ABSTRACT

CHU, FU-CHYUN. Developing Transposon Helper Strains for Two Agriculture Pests; Western Corn Rootworm and Spotted Wing Drosophila. (Under the direction of Dr. Marcé Lorenzen and Dr. Fred Gould).

Both western corn rootworm (WCR), *Diabrotica virgifera virgifera*, and spotted wing drosophila (SWD), *Drosophila suzukii*, are invasive species that have become important agricultural pests around the world. WCR is a major pest of maize that has invaded every corn-growing region in the US, and has shown a great ability of adapting to different control methods. Unlike WCR, SWD is a recently-introduced invasive species with a wide range of hosts including many important crops, which makes the control of this pest very complicated. A lack of basic molecular research has slowed down not only progress in understanding these two pests, but also the development of novel control methods. The goal of this dissertation is to develop molecular tools using transgenic techniques in these pest species. To do this, I created and established transgenic "helper strains" (i.e. express *piggyBac transposase*) for both pests. These strains will help in future transposon-based experiments and could open the door for more functional genetic research, or the development of genetic pest management based control methods in the future.

I report the first germline transformation of WCR in chapter 1, with detailed tests of the newly created transgenic strains. The transgenic WCR were produced using a *Minos* transposable element carrying both a marker gene and a *piggyBac transposase* gene. During the course of this work we developed a protocol for using digital-droplet PCR to ascertain the number of *Minos* elements integrated into a beetles' genome. Moreover, I cloned and sequenced at least one side of each insertion-site junction from all single insertion strains,

identifying nine unique insertion-sites generated from this experiment. Finally, I confirmed *piggyBac transposase* expression through reverse-transcription PCR for each of the different strains. These strains will serve as helper strains for future transformation-based experiments.

In Chapter 2, I describe the microinjection protocol for WCR and the rearing system I designed and optimized to fit my experimental needs. Although WCR has been reared in the lab for decades, the efficiency of the standard system is low. In fact, many molecular labs request samples for short-term experiments rather than establishing their own colonies. The optimized rearing system I developed will help in setting up stable, small-scale colonies that require very little space. I also discuss related experimental setups, like single-pair crosses, rearing and screening methods in this chapter.

SWD transgenesis experiments are described in Chapter 3. I created three helper strains of SWD, and tested *piggyBac transposase* expression levels in each, along with their transformation efficiencies. These results indicate that one strain (H7) is highly efficient, with a transformation rate of 80%. I further tested the H7 strain for its ability to integrate *piggyBac* elements of different sizes and demonstrated that this strain retains high efficiency, even with large inserts (10kb). Finally, I tested the ability of the H7 strain to remobilize an already integrated *piggyBac* element by hybridizing H7 with different "Donor strains", and achieved remobilization rates of 6% to 25.6%.

Finally, I conclude my work for this dissertation in the Conclusions Chapter by presenting results from other experiments which were based on the work of preceding chapters, along

with some future research directions.

Developing Transposon Helper Strains for Two Agriculture Pests; Western Corn Rootworm and Spotted Wing Drosophila

by
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BIOGRAPHY

Fu-Chyun "Clay" Chu is an international graduate student from Taipei, Taiwan and has been an insect enthusiast since elementary school, when he first discovered the beauty and diversity of beetles. In 2007 Clay received a bachelor's degree from the Department of Animal Science, National Ilan University, Ilan, Taiwan. Although Clay's undergraduate research project was on the molecular genetics of swine, his real passion was, and remains, entomology. After Clay served his mandatory year in the Taiwanese Army, he set his sights on graduate school. He knew he wanted to combine his two loves, entomology and molecular biology. NC-State's Entomology Department, and more specifically, the Genetic Pest Management Program, offered a perfect fit. Clay joined the Lorenzen lab in 2010 and has been involved in a wide variety of transgenic-based projects. He was awarded a Southern SARE Graduate Student Grant for developing a novel transgenic control strategy for *Drosophila suzukii* in 2014. In addition to his molecular skills, he also has expertise in insect rearing and serve as "bug keeper" for NCSU Entomology Outreach Program in 2015.

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CHAPTER 1

Introduction of western corn rootworm, *Diabrotica virgifera virgifera,* and spotted-wing drosophila, *Drosophila suzukii*

Impact of invasive species on agriculture

It is estimated that there are over 50,000 non-native species in the US today (Pimentel et al., 2000). Whether an invasive species has been in the US for many years, or is a relative newcomer, it can have a significant impact on native ecosystems and managed crops. For example, the economic damages associated with crop losses due to invasive species are estimated to be on the order of 13 billion dollars per year (Pimentel et al., 2000). My dissertation work focuses on two of these non-native agricultural pests, the western corn rootworm, *Diabrotica virgifera virgifera*, and spotted wing drosophila, *Drosophila suzukii*. In this chapter I will provide background on their arrival in the US, their pest status, biology, economic impact, and current measures for controlling them on the farm. I will also discuss how genetic and genomic tools could be used to better understand these pests, and how my work fits into these studies.

Pest status of western corn rootworm

Western corn rootworm (WCR), is originally from Guatemala, Central America (Melhus et al., 1954). Although WCR was observed in the US as early as 1865 and described by LeConte in 1868, WCR wasn't reported as causing significant damage in maize until the 1940s (Wangila et al., 2015). WCR continued to expand its range, and is now a major pest of maize in North America and Europe (Kaster & Gray, 2005; Kiss et al., 2005). In North America, maize cultivation is concentrated in the Midwestern United States (South Dakota, North Dakota, Indiana, Ohio, Wisconsin, Michigan, and Kentucky) and Canada (southern, central and eastern Ontario, and southern Quebec), a region frequently referred to as the Corn

Belt. WCR has been a major pest in the US for several decades (Gray et al., 2009), but was first introduced in Europe more recently, around early 1990. It soon adapted to the European maize growing system and rapidly spread to most of the countries in early 2000 (Serbia, Greece, Bulgaria, Romania, Ukraine, Poland, Slovakia, Hungary, Croatia, Bosnia & Herzegovina, Montenegro, Slovenia, Italy, Austria, Czech Republic, Switzerland, Germany, France, United Kingdom) (Gray et al., 2009). Global cost of both crop loss and management of this pest totals in the billions of dollars annually.

Biology of WCR

In most maize-growing areas, WCR has one generation per year. They diapause as eggs in soil to overwinter, and hatch around late May to early June. WCR larvae feed on corn roots throughout their three larval instars (Fig. 1.1). Thus, this is the main life stage that damages crops. Late 3rd-instar larvae will stop feeding and start wandering to find a spot in the soil for pupation. The pupal stage lasts between one to two weeks, with adults eventually emerging around July through August. WCR adults are generalists that feed on plant tissue, leaves, flower pollen, and fruits. Adult females will feed for two weeks after emerging to become mature and start laying eggs.

Chemical control and resistance

Since most WCR damage is caused during the larval stage, soil-treatment insecticides were developed to control root-feeding larvae. However, WCR started showing resistance to the major chemical control methods around two decades after they were introduced to fields.

Cyclodiene insecticides were commonly used from the late 1940s to the early 1960s (Ball & Hill, 1952; Ball & Roselle, 1954; Muma et al., 1949). The first resistance appeared in Nebraska in 1959 (Roselle et al., 1959). Another group of chemicals, organophosphates and carbamate insecticides, which have completely different modes of actions were then used for either soil treatment or adult spray beginning in the 1970s (Pruess et al., 1974). However, resistance developed in WCR in the mid-1990s (Meinke et al., 1998; Wright et al., 1996). Moreover, after corn farmers had stopped using cyclodiene insecticides for decades, and most organophosphates and carbamate insecticides had largely been replaced by crop rotation and other control methods (ex, genetically engineered crops) for more than 15 years; resistance to both groups of chemicals still persisted in WCR field populations (Parimi et al., 2006). Since these insecticides have different modes of action and are not used at the same time, it was expected that the organophosphate-resistant population would replace the cyclodiene-resistant population. Also, in the absence of organophosphate usage, we would expect organophosphate-resistance to become less previlent since people stop using these insecticides over 15 years ago. It is surprising that the population still maintains resistance alleles in the population.

Crop rotation and resistance

The major practice of maize growers to control WCR is crop rotation. Annually switching between corn and soybeans successfully reduced WCR populations for more than 50 years in the Corn Belt (Levine et al., 2002). However, in late 1980s, a few cases of corn root injury were discovered and WCR eggs were found in fields planted with soybeans (Levine &

Oloumi-Sadeghi, 1996; Ruesink & Shaw, 1983). Originally, the hypothesis was that the eggs were the result of prolonged diapause of WCR eggs layed in the previous year's cornfield. This was later shown not to be the case by testing hatch rates after prolonged diapause using embryos from several WCR colonies collected from different locations; the hatch rate was lower than 1% after prolonged diapause (Levine et al., 1992). Another hypothesis was that WCR had changed its egg laying behavior, and this was confirmed in the early 1990s. By laying its eggs in soybean fields, WCR had developed behavioral resistance to annual crop rotations (Gray et al., 1998). Further observation confirmed this through large sampling in corn and soybean fields (O'Neal et al., 1999), which found there were more WCR females in soybean fields than in the cornfields, resulting in a higher percentage of root injury in the following year when corn was rotated on the same field. Over the next few years, the same behavior was observed throughout the Corn Belt. WCR has been introduced to Europe and has become widespread across several countries. Fortunately, resistance to crop rotations has not yet been observed in Europe, but this is a major concern, causing farmers to start to developing more comprehensive integrated pest management strategies (Gray et al., 2009).

BT corn and resistance

Besides chemicals and crop rotation for WCR control, genetically engineered (GM) crops have been available for commercial use in cornfields since 2003 (EPA, 2003). The first GM corn, also known as Bt corn, has been engineered to carry a transgene from *Bacillus thuringiensis* (Bt), which expresses the toxin, Cry3Bb1, in the corn roots to control WCR larvae. In 2009, 45% of the corn farmers in the US were growing Bt corn in their field

(James, 2009). Since 2013, there are three different Bt toxins that have been used in corn for WCR control: Cry3Bb1, mCry3A and Cry34/35Ab1. Bt corn provides a high dose of toxin and can kill around 99.99% of the WCR population what feed on it, which creates a high selective pressure, greatly increasing the risk for resistance development (Tabashnik et al., 2008). To slow the development resistance to Bt, a refuge strategy is mandated for cornfields (Gould, 1998). This method involves growing some non-Bt corn in the field to rescue the non-resistant WCR population, delaying the resistance development for generations. Although Bt corn has efficient control, significantly reducing insecticide use in the field, WCR has already begun to overcome control through Bt corn by developing resistance to Cry3Bb1, which was first seen in 2009 (Gassmann et al., 2011). Moreover, the Cry3Bb1-resistant WCR population is also resistant to mCry3A (Gassmann et al., 2014).

WCR is known to be very good at adapting to different pest-control methods and develops resistance rapidly. In fact, most insecticides are ineffective on WCR now due to field-evolved resistance in wild populations, and crop rotation has also been overcome by WCR's behavioral adaptations. Currently, Bt corn and crop rotation are still the major control methods, but the recent development of Bt resistance is a sign of the ultimate failure of this control method, as well. More importantly, Bt resistance was first detected in 2009, however, the mechanism of Bt resistance still remains unknown. A better understanding of resistance development in WCR will require basic research into the genetics and genomics of WCR. My research is aimed at the development of functional genomic tools that will allow us to gain much needed insight into the biology of this devastating agriculture pest, and provide a

means to address questions about of resistance development through studying genes, pathways and/or phenotypes associated with resistance.

Pest status of spotted wing drosophila

Spotted wing drosophila (SWD) is an invasive species in US and Europe that is originally from Asia (Kanzawa, 1935). This Drosophila is distinct from other close species as its larvae only feed on fresh fruits (Fig. 1.2), while most other Drosophila species are yeast-eaters (Sasaki & Sato, 1995). Female SWD developed a serrated ovipositor for breaking through the skins of target fruits, to give hatching larvae access to the meat of the fruit. Most of the agricultural damage SWD cause is to blackberries, blueberries, cherries, peaches, raspberries, strawberries, cranberries, and grapes, but there are more fruits and vegetables in which infestations have been reported (Kanzawa, 1939). The wide range of host food sources, and the egg laying behavior make this fly a problematic pest with zero-infestation thresholds for fresh or processed fruit brought to market in America and Europe (Lee et al., 2011). The first sign in mainland North America of SWD was in 2008 (Walsh et al., 2011), with initial damage seen in strawberries, blueberries, and cranberries for an estimated 20% of yield loss (Bolda et al., 2010). Shortly after, more than 39 states, Canada and some countries in Central America discovered infestations of SWD (Lee et al., 2013). In the US, SWD caused $500 million of damage in western states in 2011 (Goodhue et al., 2011), and $27.5 million in management costs and crop losses in eastern states in 2013 (Burrack, 2014). In Europe, SWD was first seen in Spain and Italy in 2008 (Calabria et al., 2012), rapidly spreading through several countries (France, Austria, Belgium, Bosnia-Hercegovina, Croatia, Czech Republic,

Germany, Hungary, Netherlands, Portugal, Russian, Serbia, Slovenia, Spain, Switzerland, and UK) from 2008 to 2011 (Cini et al., 2012). In Italy, the crop losses on blackberries, blueberries, and raspberries were around 30-40%. The European and Mediterranean Plant Protection Organization (EPPO) concluded that the economic consequences by SWD are high (Cini et al., 2012). This decision is base on there are wide range of host plants for this pest and many of them are important crops. Also, SWD can survive in wide range of environmental condition with short life cycle and high ability for spreading out. There are some crops are not reported to be the host of SWD, but remain the potential this pest could adapt to host on those crops.

Biology of SWD

At 25°C, SWD complete their lifecycle around 10-12 days. Each female fly can lay more than 300 eggs in her lifetime, and eggs typically hatch within 2 days of being laid (Kanzawa, 1939). SWD go through 3 larval stages (5-7 days) before pupating. After 4-5 days, pupae eclose to adults and live for few weeks. Female flies need 4-5 days to become mature and start laying eggs. In warm weather, SWD can have as many as 13 generations per season. In winter, SWD usually has a smaller population but can survive through continuous generations in non-crop habitats during cold weather due to their adaption to a variety of food sources.

Control of SWD

Cultural practices and insecticides are the major management tools used in the control of

SWD. For different groups of insecticides, bioassays testing members of the pyrethroid, organophosphate, and spinosyn classes, as well as methomyl in the carbamate class have proven these chemicals to be effective against SWD (Beers et al., 2011; Bruck et al., 2011). However, azadirachtin and organic pyrethrins are not efficient, making SWD control more challenging for organic farmers. Although some insecticides have been reported to be efficient for SWD control (Beers et al., 2011; Haviland & Beers, 2012; Van Timmeren & Isaacs, 2013), management programs are complicated by the need to target a variety of SWD life stages, on different crop fields. Non-crop hosts add to that complexity by providing the population escape from control methods (Lee et al., 2015). Culture practices also play an important role in reducing SWD damage. Remove attractants, like ripe fruits, from the field, and reducing available water sources could lower the chances of infestation. Constant sampling and use of traps to monitor the field are also suggested (Burrack et al., 2015; Lee et al., 2012). Biological control could eventually play a part in SWD integrated pest management, but there isn't yet enough evidence to support a good level of biological control of SWD (Asplen et al., 2015).

Insect molecular and genetic studies

Drosophila melanogaster, arguably the best-studied insect species, has a long history of molecular and genetic studies. It has been used as a model organism for over a century. Although the *D. melanogaster* genome was not published until 2000 (Adams et al., 2000), much was already known about its biology because genetic studies had been conducted on it since the early 20th century, and molecular genetic studies since the late 1970s. Cutting-edge

research performed on *D. melanogaster* greatly improved our understanding of basic genetic and molecular concepts, gave rise to a wealth of knowledge and resulted in several great scientists being awarded Nobel Prizes (Roberts, 2006). Many of these ground-breaking studies employed transgenic strains. Taken together, thousands of transgenic strains were created for use in a wide range of functional genomic studies, from behavioral to medical, and almost every kind of experiment purposes (Gonzalez, 2013; Jennings, 2011; St Johnston, 2002). Clearly having analogous tools for use in WCR and SWD would aid our understanding of their basic biology and a mechanism to assess resistance development.

In the field, pest-control measures put high-selection pressure on insects; as a result, individuals possessing beneficial mutations have a better chance to survive and mate, eventually these resistant insects will replace the susceptible pest population thereby causing the control measure to fail. Whether resistance is due to detoxification or a behavioral change, understanding of resistance mechanisms will require basic research into pest biology. Sometimes resistance develops through single gene mutations that affect the coding sequence, perhaps changing an amino acid that leads to a change in protein structure or function (Eissenberg et al., 1990), or perhaps results in a premature stop codon. Mutations can also occur in regulatory regions and change expression levels (Furlong et al., 2001). Therefore, genome sequencing can help reveal genes involved in resistance development and may further our understanding of the mechanisms driving these changes. For example, a *D. melanogaster* strain isolated from a field was confirmed to carry a mutation in a gamma-aminobutyric acid (GABA) receptor gene. This mutation caused them to be resistant to

picrotoxinin (PTX) and cyclodiene pesticides (Ffrench-Constant et al., 1993). Later, it was discovered that resistance to PTX and cyclodiene pesticides in an Africa population of *Anopheles funestus* mosquitoes was caused by a mutation in an orthologous GABA receptor gene (Wondji et al., 2011). This example demonstrates how insights gained in one biological system can help inform another, even if the problems arise in very different species and completely different regions. In other words, similar types of resistance and behavioral changes can be the result of mutations in similar genes between species, such as same orthologous gene, or within members of the same gene family.

Currently, farmers are having difficulties adequately controlling WCR and SWD using available control methods, given the potential of these species to adapt and overcome current treatments. However, to really make progress in understanding and controlling these species we will need to develop basic research tools for these pests, but thus far, a lack of molecular and genetic studies on both species is limiting that progress.

Fortunately, the costs associated with genome sequencing are much lower nowadays. The SWD genome has been sequenced, and an annotated assembly is available online (Chiu et al., 2013) and the WCR genome has been sequenced, but is not yet publically available (Dr. Hugh Robertson, personal communications). Both species already have published transcriptomes (Chiu et al., 2013; Kim & Sappington, 2005). These sets of data are very basic, but will prove very useful in studying these two species. For example, they have allowed us to access sequences for identifying orthologs of well-characterized genes, for

studying gene expression dynamics, and finding new genes that haven't been studied before. WCR has a genome size comparable to that of humans, which is around ten times larger than another well-studied coleopteran, *Tribolium castaneum*. WCR's amazing adaptive ability may be explained by secrets hidden in its huge genome. The genome size of SWD, on the other hand, is very similar to a closely-related Drosophila species, *Drosophila melanogaster*. A lot of orthologous genes between these two species are well conserved. Since there is over a century of research into *D. melanogaster* genetics, it is very useful to learn from this important model organism.

People use different tools for functional genomic studies in insects. One of the most common techniques is RNA interference (RNAi) (Fire et al., 1998). RNAi evolved as a cell defense mechanism against double-stranded RNA (dsRNA) viruses (Lodish et al., 2008; Obbard et al., 2009). It is a post-transcriptional gene silencing method that we can use to down-regulate a gene's expression at the mRNA level then screen or test for phenotypic changes. To trigger the RNAi effect, a sequence-specific dsRNA has to be synthesized and delivered into cells. In the cell, the dsRNA will be recognized and chopped by Dicer to many 21-23 bp pieces of small interfering RNA (siRNA). The RNA induced silencing complex (RISC) will use those siRNA to find mRNA in the cell that matches the sequence of the siRNA, then degrade that mRNA to silence gene expression. In insects, there are two different types of RNAi: systemic and non-systemic RNAi. Insects that have systemic RNAi possess a special membrane protein allows siRNAs to travel between cells and spread the RNAi effect throughout the body (Miller et al., 2012). If the insects do not have systemic RNAi, the RNAi

effect will only be observed locally at the place dsRNA is delivered. WCR has systemic RNAi and dsRNA could be delivered by feeding or microinjection; most functional genomic studies in WCR were done using RNAi (Alves et al., 2010; Baum et al., 2007). However, there is no systemic RNAi in SWD and other flies, so most RNAi experiments by injection of dsRNA in flies focus on embryonic development (Elbashir et al., 2001).

Transgenesis is another well-developed method for functional genomic studies. Different from RNAi, which can only be used for down regulation or gene silencing, transgenesis offers a wider variety of uses, such as integration of a foreign gene into the organism's genome. Moreover, parental RNAi is transient, only affects one or two generations, but mutations from transgenesis are heritable, thus are passed to successive generations, making it is more useful for long term studies or creating new strains. Insect mutagenesis experiments begin with using radiation to create mutations and study fitness cost (Crenshaw, 1965; Wallace, 1956), however, it is random and too difficult to control when trying to find desired phenotypes. After transposable elements (TE) were discovered (McClintock, 1950), they were used as tools to modify Drosophila in the early 1980s (Spradling & Rubin, 1982). TEs, like radiation, create random mutations, when they insert into the genome, however they are restricted by their specific recognition sequences. TEs are powerful tools for transgenesis because they can carry cargos of one or multiple genes, which are then integrated into the genome. In the late 2000s, additional methods were developed to facilitate transgenesis in insects; genome editing tools such as Transcription Activator-Like Effector Nucleases (TALEN) and Clustered Regularly Interspaced Short Palindromic Repeats

(CRISPR) (Bassett et al., 2013; Moscou & Bogdanove, 2009), allowed researchers to design the recognition sequences for targeting the genome with nucleases that create double stranded breaks to induce mutations. There are two pathways to repair the DNA after a double-strand break occurs in the cells, Homologous recombination (HR) and non-homologous end joining (NHEJ). If there is a new piece of the DNA flanked by sequence homologous to the target region, the repair might favor HR, enabling us to knock in one or multiple genes, or edit the original sequence. If the cell repairs the DNA through NHEJ, usually an indel (insertion/deletion) mutation will result, possibly knocking out the target gene.

My research focuses on using TEs to create transgenic strains of WCR and SWD. Importantly, current WCR rearing systems were not adequate for the propose of generating and screening transgenic WCR strains, nor were there protocols for embryonic microinjection. Therefore I develop a specialized rearing system for WCR and establish a microinjection protocol for this species. Since, WCR has a relatively long life cycle (~2 months), I focused my efforts on establishing the first transgenic WCR by creating a helper strain that expresses transposase (*piggyBac*); helper strains in the flour beetle had been shown to increase the success rate of successive transformations from 2-3% to around 20% (Lorenzen et al., 2007). Thus, a WCR helper strain could be helpful for future transgenesis studies. For my work with SWD, this fly species has a much shorter life cycle (~10-14 days) with a well-established rearing system. A protocol for embryo microinjection of SWD had also been developed, allowing the creation of transgenic lines using the *piggyBac* transposon

(Schetelig & Handler, 2013). Here I take a further step to create a transposon-based mutagenesis system in SWD. For this system, I created both helper (expressing *piggyBac* transposase) and donor strains (carrying a *piggyBac* insertion), which can be used to set up hybrid crosses that result in remobilization of the insertion to other genomic locations. This system takes advantage of the ability of TEs to insert randomly to create new enhancer trap expression lines or new mutational lines with interesting phenotypes for functional genomic studies (Cooley et al., 1988).

This dissertation is composed of three manuscripts, each written in the format of the journal to which it will be submitted. The first manuscript (Chapter 1) has been submitted to Insect Molecular Biology, and describes stable germline transformation of the WCR with a DsRed-marked *Minos* element, and expression of a dual transgene, *piggyBac transposase*. The second manuscript (Chapter 2) details the design, creation and optimization of a specialized rearing system for handling the production and screening of transgenic WCR. This manuscript will be submitted to Journal of Insect Science. The third manuscript (Chapter 3) describes production of SWD helper strains using the same DsRed-marked *Minos* element, however, unlike chapter one, here I outline the use of the dual transgene (*piggyBac transposase*) to help generate new *piggyBac*-based transgenic strains, as well as to catalyze remobilization of an already inserted marked *piggyBac* element. This manuscript will also be submitted to Insect Molecular Biology. This dissertation closes with additional data I plan to use in future manuscripts, as well as some ideas for future research directions.

Figure 1.1. Corn roots damaged by WCR larvae. The primary damage done by WCR is when its larvae feed on corn roots. Healthy corn roots (right) v.s. damaged corn roots (left). Figure produced by Monsanto (http://www.americasfarmers.com)

Figure 1.2. Damage from SWD. SWD larvae (arrows) discovered in fresh fruit. Figure produced by Cooperative Extension - University of Maine (https://extension.umaine.edu/highmoor).

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CHAPTER 2

Germline transformation of the western corn rootworm, *Diabrotica virgifera virgifera*

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Abstract

The western corn rootworm (WCR), a major pest of maize, is notorious for rapidly adapting biochemically, behaviorally, and developmentally to a variety of control methods. Despite much effort, the genetic basis of WCR adaptation remains a mystery. Since transformation-based applications such as transposon tagging and enhancer trapping have facilitated genetic dissection of model species such as *Drosophila melanogaster*, we have developed a germline-transformation system for WCR in an effort to gain a greater understanding of the basic biology of this economically important insect. Here we report the use of a DsRed-marked *Minos* element to create transgenic WCR. We demonstrate that the transgenic strains express both an eye-specific DsRed marker, as well as, *piggyBac transposase*. We have identified insertion-site junction sequences via inverse PCR and assessed insertion copy number using digital droplet PCR (ddPCR). Interestingly, most WCR identified as transgenic via visual screening for DsRed fluorescence proved to carry multiple *Minos* insertions when tested via ddPCR. A total of eight unique insertion strains were created by outcrossing the initial transgenic strains to non-transgenic WCR mates. Establishing transgenic technologies for this beetle is the first step towards bringing a wide-range of transformation-based tools to bear on understanding WCR biology.

Introduction

The western corn rootworm (WCR, *Diabrotica virgifera virgifera*) is a devastating pest of maize. More pesticides are used to control WCR and other Diabrotica species than in programs aimed at the control of other agriculture pests (EPA, 2005). Larvae are the most damaging life stage because they feed on roots, causing reduced yields and plant lodging (Krysan, 1986). Before widespread adoption of transgenic corn, Diabrotica species were estimated to cost US farmers over $1 billion per year in lost revenue (Marra et al., 2012). Unfortunately, yield loss due to this pest is once again on the rise due to the ability of WCR to rapidly adapt to control methods, including development of resistance to the Cry3Bb1 transgene used in transgenic corn (Gassmann et al., 2011; Narva et al., 2013), to chemical insecticides (Meinke et al., 1998), and to crop rotation (Ball & Weekman, 1963; Gray et al., 2009; Levine & OloumiSadeghi, 1996; Levine et al., 2002; Parimi et al., 2006). Although new control methods are on the horizon (Baum et al., 2007; Bolognesi et al., 2012), resistance to insecticides can evolve quickly (Ball & Weekman, 1963; Parimi et al., 2006), therefore the key to maintaining control against this pernicious pest lies in discovering the mechanisms by which it so readily adapts to control measures.

Unfortunately, our understanding of the genetic mechanisms that underlie adaptability lag far behind the ability of WCR to develop resistance to each new pest management strategy. Research on this topic is stymied by limited genomic resources. For example, genome sequencing efforts have made important progress, but are hampered by an expansion of repetitive sequences which has given WCR a relatively large genome, ~2.58 Gb (Coates et

al., 2012; Sappington et al., 2006). And while RNA interference (RNAi) is an effective tool for functional studies in WCR, acting systemically to knock down target gene expression (Alves et al., 2010; Baum et al., 2007), it is limited to known sequences, which is problematic for a species without a fully sequenced and assembled genome. Moreover, if a fully assembled genome sequence were available, it would still be difficult to identify poorly conserved and/or novel genes. Therefore, it is critical that new tools for functional genomics be created for this pest species.

Transposon-based applications, such as insertional mutagenesis have been the mainstay of functional genomic analysis of *Drosophila melanogaster* (Brand & Perrimon, 1993; Cooley et al., 1988; Horn et al., 2003; Robertson et al., 1988; Wilson et al., 1989). Although transgenic technologies were initially restricted to *D. melanogaster* and closely related Drosophilids due to host-range limitations imposed by the P-transposable element (Handler & James, 2000), transposons such as *piggyBac* (Cary et al., 1989) and *Minos* (Franz & Savakis, 1991) have opened the field to a much wider group of insects. Today transgenic strains have been successfully created in many insect species across several orders: Diptera (Coates et al., 1998; Grossman et al., 2001; Handler et al., 1998; Jasinskiene et al., 1998; Loukeris et al., 1995b; Rubin & Spradling, 1982), Lepidoptera (Tamura et al., 2000), Coleoptera (Berghammer et al., 1999; Lorenzen et al., 2002), and Hymenoptera (Sumitani et al., 2003). Furthermore, transgenic techniques can even be used as potential pest control methods (Nolan et al., 2011). For example, sex-specific lethal transgenic insertions, tested as a mechanism for reducing population sizes of pests like the blowfly, *Lucilia cuprina* (Scott et

al., 2004), and the human disease vector, *Aedes aegypti* (Phuc et al., 2007), could potentially be useful for controlling WCR as well.

However, successful germline transformation requires more than a functional transposon. Marker genes are needed for the detection of transgenic progeny, as are promoters to drive marker-gene expression. Early work in *D. melanogaster* used native eye-color genes such as *rosy* or *white* to "rescue" mutant eye-color phenotypes (Klemenz et al., 1987; Rubin & Spradling, 1982), but this method relies on the availability of the corresponding mutant strain. Therefore, fluorescent proteins are the most widely used markers for insect transgenesis today, and these are frequently driven by the universal eye-specific promoter, 3xP3 (Berghammer et al., 1999; Horn et al., 2000). However, even with these advancements, recognizing a few transgenic progeny amongst a multitude of wild-type siblings can be challenging. For example, the presence of wild-type eye pigments can interfere with fluorescence detection (Horn et al., 2000; Lorenzen et al., 2002) making if difficult, if not impossible to visualize eye-specific fluorescence. These issues make developing transgenic technologies for non-model organisms, such as WCR, all the more challenging.

Here we report germline transformation of WCR using a 3xP3-DsRed marked *Minos* element, which also carries an *hsp70*-driven *piggyBac transposase* gene. We demonstrate how screening WCR larvae for eye-specific fluorescence can overcome the challenge imposed by wild-type eye pigments that mask fluorescence in adults. We also report insertion-site sequence, copy number, and levels of *piggyBac transposase* expression in 1- to

5-day-old embryos. These strains represent the first step towards bringing a wide range of transformation-based tools to bear on understanding WCR biology.

Results

To establish *Minos*-mediated germline transformation in WCR, a 3xP3-DsRed marked *Minos* donor plasmid (Horn et al., 2003) was co-injected along with an *hsp70*-driven *Minos transposase* helper plasmid (Klinakis et al., 2000a) into 12- to 24-hour-old embryos. From ~9850 injected G_0 embryos we recovered 1251 larvae, but only 491 of these survived to adulthood. It is important to note that only 50% of uninjected control embryos hatched, and less than 60% of these successfully eclosed into adults (Chu and Lorenzen, manuscript in preparation). To reduce rearing efforts, G_0 adults were self-crossed (2 males x 4 females, total of 80 crosses) rather than outcrossed to wild-type mates. Most of the resultant G_1 progeny were screened for eye-specific DsRed expression (see Fig. 2.1) as adults 5 days after eclosion, but some were screened as late-stage larvae. Overall, eleven DsRed-positive G_1 offspring were identified, nine of which survived to adulthood (designated Min-1 to Min-9).

Based on these numbers we estimated our transformation efficiency was somewhere between 2 and 13%. The range is due to the fact that our overall transformation efficiency cannot be directly calculated since G_0 adults were self-crossed. For example, if we calculate based on 11 transgenic offspring from 80 crosses, transformation efficiency would be 13.7%, which is an overestimation since each parent in a cross (2 males x 4 females) had a chance to produce transformed offspring. On the other hand, if we calculate based on 11 transgenic offspring

from 491 G_0 adults, a transformation efficiency of 2.2%, we would be underestimating efficiency, since some beetles may have died or been sterile before successfully mating. The actual transformation efficiency would therefore be somewhere in-between.

To determine if transgenic beetles carried a single *Minos* insertion or multiple insertions, each DsRed-positive G_1 adult was outcrossed to a wild-type mate and the G_2 offspring screened for DsRed expression. Assuming Mendelian inheritance, we would expect a 1:1 ratio of DsRed-positive to DsRed-negative progeny if a G_1 beetle carried a single DsRed-marked *Minos* element. However, if an individual carried two or more *Minos* elements (i.e. multiple transgenic events), we would expect >50% of the offspring to have DsRed-marked eyes. Segregation analysis suggested that four of the nine insertion strains carried single insertions, while three showed clear signs of having multiple insertions (Table 2.1). Interestingly, two insertion strains had fewer DsRed-positive progeny than expected, suggesting that the presence of wild-type (black) eye pigment may completely mask visualization of weak DsRed fluorescence in some individuals.

To determine if this result was due to interference from pigments found in the adult eye, we changed our screening protocol. Rather than screening G_2 offspring as they reached adulthood, we screened 3rd-instar larvae (Fig. 2.2). While changing our screening procedure did increase the number of strains showing a 1:1 ratio of DsRed-positive to DsRed-negative offspring, there were still a few strains that had significantly fewer DsRed-positive offspring than expected.

To gain insight into this phenomenon we followed one set of G_2 offspring more closely, screening them at both the 1st-instar and 3rd-instar larval stages. We separated the DsRed negative 1st-instar larvae and screened them again once they reached the 3rd-instar larval stage. Interestingly, many larvae that failed to show discernable DsRed expression at the 1st-instar larval stage proved to be DsRed positive at the 3rd-instar larval stage (Table 2.2). Position-effect variegation (PEV) is a common phenomenon observed in germline transformation and is well studied in *D. melanogaster* (Wallrath & Elgin, 1995). In cases of PEV, the marked transposon is integrated near a heterochromatic region of the genome where transgene activity can easily be silenced in some cells, while remaining active in others. However, this seems a poor explanation for why some 1st-instar larvae show strong DsRed fluorescence, while their siblings take longer for DsRed fluorescence to appear. In our case, transgene expression is not "variegated" within an individual, but rather expression is variable amongst individuals. Therefore, despite the lower-than-expected number of DsRed positive progeny, it seems likely that these differences are due to integration of *Minos* into multiple genomic locations, each having a unique DsRed expression profile.

We also investigated DsRed expression during the pre-pupa stage. We observed the fluorescent tissues gradually shifting from the larval head capsule to the anterior region of the prothorax (Fig. 2.3). However, once these individuals pupated, fluorescence became undetectable for 1-2 days before reappearing deep in the center of the pupal eye. Taken together, these results demonstrate how screening WCR for DsRed fluorescence at different time points (e.g. pre-pupal vs. pupal stage) could confound the results. Thus far, screening

WCR for DsRed fluorescence at the 3rd-instar larval stage proved to be the most reliable method.

Since segregation analysis based on phenotypic screening of DsRed fluorescence failed to provide an accurate assessment of transgene copy number for all transgenic strains tested, we retested each transgenic strain using a molecular-based method. A recent publication (Glowacka et al., 2016) evaluated several molecular methods for determining transgene copy number and concluded that digital droplet PCR (ddPCR) was rapid, reproducible, and as accurate as Southern hybridization for this task. In ddPCR, copy number is determined by comparing amplification (concentration) of a single-copy control gene to that of the transgene. The WCR orthologs of the *D. melanogaster white* (*w*) and *ribosomal protein S6* (*RPS6*) genes were each tested for use as controls because these are known to be single-copy genes in *T. castaneum* (Grubbs et al., 2015; Park et al., 2008). This was accomplished by comparing the ddPCR results from genomic DNA isolated from males with that from females. There were no sex-specific differences in copy number of *w* or *RPS6* (Table S2.1), indicating that both genes are likely to be single-copy autosomal genes in WCR.

Typically a single-copy autosomal gene is referred to as being present only once in the genome. However, there are actually two copies, one on each homologous chromosome (see Fig. 2.4). This is an important consideration because our comparisons are to transgenic beetles that have been outcrossed to a wild-type parent, making them heterozygous for each insertion of the transgene. As a result, any transgenic strain with a single, genomic insertion

of the marked transposon will produce only half the concentration of amplicon relative to the control gene.

Of the nine DsRed-positive G_1 offspring tested via ddPCR, only one (Min-8) proved to carry a single *Minos* insertion (see Table 2.1, Table S2.2). This disparity is particularly striking for Min-9, the only transgenic strain that displayed a perfect one-to-one ratio of DsRed-to-wild-type offspring in the segregation analysis, but which appears to possess at least nine copies of *Minos* when tested via ddPCR. One possible explanation for this conflict is that our screening method may have made it impossible to detect individuals having low, or perhaps even average levels of DsRed expression. Specifically, by screening G_1 beetles as adults (in the presence of eye pigments) it is possible that DsRed fluorescence was completely masked by eye pigments, except in those cases where DsRed expression was very high, either due to insertion-site location, or the presence of multiple, DsRed-marked *Minos* elements. Another possible explanation is presented by Min-7, which appears to possess tandem or concatenated insertions of *Minos* that are inherited as a single, linked locus. While possible, it seems rather unlikely that tandem insertions would have occurred in every strain. Given the nature of the WCR genome – large and highly repetitive – it is also possible that *Minos* is incorporated into heterochromatic (i.e. transcriptionally silent) regions of the genome more frequently than into euchromatic (i.e. transcriptionally active) regions. No matter whether one or more of these occurred, the fact that eight of nine strains carried multiple copies of *Minos* suggests that additional transgenic beetles were likely produced, but could not be detected due to low, or absent DsRed expression, thus lowering our overall transformation rate.

Before assessing levels of *piggyBac transposase* expression, we first sought to retrieve as many independent, single-insertion *Minos* strains as possible. Each generation, one or more DsRed-positive males were outcrossed to wild-type mates (1 male x 3 females). After progeny were produced, the male parent was sacrificed and tested for insertion number using ddPCR. Progeny from males with reduced copy number were then screened as 3rd-instar larvae for fluorescence, and DsRed-positive males used to start the next generation. With nine insertions, creating a single-insertion strain from Min-9 proved too resource-intense, so this strain was discarded. After several generations of outcrossing and assessment, each of the seven remaining multiple-insertion strains yielded at least one single-insertion substrain. In all, a total of 17 single-insertion strains were isolated and used to establish 17 distinct WCR colonies.

We then sought to determine the site of genomic integration for each of the 17 strains. While we cloned *Minos* insertion-site junctions from all 17, we only recovered nine unique sequences. We discovered that some strains shared insertion-site identity, while others failed to provide enough junction sequence to distinguish differences. Since the only sequence requirement for *Minos* integration into the genome is the dinucleotide sequence "TA," insertion sites should be randomly located throughout the genome. The most likely explanation for multiple substrains possessing identical insertion-site sequence is that the substrains are derived from the same G_0-ancestor (i.e. same pool). Since G_0 beetles were self-crossed (small mass-selfcrosses), and all G_1 offspring were pooled for screening, irrespective of which cross they came from, multiple DsRed-positive G_1 beetles could have come from

the same initial cross, thereby inheriting the same insertional event. For example, Min-1 and Min-3 appear to share the same insertion site, while Min-1 and Min-7 share another (Table S2.3).

Another possible explanation for our finding could be that there are hot spots for *Minos* integration within the WCR genome. If *Minos* has a preference for a highly repetitive sequence, the insertion sites could be great distances from each other, or even on different chromosomes, but due to the short length of our insertion-site sequences (e.g. Min-3) we would not be able to distinguish between the various sites (Table S2.3). Moreover, despite repeated attempts to amplify both the left and right *Minos* junctions from each strain, we were only able to clone both from three strains (Fig. 2.5). We and others (Morales et al., 2007) have previously reported that some transposon insertions are simply too difficult to warrant continued attempts to clone. Given the large size and repetitive nature of the WCR genome, these characteristics may play a role in this phenomenon.

Six of the nine strains having unique single-copy insertions were healthy and fecund enough to test their embryos for *piggyBac transposase* expression via reverse transcription PCR. Since we are interested in mobilizing *piggyBac* within germline cells, we assayed embryos from each of the six strains 0-5 days post egg lay. Our results indicate that *piggyBac transposase* is expressed, but expression levels varied between strains (Fig. 2.6). Importantly, amplification of *piggyBac transposase* was never seen in RT-minus controls (results not shown). A few facts should be noted regarding these expression results. First, we used the

hsp70 promoter from *D. melanogaster* to drive expression of the *piggyBac transposase*, but did not heat shock the embryos. As a result, we did not expect high expression levels. Also, age may have been a factor. Since these were relatively young embryos it is possible that higher levels of *piggyBac* expression would be seen at later stages of development. Moreover, given the low hatch rate (~50%) it is possible that many embryos die before expressing zygotic genes. However, we have confirmed the expression of *piggyBac transposase* from these transgenic strains, and since these strains are healthy, we will be able to use them for further tests and applications in the future.

Discussion

While our findings demonstrate the utility of the *Minos* transposable element for generating heritable insertions in the western corn rootworm genome, they also highlight some of the challenges faced when developing genetic technologies for non-model organisms. Below we discuss some of these challenges, potential solutions, and steps we have or are taking to make germline transformation an efficient and effective tool for functional genomic analysis of WCR.

Although some members of our team had prior experience rearing WCR, our first challenge was securing a reliable supply of high-quality precellular embryos. Currently, we know of no research describing WCR embryonic development, therefore, based on the ~2-week period required for embryonic development reported for the non-diapausing WCR strain (Branson, 1976), and DAPI staining of 24-hour-old embryos (data not shown) we assume a 24-hour-

long window for effective germline transformation via microinjection. Also, egg lay tests ranging from 4-hours to overnight (5pm-9am) revealed that only overnight egg lays provided sufficient quantities of eggs for microinjection. So, even though the time required for WCR egg lays was more than that used for other insects, we assumed that we still had ~8 hours to complete microinjection (i.e. before cellularization occurred). However, if this assumption is wrong, and WCR embryos undergo cellularization earlier than predicted, it could explain our lower-than-expected transformation rates. Although their egg laying habits could make such research difficult, further investigations into WCR embryonic development could help improve injection efficiency.

We also found that the physical task of microinjecting WCR embryos was quite different from injecting those from *T. castaneum* or *D. melanogaster*. Unlike *T. castaneum*, 2.5% bleach damages WCR embryos, possibly due to differences in the respective egg shell structures. Also, unlike *D. melanogaster*, WCR eggs do not stick to double-sided tape, therefore we used Elmer's glue to secure embryos for microinjection. Yet, despite our best efforts, we experienced high embryonic mortality, even in our uninjected controls, meaning many more embryos had to be injected. Due to these constraints, WCR embryos were injected at a rate of ~200/day over a three month period. We noticed wide variation in the survivorship of injected embryos (2.5 to 49.2%), as well as uninjected controls (6 to 52%). Much of the variation was likely due to the inherent learning curve associated with developing microinjection methods for a new species, while some may have been due to differences in the personnel handling them (multiple people involved).

Another challenge was finding a promoter and marker gene that would enable efficient, unambiguous identification of transgenic WCR. Since some heterologous promoters function across insect orders (e.g. *hsp70* from *D. melanogaster* in *T. castaneum*; Lorenzen et al., 2007), we initially used a transformation construct known to work well in a fellow coleopteran, *T. castaneum*. This *piggyBac*-based construct carried an EGFP marker gene driven by the *Tribolium castaneum alpha tubulin-1* (*TcαTub1*) promoter. While *TcαTub1-EGFP* works well in *T. castaneum* (Siebert et al., 2008), we discovered that EGFP is a poor choice for use in WCR. The problem stems from the fact that the excitation wavelength required for EGFP fluorescence appears to be harmful to WCR embryos (i.e. exposure to EGFP screening reduced embryonic hatch rate to near-zero).

Our initial screen for eye-specific expression of the DsRed marker was not without its challenges, and our decision to only screen WCR adults for DsRed fluorescence exacerbated the problem. Given the effect of EGFP screening on embryos, this decision was based on the hypothesis that the wavelengths of light used in fluorescence microscopy were harmful to the soil-dwelling life stages of WCR. Indeed, adults were unharmed by any tested screening conditions. However, since screening adults for eye-specific fluorescence proved problematic, we tested other life stages. Interestingly, when we tested screening of larvae for DsRed or another fluorescent marker, ECFP, we discovered that the larvae reacted negatively to the high-energy wavelengths of light used for ECFP and EGFP excitation, but not that used for DsRed. Although neither ECFP nor DsRed screening had a negative impact on larval survival rate, screening larvae for EGFP was still harmful. One possibility for this is

differences in the filter sets themselves, with the EGFP excitation filter appearing to allow through more light than either of the other sets. This increased light may produce more heat, an intriguing hypothesis, given that screening larvae for EGFP while on an ice pack reduced lethality. However, we cannot eliminate the possibility that increased light exposure from our EGFP filter set may also exacerbate problems caused by higher-energy wavelengths, or somehow else hurt embryonic and larval survival in ways not experienced under our screening conditions for DsRed and ECFP. Parsing out these issues would require use of multiple filter sets of varying qualities. Regardless of the actual cause, future transgenic efforts in this or other species will benefit from considering possible lethality from fluorescent screening of different life stages when developing transgenic constructs.

The initial decision to only screen WCR adults for DsRed fluorescence also impacted our estimate of overall transformation efficiencies since we likely failed to detect DsRed fluorescence in some of the ~1000 G_1 individuals screened as adults. However, despite this obvious limitation, and the fact that we cannot directly calculate transformation efficiencies due to our crossing and screening strategy (i.e. self-crossing G_0 beetles, and pooling G_1 offspring), estimated transformation efficiencies are similar to those seen in other insects (Loukeris et al., 1995a; Loukeris et al., 1995b). It should also be noted that these estimates are based on the number of DsRed positive G_1 offspring detected, not the number of *Minos* insertions. Given the high percentage of transgenic strains possessing multiple *Minos* insertions (Table 2.1), transformation rates in WCR are likely much higher than our estimate suggests.

The first reason to believe that the real number of transformation events was greater than detected is the fact that it takes up to five days for 3xP3-driven DsRed to accumulate to an observable level in WCR adults having wild-type eye pigmentation. It is impossible to know how many transgenic G_1 beetles went undetected before we discovered this issue. The problem was discovered when we started screening G_2 offspring (more than two months into the screen). Since we knew 50% of the progeny from a transgenic G_1 beetle should inherit the DsRed-marked *Minos* element, we knew something was wrong when only a few adults proved to be DsRed positive. Given the fact that we discovered this problem over halfway through our screen, it is possible we mis-scored many G_1 beetles, in which case the actual transformation efficiency could be twice that reported.

The second reason to believe that the real number of transformation events was greater than detected is the fact that we believe several "silent" insertions were present in some of the DsRed-positive strains (Min-4, Min-6, and Min-9). These strains had a relatively low percentage of DsRed-positive offspring, yet ddPCR results indicate each strain had several insertion events (Table 2.1). While silent insertions are of little value, due to difficulties associated with tracking carriers, knowing how many such insertions were generated would provide a better estimate of *Minos* activity in WCR. While silent insertions and position effects are known possibilities when using transposable elements to generate genomic insertions (Spradling & Rubin, 1983), these types of insertions could be more likely in WCR than in species with smaller genomes. Specifically, the WCR genome is estimated to be around 2.58 Gb (Coates et al., 2012; Sappington et al., 2006), which is ~10x the size of the *T.*

castaneum genome (Tribolium Genome Sequencing Consortium, 2008). The canonical recognition sequence for *Minos* is TA, and this was always present in our sequenced insertion-site junctions. However, many of our junction sequences have high A+T content or short repetitive elements (Table S2.3). Since much of the additional sequence is highly repetitive, it is likely to be heterochromatic. While insertions within euchromatic regions are likely to have reasonable levels of marker-gene expression, insertions in heterochromatic regions tend to have low, or no, expression. As other research has shown (Klinakis et al., 2000b), insertions may occur disproportionately within AT-rich genomic areas, and thus could be more likely to be silenced due to their heterochromatic location. In our case, it is very hard to determine uniqueness of insertion-site junctions when they are highly repetitive, or when their sequence reads are short. On the other hand, comparing the data between insertion numbers and junction sequences shows that there are likely some insertion sites we have not yet successfully cloned and sequenced, which would further indicate our actual transformation rate is higher than first predicted. Interestingly, Schulte and colleagues (2014) suggested that their higher-than-usual transformation rates in honeybees using the *piggyBac* element, which has a recognition sequence of TTAA, may have been due to the high A+T content of the honeybee genome.

For short insertion-site junction sequences, it is important to note that our cloning process favors shorter fragments, because PCR is more likely to produce an amplification product when *Minos* is inserted near the corresponding restriction site. However, while our use of restriction enzymes with 4- to 5-bp recognition sites may have prevented us from acquiring

longer sequences, attempts with restriction enzymes having longer recognition sequences (6-bp) failed to produce amplification products. This issue could likely be overcome by performing repeated tests with a wide variety of restriction enzymes, but based on the effort required verses possible gains, we chose to limit our tests to a small number of enzymes. The drawback to having very short insertion-site sequences is that we cannot reliably identify the location of genomic integration, or determine what genes might be disrupted. We can, however, homozygose the single insertion strains and create larger colonies in order to perform fitness tests. Such tests could deliver a better understanding of how the different insertions affect WCR health and reproduction.

Greatest among our many challenges was development of a WCR rearing protocol suitable for transgenic research. Since WCR larvae live in the soil and feed on the roots of corn plants, an appropriate artificial diet (i.e. one on which larvae can survive until adulthood) has not yet been successfully developed. Therefore production of high-quality WCR requires rearing injected and transgenic individuals on corn plants. While most rearing protocols (Branson et al., 1975; Branson et al., 1988; Dominique & Yule, 1983; Georce & Ortman, 1965; Jackson & Davis, 1978) have shown that open-container rearing is the best method for growing healthy corn, it is not suitable for rearing transgenic insects due to containment issues. However, we found that closed-container rearing increased the rates of harmful molds and mites. To help reduce these issues, we added an additional transfer step, moving the insects to a new container while they are at the 3rd-instar larval stage or older. This extra step greatly reduced the number of mites. It also provides an opportunity for us to check and

separate sexes during the pupal stage if necessary.

We have observed *piggyBac transposase* expression in 0 to 5-day-old embryos from the single-insertion strains via RT-PCR. Since WCR require 12-14 days to complete embryonic development, the first few days are probably the most important for integration, or reintegration of transposable elements into the genome for the propose of germline transformation or transposon-based mutagenesis. Therefore we are currently in the process of selecting the healthiest single-insertion strain with the strongest *transposase* expression. This "helper" strain will be used to create transgenic WCR that carry a marked *piggyBac* transposon. Together, one of the *Minos* strains reported here, and a strain containing a mobilizable *piggyBac* insertion, will serve as the tools necessary for forward-genetic screens in WCR.

Beyond building new tools for WCR genetic research, we have demonstrated useful, and not-so-useful, techniques that will help advance genetic studies of this economically important pest. This work demonstrates the limitations of fluorescent marker genes in this species, and highlights the need for new promoters to improve transgene expression. It is also critical for researchers to give thought to rearing techniques, since these are likely to differ between small academic-scale rearing systems and medium-to-large industrial-scale systems. Seemingly simple points, such as whether rearing containers should be open or closed, can have a major impact on the overall efficiency of a system. While our microinjection and rearing system were designed to enable germline transformation of WCR, it is important to

note that the system we describe can be used for other techniques, including CRISPR/Cas9 genome editing. These data should be taken into consideration as researchers move forward in understanding WCR at a genetic level.

Experimental Procedures

Insects, rearing and egg collection

A non-diapausing, but otherwise wild-type, western corn rootworm strain (Kim et al., 2007) was used in this work and is referred to as wild-type. To minimize the effects of inbreeding, we established our laboratory colony using WCR from Dr. Wade French (USDA-ARS-NGIRL, Brookings, SD), as well as from Crop Characteristics, Inc. (Farmington, MN, USA), and we kept colony population size at >500 individuals.

WCR larvae were reared in plastic containers with soil (Scotts® Premium Topsoil, The Scotts Company, Marysville, OH, USA) and organic corn (Trucker's Favorite yellow, Coor Farm Supply, Smithfield, NC, USA). Young larvae were housed in 16 oz containers (Item: #128HRD16, WebstaurantStore, Lancaster, PA, USA), while older larvae and pupae were housed in 38 oz containers (Item: #128NC888, WebstaurantStore, Lancaster, PA, USA). Adults were housed in 30 cm^3 cages (BugDorm, MegaView Science, Taiwan) and held at 26^oC, 60% humidity with a 14:10 h (light:dark) photoperiod and fed an artificial diet (Product# F9766B; Frontier Agricultural Services, Newark, Delaware, USA).

Fertilized eggs were collected from adults by placing a 100 x 15 mm Petri dish containing

1% agar, a filter paper disc, and four layers of cheesecloth into their cage overnight. Eggs for microinjection were washed with ddH_20 and handled as outlined below, while eggs for establishing and/or maintaining colonies were pipetted into soil-filled containers, ~200 eggs per 1 oz cup, covered, and held at 26°C. The number of egg collections per colony each week varied based on experimental needs. After 7 days, the contents of each 1 oz cup (soil and embryos) was transferred to separate 16 oz containers along with fresh soil and newly sprouted corn. Embryos were allowed to hatch and grow to 2nd-instar larvae in these containers (~2 weeks), before being moved to new containers with fresh soil and 4-day-old corn sprouts. After an additional 10−12 days, insects were carefully removed from these containers and sorted by life stage.

For transgenic WCR, all insects were screened for fluorescence before being placed in new containers, and all DsRed negative insects were discarded. Insects from the wild-type colony were directly moved into the next container. 3rd-instar larvae were placed in containers (~85 larvae/38 oz container) with fresh soil and 4-day-old corn sprouts. Pre-pupae and pupae were placed in soil (38 oz container without corn) and the containers checked daily until no more adults eclosed. Newly-eclosed adults were collected and put in a 30 cm^3 cage (BugDorm, MegaView Science, Taiwan).

Plasmids and microinjection of WCR embryos

Transgenic WCR strains were established using a *Minos* helper plasmid pHSS6hsILMi20 (Klinakis et al., 2000a), which supplies a source of *Minos* transposase, and DsRed-marked

Minos donor plasmid pMi{3xP3-DsRed, hsp70-piggyBac} (Horn et al., 2003) which also carries a *piggyBac transposase* gene. Injection-ready plasmids were isolated from Top 10 cells (Life Technologies, Carlsbad, CA, USA) using a Plasmid DNA Midi Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol, and eluted in water. Plasmid concentrations were determined using a Nanodrop 1000 (Thermo Scientific, Waltham, MA, USA) and injection-ready solution prepared by adding a buffered phenol red solution (Cat #143-74-8; Sigma-Aldrich, St. Louis, MO, USA) to aid visibility.

Fertilized eggs were collected from wild-type beetles via an overnight egg lay (see above) and individually affixed to a strip of black filter paper (Ahlstrom, Helsinki, Finland) using a glue (Elmer's GlueAll, non-toxic, Inc., Statesville, NC, USA). After the glue dried (~10 mins) distilled water was brushed onto eggs to help soften the eggshell/chorion, allowing the injection needle to penetrate into the preblastoderm embryos, delivering helper and donor plasmids at a final concentration of 350 ng/µl and 250 ng/µl, respectively.

Injected embryos, filter paper and all, were transferred to a Petri dish containing 1% agar, covered, and sealed with parafilm. Injectees were held at 26^{o}C in the dark for 10 days. Embryos were checked daily starting 11 days post-injection; to maximize our numbers, each Petri dish of injectees was screened for up to two weeks to catch all surviving larvae. Newly hatched larvae were transferred to 16 oz rearing containers with soil and newly sprouted corn, no more than 20 larvae per container, and kept at 26^{o}C with 60% humidity for two weeks. Then, insects were carefully removed from these containers and placed into new 16

oz containers with fresh soil and 4-day-old corn sprouts and stored in the same environment for another two weeks. Containers were then checked daily, and newly emerged adults transferred to 6 oz containers with artificial diet and an agar dish (90 mm) with 1% agar for a water supply. G_0 adults were mated in small groups (2 males x 4 females) per container. Egg collection began after two weeks by adding four layers of cheesecloth on the agar dish. G_1 offspring were screened for DsRed expression, and transgenic individuals transferred to their own chamber until ready to mate. DsRed-positive G_1 beetles were crossed with two wild-type mates.

Fluorescence microscopy

DsRed expression was observed using a Leica M165 FC fluorescence stereomicroscope (Leica Microsystems Inc., Wetzlar, Germany) equipped with a DsRed (DSR) filter set (excitation filter: 510-560 nm, emission filter: 590-650 nm). Photography was performed with a Leica digital camera (model DFC290). All potentially transgenic WCR (G_1 generation onward) were screened for eye-specific DsRed fluorescence either during the 1^{st}-instar or 3^{rd}-instar larval stage, or on the 5^{th} day of adulthood.

DNA isolations, digital droplet and inverse PCR

Genomic DNA (gDNA) was extracted from individual transgenic or wild-type beetles using a Wizard Genomic DNA Isolation Kit (Promega, Madison, WI, USA). We employed a modified version of the manufacturer's protocol (Lorenzen et al., 2007) with homemade cell lysis and protein precipitate buffers (Onate-Sanchez & Vicente-Carbajosa, 2008). Purified

gDNA was then used as template for inverse and/or digital droplet PCR (ddPCR).

Insertion-site junctions were amplified via inverse PCR (Ochman et al., 1988) using the *Minos*-specific primers IMio1, IMii1, IMio2 and IMii2 (all primer sequences listed in Table S2.4) (Klinakis et al., 2000b). DNA templates for inverse PCR were generated by digesting ~500 ng of gDNA from each individual transgenic beetle with the restriction enzymes *Alu*I or *Tfi*I (New England Biolabs, Ipswich, MA, USA), diluting the digested DNA to a concentration of ~2.5 ng/µl, and creating circularized fragments through self-ligation using T4 DNA ligase (New England Biolabs, Ipswich, MA, USA). Inverse PCR was performed using 1 µl of the ligation product as template in a 10 µl PCR reaction, along with the *Minos*-specific primers IMio1 and IMii1 (Table S2.4) (Klinakis et al., 2000b). A second round of PCR was performed using 1 µl of a 1:10 dilution of the first-round PCR product as template, along with the "nested" *Minos*-specific primers IMio2 and IMii2 (see (Klinakis et al., 2000b). PCR products larger than 200 bp were gel purified (QIAquick® Gel Extraction Kit, QIAGEN, Hilden, Germany) and sequenced at the Genomic Sciences Laboratory (North Carolina State University, Raleigh, NC, USA).

Copy number was assessed via digital droplet PCR (ddPCR) using a QX200 Droplet Digital™ PCR System (BioRad, Hercules, CA, USA). Genomic DNA (gDNA) from individual transformed beetles (progeny were always from an outcross) was used as template in each ddPCR reaction in accordance with the manufacturer's protocol for EvaGreen® (BioRad, Hercules, CA, USA). In brief, each 20 µl reaction included 1x EvaGreen ddPCR

supermix, 60 ng gDNA, and 200nM gene specific primers. The qTrans F3/R3 primer set was used to amplify the transgene, while the qWhite F1/R1 and qRPS6 F1/R1 primer sets were used to amplify the control gene (Table S2.4). Each reaction was mixed in the QX200 Droplet Generator (Bio-Rad) with 50 µl of Droplet Generation Oil (Bio-Rad) to create droplets, then transferred to a twin.tec PCR plate 96 (Eppendorf) and sealed by the PX1 PCR Plate Sealer (Bio-Rad). The PCRs were performed in a T100 Thermal Cycler (Bio-Rad) with the following cycling conditions: 1× (95°C for 5 min), 40× (95°C for 30 s, 58°C for 30 s, 72°C for 45 s), 1× (4°C for 5 min, 90°C for 5 min). After the PCR, the plate was put into a QX200 Droplet Reader (Bio-Rad) to count individual droplets, and quantify their fluorescence intensity. The data analysis was performed with QuantaSoft droplet reader software (Bio-Rad). Use of a single-copy, control gene (*white*) allowed us to determine absolute quantification of the inserted transgene (*piggyBac transposase*) by comparing the ratio between the concentration data (generated by QuantaSoft software from the values of the transgene vs. the control gene) which indicated how many copies of the transgene (i.e. *Minos* element) were present in each sample.

RNA isolations and Reverse Transcription-PCR

Total RNA was isolated from 500 1-5 day-old WCR embryos by homogenizing in 300 µl of QIAzol Lysis Reagent (QIAGEN, Hilden, Germany) and extracted using the RNeasy Mini Kit (QIAGEN) following the manufacturer's recommendations. Samples were treated with DNase (QIAGEN) directly on the column before elution. Each RNA sample was reverse transcribed using the SuperScript® III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) and an

anchored oligo(dT) primer (RT-Uni) (Grubbs et al., 2015), and RT-PCR performed with the *piggyBac transposase*–specific primer qTrans F2 /R3 (Table S2.4) to detect the expression of *piggyBac transposase.*

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Table 2.1. DsRed segregation analysis. Result of screening progeny from the Min-1 to Min-9 outcrosses for DsRed fluorescence. Number of individuals having DsRed-positive eyes ($DsRed^{pos}$) and number of individuals having DsRed-negative eyes ($DsRed^{neg}$) were counted, and the ratio calculated to determine the likely number of *Minos* elements (expected events) in each strain. We also include results from ddPCR as a comparison to show a more accurate estimate of *Minos* insertions in each strain.

Strain	DsRed pos/neg	Ratio*	Total	% $DsRed^{pos}$	Expected Events	Events detected by ddPCR
Min-1	225/119	1.89 : 1	344	65%	2	4
Min-2	45/35	1.28 : 1	80	56%	1	4
Min-3	731/176	4.15 : 1	907	81%	3	4
Min-4	54/98	0.55 : 1	152	36%	1	6
Min-5	137/65	2.11 : 1	202	68%	2	5
Min-6	184/194	0.95 : 1	378	49%	1	5
Min-7	80/90	0.89 : 1	170	47%	1	3
Min-8	46/128	0.36 : 1	174	26%	1	1
Min-9	95/95	1.00 : 1	180	50%	1	9

* Ratio = DsRed-positive eyes / DsRed-negative eyes.

Table 2.2. Age-related change in DsRed fluorescence. Result of screening previously DsRed-negative 1st-instar larvae (1st Instar DsRed^{neg}) upon survival to the 3rd-instar larval stage. Percent 3rd Instar DsRed^{pos} was calculated as the number of newly identified, DsRed-positive 3rd-instar larvae (3rd Instar DsRed^{pos}) divided by the number of previously DsRed-negative 1st-instar larvae surviving to the 3rd-instar larval stage (3rd Instar Total).

Strain	1st Instar DsRed^{pos}	1st Instar DsRed^{neg}	3rd Instar Total	3rd Instar DsRed^{pos}	% 3rd Instar DsRed^{pos}
Min-1	25	30	25	9	36%
Min-2	20	25	24	3	12.5%
Min-3	9	25	18	3	16.67%
Total	54	80	67	15	22.39%

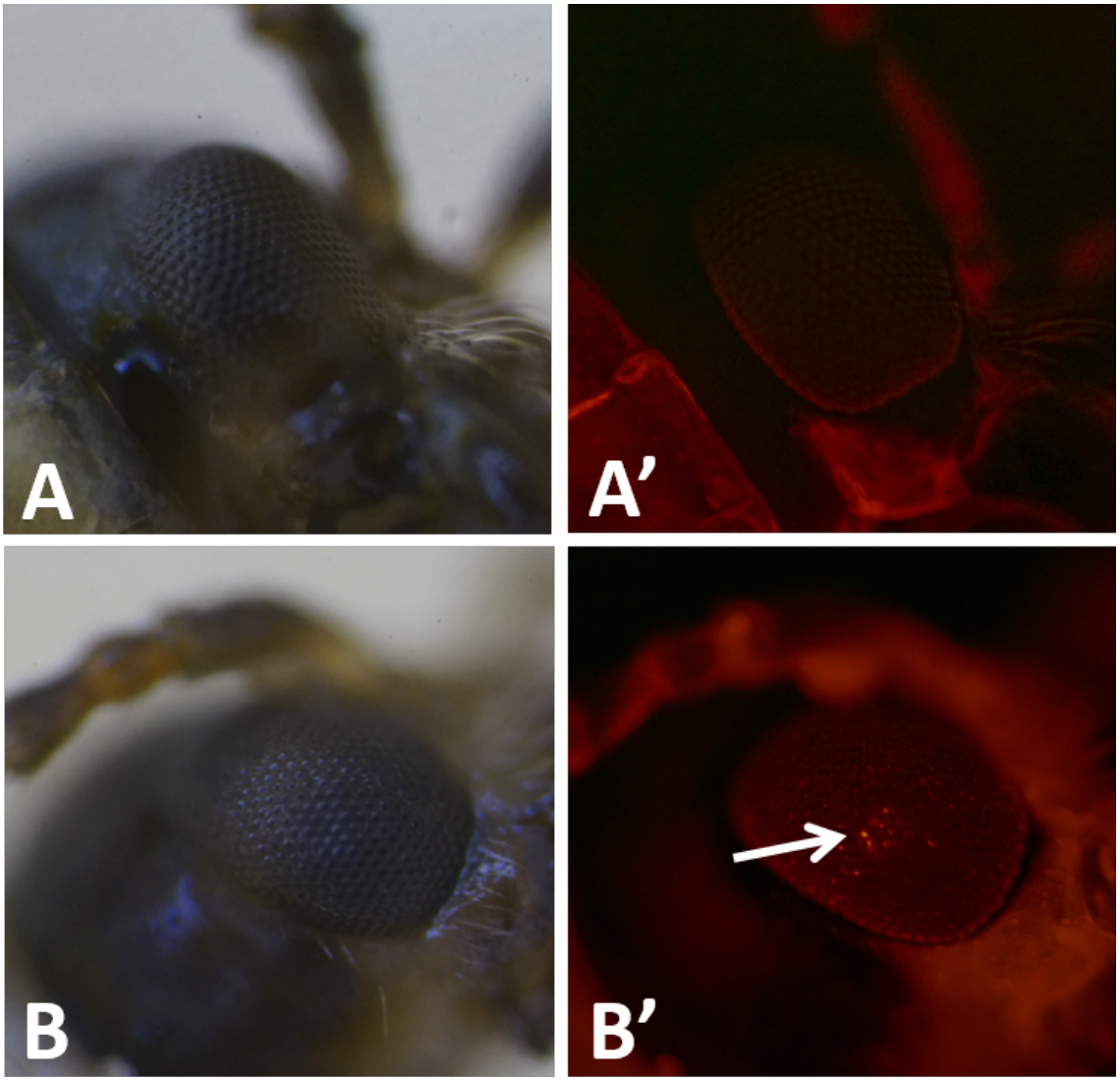


Figure 2.1. Comparison of wild-type and transgenic eye fluorescence phenotypes. Eye-color phenotype observed in A) wild-type and B) transgenic adult WCR with white light. The same individuals, A’) wild-type and B’) transgenic, viewed with DsRed filter set. Arrow indicates location of DsRed-fluorescent ommatidia.

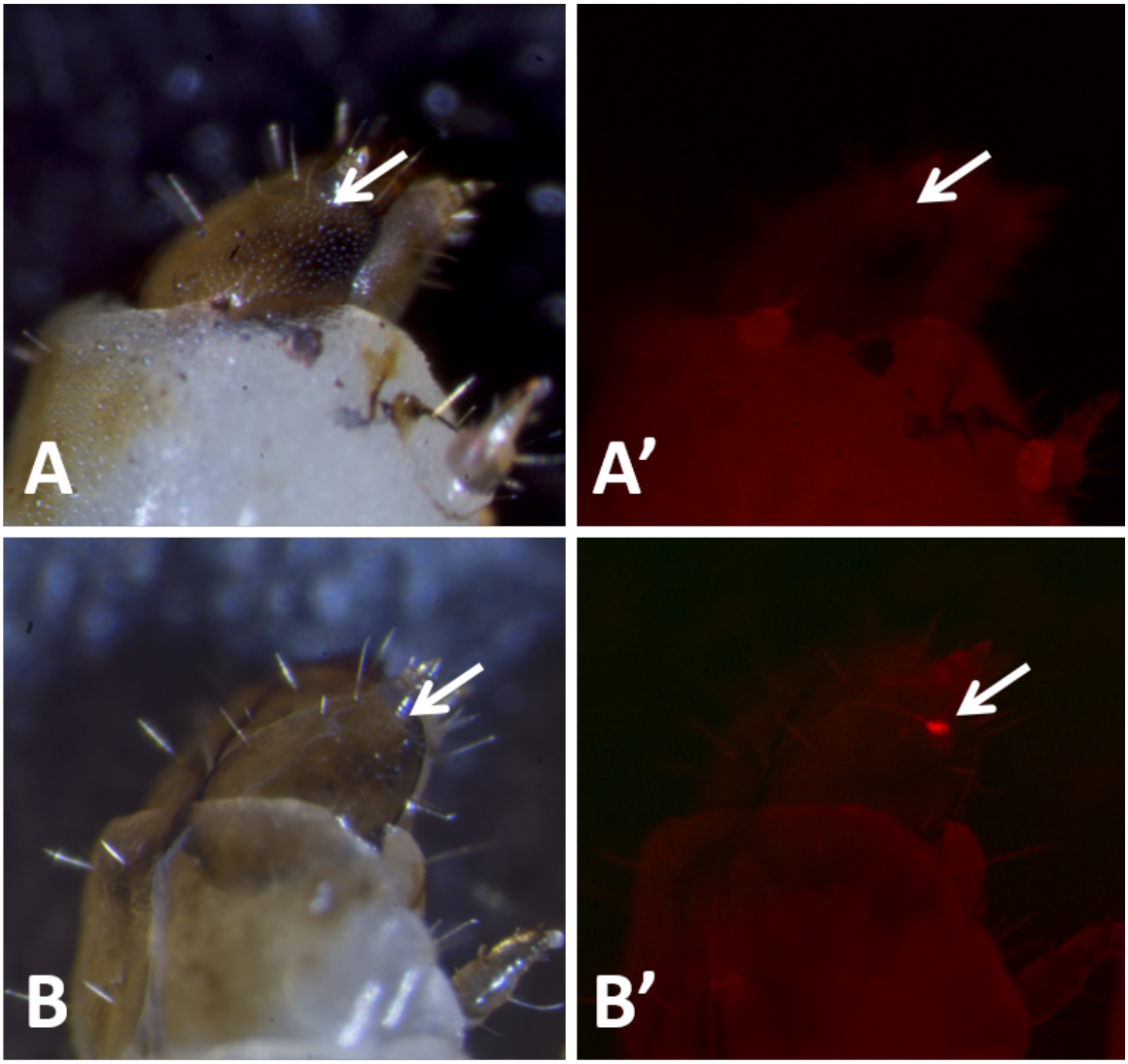


Figure 2.2. Comparison of larval-eye spots in 3rd-instar wild-type and transgenic WCR larvae. A) Wild-type and B) transgenic 3rd-instar larvae viewed with white light. The same individuals, A') wild-type and B') transgenic, viewed with DsRed filter set. Arrows indicate region of larval eye.

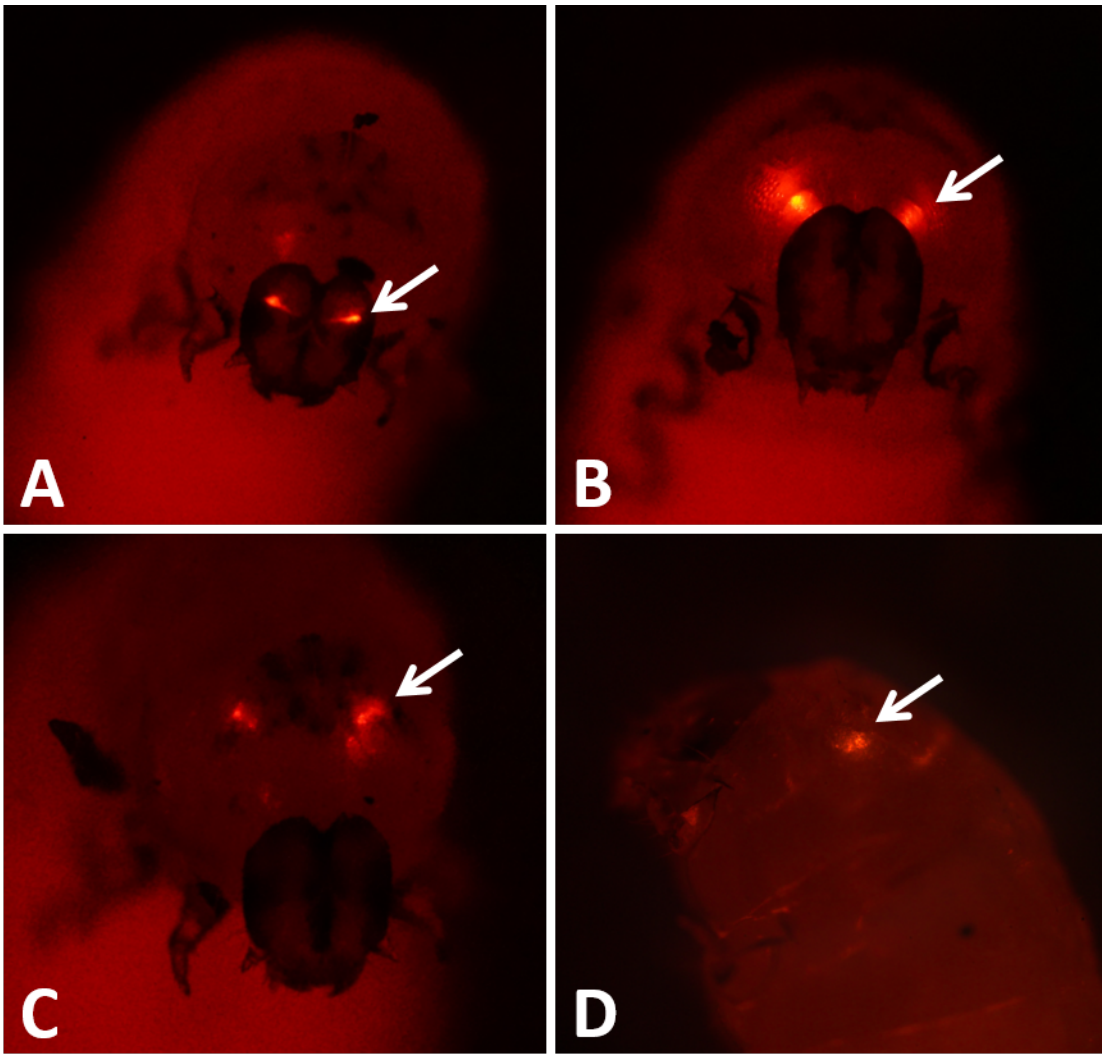


Figure 2.3. Migration of eye tissue during WCR pre-pupal development. DsRed expression observed over time in A) early, B) early mid-stage, C) late mid-stage and D) late stage pre-pupa. Arrows indicate region of presumptive eye tissue.

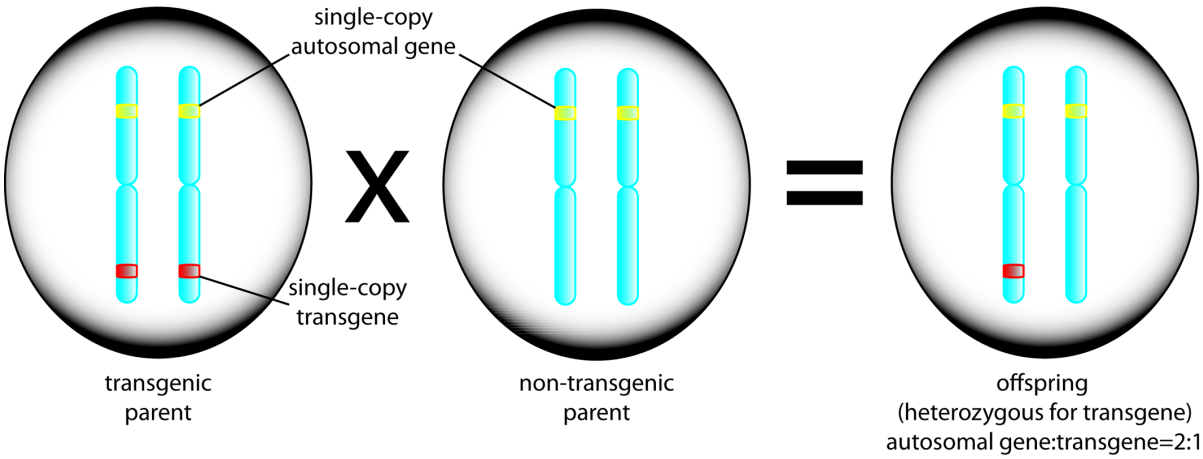
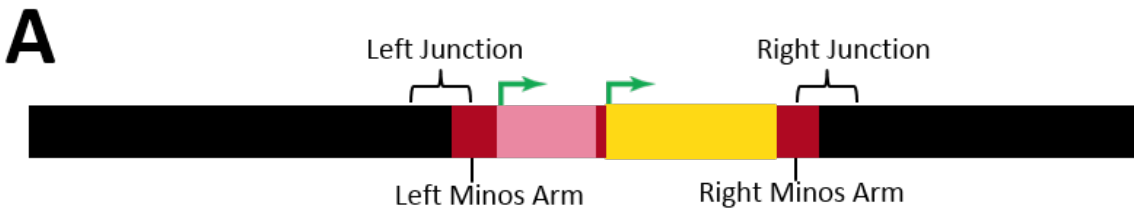


Figure 2.4. Comparison of single-copy autosomal gene vs. single-copy transgene. Transgene copy number was determined by comparing ddPCR results for a single-copy autosomal gene (yellow boxes on blue chromosomes) to that from that transgene itself (red boxes on blue chromosomes). Since ddPCR was performed on DNA isolated from offspring of an outcross (far left), individuals possessing a single copy of the transgene will appear to have twice as much amplification product from the control gene (single-copy autosomal gene) as from the transgene – i.e. homozygous for the control gene, but heterozygous for the transgene.



B

```
Min-1a TTTGACTATTA - Minos - TA---------
Min-1b ---AGCTTATA - Minos - TA---------
Min-2a ---------TA - Minos - TATCAGATCTT
Min-3a CCACATACGTA - Minos - TACGTATGTGG
Min-3b CATTTCTATTA - Minos - TAGATATAAAA
Min-3c TCATTATATTA - Minos - TA---------
Min-4a TATAAACCATA - Minos - TA---------
Min-7a AACAGAATATA - Minos - TA---------
Min-8  AATTTGTAATA - Minos - TAGGTACATTT
```

Figure 2.5. Schematic of *Minos* insertion-site junctions. A) Diagram of *Minos* (red bars) carrying a marker gene (pink bar) and transposase gene (yellow bar) inserted into the genome (black bar). Left and right junctions are distinguished based on slight differences in sequence of the left and right *Minos* arms. B) Nine unique junction sequences from 17 single insertion strains. Most of the sequences were from the left *Minos* junction. We successfully cloned both sides of the junction sequences of the strains Min-3a, Min-3b, and Min-8. Unknown sequences are designated with dashes. Note the brevity of the Min-1b sequence, as well as the high AT content of all the sequences.

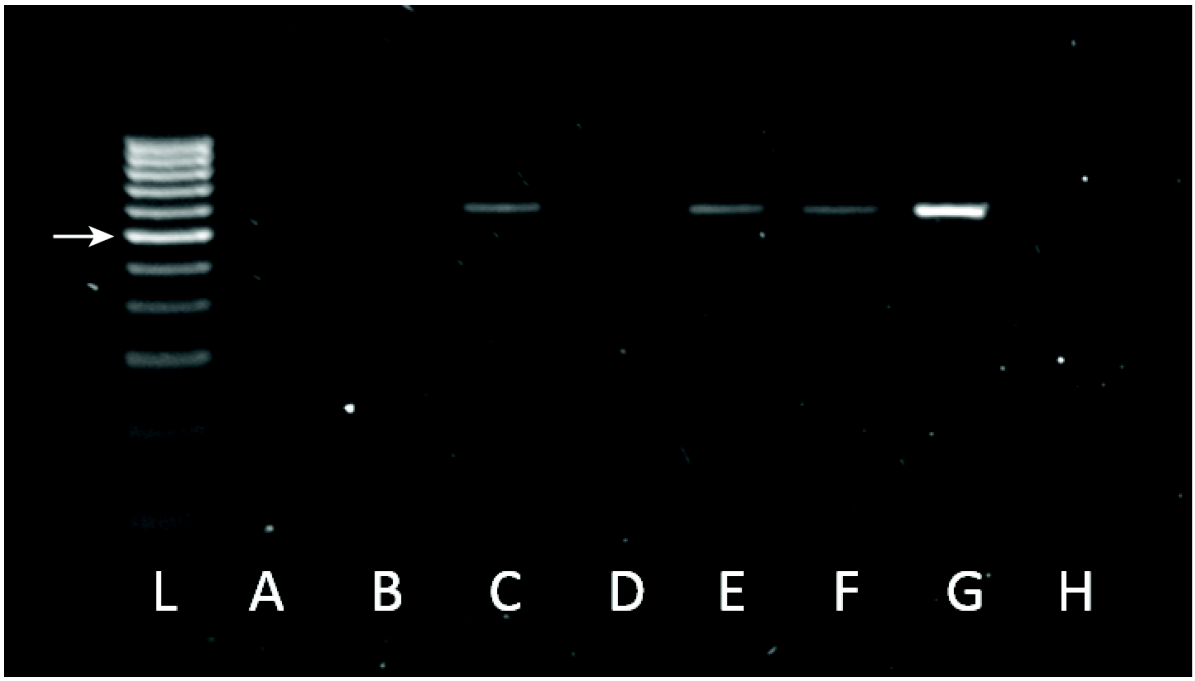


Figure 2.6. Expression levels of *piggyBac transposase* in 1- to 5-day-old embryos from transgenic WCR strains. Lane L) 100-bp ladder (exACTGene, Fisher), A) Min-3a; B) Min-3b; C) Min-8; D) Min-2a; E) Min-1a; F) Min-3c; G) Min-3a gDNA as the positive control; H) ddH_2O. Expected PCR product = 592 bp (arrow marks 500 bp band). Note low level of amplification in lanes A, B, and D.

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SUPPORTING INFORMATION

Table S2.1. ddPCR comparison of two single-copy control genes. We used gDNA from a single male or female to test the copy number for the control gene, *white*. The concentrations are calculated by QuantaSoft.

Sample	Target	Concentration	Positive Droplets	Total Droplets	Ratio*
Male1	*White*	436	4466	14433	1.05:1
	RPS6	417	4457	14948	
Male2	*White*	271	3251	15825	0.93:1
	RPS6	290	3543	16225	
Female1	*White*	360	4213	15997	1.02:1
	RPS6	353	3728	14395	
Female2	*White*	291	3109	14182	0.93:1
	RPS6	314	3619	15434	

* Ratio = Concentration of *White* / Concentration of *RPS6.*

Table S2.2. ddPCR results confirming each single insertion strain.

Sample	Target	Concentration	Positive Droplets	Total Droplets	Ratio*
Min-1a #1	Transgene	196	2671	17383	0.65:1
	White	301	4387	19421	
Min-1a #2	Transgene	187	2452	16655	0.52:1
	White	360	4310	16350	
Min-1a #3	Transgene	180	2363	16639	0.46:1
	White	391	4885	17290	
Min-1b #1	Transgene	172	2161	15899	0.53:1
	White	323	3680	15345	
Min-1b #2	Transgene	229	2604	14717	0.44:1
	White	515	5691	16046	
Min-1b #3	Transgene	288	3293	15167	0.43:1
	White	671	7371	16953	
Min-1c #1	Transgene	247	3155	16632	0.5:1
	White	496	6217	18063	
Min-1c #2	Transgene	234	2948	16352	0.47:1
	White	497	5487	15912	
Min-1d #1	Transgene	171	740	5468	0.44:1
	White	390	4542	16097	
Min-2a #1	Transgene	168	2272	17104	0.54:1
	White	311	4238	18251	
Min-2a #2	Transgene	163	2291	17683	0.58:1
	White	282	4068	19108	
Min-2a #3	Transgene	162	2302	17883	0.46:1
	White	353	4735	18268	
Min-3a #1	Transgene	180	2168	15252	0.43:1
	White	418	4935	16503	
Min-3a #2	Transgene	166	2559	19470	0.4:1
	White	420	5657	18860	
Min-3a #3	Transgene	199	2197	14147	0.5:1
	White	399	4082	14204	
Min-3b #1	Transgene	143	1638	14289	0.5:1
	White	287	2965	13700	
Min-3b #2	Transgene	253	3333	17218	0.48:1
	White	529	6737	18589	
Min-3b #3	Transgene	226	3010	17243	0.47:1
	White	479	6248	18684	

Table S2.2. Continued

Sample	Target	Concentration	Positive Droplets	Total Droplets	Ratio*
Min-3c #1	Transgene	191	2294	15334	0.55:1
	White	346	3938	15454	
Min-3c #2	Transgene	284	2905	13552	0.52:1
	White	545	5597	15098	
Min-3c #3	Transgene	148	1841	15531	0.36:1
	White	407	5004	17100	
Min-3d #1	Transgene	112	1378	15223	0.47:1
	White	238	2780	15173	
Min-3d #2	Transgene	149	1995	16736	0.48:1
	White	310	3883	16746	
Min-3d #3	Transgene	153	2305	18928	0.49:1
	White	310	4500	19406	
Min-3e #1	Transgene	240	3494	18916	0.52:1
	White	458	6108	18956	
Min-3e #2	Transgene	292	4215	19176	0.55:1
	White	535	6753	18477	
Min-3f #1	Transgene	206	2642	16449	0.56:1
	White	368	4361	16248	
Min-3f #1	Transgene	180	2150	15148	0.44:1
	White	408	4698	16017	
Min-3g #1	Transgene	221	2653	15487	0.48:1
	White	456	4833	15049	
Min-4a #1	Transgene	280	3458	16342	0.51:1
	White	553	6371	16986	
Min-5a #1	Transgene	218	3044	17959	0.44:1
	White	497	6665	19340	
Min-5a #2	Transgene	162	2438	18977	0.49:1
	White	330	4610	18858	
Min-7a #1	Transgene	218	2483	14660	0.45:1
	White	487	6242	18430	
Min-7a #2	Transgene	220	2794	16399	0.45:1
	White	488	6381	18808	
Min-7a #3	Transgene	52.3	629	14454	0.47:1
	White	112	1326	14607	
Min-7b #1	Transgene	116	824	8986	0.62:1
	White	186	2177	14921	

Table S2.2. Continued

Sample	Target	Concentration	Positive Droplets	Total Droplets	Ratio*
Min-8 #1	Transgene	77.5	1031	16177	0.63:1
	White	124	1691	16854	
Min-8 #2	Transgene	80.8	1111	16741	0.58:1
	White	140	1952	17351	
Min-8 #3	Transgene	142	1915	16832	0.52:1
	White	272	3503	16980	

We used single individual's gDNA to detect the copy number of the transgene compared to the control gene, *white*. The concentrations are calculated by QuantaSoft. Note: since all samples were offspring from outcrosses, the concentration of the transgene (heterozygous) will be half the amount compared to the control gene (homozygous).
* Ratio = Concentration of Transgene / Concentration of *White.*

Table S2.3. Junction sequences for 17 single insertion strains.

Insertion*	Strain	Junction	Sequence
1	Min-1a	Left *Minos*	**TA**ATAGTCAAAAGTTGAATGGGTTCAACAAGTC
1	Min-5a	Left *Minos*	**TA**ATAGTCAAAAGTTGATGGGTTCACAAGTC
2	Min-1c	Left *Minos*	**TA**ATAGAAATGAGTGTAAAATGCACAAGAATA
2	Min-3f	Left *Minos*	**TA**ATAGAAATGAGTGTAAAATGCACAAGAATA
2	Min-3b	Left *Minos*	**TA**ATAGAAATGAGTGTAAAATGCACAAGAATA
3	Min-2a	Right *Minos*	**TA**TCAGATCTTTTGTAGCTTT
4	Min-3a	Left *Minos*	**TA**CGTATGTGGTAAAGGTGCGAGCGTAAGACCT
4	Min-3e	Left *Minos*	**TA**CGTATGTGGTAAAGGTGCCAGCGTAAGACCT
4	Min-7b	Left *Minos*	**TA**CGTATGTGGTAAAGGTGCGAGCGTAAGACCT
5	Min-3c	Left *Minos*	**TA**ATATAATGAGTGTAATAT
6	Min-4a	Left *Minos*	**TA**TGGTTTATACCCATGATACATTAACAATTATT
7	Min-7a	Left *Minos*	**TA**TATTCTGTTTACATCCGTGGACTACCGCATTC
8	Min-8	Left *Minos*	**TA**TTACAAATTATCTGTCAGAAATTAACCGCAA
9	Min-1b	Left *Minos*	**TA**TAAGCT
9	Min-3d	Left *Minos*	**TA**TAAGCT
9	Min-3g	Left *Minos*	**TA**TAAGCT
9	Min-1d	Left *Minos*	**TA**TAAGCT

Insertion*: Different number indicates different insertion sequence in the indicated single insertion strain.

Table S2.4. Primer sequences.

Name:	Sequence:	Purpose:
qTrans F2	AAGAGGAACACAGACCAACGG	RT-PCR
qTrans F3	GTAGGAAGACGAATAGGTGG	ddPCR & RT-PCR
qTrans R3	CGTCAGGCTCATGTAAAGGTT	ddPCR & RT-PCR
qWhite F1	GACGGACTAGCGTGCTCAGG	ddPCR
qWhite R1	TCCACGGGCACACCATTTATAC	ddPCR
qRPS6 F1	TTGAAGAAGAAGAGGTGC	ddPCR
qRPS6 R1	TCTGCGTGCTGGATTTACTA	ddPCR
RT-Uni	CGTCAGCTTGATTAAGTCAACGATCTTTTTTTTTTTTTTTTTTTTV	RT-PCR
IMio1	AAGAGAATAAAATTCTCTTTGAGACG	Inverse PCR
IMio2	GATAATATAGTGTGTTAAACATTGCGC	Inverse PCR
IMii1	CAAAAATATGAGTAATTTATTCAAACGG	Inverse PCR
IMii2	GCTTAAGAGATAAGAAAAAAGTGACC	Inverse PCR

CHAPTER 3

An optimized small-scale rearing system and embryonic microinjection protocol for western corn rootworm, *Diabrotica virgifera virgifera*

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Abstract

Western corn rootworm (WCR), a world-wide pest of corn, has been successfully reared in laboratories since the 1960s. While established rearing methods are excellent for maintaining WCR colonies, they are not optimal for generating and screening transgenic WCR. Here we report the development of an optimized rearing system for use in WCR functional genomics research, specifically the development of a system that facilitates embryonic microinjection and downstream phenotypic screening for transgenic progeny. Transgenics-based experiments require smaller populations, stable survival rates, and the ability to manipulate insects at every life stage. In our system, the WCR life cycle (egg to adult) takes approximately 42.6 days, with the majority of individuals eclosing between 41 and 45 days post egg lay. Over the course of one year our overall survival rate was 67.44%. We used this data to create a quality control system. Herein we also report a detailed description of our protocol for microinjection of pre-cellular WCR embryos, and methodology for single-pair crosses.

Introduction

Western corn rootworm (WCR), *Diabrotica virgifera virgifera*, is a major pest of corn that has invaded many corn-producing regions around the world (Gray et al., 2009). To better understand this pest, researchers established laboratory rearing protocols for WCR in the mid-1960's (Georce & Ortman, 1965). Their rearing protocols were based on methods developed for rearing a closely-related species, the southern corn rootworm, *Diabrotica undecimpunctata howardi*, (Bigger & March, 1943), and were updated many times during the 1970s and 1980s (Branson et al., 1975; Branson et al., 1988; Dominique & Yule, 1983; Jackson & Davis, 1978). Importantly, many of the advances made to date in WCR research owe their success to the availability of lab reared WCR, thus demonstrating the critical importance of finely-tuned rearing protocols.

While the aforementioned WCR rearing protocols were effective for rearing beetles in the laboratory, it still took at least seven months to rear a single generation of beetles because eggs needed to be chilled for five months (Branson et al., 1988). Therefore the discovery and establishment of a non-diapausing strain of WCR was a major advancement in WCR rearing because this strain could produce an average of six generations per year, thereby providing a continuous supply of insects for experimental use (Branson, 1976).

Most rearing protocols focused on supplying WCR for use in applications such as pesticide trials (Meinke et al., 1998; Miota et al., 1998; Wright et al., 2000) or for testing resistance to transgenic crops (Gassmann et al., 2014; Meihls et al., 2008). However, the large-production

scale required to generate the numbers of insects needed for these experiments (e.g. hundreds or thousands of insects) could withstand inefficiencies in the system as these had negligible impact on overall output. Researchers that required fewer insects developed smaller-scale rearing and mating protocols for use in behavioral experiments (Branson & Johnson, 1973; Hill, 1975), and some tried to optimize rearing protocols for enabling manipulation of specific life stages (Jackson, 1986). Each of these protocols is extremely useful, and serves as the basis for laboratory rearing of WCR today.

The best rearing protocols are those that have been linked with the insect's behavior, and the aforementioned rearing protocols were developed following this principal. They not only pay close attention to the WCR life cycle, described by Krysan and Miller (2012), but also take into account WCR characteristics such as egg laying and mating behaviors. The two most common egg-collection methods use either soil dishes (Meihls et al., 2008), or agar plates with cheese cloth (Lefko et al., 2008) for oviposition. Eggs can be collected overnight, or for as long as seven days. Collected eggs should be washed to remove soil and other particulates either by washing the collected eggs on a sieve, or by washing them through/off the cheese cloth they were collected on, into water. Both methods are straightforward, and have good egg recovery rates.

A major challenge for rearing WCR is providing them high-quality corn roots to feed on (i.e. larvae can only feed on corn roots). Most rearing protocols do this by co-localizing the insect with its host (i.e. rearing larvae in soil along with the growing corn plants). Once WCR

larvae are ready to pupate, they stop feeding and start searching for a suitable location in the soil for pupation. To make allowances for this behavior, many protocols suggest moving late-stage larvae to a soil-only environment. In the absence of satisfactory locations for pupation, larvae will remain in the wandering stage for longer periods of time, up to a few weeks. For mating and egg laying, adult WCR are usually kept in cages that accommodate population sizes of 100-1000 individuals.

Although there are several artificial diets that are known to work well for rearing adult WCR (Frontier Insect Diet, Newark, DE, USA), there are no good artificial diets for long-term use with WCR larvae. Artificial diets, while good for short term larval feeding, have very low efficiency when trying to rear larvae to adulthood (Pleau et al., 2002). Therefore, many researchers simply purchase WCR as needed rather than keeping them in colony. A good artificial diet, one that could support WCR from larva to adult, would greatly benefit researchers by reducing the space required to rear WCR, while also permitting better control of rearing conditions (e.g. contamination from soil, corn, and other soil pests).

While artificial diet is not ideal, researchers frequently use these when conducting molecular genetic tests such as oral RNA interference (RNAi). The diet is sufficient for short-term use, and enables better uniformity. However, these experiments are limited in scope due to the fact that they have to be completed within the same generation. All tests requiring more than one generation must be performed on corn, rather than artificial diet.

Microinjection in WCR has been reported before, but with larvae (Alves et al., 2010). Larvae microinjected with double-stranded RNA (dsRNA) is another way to carry out RNAi experiments in WCR. It is a better way to observe RNAi phenotypes from larval to adult stages, because of the limitation of feeding on artificial diet. However, for other molecular methods, such as transgenic techniques, embryonic microinjection is required. The conditions for microinjection of embryos can vary widely for different insect species, for example microinjecting embryos directly (Lorenzen et al., 2007) vs. injecting them under oil (Cooley et al., 1988). A critical factor is the timing of injection. Specifically, embryos must be injected before they cellularize (i.e. during the syncytial blastoderm stage) because unlike dsRNA, used for RNAi, DNA cannot cross cell membranes. For example, in Drosophila, embryos need to be injected within two hours after egg lay. Therefore development of a successful embryonic microinjection protocol for use in WCR needs to take into account the best conditions for female egg laying, as well as the best method for capturing sufficient quantities of precellular embryos.

Functional genomic experiments, including the development and maintenance of transgenic strains, have certain requirements not currently met by standard WCR rearing protocols. Therefore we have developed a specialized rearing system that has been optimized for transgenics-based studies. Herein we also describe an embryonic microinjection protocol for production of transgenic WCR, and methods for downstream screening for transgenic offspring. Importantly, the rearing protocol described here is geared towards small-scale molecular genetic studies requiring multiple strains or treatments, with the added requirement

of having to fit within the confines of an average molecular biology laboratory. Finally, we describe a simplified system for single-pair mating that reduces the labor involved in egg collection, while still providing sufficient offspring for analyses. It is our hope that the availability of small-scale rearing protocols aimed at functional genomic studies will help broaden the scope and application WCR research.

Materials and Methods

Insect strains

The non-diapausing strain of WCR used in this work is a mixed colony generated from WCR received from Dr. Wade French (USDA-ARS, North Central Agricultural Research Laboratory, Brookings, SD) and Crop Characteristics (Farmington, MN, USA). Transgenic WCR were developed by Fu-Chyun Chu in 2015 from this non-diapausing colony following the protocols established here and in our previous work (Chu et al., 2017). All insects were maintained at 26^{o}C in 60% humidity with 14:10 light cycle. Adults were fed artificial diet (Frontier Insect Diet, Newark, DE, USA), while larvae were fed on organic corn (Trucker's Favorite yellow, Coor Farm Supply, Smithfield, NC, USA) grown in topsoil (Scotts® Premium Topsoil, The Scotts Company, Marysville, OH, USA) following the protocols outlined below.

Details of Rearing Protocol

This rearing system was designed to allow multiple strains to be reared in the same area, without cross contamination. Specifically, since WCR larvae have a wandering stage where

they could potentially escape their containers, a closed-lid system was created to keep each strain isolated. To determine if this system produced WCR of similar or better quality than established rearing systems (Branson et al., 1988), we monitored our closed-lid system for larval growth and adult eclosion rates.

1. Single pair and colony level egg collection

Egg collection was conducted on 1% agar (Drosophila agar, type II, Apex, San Diego, California, USA) in Petri dishes (100 mm x 15 mm, VWR, Radnor, PA, USA) having one layer of filter paper (Fisher, Pittsburgh, PA, USA) on the surface of the agar, followed by an additional four layers of cheesecloth.

For small-scale egg collection, such as from a single-pair cross, WCR adult diet (Product# F9766B; Frontier Agricultural Services, Newark, Delaware, USA) was added to a small Petri dish (35 mm x 10 mm) and placed on top the cheesecloth. A 6 oz container with a 100 mm opening (to fit Petri dish) was placed upside-down on the agar dish to act as a lid, and very small holes were poked into the top for air exchange. Each of these single-pair chambers can hold two to seven adults (note, death rate increased when placing more than eight adults in one chamber). Fresh adult diet was provided every two to three days (note, mold growth becomes an issue if diet remains more than three days). These chambers were used for overnight egg collections, as well as for week-long egg collections. Note: agar dishes start to dry out after seven to ten days, therefore we changed them every week.

Colony-level egg collection was performed in a 30 cm^3 cage (BugDorm, MegaView Science, Taiwan) from 500-1000 adults. A flask (300 ml) of water, covered with a cotton ball which holds in place a cotton roll (6" x 3/8", TIDI Products, Neenah, WI, USA), was used as the colony's water source. A Petri dish (100 mm x 15 mm) with adult diet was placed in the cage, with fresh diet added as needed (i.e. when the diet gets low or too dry). To collect eggs, an agar egg collection dish (as described above) was placed into the cage and covered with a tent of tin foil. Overnight egg collect is recommended, but the system is also good for collecting embryos over a few days.

2. Egg collection and incubation

Cheesecloth was removed, one layer at a time, with forceps and put in a beaker (500 ml) of water. Eggs were washed by gently stirring. Eggs were transferred to filter paper or soil using a bulb pipette, taking care to minimizing water carry-over.

Eggs that were still covered in unwanted material were transferred to a new cup of water and washed up to three more times. For standard rearing, 300-500 eggs were transferred directly into a 1 oz cup (WNA, Covington, KY, USA) with soil and covered with a lid (Fig. 3.1). These were held for seven days in a 26^{o}C incubator. Two to three cups of eggs per week were used to maintain a colony of around 500-1000 adults. Note, colony size needed to produce 10,000 or more eggs per week.

For smaller-scale egg collections, the desired number of eggs were transferred to filter paper and keep in an agar dish at 26^{o}C. Eggs were incubated until they hatched. Note, sometimes mold grew during incubation, but most of the molds had little to no affect on hatch rates.

3. Preparation of sprouted corn

Corn kernels were washed with 1% bleach and rinsed well before soaking them in water over night. The next day the kernels were spread out on a wet tissue paper in a large Petri dish (150mm x 15mm) and left for one to two days. Corn was monitored for sprout growth starting at ~24 hours. Once sprouts reached 30 mm in length they were placed into a larval-rearing box (38 oz, Item: #128NC888, WebstaurantStore, Lancaster, PA, USA) and stored in 4 ^{o}C until needed. One box of sprouted corn created 40-50 "primary" larval-rearing boxes (16 oz, Item: #128HRD16, WebstaurantStore, Lancaster, PA, USA) or 20-25 "secondary" larval-rearing boxes (38 oz, Item: #128NC888, WebstaurantStore, Lancaster, PA, USA).

4. Preparation of primary larval-rearing boxes

Collected embryos were incubated for seven days, then transferred along with soil from the 1 oz cup to a primary rearing box (16 oz, Item: #128HRD16, WebstaurantStore, Lancaster, PA, USA) with sprouted corn on the bottom (Fig. 3.2). Additional soil was added to fill ~60% of the container. Water was applied as needed. Note, for larval rearing conditions, too much moisture will increase issues with mold and mites, but too little causes dry roots and/or larval death. Soil moister levels were tested by sampling soil by hand (i.e. squeezing with finger tips). Ideally there should be enough moister to hold the soil together without squeezing out

any water. However, the best conditions may differ depending on rearing environment and/or soil. Eggs hatched two to four days later and First-instar larvae remained in primary rearing boxes were they fed on the corn roots for up to one week. Although primary boxes are small,, the corn roots were enough to support larvae until mid to late second instar. Each primary box was covered with a lid that had small holes for air exchange, and stored at 26^{o}C.

5. Preparation of secondary larval-rearing boxes

Secondary larval-rearing boxes (32 oz, Item: #128NC888, WebstaurantStore, Lancaster, PA, USA) were prepared by adding sprouted corn covered with soil to one side of each box (Fig. 3.3 A) at least three days before use. Note, when prepared more than three days prior to use, we placed them in 4^{o}C after day three. On the day of transfer, everything (soil, corn and larvae) from the primary rearing box was moved to the empty half of the secondary rearing box (Fig. 3.3 B). Any remaining larvae were moved from the primary rearing box to the new box using a small brush. Additional soil was added to fill ~60-70% of the new box and keep stored at 26^{o}C for two weeks.

After two weeks, the secondary rearing boxes were opened and larvae and pupae were transferred to age-specific rearing boxes (see below). Note, if insects were healthy most were at the late third-instar larval, pre-pupal, or pupal stage. Since third-instar larvae may have still been feeding, these were moved to a new secondary rearing box with sprouted corn evenly spread out in the container and covered by soil. Note, a secondary rearing box can house 100-150 large larvae, and is sufficient to support individuals until they eclose to adults.

Note that the late third-instar larval stage was the best age for screening and/or microinjecting, since later stage larvae are both large and strong enough for handling, and tolerate some degree of damage caused by microinjection. Moreover, it only takes about 10-14 days for those larvae to become adults, so it is easier to set up and track the experimental individuals or groups for data collection. However, pre-pupae and pupae are not good for microinjection and need to be handled carefully. Too much force from the brush or forceps might lead to deformed adults or unsuccessful eclosion.

6. Preparation of pupal-rearing boxes for individuals or groups

Pre-pupae and pupae were placed into new secondary rearing boxes with soil (no corn). Each box held 150-200 individuals. Insects were covered with soil to keep them moist. In cases were individual pupae needed to be kept separately, they were placed in 1 oz cups with soil, like those used for embryos, and held until they eclosed. Pupae were screened and sexed, either individually, or in groups, and separated by sex.

7. Collection of adults for colony- or single-pair scale mating

After an additional week of rearing, individuals that had eclosed were moved into colony cages or used in single-pair crosses (described above). Each rearing box was checked daily until no new adults emerged for three consecutive days. In cases where every individual was important (i.e. transgenics), we removed soil and plants and careful went through each rearing box looking for any remaining larvae, pupae or adults. All larvae or pupae found during this search were transferred to an appropriate rearing box to complete development.

Developmental time, efficiency of eclosion after handling, and quality control system

Data on growth rate and total developmental time (egg to adult) was collected for WCR reared following the above protocol. We first determined growth rate and total developmental time based on "normal" rearing, then collected the same data for "disturbed" rearing to determine if handling affecting insect survival rate. After we sorted out the insects from secondary rearing boxes, we used the larvae and pupae to set up separate rearing boxes according to the protocol, and keep rearing the individuals to adulthood. Third-instar larvae were determined by the individual's size and activity (moving and/or feeding), while individuals that still looked like larvae, but had already built a soil cocoon or were otherwise showing little movement were recorded as being prepupae. Survival rates were calculated based on numbers of larvae and eclosion of healthy adults over a 19-week period. Larval and prepupal/pupal survival rates were compared to determine which life stage is most sensitive to handling. A quality control scheme for monitoring overall health of the rearing system was based on a one-year record of survival rates. Since survival from third-instar larval stage to the adult stage was the critical part of the system, quality control was set by survival rate after the third-instar larval stage. We used JMP (SAS labs, Cary, NC, USA) to calculate and graph the quality control system. The data set was used for calculating the mean ($\bar{x}$) and standard deviation (σ). The mean was set as the standard for the system. The upper control limit (UCL) was calculated as ($\bar{x} + 3\ \sigma$) and lower control limit (LCL) was calculated as ($\bar{x} - 3\ \sigma$).

Embryonic microinjection protocol

Germline transformation was accomplished by microinjection of plasmid DNAs into pre-cellular embryos. Newly laid eggs were collected on an agar dish (see above) inside an adult cage from ~5PM to ~8AM the next day (i.e. overnight egg lay). Agar dish of eggs was removed by no later than 9AM and all embryos were microinjected by noon (12PM). Therefore the oldest embryo would be no more than 19 hours old. Note, microinjection of dsRNA lacks this time limitation because RNAi is systemic in WCR, meaning that dsRNA can move between cells. Therefore, if performing RNAi it may be better to use older embryos because this could increase survival rates.

Collected embryos were washed (see rearing protocol above) before transferring them to filter paper. Black filter paper (Ahlstrom, Helsinki, Finland) was cut into strips as wide as a glass slide and taped firmly to the slide to ensure the paper laid flat. Note, black filter paper improves visibility under the microscope by reducing the amount of reflected light.

Glue (Elmer's GlueAll, non-toxic, Inc., Statesville, NC, USA) was applied to the filter paper in fine lines, and a fine paintbrush was used to gently move WCR eggs one-by-one from the filter paper to the glue line. Note, it is important to lay eggs on the glue before the glue dries out, and to keep at least one egg's distance between eggs. Also note that eggs will sink into the glue if too much is applied, which will make the eggs very difficult it inject once the glue is dry. Multiple lines of eggs were placed on a single filter paper slide. Once all eggs were placed on the filter paper, we waited until all the glue was dry before microinjecting them.

Microinjection needles were formed from borosilicate glass capillaries (4 in, OD: 1 mm, ID: 0.58 mm, World Precision Instrument, Inc., Sarasota, FL, USA) using a P-2000 Laser Based Micropipiette Puller System (Sutter Instrument, Novato, CA, USA). For WCR embryos we used a setting of: Heat = 335, FIL = 4, VEL = 40, DEL = 200, PUL = 100. DNA in injection buffer was backfilled into the glass injection needle and placed into a needle holder. The tip of the needle was broken very gently using a fine pair of forceps. Due to the hard surface of WCR eggs, each egg was wetted with autoclaved water using a fine paintbrush and microinjected before the surface dried. After injection, the filter paper of eggs was removed from the glass slide and placed on a 1% agar dish (see above). Dishes were then sealed with parafilm (Bemis NA, Neenah, WI, USA) and stored at 26℃ for 10 days.

Eggs were checked after ten days and newly hatched larvae were transferred to a primary rearing box with sprouted corn and soil (see above, ~20 larvae per box). Eggs were checked daily for up to two weeks, transferring larvae each day. Note, mold sometimes grew on the agar dish, but larvae still hatched in most cases. Also note that larvae may move to the lid or get stuck in the water, or mold so it's important to look carefully to find all of them.

After ~two weeks we opened the primary rearing boxes and transferred ~ 20 larvae each into new primary rearing boxes with sprouted corn and soil, then reared them at 26℃, checking for adults at the appropriate time.

DNA plasmids and controls

To generate transgenic WCR, precellular embryos were microinjected with plasmid DNA, pBac{3xP3-EGFPaf} and phspBac (Horn et al., 2000), with a concentration of 750 ng/µl in 20% phenol red buffer (Cat #143-74-8; Sigma-Aldrich, St. Louis, MO, USA). Controls consisted of either uninjected embryos, or embryos injected with buffer alone. After injection, embryos were monitored for hatch rates from each treatment and compared between experiential and control groups.

Fluorescent light screening

Rearing boxes were opened and sorted ~4-weeks post injection (phenotypic screening was expected to be clearest in pre-pupae or pupae). Larvae were placed at 4^{o}C to slow them down for screening. Note, screening a single secondary-rearing box of larvae took around 20-30 minutes. Insects were separated into groups of larvae, pre-pupae, or pupae for convenience. Either separated or mixed stages were placed into separate containers and reared to adulthood following the rearing protocol, and survival rates calculated.

Handling of individual pupae and survival rates

Pupae were separated based on sex as described by (Krysan, 1986), and eclosion rates determined. Pupae were removed from rearing boxes, handled with pointed featherweight forceps (Catalog #4748, Bioquip, Rancho Dominguez, CA, USA), and viewed with a dissecting microscope (Leica). Each pupae was placed into a small cup (same as eggs cup), one pupa per cup. These were monitored for eclosion rate to determine if the handling

damaged the pupae, and each was re-sexed as an adult to validate the result of pupal sexing. Note, while handling and screening pupae can damage them, this step is unavoidable because WCR larval development can vary greatly. Specifically, while some individuals in a rearing box may be early in development (second-instar larvae), others from the same egg collection may have already pupated. Moreover, for some experiments such as RNAi or transgenic, individuals may need to be screened for phenotypes and separated for monitoring at the pupal stage.

Assessment of single-pair cross protocol

We determined efficiency of single-pair matings and egg lays by recording results from ten such crosses. Each mating pair was housed separately in single-pair chambers (see above), and cheesecloth changed daily to monitor female egg laying behavior. In cases where the male died before the female started laying eggs, a new male was added to the chamber. If the male died after the female started laying eggs, no new male was added. Eggs were counted every day, and female survival was recorded in days.

To determine if mating occurred before adults were collected, 15 outcrosses were performed with one male beetle from a transgenic WCR strain that carried a marker gene, DsRed, driven by the common eye expression promoter, 3xP3 promoter (Berghammer et al., 1999), crossed to two to three wild-type females per container (single pair chamber). Four outcrosses of one female beetle and one wild-type male were also set up as the control. We screened third-instar G_1 larvae for marker gene expression with a Leica M165 FC

fluorescence stereomicroscope using a DsRed filter (excitation filter: 510-560 nm, emission filter: 590-650 nm), and calculated the ratio between transgenic G_1 to total G_1 recovered from each outcross to determine if the wild-type females were already pre-mated with wild-type males.

Results

This is a relatively small scale rearing system but works for multiple purposes or strains in a regular molecular and genetic lab space (Fig. 3.5). We designed these colony tests for this system, along with some related experiments, such as microinjection, screening, and single pair crosses to determine if this system can fit and work for the requirements for transgenic strains or functional genomic-related experiments.

Developmental time

Six cohorts of WT WCR, each consisting of 300-500 individuals, were tracked as they moved through our small-scale rearing system. Since WCR routinely have relatively low hatch rates in the lab, individuals weren't counted until they were sorted from secondary rearing boxes, 21-24 days post egg lay (pel). At this time there were 1300 WCR larvae and pre-pupae/pupae, with the majority being third-instar larvae, and 13.8% (180 out of 1300) being pre-pupae/pupae. All insects were placed into new rearing boxes and monitored until eclosion. Of these, 1097 successfully eclosed (84.4%). The average time from egg to adult was 42.66 days, with more than 80% eclosing between 41 and 45 days pel (Fig. 3.6). This

short, high peak of adult emergence indicates that the system is stable, and that the insects develop at somewhat similar rates.

To determine if developmental times and eclosion rates remained more or less constant we tracked additional cohorts. A total of 25 cohorts, a total of 3260 third-instar larvae, were tracked from 16 rearing boxes consisting of 2184 pre-pupae/pupae, and 13 rearing boxes consisting of 2146 mixed stage WCR (1544 larvae and 602 pre-pupae/pupae) for a period of 19 weeks (Table 3.1). Survival to adulthood was higher (82.1%) for cohorts tracked from an earlier stage (larvae), while survival of pre-pupae/pupae was much lower (56.0%). However, the average of the two (71.6%) is comparable to the results from the mixed-stage rearing boxes (73.9%), indicting that both methods are similar in efficiency. Taken together, the overall survival rate is 72.25%.

Quality control system

Since quality control systems can provide insight into how well a rearing system is working, we sought to analyze our data based on quality control standards. Base on our survivorship data, mean survival in our system is 66.3%. Therefore this value was used as the standard to indicate a healthy colony. Upper and lower control limits (UCL and LCL) are used to indicate when the system is out of control and needs input or change to increase the health of the colony. Analysis of our data (Table S3.1) via quality control standards identified a single time point where the system did not pass quality control standards (Fig. 3.7). Upon closer inspection it was clear that this was due to one survival rate that was lower than the LCL.

Because all of the other time points clearly passed, we concluded that our system was basically stabile and that it only occasionally encountered situations with lower-than-expected survivorship. However, our data also suggest that if a low-survivorship situation doesn't recover for more than two consecutive weeks, that the system will become unstable, and will need intervention and/or adjustment to the rearing process to re-stabilize.

Embryonic microinjection

Four sets of embryos were tracked from egg lay to hatch. Of these, some were handled but not injected, while others were injected with either plasmid DNA in buffer, or buffer alone. Of the 485 embryos injected with buffer alone, 148 hatched (hatch rate=30.5%, Table 3.2), while the 1450 embryos injected with DNA injections only had a slightly lower (26.8%) hatch rate. While there was little difference between hatch rates from buffer injection vs. DNA injection, these initial tests of embryonic injection had much lower hatch rates than embryos that were handled in the same way, but not injected (53.2% see Table 3.2). Although this is 20% higher than the microinjected egg survival rate, we had expected to see more damage and lower survival rate in injected embryos. The similar survivorship seen in buffer vs. DNA injected embryos indicates that the amount of DNA being microinjected is acceptable for WCR embryos. Moreover, our microinjection protocol works and can be used for other applications.

Fluorescent light screening

To determine if the fluorescent light or cold temperatures (4^{o}C) could potentially damage the insects, we use an experimental wild-type WCR strain for the screening test. Of the 480 screened larvae, 438 (91.3%) successfully emerged as adults (Table 3.3). Pre-pupae had a 68.4% (1789 out of 2630) survival rate, pupae had an 82.9% (3950 out of 4766) survival rate, and a group containing both of these stages had a 79.7% (652 out of 818) survival rate. This number is slightly better than the longer observation results, which means that neither the screening method nor the cold temperatures caused significant damage to those insects.

Handling and survivorship of individual pupae

Survivorship from the pupal stage to the adult stage was much lower than that of larvae in our previous tests. However, it is nearly impossible to avoid working with pupae. Indeed, many applications might need to screen pupae. Therefore we designed and tested a protocol for working with pupae, and for separating them for individual monitoring. A total of 178 pupae were tested, with 141 successfully emerging as adults (79.2%) from their individual cups. Within that, 140 out of 141 pupae were correctly sexed as both pupae and adults. This result was higher than, or similar to our other results (Table 3.1 and 3.3), suggesting that additional handling can be done without causing excessive damage to the pupae. This also demonstrates that pupae can be reared successfully in individual containers if need be.

Single-pair crosses

Since germline transformation and other applications require single-pair crosses to be established, we assessed the efficiency of our single-pair crossing scheme, as well as the respective egg-collection method. From a total of 10 single-pair crosses, two males died before the female started laying eggs. After introducing a new male to each of these chambers, the females started to lay eggs. Pair #10 had the longest delay, with the first eggs being laid 40 days after mating. The average delay in egg lay for all 10 mating pairs was 19.3 days. After the first egg lay, there was gap of 4.63 days on average before the next clutch was laid (Table 3.4). In this system, the females laid an average of eight egg clutches during their lifetime, with the most fecund female laying 17 clutches, and on the opposite side of the spectrum, a few females had only three clutches (Table 3.4). On average females produced 414 eggs, with an average clutch size of 54.42 eggs. The shortest-lived female survived for 31 days, while the longest-lived survived for 132 days, with an overall average adult lifespan of 78.4 days (Table 3.4).

We also tested our single-pair crossing scheme using transgenic males outcrossed to wild-type females, because, if pre-mating occurred, it would result in clutches of non-transgenic offspring. Therefore, if our single-pair crossing scheme is not efficient or favorable for mating, we won't recover transgenic offspring. Our results demonstrate that all 15 of the crosses produced transgenic offspring in the first egg lay (Table 3.5). This indicates that the transgenic males successfully mated. However, since we have multiple females in each chamber, we would not be able to tell if only some of the females were pre-mated. We

compared these results with control outcrosses, which used one transgenic female mated with one wild-type male in each chamber. These results demonstrate that 28.4% of the offspring from these crosses were DsRed-positive, similar to single-pair crosses of transgenic males to wild-type females (23% see Table 3.5). The ratios of transgenic G_1 offspring obtained from the control outcrosses ranged from 16% to 40%. However, two of the experimental outcrosses (#8 and #11) had transgenic G_1 ratios below 10%, suggesting that pre-mating of wild-type females possibly occurred in these two outcrosses. On the other hand, since all of our outcrosses provided transgenic offspring in the first week of successful egg laying, pre-mating appears to be rare.

Taken together, the data demonstrate that our modified WCR rearing system enables manipulation of WCR at all life stages, and that it is efficient for small-scale rearing of multiple strains. We also describe establishment of an embryonic microinjection protocol suitable for germline transformation of this important pest species, and outline an efficient system for single-pair matings, small-scale egg collection, and screening and handling of transgenic offspring. Our rearing system can serve as the basis for developing additional transposon-based applications such as over-expression studies or for CRISPR/Cas9-mediated genome editing, and will hopefully aid future research into WCR biology.

Discussion

The goal of this research was to develop a small-scale rearing system for WCR that could supply high-quality precellular eggs for embryonic microinjection, facilitate downstream

phenotypic screening, and produce high-quality adults for molecular genetic studies. The results presented herein indicate that our rearing system achieved stable survival and developmental rates, and provided sufficient quality control. Each of the tests we performed demonstrated that our manipulation processes and protocols are efficient for obtaining reliable results. Moreover, the single-pair crossing scheme we outline resulted in successful mating and egg laying and fit easily into the overall system.

Since our rearing system was specifically designed for the production and maintenance of transgenic WCR, it necessitated the use of closed rearing containers. The main concern for such a system is the environment inside the larval-rearing boxes (both primary- and secondary-rearing boxes). Since larvae require corn plants, corn had to be grown within the confines of the box, therefore the size of the rearing box, with the lid closed limited the space for plants to grow. This situation can cause issues that threaten the health of the plants. While developing this rearing protocol we had problems with corn leaves rotting quickly due to high moisture content, which killed the insects and created mold and mite problems. However, planting fewer corn plants in each box and increasing the size of the opening for aeration in the lids reduced the occurrences of these problems.

After the larval-rearing boxes were stabilized, we observed the developmental rate from eggs to adults, and found that most eclosed between 41 and 45 days after egg lay. This indicates the insects were healthy, and the developmental rates were very similar. Adult females will needed another 10-15 days before they started laying eggs. As a result, the total generation

time is around 55-60 day, or around two months. This is similar to what others have reported (Branson et al., 1988; Li et al., 2014; Meihls et al., 2008). Since those insects were also been handled by sorting out from the boxes as larvae or pupae, our rearing process is comparable to previous methods. Moreover, this work was carried out by three different workers (one lab assistant, one graduate student, and one undergraduate student), which demonstrates that any level of laboratory worker can be trained to use or handle this system.

Our survival and eclosion rates are similar to those reported by others (Branson et al., 1988). Moreover, the rates were good enough to keep the colony healthy and producing enough adults to collect sufficient quantities eggs for use in microinjection-based studies. We were able to keep colony size around 500-1,000 adults at all times, and were able to collect over 10,000 eggs per week when needed. Another key indicator is the fact that analysis of our rearing data using the quality control standard revealed that our colony is relatively stable. A benefit of this stability is that the rearing system is able to endure occasional periods of low survival rates. The primary reason for the quick recovery is the fact that we are consistently collecting eggs (i.e. every week) to maintain the colony instead, of operating on a generation-to-generation capacity, as is done with other species. As a result, one week of high losses has a much smaller impact on the system as a whole. On the other hand, continuously low survival rates will indicate that the environment has changed or that some part of the system no longer works. If this has been determined, immediate actions are needed to find where the problem lies. Our quality control system, does not account for colony-level hatch rates. This is because eggs from colony-level collection are placed directly into the soil rather than being

counted (i.e. estimated the number of eggs per container). While we have tested hatch rates by placing eggs on moist filter paper and monitoring hatching (data not shown), this is not the same as if the eggs hatched in the soil. Expected differences are due to: 1) Eggs on filter paper will encounter more mold growth than they would in soil. 2) Eggs on filter paper will also encounter higher rates of bacterial growth. Although the growth of mold and bacteria did not appear to negatively impact hatch rates, they did change the environment the eggs hatched in.

One of the main challenges for development of a successful microinjection protocol was obtaining sufficient quantities of pre-cellular WCR embryos. This required acquiring embryos at very early time points, which in turn required the development of methods for collecting embryos. First, we monitored the agar dishes used for egg lays in the lab, and found that most females will only lay their eggs in the dark. This result is not surprising since researchers have long used foil or other materials to create dark environments for WCR to lay their eggs (Georce & Ortman, 1965). To accommodate egg collection for use in microinjection experiments we set the light-dark cycle for our incubators to be dark from 12AM to 10AM, encouraging the vast majority of females to lay their eggs after midnight. This ensured that harvested embryos would be 0-10 hr old, providing plenty of time to complete microinjections before cellularization.

Developing a microinjection method with acceptable survival rates took some time. One of the first issues we had to overcome was finding a reliable method for washing embryos and

affixing them so an injection needle could penetrate the eggshell. Unlike Drosophila, dechorionation of WCR eggs results in a complete loss of cohesion, perhaps due to differences in the structure of the eggshell or disruption of a vital embryonic membrane (Margaritis et al., 1980). While the use of glue to affix embryos to filter paper takes more time than simply placing embryos on a slide, as done for *Tribolium castaneum*, we have found this to be the most reliable method for WCR. Another time-consuming step is the need to pre-wet each WCR embryo, one-by-one, before injection, but again, we found this method worked best (i.e. reduced needle breakage).

The importance of having the ability to microinject pre-cellular WCR embryos with plasmid DNAs cannot be overemphasized because it will enable researchers to adapt sophisticated molecular genetic tools currently used in *T. castaneum*, including genome-wide mutagenesis (Lorenzen et al., 2007) and CRISPR/Cas9-mediated genome editing (Gilles et al., 2015) to bear on WCR biology. These genetic technologies may also hasten production of genetic pest control strategies (Horn & Wimmer, 2003; Phuc et al., 2007) for use in WCR.

Another hurdle we had to overcome was the use of fluoresce-based screening. Specifically, unlike most of the insects we work with, WCR spend the bulk of their lives in the soil. We quickly discovered that when WCR larvae and pupae are exposed to high-intensity fluorescent light (i.e. that used in microscopy), they reacted negatively and tried to avoid and/or move away from the light. While chilling them at 4^{o}C slowed them down enough to allow screening, the process may be somewhat harmful and could reduce overall fitness. That

said, our data indicate that they are sufficiently fit and fecund after exposure to both the light and to the cold.

Few research groups have examined and reported on the use of single-pair crossing schemes in WCR. Hill et al. (1975) reported data from 11 such crosses (Table 3.4), and a quick comparison shows that our data is very similar to theirs in most categories. However, they provided adults a natural food source rather than an artificial diet, and their experimental insects were larvae collected from the field, rather than insects reared for countless generations in a lab. Another group, Branson and Johnson (1973) (Table 3.4), reported longer female longevity, and obtained twice as many eggs per female. Another important difference is that both groups used a diapausing strain of WCR, either from the wild or reared in the lab for ~10 generations. These differences make comparisons to our work rather unreliable because our strain is a non-diapausing one that has been kept in the lab for over 200 generations. Despite these differences, our data suggest that our small-scale rearing system is suitable for many types of assays requiring healthy WCR of any life stage.

Although we collected adults daily males and females shared the same box for up to 24 hours before collection. Previous reports describe WCR mating as more likely to occur in the early morning and evening (Cates, 1968). Since we usually collected adults in the late morning (10AM-12PM), it is possible that some beetles mated before we collected them. For WCR, males can mate multiple times, however, females usually only mate once during their lifetime, though some have been observed to mate twice (Hill, 1975). Interestingly, another

report showed that if female WCR first mated with sterile males, and were then allowed to mate with fertile males, only 7 out of 30 females produced viable embryos (Branson et al., 1977). Since these reports indicate that it is rare for WCR females to have successful second matings, and the eggs laid by more than two thirds of those females could still be from the first mating, we used marker-gene screening of offspring to determine if the females we collect were virgins. All crosses of wild-type females to transgenic males produced transgenic offspring, suggesting that; 1) females successfully mated with transgenic males, and 2) most females were likely virgins when we collected them. However, some of our crosses had low percentages of transgenic offspring. Since each male was mated with three females, it is possible that some females pre-mated with wild-type males, however the percentage of non-virgin females is likely very low. Another important point to consider is the fact that some of the transgenic strains used in this assay were very weak expressers, and it is common to observe low percentages of transgenic offspring from some low-expressing strains. Therefore it is more likely that the low number of transgenic offspring was due to poor expression of the transgene, rather than due to non-virgin females. This conclusion is supported by control crosses (transgenic female crossed with wild-type male), which also showed low percentages of transgenic offspring.

In this study, we established and optimized a small-scale rearing system for WCR that can be monitored for colony healthy using a quality control system. We also describe methods for embryonic microinjection that is suitable for producing transgenic WCR. This work also demonstrates that screening WCR for transgene expression using fluoresce-based

microscopy, exposure to cold temperature, and handling are all acceptable for WCR larval and pupal survival. Finally, we describe a method for setting up single-pair crosses that is efficient for WCR mating and egg collecting. Taken together these methods should aid in the development of new genetic technologies for use in the study of WCR biology.

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Table 3.1. Survival rates among different life stages. WCR survival rates from different life stages after sorting from secondary rearing boxes. Pre-pupae and pupae are grouped together since they no longer require food.

Strain	Stages	# of Insects	# of Adults	Survival Rate
WT	Larvae	3260	2675	82.06%
	Pre/pupae	2184	1223	56.00%
	Mix*	2146	1586	73.90%
	Total	7590	5484	72.25%

*Mix = a mixture with larvae, pre-pupae, and pupae in the same container.

Table 3.2. Effect of embryonic microinjection on hatch rate. Microinjection tests on WCR embryos from overnight egg lay. The eggs from non-injection treatment were treated the same as injected eggs without microinjections.

Strain	Treatment	Eggs	Hatched	Hatch Rate
WT	Non-inject	100	63	63.00%
		150	76	50.67%
		100	57	57.00%
		120	54	45.00%
	Buffer-inj.	145	60	41.38%
		120	38	31.67%
		100	12	12.00%
		120	38	31.67%
	DNA-inj.	250	41	16.40%
		450	75	16.67%
		500	126	25.20%
		250	147	58.80%

Table 3.3. Effect of fluorescent-marker screening on adult eclosion rate. The adult eclosion rates for different life stages of WCR screening under the fluorescent light (CFP). The Mix stages contained pre-pupae and pupae only.

Type of light	Stages	Sets of screening	# of insects	# of adult	Range	Eclose Rate
CFP	Larvae	5	480	438	83%-94%	91.25%
CFP	Pre-pupae	25	2630	1798	30%-90%	68.37%
CFP	pupae	42	4766	3950	68%-95%	82.88%
CFP	Mix(pre+pu)	8	818	652	52%-95%	79.71%

Table 3.4. Female fitness in single pair set up. Different bioassay of female fitness cost tests on females from single pair containers. We also compare with some data from previous paper.

	Single Pair		1973*		1972+	
	Range	Mean	Range	Mean	Range	Mean
Female longevity (days)	31-132	78.4±32.6	6-163	67.7± 30.6	19-126	94.8±12.5
No. eggs oviposited/ F	83-1070	414±290.3	237-912	593.l±231.3	85-1913	1023±240
No. clutches/ F	3-17	8.1±5	3-20	11.3± 5.4		
Avg no. eggs/clutch/ F	1-128	54.42±25.7	39-79	56.9± 14.2		
Avg days between ovarian cycles	1-11	4.63±2.3	4.2-6.0	4.9± 0.4		
Preoviposition period (days)	13-40	19.3±8	1I-19	15.3± 2.0		

*1973= (Hill, 1975)

+1972= (Branson & Johnson, 1973)

Table 3.5. Outcross G1 screening for marker gene. Use transgenic strain to test the single pair outcross to determine if pre-mating happening in the wild-type strain females. Control crosses were set up with transgenic female crossed to wild-type male.

Cross #	1st week DsRed+ Larvae	Total Larvae	Total Red	Total Larvae	Rate
1	4	29	53	412	12.86%
2	34	141	75	415	18.07%
3	43	146	121	432	28.01%
4	28	115	153	465	32.90%
5	54	118	66	149	44.30%
6	28	79	59	180	32.78%
7	23	42	30	68	44.12%
8	12	184	47	608	7.73%
9	22	200	24	239	10.04%
10	39	146	216	693	31.17%
11	8	97	37	463	7.99%
12	87	255	266	728	36.54%
13	28	111	107	636	16.82%
14	11	113	80	558	14.34%
15	57	167	163	469	34.75%
Control 1	61	139	85	211	40.28%
Control 2	58	133	87	268	32.46%
Control 3	38	105	70	272	25.74%
Control 4	22	104	41	244	16.80%

Figure 3.1. Egg rearing containers. Eggs are placed in the middle of a 1 oz cup and covered with soil.

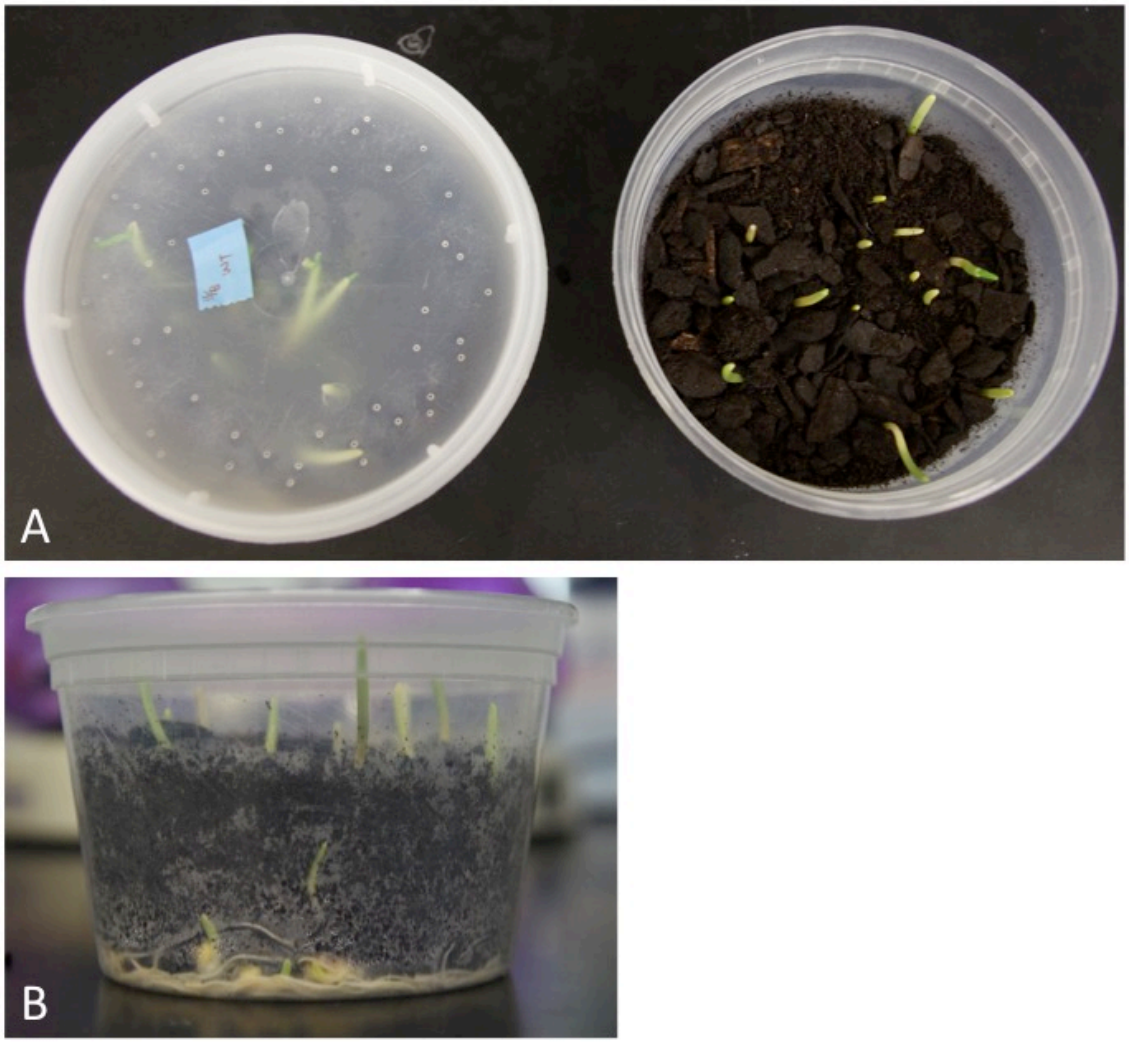

Figure 3.2. Primary larval-rearing containers. A) Each container possesses soil and newly sprouted corn, lids have wholes to allow air exchange. B) Minimum level of root growth required for larval to feed.

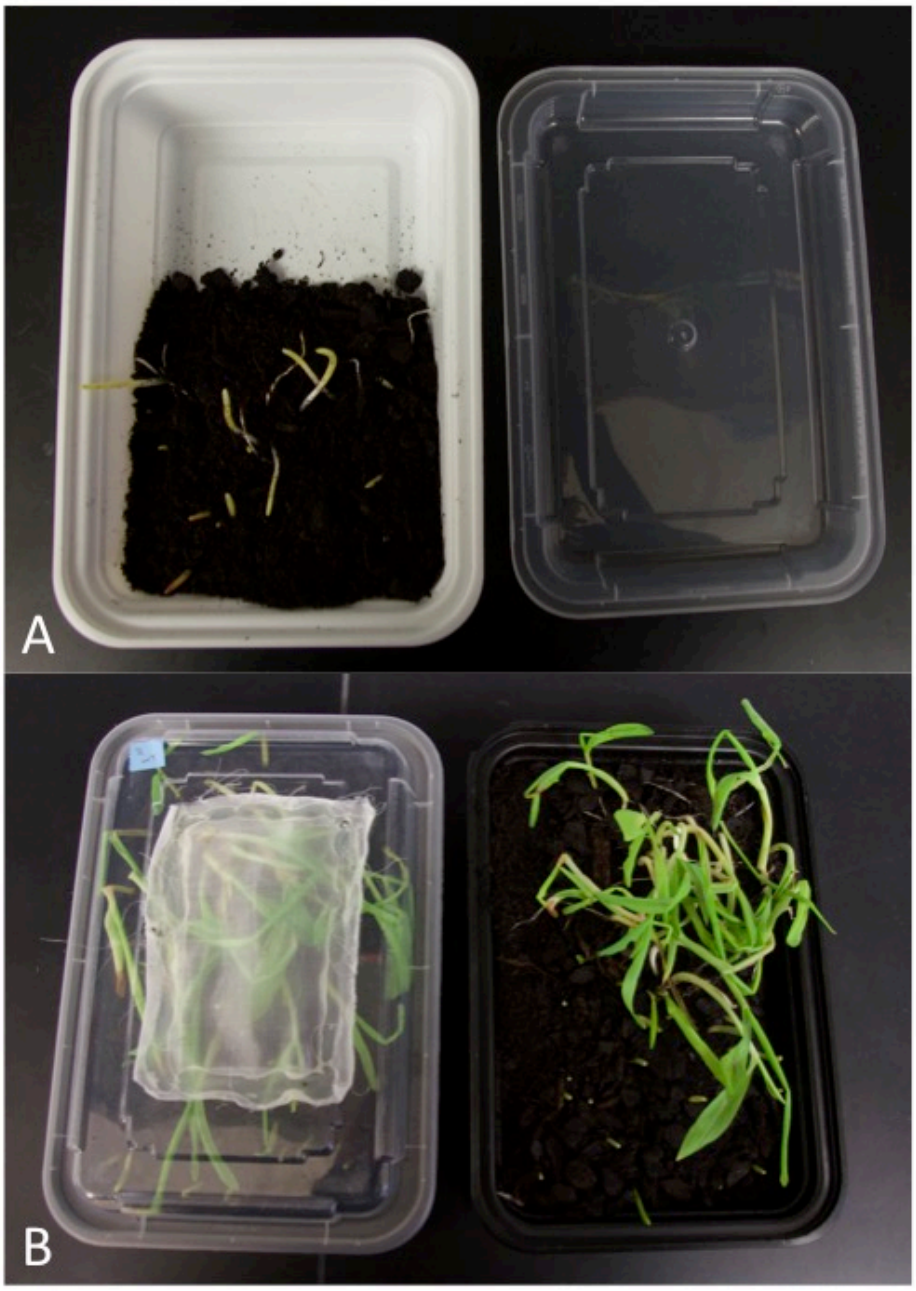

Figure 3.3. Secondary larval-rearing containers. A) The half box set up, the empty half will be used for transfer the contents of primary larval-rearing box. Use the complete lid when growing the corn without feeding. B) After adding the contents from primary larval-rearing box, use a lid with fine screen for air exchange.

Figure 3.4. Adult collection containers. Most adults exit the soil and can be seen through the lid (arrows pointed newly eclosed adults). Once the adults start eclosing, all plates can be cut and removed for convenient collecting of adults.

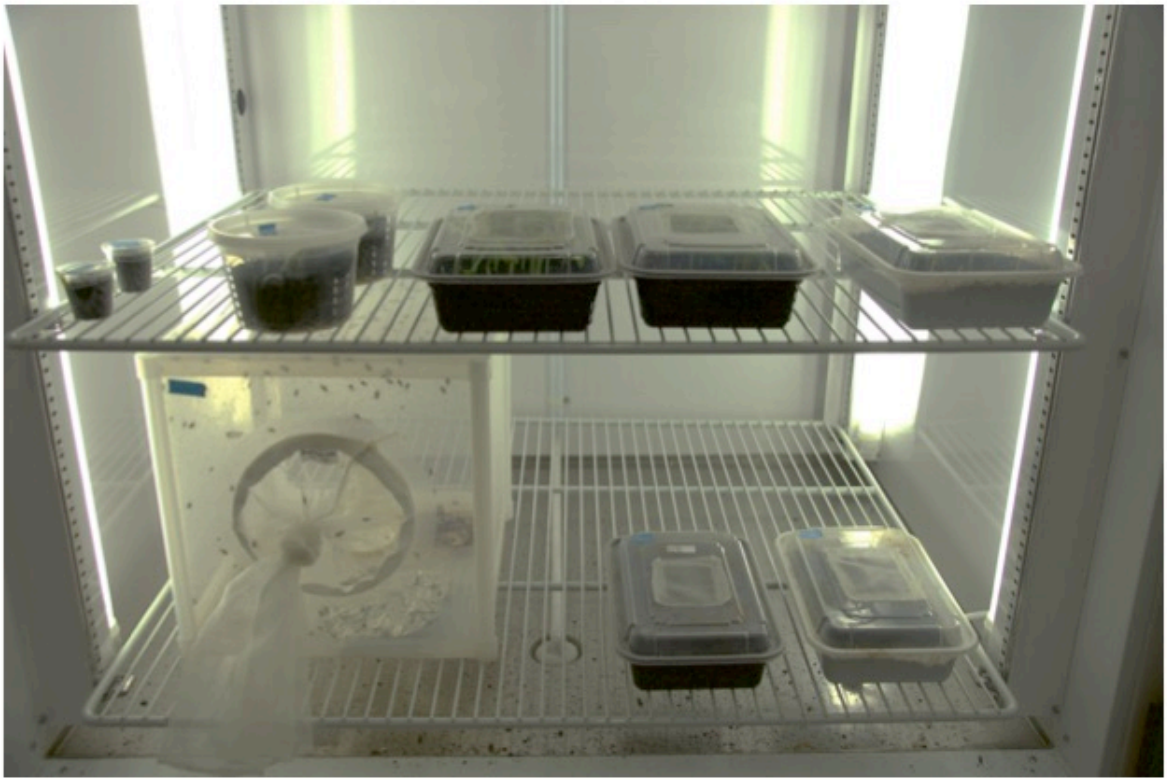

Figure 3.5. All containers needed for keeping a single WCR colony. The minimum requirement of space and containers for one colony from this rearing system. The upper left to right: Egg cups, primary larval-rearing boxes, two secondary larval-rearing boxes, and one after screening box. The lower level left is the adult colony and two adult collection boxes on the right.

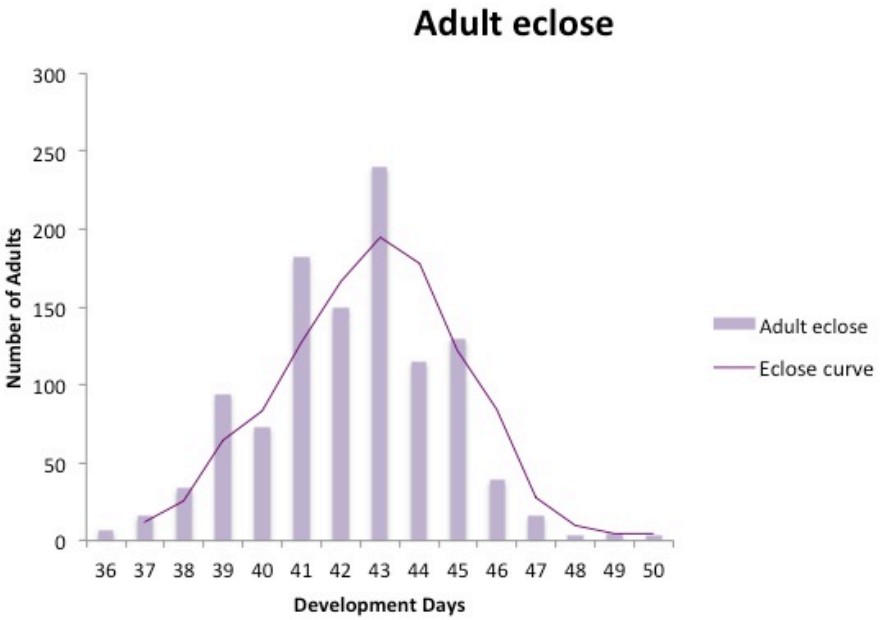

Figure 3.6. WCR development time. Each bar indicates how many insects had eclosed by each of the given total days of development time (days). The average is around 43 days.

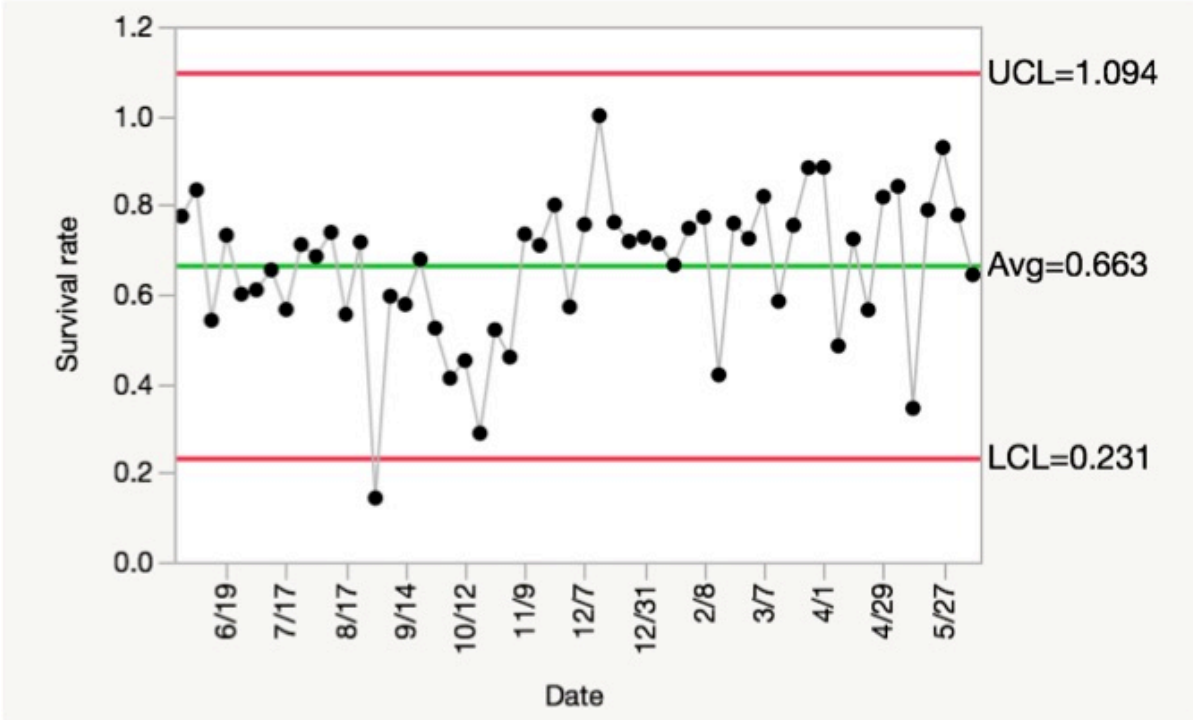

Figure 3.7. Quality control assessment. This quality control system is based on the survival rates for WCR (Table S3.1). Data within the upper red line (UCL) and lower red line (LCL) are within quality control standards, however, those above or below are considered to be out of control. UCL (Upper control limit) = $x + 3\sigma$ (x= mean, σ= standard deviation). LCL (Lower control limit) = $x - 3\sigma$.

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SUPPORTING INFORMATION

Table S3.1. WCR survival rate on secondary larval-rearing boxes.

Date	# of Insects	Adult	Survival Rate%
6/1	386	299	77.46%
6/5	132	110	83.33%
6/15	423	229	54.14%
6/19	97	71	73.20%
6/29	130	78	60.00%
7/6	415	253	60.96%
7/13	790	517	65.44%
7/17	419	237	56.56%
7/27	1104	785	71.11%
7/31	355	243	68.45%
8/7	195	144	73.85%
8/17	283	157	55.48%
8/24	120	86	71.67%
8/31	21	3	14.29%
9/4	655	390	59.54%
9/14	487	281	57.70%
9/21	1498	1016	67.82%
9/28	628	329	52.39%
10/2	272	112	41.18%
10/12	144	65	45.14%
10/19	323	93	28.79%
10/26	569	296	52.02%
11/2	366	168	45.90%
11/9	885	650	73.45%
11/16	767	544	70.93%
11/23	370	296	80.00%
11/30	77	44	57.14%
12/7	82	62	75.61%
12/14	12	12	100.00%
12/21	774	589	76.10%
12/28	969	696	71.83%
12/31	1307	951	72.76%
1/11	307	219	71.34%
1/15	209	139	66.51%

Table S3.1. Continued

Date	# of Insects	Adult	Survival Rate%
1/25	218	163	74.77%
2/8	264	204	77.27%
2/12	198	83	41.92%
2/22	589	447	75.89%
2/29	247	179	72.47%
3/7	293	240	81.91%
3/14	113	66	58.41%
3/21	346	261	75.43%
3/28	343	303	88.34%
4/1	519	459	88.44%
4/8	659	319	48.41%
4/15	489	354	72.39%
4/22	572	323	56.47%
4/29	263	215	81.75%
5/6	366	308	84.15%
5/13	375	129	34.40%
5/20	719	567	78.86%
5/27	491	456	92.87%
6/3	377	293	77.72%
6/10	432	278	64.35%
Total	23444	15811	67.44%

This is the data set for creating the quality control system. The data presenting one year monitoring of the rearing system. Survival rate is calculated by total adults eclosed divided by the number of insects (larvae, pre-pupae, and pupae) from sorting out the secondary boxes of the same group. The data was collected on a weekly base but sometimes the date is shifting due to the experimental schedule or holidays.

CHAPTER 4

Development and use of a *piggyBac*-based jumpstarter system in *Drosophila suzukii*

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Abstract

Spotted wing drosophila, *Drosophila suzukii*, are an invasive pest that primarily attack fresh, soft-skinned fruit. While others have reported successful integration of the *piggyBac* transposable element into the *D. suzukii* genome, with a very respectable transgenesis rate of ~16%, here we take this work a step further by creating *D. suzukii* jumpstarter strains. These were generated through integration of a DsRed-marked *Minos* element carrying an *hsp70*-driven *piggyBac transposase* gene. We demonstrate that there is a dramatic increase in transformation rate when *piggyBac*-based transformation is performed in a *piggyBac transposase*-expressing background. For example, we achieved transformation rates as high as 80% when microinjecting *piggyBac*-based plasmids into embryos derived from a *D. suzukii* jumpstarter strain. We also investigate the effect of insert size on transformation efficiency by testing the ability of the most efficient jumpstarter strain to catalyze integration of differently-sized *piggyBac* elements. Finally, we demonstrate the ability of a jumpstarter strain to remobilize an already integrated *piggyBac* element to a new location. We tested two donor strains, each possessing a different EGFP-marked *piggyBac* insertion. The first exhibits an enhancer-trap phenotype (i.e. expresses EGFP in additional tissues) and displays EGFP fluorescence throughout the entire body, while the other only expresses EGFP in the expected tissues, but at a lower-than-expected level. Remobilization rates differed by donor strain, with the latter donor being more efficient at 25.6% than the former. This demonstrates that our jumpstarter strains not only increase transformation efficiency when creating new *piggyBac*-based insertions, but could also be used in conjunction with a *piggyBac*-based donor strain for genome-wide mutagenesis of *D. suzukii*.

Introduction

Drosophila suzukii (aka spotted wing drosophila) was first described in Asia (Kanzawa, 1935), but has recently become an invasive pest in both the US and Europe (Bolda et al., 2010; Calabria et al., 2012). Its movement into these new regions poses a substantial risk to the fresh-fruit industry because, unlike most *Drosophila* species, *D. suzukii* larvae feed on ripening fruit rather than overripe or rotting fruit (Walsh et al., 2011). Moreover, *D. suzukii* is highly polyphagous and has been recorded feeding on many economically important crops (Cini et al., 2012). Therefore the dramatic expansion of *D. suzukii's* range has been accompanied by significant crop losses and large increases in pesticide usage, hitting the US fruit industry substantially, with $27.5 million of additional costs and losses in eastern states in 2013 (Burrack, 2014), and $500 million reported in western states in 2011 (Goodhue et al., 2011).

Research on *D. suzukii* focuses primarily on its pest status, control measures, behavior and basic biology (Asplen et al., 2015; Burrack et al., 2015), but gaining a broad understanding of *D. suzukii* genetics will require sophisticated molecular genetic studies. Current resources and tools for *D. suzukii* include a sequenced and assembled genome and several transcriptomes (Chiu et al., 2013), germline transformation (Schetelig & Handler, 2013), and Cas9-mediated genome editing (Li & Scott, 2016). However, like other Drosophilids, *D. suzukii* lacks systemic RNA interference (RNAi), forcing researchers to consider alternative molecular genetic research methods, such as siRNA expression via germline transformation

(Dietzl et al., 2007; Haley et al., 2008).

Most of the above-mentioned functional genomic tools rely on a candidate-gene approach. However, we sought to create a sequence-independent method that would enable an unbiased mechanism for querying the genome for genes involved in species-specific traits, such as host attractiveness. Since *D. suzukii* is closely related to *D. melanogaster,* arguably one of the most studied genetic model organisms, there is a wealth of molecular genetic tools that could be readily adapted to *D. suzukii*. Therefore, using *D. melanogaster* as a paradigm, we chose to build a transposon-based mutagenesis system (Horn et al., 2003), that has also been successfully used in non-Drosophila species, including *Tribolium castaneum* (Lorenzen et al., 2007) and *Anopheles stephensi* (O'Brochta et al., 2011). The system is frequently referred to as a helper/donor system, or more simply, a jumpstarter system.

The jumpstarter system employs two transgenic strains, each carrying a distinct transposon with unique marker genes to enable rapid identification of the elements. The first strain, the "donor", is stable in the absence of the required transposase. The second strain, the "helper", carries the necessary *transposase* gene. Each strain is stable on its own, but when crossed, the helper can remobilize the donor, provided the helper produces sufficient levels of *transposase* and that the donor is integrated in an accessible region of the genome. When the donor transposon is remobilized, and subsequently reintegrated into the genome, it is capable of disrupting genes in or near the new integration site. Thus, together, these strains can be used as a powerful mutagenic tool, one that can be used for genome-wide mutagenesis. An

advantage of such a system is that it is not limited to predictable targets, so can be used for broad, unbiased genetic screens, while allowing novel mutations to be quickly tracked down by identifying the genomic sequences flanking the transposon.

Here we report the generation and use of a similar system in *D. suzukii*. The donor strain was created using a *piggyBac* element (Cary et al., 1989; Fraser Jr, 2000), while the helper was generated using a *Minos* element (Klinakis et al., 2000a). We then demonstrate the utility of using the helper strains for establishing new *piggyBac* strains via microinjection of *piggyBac*-based donor plasmids into pre-cellular embryos derived from the helper strain, as well as for remobilization of an already integrated *piggyBac* element.

Results

Minos insertion strains

Transgenic *D. suzukii* were generated by coinjection of a 3xP3-DsRed marked *Minos* donor plasmid (Horn et al., 2003), along with a *Minos* helper plasmid (Klinakis et al., 2000a), into pre-cellular *D. suzukii* embryos. Of the 430 injected G_0 embryos, 124 hatched, with 45 surviving to adulthood. Each of the 45 G_0 adults were backcrossed to 2-3 wild-type mates. Two of the 45 crosses yielded transgenic offspring, with one G_0 parent producing two DsRed-positive G_1 offspring. We kept all three in case each was an independent transformation event. Our overall transformation rate was calculated at 4.4% (2/45), which is much lower than the rate Schetelig and Handler (2013) observed using *piggyBac*.

To determine if the transgenic G_1 flies carried a single *Minos* insertion or multiple insertions, each DsRed-positive G_1 adult was outcrossed to a wild-type mate and the G_2 offspring screened for DsRed expression. The two G_1 flies, H7 and H10, that were derived from the same G_0 parent, both produced a 1:1 ratio of DsRed-positive to DsRed-negative progeny (see Table 4.1), suggesting that a single *Minos* element had been integrated into their genomes. However, H28 had a lower proportion of transgenic offspring (Table 4.1).

Since segregation analysis indicated that each of the three G_1 flies carried only a single *Minos* element, we mated full-sibling, DsRed-positive G_2 flies and selected the brightest DsRed-positive G_3 offspring, as these were likely to be homozygous for the DsRed-marked *Minos* element. This process was repeated with the G_3 flies to establish pure-breeding *D. suzukii* helper strains. During the process, the H7 and H10 strains continued to produce healthy offspring, while the H28 strain generated fewer offspring by comparison (data not shown).

Cloning Insertion-site Junctions

Once homozygous strains were established, we cloned and sequenced insertion-site junctions from each of the three lines (Fig. 4.1). Sequence comparison revealed that the two G_1 flies (H7 and H10) derived from the same G_0 parent possessed identical insertion-site sequences, suggesting they resulted from the same transformation event. BLASTN analysis of the *D. suzukii* genome sequence indicates that this *Minos* insertion is located in an intron of the *D. suzukii* ortholog of a *Ca^{2+}-channel protein α1 subunit D (Ds Ca-α1D)* gene. Casual

observation has not revealed any obvious changes in fitness, physiology, or behavior in either strain, but we cannot fully eliminate the possibility that there is a phenotypic effect resulting from this insertion that makes them less fit. Conversely, the H28 strain has consistently produced fewer than expected DsRed progeny, a finding that may be explained by the site of genomic integration. Specifically, the *Minos* element inserted approximately 300-bp upstream of the 5' end of the *D. suzukii* ortholog of *Hsp70Bbb (Ds Hsp70Bbb)*. Since it is highly likely that this location is in the promoter region of this gene, it is possible that the insertion might be disrupting normal gene expression, which could explain why the H28 insertion seems to have affected survival (see Table 4.1).

PiggyBac transposase expression assay

To determine if the helper strains were expressing *piggyBac transposase* as expected, as well as to compare the expression levels between different strains, we extracted RNA from *D. suzukii* embryos at three different time points, 6 hr-, 15 hr-, and 24 hr-post egg lay (pel). *D. suzukii* generally hatch around 36 hr-pel. We used digital-droplet PCR (ddPCR) to assess levels of *transposase* expression, and normalized the data to a control gene, *D. suzukii white* (*DsWhite*) for each time point.

Interestingly, ddPCR results indicated that the H7 and H10 strains have slightly different expression profiles (for raw ddPCR data see Table S4.1). This finding is unexpected, since insertion-site junction sequence suggests that these strains are the result of the same transformation event. Specifically, H7 had a higher level of *transposase* expression during

early embryogenesis (6 hr-pel) than H10 (Fig. 4.2 A), although this difference was not significant. However, both H7 and H10 had significantly higher expression levels than the H28 strain (Fig. 4.2 A). By the middle time point (15 hr-pel), *transposase* expression was no longer significantly different among the strains, as *transposase* expression in the H28 strain had increased relative to the other two strains (Fig. 4.2 B). By the later time point (24 hr-pel), *transposase* expression in embryos was not significantly different among all strains (Fig. 4.2 C). Overall, embryos from the H7 strain start out with the highest levels of *transposase* expression, while embryos from the H10 strain have lower, but more consistent levels of *transposase* expression. Interestingly, *transposase* expression appears to increase over time in embryos from the H28 strain.

Transformation efficiency assay through microinjection

Since *transposase* (mRNA) levels are not necessarily indicative of activity, we tested the ability of each helper strain to catalyze integration of a simple *piggyBac* element into the *D. suzukii* genome. For this, we used a *piggyBac* donor plasmid (total insert size = 3783 bp) marked with 3xP3 driving enhanced cyan fluorescent protein (ECFP) . Numbers of injected individuals and percent hatch are shown in Table 4.2. All surviving G_0 flies were backcrossed to wild-type mates, and the G_1 offspring screened for ECFP in their eyes and ocelli. Transformation rates were most closely associated with *piggyBac transposase* levels observed in early embryos. The H7 strain had the highest transformation rate (80%), with 12 of 15 backcrosses generating transgenic offspring. Despite the fact that the H7 and H10 strains appear to possess identical insertion sites and similar early expression levels, they had

differences in transformation efficiencies, with H10 have a 60% transformation rate. H28 had a lower level of transposase expression during the early time point (see Fig. 4.2), and had the lowest transformation rate (25%), making it a comparatively poor helper, and suggesting that early expression of the *transposase* is likely the most important for effective transformation.

Since the H7 helper strain had the best transformation efficiency, we sought to further test its efficiency using insertions of different sizes. Each donor plasmid included the enhanced green fluorescent protein (EGFP) marker gene driven by 3xP3, but the total size of the cargo differed, with the smaller being 6586 bp in length, and the longer 9922-bp (see Table 4.3). It is well known that transformation efficiency is reduced as the size of element increases (Karsi et al., 2001). This was borne out by our results, with the smaller having a much higher rate of transformation (86.7%) than the larger element (15%).

piggyBac remobilization assay

To test the ability of the H7 helper strain to remobilize an already integrated *piggyBac* element, we utilized two transgenic donor strains. Both donor strains possess 3xP3-EGFP-marked *piggyBac* elements, but these differ in size, and most importantly, in location of genomic integration. Moreover, the two donor strains have vastly different expression patterns. Specifically, one donor strain, S16, exhibits an enhancer-trap phenotype (i.e. expresses EGFP in additional tissues) and displays EGFP fluorescence throughout the entire body, while the other donor, G3W, only expresses EGFP in the expected tissues, but at a lower than expected level. These donor strains were selected because movement of their

respective EGFP-marked *piggyBac* elements to new locations in the genome should result in visible changes in EGFP expression levels and/or EGFP expression patterns (Lorenzen et al., 2007). Remobilization was catalyzed by crossing each of the two donor strains to the H7 helper strain. The hybrid G_1 progeny were outcrossed to wild-type mates and the G_2 offspring screened for differences in EGFP expression patterns (see Table 4.4).

As mentioned above, the G3W donor strain possess weak EGFP expression, therefore G_2 offspring were screened for remobilization events based on gain of "normal" levels of EGFP in their eyes, or possession of new enhancer-trap phenotypes. A total of 94 G_2 flies were screened, and 43 of these were EGFP positive (45.7% of G_2s were donor, expected: 50%), and 11 of these displayed differences in EGFP expression levels (Table 4.4), one of which was dramatically different (see Fig. 4.3). To confirm phenotypic changes, and to ensure that no new insertion events were missed, we screened all G_2 offspring twice, first as 1^{st}-instar larvae and again as adults. Although fly larvae doesn't have visible eye structures, we can usually detect expression of marker genes driven by the 3xP3 promoter in the central nervous system at the anterior end of the larvae (see Fig. 4.3 B and C). To confirm that the EGFP-marked *piggyBac* element was now in a new genomic location we cloned insertion-site junctions from five of these flies. As expected, sequence data demonstrates that all five are the result of new events - i.e. possess novel insertion-site junction sequence compared to the original G3W junction sequence (Fig. 4.4). Surprisingly, sequence data demonstrated that four out of five samples had multiple new insertion events. Since the original fly only yielded the sequence of a single insertion-site junction via inverse PCR, the multiple events seen in

these G_2 individuals are indicative of new piggyBac insertions, and suggest that remobilization may be too active due to high *piggyBac transposase* expression.

Since the second donor strain, S16, had strong EGFP expression at all life stages,G_2 offspring were screened for remobilization events based on loss of enhancer-trap phenotype, or gain of a new enhancer-trap phenotype. A total of 210 G_2 flies were screened, and 117 of these were EGFP positive (55.7% of G_2 were donor, expected: 50%), and seven of these displayed a change in EGFP expression, which is a remobilization rate of around 6% (Table 4.4). We didn't follow these flies or test junction sequences.

Discussion

This work demonstrates that microinjection of *piggyBac*-based constructs into *D. suzukii* helper strains that express *piggyBac transposase* can increase transformation rates by two- to five-fold compared to a previous study that used traditional microinjection methods in a wild-type background (Schetelig & Handler, 2013). Further tests with our best performing strain (H7) showed that this helper strain was capable of effecting efficient transformation of inserts up to 10 kb in size. Finally, we have shown that our helper strains are capable of remobilizing *piggyBac* insertions when crossed to strains possessing a marked *piggyBac* insertion (donor strain). In addition, these data provide further evidence that different donor strains can also affect remobilization efficiency.

When the G_0 founders of our helper strains were initially backcrossed, H7 and H10 both

produced around 50% transgenic offspring, which is expected for a single insertion, but H28 had fewer transgenic offspring (37.5%) (Table 4.1). Moreover, results from sequencing the H28 insertion-site junction suggest that the reason the H28 strain produces fewer than expected transgenic offspring may be due to fitness costs caused by the genomic location of the *piggyBac* insertion. It isn't surprising that foreign DNA integrated into an organism's genome could negatively impact their fitness, and this effect is expected to be compounded by the location of the insert, should it occur near or within critical genes. While the H28 insertion is not located within known coding sequence, it does appear to be very close to the 5' end of a gene, which could negatively impact that gene's expression by interfering with the its primary promoter. Critically, this gene appears to be an ortholog of *Dm Hsp70Bbb*, which is a member of the ubiquitin protein superfamily, so it would not be surprising to see a high fitness cost if this insertion is disrupting normal gene expression. Indeed, when we examined levels of *piggyBac transposase* expression in this strain, we observed differences relative to H7 and H10 (Fig. 4.2). This may be the result of position-effect variegation (PEV) (Wallrath & Elgin, 1995) altering how *transposase* is expressed from this region of the *D. suzukii* genome through shifting epigenetic markers. The H28 pattern of *transposase* expression could also be the result of enhancer-trap effects from its position within a promoter region. It is also possible that these mechanisms are responsible for the reduced fitness of H28, if they result in the maintenance of high levels of *transposase* expression after the observed embryonic stages. While we do not yet have sufficient data to distinguish these possibilities, the low fitness and low transformation rate of H28 make it a poor choice for further research requiring a helper strain.

Although insertion-site junction sequence indicated that the H7 and H10 strains resulted from the same insertional event, we selected H7 as the better helper strain based on outcomes from microinjecting *piggyBac*-based constructs, and the slight differences in transposase expression. Unlike H28, both H7 and H10 lack obvious health problems, despite the fact that *piggyBac* inserted into an intron of a gene orthologous to *Dm Ca-α1D*.

It is interesting to note that in spite of the high *transposase* expression in the late stage of H28 embryogenesis, this strain had the lowest transformation rate. The H7 and H10 strains had significantly higher *transposase* expression early in embryogenesis, and better overall transformation rates compared to the H28 strain. Interestingly, as mentioned above, these strains had slightly different expression patterns (Fig. 4.2) despite having the same insertion, with H7 having the higher level of *transposase* expression at the earliest observed stage. Moreover, H7 had a markedly better transformation rate (80%) relative to H10. H10 had the most consistent pattern of *transposase* expression, and was still reasonably efficient at transformation, with a rate of 60%, as well as the highest average transgenic offspring rate (15.9%) among the three strains. Altogether, these results indicate that timing of expression, more than levels, is important for helping the integration of injected *piggyBac* transposons. We have not yet determined if there is any inheritable factor that has created the observed transformation differences between the H7 and H10 strains. However, based on fitness and transformation rates, either would make a useful helper strain.

In comparing the expression of ECFP and EGFP under the control of the eye-specific

promoter 3xP3, we found that ECFP is more difficult to view in *D. suzukii*. The *D. suzukii* strain that we used has the wild-type eye color. As a result, the regular red pigmentation blocks most of the fluorescence in the compound eye. We can only reliably screen by examining the ocelli to identify transgenic individuals. When screening for EGFP under the GFP2 filter, the fluorochrome showed up brightly. However, ECFP does not show up as brightly under its own filter, reducing the efficiency for screening ECFP-bearing transformants compared to those carrying the EGFP marker. Fortunately, ECFP is visible in most cases, so this situation is only problematic when the insertion is located in an expression-quenching genomic region, so ECFP is still a useful marker. The most obvious difference between these two marker genes during screening is how many enhancer traps were found during each assay (data not show), at least partly because it is easier to identify an enhancer trap under EGFP screening.

The assay of the efficiency of the H7 strain at integrating different-sized inserts indicated similar transformation rates for inserts smaller than 6.5 kb. Importantly, this assay demonstrated the utility of the H7 helper strain to integrate transposons of a wide range of sizes, which will allow future researchers to include one or two genes of interest in their cargo, along with the marker. Even when the insertion size was increased to nearly 10 kb, we still got a good transformation rate of 15%. This rate is efficient enough to get positive results with even a small set of injections (around 30 eggs). Different cargo sizes did not appear to cause any obvious differences in health, fecundity, or longevity, so we do not expect extra insertion length to greatly increase fitness costs, although different genes-of-

interest included in those cargos could still affect transgenic *D. suzukii* in unexpected ways. It is important to note that several of the G_0 crosses yielded percentages of transgenic offspring significantly higher than 50%, indicating multiple insertions in these injected individuals. Given the high transformation efficiency, this result was not that surprising. As long as transgenic G_1 offspring are outcrossed, multiple insertions can be teased apart, allowing researchers to focus on individual transposon insertions that yield the desired phenotype (e.g. homozygous-lethal insertion if conducting a screen for vital genes).

The two donor strains used in this work have a few major differences. First, the site of genomic integration of the 3xP3-EGFP marked *piggyBac* element is different, which could have epigenetic or other structural differences that may affect insert mobility. Indeed, the weaker expression of the G3W strain may indicates that this insertion is located in an area with secondary structures or other factors which reduce the function of the marker gene promoter (Spradling & Rubin, 1983; Wallrath & Elgin, 1995); these same factors might impact remobilization. The second major difference is the size of the *piggyBac* element in these two strains (Karsi et al., 2001). Although our transformation data shows that there is no appreciable difference between 3-kb and 6.5-kb cargo sizes for integrating *piggyBac* elements into the genome, it is still not clear if this is true for remobilizing elements that have already integrated into the genome. The third difference was that the two elements we sought to remobilize were in different locations in the genome. Although the S16 strain possessed an enhancer-trap phenotype, it is possible that the *piggyBac* element moved, but that EGFP-expression remained the same. This could explain why we detected fewer remobilization

events using the S16 donor. Specifically, *piggyBac* is known to prefer local reintegration (Lorenzen et al., 2007) and enhancers can act over very long distances, therefore remobilization may have occurred far more frequently due to the lack of change in the pattern of EGFP expression. It was not always easy to identify expression changes in the S16 G_2 offspring, because we were expecting to see the loss of EGFP expression in the body (e.g. only maintain 3xP3 pattern of expression). However, most the new-insertion individuals we identified only had reduced EGFP expression, so we may have missed some individuals carrying new *piggyBac* integrations because their phenotypes weren't obvious. On the other hand, because we were looking for increased expression in the G3W offspring, we had an easier time identifying new *piggyBac* integrations, although we may have missed remobilization events that maintained low expression. If we had been engaging in a mutagenesis screen, looking for a morphological phenotype of interest might have overcome these limitations that result from identifying remobilization events using changes in marker gene expression alone. While, it is important to note that we still identified remobilization events from both donor strains using only the marker gene expression as evidence, these efforts could be improved by creating an enhancer trap with a more specific expression pattern that is lost with any jump, as was done for *T. castaneum* (Lorenzen et al., 2007). Such a donor would be particularly useful in mutagenesis screens for a more cryptic trait, such as pesticide resistance, by allowing researchers to more accurately identify individuals with successful remobilization events.

It is also important to note that insertion-site junction sequences indicated that multiple

remobilization events occurred in some individuals (Fig. 4.4). We believe this is due to overly high levels of *transposase* expression and/or *transposase* expression during later stages of embryonic development. For example, even if there is a single event in an early embryo, if *transposase* expression is maintained, additional insertion events can occur at later developmental stages. This is particularly problematic when a transposon "moves" during DNA replication. It is known that even non-replicative transposons (i.e. cut and paste type) can replicate under such conditions (Charlesworth et al., 1994). Therefore, as long as the donor and helper elements remain in the same individual, remobilization can continue. This could be problematic for mutagenesis screens and other applications. The simplest fix would be to follow up every helper/donor cross with a few generations of outcrosses to separate the helper and donor elements, as well as any multiple-donor insertions that have accumulated. However, this would add time and resources to mutagenesis screens that may not be proportional to the trait being studied. A better fix would be to replace the current *transposase* promoter with an early or tissue-specific promoter, which should limit the ability of the donor to remobilize, reducing the potential for multiple insertions.

In this study, we successfully created three *D. suzukii* helper strains, H7, H10, and H28, and have shown H7 is the most efficient for transformations, which is five times better than the traditional methods previously employed by another lab (Schetelig & Handler, 2013). While this strain is useful on its own, improving the efficiency of creating new transgenic strains using *piggyBac*, the real power of the helper strain is in combining it with a donor strain for the jumpstarter system. Indeed, we also showed the successful remobilization of a *piggyBac*

donor in *D. suzukii* using our helper strain. Finally, we developed effective donor strains, which are marked with visible enhancer traps that show successful remobilizations of *piggyBac*. Altogether, we have created the tools necessary to employ a jumpstarter mutagenesis system in *D. suzukii*, allowing researchers to deploy high-throughput screening methods to study this increasingly problematic pest.

Experimental Procedures

Insect strain and rearing

All insects used in this experiment were a mixture of wild-derived colonies from the laboratories of Dr. Hannah Burrack and Dr. Max Scott (NC-State University). All flies were kept at 25^{o}C and fed a standard cornmeal-molasses-yeast diet (Bloomington Drosophila Stock Center). Embryos for microinjections were collected using grape plates (Drosophila fruit juice egg plates, Cold Spring Harbor Protocols).

Plasmid DNAs and microinjection

The *Minos* donor DNA plasmid, pMi{3xP3-DsRed, hsp70-piggyBac} (Horn et al., 2003), included a red fluorescence protein (DsRed) marker gene driven by the 3xP3 promoter (Berghammer et al., 1999), and a *piggyBac transposase* coding sequence driven by a *D. melanogaster hsp70* (*Dmhsp70*) promoter. The *Minos* helper DNA plasmid, pHSS6hsILMi20 (Klinakis et al., 2000a), included a *Dmhsp70* promoter driving the *Minos* transposase coding sequence. Both donor and helper DNA plasmids were purified using the Qiagen midi-prep kit, then stored at -20^{o}C. This set of DNA plasmids was used to create the *D. suzukii* helper

strains.

Three distinct *piggyBac* transposable element plasmids were used to test the *D. suzukii* helper strains for transformation efficiency. The first, pBac{3xP3-EGFPaf}, which contained a 3xP3 promoter driving enhanced cyan fluorescence protein (ECFP) (Horn & Wimmer, 2000), was used to test all three helper strains. The other two plasmids both contained a 3xP3 promoter driving enhanced green fluorescent protein (EGFP), and were only used to test the helper strain that had the best transformation rate based on its performance with the ECFP plasmid. These EGFP-bearing *piggyBac* plasmids each carried an additional element: One contained a gene from *T. castaneum* to form a cargo of 6582 bp in length, while the other included a bacterial gene which made the transposon's cargo 9986 bp long. Neither insert is known to have any effect on *D. suzukii* fitness.

All embryo microinjections followed the *D. melanogaster* protocol described in Campos-Ortega and Hartenstein (2013), and all injections were done within 1.5 hr post-egg lay. To make *Minos* transgenic flies, we injected 350 ng/µl of donor DNA and 250 ng/µl of helper DNA in nuclease-free water, mixed with Phenol Red (Cat #143-74-8; Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 20% for coloration. To make *piggyBac* transgenic flies, 400 ng/µl of the transposon in nuclease-free water, mixed with phenol Red at a final concentration of 20% for coloration, was injected into our helper strains. Following injection, newly hatched larvae were transferred to cornmeal diet and kept at 25^{o}C. All injected flies that survived to adulthood were separated and each was backcrossed to 2-3 wild

type flies of the opposite sex. We calculated the transformation rate by the number of backcrosses which produced transgenic offspring divided by the number of backcrosses which successfully produced offspring.

Fluorescent microscopy and crosses

Expression of fluorescent proteins was observed using a Leica M165 FC fluorescence stereomicroscope (Leica Microsystems Inc., Wetzlar, Germany) equipped with DsRed, ECFP, and GFP2 filter sets (Leica Microsystems Inc., Wetzlar, Germany). The fluorescent protein was expected to be found in the eyes, but since the natural eye pigmentation of *D. suzukii* could interfere with weak fluorescent signal, we focused on screening for evidence of fluorescence in the ocelli, which also show expression from the 3xP3 promoter. Once confirmed, a transgenic individual was backcrossed with wild-type mates to begin establishing a strain. To confirm single insertions, G_2-individuals were screened to determine the ratio of transgenic-to-wild-type offspring. If the ratio was consistent with a single insertion (~1/2), then the G_2-individuals were selfed to develop a homozygous strain.

DNA isolations, digital droplet and inverse PCRs

Genomic DNA was extracted from pools of five transgenic flies per strain using a modified Wizard DNA Purification protocol (Lorenzen et al., 2007) with lab-prepared cell lysis and protein precipitate buffers (Onate-Sanchez & Vicente-Carbajosa, 2008).

Insertion-site junctions for each of the homozygous *Minos*-based helper strains were

amplified via inverse PCR (Ochman et al., 1988) using the *Minos*-specific primers IMio1, IMii1, IMio2 and IMii2 (Klinakis et al., 2000b), while insertion-site junctions for selected *piggyBac* strains were amplified using the *piggyBac*-specific primers pBo-L1, pBi-L 1, pBo-L2 and pBi-L2 (all primer sequences listed in Table S4.2).

DNA templates for inverse PCR were generated by digesting ~500 µg of gDNA from each sample with *Alu*I (for *Minos*) or *Bgl*II (for *piggyBac*), then diluting the digested DNA to a concentration of ~2.5 ng/µl, and creating circularized fragments through self-ligation using T4 DNA ligase (New England Biolabs, Ipswich, MA, USA). Inverse PCR was performed using 1 µl of the ligation product as template in a 10 µl PCR reaction, along with the *Minos*-specific primers IMio1 and IMii1. A second round of PCR was performed using 1 µl of a 1:20 dilution of the first-round PCR product as template, along with the "nested" *Minos*-specific primers IMio2 and IMii2.

For *piggyBac*, first-round PCR primers were pBo-L1 and pBi-L1, while the second- round PCR primers were pBo-L2 and pBi-L2. PCR products larger than 200 bp were gel purified (QIAquick® Gel Extraction Kit, Qiagen, Hilden, Germany) and sequenced at the Genomic Sciences Laboratory (North Carolina State University, Raleigh, NC, USA). Junction sequences were compared to the publicly available *D. suzukii* genome (http://spottedwingflybase.oregonstate.edu) using BLASTN to identify the location of each transposon insertion.

To quantify expression of *piggyBac transposase*, we collected three sets of 50 embryos from each of our helper strains. Our flies were allowed to lay eggs for 30 min, then aged for 6 hr, 15 hr, or 24 hr before 50 embryos were collected for extraction. Total RNA was extracted from each pool by homogenizing the embryos in 300 µl of Qiazol (Qiagen, Hilden, Germany), then purified using the RNeasy mini kit (Qiagen, Hilden, Germany). The RNA template was reverse transcribed into a complementary DNA (cDNA) pool using SuperScript III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) using an oligo (dT) primer, RT-Uni (Grubbs et al., 2015).

Expression levels for *piggyBac transposase* were determined using digital-droplet PCR (ddPCR), using three biological replicates and two technical replicates. As a result, six data points were generated for each strain, at each of the 3 time points. For ddPCR reactions, we used EvaGreen® (BioRad, Hercules, CA, USA) following the manufactures protocol. In brief, each 20 µl reaction included 1x EvaGreen ddPCR supermix, 60 ng cDNA, and 200nM gene-specific primers. The primers qTrans F3 and qTrans R3 (Table S4.2) were used to quantify *piggyBac transposase* expression. The control gene, *Dswhite*, primer set was qDsW F1/R1 (Table S4.2). Each reaction was mixed in the QX200 Droplet Generator (Bio-Rad) with 50 µl of Droplet Generation Oil (Bio-Rad) to create droplets, then transferred to a twin.tec PCR plate 96 (Eppendorf) and sealed by the PX1 PCR Plate Sealer (Bio-Rad). The PCRs were performed in a T100 Thermal Cycler (Bio-Rad) with the following cycling conditions: 1× (95°C for 5 min), 40× (95°C for 30 s, 58°C for 30 s, 72°C for 45 s), 1× (4°C for 5 min, 90°C for 5 min). After the PCR, the plate was put into a QX200 Droplet Reader

(Bio-Rad) to count individual droplets, and quantify their fluorescence intensity. The data analysis was performed with QuantaSoft droplet reader software (Bio-Rad). We normalized the data using a control gene, *D. suzukii white* (*Dsw*) and calculated *transposase* expression levels for each strain, and at each time point, using Dixon's Q test (Dean & Dixon, 1951) with 95% confidence to remove the outliers. Finally, ddPCR data was analyzed using ANOVA and Tukey to determine if differences were significant ($P<0.05$) from the expression level between each strain at the same time point.

piggyBac remobilization assay

We backcrossed the EGFP enhancer-trap strains (G3W and S16) to the *D. suzukii* helper strain (H7), then outcrossed the G_1 hybrid flies to wild-type mates. The G3W strain is an enhancer-trap strain that carries the 3xp3 promoter driving EGFP, but expression of EGFP in the eye and brain are abnormally low. S16 carries the same promoter and marker gene, but has very strong EGFP expression in the whole body. G_2 individuals were screened for change of EGFP enhancer-trap expression, which indicated successful remobilization of *piggyBac*. Remobilization efficiency was calculated as the number of new insertions divided by total number of EGFP-positive G_2 progeny. We confirmed the remobilization events by using inverse PCR to collect junction sequences from selected G_2 flies with altered EGFP expression, and comparing these sequences to the junction sequences from the original enhancer trap strains (http://spottedwingflybase.oregonstate.edu).

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Table 4.1. DsRed segregation analysis. Results of screening progeny from H7, H10, and H28 *Minos* strains for DsRed fluorescence. The total number of progeny and number of individuals possessing DsRed-positive eyes ($DsRed^{pos}$) were counted for calculating the ratio and estimated *Minos* insertion number in each strain.

Strain	DsRed ^{pos}	Total	Ratio*	% Red^{pos}	Events
H7	16	29	1.23:1	55.17%	1
H10	35	67	1.09:1	52.24%	1
H28	12	32	0.60:1	37.50%	1

* Ratio = DsRed-positive eyes / DsRed-negative eyes.

Table 4.2. Transformation efficiency of each helper strain.

Strain	Injected eggs	Hatched	G_0 Adult	Valid Crosses	# pairs w/ ECFP G_1	Transformation Rate %†	Range*	Average+
H7	65	35	19	15	12	80.00%	0.78%-41.17%	13%
H10	116	45	26	20	12	60.00%	2.78%-54.54%	11.98%
H28	63	41	24	20	5	25.00%	0.68%-14.29%	4.52%

†Transformation rate= the number of crosses that produced transgenic (ECFP positive) progeny divided by total number of crosses that produced progeny (Valid Crosses).
*Range= the percentage of transgenic offspring produced by the crosses with the least and most transgenic progeny, respectively.

Table 4.3. Transformation efficiency of different cargo sizes in the H7 strain.

Strain	DNA/size	Injected Eggs	Hatched	G_0	Valid Crosses	# pairs w/ EGFP G_1	Transformation Rate %
H7	TcSid1/6586bp	76	32	17	15	13	86.7%
H7	UbCas-9/9922bp	141	61	25	20	3	15%

Table 4.4. *piggyBac* insertion remobilization following cross with H7.

Strain	Total G_1	GFP^{pos} G_1	Ratio*	*piggyBac* Remob. G_1	Remobilization Rate†
S16	210	117	55.71%	7	5.98%
G3W	94	43	45.74%	11	25.58%

*Ratio= number of GFP^{pos} G_1 divided by number of total G_1; ~50% transgenic progeny from heterozygous parents indicates both of the donor strains were single-insertion strains
†Remobilization Rate= number of *piggyBac*-remobilized G_1 divided by number of GFP^{pos} G_1.

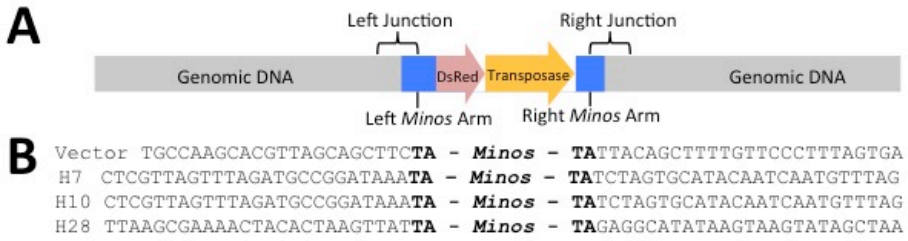

Figure 4.1. Schematic of *Minos* insertion-site junctions. A) Diagram of *Minos* (blue bars) carrying a marker gene (pink bar) and transposase (yellow bar) inserted into the genome (gray bar). Left and right junctions are distinguished by slight differences in the sequences of the left and right *Minos* arms. B) Two unique junction sequences from three single insertion strains. We successfully cloned both sides of the junction sequences of all three strains.

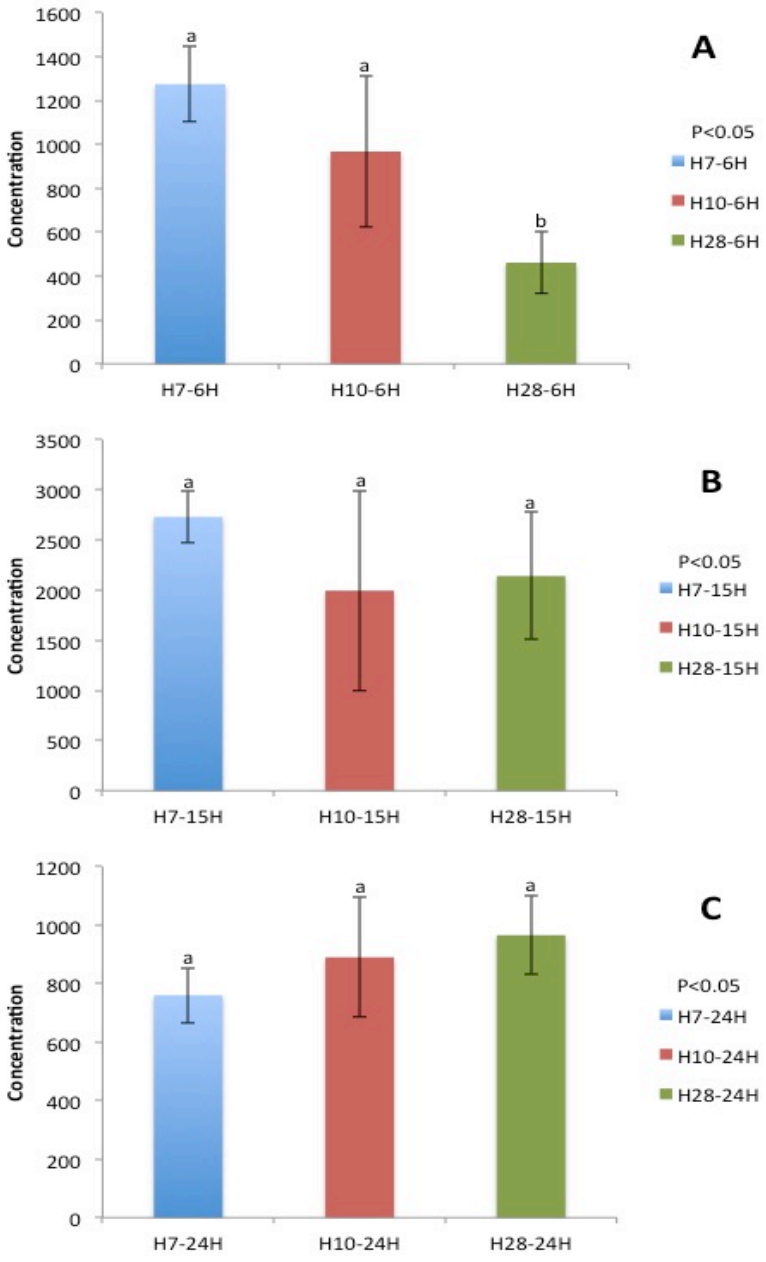

Figure 4.2. *piggyBac transposase* expression levels. Embryos from each helper strain was tested for *transposase* expression at A) 6 hr-pel, B) 15 hr-pel, and C) 24 hr-pel. Data were analyzed using ANOVA and Tukey (95% confidence) to determine if differences were significant.

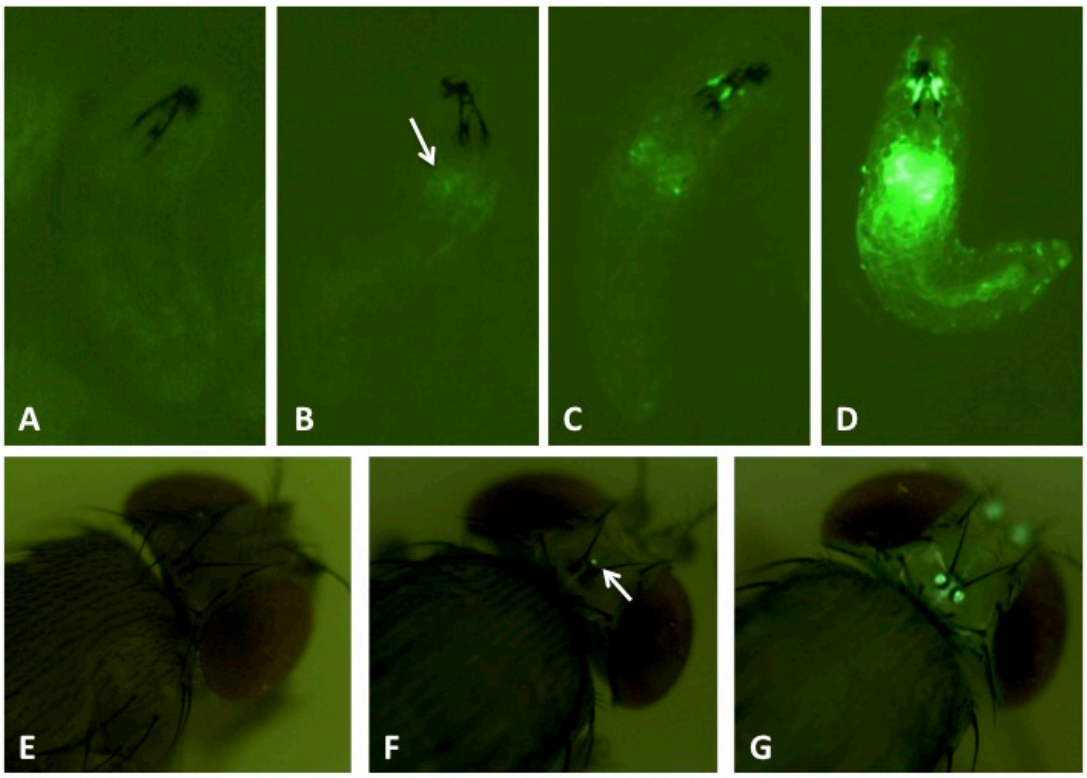

Figure 4.3. EGFP expression observed in *piggyBac* remobilization assay. A-D) Larvae. E-G) Adults. A) and E) wild-type (no transgene); B) and F) original EGFP expression patterns of the W3G strain (arrows point out EGFP expression area); C), D), and G) are examples of new EGFP expression in remobilized progeny. Note the increase in intensity relative to the original strain.

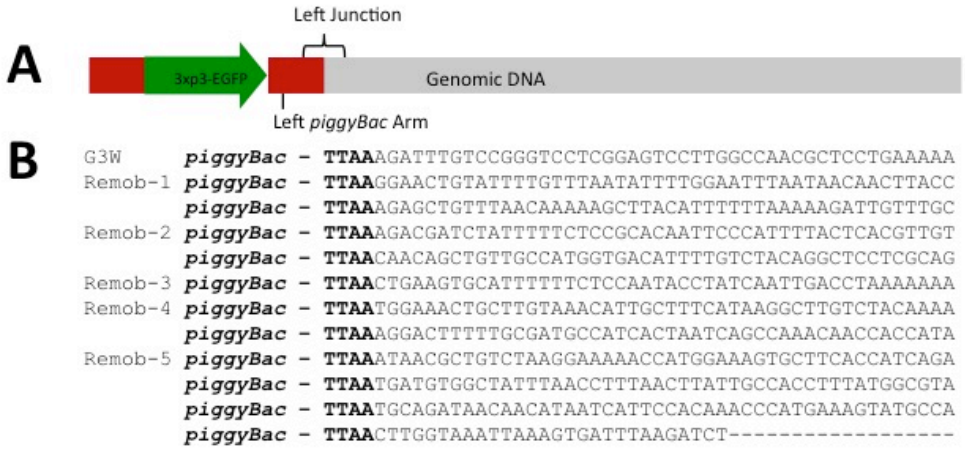

Figure 4.4. Cloning and sequencing of *piggyBac* junctions following remobilization. A) Diagram of *piggyBac* (red bars) carrying a marker gene (green bar) inserted into the genome (gray bar). B) Eleven unique junction sequences from five insertion-remobilized single flies, with the original junction sequence from G3W for comparison.

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SUPPORTING INFORMATION

Table S4.1. ddPCR results for *piggyBac transposase* expression in each helper strain.

Sample	Target	Concentration	Positive Droplets	Total Droplets	Calaclated Trans. Conc.	Q-test (95%)*
H7-6H-1-1	Trans	884	8060	15252	1449.20	
	White	60.2	817	16377		
H7-6H-1-2	Trans	1362	10581	16222	1371.59	
	White	98	1016	13512		
H7-6H-2-1	Trans	1550	9919	13549	1108.47	
	White	138	1490	13494		
H7-6H-2-2	Trans	1243	10259	17130	1333.39	
	White	92	1141	17863		
H7-6H-3-1	Trans	1175	9092	14393	1017.20	
	White	114	1376	14946		
H7-6H-3-2	Trans	1075	11232	16380	1365.40	
	White	77.7	1366	17138		
H10-6H-1-1	Trans	713	6769	14888	469.11	
	White	150	1681	14064		
H10-6H-1-2	Trans	1135	10044	16230	727.36	
	White	154	2119	17225		
H10-6H-2-1	Trans	1180	9248	14605	1200.56	
	White	97	1099	13924		
H10-6H-2-2	Trans	949	8552	15446	1440.87	
	White	65	954	17738		
H10-6H-3-1	Trans	990	7476	13138	930.51	
	White	105	1154	13464		
H10-6H-3-2	Trans	876	4802	9145	1035.36	
	White	83.5	1189	17363		
H28-6H-1-1	Trans	454	4822	15067	599.80	
	White	74.7	1043	16951		
H28-6H-1-2	Trans	694	7014	15733	547.93	
	White	125	1703	16927		
H28-6H-2-1	Trans	439	4169	13387	277.72	
	White	156	1951	15696		
H28-6H-2-2	Trans	390	4484	15879	501.16	
	White	76.8	1120	17715		

Table S4.1. Continued

Sample	Target	Concentration	Positive Droplets	Total Droplets	Calaclated Trans. Conc.	Q-test (95%)*
H28-6H-3-1	Trans	216	2361	14087	291.61	
	White	73.1	836	13870		
H28-6H-3-2	Trans	202	2814	17846	547.68	
	White	36.4	536	17616		
H7-15H-1-1	Trans	429	4715	15440	287.94	Outlier
	White	652	6961	16363		
H7-15H-1-2	Trans	505	5819	16664	269.83	Outlier
	White	819	8363	16678		
H7-15H-2-1	Trans	1500	8776	12179	2505.40	
	White	262	3062	15319		
H7-15H-2-2	Trans	2260	12972	15198	2770.30	
	White	357	4574	17457		
H7-15H-3-1	Trans	2440	12780	14616	2566.75	
	White	416	4942	16580		
H7-15H-3-2	Trans	2890	14583	15946	3077.11	
	White	411	5272	17866		
H10-15H-1-1	Trans	1336	9356	13784	814.27	
	White	718	6622	14493		
H10-15H-1-2	Trans	2017	11437	13948	1220.83	
	White	723	7331	15963		
H10-15H-2-1	Trans	1554	11793	16087	3091.12	
	White	220	2211	12076		
H10-15H-2-2	Trans	1964	12716	15667	3159.80	
	White	272	1951	9438		
H10-15H-3-1	Trans	1014	4368	7560	1404.23	
	White	316	3702	15732		
H10-15H-3-2	Trans	1795	12982	16591	2276.84	
	White	345	4674	18401		
H28-15H-1-1	Trans	1604	11257	15126	1480.86	
	White	474	5443	16407		
H28-15H-1-2	Trans	2041	12922	15690	1367.78	
	White	653	7542	17707		
H28-15H-2-1	Trans	1116	8340	13610	2872.78	
	White	170	1787	10704		
H28-15H-2-2	Trans	1528	11660	16035	2440.39	
	White	274	3629	17440		

Table S4.1. Continued

Sample	Target	Concentration	Positive Droplets	Total Droplets	Calaclated Trans. Conc.	Q-test (95%)*
H28-15H-3-1	Trans	1729	10670	13857	1960.18	
	White	386	4498	16089		
H28-15H-3-2	Trans	2540	13779	15576	2717.68	
	White	409	4796	16335		
H7-24H-1-1	Trans	865	8206	15766	687.18	
	White	237	2935	16084		
H7-24H-1-2	Trans	1092	10373	17149	667.54	
	White	308	4264	18505		
H7-24H-2-1	Trans	986	8887	15665	722.35	
	White	257	3149	16039		
H7-24H-2-2	Trans	1159	10432	16649	869.39	
	White	251	3461	18015		
H7-24H-3-1	Trans	1100	7178	10447	852.30	
	White	243	2725	14597		
H7-24H-3-2	Trans	2397	14621	16812	1864.91	Outlier
	White	242	3320	17863		
H10-24H-1-1	Trans	725	6348	13804	672.43	
	White	203	2281	14409		
H10-24H-1-2	Trans	890	8753	16493	768.67	
	White	218	3007	17749		
H10-24H-2-1	Trans	625	6226	15113	1005.77	
	White	117	1578	16642		
H10-24H-2-2	Trans	868	8851	16956	1111.75	
	White	147	2072	17606		
H10-24H-3-1	Trans	1707	10808	14115	2453.39	Outlier
	White	131	1592	15138		
H10-24H-3-2	Trans	3250	14212	15166	4191.16	Outlier
	White	146	1911	16375		
H28-24H-1-1	Trans	4820	14296	14538	6131.82	Outlier
	White	148	1800	15236		
H28-24H-1-2	Trans	5040	15684	15903	5648.40	Outlier
	White	168	2082	15666		
H28-24H-2-1	Trans	782	7351	15141	1150.27	
	White	128	1417	13704		
H28-24H-2-2	Trans	951	9160	16524	957.51	
	White	187	2620	17805		

Table S4.1. Continued

Sample	Target	Concentration	Positive Droplets	Total Droplets	Calaclated Trans. Conc.	Q-test (95%)*
H28-24H-3-1	Trans	563	5459	14358	834.66	
	White	127	1605	15698		
H28-24H-3-2	Trans	846	9071	17685	918.40	
	White	147	1177	10016		

The ddPCR result from three time points of embryonic development from three helper strains. Three biological replicates and two technical replicates were used to generate these results. We use the mean of the control gene (*White*) from each time point to normalize and calculate *transposase* expression. Dixon's Q-test was used to remove the outliers with 95% confidence.
*Q-test (95%)= After the calculation, if the result for any data point is larger than Q table 95% confidence, this data point will be called an outlier and removed from the statistical analysis of the data set.

Table S4.2. Primer sequences

Name:	Sequence:	Purpose:
qTrans F2	AAGAGGAACACAGACCAACGG	RT-PCR
qTrans F3	GTAGGAAGACGAATAGGTGG	ddPCR & RT-PCR
qTrans R3	CGTCAGGCTCATGTAAAGGTT	ddPCR & RT-PCR
qDsW F1	AGAACCTCACCTACGCCTGG	ddPCR
qDsW R1	TCGCTCGTTGCAGAATAGTC	ddPCR
RT-Uni	CGTCAGCTTGATTAAGTCAACGATCTTTTTTTTTTTTTTTTTTTV	RT-PCR
IMio1	AAGAGAATAAAATTCTCTTTGAGACG	Inverse PCR
IMio2	GATAATATAGTGTGTTAAACATTGCGC	Inverse PCR
IMii1	CAAAAATATGAGTAATTTATTCAAACGG	Inverse PCR
IMii2	GCTTAAGAGATAAGAAAAAAGTGACC	Inverse PCR
pBo-L1	ATCAGTGACACTTACCGCATTGACA	Inverse PCR
pBo-L2	CTCCAAGCGGCGACTGAG	Inverse PCR
pBi-L1	GAATCTTGACCTTGCCACAG	Inverse PCR
pBi-L2	CAAAGTCCACGAGCGGTAGC	Inverse PCR
pBi-Lseq	GCACAGCGACGGATTC	Sequencing

CHAPTER 5

Conclusion and applications in WCR and SWD

Overview

The main goal of my research was to create and adapt molecular tools for western corn rootworm (WCR), *Diabrotica virgifera virgifera*, and spotted wing drosophila (SWD), *Drosophila suzukii*. Importantly, I have established transgenic strains in each of these species by integrating DsRed-marked *Minos* elements that carry a *piggyBac transposase* gene, which will allow these strains to serve as "helpers" (i.e. sources of *piggyBac transposase*) for future *piggyBac*-centered, transposable element (TE)-based studies. Use of TEs for functional genomic studies began in *D. melanogaster* more than 30 years ago (Ohare & Rubin, 1983; Spradling & Rubin, 1982), and has since been adapted for use in a few non-model organisms (Fraser et al., 1995; Grossman et al., 2001; Sumitani et al., 2003). However, the first report of germline transformation in SWD was only a few years ago (Schetelig & Handler, 2013), despite the fact that SWD is a close relative of *D. melanogaster*. While *D. melanogaster* is not considered a pest, SWD has evolved very different feeding and ovipositing behaviors (Asplen et al., 2015), which have elevated this fly to pest status. Unlike SWD, WCR lacks a well-studied, close relative to borrow tools from. However, WCR has a long history as a major pest of corn, and is known to have a strong ability to adapt to control methods (Gray et al., 2009), thus making it an important species to study. Both species are invasive to a large part of the world, with the potential to cause major damage to crops.

Researchers have addressed a number of fundamental questions about these two species, but critical questions, like the source and extent of their adaptive abilities, still remain. Application-based studies have made great progress in developing more efficient control

methods, but more information is needed for the future. The molecular tools I have developed will provide more flexibility for researchers to get a deeper understanding of these pests. Importantly, my helper strains could reduce the amount of work involved in germline transformation, including microinjections, screening, and rearing, as well as reducing the number of individuals needed for successful transformation by increasing transformation efficiency. The protocols developed to carry out this work also have the potential to be adapted to other applications in these two species. Moreover, the helper/donor mutagenesis system in SWD could be directly used in any type of phenotype screening, such as gene-specific enhancer traps or resistance screening. However, there are always ways to improve or optimize these systems. Here, I present results from some experiments we carried out to test the efficacy of our new systems, to attempt to improve their efficiency, or to broaden their applications.

WCR rearing and microinjection system applications

WCR has been reared in the lab since the 1960s (Georce & Ortman, 1965; Jackson, 1986), but there are still major problems when it comes to survival rates. Most functional genomic experiments only require a relatively small number of insects, but the survival rates must be much better than previous WCR rearing protocols have achieved. I have developed a small-scale rearing system which should fit in most molecular and genetic laboratories, as well as a simplified single-pair mating setup with an egg collection system that can speed up experiments and permit tracking of offspring. The embryonic microinjection protocol I developed was successfully used to create multiple new transgenic helper strains in WCR.

This rearing and microinjection system would also be useful for other applications, such as RNAi or CRISPR-Cas9 genome editing.

I tested the system, to determine if my WCR rearing protocols were, in fact, useful for larval RNAi experiments. Because the rearing system already includes sorting of insects from secondary-rearing boxes (rearing protocol), there were no extra steps needed to reliably find sufficient quantities of large, healthy third-instar larvae for use in RNAi experiments. It was very convenient to collect larvae for dsRNA microinjections, and then continue rearing them according to the protocol without setting up another modified protocol.

For our larval RNAi experiment, we decided to target the ortholog of the Drosophila *white* gene, which is critical for proper eye pigmentation. This target was selected based on a major problem encountered during screenings for eye-specific expression of the fluorescent marker gene, DsRed. The natural dark pigmentation in the adult WCR eye blocked most of the fluorescent light coming from marker gene expression (Fig. 5.1 A and A'; see also Fig. 2.1 A). To overcome this problem, our lab successfully identified the *Dvv white* (*Dvvw*) gene and microinjected *Dvvw*-specific dsRNA into transgenic larvae to knock down its function (Grubbs, Chu, and Lorenzen, unpublished data). Injected individuals showed a reduction in eye pigment (Fig. 5.1 B). When transgenic individuals with the RNAi phenocopy were screened under fluorescent light, the DsRed expression was much more visible (Fig. 5.1 B').

To further optimize the helper strain, we sought to create a white-eye mutant strain by using the CRISPR-Cas9 system to target *Dvvw*. The CRISPR gene-editing system, like germline transformation, requires embryonic microinjection of precellular embryos, so our microinjection protocol and single-pair cross system were directly useful for this application. This experiment was successful, and we have created a white-eyed colony of WCR in our lab (Fig. 5.1 C and C'). However, a mutation has not been detected in the expected genomic target location. Since WCR has a huge genome with repetitive sequences, it is possible that our white-eye mutation is the result of an off-target effect, so we are continuing to work in the lab to figure out the source of the mutation.

To develop a better CRISPR system for WCR, I constructed a *piggyBac* plasmid carrying the WCR *Ubiquitin* promoter driving the Cas9 protein coding sequence, since it is known that innate expression of Cas9 improves the efficiency of CRISPR-based gene editing (Gratz et al., 2014). Our helper strains should be useful in establishing a Cas9-expressing strain, because early expression of *piggyBac transposase* is expected to increase transformation efficiency (Lorenzen et al., 2007). Hopefully this first WCR Cas9-expressing strain will open up WCR to more CRISPR-Cas9 research in the future.

Donor strain for WCR mutagenesis system

The efficiency of the WCR helper strains still needs to be tested to define which strain is the best or the most suitable for other TE-based applications. This could also be a good opportunity to generate a potential donor strain for transgenic mutagenesis (Material and

method: see Appendix A). I have already attempted a transformation using a *piggyBac* plasmid carrying an Enhanced Cyan Fluorescent Protein (ECFP) marker gene driven by the 3xP3 promoter. I injected this plasmid into the most healthy helper strain, Min-8. However, I later discovered that ECFP expression is very weak in another insect (SWD), so, in the presence of wild-type eye pigments, we would be unlikely to detect transgenic offspring (i.e. the white-eye mutation had not yet been incorporated into the Min-8 helper strain). As a result, it was too difficult to screen for this marker gene. We continued to screen for good ECFP expressers for more than three generations of self-crosses, but never found evidence of an ECFP-positive individual. I am now in the process of trying another *piggyBac* plasmid, which carries Enhanced Green Fluorescent Protein (EGFP) driven by the 3xP3 promoter. The SWD experiments demonstrate that this marker gene is good, even when eyes are fully pigmented. It is important to note that screening WCR embryos for EGFP expression is harmful to embryos and larvae, but such screening is not harmful for pupae or adults, and the harmful effects on larvae can be mitigated by screening them on an ice block. However, since it is sometimes necessary to examine eggs in a mutagenesis screen, we are still looking for alternative markers.

SWD helper strain optimization and applications

The SWD helper strains are highly efficient for creating new transgenic strains (see Table 4.2). This could speed up experimental time and reduce the work of screening and rearing. So far, the H7 strain has the highest efficiency in creating new *piggyBac* transgenic strains, so, for most tests, I only used the H7 strain. For more TE-related applications, like promoter tests

or protein expression/function tests, H7 is an excellent strain to use. To further optimize the helper strain, our collaborator, Dr. Max Scott, has suggested we move the *Minos* insertion onto the X chromosome. This could help speed up downstream processes, such as removing the helper chromosome from the background of new transgenic strains. One way we might increase the efficiency of screening for the marker gene in this system is to have a white-eye background in SWD. A white-eyed strain has been created using CRISPR (Li & Scott, 2016), so simple crosses are all that would be needed to integrate the white-eyed mutation into our helper strain.

As with SWD, I have made a *piggyBac* construct carrying the Cas9 protein coding sequence (Gratz et al., 2014) driven by a *Ds Ubiquitin* promoter. I have also been able to integrate this construct into the SWD genome (10-kb construct, Chapter 3). Several strains were made, and I tested one of these strains (DsCas9-4) and found that the mRNA is expressed (Appendix B). I have used this strain to successfully knock out an EGFP marker gene by injecting embryos with a guide RNA plasmid (Dm U6 promoter driving sgRNA) (Bassett et al., 2014) targeting the EGFP sequence (Fig. 5.2 A). The 12 viable G_0 adults were outcrossed to wild-type mates, and seven crosses (58%) produced progeny having EGFP knock-out phenotypes (EGFP gene present, but no EGFP expression), with one producing 100% non-EGFP offspring (See Fig. 5.2 B). This data shows that the DsCas9-4 strain can be used successfully for CRISPR applications.

I also tested a tissue-specific SWD promoter, cloned from the *Ds β-2-tubulin* gene (Appendix C). This gene has been reported to be expressed in male testes in *D. melanogaster* (Michiels et al., 1989; Rudolph et al., 1987). To test this putative promoter sequence, I used it to drive a marker gene, DsRed, carried by the *piggyBac* transposon. As expected, DsRed expression is only seen in SWD testes (Fig. 5.3). I further tested the RNA from both male and female adult flies to determine if the DsRed mRNA is expressed only in males (Table 5.1). We did detect some low-level expression in the female samples, but never saw any DsRed fluorescence from screening (See appendix Table C.1). Thus, the detected "expression" most probably came from genomic contamination in the RNA sample, although there could be very low expression in females. Since we weren't able to normalize our results between samples, we can only determine that the DsRed expression in testes was very high, but we cannot eliminate the possibility that DsRed was expressed at low levels in females or other parts of the male body. Based on these results, we concluded that this promoter could potentially be useful for male-specific applications. To test this, I replaced the DsRed marker gene with a restriction endonuclease gene, *Eco*RI (Addgene plasmid #40190) (Wright et al., 1989), and successfully created multiple transgenic strains carrying this insertion. The expectation was that the endonuclease would degrade the DNA in the sperm, thereby making the male flies sterile. However, none of the strains had a sterile-male phenotype or other major fitness problems. In fact the *Eco*RI gene is not expressed in the adult flies (neither male nor female) in the strains we tested. Since multiple strains all lacked *Eco*RI expression, but still expressed the marker gene (3xP3-EGFP), we believe this problem is not caused by the insertion location or a random mutation. It is also unlikely that our screening methods inadvertently

selected for non-expressers, since, at every generation, we identified and used females, as well as males, to produce the next generation (see cross methods in Appendix C). Further tests are required to figure out the reason(s) for this apparent repression of transgene expression. Another way this promoter could be used is to drive the Cas9 protein for the CRISPR system. Since this is a testes-specific promoter, we could use this system to drive male-specific mutation of germ cells (sperm) by CRISPR. Such an application would be particularly useful for use in a CRISPR gene-drive system, and may also be useful for a sterile-male system without the complications caused by EcoRI.

The transposon-based mutagenesis system in SWD was capable of remobilizing the donor element, although different donor strains remobilized with different efficiencies. It is important to have a good donor strain for this system, since better remobilization rates mean less work and time are needed before getting the desired phenotype. A good example of this comes from *T. castaneum*, which has a high remobilization rate and an easily-observed phenotypic change (Lorenzen et al., 2007). This donor strain has EGFP expression in all muscles, but remobilization results in loss of muscle-specific EGFP expression without loss of eye-specific expression. Such a system is not only good for phenotype screening after remobilization, but would also be good for applications like resistance screening. My results indicate that one donor strain, G3W, is better than the other. While the G3W strain could be directly employed for applications like phenotype or enhancer screens, we would like to test more donor strains, as well as get more information about their fitness. On the other hand, from the junction sequence results, the multiple junctions from the same individual indicate

that the helper strain could be too efficient, causing the insertion to move to multiple locations. While crossing out the helper will restore the stability of the new donor location, germline-specific expression of the *transposase* could help fix this problem.

Conclusion

I have presented some of the downstream work necessary for optimizing these systems, as well as some of the ways my work could be applied in these two pest species. I am confidant that these systems work well and that they hold great potential to for advancing both fundamental and applied research. Since both species are agricultural pests, these systems could even be used for pest control (Nolan et al., 2011), perhaps through creation of a CRISPR-Cas9 gene-drive system (Gantz & Bier, 2015). Such a control system could work as part of an IPM program to suppress the pest population, or to slow down the development of resistance to current control methods.

Table 5.1. ddPCR results for DsRed expression in *D. suzukii*. This transgenic strain expressed DsRed in the testes. The ddPCR results show DsRed expression could be detected in larval, pupal, and adult stages. The concentrations were calculated by QuantaSoft base on the ratio of positive and total droplets (also see Chapter 2).

Sample	Concentration	Positive Droplets	Total Droplets
Mixed Pupae	830	8074	15954
Mixed Larvae	2480	15148	17243
Female Adult	89	1195	16358
Male Adult Body	144	1912	16569
Male Adult Testes	6500	16298	16363

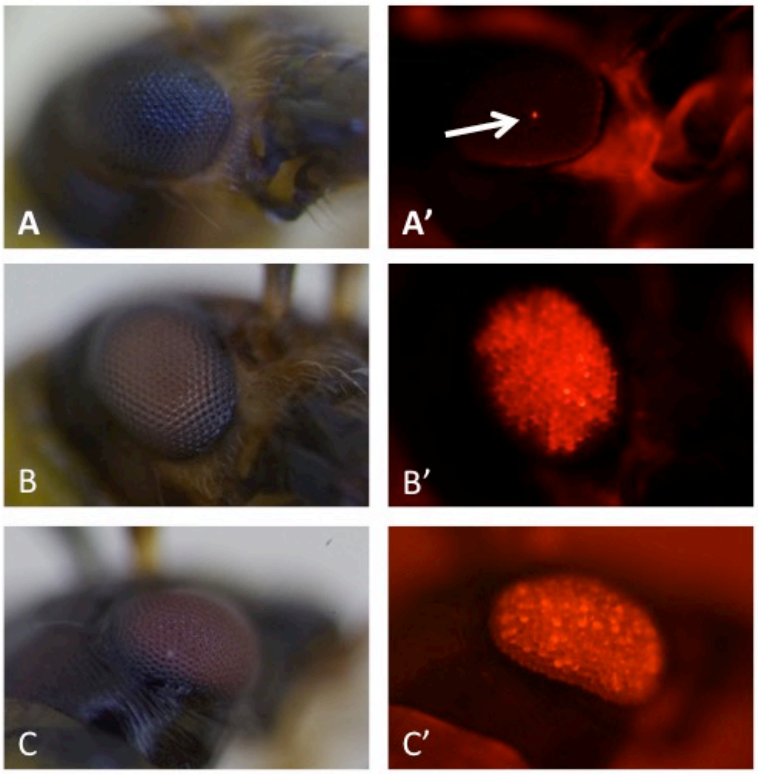
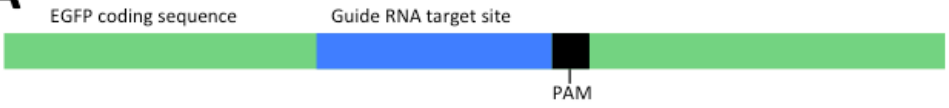


Figure 5.1. Comparison of WT, RNAi, and CRISPR eye-color phenotypes in a transgenic WCR strain. Eye-color phenotype observed in A) wild-type, B) RNAi, and C) CRISPR adult WCR with white light. The same individuals (A'-C') viewed with DsRed filter set. Arrow indicates location of DsRed-fluorescent ommatidia.

A




```
EGFP      TTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCA
CRISPR1   TTCAGCGTGTCCGGC----------C-ATGG---CTACGGCAATCTGACCCTGAAGTGCATCTGCACCA
CRISPR5   TTCAGCGTGTCCGGCGAGGGCGAGGGCGATG---CC-ACGGCAAGCTGACCCTGAAGTTCATCTGCACCA
CRISPR6   TTCAGCGTGTCCGGCGAGGGCGAGGGCGATG---CC-ACGGCAAGCTGACCCTGAAGTTCATCTGCACCA
CRISPR11  TTCAGCGTGTCCGGCAAGGG-GAAGG---TG----------AA--TG-C---------CATCTGCACCA
CRISPR12  TTCAGCGTGTCCGGCGAGGGCGAGGGCGATG---CC-ACGGCAAGCTGACCCTGAAGTTCATCTGCACCA
CRISPR14  TTCAGCGTGTCCGGCGAGGGCGAGGGCGATG----------CTGACCCTGAAGTTCATCTGCGCCA
```

B

	G_0 sex	Total G_1	Knock out	%
CRISPR1	Female	46	37	80.43%
CRISPR2	Male	71	0	0%
CRISPR3	Female	52	0	0%
CRISPR4	Male	49	0	0%
CRISPR5	Female	23	11	47.83%
CRISPR6	Male	52	4	7.69%
CRISPR7	Male	77	0	0%
CRISPR8	Male	73	4	5.48%
CRISPR9	Female	9	0	0%
CRISPR10	Female	-	-	-
CRISPR11	Female	35	9	25.71%
CRISPR12	Male	-	-	-
CRISPR13	Female	37	18	48.65%
CRISPR14	Female	38	38	100%

Figure 5.2. CRISPR knock out of EGFP in a Cas9-expressing transgenic SWD strain. A) Diagram of CRISPR target site (blue bars) and PAM sequence for the sgRNA (black bar) in the EGFP coding sequence (green bar). We used genomic DNA from individual G_1 knock-out flies from each G_0 outcross to amplify and clone the sgRNA target area (blue letters), which was then sequenced to find insertions (red letters) and deletions (dashes). B) The numbers and percentages of knock-out offspring found in each G_0 outcross. Outcross CRISPR12 and 13 did not produce offspring.

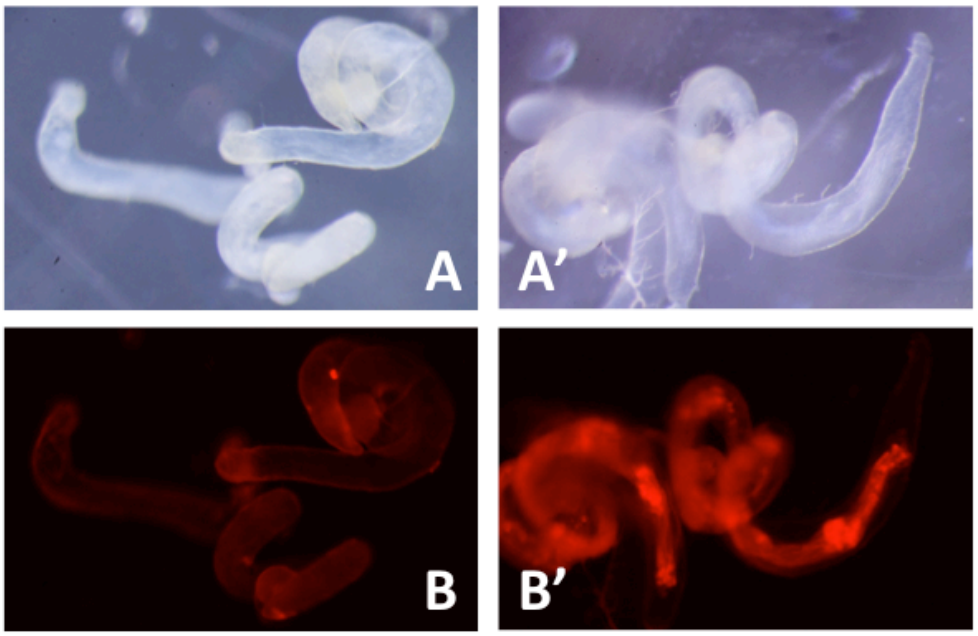


Figure 5.3. DsRed expression of *D. suzukii* testes. A) Wild-type and A') transgenic *D. suzukii* testes viewed with white light, B) wild- type and B') transgenic *D. suzukii* testes viewed with DsRed filter set.

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APPENDICES

Appendix A.

Donor strain for WCR mutagenesis system

Background

The transposable element (TE)-based mutagenesis system referred to as "jumpstarter" requires two transgenic strains: a helper strain, which produces transposase, and a donor strain, which supplies a remobilizable transposon. Crossing the helper and donor strains generates hybrid individuals in which the transposase can remobilize the donor transposon; the hybrid insects, in turn, produce offspring that can carry the transposon in new locations of their genomes. This allows for the jumpstarter system to serve as a tool for functional genomic studies by providing individuals that can be screened for altered gene function or altered marker-gene expression resulting from disrupted genes or enhancer traps (Lorenzen et al., 2007). Chapter 2 describes WCR helper stains that express *piggyBac transposase*, however we currently lack a suitable donor strain (marked *piggyBac transposon*). The ideal donor strain would display an enhancer-trap-like phenotype that would be lost during remobilization, even if the transposon reintegrated nearby. Since, any *piggyBac*-bearing strain can be used as a donor, although the efficiency may be lower, I sought to generate transgenic WCR by microinjection plasmid DNAs possessing marked-*piggyBac* elements into precellular embryos derived from my WCR helper strains.

Materials and methods

Insect strains

The WCR strains I used for this experiment were the single-insertion helper strains Min-7 and Min-8, which were described in Chapter 2. These strains were selected based on the relative health and size of their respective colonies. According to a previous report, using a helper strain should increase transformation efficiency (Lorenzen et al., 2007). Colony maintenance and insect rearing methods were carried out as described in Chapters 2 and 3.

Plasmid DNAs and microinjection

The two marked-*piggyBac* elements used in this experiment, pBac{3xP3-ECFPaf} and pBac{3xP3-EGFPaf}, each contained the eye-specific 3xP3 promoter driving expression of either an enhanced cyan fluorescence protein (ECFP) gene or an enhanced green fluorescence protein (EGFP) gene, respectively (Horn & Wimmer, 2000). Germline transformation was done by injecting 500 ng/µl pBac{3xP3-ECFPaf} in 20% Phenol Red (Sigma-Aldrich, St. Louis, MO, USA) into pre-cellularized embryos from the Min-7 strain, or 500 ng/µl pBac{3xP3-EGFPaf} and 250 ng/µl pBacHelper in 20% Phenol Red into pre-cellularized embryos from the Min-8 strain.

Crosses and screening

All insects were reared according to the methods described in Chapters 2 and 3. G_0 adults from each microinjection set were placed into group-mating setups with three males and five females, then all setups were transferred into one big colony for egg-lay. The mating setup

increased the opportunity for each male to mate, while the colony-sized egg-lay reduced the amount of work required to keep up with collecting G_1s. All G_1 individuals were screened for either ECFP or EGFP fluorescence as third-instar larvae or as pupae; the possible fluorescent-positive individuals were isolated, screened again as adults, and outcrossed to wild-type mates so that their G_2 progeny could be screened to confirm the phenotype. All fluorescent-negative G_1 individuals were kept together to create a new colony. Screening continued in the negative colonies for three generations.

Result

Injectioned embryos from the Min-7 strain had lower hatch and survival rates compared to those from the Min-8 strain (Table A.1). After three generations of screening, no fluorescent-positive individual were found from either set of microinjection.

Table A.1. Hatch and survival rates after microinjection.

Strain	DNA plasmid	Eggs Injected	Hatched	Hatch Rate	Adults	Survival Rate
Min-7	pBac{3xP3-ECFPaf}	1580	179	11.33%	49	27.37%
Min-8	pBac{3xP3-EGFPaf}	1200	242	20.17%	98	40.50%

Appendix B.

Cas9-mediated gene knock-out in EGFP positive SWD

Background

To know if the Cas9-expressing SWD strain (DsCas9-4) could be used for CRISPR experiments, I assessed Cas9 function by targeting a fluorescent marker gene in transgenic SWD. Because the Cas9 gene is driven by a *Ds Ubiquitin* promoter, I expected the Cas9 protein to be expressed at high, consistent levels throughout all life stages. The target gene for CRISPR was EGFP driven by the eye specific promoter, 3xP3.

Materials and methods

Insect strain

DsCas9-4 is a homozygous strain with one insertion of *piggyBac* carrying one copy each of Cas9 and EGFP transgenes. All rearing methods and conditions were followed according to Chapter 4. We pooled four adults for RNA extractions. These extractions were then reverse transcribed to cDNA (Chapter 4) for PCR, to confirm Cas9 mRNA expression, using the primer set: Cas9 F4 and Cas9 R5 (Table B.1).

Plasmid DNAs and microinjection

The small-guide RNA (sgRNA) sequence for targeting EGFP was based on a previous publication (Gilles et al., 2015). The oligos (sgEGFP7F and sgEGFP7R, Table B.1) were ordered and cloned into the plasmid pAc-sgRNA-Cas9 (Addgene plasmid # 49330) (Bassett

et al., 2014) and confirmed by sequencing (Primer: pAC-sgRNA-R, Table B.1). We injected 400 ng/µl of the sgRNA plasmid with 20% Phenol Red (Sigma-Aldrich, St. Louis, MO, USA) into eggs from 0.5 hour egg-lays. All hatched larvae were reared to adulthood, then outcrossed to wildtype.

Screening and molecular verification

G_1s from each cross were screened as adults. We collected all EGFP negative flies from each cross for DNA extraction (Chapter 4), individually, or in pools of three. Using PCR, we amplified the target region with the primers 3xp3 F1 and EGFP R1 (Table B.1). We then Sequenced the PCR product using the primer EGFP F1 (Table B.1). Finally, we used ContigExpress (Thermo Fisher Scientific, Waltham, MA, USA) to align the sequences to determine the deletions in each cross.

Result

Expression of Cas9 mRNA was detected by RT-PCR, and no genomic contamination was observed in the RNA samples (Fig. B.1). Out of 14 G_0 outcrosses, 12 produced G_1 offspring, and seven crosses had EGFP knock-out progeny (Fig. 5.2 B). The aligned sequences also show the deletion from each cross (Fig. 5.2 A).

Table B.1. Primer sequences.

Name:	Sequence:	Purpose:
sgEGFP7 F	TTCGGCGAGGGCGATGCCACCTA	Clone
sgEGFP7 R	AACTAGGTGGCATCGCCCTCGCCC	Clone
pACsgRNA R	GTTCGACTTGCAGCCTGAAATACG	Sequencing
3xp3 F1	AGCTCGCCCGGGATCTAATTCA	PCR
EGFP R1	TTTACTTGTACAGCTCGTCCATGC	PCR
EGFP F1	ATGGTGAGCAAGGGCGAGGAGC	Sequencing
Cas9 F4	AGAAGGGACAGAAGAACAGC	RT-PCR
Cas9 R5	AAGCCGCCTGTCTGCACCTC	RT-PCT

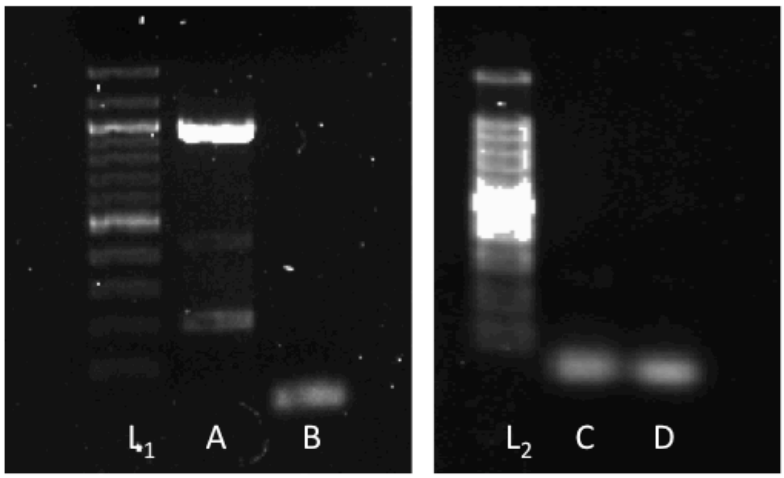

Figure B.1. PCR for Cas9 mRNA in the DsCas9-4 strain. The PCR shows that Cas9 mRNA is expressed in this strain. Lane L_1) 100-bp ladder (exACTGene, Fisher); A) DsCas9-4 cDNA; B) ddH_2O; L_2) 100-bp ladder (100bp DNA Ladder, Promega); C) DsCas9-4 RNA (not reverse-transcribed); D) ddH_2O.

Appendix C.

Characterization of the testes-specific promoter, *Ds β-2-tubulin*, in SWD

Background

To expand the promoter options that can be used in SWD, I tested the promoter from the *Ds β-2-tubulin* gene. This is a testes-specific gene in *D. melanogaster* (Michiels et al., 1989; Rudolph et al., 1987). If the SWD ortholog was indeed a testes-specific promoter, it can be used to drive expression of a restriction endonuclease gene, such as *Eco*RI, to create a sterile-male strain.

Materials and methods

Insect strains

Wild-type SWD and the helper strain, H7 (see chapter 4), were used in these experiments following standard rearing and microinjection protocols described in Chapter 4.

Plasmid DNAs and microinjection

The DNA plasmid pBac-*Ds β-2-tubulin*-Red was based on the pBac{3xP3-EGFPaf}(Horn & Wimmer, 2000), but I added the *Ds β-2-tubulin* promoter, the DsRed coding sequence, and an SV40 poly-A signal, followed by the restriction enzymes sites for *Asc*I, *Afl*II, and *Fse*I (New England Biolabs, Ipswich, MA, USA). These pieces were PCR-amplified using the following primers and templates: *Ds β-2-tubulin* promoter by Dsb2tub F2 and R1 from genomic DNA, DsRed by DsRed F1 and R1 from the plasmid pHSS6hsILMi20 (Klinakis et

al., 2000), and SV40 by SV40 F1 and R1 from pBac{3xP3-EGFPaf} with restriction sites built into the primers (for primer sequences, see Table C.1). I also made a second plasmid, pBac-*Ds β-2-tubulin*-EcoRI from the pBac-*Ds β-2-tubulin*-Red by replacing the DsRed sequence with one for *Eco*RI, which was PCR amplified from pEQ111m (Addgene plasmid # 40190) (Wright et al., 1989) using EcoRI F1 and R1 (Table C.1). I injected 500 ng/µl of the pBac-*Ds β-2-tubulin*-Red with 250 ng/µl pBacHelper and 20% Phenol Red (Sigma-Aldrich, St. Louis, MO, USA) into eggs from wildtype SWD, and 500 ng/µl of the pBac-*Ds β-2-tubulin*-EcoRI with 20% Phenol Red was injected into eggs from H7 strain.

Screening and molecular confirmation of promoter function

We outcrossed all G_0 flies individually and screened G_1 flies as adults for expression of the EGFP marker gene. EGFP-positive flies from the pBac-*Ds β-2-tubulin*-Red injection were outcrossed individually again, then EGFP-positive G_2 flies from each cross were kept together to create colonies. The male adult flies from EGFP-positive colonies were dissected, and the testes screened for DsRed fluorescence. The specificity of DsRed expression was confirmed by extracting RNA from multiple samples: pooled larvae, pooled pupae, adult females, adult male bodies (no testes), and adult testes. The RNA was reverse-transcribed into cDNA, and amplified in PCR for ddPCR (for protocols, see chapter 2), using the primer set qDsRed F1 and DsRed R1 (Table C.1) and 30 ng of cDNA from each sample as template.

Crosses to confirm sterile-male phenotype

Both male and female pBac-*Ds β-2-tubulin*-RcoRI G_0 flies produced transgenic G_1 offspring of both sexes. Each G_1 male was outcrossed with wild-type mates, and screened for sterile-male phenotype. Transgenic G_2 males from G_1 females were used to set up both outcrosses and self-crosses (with transgenic sisters), and screened for sterile-male phenotype. Finally, homozygous G_3 males from the self-crosses were mated to their sisters, and screened for sterile-male phenotype. For each generation, we set up female outcrosses from each strain in case the sterile-male phenotype resulted in no offspring. EcoRI expression was tested in EGFP-positive males with PCR using the primers EcoRI F1 and R1 (Table C.1).

Result

I found EGFP positive G_1 flies from both transgenic experiments, so I set up individual colonies. The *Ds β-2-tubulin*-Red males were dissected, and their testes extracted to check for DsRed fluorescence (Fig. 5.3). The ddPCR results showed very high expression of DsRed in testes, but very low expression in female and testes-removed male samples (see Table 5.1). The *Ds β-2-tubulin*-RcoRI strains did not exhibit any sterile phenotype, and RT-PCR results indicated low-to-no EcoRI mRNA in either males or females (Fig. C.1).

Table C.1. Primer sequences.

Name:	Sequence:	Purpose:
Dsb2tub F2	ACTGGTCTCGCGCGCCACACCCATGTCAATATCAATCGTA	Clone
Dsb2tub R1	ACTGGTCTCCTTAAGCTTAACCGACTGTCAAGGATCAA	Clone
DsRed F1	GATCTTAAGCCATGGTGCGCTCCTCCAAG	Clone
DsRed R1	ATTGGCCGGCCCTACAGGGAACAGGTGGTGG	Clone & ddPCR
SV40 F1	AGTGGCCGGCCCGACTCTAGATCATAATCAGCCAT	Clone
SV40 R1	AGTCGTCTCGCCGGATCGATACATTGATGAGTTTGGAC	Clone
EcoRI F1	AAGCTTAAGATGTCTAATAAAAAA	Clone
EcoRI R1	GTCGGCCGGCCTTAGTCTAGTTTAACGCGTTTGGCAGCAG GTCGCTTAGATGTAAGCTG	Clone
qDsRed F1	ATCCCCGACTACAAGAAGC	ddPCR
EGFP 3F1	CGCCGCCGGGATCACTCTCG	ddPCR
EGFP R1	TTTACTTGTACAGCTCGTCCATGC	ddPCR

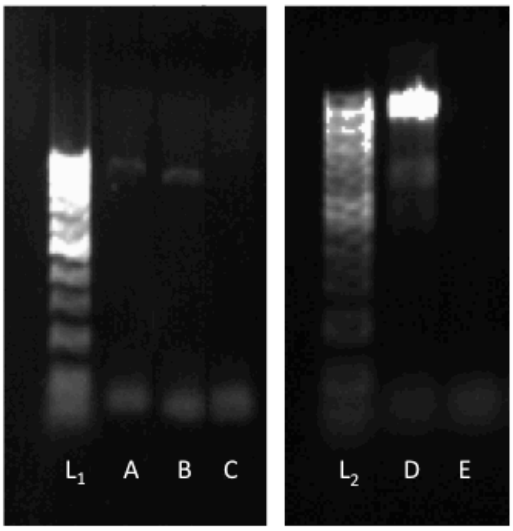

Figure C.1. PCR of EcoRI cDNA from a *Ds ß-2-tubulin*-RcoRI strain. The PCR shows that the EcoRI mRNA is expressed at very low levels in this strain, while the positive control shows that the DNA was successfully intergraded into the genome. Lane: L_1) 100-bp ladder (HyperLadder™ 100bp, Bioline); A) Male cDNA; B) Female cDNA; C) ddH_2O; L_2) 100-bp ladder (HyperLadder™ 100bp, Bioline); D) gDNA; E) ddH_2O.

Appendix D.

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