The effects of RNAi soybean lines on *Dectes texanus* larval development

by

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Major Professor
Harold N. Trick

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Abstract

Dectes stem borer, *Dectes texanus,* is a coleopteran arthropod pest that infests sunflowers and soybeans. The larvae, the damaging life stage, feed downward through the stem to its base where they girdle the stem to create an overwintering site. It has been observed that *Dectes* stem borer causes significant soybean yield loss due to lodging, and few control methods are available. Contact insecticides will not kill stem burrowed larvae and multiple sprays are needed to negatively impact adult populations. Producers may employ cultural practices, such as early harvest before lodging can occur; however, cultural control is dependent upon uncontrollable factors, such as weather. RNAi was evaluated as a possible strategy for *Dectes* stem borer larval control. Previous *in vitro* experiments were conducted and demonstrated that dsRNA injection or dsRNA diet, using dsRNA that targets critical genes in *Dectes*, could inhibit development of *Dectes* larva. Our research continued this project to determine if host induced gene silencing (HIGS) could be an effective method to control *Dectes* stem borer. Three genes (Laccase 2, Chitin Synthase II, and CYP9E) in *Dectes* were targeted for these experiments. Through host-induced gene silencing, the RNAi lines were hypothesized to cause cuticle deformations in the adult beetle, lower chitin content in the midgut of the larvae, and cause delayed growth and mortality in the larvae, respectively. Those negative effects would in turn cause slowed or no tunneling in soybean stems. Transgenic events were created expressing these genes and were used for both greenhouse and field-based experiments. The field research trial was deployed at the Agronomy North Farm in Manhattan, Kansas where high *Dectes* pressure has been observed in previous years. The transgenic RNAi soybean lines, as well as a control non-transgenic line, were planted, and stem tunneling data was collected and analyzed to compare stem tunneling

between each line and the non-transgenic control. The data suggests more research needs to be done to determine if RNAi can be an efficacious control method of *Dectes* stem borer.

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Chapter 1 - Introduction

Introductory Statement

A potential threat to soybean production in several states of the U.S, *Dectes* stem borer or *Dectes texanus* LeConte (Coleoptera: Cerambycidae) is a long-horned beetle species native to North America (Bezark, L. G., 2010) . Prior to soybean production, wild sunflower *Helianthus annus* (L.) and giant ragweed *Ambrosia trifida (*L.) were the typical hosts of *D. texanus* (Campbell, 1980, Hatchett *et al.* 1977, Patrick, 1973, Campbell and Van Duyn, 1977). However, over the past half century soybean production has advanced into the insect's native range. Increased soybean production, together with the reduction of native host plants due to agricultural practices, has steered *D. texanus* to soybean as a suitable host. The larval stage of *D. texanus* damages the stems by tunneling and girdling, causing the plant to lodge before the soybeans can be harvested (Campbell, 1980, Hatchett *et al.* 1977, Patrick, 1973, Campbell and Van Duyn, 1977). This lodging can cause significant reductions in soybean yield (FMC, 2009, Buschman *et al.*, 2009).

Taxonomy

Dectes stem borer, *Dectes texanus*, are insects, belonging to the kingdom Animalia, phylum Arthropoda, and class Insecta. The class Insecta is very diverse, containing more described species than any other class. Beetles are the most abundant and diverse order, Coleoptera. It has been estimated that there are 300,000 to 450,000 existing species of beetles (Nielsen and Mound, 1999). *Dectes* stem borer belongs to the order Coleoptera, which gives insight into their general biology and life cycle. *Dectes* stem borer belongs to the suborder Polyphaga and the family Cerambycidae (Bouchard *et al.*, 2017). Cerambycidae are known as the long-horned beetles. General characteristics of long-horned beetles include an elongated

body and antennae that are typically longer than the body, (Choate, 1999). *Dectes* stem borer belongs to the genus *Dectes* and the species *texanus*.

Biology

Dectes stem borer goes through a complete metamorphosis. The cycle begins with an egg, which then hatches into a larva. The larva goes through multiple larval instars, or growth stages, before becoming a pupa. A sexually mature adult beetle will emerge from the pupal stage.

Dectes are univoltine, which means they produce only one generation per year. Adults will emerge over an extended period beginning in mid-June and continuing through July. After emerging, adult females will mate and lay their eggs within a week. Larvae will hatch from the eggs in about a week and begin developing through instars. *Dectes* overwinter as larvae and typically pupate in late spring (Whitworth *et al.*, 2010). Once adults emerge from pupae in their overwintering sites, the life cycle begins again.

Females have a unique way of ovipositing. They start by chewing a hole into either a petiole or a stem. Then, they poke their ovipositor around in the hole until they find the central pith. They lay their eggs in the central pith to protect them from desiccation. Females create multiple oviposition sites, but not all of their eggs will hatch. The larvae that do hatch begin to eat and tunnel their way down into the main stem. Their goal is to make it to the base of the stem to create their overwintering site. The larvae aggressively compete with each other to make it to the base of the stem, so they kill each other on their way down (Michaud, 2013). Once one larva makes it to the base of the stem, it will create its overwintering site.

Dectes have a few distinct characteristics and cause distinct plant symptoms. The adults themselves are rather recognizable. Their bodies are gray with long antennae. The antennae are longer than their bodies and have dark bands wrapping around them (Michaud, 2013). Adult *Dectes* can be hard to find though, so identifying an infestation in the field may take looking for warning signs. Oviposition scars are small holes found along petioles and stems. The most obvious diagnostic symptoms are dead petioles and entrance holes. As larvae eat through petioles and into the main stem, the petioles die and fall off due to disruption of their vascular structure. Once the petioles fall off, entrance holes remain in the stems that show the larvae have passed through. Some will have frass from the larvae present.

Damage

Adults are minor foliar feeders and cause minimal damage. The most damaging life stage of *Dectes* stem borer is the larvae. Larvae cause the most damage through their creation of an overwintering site. As mentioned above, one larva will reach the base of the plant and switch from feeding to overwintering site creation. This is done when the plant is nearing physiological maturity. The overwintering site is created by the larva using its mandibles to cut horizontally around the inside of the stem. This incision is called a girdle. Chewed up stem and pith tissues are then used to pack the larval tunnel beneath the girdle, sealing off an overwintering pocket for the larva (Michaud, 2013).

The most significant yield losses come from lodging after the creation of an overwintering site. Girdling by the larvae causes the stalk to become weak and break over at the end of the season; this process is called lodging. Lodging is an issue when farmers harvest; it is

a challenge to harvest plants that have fallen over. Yields can be reduced by up to 12% due to larval tunneling and lodging (Michaud, 2013).

Approximately 4.3 billion bushels of soybeans were produced in the United States in 2022. The average soybean yield across the U.S. was estimated to be 49.5 bushels per acre (NASS, 2022). The USDA Economic Research Service is projecting a season-average soybean price of $14.30 per bushel (USDA ERS, 2023). A theoretical situation using those statistics illustrates how large *Dectes* stem borer losses can be. With a 12% yield reduction due to *Dectes* damage and an average soybean yield of 49.5 bushels per acre, the average bushel loss per acre would be approximately 6 bushels per acre. Using a price of $14.30 per bushel, that is a loss of $85.80 per acre. That yield and financial loss could be devastating to farmers.

Distribution

Dectes are native to the southeastern U.S. and the High Plains region, and they originally infested sunflowers as their preferred host. Infestation began moving to soybeans once soybean cultivation grew in the 1960s (Michaud, 2013). In 1985, *Dectes* stem borer was first documented in Edwards County, Kansas (Bell, 1985a). To investigate *Dectes* distribution throughout the state of Kansas, a study was conducted from 1999 to 2008.

In this study, field surveys were taken in 1999 and 2008 in Kansas soybean fields. The goal was to look at two to four fields in each county, and ten to twenty plants per field were checked for *Dectes* larval presence. The results of the 1999 field survey showed the highest level of infestation, 15.7%, was in the south central region of the state. In the northwest, northeast, southeast, and east central crop reporting districts, there were no *Dectes* stem borers detected. The results of the 2008 field study showed the highest level of infestation, 40.3%, was

in the southwest region of the state. The next highest levels of infestation were recorded in the south central, central, and north central crop reporting districts. Lower infestation levels were recorded in northwest and west central crop reporting districts. There was little to no *Dectes* stem borer infestation in the eastern third of the state (Buschman & Sloderbeck, 2010). This study showed that over a ten year period, *Dectes* stem borer distribution in the state of Kansas greatly increased and spread.

A figure (Figure 1) from the previously mentioned study (Buschman & Sloderbeck, 2010) showed the spread of infestation through the state by color-coding counties. It shows the original five counties infested in the south central part of the state: Edwards, Ford, Kiowa, Barton, and Pawnee. It then shows new counties infested between 1985 and 2005, totaling 47 new counties. Finally, it shows counties added from 2005 to 2010 when the paper was published, totaling 12 new counties (Buschman and Sloderbeck, 2010). It is clear from the Figure 1 that *Dectes* distribution has spread from the south central part of the state to a large portion of the central and western two thirds of the state. At the time of the study publication, there was also some spread into the eastern third of the state. Currently, based on heavy infestations personally observed in Riley county and the spread documented from 1985 to 2010, one can infer that it is highly probable that distribution has continued to increase and expand into the eastern third of the state.

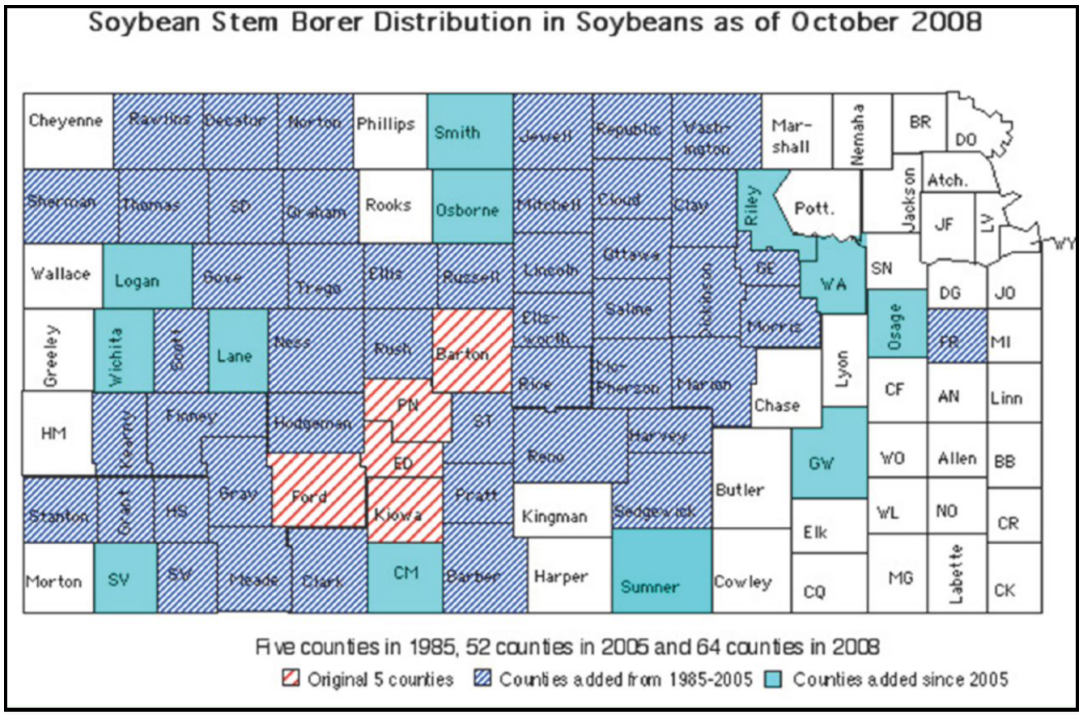

Figure 1. Soybean Stem Borer Distribution in soybean as of October 2020 (from Buschman & Sloderbeck, 2010).

Kansas is not the only state infested with *Dectes* stem borer. In 2018, Pioneer Field Agronomists observed infestations across the United States (Figure 2). Many states are infected, including Alabama, Arkansas, Colorado, Delaware, Georgia, Illinois, Indiana, Iowa, Kansas, Kentucky, Louisiana, Maryland, Mississippi, Missouri, Nebraska, North Carolina, Ohio, Oklahoma, Pennsylvania, South Carolina, South Dakota, Tennessee, Texas, Virginia, and West Virginia (Jeschke, 2018). Based on the Kansas distribution study, as well as the distribution across the United States as a whole, it is clear that *Dectes* stem borer are present and able to causes losses. This makes management of this pest critical.

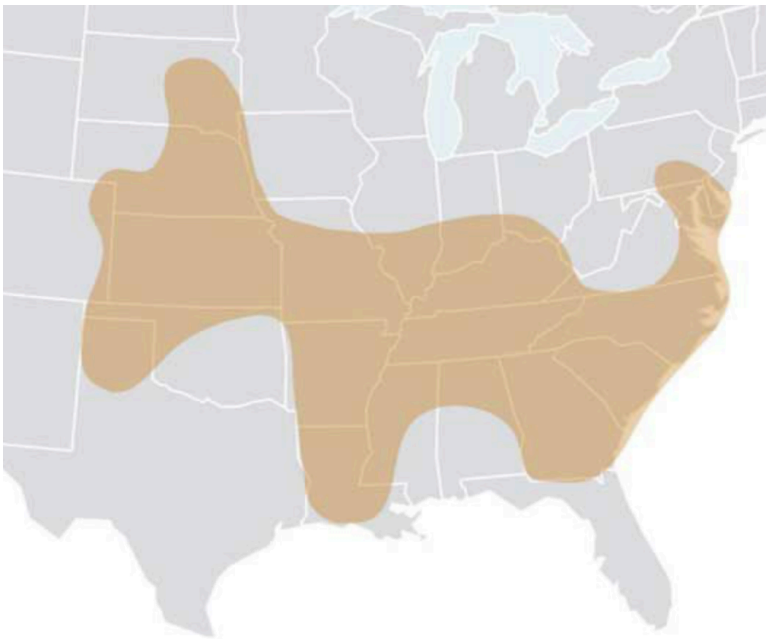

Figure 2. 2018 Soybean Stem Borer Distribution in the US (from Jeschke, 2018).

Current Management

Currently, cultural control methods are the best management practices available for *Dectes* stem borer control. One method of control is crop rotation between soybeans and another non-host crop. Sunflowers are the preferred host of *Dectes* stem borer, so a rotation between soybeans and sunflowers should be avoided. Planting next to fields, whether sunflower or soybean, with infested stubble should be avoided (Michaud, 2013). Deep plowing in the winter to bury stems can decrease survival of larvae and the number that emerge as adults. A study found that stems plowed two inches under the soil surface sufficiently decreased *Dectes* stem borer populations (Campbell and Duyn, 1977). *Dectes* have weed hosts, such as ragweed and cocklebur, so control of these weeds is important. Farmers can harvest as early and promptly as possible before plants lodge (Michaud, 2013).

Use of insecticides is currently not economically or efficaciously feasible in controlling *Dectes* stem borer. Contact insecticides do not kill larvae bored and feeding within the plant.

While adult beetles are vulnerable to insecticide sprays, they cannot be controlled with a single insecticide treatment. Adults emerge over an extended period of time through June and July. Multiple sprays throughout this emergence period would not be affordable (Michaud, 2013). A study conducted in 2015 tested the efficacy of insecticides on *Dectes* stem borer adult survival. One trial tested two different insecticide treatments sprayed at R1 and R3 compared to an untreated check. The second trial tested two different insecticides applied at V8, R1, R3, and a V8 and R3 compared to an untreated check. Observations of stem tunneling damage to the first 12 inches of the main stem were used to show adult survival into the larval stage. Statistical analysis in both trials found no significant differences between the different treatments. Even with multiple sprays at V8 and R3, there was no significant control of *Dectes* stem borer (Cook and Gore, 2015).

The EPA states that integrated pest management is the combined use of common-sense management practices, while being effective and environmentally sensitive (EPA, 2022). In other words, integrated pest management is the use of all facets of control: biological, chemical, cultural, mechanical, and resistance. Using multiple different control strategies puts different forms of pressure on the pest, not just one selective pressure. Currently, cultural control is the main source of control against *Dectes* stem borer. Novel strategies, like resistance, need to be explored to add to the integrated pest management control strategy of *Dectes* stem borer.

RNAi as a New Management Strategy

The central dogma of molecular biology is the unidirectional movement of genetic information from DNA to RNA to protein. DNA is transcribed into RNA, and RNA is translated into proteins. A possible strategy for resistance against *Dectes* stem borer is to disrupt the flow

of genetic information through RNA interference, RNAi. RNAi is a form of post-transcription gene silencing. This is done through introduction of dsRNA into target cells to hybridize with target messenger RNA, mRNA, to interfere with translation into proteins (Fire, 1998). To begin with, dsRNAs interact with Dicer-like proteins, which cleave them into small interfering RNAs or micro RNAs (Ketting *et al.*, 2001). The siRNA or miRNA interact with multiple protein components to form an RNA-induced silencing complex, or RISC. The RISC then moves to the target mRNA sequence to cause degradation, thus inhibiting translation (Martinez *et al.*, 2002). The small, dsRNA molecules can be introduced to a target organism in multiple ways.

Host-Induced Gene Silencing (HIGS)

One mechanism of introducing RNAi to a target organism is through host-induced gene silencing, HIGS. In the case of plants, the genetically transformed plant host produces dsRNAs that target a specific pathogen or pest. It is common that the dsRNAs are formed in a hairpin, which are then chloroplast-expressed. Pests feed, insects for example, consuming the hairpin structures (Koch and Wasseneggar, 2021).

When insect pests ingest dsRNA expressed in host tissues, the midgut cells take it up. The native RNAi machinery in the insect then processes it. This RNAi machinery includes the Dicer nucleases and proteins that form RISCs. It is RNAi delivered via ingestion (Bolognesi *et al.*, 2012).

Through a previous study, it was found that HIGS can be an effective control strategy against western corn rootworm larvae. In this study, second instar western corn rootworm larvae were fed a diet of dsRNA at a concentration of 1000 ng/mL dsRNA. The dsRNA in the diet targeted a component of the endosomal sorting complex required for transport. After five days

of feeding, growth inhibition in the larvae was noted. To take it a step further, percent mortality was observed depending on concentration of dsRNA in the diet and time of exposure to said diet. Two different diets, dsRNA concentration of 50 ng/mL and 1000 ng/mL, were fed for 2, 3, 6, 12, and 24 hours over the course of 12 days. With the lower concentration diet, percent mortality after 12 days ranged from 0 to 90.39% from short to long exposure times respectively. With the higher concentration diet, percent mortality after 12 days ranged from about 60 to 96.49% from short to long exposure times respectively. With a higher dsRNA concentration and a longer exposure time, a diet of dsRNA caused a high percent mortality of western corn rootworm larvae (Bolognesi *et al.*, 2012).

Currently, Bayer has an RNAi product on the market: SmartStax Pro with RNAi Technology. This genetically transformed corn variety contains three modes of action against corn rootworm. Two modes of action are insecticidal proteins Cry34Ab1/Cry35Ab1 and Cry3Bb1. The third mode of action is RNAi targeting the snf7 gene, which codes for a protein required in the insect midgut (Bayer, n.d.). Since RNAi is being used in this crop and insect system, it is possible it can be used in other systems, specifically in soybeans and *Dectes* stem borer

PREVIOUS RESEARCH IN DECTES

Previously, research on RNAi silencing of the Laccase2 gene in *Dectes* stem borer has been completed. *Dectes* eggs were hatched and either fed a dsLac2 diet or injected with dsLac2. Older instars were injected with dsLac2, and there was a clear difference in Lac2 transcript level at 1 day post injection compared to 8 days post injection. The data showed a dramatic lack of Lac2 transcript 1 day post injection compared to the level of Lac2 transcript 8 days post

injection. Larvae at the 1st instar stage were fed a dsLac2 diet and observed for abnormal morphology and survival. The larvae fed dsLac2 had 100% abnormal morphology leading to no survival compared to control larvae fed a dsGFP diet (Rojas, 2019). The dsLac2 diet shows that RNAi can be effective in decreasing *Dectes* stem borer survival.

This research showed promising results, and the next step would be to test the plant and insect interaction through HIGS.

Chapter 2 - Materials and Methods

Selection of target genes for RNAi experiments

Laccase 2 (*Lacc2*) was selected as a target gene as in other insects, suppression of Laccase 2 impairs cuticle tanning which results in thin and soft cuticles and proper molting of insects (Du *et al*., 2017; Rojas, 2019). Rojas (2019) used degenerate primers based on conserved regions detected in *Lac2* and *β-actin* mRNA sequences of *Monochamus alternatus* and *Tribolium castaneum* to amplify a portion of the Laccase2 gene (Figure 3). Primers were designed from this sequence to amplify an 256bp fragment of the gene and this fragment was cloned using the Dpilot topoF and Dpilot R primers (Table1) into the *pANDA35HK* vector system (Figure 4) (Miki and Shimamoto, 2004) as described by Li *et al*., (2009).

Chitin synthase 2 (*CHS2*) was selected as it is required for chitin synthesis for the peritrophic matrix (PM) and is expressed in epithelial cells of the midgut (Zhu et al., 2016). Silencing of *CHS2* results in feeding cessation and midgut-shrinkage a number of species including *T. castaneum* (Arakane *et al.*, 2005), *L. decemlineata* (Shi *et al.*, 2016), and *Anthonomus grandis* (Macedo *et al.*, 2017). Rojas (2019) used degenerated primers designed based on conserved regions detected in *CHS2* mRNA sequences of *M. alternatus* and *T. castaneum* to amplify a portion of the *CHS*2 gene (Figure 5). Primers were designed from this sequence to amplify a 301bp fragment of the gene and this fragment was cloned using the Dchitin topo F and Dchitin R primers (Table1) into the *pANDA35HK* vector system (Figure 4).

The cytochrome P450 gene was selected as it plays a vital role in growth, development, feeding, and protection against foreign substances (Scoot and Wen, 2001). The 305bp DtCP8E2 sequence (aka cytochrome P450) (Figure 6) was provided by Lina Rojas and was synthesized by

IDT as a gene block and then subsequently cloned using the DtCP8E2-RP topo F and DtCP8E2-RP primers (Table1) into the *pANDA35HK* vector system (Figure 4).

>selected portion of the Lac2 sequence (from Rojas, 2019)

CGGGGACACCGGACCACGCCAGTTCGCAATTCCGACGCAGGGGGCGCCGCAATTCGCGCTTGCAG
ACATAAGAGTTAGTGGAGGAGCACAAGGTTTTATAAAGCCGGTGAACGCAAGAAGAAGTAGTAA
CCGCGGTAAAAATATAGGGCAGTGTTAAACAGTGTTTGCCGCAGTTCGGACGTAGTGGAAGTCGC
TTAACGGTGACTTGTGATTGATGATGATGGACACTAGATGGTTTGCGATAGCTTCTGCGGCTCTAT
TGTTCGTTCTTCTGCTTGTGGACAGCATCAACGCTGTTAGGGTCGGAGCTAAGAAGAAAGTAGCTG
ACCAGTCAGCATCAGCCTCGTGGATCCAAGGTGACGCCATCAGCACTAATGGCGCTTTCGATTCCT
CAGACTTCTTTTCTTCATCCCATGGGGTATTCCAGACACACCCTGAGGGATCTTTCGGCGTCAGGGC
CAATTCCGGTTCTGGTTCCGGTTCCGGAGGAGGTAGAAGAAACACGGGCTCAATGAGGTCTAAAC
ACTTGGATTACAGAACATCCTCTACAGCTGAGTTGAGGAAGAACCCTTCATTGTCATCCCCAGAGG
AATGCGCGAGGGCTTGCAGAGAAGGGGAACCCCCAAGAATTTGTTATTATCATTTCACACTGGAG
TTCTACACAGTCCTTGGAGCTGCCTGTCAAGTTTGTACACCAAATGCCACCAACACTGTATCGTCAC
ACTGTCAATGTGTCTTGGCTGACGGTGTGGAAAGAGGTATCCTTACAGCAAATCGTATGGTACCAG
GACCTAGTATACAGGTTTGTGAAGGAGACAAGGTTGTGATAGATGTGGAAAATCACATGGAAGGT
ATGGAAGTTACCATCCATTGGCACGGAATCTGGCAGAGAGGCACTCAGTACTACGATGGTGTGCC
ATTTGTAACACAGTGTCCTATACAGCAAGGAAACACCTTCAGATACCAATGGATAGCAGGAAACGC
AGGAACCCACTTCTGGCACGCTCACACTGGTCTCCAGAAGATGGACGGTCTCTACGGCAGTATTGT
CAT**CCGTCAACCACCTTCCAAAGACCCCAACAGCCACCTCTACGACTTCGATCTCACCACCCACGT**
GATGCTTCTCAGTGATTGGATGCACGAAGACGCCGCTGAAAGGTTCCCAGGTAGATTAGCCGTC
AATACTGGACAGGATCCCGAAAGTTTGCTCATCAACGGAAAGGGTCAGTTTAGAGATCCAAACA
CTGGTTTCATGACGAATACGCCATTGGAAGTCTTCACCATGACTCCAGGAAGAAGGTACAGATTC
AGAATGATCAATTCTTTTGCCTCTGTGTGTCCGGCTCAACTGACCATTCAAGGACACAATCTGACTT
TGATCGCGACAGATGGTGAACCTGTCCATCCAGTATTAGTCAACACTATTATATCGTTCTCTGGGG
AAAGATATGATTTCGTGATAAACGCCGATCAGGCTCCTACAGCTTACTGGATCCAACTAAGAGGAC
TGGGTGAGTGTGGTATCAGAAGAGTTCAGCAACTTGGTATCCTTCGTTACGCAAGAGGTCCGTATC
AACCATCATCGAATCCTCCAACTTACGACTACGGCATACCTCAGGGAGTGGTACTAAATCCTTTGG
ACGCCATATGTAATAACAGAAGAACTGATGCTGTATGCATAAATCAATTAAAGAATGCAAGAGAT
ATTGATCGTGGACTTCTAGCAGAAAGACCAGATGTCAAGATTTTCTTACCATTCAGATTCCACGTTT
ACACACCTGAAGATATATTTGCCCCCAAAACATACAACAGATTTTTAGTCGCGCCAAACGGAGATC
ATGTAATTAGTTTGATAGACGAAATTTCGTATTTGTCAGCACCTTCCCCTTTACTTTCCCAATATGAT
GACATAAACCCCGATCAGTTCTGTAATGGAGACAATATACCACCAGATTGTGGACAGAACTGCATG
TGTACCCATAAAATTGACATACCTTTAAATGCAGTTGTTGAAGTCGTATTGGTAGATGAAGTCCAA
CAACCTAACTTGAGTCATCCTTTCCATCTTCACGGTTACGCCTTCAACGTTATTGGTATTGGTAGATC
ACCCGACCAAAATGTCAAAAAAATTAATTTGAAACATGCCCTTGACCTAGATAGACAAGGTCTCCT
CCATCGTCAATTTAACTTACCTCCAGGAAAGGATACAATTGCTGTGCCAAATAACGGATACGTTGTT
CTCAGATTCAGAGCTGATAATCCTGGCTTTTGGCTATTCCACTGTCACTTTTTGTTCCACATTGTAAT
TGGAATGAATTTGATATTCCAGGTTGGCAGTCTAACTGACCTGCCACCTATTCCACCAGGTTTTCCA
ACCTGTGGTGATCACAAAGCAGAAATTTCACTTGACATTCAAAATGAATATCCCAAGCAAT

*Sequence highlighted in yellow and bold fonts is used for RNAi construct

Figure 3. Selected sequence of the Laccase 2 gene used in this study.

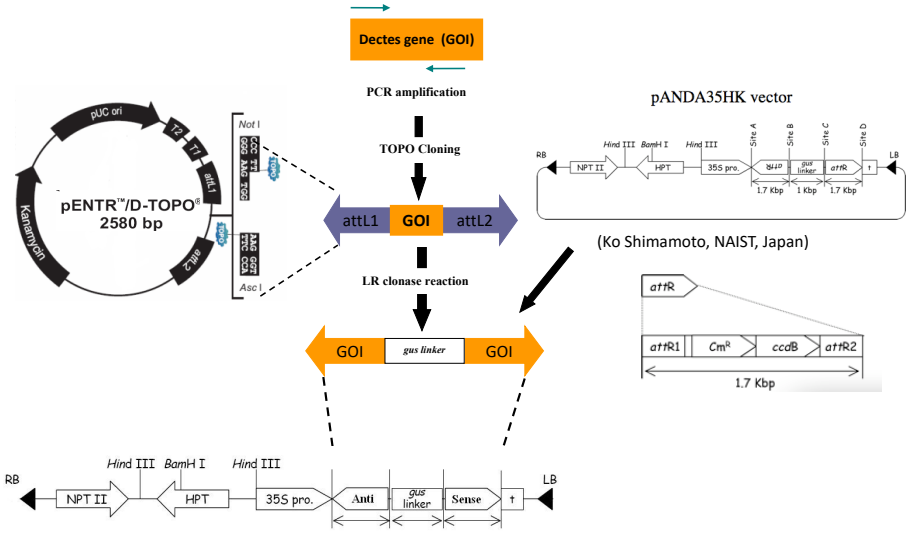

Figure 4. Vector construction used for producing transgenic soybean lines.

>Chitin synthase (DtCP8E2)

GATTTTCATAAGTATTAATTGTTTAGCGATATTCCAGACTACCAATTGAAAAAAATATGTGTGGTTCATACAAAGCA
TAGTTTTCTCTTATGGTGATGAGATAAAGTTTATTTTAAAATTCACTTAATCGAAGACAGTCAAAATGAGAAGACGC
GAAATTAATGAGGAAGATGAACCGCTTATAGAACAAGATGGGGAAGAACCTGACACACAAAAAGGTTGGAATAT
ATTTAGAAACAATCCCAGAAAAATCGATTCTGGTTCTACAGCTGAATCAAAGTTCATTGATACTGGAGTAAAATGT
TTAAAATGCTTCACTGTAATATTTACTTACTGTGTAGTGTTAGGTATGGGGGTTCTATCAAAAGGAACGTTATTGTT
TATGACATCACAAATCAGAAATATTACTAGACCATATTGTAACAAAGACATAGAAGTCAATCAACAATTTCAAGTG
AACCTACCAACAGAAGAAAGAGTAGTTTGGATGTGGTTGATTATTTTCGCCTACATGGTTCCAGATTTGGGTACAT
TCTTGAGATCTGTTAGAATAGTATGTTTTAAATCTTGGGAATATCCTAACCAATGGAAATTTTTGTCCGTCTTCATCA
CAGAAACACTCCCTCCTATAGGGAGTGCAATTTTGGTTTTCGTAATTTTTCCGGAACTAGACGTAATTAAGGCGGC
GATGTTGACCAATGCTGTGTGCATCGTACCTGCTTTTGTTGGCTTCTTATCTAGAAATCCACATTCGTTCAATAGTAC
CCTAAAAGTGGTGTTAGATGTATTTGCCATCGTAGCGCAAGCCAGTGCTTTCGTGATATGGCCTTTACTTACAAAAC
AAAACATTTTGTATTTAATACCAGTCAGTTTAATTCTAAT**ATCATTTGGATGGTGGGAAAACTTTTTATCGGAGAA**
AAGCCCTATTAAAGTCATTAGTGAAATGGCTATAGCGAGAAAACATTTAAAAAATGATAGCTATTTCATGTATG
CTTTCATTGCTCCATGGAAGTGTTTAGTATTCTTGATTACAACTGTAATTATCCTGGTAATACAAGGAGAGGCTTA
CTTATTTCTGTTTTCAAATTTCCAAGCTGCTTTCCAGTCACACAGTATTAACATTACTGAAACTGGACCAAACATAG
CCATTATCAATGCAAGTGTTGATGGTGCTCTTGCAAGTGGGTACGGCACAGTAATTGAAACATCAATTTGGACTCC
TTTATCCGTATTTTTGATAAATATAATAAGTACTTTCCTATGTTATGCTTTTGGAAAATTCGCATGCAAGATCATGAT
CCAATCATTTGGCTTTGCTCTCCCCATTAACTTGGTGGTTCCAGTTCTTCTCACAGCATTGATAGCAGTTTGCGGTAA
CTACAATAAAGATGAATGTGCTTATGCTGACACAATTCCTGCGTATCTCTTCTTCAATACACCATCCGTGACTGATTT
AAATAACTTTATTGCTTATCAACATTCTTGGATATGGTTAGTGTGGCTATTATCTCAGTGTTGGATTACGGTACATAT
ATGGAAAAACAACAATGAAAAACTGGCTTCTACCGAAAAGCTGTTCTTTAGACCCATGTATGATGCCTACCTTATC

*Sequence highlighted in yellow and bold fonts is used for RNAi construct

Figure 5. Partial sequence of the CHSN2 gene used in this study.

>DtCP8E2

TTCGTCCAGCATTTCCTGCAGAAGGACGAAGACGTGATAACCATCGAGTTCAAGGACACCTTTACGCGTTTCACC
AACGATGTGATTGCCACCACGGCTTTCGGTATTAAAGTGGATTCGTTGGGCGACCCGCAAGTAGTACGACCAGA
TCTGTTGAACATCCTTCTGGACGCTAGGAAAAACGCGCGGAAGATGCAAGAAAAAGGACTAGCTCACGACAGA
ATCACGAAAATCGCCGCCGAATTACAAAACGAAGACATCACGGCCCACGCTTTGATTTTCTTCATAGCCGGCTTC
GAATCCGT

*Sequence highlighted in yellow and bold fonts is used for RNAi construct. This sequence was synthesized as gene block from IDT.

Figure 6. Sequence of Cytochrome P450 gene used in this study.

Plant material and tissue culture

Soybean plants of the cultivar 'Jack X' (Jack/PI417138) (a kind gift from Wayne Parrott, University of Georgia) were grown in the greenhouse at 26 °C day/22 °C night with a 16:8 hr photoperiod. Immature pods were collected and surface-sterilized in a 20% commercial bleach solution (5.25% sodium hypochlorite) containing 1 to 2 drops of Tween 20 for 15 min with gentle agitation on an orbital shaker. The soybean pods were washed in a laminar flow hood 3 times with sterile distilled water. Initiation and maintenance of soybean somatic embryos followed the procedure outlined by Finer (Finer and Nagasawa, 1988) and modified by Bailey *et al.* (1993). Immature seeds between 4 and 6 mm in size were aseptically remove from the sterilized pods and placed into a sterile Petri plate. The cotyledons were separated from the seed coat by excising the end of the seed containing the embryonic axis and then placed abaxial side-down on D40 induction medium (Appendix 1). Cotyledons were cultured in the growth chamber at 23 °C with a 16:8 hr photoperiod and a light intensity of 70-120 $\mu Em^{-2}s^{-1}$. After approximately two to three weeks on D40 medium, cotyledons exhibiting embryo formation were transferred to D20 proliferation medium. Small clusters of green or translucent globular embryos were selectively subcultured every two weeks onto fresh D20 medium and maintained in the growth chamber at 23 °C with a 16:8 hr photoperiod and a light intensity of 70-120 $\mu Em^{-2}s^{-1}$. Somatic embryos were proliferated on D20 medium for approximately three months before being used in transformation experiments.

For transformation, approximately 25 embryogenic clusters were arranged uniformly in the center of a Petri dish containing D20 medium and bombarded with DNA-coated tungsten particles using the particle inflow gun (Finer & McMullen 1991). One week after transformation, embryos were subcultured onto D20 medium supplemented with 15 mg/ml hygromycin to select for transformed embryos. Healthy embryogenic clusters were subcultured every 2 weeks onto fresh

D20 medium containing either 15 mg/ml or 7.5 mg/ml hygromycin (Appendix 1). Putative transformed embryogenic clusters were obtained after approximately 6 to 8 months of selection and screened with PCR to confirm DNA integration. During selection, embryos were maintained in the growth chamber at 23 °C with a 16:8 hr photoperiod and a light intensity of 70-120 $\mu Em^{-2}s^{-1}$. Embryogenic clusters with confirmed DNA integration were transferred to M6 maturation media (Appendix 1) for one month to induce elongation of the embryos. Elongated embryos were gently separated and transferred to fresh M6 medium for an additional month to promote maturation. During maturation, embryos were maintained in the growth chamber at 23 °C with a 23:1 hr photoperiod and a light intensity of 20-35 $\mu Em^{-2}s^{-1}$. Maturation was considered complete once the embryos became a cream to yellow color. Mature embryos were desiccated for 2 to 3 days in 100X15 mm Petri dishes before being transferred to OMS medium (Appendix 1) for germination. Germination was conducted in the growth chamber at 25 °C with a 23 hr photoperiod and a light intensity of 70-120 $\mu Em^{-2}s^{-1}$. Embryos remained on OMS medium until roots and shoots formed. Plantlets exhibiting branched roots and elongated shoots were transferred to Magenta GA-7 boxes (Magenta Corp., Chicago, IL) containing vermiculite and ½ OMS medium (Appendix 1) and grown in the growth chamber at 25 °C with a 23 hr photoperiod and a light intensity of 70-120 $\mu Em^{-2}s^{-1}$. As soon as mature leaves appeared, plants were transferred into small pots filled with soil-less potting mix (Pro-Mix BX, Hummert International, Earthcity, MO), placed into a clear 81.3-liter box (Rubbermaid Commercial Products, Inc., Winchester, VA) containing 1 cm of water, and covered with a clear lid to maintain high humidity. The box was kept in the growth chamber at 25 °C and 75% relative humidity with a 23:1 hr photoperiod and a light intensity of 200 $\mu Em^{-2}s^{-1}$. The lid was adjusted over a 2-week interval to acclimate the plants to lower humidity conditions. Once acclimated, the plants were transplanted into 5-gallon pots

filled with a soil mix and grown to maturity in the greenhouse at 26 °C day/24 °C night with a 16:8 hr photoperiod.

Plant transformation

Transformation of soybean somatic embryos was performed using the particle inflow gun (PIG) (Finer *et al.*, 1992). Prior to transformation, 25 embryogenic clusters were uniformly arranged in the center of a 100X15 mm Petri dish containing D20 medium and air dried uncovered in a laminar flow hood for 15 minutes. For each transformation experiment, 50 mg of 0.2 µm tungsten particles were sterilized in a 1.5 ml microcentrifuge tube with 500 µl of 95% ethanol for 20 min, washed five times with sterile deionized water, and re-suspended in 500 µl of sterile deionized water. All transformation experiments were performed with co-bombarded plasmid DNA mixed at a 1:1 ratio. Plasmid DNA (5 µg) was mixed with 25 µl of the re-suspended tungsten particles and allowed to incubate on ice for 1 min. Plasmid DNA was precipitated onto the tungsten particles by adding 25 µl of 2.5 M $CaCl_2$ and 10 µl of 100 mM spermidine and incubating the mixture on ice for 4 min. Following incubation, 50 µl of supernatant was discarded, leaving a total of 15 µl of DNA-coated tungsten particles for bombardment. The mixture was re-suspended by vortexing just prior to bombardment. For bombardment, a 2 µl aliquot of DNA-coated particles were applied to the surface of a Swinney filter holder which was then secured to the leur-lock adapter located at the top of the PIG chamber. The arranged embryogenic clusters were covered with a 500 µm mesh baffle and placed in the PIG chamber 15 cm below the filter holder and the chamber was placed under a vacuum. Once the vacuum of -94 kPa was obtained, the DNA-coated particles were propelled towards the embryogenic tissue with quick burst a 60 psi of helium gas. Each plate of embryos underwent two successive bombardments.

Greenhouse Trial

In 2021, a trial was placed in the greenhouse to test the effects of HIGS on the Lac2 (Laccase 2), ChSy2 (Chitin Synthase II), and CYP9E (Cytochrome P450) genes in *Dectes* stem borer larvae. Twenty seeds from plants independently producing dsLac2, dsChSy2, and dsCYP9E were planted in three-gallon plastic pots (Hummert International, Earth City, MO) filled with steam sterilized soil. One-half to one whole trifoliate leaf was collected from the newest emerged trifoliate of each plant; approximately 100 mg. Each sample was placed in a 1.5 mL microcentrifuge tube (MidSci, St.Louis, MO) and placed in liquid nitrogen. One by one, each sample was ground using a sterile pestle and placed back into liquid nitrogen. Samples were placed in –80 °C for storage.

DNA extraction of the greenhouse samples was completed using the Omega Bio-tek E.Z.N.A. Plant DNA Kit Short Spin Protocol (Omega Bio-tek, Norcross, GA). DNA concentration was quantified using a NanoDrop. PCR positive plants were placed in mesh tents, two to a tent, and approximately 3 adult *Dectes* stem borers were placed in each tent. Adult beetles were not able to escape the tents. Once adults had finished oviposition and died, tents were removed.

Oviposition data was collected from these plants. Each plant was fully observed for oviposition scars, and the number of oviposition scars was recorded. After plants were fully grown and beginning to senesce, stem tunneling data was collected from these plants. Petioles, lateral stems, and the main stem were split using a box cutter. The number of tunnels present in lateral stems and the main stem was recorded, as well as number of larvae found.

DNA Extraction of Field Samples

DNA extraction of field samples was completed using the Omega Bio-tek E.Z.N.A. Plant DNA Kit Short Spin Protocol. DNA concentration was quantified using a NanoDrop100, and samples were stored at –20 °C.

DNA Extraction of Growth Chamber Samples

DNA extractions were performed using a modified CTAB procedure (Saghai-Maroof et al. 1984). Prior to DNA extraction, samples were taken from –80 °C storage and immediately placed in liquid nitrogen. The microcentrifuge tubes with samples contained appropriately sized beads in a sufficient number for bead beating. To grind plant tissue, four rounds of dry bead beating for 15 seconds at speed 5 was completed while keeping the tissue frozen the whole time. Once done, samples were placed in –80 °C for storage. To begin DNA extraction, each sample was removed from storage in –80 °C, quickly spun down in the centrifuge, and placed in liquid nitrogen. Four samples were removed at a time and 500 µL CTAB Buffer was added to each sample. The samples were vortexed and incubated at 65 °C for 20 minutes, gently mixing every 5 minutes. Samples were removed from incubation, and 500 µL of Chloroform IAA (24:1) was added to each sample and vortexed. Samples were incubated at room temperature for 5 minutes and centrifuged for 15 minutes at 13,000 rpm. Samples were removed from the centrifuge and 350 µL of the liquid layer was transferred to new 1.5 mL microcentrifuge tubes. 300 µL of isopropanol was added to each sample and mixed gently. Samples were centrifuged for 20 minutes and 13,000 rpm. Isopropanol was discarded, leaving a DNA pellet in each tube. The DNA pellets were washed with 500 µL of 75% ethanol. Tubes were centrifuged for 5 minutes at 13,000 rpm, and ethanol was discarded. Using a pipette, as much of the ethanol as possible was

removed from each tube. Tubes were left open to air-dry. Once the DNA pellets were dry, 100 µL of sterile, double distilled water was added to each tube and let sit to dissolve. DNA concentration was quantified using a NanoDrop100, and samples were stored at –20 °C.

PCR Detection of dsTransgenes

The DNA extracted using the procedure above was analyzed using PCR. Promega GoTaq Flexi DNA Polymerase (Promega, Madison, WI) and Promega dNTP Mix (Promega, Madison, WI) were used. Each PCR reaction totaled 25 µL and 5 µL Promega 5X Green GoTaq Flexi Buffer, 2.5 µL Promega $MgCl_2$ Solution 25 mM, 1 µL Promega dNTP Mix 40 mM, 1 µL forward primer, 1 µL reverse primer, 0.2 µL GoTaq DNA Polymerase (5u/µL), 0.5 to 1 µL DNA (depending on DNA concentration), and sterile, double distilled water to reach 25 µL. Due to the amount of DNA samples, 0.5 µL DNA was added to the PCR mix if the concentration was greater than 1000 ng/µL, and 1 µL of DNA was added to the PCR mix if the concentration was less than 1000 ng/µL. The samples were amplified in a MultiGene thermocycler (T9600G, Labnet International) using 1 initial denaturation cycle at 94 °C for 3 minutes; 30 denaturation, annealing, and extension cycles at 94 °C for 30 seconds, 56 °C for 30 seconds, and 72 °C for 45 seconds; and a final extension cycle at 72 °C for 10 minutes. Once finished, the PCR products were held at 4 °C.

Table 1. Primer sequence and descriptions of sequences used in these studies.

	Sequence (5'-3')	Annealing Temperature (°C)	Product size	Description
gus F1	CACGTAAGTCCGCATCT TCA	54.9	216 bp +GOI	Used with the CP specific primers
gus R1	ATCTCTTTGATGTGCTGTGCC	55.6	353 bp + GOI	to determine presence of GOI
gus Sense	CATGAAGATGCGGACTTCCG	50	636 bp	RT-PCR primers to establish *gus*
gus Antisense	ATCCACGCCGTATTCGG	52		linker expression
CaMV35SF	GCACAATCCCACTATCCTTCGCAA	60		determine presence of marker gene
HYGR	CTTCTACACAGCCATCGGTCCAG	60	800bp	determine presence of marker gene
Dpilot_F_topo	CCGTCAACCACCTTCCAAAG	56		
DPilot_R	CCGGACACACAGAGGCAAAAG	56		With *gus*R1
DChtnF_topo	ATCATTTGGATGGTGGGAAAA	56		
DChtn_R	ACTTGCAAGAGCACCATCAA	56	1054 bp	With *gus*R1
DtCP8E2-RP	GATTCGAAGCCGGCTATGAAG	56	305bp	
DtCP8E2F-top	CACCTTCGTCCAGCATTTCCT	52		

PCR products were run on a 1% agarose gel made with Bullseye General Purpose Agarose GP2 (MidSci, St. Louis, MO) and 1X TAE Buffer. 1X TAE Buffer was diluted from 50X TAE Buffer Stock, which contains 242 g Tris base, 57.1 mL glacial acetic acid, 100 mL 0.5M EDTA solution, and double distilled water to 1 liter. The agarose gel was placed in an electrophoresis box containing enough 1X TAE Buffer to cover the gel. PCR products and 100 bp DNA ladder were loaded onto the gel and run. The gel was then removed and placed on an ultraviolet light box. Using a digital camera setup, a picture of the gel was taken and imported into the DigiCam program. The image was then imported into GIMP, an open software graphics program for image editing (https://www.gimp.org/).

RNA Extraction

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and was performed by modifying the manufacturer's protocol as below. Approximately 100 mg of leaf tissue was collected in tubes and immediately frozen in liquid nitrogen. The microcentrifuge tubes contained appropriately sized beads in a sufficient number for bead beating. To grind sample tissue, four rounds of dry bead beating for 15 seconds at speed 5 was completed while keeping the tissue frozen. To begin RNA extraction, one sample was removed from liquid nitrogen and 1000 µL Trizol was added and vortexed immediately. The tube was then placed on ice, and this process was repeated one by one with the rest of the samples. After all samples contained Trizol, tubes were shaken vigorously for 15 seconds and incubated at room temperature for 5 to 10 minutes. This allowed for separation of nucleo-protein complexes. 200 µL of chloroform was added to each tube, mixed vigorously, and incubated at room temperature

for 10 to 15 minutes. Next, tubes were centrifuged at 4 °C for 15 minutes at 12,000 x g for phase separation. Using new 1.5 mL microcentrifuge tubes, approximately 50-60% of the top aqueous layer was transferred to the new tubes. An equal volume of isopropanol was added to the tubes, mixed well, and incubated at room temperature for 10 minutes for RNA precipitation. The tubes were then centrifuged at 4 °C for 20 minutes at 12,000 x g. Isopropanol was removed carefully, and the RNA pellet was washed with 1000 µL of 75% ethanol by centrifugation at 4 °C for 5 minutes at 8,000 x g. Ethanol was removed carefully, and the tubes were briefly spun. A pipette was used to remove the remaining ethanol. The tubes air dried at room temperature for 5 to 10 minutes. The pellet was dissolved in 50 µL of DEPC treated or RNase free water and incubated on ice for 15 to 20 minutes to rehydrate RNA. Tubes were incubated at 55-60 °C to completely dissolve the RNA. RNA samples were quantified using the NanoDrop100, and 1000 ng of total RNA was used for cDNA preparation.

cDNA Synthesis

The synthesis of cDNA was performed following manufacturer protocol (Promega, Madison, WI). Briefly, the RNA samples were subjected to a DNase treatment with 1 unit of Promega RQ1 RNase-free DNase in 1X DNase buffer for 1 hour at 37 °C. After the DNase treatment, 1 µL of RQ1 DNase stop solution was added to each sample and the tubes were incubated for 10 minutes at 65 °C for DNase deactivation. Next, 1 µL of 10 mM dNTPs and 1 µL of random hexamers were added to each sample, and the tubes were incubated for 5 minutes at 65 °C. Immediately after incubation, tubes were transferred to ice for 5 minutes. Next, 13 µL RNA + dNTPs + Random Hexamers, 2 µL 10X M-MuLV Buffer, 0.2 µL RNase inhibitor, 1 µL M-MuLV-Reverse Transcriptase, and 3.8 µL of nuclease-free water were mixed for each sample,

creating a total volume of 20 µL. cDNA synthesis was then performed in a thermocycler using 1 cycle at 25 °C for 10 minutes; 1 cycle at 42 °C for 60 minutes; 1 cycle at 80 °C for 5 minutes; and 1 cycle at 4 °C for 10 minutes.

rtPCR

The cDNA was analyzed using PCR. Promega GoTaq Flexi DNA Polymerase and Promega dNTP Mix were used. Each PCR reaction totaled 25 µL and 5 µL Promega 5X Green GoTaq Flexi Buffer, 2.5 µL Promega $MgCl_2$ Solution 25 mM, 1 µL Promega dNTP Mix 40 mM, 1 µL forward primer, 1 µL reverse primer, 0.2 µL GoTaq DNA Polymerase (5u/µL), 2 µL DNA, and sterile, double distilled water to reach 25 µL. The samples were amplified in a thermocycler using 1 initial denaturation cycle at 95 °C for 3 minutes; 35 denaturation, annealing, and extension cycles at 95 °C for 30 seconds, 53 °C for 30 seconds, and 72 °C for 45 seconds; and a final extension cycle at 72 °C for 5 minutes. Once finished, the PCR products were held at 4 °C.

PCR products were run on a 1% agarose gel made with Bullseye General Purpose Agarose GP2 and 1X TAE Buffer. 1X TAE Buffer was diluted from 50X TAE Buffer Stock, which contains 242 g Tris base, 57.1 mL glacial acetic acid, 100 mL 0.5M EDTA solution, and double distilled water to 1 liter. The agarose gel was placed in an electrophoresis box containing enough 1X TAE Buffer to cover the gel. PCR products and 100 bp DNA ladder were loaded onto the gel and run. The gel was then removed and placed on an ultraviolet light box. Using a digital camera setup, a picture of the gel was taken and imported into the DigiCam program. The image was then imported into the GIMP an open software graphics program for image editing (https://www.gimp.org/).

Field Experimental Design

The field trial was set up as randomized complete block design (RCBD) with four replications. Transgenic events regenerated from transformation experiments were used in the field trial (Figure 7). The dimensions of the trial were 25' x 55'. It was 10 plots wide, with each plot consisting of 1 row, and 4 replications long (Figure 8). Each Plot was 10' long, with 2.5' between plots and 5' between replications. A 10' fallow border surrounded the trial.

ENTRIES	ENTRY NUMBER:
Treatment 1: JackX	104; 205; 308; 408
Treatment 2: Prp17 T2 P6 01 (RNAi control)	108; 209; 301; 409
Treatment 3: Chamber (CHITIN)	101; 204; 303; 407
Treatment 4: Piping (LAC2)	107; 206; 310; 406
Treatment 5: Pizza (LAC2)	103; 207; 304; 405
Treatment 6: Carpel (CYTOP450)	105; 203; 305; 402
Treatment 7: Coup (CYTOP450)	110; 201; 309; 401
Treatment 8: Carpel (CYTOP450)	106; 208; 302; 410
Treatment 9: Coup (CYTOP450)	102; 210; 306; 403
Treatment 10: Chamber (CHITIN)	109; 202; 307; 404

Figure 7. Entries used in the randomized complete block design (RCBD) field trial.
JackX and Prp17T2P601 were the non-transgenic and null control lines respectively. Piping and Pizza denote two different Lac2 lines. Carpel and Coup denote two different CytoP450 lines.

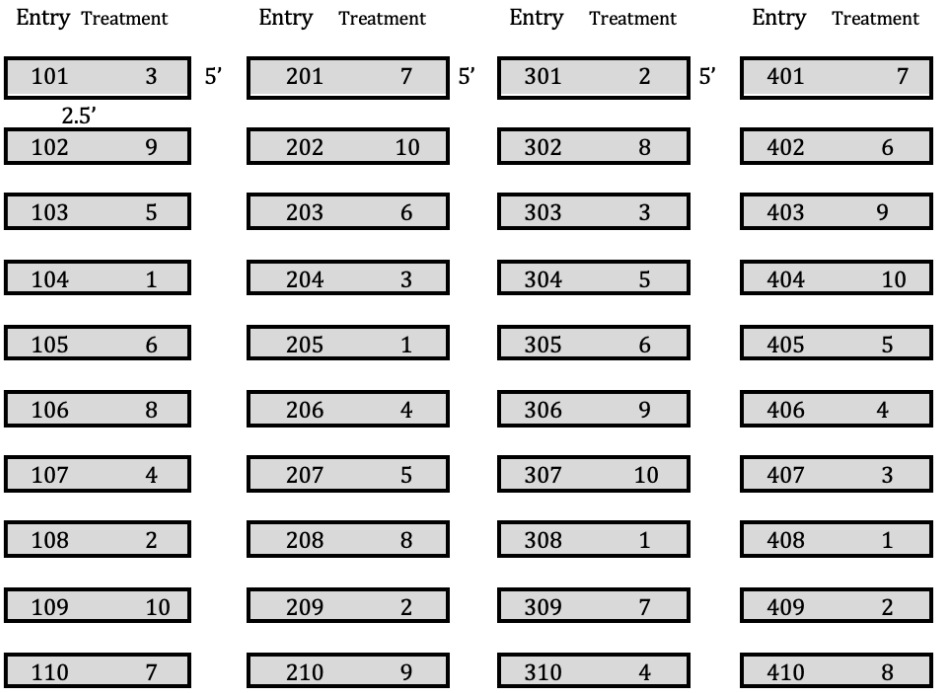

Figure 8. The field trail set up of the Randomized complete block design experiment. Each entry row was 10 feet long and 2.5 feet between entries and 5 feet between blocks.

The trial was planted on June 3rd, 2022. A garden hoe was used to open a shallow 10' long furrow in each plot, and 70 seeds were planted per plot and covered gently. Approximate seed spacing was 1 seed per 1.75". Plots were maintained through hand-weeding between rows and tilling between replications.

Field Sampling

Before sampling, 8 plants per plot were tagged with tape colors corresponding to their treatment. Each tag had a plot number and a plant letter from A through H (for example: 101A). Plants were chosen at random. Leaf tissue samples were collected from the field on August 9th, 2022. Treatments 3 through 10 were sampled for DNA extraction. In each plot, 8 samples were collected, which added up to 64 samples per block and 256 samples total. Samples were

collected in microcentrifuge tubes with the appropriate size and number of beads for later grinding. Depending on the size, ½ to 1 whole trifoliate leaf was collected from the newest emerged trifoliate; approximately 100 mg of tissue was collected. Once collected, each sample was immediately placed in liquid nitrogen. Samples were transported to the lab and place in –80 °C for storage.

After DNA extractions and PCRs to test for transgene presence were completed, whole plant samples were collected from the field trial for stem splitting. Three PCR positive plants per plot, as well as 3 random plants per control plot, were sampled by using hand pruners to clip the base of the plant as close to the ground as possible. Samples were collected by treatment and bagged together.

Data Collected and Analysis

Once whole plant samples were collected, stems were split to gather *Dectes* larval tunneling data. The main stem of each plant was split, and data on whether there was a tunnel in the main stem, if there was a tunnel in the bottom 6 inches of the main stem, or if the tunnel reached the base of the stem was collected.

The stem tunneling data was analyzed using the Statistical Analysis System (SAS). The generalized linear mixed models theory (PROC GLIMMIX) was used. When making comparisons in differences of LS-means, the Dunnet adjustment (ADJUST=DUNNETT) was used. PROC GLIMMIX performs all pairwise differences between treatments, so the ADJUST=DUNNET specification will analyze differences with one control. The statistical model used was $yijk = n + Bi + \alpha j + B\alpha ij + eijk$, where B = i^{th} block, α = Trt = j^{th} row, Bα = Block * Row interaction, and e = residual = k^{th} measurement from the j^{th} row in the i^{th} block.

Chapter 3 - Results and Discussion

Greenhouse Trial

Due to the sensitive nature of the insect, transport and release into the greenhouse environment caused variable adult mortality. This was shown through highly variable oviposition levels between plants. There were 0-5 oviposition scars in 24 out of the 43 plants, 6-15 oviposition scars in 6 out of 43 plants, and 16+ oviposition scars in 13 out of the 43 plants. Over half of the plants had very low levels of oviposition scarring. Keeping in mind that not all eggs laid will hatch, tunneling data was inconsistent, and a trial in the insect's natural, field environment appeared to be the more logical route.

Field Trial: PCR for Clone Detection

Due to the use of seeds from T_2 generation soybean plants, a 3:1 gene segregation ratio, or 75% plants positive for transgene presence, was expected from the samples. For treatment 3, dsChSy2, 25 out of the 32 tested plants were positive for transgene presence; 78.1% of the plants were positive for transgene presence (Figure 9). For treatment 6, dsCYP9E, 12 out of the 32 tested plants were positive for transgene presence; 37.5% of the plants were positive for transgene presence (Figure 10). This is much lower than the 3:1 expected gene segregation ratio and could be due to the random selection of seeds planted and plants chosen for sampling. For treatment 7, dsCYP9E, 22 out of the 32 tested plants were positive for transgene presence; 68.8% of the plants were positive for transgene presence (Figure 11). For treatment 8, dsCYP9E, 25 out of the 32 plants were positive for transgene presence; 78.1% of the plants were transgenic (Figure 12). For treatment 9, dsCYP9E, 27 out of the 32 tested plants were positive for transgene presence; 84.4% of the plants were positive for transgene presence (Figure 13). For

treatment 10, dsChSy2, 22 out of the 32 tested plants were positive for transgene presence; 68.8% of the plants were positive for transgene presence (Figure 14). Overall for treatments 3, 7, 8, 9, and 10, the 3:1 gene segregation ratio was shown through the PCR analyses.

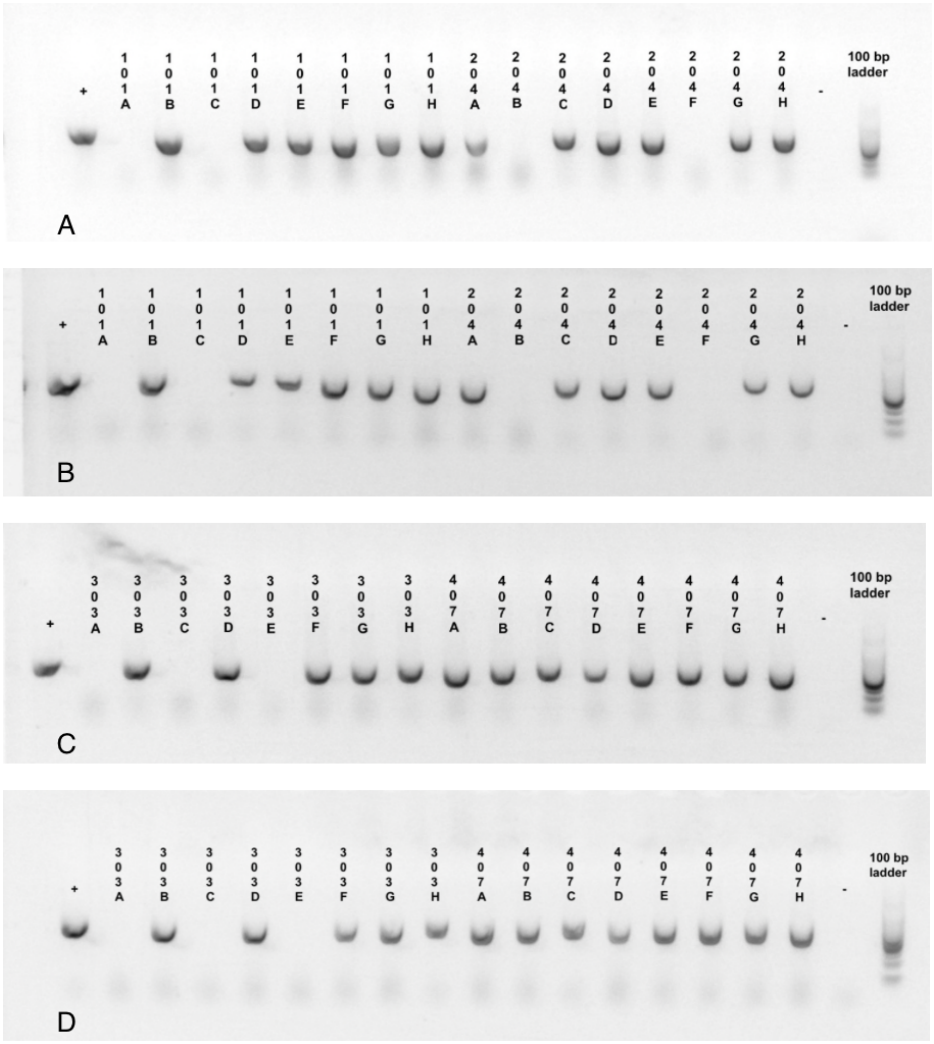

Figure 9. PCR analysis of individual field plot plants for dsChSy2 events, treatment 3.

(A) Samples taken from plot 101 plants A-H and plot 204 plants A-H using gusF1/ DChtn_R primer pair. **(B)** Samples 101 A-H and 204 A-H using gusR2/DChtn_R primer pair. **(C)** Samples 303 A-H and 407 A-H using gusF1/DChtn_R primer pair. **(D)** Samples 303 A-H and 407 A-H using gusR2/DChtn_R primer pair.

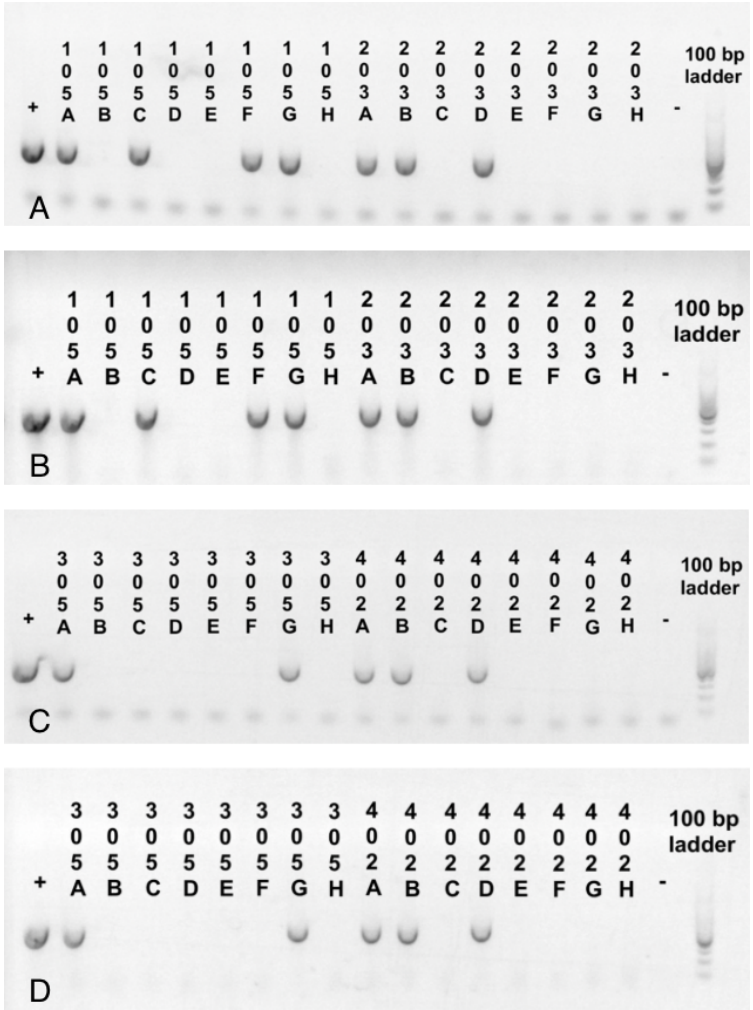

Figure 10. PCR analysis of individual field plot plants for dsCYP9E events, treatment 6.

(A) Samples taken from plot 105 plants A-H and plot 203 plants A-H using gusF1/ DtCP8E2-RP primer pair. **(B)** Samples 105 A-H and 203 A-H using gusR2/ DtCP8E2-RP primer pair. **(C)** Samples 305 A-H and 402 A-H using gusF1/ DtCP8E2-RP primer pair. **(D)** Samples 305 A-H and 402 A-H using gusR2/DtCP8E2-RP primer pair.

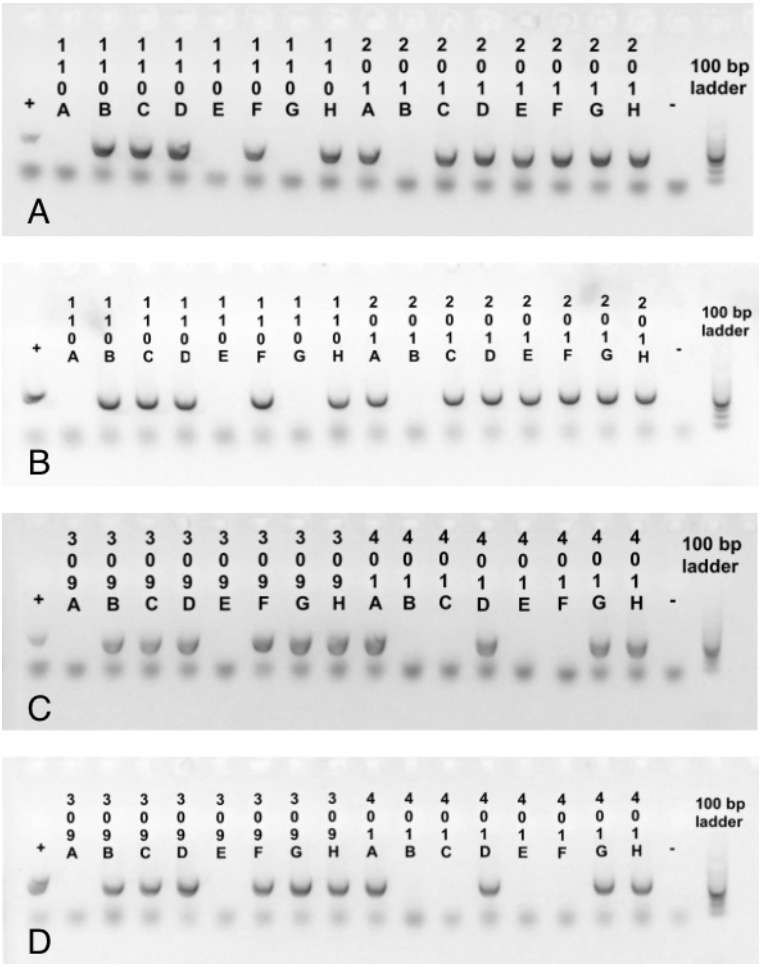

Figure 11. PCR analysis of individual field plot plants for dsCYP9E events, treatment 7.

(A) Samples taken from plot 110 plants A-H and plot 201 plants A-H using gusF1/ DtCP8E2-RP primer pair. **(B)** Samples 110 A-H and 201 A-H using gusR2/ DtCP8E2-RP primer pair. **(C)** Samples 309 A-H and 401 A-H using gusF1/ DtCP8E2-RP primer pair. **(D)** Samples 309 A-H and 401 A-H using gusR2/DtCP8E2-RP primer pair.

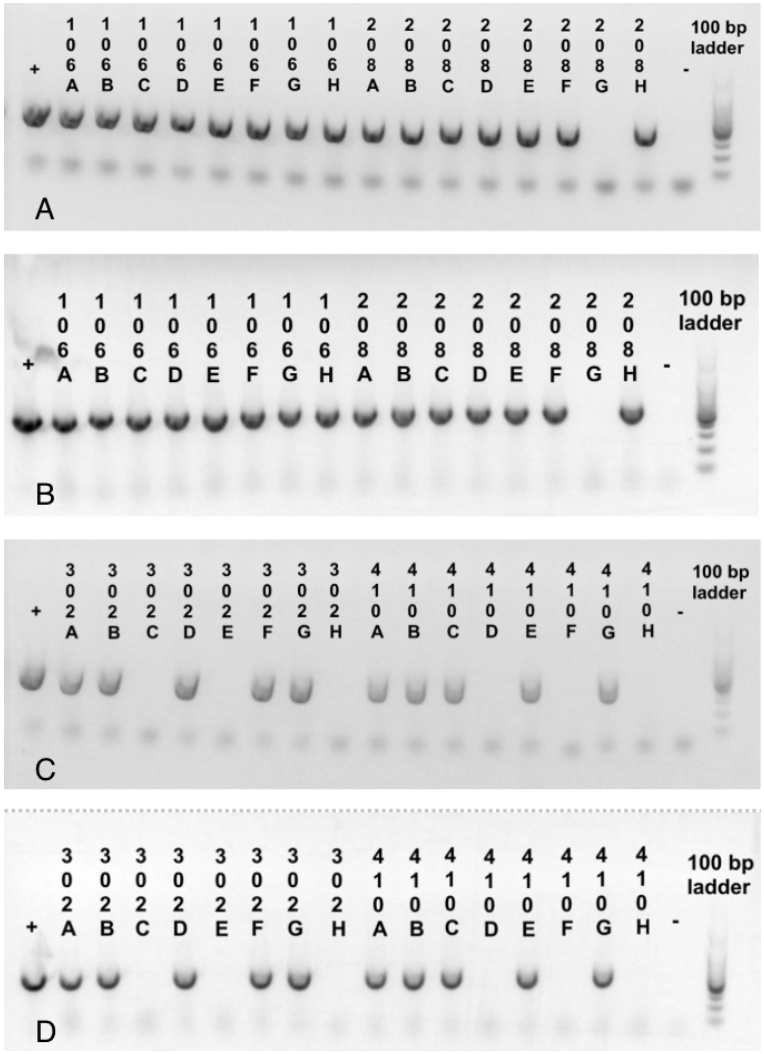

Figure 12. PCR analysis of individual field plot plants for dsCYP9E events, treatment 8.

(A) Samples taken from plot 106 plants A-H and plot 208 plants A-H using gusF1/ DtCP8E2-RP primer pair. **(B)** Samples 106 A-H and 208 A-H using gusR2/ DtCP8E2-RP primer pair. **(C)** Samples 302 A-H and 410 A-H using gusF1/ DtCP8E2-RP primer pair. **(D)** Samples 302 A-H and 410 A-H using gusR2/DtCP8E2-RP primer pair.

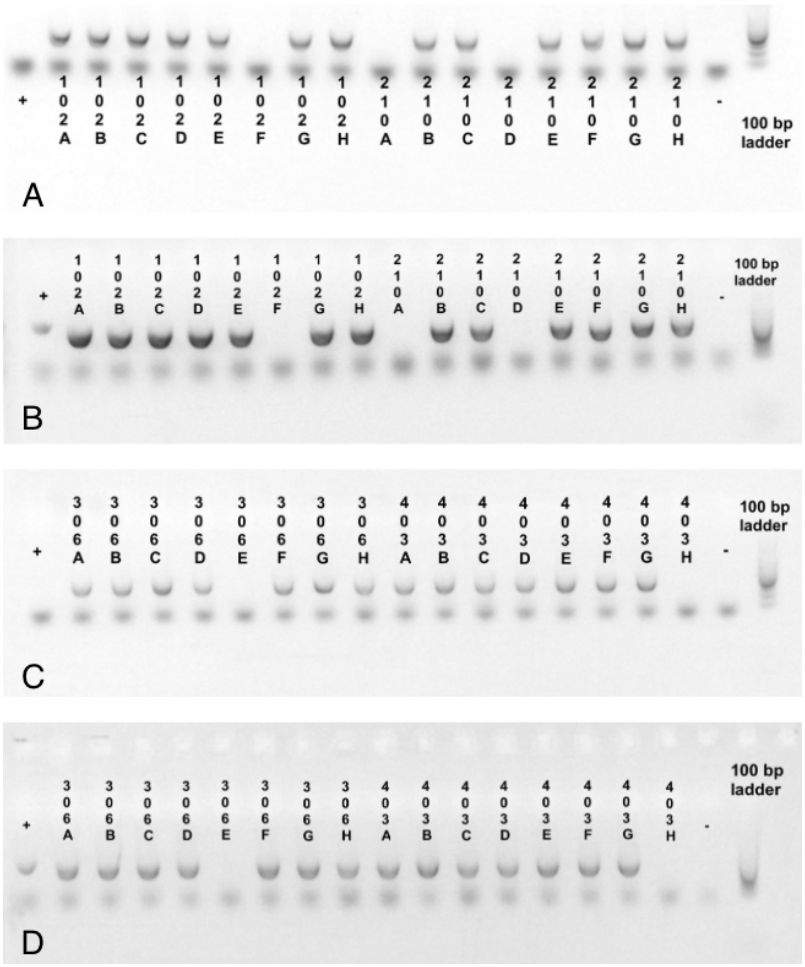

Figure 13. PCR analysis of individual field plot plants for dsCYP9E events, treatment 9.

(A) Samples taken from plot 102 plants A-H and plot 210 plants A-H using gusF1/ DtCP8E2-RP primer pair. **(B)** Samples 102 A-H and 210 A-H using gusR2/ DtCP8E2-RP primer pair. **(C)** Samples 306 A-H and 403 A-H using gusF1/ DtCP8E2-RP primer pair. **(D)** Samples 306 A-H and 403 A-H using gusR2/DtCP8E2-RP primer pair.

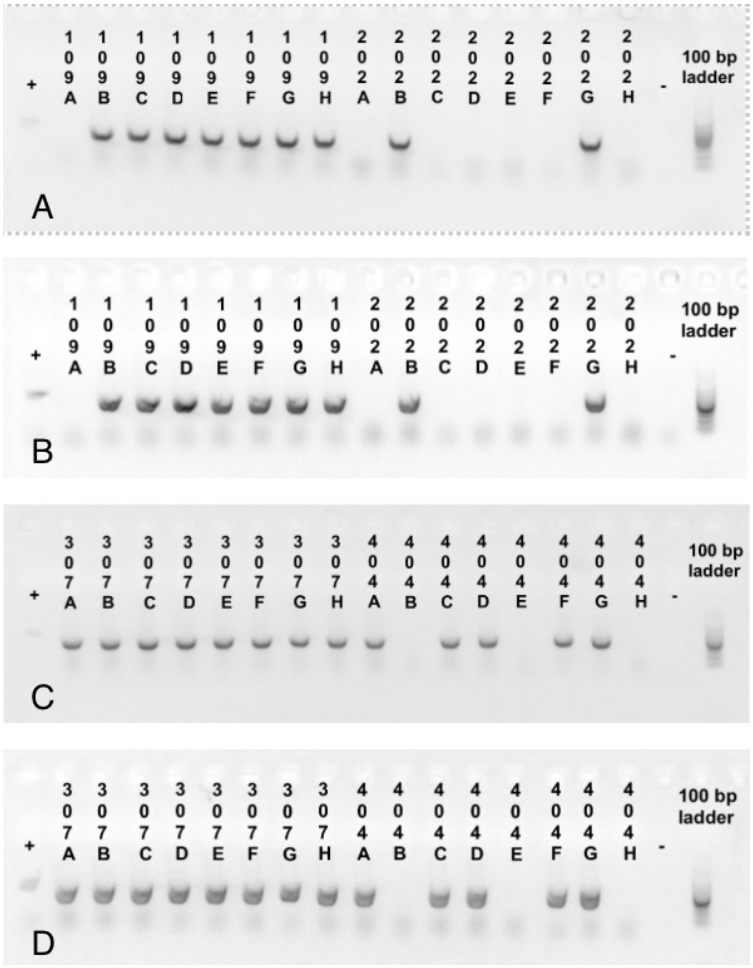

Figure 14. PCR analysis of individual field plot plants for dsChSy2 events, treatment 10. **(A)** Samples taken from plot 109 plants A-H and plot 202 plants A-H using gusF1/ DChtn_R primer pair. **(B)** Samples 109 A-H and 202 A-H using gusR2/DChtn_R primer pair. **(C)** Samples 307 A-H and 404 A-H using gusF1/DChtn_R primer pair. **(D)** Samples 307 A-H and 404 A-H using gusR2/DChtn_R primer pair.

The PCRs for transgene presence for treatments 4 and 5, dsLac2, showed no positive transgene amplification. After multiple replications, treatments 4 and 5 still showed no positive transgene amplification, as there were no positive bands on the gels. When looking at research the research done by Lina Rojas (Rojas, 2019), the Lac2 gene was a very promising RNAi target. It was determined that these plants were escapes and not transgenic. Analysis of the efficacy of dsLac2 through HIGS was not able to be tested.

Field Trial: Growth Chamber PCR for Clone Detection

Plants in the field trial were senescing and losing leaves before more sampling could take place, so plants were grown in the growth chamber to use for rtPCR analysis. Plants 1-4 were grown from dsChSy2 seed stock, the same that were planted in the field. Only 4 plants were planted due to minimal seed left over from the field trial. Plants 13-30 were grown from dsCYP9E seed stock, the same that were planted in the field. Plants 5-12 were grown from dsLa2 seed stock, the same that were planted in the field; however, these plants were excluded from the analysis as they were determined to be not transgenic.

As with the field samples, the PCRs of the growth chamber samples showed a similar 3:1 positive to negative gene segregation ratio. For dsChSy2, 3 out of the 4 tested plants were positive for transgene presence; 75% of the plants were positive for transgene presence. For dsCYP9E, 11 out of the 18 tested plants were positive for transgene presence; 61.1% of the plants were positive for transgene presence. The plants positive for transgene presence were tagged for later RNA extraction and rtPCR analysis.

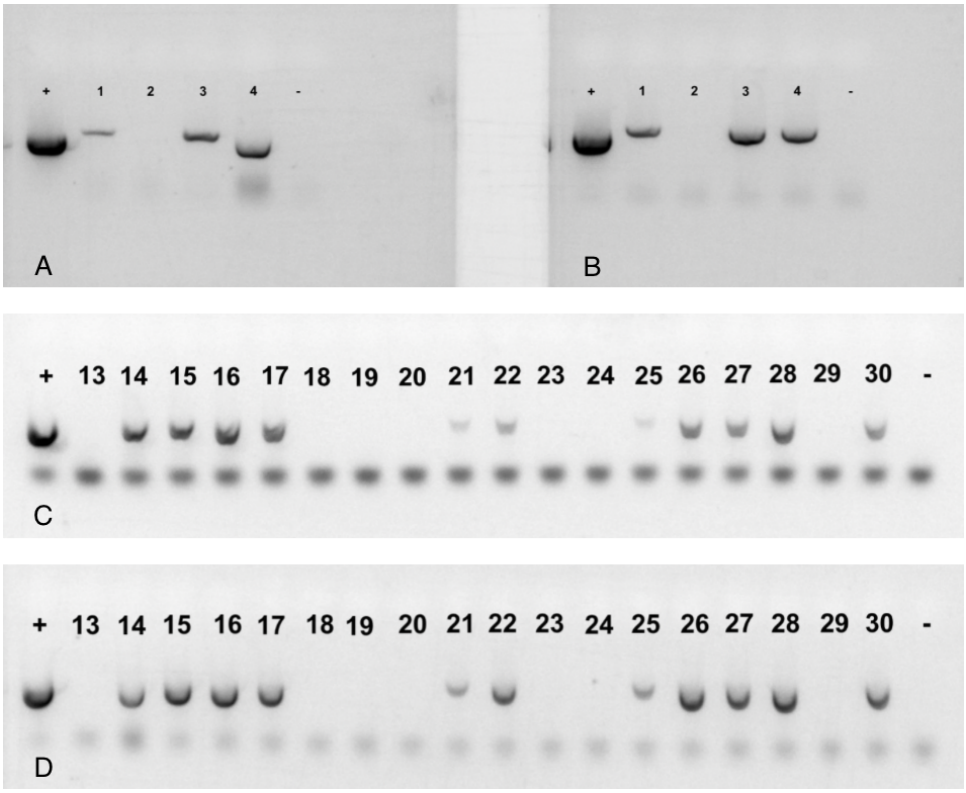

Figure 15. PCR analysis of individual growth chamber plants for dsChSy2 events and dsCYP9E events.

(A) Samples taken from plants 1-4 using gusF1/ DChtn_R primer pair. **(B)** Samples 1-4 using gusR2/DChtn_R primer pair. **(C)** Samples 13-30 using gusF1/ DtCP8E2-RP primer pair. **(D)** Samples 13-30 using gusR2/ DtCP8E2-RP primer pair.

Field Trial: Growth Chamber rtPCR

The plants grown in the growth chamber found positive for transgene presence were sampled again for RNA extraction. As with other samples, these samples were collected from the newest trifoliate on the plant; approximately 100 mg of tissue was collected. After RNA extraction, the samples were converted to cDNA and an rtPCR was performed.

Since actin is a highly conserved protein that is constitutively expressed in proteins, actin primers were used to make sure the cDNA made was high-quality. All cDNA samples showed strong positive bands, and these cDNA samples were compared to genomic DNA (Figure 16A).

Since the genomic DNA sample still contained introns, and the cDNA samples had the introns spliced out, the genomic DNA sample did not move as far on the gel due to its weight. The cDNA appeared to be high quality.

When the cDNA samples were amplified with their respective transgene primers, there were varying levels of gene expression. The samples containing the dsChSy2 gene, samples 1-3, showed strong positive bands inferring high levels of transgene expression (Figure 16B). On the other hand, the samples containing the dsCYP8E gene, samples 4-16, showed much weaker positive bands; dsCYP8E transgene expression was highly variable (Figure 16B). This variable gene expression in dsCYP8E plants could affect the efficacy of RNAi against *Dectes* stem borer larvae.

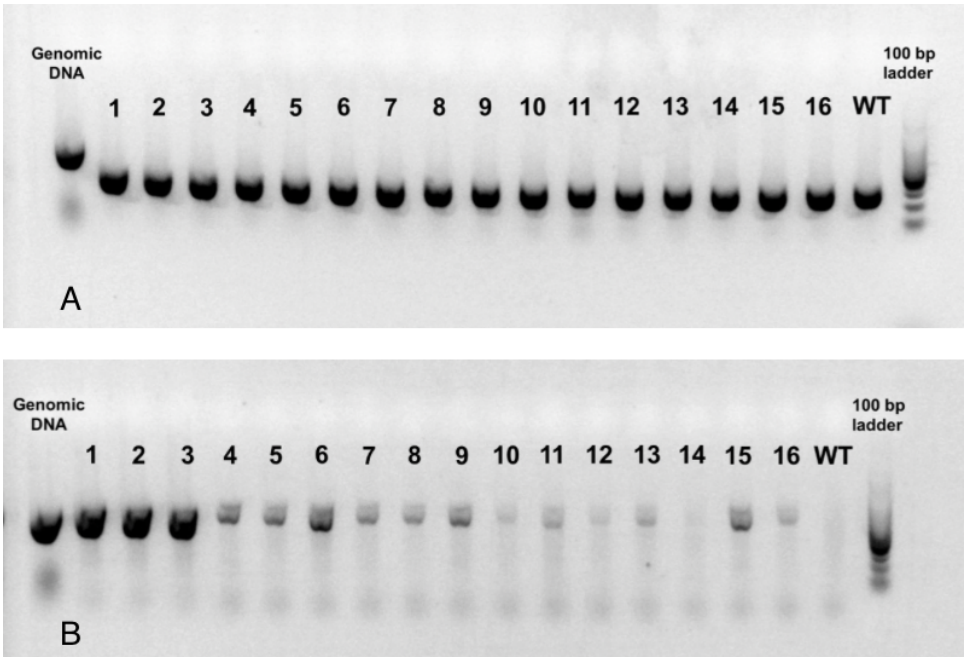

Figure 16. Gels run with treatment rtPCR products.

(A) Samples 1-3, dsChSy2, and samples 4-16, dsCYP8E, using Actin primers. **(B)** Samples 1-3, dsChSy2, and samples 4-16, dsCYP8E, using respective transgene primers.

Field Trial: Stem Tunneling Analysis

Once it was determined which plants in the field trial were positive for transgene presence, three plants from each plot were randomly chosen for stem splitting. The three plants from each plot were cut at the base as close to the ground as possible. The plants from all 4 replications, since they were tagged by plot number and plant letter, could be bagged together for stem splitting. Working treatment by treatment, stems were split from the top of the main stem down to the base. Data was gathered on whether any tunneling was present in the main stem, if tunneling reached the bottom six inches of the main stem, and if the tunneling reached the base of the main stem. All data was scored either yes or no, therefore it was analyzed as binary data.

Figure 17. Stem tunneling examples.

(A) Stem with no tunneling. **(B)** Stem with tunneling to the lower 6 inches of the stem but not reaching the base. **(C)** Stem with tunneling down to the base.

When statistically compared to each other, the mean main stem tunneling of treatment 1 (control) was not significantly different than the mean main stem tunneling of treatment 2 (RNAi control) (Table 2). No differences were expected between the two control treatments, as the null RNAi construct has no target sequence in *Dectes*. When treatments 3 and 10 (dsChSy2) were compared to treatment 1, there were no statistically significant differences between the main stem tunneling means of the transgenic lines and the main stem tunneling mean of the control (p-value > 0.05). When treatments 6, 7, 8, and 9 (dsCYP8E) were compared to treatment 1, there

were no statistically significant differences between the main stem tunneling means of the transgenic lines and the main stem tunneling mean of the control (p-value > 0.05).

Table 2. Results of the statistical analysis comparing main stem tunneling presence in the transgenic lines to the control line.

Main Stem Tunneling Analysis

Control Line	Treatment	Pr>|t|
1	**2 (RNAi null)**	**1.0000**
1	**3 (ChSy2)**	**0.9840**
1	**6 (CYP8E)**	**0.9828**
1	**7 (CYP8E)**	**0.9829**
1	**8 (CYP8E)**	**0.9834**
1	**9 (CYP8E)**	**0.9834**
1	**10 (ChSy2)**	**0.9828**

When statistically compared to each other, the mean six-inch stem tunneling of treatment 1 (control) was not significantly different than the mean six-inch stem tunneling of treatment 2 (RNAi control) (Table 3). No differences were expected between the two control treatments. When treatments 3 and 10 (dsChSy2) were compared to treatment 1, there were no statistically significant differences between the six-inch tunneling means of the transgenic lines and the six-inch tunneling mean of control (p-value > 0.05). When treatments 6, 7, 8, and 9 (dsCYP8E) were compared to treatment 1, there were no statistically significant differences between the six-inch stem tunneling means of the transgenic lines and the six-inch stem tunneling mean of the control (p-value > 0.05).

Table 3. Results of the statistical analysis comparing presence of tunneling in the bottom six inches of the stem in the transgenic lines to the control line.

Six Inches Tunneling Analysis

Control Line	Transgenic Line	Pr>|t|
1	**2 (RNAi null)**	**0.9737**
1	**3 (ChSy2)**	**0.9737**
1	**6 (CYP8E)**	**0.9728**
1	**7 (CYP8E)**	**0.9711**
1	**8 (CYP8E)**	**0.9745**
1	**9 (CYP8E)**	**0.9724**
1	**10 (ChSy2)**	**0.9728**

When statistically compared to each other, the mean base stem tunneling of treatment 1 (control) was not significantly different than the mean base stem tunneling of treatment 2 (RNAi control) (Table 4). No differences were expected between the two control treatments. When treatments 3 and 10 (dsChSy2) were compared to treatment 1, there were no statistically significant differences between the base tunneling means of the transgenic lines and the base tunneling mean of the control (p-value > 0.05). When treatments 6, 7, 8, and 9 (dsCYP8E) were compared to treatment 1, there were no statistically significant differences between the base stem tunneling means of the transgenic lines and the base stem tunneling mean of the control (p-value > 0.05).

Table 4. Results of the statistical analysis comparing presence of tunneling to the base of the stem in the transgenic lines to the control line.

Base of Stem Tunneling Analysis

Control Line	Transgenic Line	Pr>|t|
1	**2 (RNAi null)**	**0.9737**
1	**3 (ChSy2)**	**0.9373**
1	**6 (CYP8E)**	**0.9714**
1	**7 (CYP8E)**	**0.9711**
1	**8 (CYP8E)**	**0.9711**
1	**9 (CYP8E)**	**0.9724**
1	**10 (ChSy2)**	**0.9714**

There were no statistically significant differences found between any of the treatments compared to the empty control on any of the data points collected. There are a few possible reasons for this observation. First, the sample size analyzed was rather small, as it was only 12 plants per treatment. With a larger sample size, it is possible that more of a difference between treatments would have been shown. Another possible reason for the lack of statistical differences is the variable expression of the transgenes. It was shown through rtPCR analysis that the transgenes, particularly dsCYP8E, were expressing at different levels between plants. Plants expressing the transgene at much lower levels could have much smaller of an effect on *Dectes* stem borer larvae. It is plausible that a threshold of siRNA is required to demonstrate an effect on *Dectes* larvae feeding on the transgenic lines. Results from experiments with host-derived gene silencing of parasite fitness genes of soybean cyst nematodes suggest that a threshold of small interfering RNA molecules are needed to down regulate target genes (Tian et al. 2019). To confirm this hypothesis one would need to isolate mRNA from individual stem borer larva and perform real time PCR on the target genes. A housekeeping gene would be used as an internal control to compare larval feeding on control and transgenic events. Previous

research (Bolognesi et al, 2012) showed that a sufficient concentration of dsRNA as well as a long exposure time is necessary for RNAi to be effective. It is possible that the transgenic plants used in the field did not produce a high enough level of dsRNA to affect the *Dectes* larvae significantly.

To show if RNAi can be an efficacious control method against *Dectes* stem borer, additional research is required. It would be helpful to work with a much larger trial and larger sample sizes. Also, all samples in this trial were taken from leaves. Analysis of transgene expression in stems compared to transgene expression in leaves would show whether larvae are consuming dsRNA or not. Although a constitutive promoter was driving gene expression and previous reports have demonstrated the 35SCaMV promoter does express in stems, expression does vary between events. Leaves and stems could have different levels of transgene expression. A visual comparison between larvae found in transgenic stems and larvae found in control stems could reveal any morphological differences due to dsRNA consumption. Even more telling, though, would be a real-time PCR analysis of the larvae themselves. The real-time PCR would show whether there was a downregulation of the genes being targeted by the RNAi.

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