75,76]. The type of distribution used for each response variable is listed on table 2.17. The type III test of fixed effects was used to compare data of both genotypes with the PROC GLIMMIX procedure[77] and the Kenward-Rogers approximation[78] for estimation of degrees of freedom. Each sampling date was analyzed

independently where genotype was considered the fixed effect in the model. Sampling day after 120 d post infestation was considered a blocking factor and a random effect in the model[79]. When the F-test was significant ($P < 0.05$) means were given a different uppercase letter.

The percentage of larvae per instar, stem girdling and stem-tunnel reaching the plant base were analyzed using a Pearson's chi-square test using the PROC FREQ procedure[80]. When the percentage of larvae per instar was significant ($P < 0.05$), Fisher's exact tests were conducted per instar between genotypes. A chi-square distribution was used because data convergence failed using a binomial distribution. SAS v. 9.2 was used to perform all statistical analyses.

Table 2.1. OL antibiosis ratio was significantly different between genotypes at 7 d post infestation.

Genotype	Mean (lower, upper CI)					
	OEL antibiosis ratio (OvP/[Eggs+Larvae])	OL antibiosis ratio (OvP/Larvae)	# Oviposition punctures	# Eggs	# Larvae	Head capsule width (mm)
K07_1544	1.9 (1.2, 3.1) A	2.3 (0.4, 4.1) A	12.0 (2.6, 21.4) A	5.1 (1.7, 8.5) A	2.1 (0.6, 7.4) A	0.43 (0.39, 0.47) A
PI165673	2.3 (1.6, 3.8) A	4.7 (3.5, 5.9) B	22.2 (12.8, 31.6) A	5.6 (2.2, 9.0) A	4.7 (1.4, 15.2) A	0.5 (0.47, 0.52) A

Means within a column with different uppercase letter are significantly different based on an F-test ($P < 0.05$). CI= confidence interval

Table 2.2. Percentage of larval instars per genotype 7 d post infestation.

Genotype	% larvae per instar (± SE)	
	I	II
K07_1544	100 ± 1 A	0 ± 1 A
PI165673	93.3 ± 1 A	6.7 ± 1 A

Percentages within a column are not significantly different based on a Pearson's chi-square test ($P > 0.05$)

Table 2.3. No differences detected between genotypes at 9 d post infestation.

	Mean (lower, upper CI)					
Genotype	**OEL antibiosis ratio (OvP/[Eggs+Larvae])**	**OL antibiosis ratio (OvP/Larvae)**	**# Oviposition punctures**	**# Eggs**	**# Larvae**	**Head capsule width (mm)**
K07_1544	1.9 (1.5, 2.3) A	5.7 (3.0, 10.8) A	25.2 (15.4, 41.4) A	6.9 (2.1, 11.6) A	6.0 (2.9, 12.4) A	0.48 (0.42, 0.53) A
PI165673	1.6 (1.2, 2.1) A	10.0 (4.3, 23.5) A	15.0 (8.0, 28.0) A	8.2 (2.3, 14) A	2.0 (0.7, 5.6) A	0.48 (0.40, 0.55) A

Means within a column with same uppercase letter are not significantly different based on an F-test ($P > 0.05$). CI= confidence interval

Table 2.4. Percentage of larval instars per genotype 9 d post infestation.

	% larvae per instar (± SE)	
Genotype	**I**	**II**
K07_1544	92.3 ± 1 A	7.7 ± 1 A
PI165673	100 ± 1 A	0 ± 1 A

Percentages within a column are not significantly different based on a Pearson's chi-square test ($P > 0.05$)

Table 2.5. No differences detected between genotypes at 11 d post infestation.

Genotype	Mean (lower, upper CI)					
	OEL antibiosis ratio (OvP/[Eggs+Larvae])	OL antibiosis ratio (OvP/Larvae)	# Oviposition punctures	# Eggs	# Larvae	Head capsule width (mm)
K07_1544	1.5 (0.8, 2.9) A	5.7 (2.8, 11.6) A	16.1 (5.7, 26.6) A	5.4 (1.2, 9.6) A	5.3 (2.7, 7.8) A	0.5 (0.4, 0.6) A
PI165673	2.1 (1.3, 3.5) A	5.4 (2.5, 11.7) A	15.4 (6.2, 24.6) A	5.9 (2.2, 9.6) A	2.4 (0.3, 4.6) A	0.5 (0.47, 0.6) A

Means within a column with same uppercase letter are not significantly different based on an F-test ($P > 0.05$). CI= confidence interval

Table 2.6. Percentage of larval instars per genotype 11 d post infestation.

Genotype	% larvae per instar (± SE)	
	I	II
K07_1544	94.1 ± 1 A	5.9 ± 1 A
PI165673	86.4 ± 1 A	13.6 ± 1 A

Percentages within a column are not significantly different based on a Pearson's chi-square test ($P > 0.05$)

Table 2.7. OEL antibiosis ratio and % of larvae per instar are significantly different between genotypes at 13 d post infestation.

	Mean (lower, upper CI)					
Genotype	**OEL antibiosis ratio (OvP/[Eggs+Larvae])**	**OL antibiosis ratio (OvP/Larvae)**	**# Oviposition punctures**	**# Eggs**	**# Larvae**	**Head capsule width (mm)**
K07_1544	1.4 (0.7, 2.8) A	3.4 (0.1, 6.8) A	23.6 (8.9, 38.3) A	8.4 (4.6, 12.2) A	6.4 (2.3, 10.4) A	0.48 (0.4, 0.55) A
PI165673	3.7 (2.4, 5.6) B	6.1 (3.7, 8.5) A	28.3 (15.2, 41.4) A	4.5 (1.1, 7.9) A	6.1 (2.5, 9.7) A	0.49 (0.4, 0.55) A

Means within a column with different uppercase letter are significantly different based on an F-test ($P < 0.05$). CI= confidence interval

Table 2.8. Percentage of larval instars per genotype 13 d post infestation.

	% larvae per instar (± SE)	
Genotype	**I**	**II**
K07_1544	97.9 ± 1 A	2.1 ± 1 A
PI165673	88.1 ± 1 A	11.9 ± 1 A

Percentages within a column are not significantly different based on a Pearson's chi-square test ($P = 0.07$)

Table 2.9. OEL antibiosis ratio and % of larvae per instar are significantly different between genotypes at 15 d post infestation.

	Mean (lower, upper CI)					
Genotype	**OEL antibiosis ratio (OvP/[Eggs+Larvae])**	**OL antibiosis ratio (OvP/Larvae)**	**# Oviposition punctures**	**# Eggs**	**# Larvae**	**Head capsule width (mm)**
K07_1544	1.5 (1.2, 2.0) A	6.3 (0.4, 12.1) A	10.5 (1.2, 22.2) A	3.1 (1.3, 7.2) A	3.1 (1.2, 7.9) A	0.5 (0.4, 0.7) A
PI165673	2.4 (1.8, 3.1) B	12.2 (6.9, 17.5) A	24.5 (14.0, 34.9) A	6.4 (3.2, 12.9) A	5.8 (2.6, 12.7) A	0.6 (0.4, 0.7) A

Means within a column with different uppercase letter are significantly different based on an F-test ($P < 0.05$). CI= confidence interval

Table 2.10. Percentage of larval instars per genotype 15 d post infestation.

	% larvae per instar (± SE)		
Genotype	**I**	**II**	**III**
K07_1544	58.3 ± 1 A	41.7 ± 1 B	0 ± 1 A
PI165673	86.4 ± 1 B	10.2 ± 1 A	3.4 ± 1 A

Percentages within a column with different uppercase letter are significantly different based on Fisher's Exact tests per instar ($P < 0.05$)

Table 2.11. PI165673 high antibiosis ratio indicated resistance against *Dectes texanus* larvae at 21 d post infestation.

	Mean (lower, upper CI)					
Genotype	**OEL antibiosis ratio (OvP/[Eggs+Larvae])**	**OL antibiosis ratio (OvP/Larvae)**	**# Oviposition punctures**	**# Eggs**	**# Larvae**	**Head capsule width (mm)**
K07_1544	1.8 (1.1, 2.5) A	2.3 (1.1, 4.9) A	18.3 (7.3, 29.3) A	2.0 (1.0, 5.0) A	7.8 (2.7, 12.9) A	0.57 (0.52, 0.62) B
PI165673	2.3 (1.8, 2.9) A	6.4 (3.6, 11.3) B	19.1 (10.1, 28.1) A	4.0 (1.6, 6.4) A	4.7 (0.5, 8.9) A	0.46 (0.4, 0.51) A

Means within a column with different uppercase letter are significantly different based on an F-test ($P < 0.05$). CI= confidence interval

Table 2.12. Percentage of larval instars per genotype 21 d post infestation.

	% larvae per instar (± SE)		
Genotype	**I**	**II**	**III**
K07_1544	67.4 ± 1 A	26.1 ± 1 A	6.5 ± 1 A
PI165673	84.4 ± 1 A	14.6 ± 1 A	0 ± 1 A

Percentages were not significantly different based on a Pearson's chi square test ($P > 0.05$)

Table 2.13. Percentage of stem tunneling by *Dectes texanus* is lower in PI165673 at 120 d post infestation.

	Mean (lower, upper CI)					
Genotype	**Stem tunneling (%)**	**OL antibiosis ratio (OvP/Larvae)**	**# Oviposition punctures**	**# Larvae**	**# Stem entrance holes**	**Head capsule width (mm)**
K07_1544	71.7 (62.2, 81.1) A	13.4 (9.9, 18.1) A	17.7 (11.2, 28.2) A	1.3 (0.5, 3.3) A	5.3 (3.9, 6.7) A	2.0 (1.9, 2.1) A
PI165673	50.4 (40.4, 59.7) B	12.4 (9.1, 16.7) A	27.3 (17.1, 43.5) A	2.4 (0.9, 5.9) A	3.6 (2.8, 4.7) A	2.0 (1.9, 2.1) A

Means within a column with different uppercase letter are significantly different based on an F-test ($P < 0.05$). CI= confidence interval

Table 2.14. Percentage of larval instars per genotype at 120 d post infestation

	% larvae per instar (± SE)		
Genotype	**IV**	**V**	**VI**
K07_1544	0 ± 1 A	2.9 ± 1 A	97.1 ± 1 A
PI165673	1.8 ± 1 A	1.8 ± 1 A	96.4 ± 1 A

Percentages were not significantly different based on a Pearson's chi-square test ($P > 0.05$)

Table 2.15. Summary of sample size per sampling date.

	# Sampled plants		# Plants oviposited by *Dectes texanus*	
Sampling date (d post infestation)	**K07_1544**	**PI165673**	**K07_1544**	**PI165673**
7	10	10	10	10
9	10	10	9	6
11	10	10	7	9
13	10	10	9	9
15	10	10	8	10
21	10	10	6	9
120	50	50	26	24
Total	110	110	75	77

Table 2.16. Summary of normality and homogeneity of variances verification before F-test per sampling date.

	d post infestation						
Variable	**7**	**9**	**11**	**13**	**15**	**21**	**120**
# Oviposition punctures	P	F	P	P	P	P	F
# Eggs	P	P	P	P	F	P	-
# Larvae	F	F	P	P	F	P	F
Antibiosis ratio OEL (OvP/[Eggs+Larvae])	F	P	F	F	F	P	-
Antibiosis ratio OL (OvP/Larvae)	P	F	F	P	P	F	F
Larval head capsule width	F	P	P	P	P	P	P
# Stem entrance holes	-	-	-	-	-	-	F
% Stem tunneling	-	-	-	-	-	-	P

P= passed; F= Failed the tests

- = none applied

Table 2.17. Summary of distributions used per sampling date to analyze each data.

	d post infestation						
Variable	**7**	**9**	**11**	**13**	**15**	**21**	**120**
# Oviposition punctures	N	P	N	N	N	N	NB
# Eggs	N	N	N	N	NB	N	-
# Larvae	NB	NB	N	N	NB	N	NB
Antibiosis ratio OEL (OvP/[Eggs + Larvae])	NB	N	P	NB	NB	N	-
Antibiosis ratio OL (OvP/Larvae)	N	NB	NB	N	N	NB	NB
Larval head capsule width	P	N	N	N	N	N	N
# Stem entrance holes	-	-	-	-	-	-	P
% Stem tunneling	-	-	-	-	-	-	N

N= normal; P = Poisson; NB= negative binomial distribution

- = none applied

Chapter 3 - Differential gene expression in larvae of *Dectes texanus* LeConte (Coleoptera: Cerambycidae) fed soybean, giant ragweed or sunflower

Introduction

Dectes texanus LeConte (Coleoptera: Cerambycidae), commonly known as the *Dectes* stem borer, is a native long-horned beetle species of North America[14] and a pest of soybeans (Fabaceae: *Glycine max* (L) Merr.) in several states of the U. S. A. The larval stage damages the stems by tunneling and girdling[15,19–21], causing a 15% reduction in soybean yield [81,82]. Although giant ragweed (Asteraceae: *Ambrosia trifida* L.) and wild sunflower (Asteraceae: *Helianthus annus* L.) are native hosts of *D. texanus*[15], this beetle has been colonizing and using soybean as host for the last 50 years[11,22]. Expansion of soybean production, reduction of native wild hosts, and adoption of non-tillage farming practices are most likely associated with the use of soybeans as a host by *D. texanus*[13,15].

Although detoxification enzymes and gene families important for host plant selection and the ability to switch plant hosts have been identified in other cerambycids[83–85] and insects[86], it is unknown what genetic factors enable *D. texanus* to colonize, feed and survive on soybeans. To understand how *D. texanus* has adapted to soybean as a new host, we compared the global transcriptome profiles of larvae fed on soybean to larvae fed on sunflower and giant ragweed. Through this approach, we produced the first *de novo* transcriptome assembly from *D. texanus* larvae and identified unigenes differentially expressed in larvae fed soybean that could be

associated with its success in feeding on a novel host. Ultimately, this research will help us to develop novel management tools to make soybean unsuitable for this pest.

Results and discussion

Sequencing and de novo transcriptome assembly

A total of 355.6 million reads were obtained from the 12 RNA-seq libraries derived from third instar larvae. These libraries were subjected to Illumina HiSeq2500 sequencing with a total number of read pairs per sample ranging from 25.7 to 33.8 million reads (Table 3.1). Approximately ~335.4 million (99%) reads from all samples were retained after removing low quality bases and reads. Approximately 11.8 million high quality reads remained after *in silico* normalization and were used by Trinity to construct the *de novo* transcriptome assembly.

The raw *D. texanus* transcriptome assembly yielded 127,878 putative transcripts and 65,979 unigenes with an N50 length of 2,387 and 1,877 bp, respectively (Table 3.2). A total of 19,791 (15.5%) transcripts had low abundance and expression value compared to the dominant isoform (see methods), and 66,626 (52.1%) transcripts lacked an open reading frame (ORF). The final filtered assembly contained 41,461 transcripts and 14,504 unigenes with an N50 length of 3,025 and 3,195 bp, respectively (Table 3.2). The size of the filtered assembly was 97 Mb based on the sum of the transcript lengths and 33.3 Mb based on the sum of the longest transcript per unigene. Transcripts with predicted complete, 5’ and 3’ partial ORFs represented 73.8, 7.9, and

12.5% of the filtered assembly, respectively. Unigenes with predicted complete, 5' and 3' partial ORFs represented 68.8, 10.9, and 9.8% of the filtered assembly, respectively.

Though ~93.6% of the assembly matched insect-derived sequences, 5 unigenes (0.03%) were derived from plants, 11 unigenes (0.08%) were derived from bacteria, 4 unigenes (0.03%) were derived from fungi, and 26 unigenes (0.2%) were derived from viruses. These were removed from the final assembly. Approximately 86% of the *D. texanus* protein coding unigenes matched sequences derived from the order Coleoptera, where 54.1% of the unigenes had highest scoring matches to sequences derived from the family Cerambycidae (Fig. 3.1).

The filtered transcriptome was functionally annotated using BLASTp and HMMER against the Uniprot/SwissProt and PFAM-A databases, respectively, where 10,307 (71.1%) and 10,471 (72.2%) of the unigenes had at least one BLASTp match to an Uniprot/SwissProt protein and a Pfam-A domain, respectively (Table 3.3). In addition, at least one gene ontology (GO) and/or KEGG (Kyoto Encyclopedia of Genes and Genomes) orthology (KO) terms was predicted for 6,772 (46.7%) and 5,362 (37%) of the transcripts, respectively (Table 3.3).

BUSCO analysis was performed and the KEGG pathway representation was compared to *A. glabripennis* to gauge the completeness of the *D. texanus* transcriptome assembly in terms of recovered gene space. The analysis led to the recovery of 1,570 (94.6%) complete BUSCOs of the Insecta ODB9 linage gene set with 93.8%, 0.8%, and 1.6% complete single-copy, duplicated, and fragmented BUSCOs, respectively, indicating that the majority of the conserved insect genes were captured in the assembly. Providing further support to the high quality of the *D. texanus*

transcriptome assembly, the number of *D. texanus* unigenes assigned to core KEGG metabolic pathways that are expected to be represented in most of the insect taxa were fairly similar to those previously annotated in the *A. glabripennis* genome (Fig. 3.2). The number of unique KO terms found in the *D. texanus* transcriptome were similar to those represented in the genomes of other beetle taxa, including *Tribolium castaneum*, *Dendroctonus ponderosae*, and *A. glabripennis* (Table 3.4). These data indicated that several conserved metabolic pathways were well represented in the transcriptome assembly, suggesting that the *D. texanus* transcriptome contains a comprehensive representation of the majority of KEGG metabolic pathway genes.

Glycoside hydrolases involved in plant cell wall degradation

Cerambycids rely on plant cell wall polysaccharides, including cellulose, pectin, and hemicelluloses, as the main carbon source for their growth and development[64]. Glycoside hydrolases (GH) are important enzymes in stem and wood-boring insects that facilitate digestion of major classes of plant cell wall polysaccharides into absorbable monosacharides[85,87]. Also, GH are relevant for the biotransformation of plant defensive compounds that contain glycosidic linkages[88,89]. A brief descriptive summary of these families in the *D. texanus* assembly are described herein since they may be relevant in its ability to use soybean as a host plant.

Twenty-two different glycoside hydrolase families, spanning 120 unigenes, were identified from the *D. texanus* transcriptome (Fig. 3.3). The number of GH families identified is 1.6 times higher than those previously identified in the *A. glabripennis*-midgut *de novo* transcriptome[63], most likely due to assembling the *D. texanus* transcriptome with RNA-seq data

from larvae fed three different plant species while the *A. glabripennis* transcriptome was constructed from insects feeding only on one tree species. However, the number of GH families identified in the *D. texanus* transcriptome was ~4% lower than the number of GH families encoded in the *A. glabripennis* genome[83].

Overall, GH1 was the most highly represented family in the *D. texanus* transcriptome associated with plant cell wall degradation (Fig. 3.3a, 3.4); however, the number of unigenes identified in this family is 35% and 39% less than those previously reported in the *A. glabripennis* transcriptome[63] and genome[83]. *D. texanus* GH1 peptides had highest scoring BLASTp matches to myrosinase 1, myrosinase 1-like, lactase-phlorizin hydrolase and lactase from the Uniprot database. Myrosinases have roles in degrading glucosinolates in insect taxa that feed on members of the plant order Brassicales; however, glucosinolates are not known to occur in any of *D. texanus* hosts from the families Fabaceae and Asteraceae. Therefore, GH1 enzymes coded by *D. texanus* could be acting as β-glucosidases to degrade di- and tri-saccharide sugars found in plant tissues or act on other plant secondary compounds[90,91].

Interestingly, the GH5 and GH48 families had two and five more *D. texanus* unigenes identified relative to those annotated in the *A. glabripennis* genome (Fig. 3.3a), respectively. Members of the GH5 family had highest scoring BLASTp matches to proteins annotated as endoglucanase Z and endoglucanase 5A while members of the GH48 family had matches to exoglucanase B. However, genome sequencing would be required to validate whether these unigenes are coded by separate loci/genes in *D. texanus*. Other, endoglucanase unigenes assigned to the GH9 and GH45 families (Fig. 3.3a) most likely confer the ability to digest cellulose and

xylan in *D. texanus*, which are the two most prominent polysaccharides in secondary plant cell walls, as indicated in *A. glabripennis* through *in-vitro* functional characterization[83].

The GH28 and GH38 families had 12 and 10 *D. texanus* unigenes with high scoring BLASTp annotation to poly- and endo-galacturonases, and α-mannosidases, respectively. Putatively, these enzymes most likely are important for *D. texanus* in the hydrolysis of polygalacturonan (pectin) and hemicellulose (glycan bonds) when digesting stem and petiole pith, respectively. The GH18 was the second most abundant GH family in the *D. texanus* transcriptome and had 18 unigenes with high scoring BLASTp matches to chitinases (Fig. 3.4) which may be involved in the digestion of chitin from conspecifics since *D. texanus* larvae are cannibalistic.

Enzymes involved in biotransformation of plant allelochemicals

UDP-glucuronosyl-transferases (UGTs), glutathione S-transferases (GSTs), carboxylesterases (COesterases), and cytochrome P450 (P450s) are key enzymes involved in the biotransformation of plant allelochemicals and confer the ability to use a plant host in many insect species[92]. The most prominent class of biotransformation enzymes identified in the *D. texanus* transcriptome was P450, followed by COesterases, UGTs, and GSTs (Fig. 3.3b). The total number of unigenes coding for each of these classes was lower than those reported in the *A. glabripennis* genome[83], or the transcriptomes of the cerambycids *Monochamus alternatus*[65] and *Batocera horsfieldi*[66].

The four clades of P450s typically found in insect genomes and transcriptomes[93] were represented in the *D. texanus* transcriptome, and family members of clades 3 and 4 were abundant among the unigenes coding for P450s. Within clade CYP3, 34.5% of the P450s unigenes were assigned to the CYP6 family, and 15.5% were assigned to the CYP9 family; and within clade CYP4, 31% were assigned to the CYP4 family. In contrast, only nine enzymes coding for mitochondrial P450 unigenes that spanned six families were identified in the *D. texanus* assembly. Among these unigenes, those coding for peptides in the CYP49 family were the most frequent. The CYP4, 6, and 9 families include many enzymes associated with insect-plant interactions in other insect taxa[94]. However, the interaction between soybean substrates and *D. texanus* P450s is unknown.

Differentially expressed unigenes in soybean-fed larvae

The average overall alignment rate for each library was 66.8% when it was mapped against the filtered transcriptome assembly containing only transcripts that coded for proteins. The three replicates within soybean, sunflower, and artificial diet-fed larvae were correlated ($R^2 > 0.5$) with one another based on global expression profiles and Pearson correlations (Fig. 3.5 and 3.6). However, the three replicates from giant ragweed treatment were not as strongly correlated ($R^2 < 0.3$) with one another and the expression profiles were more variable (Fig. 3.6). This variability may be attributed to environmental effects, plant age differences, or the length of time it took to dissect the stems.

Overall, 478 *D. texanus* unigenes were differentially expressed in at least one of the plant diet treatments using fold change (FC) thresholds of ≥ ±1.5 and False Discovery Rate (FDR) adjusted p-values of ≤ 0.05. Soybean-fed larvae up-regulated 189 and 75 unigenes compared to larvae fed sunflower and giant ragweed, respectively (Fig. 3.7a); and down-regulated 127 and 111 unigenes compared to either native host, respectively (Fig. 3.7b). There were 46 and 19 unigenes commonly up and down-regulated in soybean-fed larvae compared to those fed the other two plant hosts, respectively (Fig. 3.7a and b). Also, soybean-fed larvae differentially up-regulated and down-regulated 51 and 71 unigenes compared to those fed artificial diet, respectively (Fig. 3.7 c and d).

Of the commonly differentially expressed unigenes in larvae fed soybean compared to those fed in both natural hosts, only five and six unigenes were also up- and down-regulated compared to those fed artificial diet, respectively (Fig. 3.7c, and d). Therefore, 41 and 13 unigenes were exclusively up-regulated and down-regulated, respectively, in soybean-fed larvae compared to those fed sunflower or giant ragweed. Unigenes involved in transport of small hydrophobic molecules and solutes; and phosphorylation of ecdysteroids were among the five up-regulated unigenes in soybean-fed larvae compared to all diet treatments (Fig 3.8a). Unigenes coding for insect cuticle proteins, GH45, chitin binding peritrophin-A domain, and transglutaminases were among the six down-regulated unigenes in soybean-fed larvae compared to those fed either native host and artificial diet (Fig.3.8b).

Four molecular function GO categories were significantly (FDR < 0.05) enriched among the 41 up-regulated unigenes in larvae fed soybean compared to those fed either native host

(Table 3.5). These categories were oxidoreductase activity (GO:0016705), iron-ion binding (GO:0005506), tetrapyrrole binding (GO:0046906), and heme binding (GO:0020037), which included five P450 unigenes belonging to the families CYP6 and 9. No GO categories were significantly enriched among the 13 down-regulated unigenes in larvae fed soybean compared to the other plant hosts. GO categories significantly enriched in specific comparisons between larvae fed soybean and those fed the other diet treatments are listed in Table 3.6.

Approximately 60% of the unigenes commonly up-regulated in soybean fed-larvae compared to those fed either native host had a significant (e-value < 0.00001) BLASTp matches to annotated proteins or contained known Pfam-A domains. Unigenes that encoded P450s, COesterases, and the Major Facilitator Superfamily (MSF) were the most represented protein families among the 41 up-regulated unigenes in soybean fed-larvae (Fig. 3.9a). Protein coding unigenes related to digestion and protein binding/transport included a GH1, a lytic polysaccharide monooxygenase, a short chain dehydrogenase, a chitin binding peritrophin-A, and an apolipoprotein (Fig 3.9a). Protein families that function at the nuclear level were also represented among the up-regulated unigenes, and they included a transcription activator MBF2, a transposase IS4, a methyltransferase MT-A70, and a DDE endonuclease (Fig 3.9a). P450s, COesterases, MFS transporters, and other unigenes related to digestion may be associated with the utilization of soybean as a host by *D. texanus* third-instar larvae.

Among the 13 down-regulated unigenes in larvae fed soybean compared to native hosts, 46% contained a significant match to a Pfam-A domain. These down-regulated protein coding

unigenes included the following protein families: an insect cuticle protein, a transglutaminase, a collagen triple helix repeat, an UGT, a MFS, and a FAD binding domain (Fig 3.9b).

K-means analyses of up-regulated and down-regulated unigenes in soybean-fed larvae

When data were partitioned into clusters of unigenes with similar expression profiles across replicates in each diet treatment, 28 and 51 unigenes were more highly expressed in soybean-fed larvae compared to larvae fed sunflower, giant ragweed, or artificial diet, respectively (Fig. 3.10a and b). Further, soybean fed larvae differentially down-regulated 29 and 43 unigenes compared to those fed either native host or artificial diet, respectively (Fig. 3.11a and b). Only two and five unigenes were consistently up- and down-regulated in soybean fed larvae compared to those fed sunflower, giant ragweed or artificial diet, respectively (Fig. 3.10c and 3.11c); whereas only 26 and 24 unigenes were up- and down-regulated in soybean fed larvae compared to those fed either native host (Fig 3.10c and 3.11c).

Lipocalin, a protein family involved in transport of small hydrophobic molecules across membranes, was among the two commonly up-regulated unigenes in soybean-fed larvae compared to those fed either native host or artificial diet (Fig. 3.12a). The other commonly up-regulated unigene lacked a Pfam-A domain match. Two unigenes coding for insect cuticle proteins, one coding for an GH45, other coding for a transglutaminase, and one coding for a FAD binding domain were among the five commonly down-regulated unigenes in soybean-fed larvae compared to those fed either native host or artificial diet (Fig. 3.12b).

Five molecular function GO categories were significantly enriched among the unigenes up-regulated in soybean-fed larvae compared to those fed sunflower or giant ragweed (Table 3.7). These enriched categories included three glycoside hydrolases and four P450 unigenes belonging to the families GH28, CYP6 and 9, respectively. No GO categories were significantly enriched among the down-regulated unigenes in soybean fed larvae compared to those fed either native host, and among the up-regulated and down-regulated unigenes in larvae fed soybean compared to those fed artificial diet.

Protein families involved in biotransformation of plant allelochemicals, digestion of plant cell walls, transport of small solutes and lipids, and protein binding were among the 26 unigenes up-regulated in soybean-fed larvae compared to those fed either native host (Fig. 3.10, 3.13a). P450, COesterases, GH28, and MFS protein families were represented at least by more than two up-regulated unigenes (Fig. 3.13a). A GH1, a lytic polysaccharide monooxygenase, a short chain dehydrogenase, an apolipoprotein, a chitin binding peritrophin-A, a WD domain-G-beta repeat, and an AMP-binding protein family were represented by only one unigene, respectively (Fig. 3.13a). Also, two protein families that function at the nuclear level were up-regulated, and they included an unigene coding for a DNA-binding endonuclease, and other coding for a transcription activator factor (Fig 3.13a). Approximately, 80% of the 26 up-regulated unigenes had a Pfam-A domain.

Unigenes involved in the addition of glycosyl groups to hydrophobic substrates, hydrolysis of ester bonds and pectin, proteolysis, transport of small solutes across cell

membranes, storage of amino acids, chitin binding, oxidoreductase activity, and cell signaling were among the 24 down-regulated unigenes in soybean-fed larvae compared to those fed sunflower and giant ragweed (Fig. 3.13b). MFS was the only protein family represented by more than one unigene. Fifty percent of the 24 down-regulated unigenes contained a Pfam-A domain.

Summary of up-regulated unigenes in larvae fed soybean

In the comparison between soybean fed larvae and those fed either native host or artificial diet, only one up-regulated unigene coding for lipocalin was consistently identified in the differential gene expression and K-means analyses. Lipocalins are proteins involved in the transport of small hydrophobic molecules across membranes. Up-regulation of lipocalin by larvae fed soybean could be associated with the maximization of lipid transport and storage when feeding in a host with low lipid content[32]. Lipocalins may be important for the development of *D. texanus* third-instar larvae feeding on soybean.

Interestingly, other commonly up-regulated unigenes in larvae fed soybean compared to those fed sunflower or giant ragweed were not differentially expressed compared to those fed artificial diet. This result may be attributed to the induction of larval gene expression by locust bean gum contained in the artificial diet. Locust bean gum is obtained from seeds of the locust tree (Fabaceae: *Ceratonia siliqua* L.) and used as a thickening agent in the diet.

In the comparison between soybean fed larvae and those fed either native host, up-regulated unigenes coding for three CYP9 and two COesterases were consistently identified in

the differential gene expression and K-means analyses. The up-regulated unigenes coding for proteins belonging to the CYP6 family only appeared once in either transcriptomic analyses. The P450 unigenes also were consistently detected in the GO enrichment analyses Although it is unknow which substrates are binding to these P450s, they may be relevant for *D. texanus* to use soybean as a host. The up-regulated unigenes coding for COesterases had a significant BLASTp match to an esterase 1 and S, respectively. They may be involved in the hydrolysis of ester bonds in pectin and digestion of other plant compounds in *D. texanus* larvae.

Unigenes coding for a GH1 and a lytic polysaccharide monooxygenase were up-regulated in soybean-fed larvae compared to either native host in both methods of gene expression analyses. These genes most likely are involved in the digestion of plant cell walls by hydrolysis and oxidation of glycosidic bonds, and their expression may be up-regulated in soybean-fed larvae in response to high carbohydrate content in soybean stem pith[32]. Although the unigene coding for a GH1 has a significant BLASTp match to a myrosinase 1, it is most likely acting as β-glucosidase on cellulose and hemicellulose, since glucosinolates are not known to occur in soybeans. Three up-regulated unigenes coding for polygalacturonase (GH28) were also identified in soybean-fed larvae in the K-means and GO enrichment analyses. These genes are probably involved in the hydrolysis of glycosidic bonds of de-esterified pectins. An up-regulated unigene coding for a chitin binding peritrophin-A was identified in both methods of gene expression analyses. This unigene is most likely coding for a glycoside hydrolase 18 (chitinase) based on significant BLASTp matches to probable *Drosophila melanogaster* and *A. glabripennis* chitinases. This domain is found in peritrophic matrix proteins and chitinases. This unigene may

be involved in maintenance of the *D. texanus* larvae peritrophic matrix integrity or digestion of conspecifics.

An unigene coding for an apolipoprotein was identified in both methods of gene expression analyses, but different unigenes coding for MFS transporters were detected in each method. These unigenes along with the one coding for lipocalin may be involved in regulating levels and transport of small carbohydrates and lipids in *D. texanus* larvae fed soybean.

Up-regulated unigenes coding for a tryptophan-aspartic acid dipeptide (WD) domain, a short chain dehydrogenase, an aspartic acid-aspartic acid-glutamic acid motif (DDE) endonuclease, and a transcription activator multiprotein-bridging-factor 2 (MBF2) were identified consistently in soybean-fed larvae in both gene expression analyses. The relationship of these unigenes in the use of soybean as a host is unclear, but they may be involved in binding phosphorylated substrates, oxidoreductase activity in lipid metabolism, nuclease activity, or transcription activation, respectively.

Summary of down-regulated unigenes in larvae fed soybean

In the comparisons between soybean fed larvae and those fed either native host or artificial diet, down-regulated unigenes coding for two insect cuticle proteins, a GH45 and a transglutaminase were consistently identified in both the differential gene expression and K-means analyses. These unigenes are involved in keeping the structural integrity of cuticle (chitin

binding), hydrolysis of glycoside linkages in cellulose, and linking proteins, respectively. Differences in pith texture and composition between soybean and the native hosts may be associated to the down-regulation of these unigenes. Soybean stem pith is softer and frothier than that from sunflower and giant ragweed.

Down-regulated unigenes coding for an UGT, an MFS transporter, a collagen triple helix repeat, and a FAD binding domain were consistently identified in both methods of gene expression analyses. These unigenes are involved in adding glycosyl groups to hydrophobic substrates, transport of small solutes, formation of connective tissue structures, and oxidoreductase activity, respectively. Down-regulation of these genes may be attributed to differences in composition of defensive compounds and low lipid content in soybean.

Overall, up-regulation of unigenes involved in digestion, biotransformation of plant allelochemicals and transport of small hydrophobic molecules most likely are important for *D. texanus* in using soybean as host. Up-regulation of these unigenes may be associated to high carbohydrate and low lipid content in soybean piths. These unigenes could be used in the development of novel management tools for *D. texanus* and in understanding the process of adaptation of this species to a new host.

Genome size of *D. texanus*

The mean genome size of *D. texanus* females and males were 466.4 Mb (SE=1) and 463.2 Mb (SE=0.7), respectively, for an overall mean size of 464.7 Mb (SE= 0.6), which is smaller than the genome of *A. glabripennis* (710 Mb)[83]. *D. texanus* has the smallest known genome size of any other Cerambycid[95] measured by flow cytometry and reported in the Animal Genome Size Database[96] (March 25th, 2019).

Materials and methods

Plant material

Seeds of the *D. texanus*-susceptible soybean genotype K07_1544, common sunflower and giant ragweed were provided by the Kansas State University soybean breeding program and weed ecology lab, respectively. Sunflower and giant ragweed seeds were pre-germinated in soil filled-flats in a cold room (4°C) for 21 d and moved to the greenhouse for germination 7 d before planting in the field. Giant ragweed seeds did not germinate. Thus, seedlings were collected from giant ragweed populations around the experimental plots at the Kansas State University Ashland Bottoms Research Station, near Manhattan, KS and transplanted to cylindrical pots (10 cm wide x 8.5 cm deep) with soil 7 d before planting in the field. Giant ragweed seedlings were identified based on the spoon-shaped cotyledons and first true leaf in the greenhouse before planting in the field.

RNAseq experiment

The RNAseq experiment was arranged in nine 3 x 3 m plots at the KSU Ashland Bottoms Research Station where each plot consisted of four 2.3 m long rows with five planting spots per row spaced 30 cm apart, for a total of 20 planting spots (plants) per plot. All plants in a plot were of the same species to prevent any host bias during oviposition by *D. texanus*. Three plots (replicates) were planted for each plant species. Soybean seeds, and sunflower and giant ragweed seedlings were hand-planted about 2.5 cm and 10 cm deep, respectively, in late May 2017.

Plants were caged 21 d after planting in 3 x 3 x 1.8 m polyvinyl chloride (PVC) frames covered with mosquito mesh to prevent other insects from colonizing the plants and prevent beetles from escaping. Caged plants were infested with unsexed *D. texanus*-adults at a rate of four beetles per plant 7 d later. Adults were collected from soybean fields at the research station and were evenly distributed in each cage. A sex ratio of 1 female to 1 male was assumed since adults have sexual monomorphism. *D. texanus* larvae were collected 21 d post infestation by cutting three plants per cage at soil level and splitting the stems. Three third-instar larvae collected within the same plot were pooled together and represent one biological replicate. A total of three biological replicates per plant species were collected for analysis. Larval instar[17] was estimated based on larval head capsule width, and samples were stored at -80°C until RNA extraction

D. texanus third instar-larvae fed pink bollworm artificial diet (Frontier Scientific Services, Newark, DE USA) since egg hatch were also collected for RNA extraction. *D. texanus* field-collected adults were provided green beans as an oviposition substrate and kept inside mite-

proof cages (20.2 wide x 20.8 long x 20.2 tall cm, 35-micron mesh). These adults were collected from the same soybean fields as the adults used in the field experiment described above. Pods were dissected to harvest eggs that were then stored on petri dishes with moist filter paper until eclosion (4 d later). After egg hatch, larvae were fed diet and reared following Hatchett's rearing protocol[20]. *D. texanus* adults, eggs and larvae were kept in mite-proof cages inside a Thermo Scientific growth chamber (27°C, 14L:10D) at the Department of Entomology, Kansas State University, Manhattan, KS. Three biological replicates were collected for analysis, each replicate consisted of three pooled third-instar larvae collected within the same cage. Larval head capsules were measured to estimate larval instar[17] before storage inside a -80°C freezer until RNA extraction.

RNA extraction and mRNA sequencing

Total RNA was extracted from whole bodies using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol with the addition of a DNA elimination step. Three biological replicates were prepared for each of the four-diet treatments (three plant species and one artificial diet). RNA quality and quantity were measured with an RNA 6000 Nano Assay on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA USA) and a NanoDrop ND-ONE Spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA), respectively, before construction of cDNA libraries. RNA was of high quality and showed no degradation based on electropherograms and spectrophotometry. Separate cDNA libraries for each biological replicate per diet treatment were constructed using 2 ug of total RNA with a

TruSeq Stranded mRNA Library Preparation Kit v2 (Illumina, San Diego, CA USA) according to manufacturer's instructions that included indexed-adaptor ligation and oligo-dT beads to capture polyA tails. Libraries were amplified by PCR for 8 cycles with a KAPA-Library Quantification Kit for Illumina (KAPA Biosystems, Wilmington, MA USA) using a PE9700 thermal cycler (Perkin Elmer, Waltham, MA USA). cDNA library quality and quantity were measured with the DNA High Sensitivity Assay on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA USA) and qPCR on an Applied Biosystems Step One instrument (Thermo Fisher Scientific, Waltham, MA USA), respectively. The average cDNA lengths ranged from 482 to 515 bp for the 12 libraries, and the overall average was 497 bp. Indexed Illumina libraries were combined into a single library pool and sequenced as 2 x 100 PE reads in one lane of a 2-lane Rapid Flowcell on an Illumina HiSeq2500. Illumina library preparation and sequencing were conducted at Purdue University Genomics Core Facility, West Lafayette, IN USA.

De novo transcriptome assembly

Reads were trimmed to remove low quality bases (< 20) and residual Illumina adapters using the program Trimmomatic[97] v.0.38, and reads shorter than 30 nt after quality trimming were discarded. FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to assess the quality of the reads before and after quality filtering.

A *de novo* transcriptome assembly was performed using the trimmed and filtered reads from all twelve *D. texanus* samples with Trinity v.2.6.5[98] including default settings and *in silico*

normalization. Afterwards, reads from each sample were mapped back to the raw transcriptome assembly using the align_and_estimate_ abundance.pl[99] script with bowtie2[100] for read mapping and RSEM[101] for abundance estimation. Transcripts with < 0.1 transcripts per million mapped reads (TPM) or transcripts representing < 5 % of the expression value of the dominant isoform for each unigene were considered low quality and were removed from the transcriptome assembly. Putative open reading frames (ORFs) of at least 100 amino acids in length were identified using Transdecoder v.5.0.2 (https://github.com/TransDecoder/TransDecoder/releases). The identification of ORFs was facilitated using BLASTp (ncbi-blast v.2.6.0+) searches against the sprot database (February 5, 2018) and hmmer searches against the Pfam-A database. The single highest scoring ORF with a BLASTp match to the sprot database or a Pfam domain for each transcript was retained using the single_best_orf, retain_pfam_hits and retain_blastp_hits options. Finally, transcripts containing no open reading frames were removed from the assembly.

Predicted ORFs were searched against the non-redundant protein database (downloaded on October 4, 2018) using BLASTp to identify any potential plant or microbial transcripts in the assembly. In brief, the top five BLASTp matches with e-values ≤0.00001 were retained for each predicted coding region and taxonomic classifications were carried out using MEGAN's[102] least common ancestor algorithm. Transcripts derived from plants, viruses, or microbial taxa were considered contaminants and were removed from the assembly prior to annotation and differential expression analyses. Remaining transcripts were functionally annotated using Trinotate[103]. Predicted proteins were annotated using BLASTp and searches against the sprot database (March 1, 2018); Pfam-A domains were predicted using HMMER[104] and the Pfam-A

database (March 1, 2018); signal peptides were predicted using signalP[105,106], and transmembrane regions were predicted using tmHMM[107].

Additionally, *D. texanus* unigenes were assigned to KEGG orthology terms using KAAS (KEGG automatic annotation server)[108] with the bi-directional best hit method for partial genomes and the *T. castaneum* genome[109] as a reference. KEGG orthology assignments were compared to KEGG annotations from the *A. glabripennis* genome using the predicted proteome from the assembly version GCA_000390285.2. Each KO term was counted once in the *D. texanus* transcriptome and in the other insect genomes (Supplemetary Table S2b). Assessment of *D. texanus* unigenes-transcriptome completeness was performed using the program BUSCO (Benchmarking Universal Single-Copy Orthologs) v3 against the Insecta ODB9 gene set[110,111].

Differential expression analyses

After removing non-coding, low abundance and contaminant transcripts from the assembly, reads from the 12 libraries were re-aligned to the filtered transcriptome assembly, individually, using the same methods described previously. RSEM counts were concatenated into two count matrixes. The first matrix contained all samples from each plant diet treatment, and the second contained samples from soybean and artificial diet-fed larvae. Differential expression analyses were conducted at the unigene level using edgeR[112] and unigenes differentially expressed among the three plant treatments were identified using the first count matrix. Unigenes differentially expressed between the non-native host (soybean) and artificial

diet were identified using the second matrix. Partitioning the samples in two matrixes helped to identify unigenes differentially expressed when *D. texanus* was feeding on the plant treatments and in response to compounds in soybean. Read counts were normalized using trimmed means (TMM) and variances were estimated using tagwise dispersions. Only transcripts with counts per million (CPM) values greater than one in at least two samples were tested for differential expression. Pairwise comparisons were used to identify unigenes that were differentially expressed in at least one sample using Fisher's Exact test. Unigenes with FC ≥ 1.5 or ≤ -1.5 (relative logFC ≥ 0.6) and FDR corrected p-values ≤ 0.05 were considered differentially expressed. Gene ontology (GO) enrichments were performed using GoSeq[113], and the entire list of unigenes with CPM>1 in at least two samples were used as a reference to determine enrichment. Nodes containing less than five unigenes were excluded from the GOSeq analysis to control false discovery rate. K-means analysis[99] was performed to identify groups of *D. texanus* unigenes with similar expression patterns across the three plant diet treatments. Unigenes with similar expression patterns in larvae fed soybean compared to either native host were further filtered using the results from the comparison between soybean-fed and artificial diet-fed larvae to identify unigenes that were specifically differentially expressed when fed soybeans.

The computing for this project was performed on the Beocat Research Cluster at Kansas State University, Manhattan, KS USA.

Estimation of genome size of *D. texanus*

The estimated genome size for *D. texanus* was determined with flow cytometry from seven females and eight males collected from a laboratory colony reared on pink bollworm artificial diet at Kansas State University. Single *D. texanus* male or female individuals were prepared for genome size estimation[114]. In brief, individual heads were separately placed into a 2 ml Kontes Dounce tissue grinder vials containing 1 ml of Galbraith buffer[115]. An internal standard (1C = 328 Mbp) consisting of the head of a female *Drosophila virilism* was included in the analysis. Samples were then ground with 15 strokes of the "B" pestle, filtered through a 40 u nylon filter, stained with 25µg/ml propidium iodide (PI), and stored at -20°C for at least 30 min. After storage, each sample and standard were scored for the relative PI fluorescence of diploid nuclei using a Partec CyFlow flow cytometry equipped with a Cobalt Samba laser emitting at 532 nm. At least 2000 nuclei were scored for each peak, with the coefficient of variation for each sample less than 3.0. Genome size (1C) was estimated as the genome size standard*mean PI–fluor beetle/ mean PI–fluor standard. An average genome size was estimated using the seven *D. texanus* females and eight males, respectively, and an overall average was estimated using both sexes. The estimation of *D. texanus* genome size was performed at the Department of Entomology, Texas A&M AgriLife Research, College Station, TX USA.

Table 3.1. *Dectes texanus* reads summary and database accessions

Sample Name	Raw Total Read Pairs	Filtered Total Read Pairs	Percent Retained
Soybean-A	29,835,970	29,820,640	99.95%
Soybean-B	29,097,180	29,076,976	99.93%
Soybean-C	33,825,722	33,804,958	99.94%
Sunflower-A	26,460,398	26,446,466	99.95%
Sunflower-B	25,787,038	25,770,590	99.94%
Sunflower-C	30,650,236	30,635,780	99.95%
Giant ragweed-A	30,301,950	30,285,848	99.95%
Giant ragweed-B	30,046,694	30,027,174	99.94%
Giant ragweed-C	31,849,712	31,830,198	99.94%
Artificial diet – B	29,461,486	29,445,310	99.95%
Artificial diet – D	27,837,534	27,822,438	99.95%
Artificial diet – E	30,492,108	30,468,730	99.92%
Total Read Pairs	355,646,028	355,435,108	99.94%
Total Gb of data	97.3	97.1	99.79%

Table 3.2. *Dectes texanus de novo* transcriptome assembly descriptive summary

Assembled Reads	**Unfiltered**	**Filtered**
Number of transcripts	127,878	41,461
Number of unigenes	65,979	14,504
N50 unigene length (longest transcript per unigene) (bp)	1877	3,195
Sum longest transcript per unigene (Mb)	57.5	33.3
N50 transcript length (bp)	2,387	3,025
Sum transcript length (Mb)	152.1	97
Isoforms per unigenes	1.9	2.9
GC %	35.77	37.19
N50 protein length (amino acids)	-	585
Number of transcripts with complete predicted ORF (% of total transcripts)	-	30,601 (73.8)
Number of transcripts with 5’ partial ORF (% of total transcripts)	-	3,267 (7.9)
Number of transcripts with 3’ partial ORF (% of total transcripts)	-	5,199 (12.5)
Number of unigenes with complete predicted ORF (% of unigenes)	-	9,975 (68.8)
Number of unigenes with 5’partial ORF (% of unigenes)	-	1,588 (10.9)

Assembled Reads	Unfiltered	Filtered
Number of unigenes with 3’ partial ORF (% of unigenes)	-	1,421 (9.8)

ORF= Open reading frame

Table 3.3. *Dectes texanus de novo* transcriptome assembly annotations summary

Annotation summary filtered *de novo* assembly	BlastP (SwissProt)	Pfam-A domain	GO	KEGG orthology
Number of transcripts with match (% total transcripts)	31,866 (76.9)	30,896 (74.5)	20,184 (48.7)	10,297 (24.8)
Number unigenes with match (% total unigenes)	10,307 (71.1)	10,471 (72.2)	6,772 (46.7)	5,362 (37.0)

GO= Gene ontology

Table 3.4. Number of unique KEGG orthology (KO) terms identified in the *Dectes texanus* transcriptome and beetle reference genomes

KO pathway	*Dectes texanus* transcriptome	*Anoplophora glabripennis*	*Tribolium castaneum*	*Dendroctonus ponderosae*
Metabolism				
Carbohydrate metabolism				
Glycolysis / Gluconeogenesis	27	27	27	27
Citrate cycle (TCA cycle)	22	22	22	22
Pentose phosphate pathway	19	19	19	19
Pentose and glucuronate interconversions	12	12	12	13
Fructose and mannose metabolism	16	16	16	16
Galactose metabolism	12	13	13	12
Ascorbate and aldarate metabolism	6	6	6	6
Starch and sucrose metabolism	15	15	15	14
Amino sugar and nucleotide sugar metabolism	29	29	29	29
Pyruvate metabolism	21	21	21	20

KO pathway	*Dectes texanus* transcriptome	*Anoplophora glabripennis*	*Tribolium castaneum*	*Dendroctonus ponderosae*
Glyoxylate and dicarboxylate metabolism	21	21	21	22
Propanoate metabolism	20	20	20	20
Butanoate metabolism	11	12	12	11
Inositol phosphate metabolism	38	37	38	38
Energy metabolism				
Oxidative phosphorylation	70	97	99	89
Nitrogen metabolism	5	5	5	5
Sulfur metabolism	6	6	6	6
Lipid metabolism				
Fatty acid biosynthesis	6	6	6	6
Fatty acid elongation	14	14	14	13
Fatty acid degradation	21	21	21	21
Synthesis and degradation of ketone bodies	4	4	4	4

KO pathway	*Dectes texanus* transcriptome	*Anoplophora glabripennis*	*Tribolium castaneum*	*Dendroctonus ponderosae*
Cutin, suberine and wax biosynthesis	1	1	1	1
Steroid biosynthesis	3	3	3	3
Glycerolipid metabolism	21	21	22	21
Glycerophospholipid metabolism	41	41	41	41
Ether lipid metabolism	12	12	12	11
Sphingolipid metabolism	18	18	18	18
Arachidonic acid metabolism	9	9	9	9
Linoleic acid metabolism	3	3	3	3
alpha-Linolenic acid metabolism	4	4	4	4
Biosynthesis of unsaturated fatty acids	7	7	7	7
Nucleotide metabolism				
Purine metabolism	94	98	102	101
Pyrimidine metabolism	62	65	66	66

KO pathway	*Dectes texanus* transcriptome	*Anoplophora glabripennis*	*Tribolium castaneum*	*Dendroctonus ponderosae*
Amino acid metabolism				
Alanine, aspartate and glutamate metabolism	22	23	23	22
Glycine, serine and threonine metabolism	23	23	23	23
Cysteine and methionine metabolism	26	27	27	26
Valine, leucine and isoleucine degradation	32	32	32	32
Lysine degradation	30	30	30	31
Arginine and proline metabolism	22	22	22	21
Histidine metabolism	5	6	7	5
Tyrosine metabolism	15	16	16	15
Phenylalanine metabolism	8	8	8	8
Tryptophan metabolism	17	17	17	16
Metabolism of other amino acids				
beta-Alanine metabolism	14	15	15	15

KO pathway	*Dectes texanus* transcriptome	*Anoplophora glabripennis*	*Tribolium castaneum*	*Dendroctonus ponderosae*
Taurine and hypotaurine metabolism	4	5	5	5
Phosphonate and phosphinate metabolism	3	3	3	3
Selenocompound metabolism	6	6	6	6
D-Glutamine and D-glutamate metabolism	2	2	2	2
D-Arginine and D-ornithine metabolism	1	1	1	1
Glutathione metabolism	21	21	22	23
Glycan biosynthesis and metabolism				
N-Glycan biosynthesis	31	32	32	33
Mucin type O-glycan biosynthesis	2	2	2	2
Mannose type O-glycan biosynthesis	4	4	4	4
Other types of O-glycan biosynthesis	9	9	9	10
Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate	8	9	9	8

KO pathway	*Dectes texanus* transcriptome	*Anoplophora glabripennis*	*Tribolium castaneum*	*Dendroctonus ponderosae*
Glycosaminoglycan biosynthesis - heparan sulfate / heparin	13	13	13	13
Glycosaminoglycan biosynthesis - keratan sulfate	1	1	1	2
Glycosaminoglycan degradation	10	10	10	9
Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	21	21	22	21
Glycosphingolipid biosynthesis - lacto and neolacto series	2	3	3	5
Glycosphingolipid biosynthesis - globo and isoglobo series	5	5	5	5
Glycosphingolipid biosynthesis - ganglio series	2	2	2	2
Other glycan degradation	11	11	11	10
Metabolism of cofactors and vitamins				
Thiamine metabolism	5	5	6	6
Riboflavin metabolism	5	5	5	5
Vitamin B6 metabolism	4	4	4	4
Nicotinate and nicotinamide metabolism	9	9	9	9

KO pathway	*Dectes texanus* transcriptome	*Anoplophora glabripennis*	*Tribolium castaneum*	*Dendroctonus ponderosae*
Pantothenate and CoA biosynthesis	8	9	9	10
Biotin metabolism	2	2	2	3
Lipoic acid metabolism	3	3	3	3
Folate biosynthesis	19	19	19	19
One carbon pool by folate	9	10	10	12
Retinol metabolism	5	6	6	5
Porphyrin and chlorophyll metabolism	18	18	18	18
Ubiquinone and other terpenoid-quinone biosynthesis	8	8	8	9
Metabolism of terpenoids and polyketids				
Terpenoid backbone biosynthesis	19	19	19	19
Insect hormone biosynthesis	13	13	13	13
Biosynthesis of ansamycins	1	1	1	1
Biosynthesis of other secondary metabolites				

KO pathway	*Dectes texanus* transcriptome	*Anoplophora glabripennis*	*Tribolium castaneum*	*Dendroctonus ponderosae*
Caffeine metabolism	1	1	1	1
Penicillin and cephalosporin biosynthesis	1	1	1	1
Monobactam biosynthesis	1	1	1	1
Neomycin, kanamycin and gentamicin biosynthesis	1	1	1	1
Prodigiosin biosynthesis	1	1	1	1
Aflatoxin biosynthesis	1	1	1	1
Xenobiotics biodegradation and metabolism				
Metabolism of xenobiotics by cytochrome P450	6	6	6	6
Drug metabolism - cytochrome P450	5	5	5	4
Drug metabolism - other enzymes	20	20	20	20
Genetic Information Processing				
Transcription				
RNA polymerase	24	26	26	27

KO pathway	*Dectes texanus* transcriptome	*Anoplophora glabripennis*	*Tribolium castaneum*	*Dendroctonus ponderosae*
Basal transcription factors	30	31	31	32
Spliceosome	94	102	102	100
Translation				
Ribosome	98	117	121	116
Aminoacyl-tRNA biosynthesis	26	26	45	26
RNA transport	113	111	114	113
mRNA surveillance pathway	55	54	55	55
Ribosome biogenesis in eukaryote	59	62	64	63
Folding, sorting and degradation				
Protein export	16	21	21	21
Protein processing in endoplasmic reticulum	102	105	106	103
SNARE interactions in vesicular transport	18	18	18	19
Ubiquitin mediated proteolysis	85	84	87	86

KO pathway	*Dectes texanus* transcriptome	*Anoplophora glabripennis*	*Tribolium castaneum*	*Dendroctonus ponderosae*
Sulfur relay system	6	6	7	6
Proteasome	36	37	37	35
RNA degradation	48	54	55	54
Replication and repair				
DNA replication	31	32	32	32
Base excision repair	20	21	22	17
Nucleotide excision repair	33	35	35	35
Mismatch repair	18	18	18	18
Homologous recombination	27	28	28	27
Non-homologous end-joining	9	9	9	8
Fanconi anemia pathway	31	31	32	32
Environmental Information Processing				
Membrane transport				

KO pathway	*Dectes texanus* transcriptome	*Anoplophora glabripennis*	*Tribolium castaneum*	*Dendroctonus ponderosae*
ABC transporters	13	13	13	13
Signal transduction				
MAPK signaling pathway - fly	78	78	80	79
Wnt signaling pathway	57	58	59	54
Notch signaling pathway	20	21	21	21
Hedgehog signaling pathway - fly	23	23	23	22
TGF-beta signaling pathway	31	32	32	33
Hippo signaling pathway - fly	46	46	46	46
Hippo signaling pathway - multiple species	15	15	15	15
FoxO signaling pathway	52	52	53	51
Phosphatidylinositol signaling system	40	39	40	41
mTOR signaling pathway	77	78	78	76
Signaling molecules and interaction				

KO pathway	*Dectes texanus* transcriptome	*Anoplophora glabripennis*	*Tribolium castaneum*	*Dendroctonus ponderosae*
Neuroactive ligand-receptor interaction	29	31	32	32
ECM-receptor interaction	14	14	14	14
Cellular Processes				
Transport and catabolism				
Endocytosis	111	111	111	108
Phagosome	38	41	42	40
Lysosome	57	58	61	55
Peroxisome	49	48	49	43
Autophagy – animal	75	76	77	73
Autophagy – other	22	22	22	20
Mitophagy – animal	31	31	33	34
Cell growth and death				
Apoptosis – fly	44	45	45	44

KO pathway	*Dectes texanus* transcriptome	*Anoplophora glabripennis*	*Tribolium castaneum*	*Dendroctonus ponderosae*
Apoptosis - multiple species	15	15	15	15
Organismal Systems				
Immune systems				
Toll and Imd signaling pathway	34	34	36	35
Sensory systems				
Phototransduction – fly	16	17	17	16
Development				
Dorso-ventral axis formation	21	21	21	21
Aging				
Longevity regulating pathway - multiple species	33	33	34	31
Environmental adaptation				
Circadian rhythm – fly	8	8	8	7

TCA= Tricarboxylic acid cycle; CoA= Coenzyme A; SNARE= Soluble N-ethylmaleimide-sensitive-factor attachment receptor; ABC= ATP-binding cassette; MAPK= Mitogen-activated protein kinase; Wnt= Wingless-Integrated; TGF= Transforming growth factor; FoxO= Forkhead box protein O; mTOR= Mammalian target of rapamycin; ECM= Extracellular matrix

Table 3.5. Enriched Gene Ontology (GO) categories from unigenes commonly up-regulated in *Dectes texanus* larvae fed soybean compared to those fed either native host

Category	Ontology	Description	# DEGs	FDR
GO:0005506	MF	Iron ion binding	5	0.01
GO:0020037	MF	Heme binding	5	0.01
GO:0046906	MF	Tetrapyrrole binding	5	0.01
GO:0016705	MF	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	5	0.01

DEG= Differentially expressed unigenes; FDR= False discovery rate; MF= Molecular function

Table 3.6. Enriched gene ontology categories in *Dectes texanus* larvae fed soybean compared to those fed sunflower, giant ragweed or artificial diet.

Fed soybean	Compared to fed	Category	Ontology	Description	# DEGs	FDR
Up regulated	Sunflower	GO:0055085	BP	Transmembrane transport	14	0.03
		GO:0055114	BP	Oxidation-reduction process	18	< 0.01
		GO:0046914	MF	Transition metal ion binding	18	< 0.01
		GO:0016491	MF	Oxidoreductase activity	17	< 0.01
		GO:0022857	MF	Transmembrane transporter activity	16	0.01
		GO:0005215	MF	Transporter activity	16	0.01
		GO:0005506	MF	Iron ion binding	16	< 0.01
		GO:0020037	MF	Heme binding	15	< 0.01
		GO:0046906	MF	Tetrapyrrole binding	15	< 0.01
		GO:0016705	MF	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	15	< 0.01
		GO:0044699	N/A	Uncharacterized	38	0.01

Fed soybean	Compared to fed	Category	Ontology	Description	# DEGs	FDR
Up regulated	Giant ragweed	GO:0006030	BP	Chitin metabolic process	7	< 0.01
		GO:0006040	BP	Amino sugar metabolic process	7	< 0.01
		GO:1901071	BP	Glucosamine-containing compound metabolic process	7	< 0.01
		GO:0006022	BP	Aminoglycan metabolic process	7	< 0.01
		GO:1901135	BP	Carbohydrate derivative metabolic process	7	< 0.01
		GO:1901564	BP	Organonitrogen compound metabolic process	7	0.02
		GO:0020037	MF	Heme binding	5	0.04
		GO:0046906	MF	Tetrapyrrole binding	5	0.04
		GO:0016705	MF	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	5	0.04
		GO:0005506	MF	Iron ion binding	5	< 0.05
		GO:0008061	MF	Chitin binding	7	< 0.01

Fed soybean	Compared to fed	Category	Ontology	Description	# DEGs	FDR
		GO:0005576	CC	Extracellular region	8	< 0.01
Up regulated	Artificial diet	N/A	N/A	N/A	N/A	N/A
Down regulated	Sunflower	GO:0003810	MF	Protein-glutamine gamma-glutamyltransferase activity	3	0.01
		GO:0016755	MF	Transferase activity, transferring amino-acyl groups	3	0.04
		GO:0018149	BP	Peptide cross-linking	3	0.01
Down regulated	Giant ragweed	GO:0042302	MF	Structural constituent of cuticle	17	< 0.01
		GO:0005198	MF	Structural molecule activity	18	< 0.01
Down regulated	Artificial diet	GO:0003968	MF	RNA-directed 5'-3' RNA polymerase activity	4	< 0.01
		GO:0042302	MF	Structural constituent of cuticle	10	< 0.01
		GO:0003724	MF	RNA helicase activity	2	0.03

BP= Biological process; MF= Molecular function; CC= Celular component; DEG= Differentially expressed unigenes; FDR= False discovery rate

Table 3.7. Enriched Gene Ontology (GO) categories from the cluster of up-regulated unigenes in *Dectes texanus* larvae fed soybean compared to those fed sunflower or giant ragweed by K-means analysis.

Category	Ontology	Description	# DEGs	FDR
GO:0004650	MF	Polygalacturonase activity	3	0.02
GO:0005506	MF	Iron ion binding	4	0.04
GO:0020037	MF	Heme binding	4	0.04
GO:0046906	MF	Tetrapyrrole binding	4	0.04
GO:0016705	MF	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	4	0.04

DEG= Differentially expressed unigenes; FDR= False discovery rate; MF= Molecular function

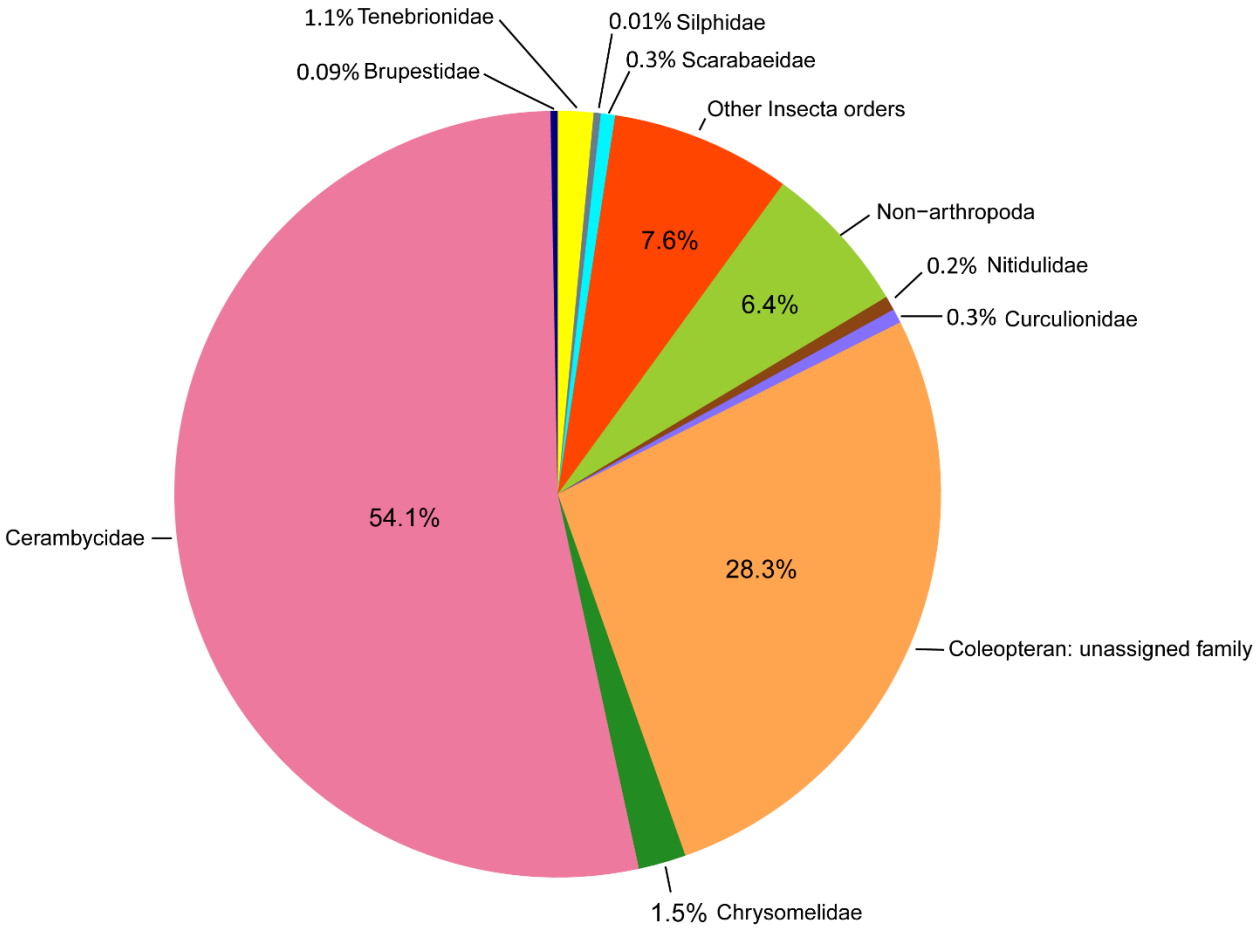

Figure 3.1. 93.6% of *Dectes texanus* protein coding transcripts match orders in the class Insecta.

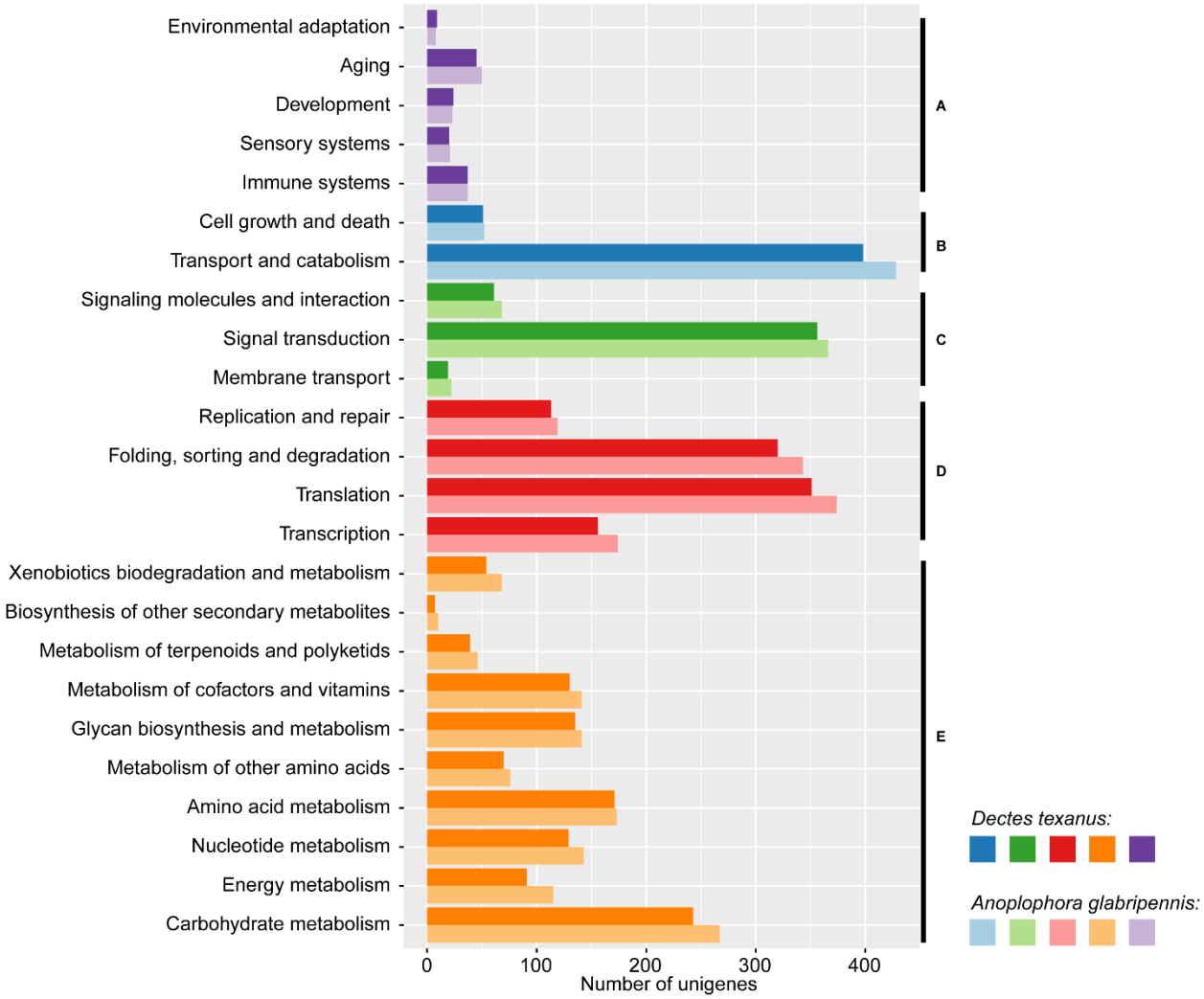

Figure 3.2. KEGG pathway classification of *Dectes texanus* transcriptome and *Anoplophora glabripennis* genome. (A) Organismal systems, (B) Cellular processes, (C) Environmental information processing, (D) Genetic information processing, (E) Metabolism.

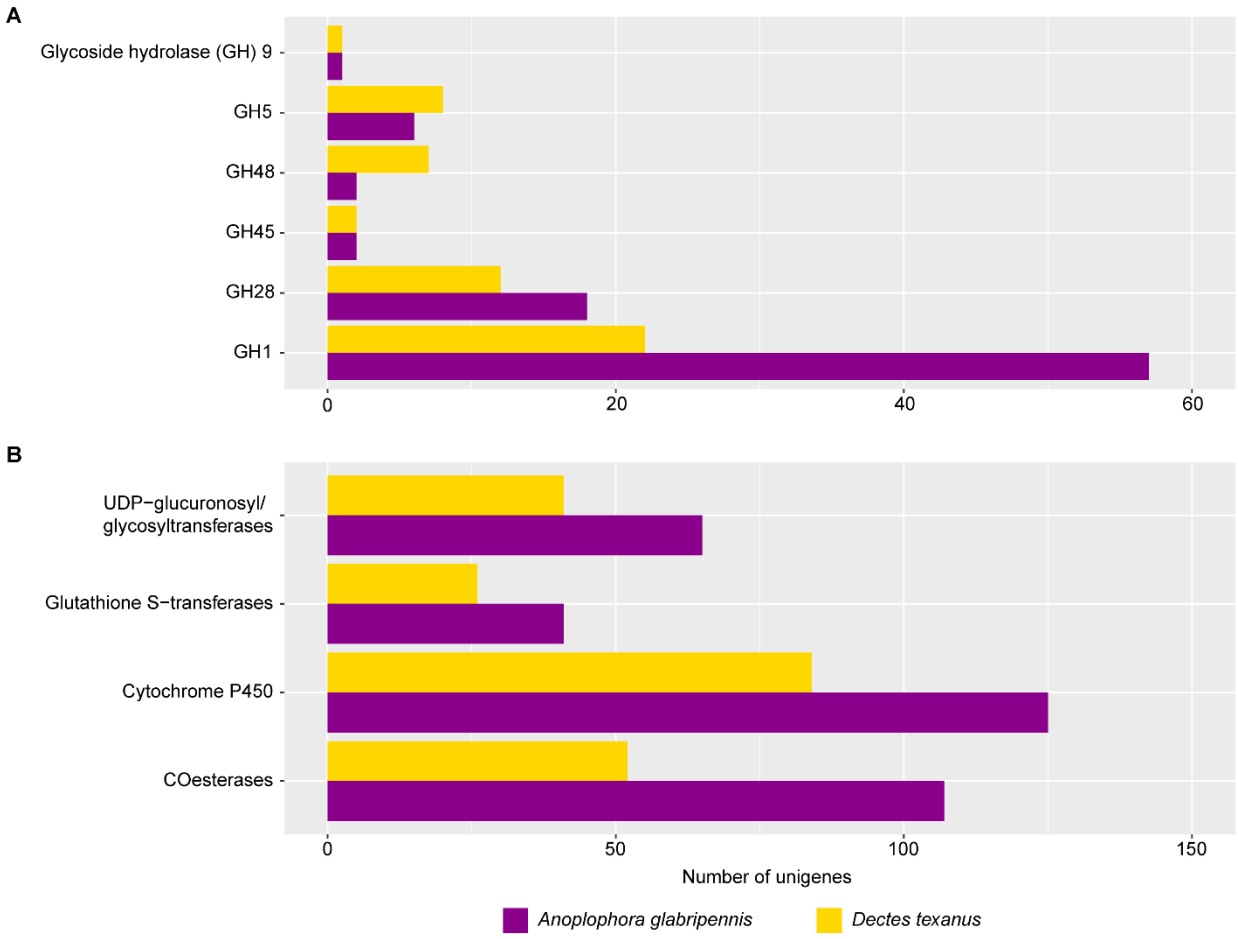

Figure 3.3. Number of *Dectes texanus* protein families and *Anoplophora glabripennis* genes coding for (A) glycoside hydrolases (GH) and (B) canonical detoxification enzymes.

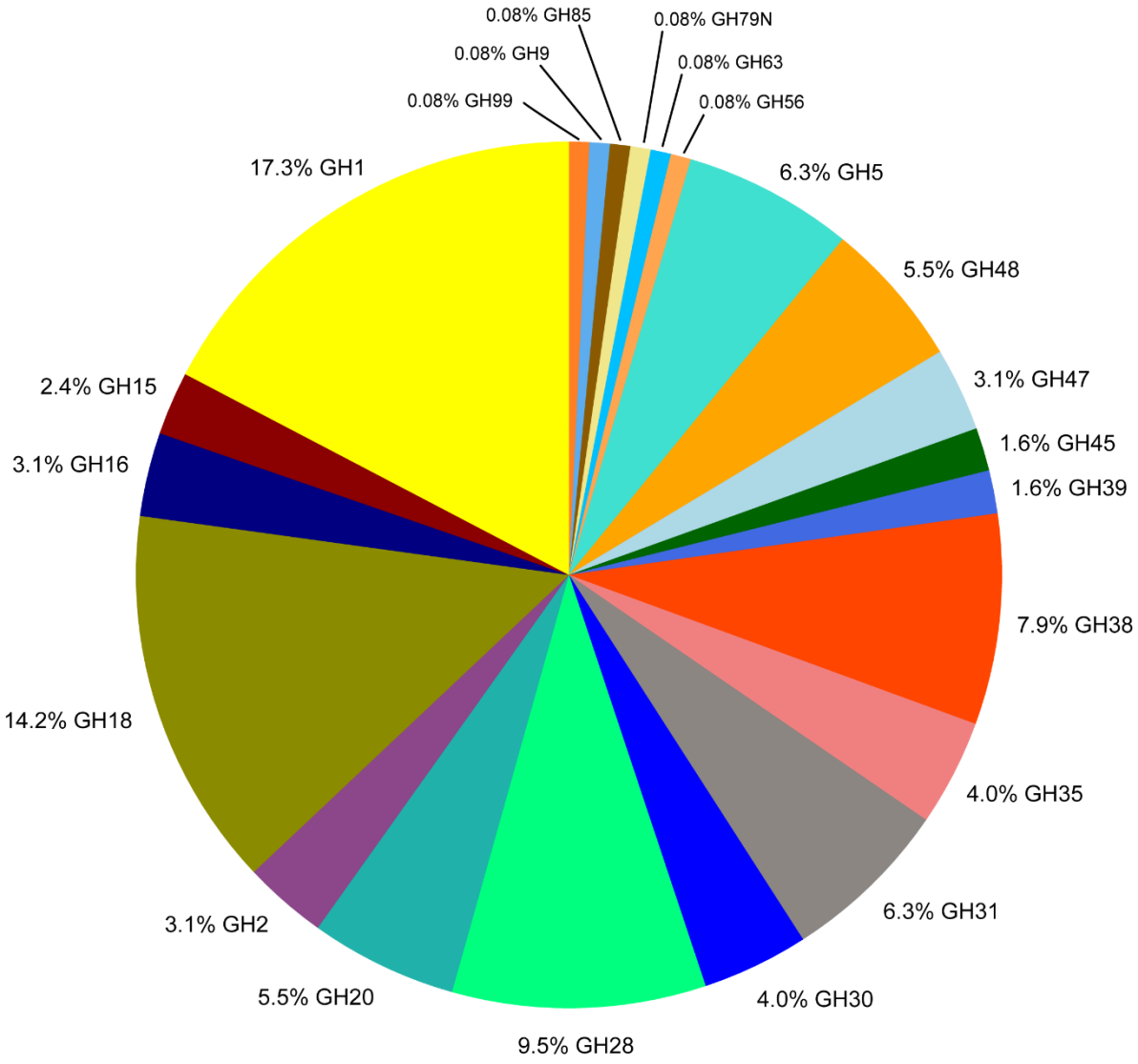

Figure 3.4. Percentage of unigenes coding for glycoside hydrolase (GH) families in the *Dectes texanus* transcriptome.

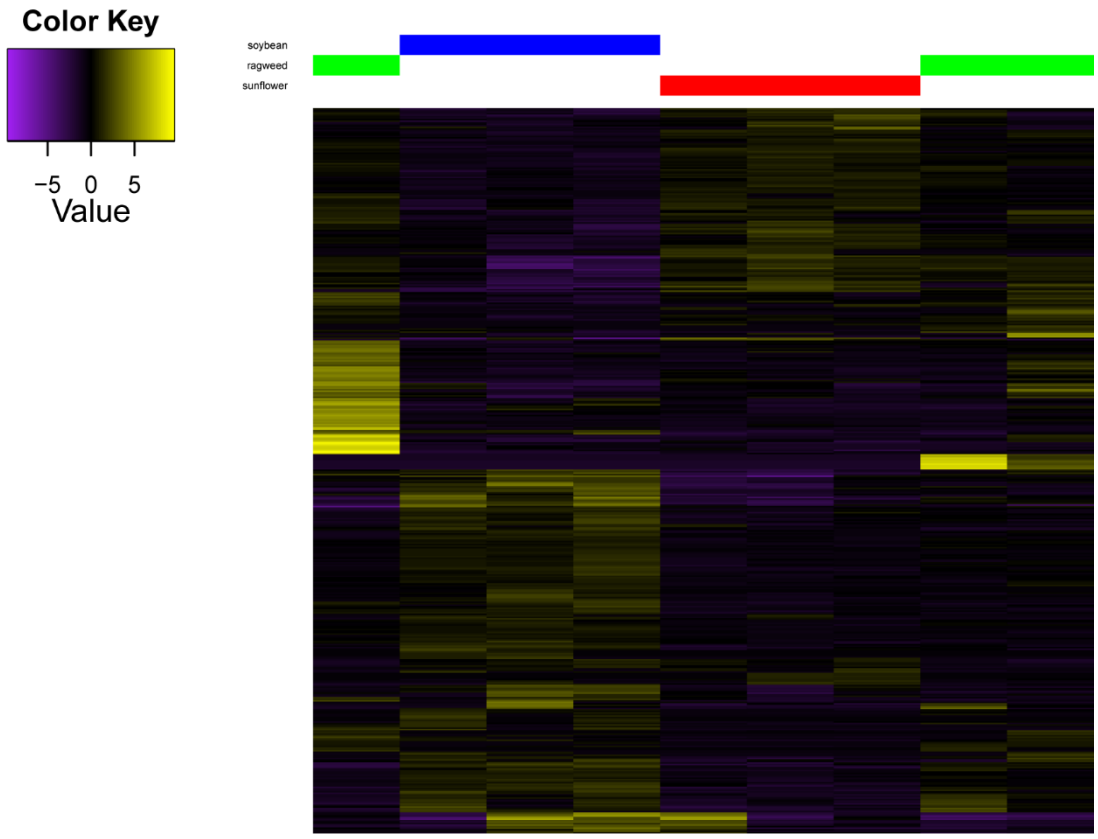

Figure 3.5. Expression profiles of *Dectes texanus* differentially expressed unigenes (fold change > ± 1.5, False Discovery Rate < 0.05) in larvae fed soybean (blue), sunflower (red), or giant ragweed (green). Each row represents a separate unigenes. Yellow and purple indicate high and low expression levels, respectively.

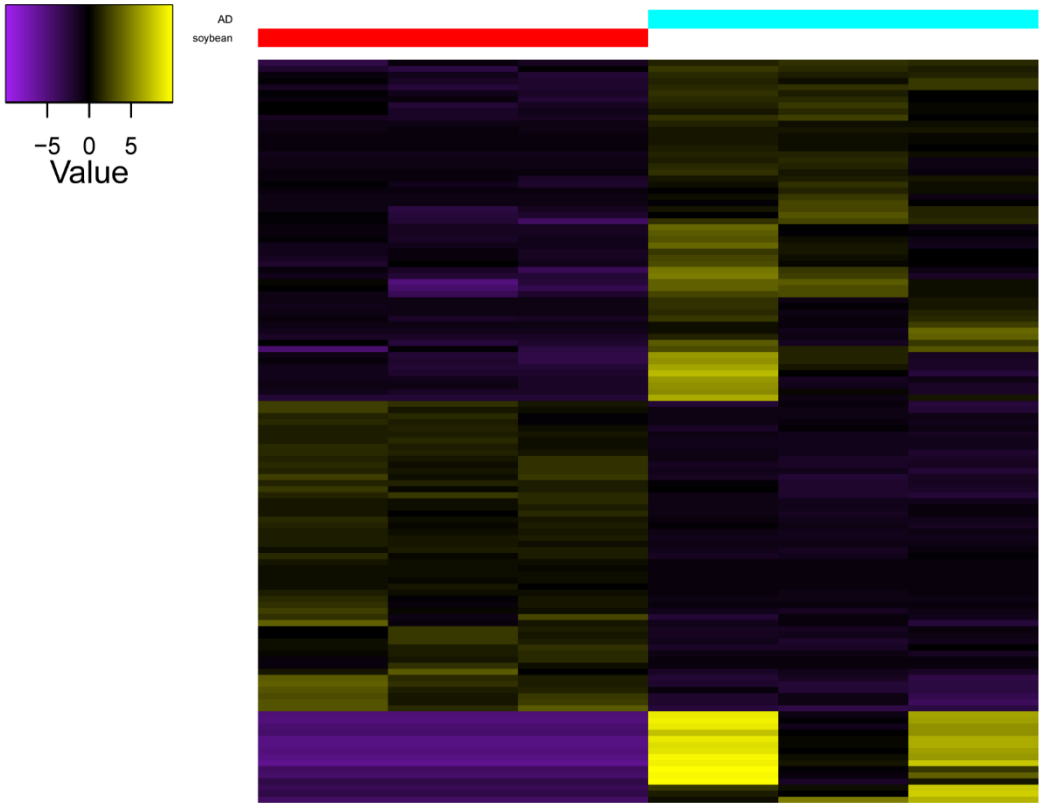

Figure 3.6. Expression profiles of *Dectes texanus* differentially expressed unigenes (fold change > ± 1.5, False Discovery Rate < 0.05) in larvae fed soybean (red) or artificial diet (AD, blue). Each row represents a separate unigene. Yellow and purple indicate high and low expression levels, respectively.

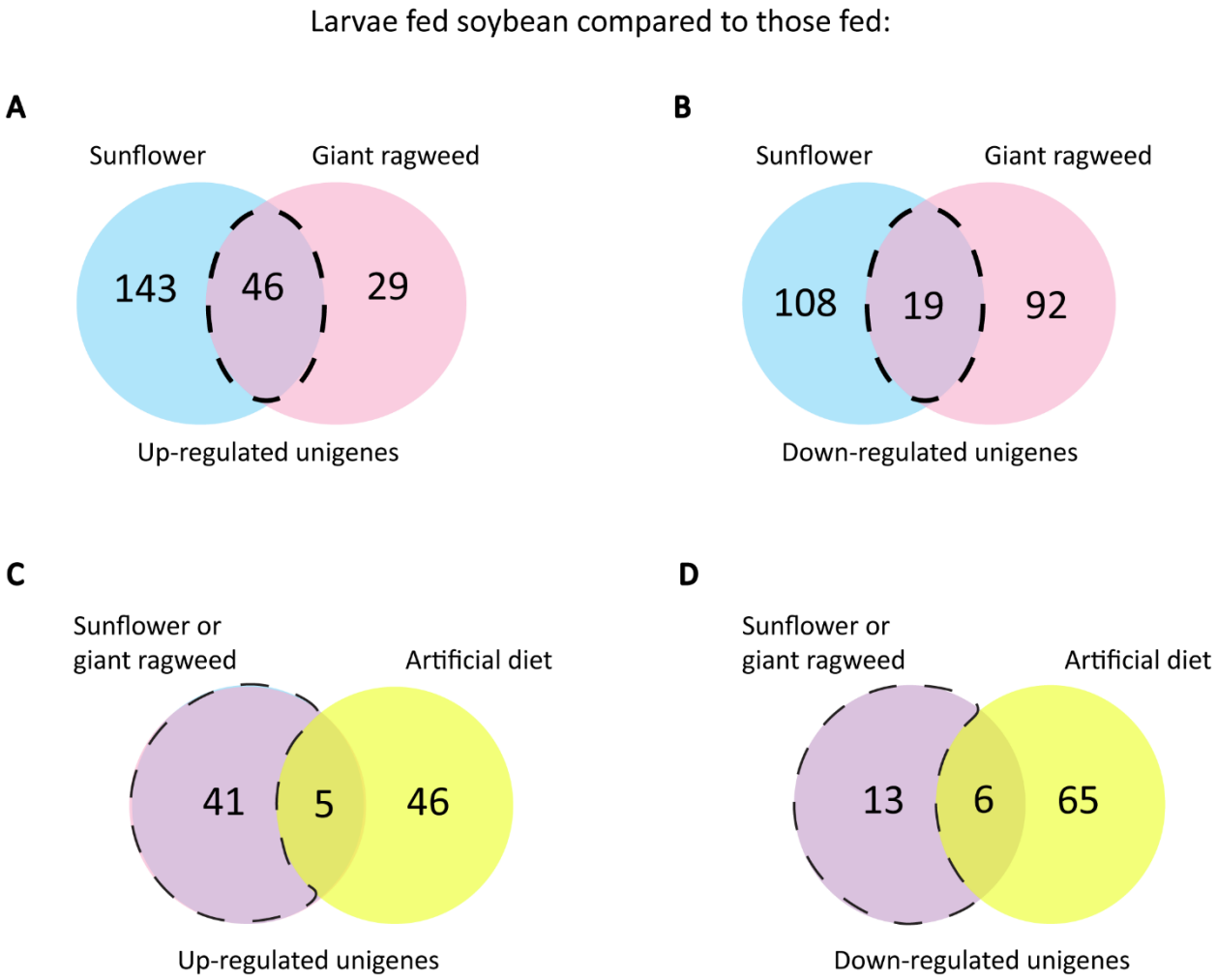

Figure 3.7. Number of differentially expressed unigenes in *Dectes texanus*-larvae fed soybean compared to those fed other diet treatments (Fold change > ± 1.5, False Discovery Rate < 0.05). (A) up-regulated and (B) down-regulated unigenes compared to those fed either native host; (C) up-regulated and (D) down-regulated unigenes compared to those fed either native host or artificial diet.

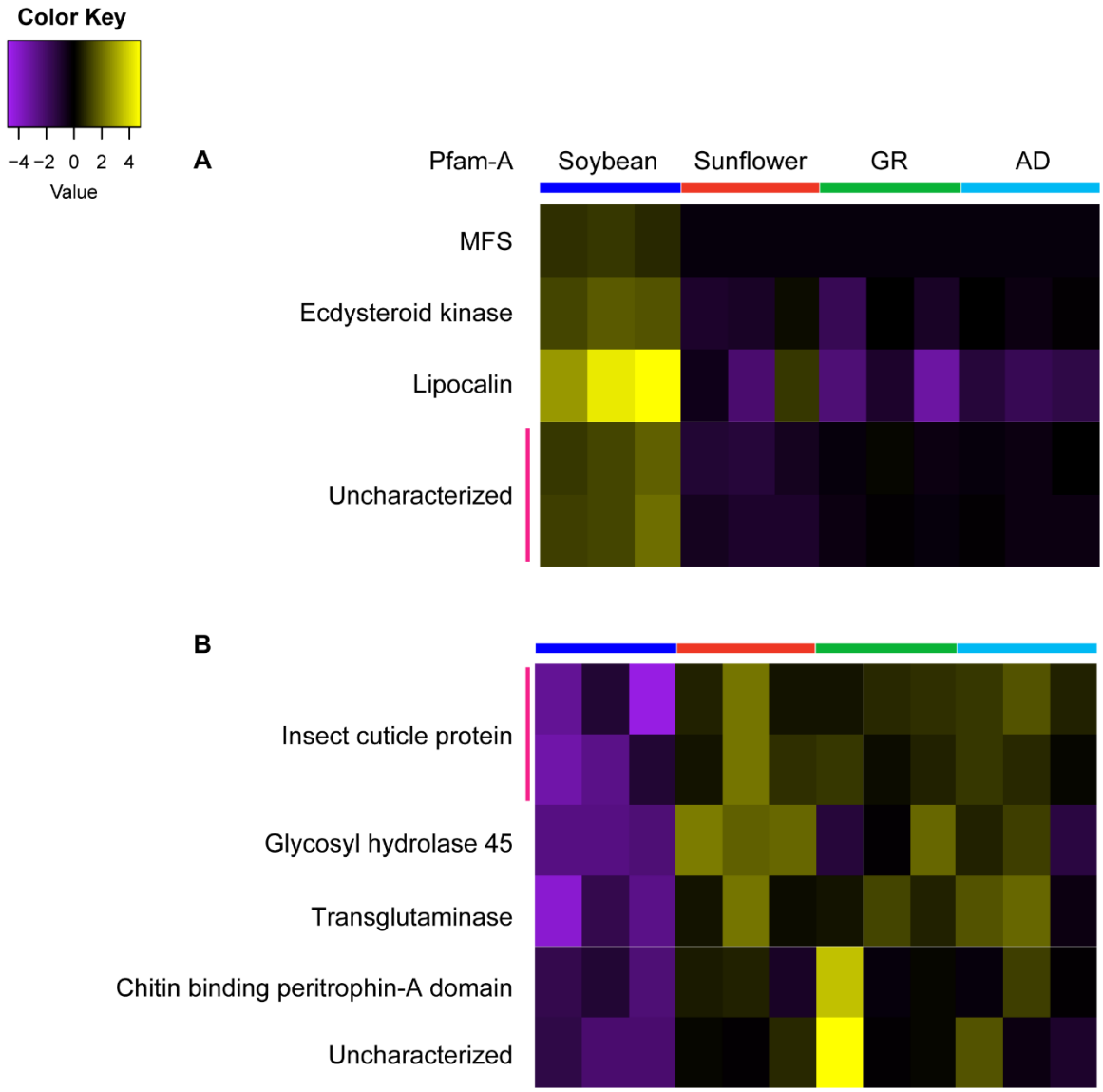

Figure 3.8. Expression patterns of commonly (A) up-regulated and (B) down-regulated unigenes in *Dectes texanus*-larvae fed soybean compared to those fed sunflower, giant ragweed (GR) or artificial diet (AD). Each row represents a separate unigene. Yellow and purple indicate high and low expression levels, respectively (Fold change > ± 1.5, False Discovery Rate < 0.05). Pfam-A= Protein family-A domain; MFS= Major facilitator superfamily.

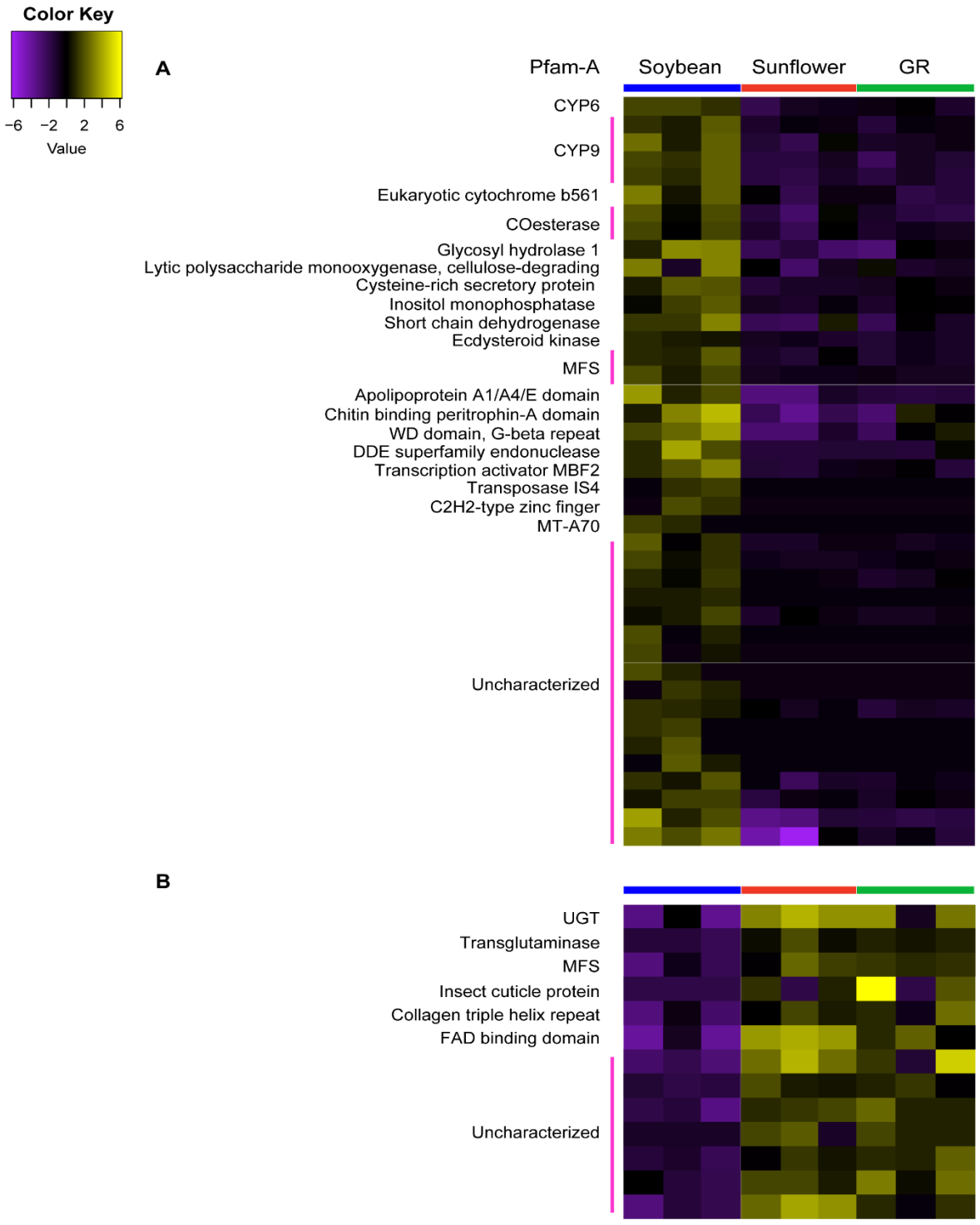

Figure 3.9. Expression patterns of (A) up-regulated and (B) down-regulated unigenes in *Dectes texanus*-larvae fed soybean compared to those fed sunflower or giant ragweed (GR).

Each row represents a separate unigene. Yellow and purple indicate high and low expression levels, respectively (Fold change > ± 1.5, False Discovery Rate < 0.05). Pfam-A= Protein family-A domain; CYP= Cytochrome P450; COesterase= Carboxylesterase; MFS= Major facilitator superfamily; WD= Tryptophan-aspartic acid dipeptide; DDE= Aspartic acid-Aspartic acid-Glutamic acid motif; MBF2= Multiprotein-bridging factor 2; IS4= Insertion sequence 4 family; C2H2= Cysteine- Cysteine -Histidine- Histidine motif; MT= Methyl-transferase; UGT=; UDP-glucuronosyl-transferase; FAD= Flavin adenine dinucleotide.

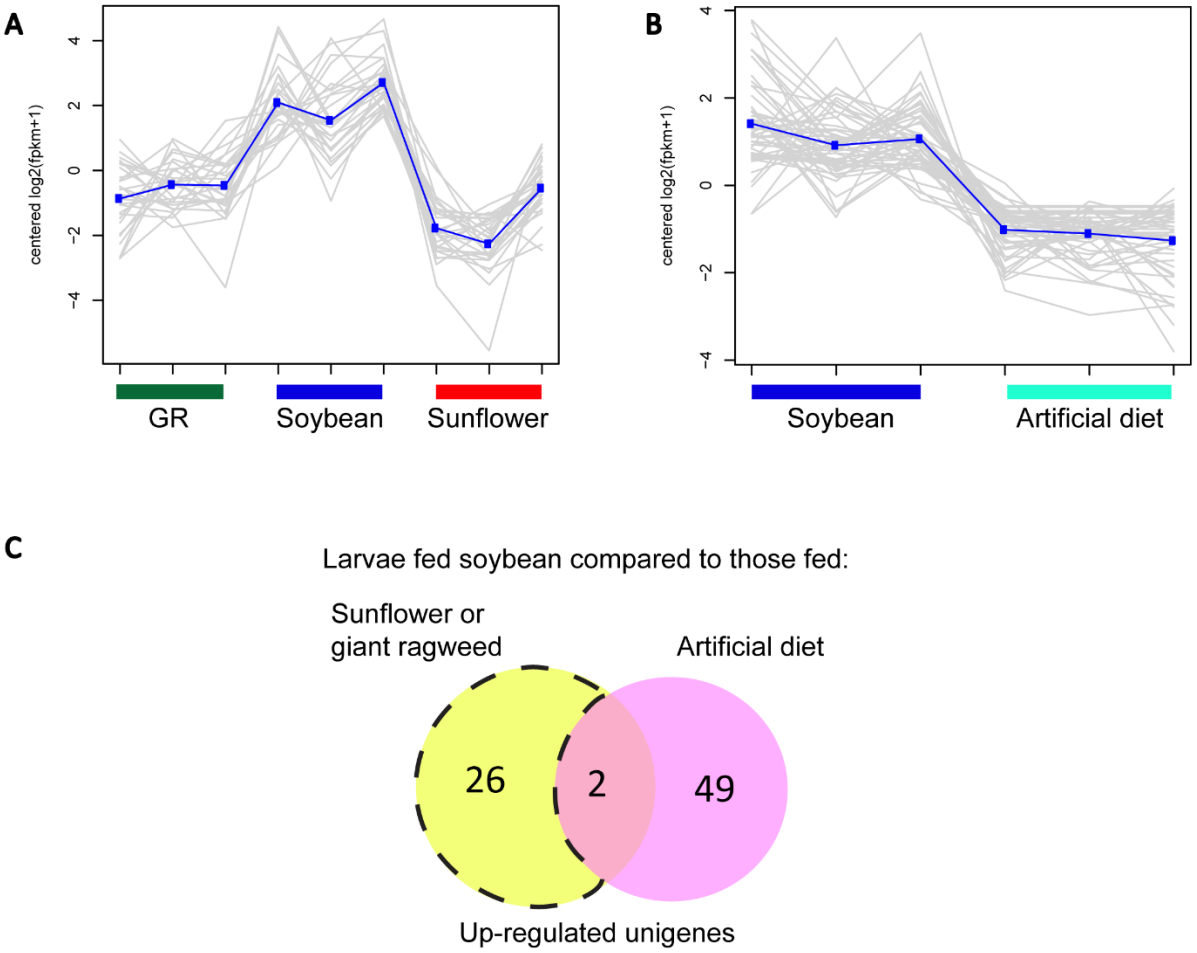

Figure 3.10. Clusters of up-regulated unigenes in *Dectes texanus*-larvae fed soybean compared to those fed (A) sunflower or giant ragweed; (B) artificial diet. (C) Number of up-regulated unigenes in cluster A and B where only two unigenes are commonly up-regulated in larvae fed soybean. Clusters were constructed with differentially expressed genes showing similar expression patterns across all treatments (Fold change > ± 1.5, False Discovery Rate < 0.05). GR= Giant ragweed.

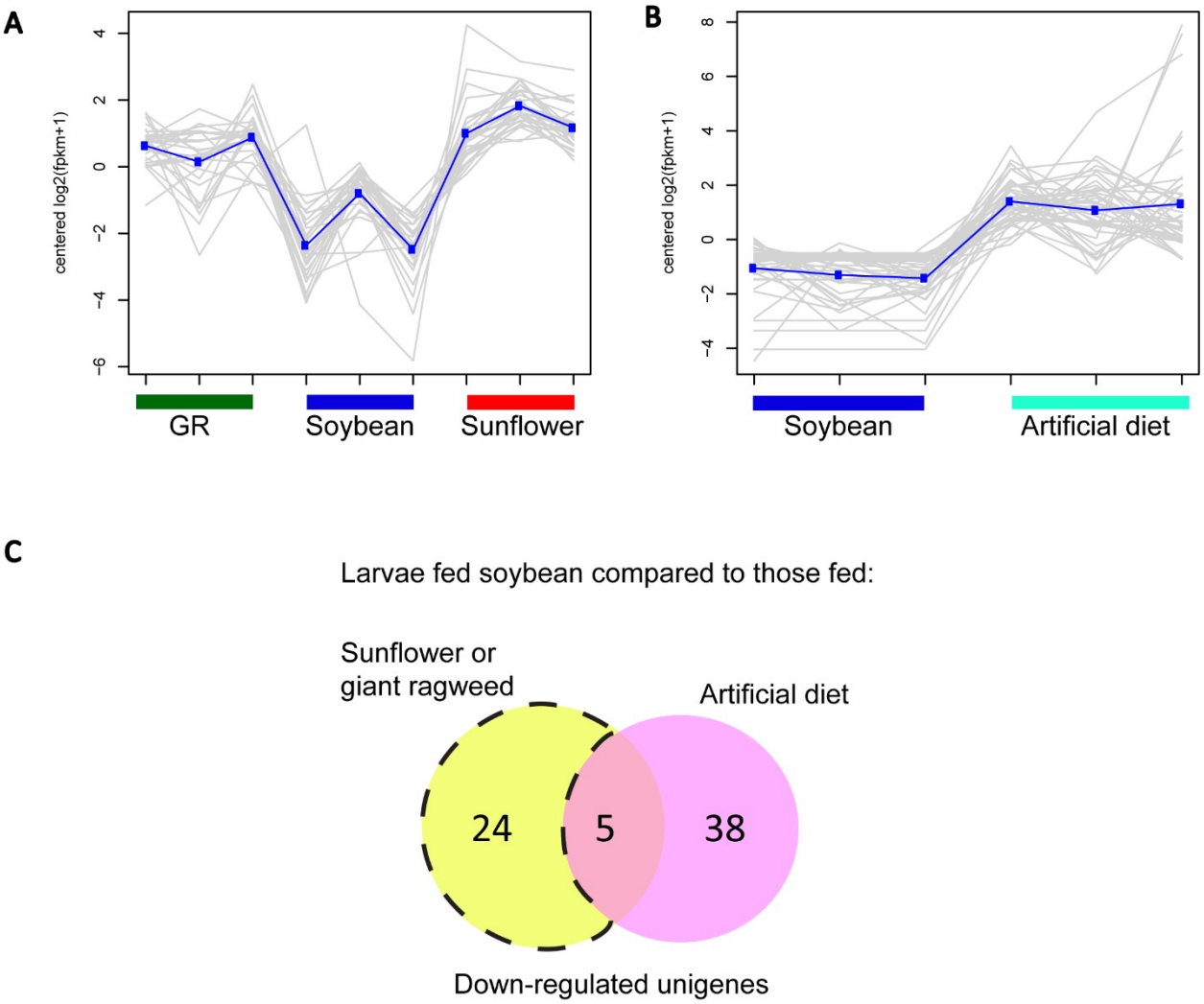

Figure 3.11. Clusters of down-regulated unigenes in *Dectes texanus*-larvae fed soybean compared to those fed (A) sunflower or giant ragweed; (B) artificial diet. (C) Number of down-regulated unigenes in clusters A and B where only five unigenes are commonly down-regulated in larvae fed soybean. Clusters were constructed with differentially expressed genes showing similar expression patterns across all treatments (Fold change > ± 1.5, False Discovery Rate < 0.05) GR= Giant ragweed.

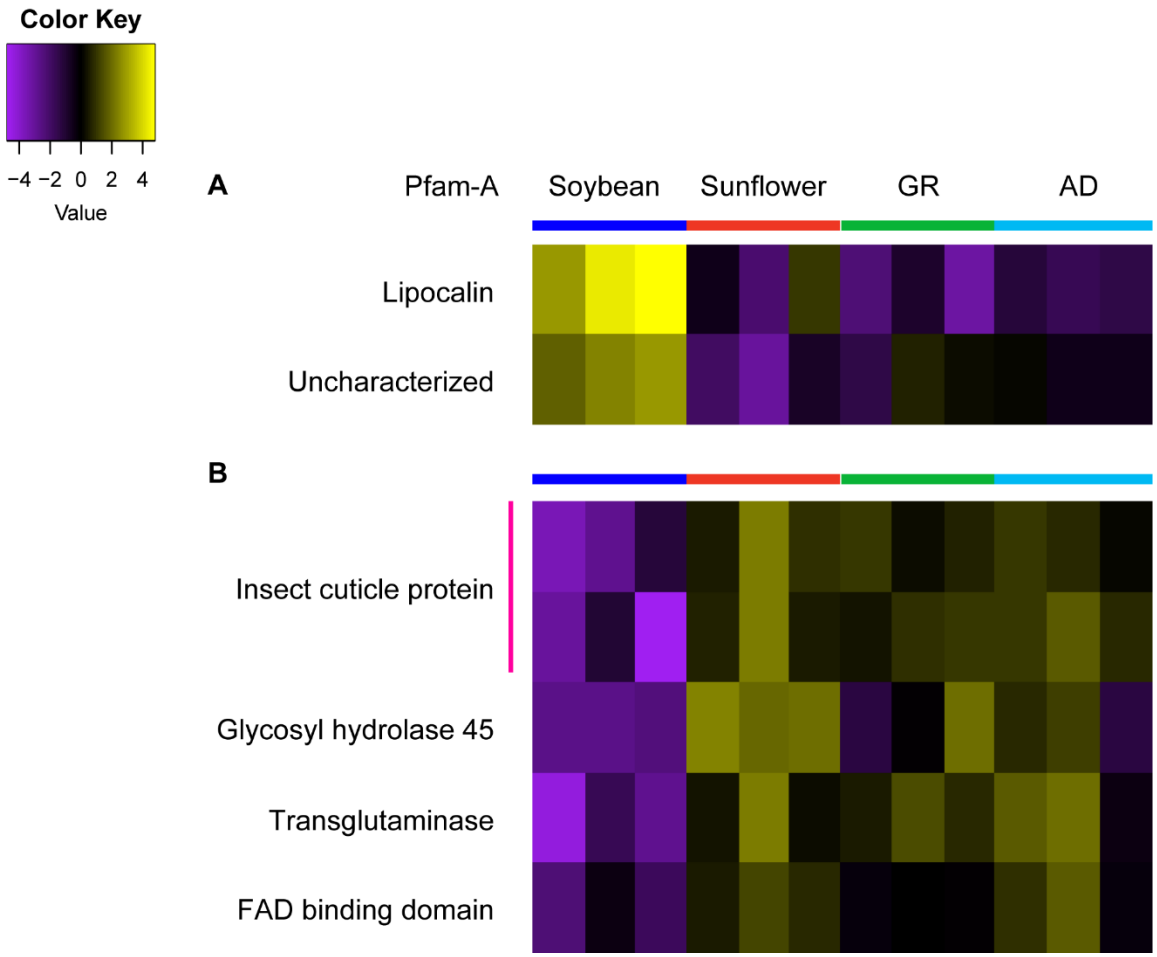

Figure 3.12. Expression patterns of unigenes commonly (A) up-regulated and (B) down-regulated in *Dectes texanus*-larvae fed soybean compared to those fed sunflower, giant ragweed (GR) or artificial diet (AD) by K-means analyses. Each row represents a separate unigene. Yellow and purple indicate high and low expression levels, respectively (Fold change > ± 1.5, False Discovery Rate < 0.05). Pfam-A= Protein family-A domain; FAD= Flavin adenine dinucleotide.

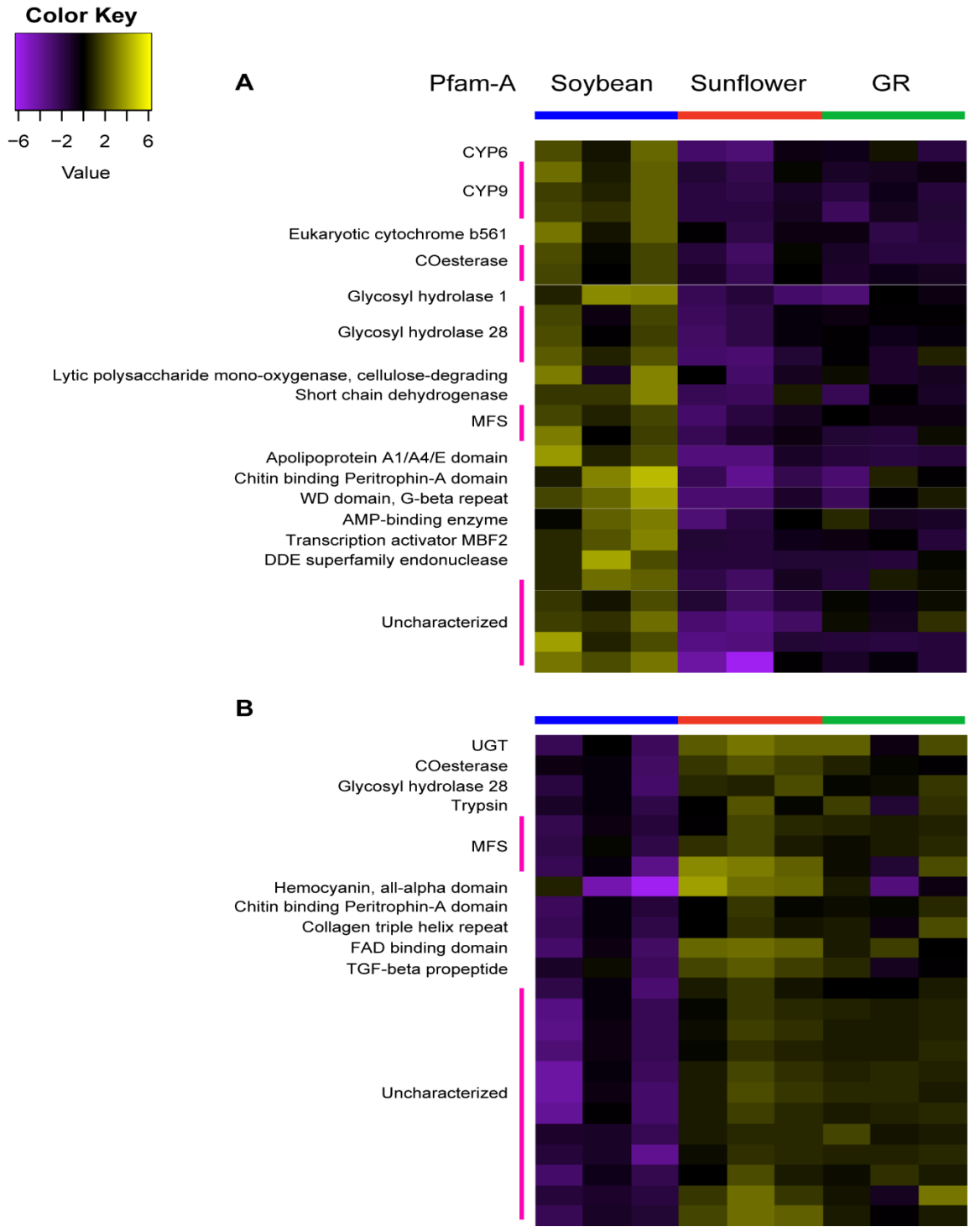

Figure 3.13. Expression patterns of unigenes (A) up-regulated and (B) down-regulated in *Dectes texanus*-larvae fed soybean compared to those fed sunflower or giant ragweed by K-

means analyses. Each row represents a separate unigene. Yellow and purple indicate high and low expression levels, respectively (Fold change > ± 1.5, False Discovery Rate < 0.05). Pfam-A= Protein family-A domain; CYP= Cytochrome P450; COesterase= Carboxylesterase; MFS= Major facilitator superfamily; WD= Tryptophan-aspartic acid dipeptide; AMP= Adenosine monophosphate; MBF2= Multiprotein-bridging factor 2; DDE= Aspartic acid-Aspartic acid-Glutamic acid motif; UGT= UDP-glucuronosyl-transferase; FAD= Flavin adenine dinucleotide; TGF= Transforming growth factor.

Chapter 4 - Silencing of *Laccase2* and *Chitin synthase2* in *Dectes texanus* (Coleoptera: Cerambycidae) by RNA interference

Introduction

Post-transcriptional gene silencing by RNA interference (RNAi) is a valuable tool for pest management due to target specificity and novel mode of action[116–118]. The RNAi pathway destroys targeted messenger RNA (mRNA) leading to a specific-suppression of gene expression[53,119]. Suppression is activated by double-stranded RNA (dsRNA) and can be summarized as follows: 1) Entry of dsRNA into the cytoplasm by endocytosis or *Sid*-like mediated transport, 2) production of small interfering RNAs (siRNAs) after cleavage of dsRNA by Dicer (RNase III-like proteins), 3) separation of siRNA base paring and retention of the antisense strand by the RNA-induced silencing complex (RISC), 4) coupling between the RISC-antisense strand complex and complimentary mRNA, and 5) degradation of mRNA (cleavage) by Argonaute proteins[53,54,120].

Host-induced gene silencing by RNAi has been used in transgenic maize and potato to successfully manage the coleopteran pests, *Diabrotica virgifera virgifera* and *Leptinotarsa decemlineata* (Chrysomelidae)[54–56], under controlled conditions. The general idea behind transgenic maize and potato based on RNAi was that larvae fed plants expressing dsRNA would absorb it through midgut cells, and that their own RNAi machinery would coordinate degradation of the target mRNA, resulting in less plant damage and possible negative effects on

the insect development[55,121]. Ingestion of transgenic maize plant roots expressing *Snf7*-dsRNA causes stunting, cessation of feeding, and death of *D. virgifera virgifera* larva, resulting in reduced maize root damage[54].

The *Dectes* stem borer, *Dectes texanus* (Coleoptera: Cerambycidae), is an important economic pest of soybeans and cultivated sunflowers in the corn-soybean belt in the U.S.A[13,23]. Damage caused by the larvae results in stem breakage and in soybean yield losses between 15 to 33% before harvest[13,17]. No insecticides or borer-resistant soybean varieties are registered to manage this pest, leaving producers with only the option of harvesting early to reduce yield losses[13]. The soybean plant introduction (PI) 165673 reduces *D. texanus* larval populations[49,50]. However, this resistance is polygenic[50] and fails to prevent larval stem damage at the end of the season (see Chapter 2). Soybean resistance to *D. texanus* can be complemented by *in planta* delivery of dsRNA to silence genes important for normal larval development[51,52]. Information about the success of gene silencing in *D. texanus* is lacking, therefore, demonstrating the ability of cells to uptake dsRNA and verification of the functionality of the RNAi pathway in *D. texanus* larvae are vital steps in determining if dsRNA can be delivered *in planta* to manage this pest.

Gene silencing by RNAi in *D. texanus* is expected to be successful based on the following evidence: 1) Injection of dsRNA has been shown to silence *Laccase2* (*Lac2*) and *inhibitor of apoptosis* (*iap*) in the cerambycids *Monochamus alternatus*[57] and *Anoplophora glabripennis*[58], respectively, 2) dsRNA transport and core RNAi pathway genes were identified in the *M. alternatus* transcriptome[65] and the *A. glabripennis* genome[58], 3) *Lac2* and *iap* are conserved in other coleopteran species[60,62], and 4) *Vacuolar-sorting protein Snf7*-dsRNA is lethal

to *D. virgifera virgifera* when is ingested from artificial diet[122,123] and transgenic maize[54]. Therefore, the objective of this research was to silence *Lac2* and *Chitin synthase 2* (*CHS2*) in *D. texanus* by feeding fifth or sixth instar larvae artificial diet coated with *Lac2* or *CHS2*-dsRNA.

Lac2 is a gene required for cuticle pigmentation and sclerotization in insects[124]. Soft bodies, exoskeleton deformations, failure of cuticle pigmentation, and mortality of pupae and adults are clear phenotypic evidences of silencing *Lac2* by RNAi in *M. alternatus*, *Cylas puncticollis* (Brentidae), and *Tribolium castaneum* (Tenebrionidae)[57,62,124]. *CHS2* is a gene required for chitin synthesis for the peritrophic matrix (PM) and is specifically expressed in epithelial cells of the midgut[125]. Feeding cessation, reduction of midgut-chitin content, amorphous PM, and midgut-shrinkage are morphological effects associated with silencing *CHS2* by RNAi in *T. castaneum*[126], *L. decemlineata*[127], and *Anthonomus grandis*[128] (Curculionidae).

Results

Double-stranded *Laccase2* (dsLac2) and double-stranded *Green fluorescent protein* (dsGFP) preliminary injection experiment

There were no significant differences in the relative *Lac2* transcript levels (%) between dsLac2 and dsGFP treated larvae at 24 h or 8 d after injection ($F_{1,4} = 4.01$, $P = 0.1159$, Table 4.1). All (100%) of dsLac2 treated larvae and 8% of dsGFP treated larvae had abnormal morphology, which included lack of cuticle pigmentation and exoskeleton deformation in the

adult stage (Fig. 4.1), shrunken larvae and failure to pupate. The proportion of abnormal morphology was significantly different between treatments based on a Pearson's χ^2 test (χ^2 = 18.3, df = 1, $P < 0.0001$).

Double-stranded *Laccase2* (dsLac2), double-stranded *Chitin synthase 2* (dsCHS2) and double-stranded *Green fluorescent protein* (dsGFP) feeding experiment

There was a significant difference in the relative *Lac2* transcript levels (%) between dsRNA treatments in the feeding experiment ($F_{3, 6} = 13.39$, $P = 0.0046$). dsLac2 treated larvae had *Lac2* transcript levels that were 6- and 15 times lower than those of dsGFP and dsCHS2 treated larvae at 8 d post dsRNA feeding (Table 4.2), respectively. However, there were no significant differences between dsLac2- and water treated larvae. There were also no significant differences in the relative % *CHS2* transcript levels, chitin content, or % abnormal cuticle/exoskeleton morphology between treatments after dsRNA feeding ($F_{3,6} = 0.74$, $P = 0.5663$; $F_{3,6} = 0.75$, $P = 0.5587$; $F_{3,6} = 3.74$, $P = 0.0796$, respectively).

Discussion

The main factor contributing to a failure of detection of statistically significant differences between treatments in injection and feeding experiments was the small numbers of samples used. Small samples sizes were related to the low availability of *D. texanus* larvae reared since egg hatch on artificial diet under controlled conditions. RNAi experiments with *M.*

alternatus and *A. glabripennis* were conducted using larvae collected from field-collected *Pinus massoniana* logs or nursery-collected *Acer rubrum* logs, respectively, and later fed on artificial diet until injection of dsRNA[57–59]. The use of field-collected *D. texanus* larvae is an option that can be used to provide larger more larvae in future RNAi experiments.

Larval ingestion of dsLac2 significantly reduced relative *Lac2* transcript levels in treated larvae compared to dsGFP and dsCHS2 controls. Interestingly, the relative *Lac2* transcript level in dsLac2 treated larvae was 10% at 24 h post-injection and 8.3% at 8 d post-ingestion of dsRNA compared to those treated with dsGFP. This difference in reduction of transcript level may be associated to the amount of dsRNA ingested and the time it took to spread the gene silencing signal across the body in dsRNA-fed larvae compared to injected larvae. Also, the type of abnormal cuticle pigmentation and exoskeleton deformations were more consistent across larvae injected with dsLac2 than in larvae fed dsLac2 (Fig. 1 and 2). Most likely, differences in dsRNA ingestion across larvae contributed to the variability in the phenotypic response.

Whole body-chitin content measurements performed in the feeding experiment likely overestimated the effects of dsCHS2 in treated larvae because *CHS2* is mostly expressed in the midgut epithelial cells. In future experiments, *D. texanus* midguts should be dissected to evaluate *CHS2* gene expression and chitin content in dsCHS2 treated- and control larvae. Also, feeding dsRNA on earlier instars and for a longer period of time can be considered since differences in relative *CHS2* transcript level were not detected.

The silencing of *Lac2* and *CHS2* in *D. texanus* by ingestion of dsRNA provides evidence required to proceed with the use of RNAi for management of this pest. Enhancing soybean resistance with host-induced gene silencing can increase the life time of this pest management strategy; increase soybean yields; and reduce development of virulent *D. texanus* biotypes, insecticide applications and non-target insect toxicity. However, testing the suppression of gene expression in *D. texanus* by delivering dsRNA *in planta* is required before an RNAi-based management strategy can be put in farmers' hands.

Materials and methods

Insects

D. texanus larvae used for experiments were fed artificial diet (Pink bollworm diet, Frontier Scientific, Newark, DE USA) since egg hatch and maintained in cardboard box-covered mite-proof cages (20.2 wide x 20.8 long x 20.2 tall cm) inside a growth chamber (27°C, 24 h D) at the Department of Entomology, Kansas State University, Manhattan, KS. Larvae were reared following the Hatchett et al. (1975) protocol. In brief, *D. texanus* adults were collected from soybean fields and giant ragweed patches at the Kansas State University Ashland Research Farm, near Manhattan, KS. Adults were kept in 35-micron mesh mite-proof cages (20.2 wide x 20.8 long x 20.2 tall cm) with green beans on moist filter paper inside a growth chamber (27°C, 14L:10D). Eggs were harvested from green beans used by *D. texanus* females to oviposit, and they were stored on petri dishes (85 diam x 15 tall mm) with moist filter paper until eclosion.

Newly hatched larvae were moved to a 0.75 oz clear plastic cup with a lid; one larva per cup. Small artificial diet pieces (0.5 cm^2) were supplied to each larva to feed on. Diet pieces were replaced daily for 14 d, and every other day thereafter, until larvae reached fifth or sixth instar. Larval head capsules were measured across their widest point before each experiment to verify larval instar[17]. Measurements were made using a Leica® MZ APO stereomicroscope at 32X. Larval instar was determined based on the head capsule width range described for each *D. texanus* instar[20].

Sequencing of *D. texanus* cDNA encoding *Lac2* and *β-Actin*

Total RNA was isolated using TRIzol (Invitrogen, Life Technologies Corporation, Carlsbad, CA USA) extraction method[129] from the whole body of a sixth instar larva. The larva was homogenized in 1000 µl TRIzol using an electric hand mortar and pestle. Chloroform (200 µl) was added to the homogenate and mixed by inversion. The homogenate mixture was incubated for 3 min at room temperature, and centrifuged at 12,000 g for 15 min at 4°C. The clear upper phase was collected, mixed with 500 µl of ice-cold isopropanol, and incubated at room temperature for 10 min. The mixture was then centrifuged for 10 min at 12,000 g, 4°C. The pellet was washed with 1000µl of 70% ethanol and centrifuged at 7500 g for 5 min at 4°C. The ethanol wash was repeated two more times, and the pellet was air dried until ethanol could no longer be smelled. The pellet was resuspended in 50 µl of nuclease free water (Ambion, Life Technologies Corporation, Carlsbad, CA USA). The RNA concentration was assessed using a Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, DE USA). RT-PCR was performed

using a High Capacity cDNA Reverse Transcription Kit with random primers (Applied Biosystems, Foster City, CA USA) according to the manufacturer's instructions using 1 µg total RNA.

Degenerated primers were designed based on conserved regions detected in *Lac2* and *β-actin* mRNA sequences of *M. alternatus* and *T. castaneum*. Sequence information of these genes were downloaded from NCBI (http://www.ncbi.nlm.nih.gov) and were aligned using MEGA6[130] to find similar regions for primer design. PCR reactions were performed using *D. texanus* cDNA, degenerate primers (Table 4.3), and GoTaq Green Master Mix (Promega, Madison, WI USA) with a thermocycler (PTC100 Thermal Cycler, MJ Research INC., Watertown, MA USA) setting of: initial denaturation at 95°C for 10 min followed by 40 cycles of 25 s of denaturation at 95°C, 25 s of annealing at 45°C, and 25 s of extension at 68°C; and a final 10 min extension at 68°C. Amplicons were gel-purified using 1% Agarose gel and a Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI USA). Amplicons were inserted into a pCR®2.1 plasmid vector (TA Cloning Kit, Invitrogen, Life Technologies Corporation, Carlsbad, CA USA), and recombinant vectors were used to transform One Shot® Chemically Competent INVF' *Escherichia coli* Cells (Invitrogen, Life Technologies Corporation, Carlsbad, CA USA). Manufacturer's instructions were followed to make recombinant plasmids, transform competent cells, and culture colonies carrying the recombinant plasmids. Recombinant plasmids were isolated from positive colonies using PureLink™ Quick Plasmid Miniprep Kit (Invitrogen, Life Technologies Corporation, Carlsbad, CA USA) and sequenced using M13 primers and Sanger sequencing at GENEWIZ (South Plainfield, NJ USA). Amplicon sequences were retrieved from the recombinant plasmid sequences and were verified against non-redundant (nr) database using

blastn at the NCBI webpage. Amplicon sequences highly matched *M. alternatus Lac2* and *β-actin*, respectively (Table 4.3). Degenerate primers designed using sequences of *M. alternatus, T. castaneum, L. decemlineata,* and *C. puncticollis* failed to amplify fragments matching *Lac2* and *β-actin* using blastn and nr database on NCBI.

D. texanus CHS2 and *Elf1a* sequences

D. texanus CHS2 and *elongation factor 1-α* (*elf1a*) mRNA sequences were retrieved from the *de novo* transcriptome assembly described in Chapter 3, as follows: *T. castaneum CHS2* and *A. glabripennis elf1a* protein sequences were downloaded from NCBI. These sequences were aligned against the *D. texanus* transcriptome using tblastn (ncbi-blast v.2.6.0+) with a cut off e-value of 0.00001. Significant matches were verified by aligning the corresponding mRNA sequences against the nr database using blastn on NCBI website. *D. texanus* sequences had significant matches to *L. decemlineata CHSII* and *elf1a*, and A. *glabripennis* probable *CHS* and *elf1a* (Table 4.4). Tblastn was run on the Beocat Research Cluster at Kansas State University, Manhattan, KS USA.

DsRNA synthesis

Total RNA was extracted from the whole body of a fifth-instar larva using RNeasy Plus Mini Kit (Qiagen) which included a DNA elimination step. First-strand cDNA synthesis was performed using 1 µg total RNA and the iScript^{TM} Reverse Transcription Supermix (Biorad,

Hercules, CA USA). RNA extraction and cDNA synthesis were performed according to the manufacturer's protocols. *D. texanus Lac2* and *CHS2* cDNA sequences, and the pGFP vector sequence (Clontech, Takara Bio, Mountain View, CA USA) were used to design primers with the T7 polymerase promoter sequence at the 5′ end by using the E-RNAi webservice[131] (Table 4.5). *D. texanus* cDNA and pGFP plasmids, GoTaq Green Master Mix, and the respective T7-tailed primers were used to amplify the DNA template for dsRNA synthesis for each mRNA target. Thermocycler (T-100, Biorad) settings are specified on Table 4.6. Amplicons were sequenced using Sanger sequencing at GENEWIZ and verified by blastn searches against the nr database on genebank and the *D. texanus* transcriptome (Table 4.5). *Lac2* and *CHS2* amplicons matched *M. alternatus* and *D. texanus Lac2* gene, and probable *CHS* from *A. glabripennis* and *D. texanus CHS2* gene. The GFP amplicon matched pGFP vector accession U17997 and failed to match any genes in the *D. texanus* transcriptome. Amplicons (1 µg) from T7-tailed primers were used for dsRNA syntheses with a MEGAscript™ RNAi Kit (Invitrogen) for the preliminary injection experiment and with a HiScribe™ T7 Quick High Yield RNA Synthesis Kit (New England BioLabs, Ipswich, MA USA) for the feeding experiment, according to manufacturers' instructions. DsRNA fragments sizes were verified using an 1% agarose gel.

DsLac2 injection experiment

Fifth-instar larvae reared on artificial diet after egg hatching were used for the dsRNA preliminary injection experiment. Before injection, ice-chilled larvae were further immobilized on double-sided sticky tape. Green-colored dsRNA (1 µg/µl) was injected on the dorsal suture

between the third and fourth abdominal segments using a glass needle mounted on a microinjector Nanoject II (Drummond, Broomall, PA, USA). The injection speed was 20 nL per sec. After injection, each larva was stored in individual 0.75 oz cup with a 0.5 cm^2 artificial Pink bollworm diet plug inside a mite-proof cage in a growth chamber at 27°C in dark conditions. All larvae were injected in one afternoon. Diet plugs were replaced daily until gene expression evaluation or they reached adulthood. Sample size per dsRNA treatment (dsLac2, dsGFP) is described in Table 4.7, according to gene expression and adult morphology evaluation. Lack of cuticle pigmentation and exoskeleton deformation were evaluated at the adult stage.

DsLac2 feeding experiment

Fifth or sixth-instar larvae reared on artificial diet after egg hatching were used for the dsRNA feeding experiment. Replications (blocks) one and two consisted of sixth-instar larvae and replication three consisted of fifth-instar larvae. One hundred milliliters of pink bollworm artificial diet were prepared with double distilled water and plated on a petri dish. A mold was used to make cylindrical plugs of equal size (0.4 cm diam x 0.5 cm tall). Red-colored dsRNA (1 µg) was added to the surface of a diet plug using a volume of 10 µl per treatment. DsRNA coated diet plugs made and replaced daily were air-dried for 30 min inside a hood before feeding the larvae. Each larva was kept in a 0.75 oz plastic cup that was also replaced daily. All replications were stored in a mite-proof cage inside a growth chamber at 27°C total darkness. Larvae were fed dsRNA coated plugs until evaluation of gene expression, larval chitin content, or adult cuticle morphology. All larvae failed completely to consume daily diet plugs. DsRNA treatments

were dsLac2, dsCHS2, dsGFP, and nuclease-free water. A no-dsRNA treatment was included to control for any possible effects coming from making the artificial diet with double distilled water or resuspending the dsRNA with nuclease-free water. Sample size per dsRNA treatment is described on Table 4.8 according to gene expression or morphological evaluation.

Gene expression measurement by qPCR

qPCR was used to evaluate the change in gene expression at 24 h and 8 d post dsRNA injection, and or 8 d post dsRNA feeding. qPCR primers were designed using NCBI primer-BLAST website (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and *D. texanus Lac2, CHS2, β-actin*, and *elf1a* sequences. 60°C was selected as the annealing and extension temperature after amplicon verification with gradient PCR. Primer efficiency and R^2 were determined using serial dilutions of *D. texanus* cDNA (0.0001 to 1 µg/µl). Primers with efficiency between 90-110% and $R^2 > 0.9$ were selected (Table 4.9). qPCR-primer amplicons were sequenced using Sanger sequencing at GENEWIZ. Amplicon sequences matched *M. alternatus Lac2* and *β-actin*, *A. glabripennis* probable *CHS*, and *Oxypeltus quadrispinosus elf1a*. Also, amplicons matched the respective mRNA sequences in the *D. texanus* transcriptome (Table 4.9).

For the dsRNA injection and feeding experiments, total RNA was extracted from the whole body of each larva using RNeasy Plus Mini Kit (Qiagen) which included a DNA elimination step. RNA concentration and quality were verified using a Nanodrop 2000 (Thermo Fisher Scientific) and an RNA Eukaryote Nano Chip (Agilent 2100 Bioanalyzer, Agilent). First-

strand cDNA synthesis was performed using 1 µg total RNA of each larva and the iScript™ Reverse Transcription Supermix (Biorad). RNA extraction and cDNA synthesis were performed according to the manufacturer's protocol, respectively.

qPCR reactions were performed using iTaq™ Universal SYBR® Green Supermix (Biorad) with a reaction volume of 10 µl that contained 5 µl of supermix, 0.25 µl of each primer (10 µM), 1 µl of cDNA, and 3.5 µl of nuclease-free water. The primers used for each gene are shown Table 4.7. Three technical replicates were used for each larval cDNA. *Lac2* and *β-actin* primers were run together in the same plate and independently from *CHS2* and *elf1a* primers, and vice versa. All biological and technical replicates per block were run together in the same PCR plate for each primer. qPCR reactions were quantified using a CFX connect Real-Time PCR Detection System (Biorad), and settings were initial denaturation at 95ºC for 3 min, 40 cycles of denaturation at 95°C for 10 s, annealing at 60 ºC for 30 s, and extension at 60°C for 30 s, and a final melt curve step with 65-95ºC and 0.5ºC increment for 5 s per step.

β-actin, and *elf1a* were selected as references genes for calculation of *Lac2* and *CHS2* gene expression using the $2^{-\Delta\Delta CT}$ method[132], respectively. DsGFP and water treatment were used as gene expression calibrators for the injection or feeding experiments, respectively.

Evaluation of abnormal morphology

D. texanus adults from dsRNA treated larvae were evaluated for the presence of morphological abnormalities in their cuticle and/or exoskeleton. These included: lack of cuticle pigmentation and exoskeleton deformations. Larvae that shrunk in size and failed to pupate were counted as abnormal phenotype. Adult morphologies were observed under a Nikon® SMZ645 stereomicroscope.

Chitin measurement

Whole bodies of dsRNA treated larvae were evaluated for chitin content following the chitin assay method[133,134]. In brief, each larva was homogenized in 0.5 ml of nuclease-free water using a plastic-pestle hand-held homogenizer. The pestle was rinsed with 0.5 ml of nuclease-free water which was combined with the homogenate. The 1.0-ml homogenates were centrifuged at 1,800 g for 15 min at room temperature and the pellet of each sample was resuspended in 0.4 ml of 3% SDS (sodium dodecyl sulfate). The samples were then incubated at 100°C for 15 min and centrifuged again for 10 min after cooling at room temperature. Later, each pellet was washed with 0.5 ml nuclease-free water, centrifuged for 10 min, and resuspended in 0.3 ml of 14 M KOH. To deacetylate chitin, the samples were incubated at 120°C for 1 h followed by cooling on ice for 5 min. After 0.8 ml of ice-cold 75% ethanol was added to each sample, the sample was mixed and incubated on ice for 15 min. Thirty µl of Celite 545 (Fisher Scientific, Pittsburgh, PA) suspension was then added to each sample and samples were centrifuged at 1,800 g for 5 min at 4°C. Each pellet containing insoluble chitosan (i.e., glucosamine polymer) was washed with 0.5 ml of 40% cold ethanol and 0.5 ml of cold nuclease-free water; each wash was followed by

centrifugation at 1,800 g for 5 min at 4°C. The chitosan in each tube was resuspended in 0.5 ml of nuclease-free water.

For colorimetric chitin content assay, 100 µl of the chitosan solution was mixed with 50 µl of 10% $NaNO_2$ and 50 µl of 10% $KHSO_4$, and gently shaken three times during a 15 min incubation period at room temperature. Mixing these chemicals allowed the generation of HNO_2 to depolymerize the chitosan and deaminate the glucosamine residues from the chitosan. After the samples were centrifuged at 1800 g for 15 min at 4°C, 60 µl of the supernatant of each sample was transferred to a new 1.5-ml microcentrifuge tube followed by the addition of 20 µl of 12.5% $NH_4SO_3NH_2$. The mixtures were then vigorously shaken for 5 min at room temperature. After 20 µl of freshly prepared 0.5% MBTH (3-methyl-2-benzothiazolone hydrazone hydrochloride hydrate) was added to each sample, mixtures were incubated at 100°C for 5 min. The samples were then cooled at room temperature for 25 min, and 100 µl of each sample was transferred to a well of a 96-well microplate. Absorbance was determined at 650 nm in a Vmax microplate reader (Molecular Devices, Menlo Park, CA USA). Chitin content was expressed as a glucosamine equivalent according to a standard curve constructed by using known concentrations of glucosamine (0.156 to 70 µg/µl).

Statistical analyses

For the injection experiment, *Lac2*-qPCR data 24 h and 8 d post injection followed assumptions of normality and homogeneity of variances based on distribution of residuals, and the

Kolmogorov-Smirnov[72], Levene's[73], and Brown and Forsythe's[74] tests. qPCR data were analyzed using a normal distribution and PROC GLIMMIX[77] (SAS v.9.2, Cary, North Carolina) where dsRNA treatment was considered a fixed effect. Abnormal morphology data was analyzed with a Pearson Chi-square test using PROC FREQ[80] (SAS).

For the feeding experiment, *CHS2*-qPCR and chitin content data followed assumptions of normality and homogeneity of variances and were analyzed using a normal distribution. *Lac2*-qPCR data did not follow these assumptions and were analyzed using a Poisson distribution with a log-link function after verification of control of overdispersion with a Pearson Chi-square/DF test[75]. Adult abnormal morphology data was analyzed using a binomial distribution with a complementary log-log link function since the nature of data was categorical (yes or no) and represented extreme categories (0 or 100% yes or no)[75,135]. Data were analyzed using PROC GLIMMIX[77] (SAS) where dsRNA treatment and blocks were considered fixed and random effects, respectively. Degrees of freedom were estimated using the Kenward-Rogers method[79] when data failed to follow assumptions of normality and homogeneity of variances. When the F-test for type III effects was significant at $P < 0.05$, pairwise comparisons were conducted a Fisher's protected least significant difference test (LSD) at $\alpha = 0.05$ significance level[135].

Table 4.1. *Dectes texanus* relative transcript levels (%) of *Laccase2* (*Lac2*) 24 h and 8 d post double-stranded RNA (dsRNA) injection.

dsRNA	Relative transcript level (%) (Mean ± SE)	
	24 h	8 d
dsLac2	10 ± 50 A	150 ± 120 A
dsGFP	160 ± 50 A	160 ± 120 A

Means with same uppercase letter within a column are not significantly different based on F-test ($P > 0.05$).

dsLac2 = double-stranded *Laccase2*; dsGFP = double-stranded *Green fluorescent protein.*

Table 4.2. *Dectes texanus* relative transcript levels (%) of *Laccase2* (*Lac2*) and *Chitin synthase* (*CHS2*), abnormal morphology (%) and chitin content (µg/larvae) post double-stranded RNA (dsRNA) feeding.

	Relative transcript level (%)		Abnormal cuticle/exoskeleton (%)	Chitin content (µg/larva)
dsRNA	***Lac2* Mean (L, U CI)**	***CHS2* Mean (± SE)**	**Mean (L, U CI)**	**Mean (± SE)**
dsLac2	8.3 (1.4, 47.6) C	90 ± 28.7 A	87.5 (49.9, 99.8) A	8.2 ± 1.9 A
dsCHS2	126.2 (45.3, 350.1) A	110 ± 28.7 A	25 (4.9, 80.5) A	6.2 ± 1.9 A
dsGFP	50.2 (16.3, 154.1) B	60 ± 28.7 A	12.5 (1.1, 78.9) A	8.9 ± 1.9 A
Water	17.8 (4.5, 70.7) CB	110 ± 28.7 A	28.6 (5.7, 85.2) A	6.2 ± 1.9 A

Means with different uppercase letter within a column are significantly different based on least significant difference (LSD) mean separation test ($P < 0.05$).

Means with same uppercase letter within a column are not significantly different based on F-test ($P > 0.05$).

dsLac2 = double-stranded *Laccase2*; dsGFP = double-stranded *Green fluorescent protein*; L, U CI = Lower and upper confidence interval.

Table 4.3. *Dectes texanus* amplicons from degenerate primers matched *Monochamus alternatus Laccase2* (*Lac2*) and *β-actin* sequences.

***D. texanus* degenerate primer**		**Blastn/nr database highest scoring match**			
ID	**Sequence[a]**	**Gene**	**Species**	**E-value**	**Bp**
Pilot 6	F 5'- ATGGACGGTCTYTACGGC -3'	*Lac2*	*M. alternatus*	6e-106	326
	R 5'- GTCARCTGAGCTGGACACAC -3'				
Pilot 11	F 5'- GTGTGTCCAGCTCAGCTGAC -3'	*Lac2*	*M. alternatus*	2e-146	526
	R 5'- CGTCGATYAAACTKATBACRTG -3'				
Pilot 1	F 5'- GCSCAAAGCAAAAGAGGTATC -3'	*β-actin*	*M. alternatus*	3e-115	275
	R 5'- GTGGTACGACCRGAAGCG -3'				

[a]IUPAC one letter code abbreviation for mixed bases: Y= C or T; R= A or G; K= G or T; B= C, G, or T; S= G or C

Bp= amplicon size in base pairs

Nr= non-redundant

Table 4.4. *Dectes texanus Chitin synthase 2* (*CHS2*) and *elongation factor 1-a* (*elf1a*) mRNA matched sequences from other cerambycids and chrysomelids.

		Blastn/nr database		
***D. texanus* gene id**	**Bp**	**Gene**	**Species**	**E-value**
DN19904_c1_g1	6410	Uncharacterized XM_023456099	*Anoplophora glabripennis*	0
		CHS II	*Diabrotica virgifera virgifera*	0
		CHS II	*Leptinotarsa decemlineata*	0
DN17316_c17_g1	1961	*Elf1a*	*A. glabripennis*	0
		Elf1a	*Colaphellus bowringi*	0
		Elf1a	*L. decemlineata*	0

Nr= non-redundant

Table 4.5. *Dectes texanus* amplicons from T7-tailed primers matched *Monochamos alternatus Laccase2* (*Lac2*) and *Anoplophora glabripennis* probable *Chitin synthase* (*CHS*). *Green fluorescence protein* (*GFP*) amplicons matched vector sequence information.

dsRNA	Primer sequence	Bp	Blastn/nr database		Blastn/*D. texanus* transcriptome	
			Species/Gene	E-value	Gene id	E-value
dsLac2	F 5'-TAATACGACTCACTATAGGCCGTCAACCACCTTCCAAAG -3'	301	*M. alternatus*/ *Lac2*	9e-109	DN20013_c0_g1	1e-150
	R 5'-TAATACGACTCACTATAGGGTCAACTGAGCTGGACACACA -3'					
dsCHS2	F 5'-TAATACGACTCACTATAGGGATCATTTGGATGGTGGGAAA -3'	294	*A. glabripennis*/ Uncharacterized XM_023456099	2-e17	DN19904_c1_g1	6e-158
	R 5'-TAATACGACTCACTATAGGGACTTGCAAGAGCACCATCAA -3'					
dsGFP	F 5'-TAATACGACTCACTATAGGGCCCGAAGGTTATGTACAGG -3'	305	Cloning vector pGFP/ U17997	1e-164	No hits	-
	R 5'-TAATACGACTCACTATAGGGTCGCCAATTGGAGTATTTTG -3'					

Bp= amplicon size in base pairs; Nr= non-redundant; dsRNA = double-stranded RNA; dsLac2 = double-stranded *Laccase2*; dsGFP = double-stranded *Green fluorescent protein*

Table 4.6. Thermocycler settings to amplify *Dectes texanus Laccase2* (*Lac2*) and *Chitin synthase 2* (*CHS2*), and *Green fluorescence protein* (*GFP*) with T7-tailed primers.

	°C, min:sec		
Thermocycler steps	***Lac2***	***CHS2***	***GFP***
1. Initial denaturation	95, 5 min	95, 5 min	95, 5 min
2 Denaturation	95, 30 s	95, 30 s	95, 30 s
3. Annealing	56, 30 s	71, 30 s	58, 30 s
4. Extension	70, 30 s	72, 30 s	70, 30 s
5. Repeat step 2-5	39 times	39 times	39 times
6. Final extension	70, 30 s	72, 30 s	70, 30 s

Table 4.7. *Dectes texanus* larval sample size per double-stranded RNA (dsRNA) treatment at each gene expression and morphology evaluations in the preliminary injection experiment.

	N			
	Gene expression			
dsRNA treatment	24 hr	8 d	Adult morphology	Total
dsLac2	3	3	12	18
dsGFP	3	3	12	18

dsLac2 = double-stranded *Laccase2*; dsGFP = double-stranded *Green fluorescent protein*

Table 4.8. *Dectes texanus* larval sample size per double-stranded RNA (dsRNA) treatment at gene expression, chitin content and morphology evaluations.

			N			
dsRNA	Replication (block)	Larval instar	Gene expression (8 d)	Larval chitin content (8 d)	Adult morphology	Total
dsLac2	1	VI	4	3	4	11
dsGFP			4	3	4	11
dsCHS2			4	3	4	11
Water			4	3	3	10
dsLac2	2	VI	3	2	2	7
dsGFP			3	2	2	7
dsCHS2			3	2	2	7
Water			3	2	2	7
dsLac2	3	V	3	2	2	7
dsGFP			3	2	2	7
dsCHS2			3	2	2	7
Water			3	2	2	7

dsLac2 = double-stranded *Laccase2*; dsGFP = double-stranded *Green fluorescent protein*; dsCHS2 = double-stranded *Chitin synthase 2*

Table 4.9. *Dectes texanus* amplicons from qPCR primers matched sequences from *Monochamus alternatus*, *Anoplophora glabripennis, Oxypeltus quadrispinosus*, and *D. texanus* transcriptome.

	qPCR primer				Blastn/nr database		Blastn/ *D. texanus* transcriptome	
Gene target	**Sequence**	**Bp**	**Efficiency (%)**	**R^2**	**Species/ Gene**	**E-value**	**Gene id**	**E-value**
Lac2	F 5'- CATCCGTCAACCACCTTCCA -3'	82	109	0.9	*M. alternatus/ Lac2*	7e-27	DN20013_c0_g1	2e-37
	R 5'- GCGTCTTCGTGCATCCAATC -3'							
β-actin	F 5'- CTCAACCCCAAGGCTAACCG -3'	110	92.4	1	*M. alternatus/ β-actin*	1e-32	DN17575_c11_g1	2e-48
	R 5'- CCGGAAGCGTACAAGGAGAG -3'							
CHS2	F 5'- AACGAAGGGAAACGGTCCAA -3'	130	95.9	0.96	*A. glabripennis/* uncharacterized XM_023456099	2e-06	DN19904_c1_g1	7e-64
	R 5'- TCTTGAATCGGTGGGTCGTC -3'							

qPCR primer					Blastn/nr database		Blastn/ *D. texanus* transcriptome	
Gene target	**Sequence**	**Bp**	**Efficiency (%)**	**R^2**	**Species/ Gene**	**E-value**	**Gene id**	**E-value**
Elf1a	F 5'- CGCCATTCTCCCACCTTCAA -3' R 5'- CACACCAGTTTCAACACGGC -3'	105	106.4	0.96	*O. quadrispinosus/ Elf1a*	9e-18	DN17316_c17_g1	3e-25

Nr= non-redundant

Bp= amplicon size in base pairs

Lac2 = Laccase2; *CHS2 = Chitin synthase 2*; *Elf1a = Elongation factor 1-a*

dsLac2 **dsGFP**

Figure 4.1. *Dectes texanus* adults from larvae injected with double-stranded *Laccase2* (dsLac2) and control double-stranded *Green fluorescent protein* (dsGFP). Larvae injected with dsLac2 lacked cuticle pigmentation and had atrophied wings and disformed antennae.

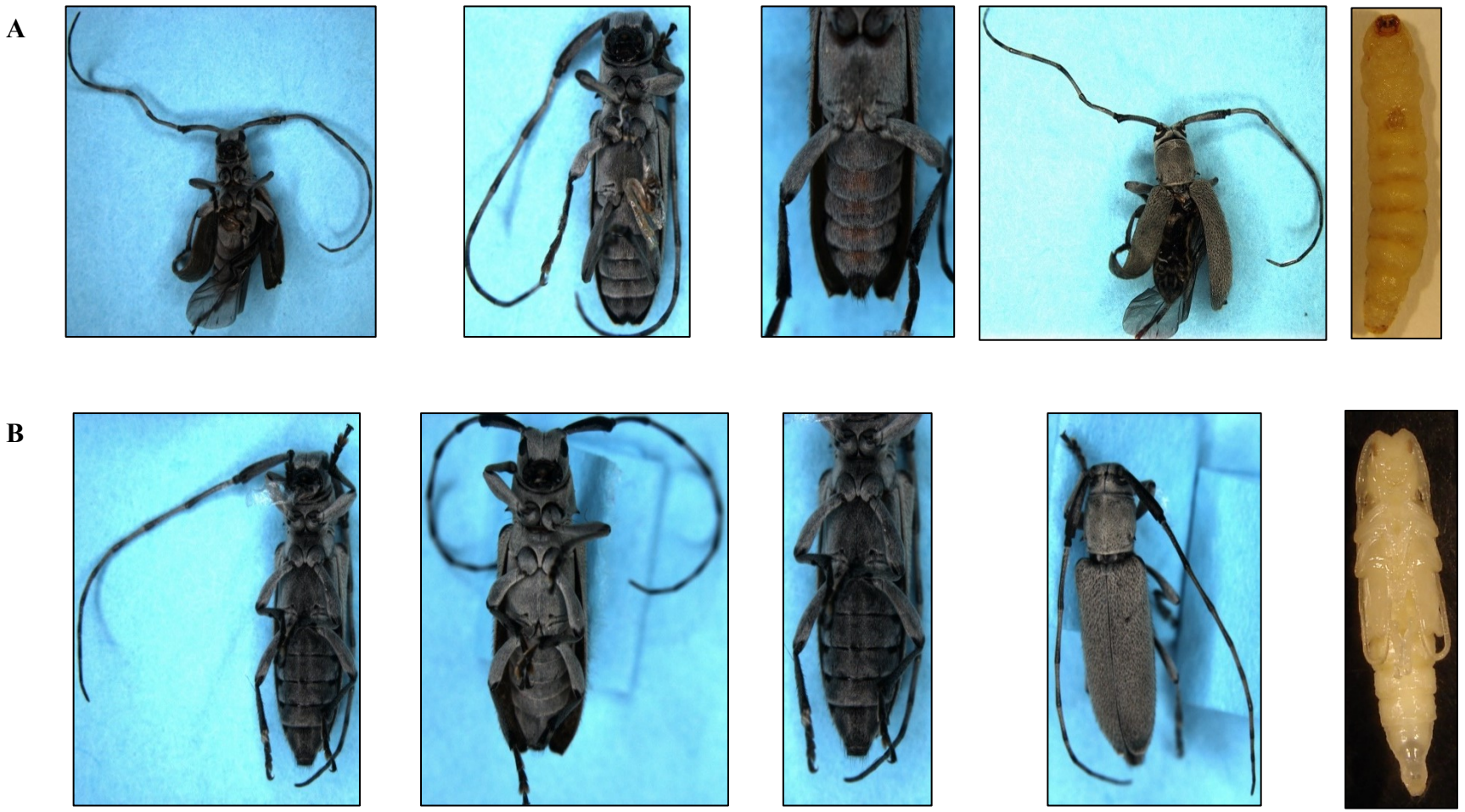

Figure 4.2. ***Dectes texanus*** **adults from larvae fed double-stranded** ***Laccase2*** **(dsLac2) (A) and double-stranded** ***Green fluorescent protein*** **(dsGFP) (B) in artificial diet. Abnormal morphology observed in dsLac2-adults included: absence of metathoracic leg and hindwing, lack of pigmentation on metathoracic leg, brown spots in abdomen, deformed forewings and antennae, and pupation failure.**

Chapter 5 - Conclusions and perspectives

The PI165673 antibiosis resistance factor(s) most likely slow development and reduce survival of *D. texanus* first instar larvae. Larval head capsule width is smaller at 21 d post infestation in the plant introduction compared to the susceptible control. The percentage of first instar larvae is also higher in larvae fed the resistant genotype at 15 d post infestation. However, surviving larvae reach the sixth instar stage, allowing the potential for them to girdle stems before harvest. It is unknown how long larvae require to reach the sixth instar compared to those feeding on susceptible control plants. The PI165673 resistance may also be accompanied by a change in the female's oviposition preference where more empty oviposition punctures are made. However, even if fewer eggs are laid only one surviving larva is needed to reach the plant base and girdle the stem. Therefore, PI165673 antibiosis resistance must be reinforced to further impair development of surviving larvae. This may be achieved by screening additional soybean germplasm, selecting for genotypes that contain larvae with small head capsule and body size, and incorporating both sources of resistance into adapted commercial varieties.

Another option that can be explored is the development of soybean varieties expressing dsRNA targeting gene families highly up-regulated in *D. texanus* fed soybean compared to native hosts or artificial diet. Cytochrome P450s, carboxylesterases and major facilitator transporters are genes most likely used by *D. texanus* to feed and survive on soybean. These protein families were up-regulated when *D. texanus* fed soybean pith compared to those fed sunflower or giant ragweed. Gene silencing by RNAi and RT-qPCR should be conducted to validate the results from the transcriptome analyses.

Larval morphological abnormalities and reduced *Lac2* transcript levels were observed in the RNAi feeding experiments. However, the larval phenotypic responses were not significantly different from larvae in the dsGFP control. Future experiments will benefit from more repetitions and a larger sample size. Field-collected *D. texanus* larvae should be considered for these experiments as well. Additional experiments should also be conducted to establish the minimum dsRNA dose required to silence genes in *D. texanus*. Soybean hairy roots expressing dsRNA sould also be considered for the feeding experiments before developing soybean plants expressing *D. texanus*-dsRNA.

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