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Professur für Insektenbiotechnologie im Pflanzenschutz
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INAUGURAL-DISSERTATION

zur Erlangung des Doktorgrades (Dr. rer. nat.)

im Fachbereich Agrarwissenschaften, Ökotrophologie und Umweltmanagement
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vorgelegt von
Syeda Azka Sehar Jaffri

Munich, 2025

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The science of today is the technology of tomorrow.

-Edward Teller

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Summary

The invasive pest *D. suzukii* causes significant economic losses to soft fruit crops worldwide. This study aims to improve the Sterile Insect Technique (SIT), an eco-friendly pest management approach, by establishing the Q system in *D. suzukii.* The Q system provides regulated expression of any gene of interest and it allows the generation of transgenic lines with the ability to induce female-specific lethality.

The objectives of this study were to isolate and characterize the endogenous pro-apoptotic and pre-embryonic genes of *D. suzukii*; to analyze gene expression profiles by RT-qPCR; to test the efficacy of pro-apoptotic genes, and embryonic promoters in S2 cells, both independently and in conjunction with Q system components; and to generate the transgenic *D. suzukii* strains carrying Q system elements for conditional lethality induction.

The results of this study provide insights into the potential of pro-apoptotic genes and early embryonic promoter that can be used in conditional expression systems to control pest populations. Notably, transgenic lines of QUAS (the effector element) were successfully generated. However, the attempts to develop any transgenic lines with QF (the activation factor) were failed in *D. suzukii*. This may pose a very serious obstacle towards the viability of the Q system in this species.

Zusammenfassung

Der invasive Schädling *D. suzukii* verursacht weltweit erhebliche wirtschaftliche Verluste bei Weichobstkulturen. Diese Studie zielt darauf ab, die Sterile Insektentechnik (SIT), ein umweltfreundliches Schädlingsbekämpfungskonzept, durch die Einführung des Q Systems in *D. suzukii* zu verbessern.

Das Q System ermöglicht die regulierte expression eines beliebigen Gens von Interesse und erlaubt die Erzeugung transgener Linien mit der Fähigkeit, eine weibliche Letalität auszulösen.

Ziel dieser Studie war es, die endogenen pro-apoptotischen und präembryonalen Gene von *D. suzukii* zu isolieren und zu charakterisieren, Genexpressionsprofile mittels RT-qPCR zu analysieren, die Wirksamkeit von pro-apoptotischen Genen und embryonalen Promotoren in S2-Zellen sowohl unabhängig als auch in Verbindung mit Q-System-Komponenten zu testen und transgene *D. suzukii*-Stämme zu generieren, die Q-System-Elemente zur Induktion bedingter Letalität tragen.

Die Ergebnisse dieser Studie geben Aufschluss über das Potenzial von pro-apoptotischen Genen und frühen embryonalen Promotoren, die in konditionalen Expressionssystemen zur Kontrolle von Schädlingspopulationen eingesetzt werden können. Insbesondere wurden erfolgreich transgene Linien von QUAS (dem Effektor-Element) erzeugt. Die Versuche, transgene Linien mit QF (dem Aktivierungsfaktor) in *D. suzukii* zu entwickeln, sind jedoch gescheitert. Dies könnte ein ernsthaftes Hindernis für die Lebensfähigkeit des Q-Systems in dieser Art darstellen.[1]

[1] Summary was translated on Deepl.com.

1. Introduction

Drosophila species are generally not considered agricultural pests because they are mostly found on rotten fruits, where they feed on decaying organic matter and associated microorganisms (Lee et al., 2015). *Drosophila melanogaster*, for example, is a common species often found near overripe bananas and is a model organism in genetics research precisely because it does not typically target commercially valuable crops. *Drosophila suzukii* stands out as a globally invasive pest that is attracted to freshly ripened soft fruits for laying eggs. This fly is generally named a 'cherry vinegar fly' or 'spotted wing *Drosophila*' (SWD). Its serrated ovipositor helps it deposit eggs inside freshly ripened soft fruits and makes it a significantly important pest to be intensively studied and efficiently controlled.

1.1 Biology of *D. suzukii* that makes it a global pest.

Adult flies of *D. suzukii* are 2-3 mm long with notable red eyes and black-striped abdomens. The eggs are oval and 0.4-0.6 mm long. A key identifying feature of male *D. suzukii* is a dark spot on each wing. However, it is observed that the species *D. biarmipes* also has such wing aberration (Atallah et al., 2014; Chiu et al., 2013). Female *D. suzukii* has a serrated ovipositor (Fig. 1) that functions like a saw and helps deposit embryos inside the fruits. This piercing causes damage to the freshly ripened fruits. The deposited eggs develop into larvae that feed on the fruit flesh (Hauser, 2011; Walsh et al., 2011). Due to physical damage to the skin, fruits are exposed to other harmful fungi and bacteria that cause additional loss. Moreover, the short generation time and overwintering ability of *D. suzukii* make it an even worse pest (Panel et al., 2018; Rendon et al., 2018). The optimum survival temperature range is 21 °C to 29 °C. A complete life cycle consists of 12 to 15 days at 21 °C (Tochen et al., 2014; Walsh et al., 2011), and the reproductive ability of *D. suzukii* is reduced at 28 °C or above. The adult lifespan is 1.5 to 2 months, but due to overwintering, some adults can also live up to 200 days (Kanzawa, 1935).

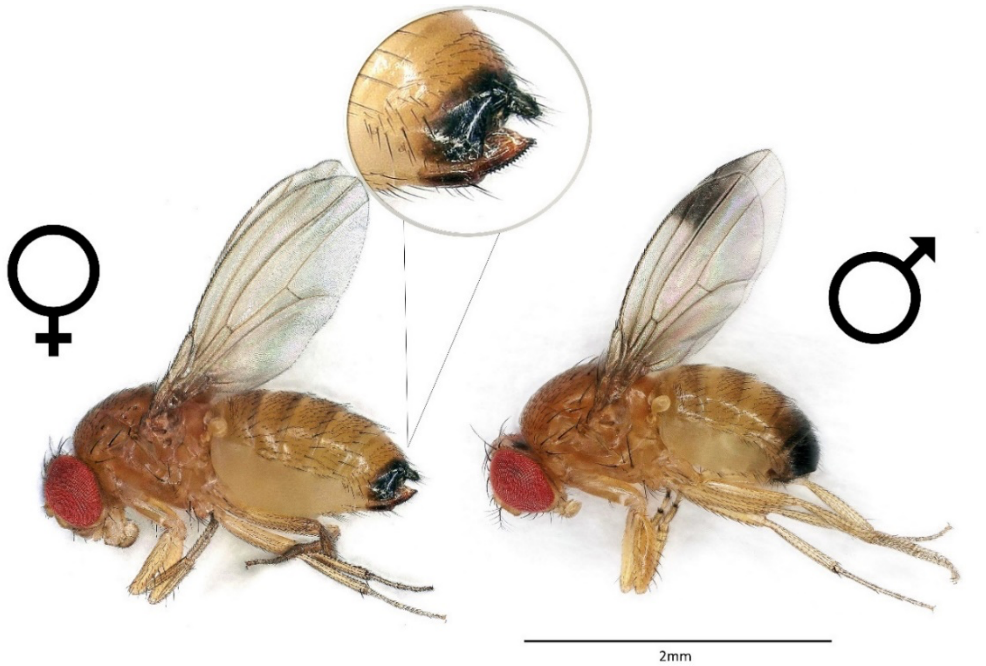

Figure 1: Distinguishing physiological traits of *D. suzukii*.
Female *D. suzukii* (left) is characterized by a serrated ovipositor (indicated by arrow), which allows egg-laying in intact fruit. Male *D. suzukii* (right) has a prominent dark spot on each wing. These features differentiate *D. suzukii* from most other Drosophilids and are critical for accurate field identification.

1.1.1 Distribution and economic impact of *D. suzukii*

The origin of *D. suzukii* is Southeast Asia, and it has become a globally expanding invasive pest. It was spotted first in Japan in 1916, and later, in 1930, it was found in other parts of Asia (Kanzawa & Kofu, 1939). Due to its short generation time and international exports of soft fruits, it has been widely spreading. Its rapid reproductive cycle enables multiple generations per year, facilitating its establishment in new regions. Further studies show its presence in Asia, America, Europe, and Africa, demonstrating its ability to thrive in diverse climates. In Europe, within three years of its initial detection in Italy (and concurrently in Spain) in 2008, the pest had spread to nine countries. i.e., France in 2009 (Calabria et al., 2012), Belgium (Mortelmans et al., 2012), Great Britain (Harris & Shaw, 2014) and Germany in 2012 (Vogt et al., 2012). Following the spread of *D. suzukii* in Western and Central Europe, it also extended to Eastern Europe and Western Asia in subsequent years. In 2014, *D. suzukii* was also reported in Turkey (Orhan et al., 2016). In 2015, the pest was detected in Iran (Parchami et al., 2015), further expanding its known distribution into Western Asia. This rapid invasion resulted in significant crop losses for berry fruits and cherries despite the use of intensive chemical control methods for prevention (Ioriatti et al., 2015).

In 2008, economical losses for California, Oregon, and Washington were estimated to be 40% on blueberries, 50% on caneberries, 20% on strawberries, and 33% on cherries, summed up to a total of $511 million loss annually due to *D. suzukii* (Bolda et al., 2010). In Brazil, the economic loss has been estimated at approximately 30 million USD (Benito et al., 2016). The potential economic losses for various fruit crops in Brazil were estimated, ranging from 45.5% for grapes to 98.3% for apples (Benito et al., 2016). The economic impact of *D. suzukii* on the production of berry crops in the Province of Trento, Italy, was calculated to be about 13% of the industry's output, which decreased to about 7% after implementing an integrated pest control strategy (De Ros et al., 2015). In recent studies in Africa, *D. suzukii* has been reported to affect 61% of raspberry crops, 22% of blueberry crops, 11% of strawberry crops, and 5% of mulberry crops (Boughdad et al., 2021).

These dreadful impacts of this species arose due to the presence of its larvae in freshly ripened fruits, which can cause an entire shipment to be rejected. It damages soft fruit crops, including cherries, peaches, raspberries, blackberries, strawberries, blueberries, and grapes. Its rapid spread has drawn much attention as a crop pest worldwide. Its global spread, high fecundity, short generation time, and overwintering capability add up to devastating damage to the fruit crops.

1.1.2 Common control practices and their limitations for *D. suzukii*

Like any other pest, many control methods have also been established for *D. suzukii*. Some of the control methods include:

- **Protective cladding** has been intensively used in Europe, especially for cherry crops. This protective cladding helps the crops avoid contact with *D. suzukii*, physically preventing the flies from laying eggs in the fruit. Furthermore, it prevents insecticide application from being washed off by rai that further maximize the efficacy of insecticides. (Chouinard et al., 2016). The effectiveness of complete enclosure systems with the help pf protective claddings has reduced *D. suzukii* damage to cherry orchards (Knapp et al., 2020).

- **Mass trapping** has also been used to control *D. suzukii* population. This method uses a dense barrier containing attractants to divert pest populations from fruit fields. Around 60 to 100 traps per acre with a distance no longer than 5 m are recommended to efficiently reduce *D. suzukii* in fruit crops (Quarles, 2015; Lee et al., 2011). The high density of traps creates a competitive environment, drawing the flies away from the fruits. Trapping attractants used for mass trapping can be homemade baits, including apple cider vinegar, fermented berries, and yeast-sugar mixtures or commercial lures like Z-Kinol®, SuzukiiTrap®, and PHEROCON® SWD (Cruz-Esteban et al., 2021a; Lasa et al., 2019; Toledo-Hernández et al., 2021). The location, crop type, and season govern the specificity and effectiveness of these traps (Burrack et al., 2015).
- **Insecticides** can act effectively to fight pest insects. The most used chemicals to control this pest are pyrethroids, spinosyns or organophosphates (Cowles, 2015; Beers et al., 2011; Timmeren & Isaacs, 2013). Pyrethroids are synthetic insecticides that offer broad-spectrum control (Ravula & Yenugu, 2021). Spinosyns, which originate from naturally occurring soil bacteria, offer a more selective mechanism of action by specifically targeting the nervous systems of certain insect species (Sparks et al., 2001). In contrast, organophosphates raise more serious environmental and health concerns, as they act by inhibiting acetylcholinesterase, a key enzyme involved in nerve signaling in both insects and mammals (Sidhu et al., 2019). While most insecticides provide crop protection for up to 14 days, and pyrethrins typically last around 7 days, their use on fruit crops involves additional complexities. Major issues around the use of insecticides include residue tolerance, harvest intervals, and crop sensitivity must be considered when planning insecticide applications in fruit production. To further enhance the effectiveness of insecticides, it can be combined with cladding (Shaw et al., 2019). Cladding, or protective netting, creates a physical barrier that reduces pest pressure, allowing insecticides to be used more effectively.

It is important to consider the adverse effects of insecticides on biodiversity (Geiger et al., 2010). Resistance to insecticides occurs when a pest population evolves the ability to survive exposure to a previously effective insecticide, requiring higher doses or alternative control methods. This can lead to increased costs for growers and potentially greater environmental impacts. *D. suzukii* can develop resistance to insecticides (Haviland & Beers, 2012), and then the development of new insecticides would be needed, which is economically not feasible (Borovok et al., 2008; Osei et al., 2003). Using insecticides for *D. suzukii* is even more critical because it lays eggs in marketable fruits. This oviposition behavior directly damages the fruit, making it unmarketable and causing significant loss for growers. A maximum residue limit (MRL) of insecticides must be achieved for exportable fruits, and balancing the allowed limit of insecticide residues becomes challenging. For example, The EU now restricts spinosad residues on strawberries to 0.30 mg kg^{-1}, whereas the U.S. still allows 0.90 mg kg^{-1} (Oregon State University Extension Service, 2024). These limits are set by regulatory agencies to make sure that food is safe for consumption. Exceeding MRLs can result in rejection of the fruit shipment. Therefore, a strategic timing for insecticide treatment is needed to prevent *D. suzukii* infestation. In addition, climate plays another vital role in the application of insecticides. High temperatures can accelerate the degradation of some insecticides, reducing their efficacy, while rainfall can wash them off the plant surface, necessitating reapplication. Timmeren & Isaacs 2013, estimated that the efficacy of insecticide treatments reduces significantly even after 2 cm of rainfall, which puts a plant at risk of infestation and needs insecticide treatment again, which again enhances the cost. Therefore, improved tactics are needed for efficient control that might include using integrated pest management (IPM) strategies, such as biological control, cultural practices, and the use of more selective insecticides, to minimize the reliance on broad-spectrum chemicals and reduce the risk of resistance development and environmental harm.

1.2 Integrated pest management

With the growing population worldwide, the need to grow natural food resources with innovative and sustainable solutions for pest control is also increasing. According to FAO (Food and Agricultural Organization of the United Nations), the Integrated pest management (IPM) is defined as

> *'The careful consideration of all available pest control techniques and subsequent integration of appropriate measures that discourage the development of pest populations and keep pesticides and other interventions to levels that are economically justified and reduce or minimize risks to human health and the environment`.*

IPM emphasizes the growth of a healthy crop with the least possible disruption to agro-ecosystems and encourages natural pest control mechanisms. Therefore, IPM is a sustainable approach to managing pests in a multidisciplinary way, including genetics, chemical repellents, cultural control (harvesting fruit crops before they ripe), and using mechanical tools to minimize health, economic, and environmental risks. Since its introduction in 1959 (Stern et al., 1959). Today, IPM offers its success in several projects on a small scale. A widely used area-wide IPM strategy is the `sterile insect technique (SIT),` which is used to repress pest populations.

1.2.1 Sterile insect technique

The sterile insect technique (SIT) is an environment-friendly, specie-specific pest control method. It reduces reliance on broad-spectrum insecticides and minimizes harm to non-target organisms that addresses growing concerns about environmental sustainability and insecticide resistance. The process of SIT begins with the mass rearing of target insect species in controlled conditions (optimized temperature, humidity, and diet to maximize production efficiency). Once a good quantity of insects have been raised, they are exposed to ionizing radiation such as gamma rays or X–rays for sterilization. The sterilization process destroys the insect's reproductive cells, making it incapable of producing fertile offspring. The optimal radiation dose is carefully calibrated to induce sterility without significantly impacting the insect’s mating competitiveness. Too high dose can reduce their ability to find mates, while too low a dose may not guarantee complete sterility. The sterilized insects are then released in bulk into the specified region (Fig. 2). In this target release area, the sterile male insects could mate with wild host female insects to produce non-viable offsprings. Over time and with repeated releases, this process can lead to significant suppression or even local eradication of the pest population (Knipling, 1955).

The classical SIT has undergone many successes, including eradication of the New World screw-worm (*Cochliomyia hominivorax*), which has been prevented and removed from North and Central America (Vargas-Terán et al., 2005). Despite its successes in various contexts, SIT faces several significant limitations that affect its broader implementation:

- **Cost of Mass rearing**: The necessity to build extensive rearing facilities and mass-rearing both sexes comes with high investment costs. These facilities require climate control, specialized equipment for egg collection, larval rearing, pupation, and adult holding, and robust biosecurity measures to prevent disease outbreaks. According to USDA, around $7.3 million were spent annually on rearing of Oriental Fruit Fly programs (USDA Animal and Plant Health Inspection Service, 2021). Since the females are later discarded, this process consumes 30–50% of total operational costs (FAO/IAEA, 2022). The USDA analysis further reveals that the absence of a temperature-sensitive lethal genetic sexing strain for OFF introduces additional costs, as females must be reared until they can be separated based on color. This requirement results in an estimated 150% increase in diet needs compared to Medfly programs that can eliminate females earlier in the rearing process (USDA Animal and Plant Health Inspection Service, 2021). These biological constraints translate directly into economic challenges, with total diet costs potentially tripling for certain species. This is because the diet needs to sustain both male and female larvae until the point of sex separation, and the diet itself can be a significant expense, often composed of specialized ingredients like yeast, sugars, and proteins. The development and implementation of effective genetic sexing strains is therefore a crucial area of research for improving the cost-effectiveness of SIT program.

- **Radiation safety:** The handling of radiation sources requires specialized facilities, equipment, and trained personnel that could operate under strict regulatory frameworks. The organizations need to implement safety measures including proper shielding, controlled access to radiation areas, and regular monitoring of radiation levels and personnel exposure (Norwegian Radiation Protection Authority, n.d.). Another important aspect to keep in mind is the public perception and acceptance about the use of radiation in pest control. This exphasizes on the need of transparent communication and education about the safety measures in place (Gauthier, 2009). Therefore, addressing public concerns about the minimal environmental impact and rigorous safety protocols is crucial for fostering support and ensuring the long-term viability of SIT.

- **The radiation effectiveness:** SIT's effectiveness highly depends on the dose of radiation that would not compromise in the mating competence of an insect. For instance, if the radiation dose is too low, the insects may not be fully sterilized and could still produce viable offspring, undermining the entire SIT program (Yamada et al., 2022). Conversely, too high a dose can severely impair their mating ability, reducing their competitiveness with wild populations and again, rendering the SIT ineffective (Yamada et al., 2014). To achieve this balance a careful calibration of radiation parameters and quality control procedures are required to deliver consistent results. This calibration often involves extensive laboratory testing to determine the optimal dose for each specific insect species. Different type of radiations (e.g., gamma, X-ray) have properties that may influence their suitability for various insect species and rearing systems. To achieve the successful sterilization, some aspects of radiation, including penetration depth and dose distribution, need to be carefully calibrated. For example, if the radiation does not penetrate uniformly throughout a container of insects, some individuals might receive a lower dose and remain fertile, leading to program failure (Bond et al., 2019;

Sassu et al., 2019). Dose mapping techniques, using dosimeters strategically placed within the rearing containers, are crucial for ensuring consistent sterilization. The selection of appropriate radiation technology, therefore, represents another technical challenge in SIT implementation (Reinhardt et al., 1989; Mastrangelo et al., 2010).

- **Male mating competitiveness:** Multiple studies have documented the adverse effects of irradiation on male mating competitiveness. A study comparing radiation-based SIT and *Wolbachia*-induced sterility in *Aedes aegypti* found that irradiation and *Wolbachia* infection reduced male mating competitiveness but to different degrees based on the specific treatment and dosage (Kittayapong et al., 2025). Similarly, another research on sterile *A. aegypti* males demonstrated that their mating competitiveness was reduced due to irradiation, necessitating higher release ratios to compensate (Helinski et al., 2021). This suggests the importance to optimize the irradiation doses to minimize negative impacts on mating performance while still achieving the desired level of sterility.

Examples of radiation doses used to sterilize *D. suzukii* for sterile insect technique (SIT) programs typically range from 120 to 220 Gy. For instance, Lanouette et al. (2017) demonstrated that irradiating pupae at 120 Gy resulted in more than 99% reduction in fertility while preserving adult viability and mating performance. Similarly, Krüger et al. (2018) and Sassù et al. (2019) found that doses up to 200–220 Gy also induced effective sterility without adverse effects on emergence rate, flight ability, or overall fly quality, making these doses suitable for operational SIT programs.

1.2.2 Genetic-based methods in modern pest control

Since the introduction of SIT, insect pest management has evolved significantly. Although classical SIT remains an essential tool, alternative genetic methods have been established. Genetic methods of pest management have proven to be more accurate and effective than the traditional strategies (Viktorov, 2017; Leftwich et al., 2016). Classical Sterile Insect Technique (SIT) differs from modern genetic SIT methods in several key aspects. While classical SIT relies on radiation to induce sterility, modern genetic approaches use transposon-based systems, CRISPR-Cas9 gene editing, and gene drives to modify insect genomes. Genetic approaches, such as the male-only population has been proven more effective as it reduces the rearing cost and eliminates the chance of additional fruit damage caused by egg deposition from released females (Rendon et al., 2004). The effects of classical SIT are limited to one generation, requiring continuous releases, while some genetic approaches can potentially persist in populations over multiple generations.

- **RIDL:** One example genetic development is the Release of Insects carrying a Dominant Lethal (RIDL) system. In RIDL, a lethal gene is introduced into the insect genome but is repressed during insect rearing. At the time of insect release and mating with wild populations, this lethal gene becomes active in the offspring, causing their death before reaching reproductive age (Thomas et al., 2000).
- **GSS:** the Genetic Sexing Strains (GSS) allows the separation of males and females based on genetically engineered traits. This separation occurs during the rearing process, supporting the release of sterile males only (Papathanos et al., 2018). This technique reduces rearing costs, eliminates chances of potential damage caused by sterile females, and, most importantly, enhances released males' mating competitiveness. For instance, the VIENNA 8 strain of Mediterranean fruit fly has been widely adopted in SIT programs due to its superior performance in quality control parameters (Augustinos et al., 2017).
- **Tsl:** The temperature-sensitive lethal mutations (tsl), wherein the change in an organism's genes causes the death of that organism under high temperatures. With this method, Females are eliminated at the rearing stage, making male release strategies more efficient (Augustinos et al., 2017).
- **pgSIT** (precision guided Sterile Insect Technique) is a novel CRISPR-based genetic control strategy that produces only sterile males by disrupting genes critical for female survival and male fertility during development. Unlike traditional SIT, pgSIT does not rely on irradiation or manual sex sorting, enabling more efficient and cost-effective mass-release programs.

Furthermore, pgSIT males do not transfer genetic material to wild populations, greatly reducing ecological and regulatory risks. This precise and self-limiting approach offers significant potential for integration into sustainable, environmentally friendly pest management strategies (Witherbee & Gamez, 2025).

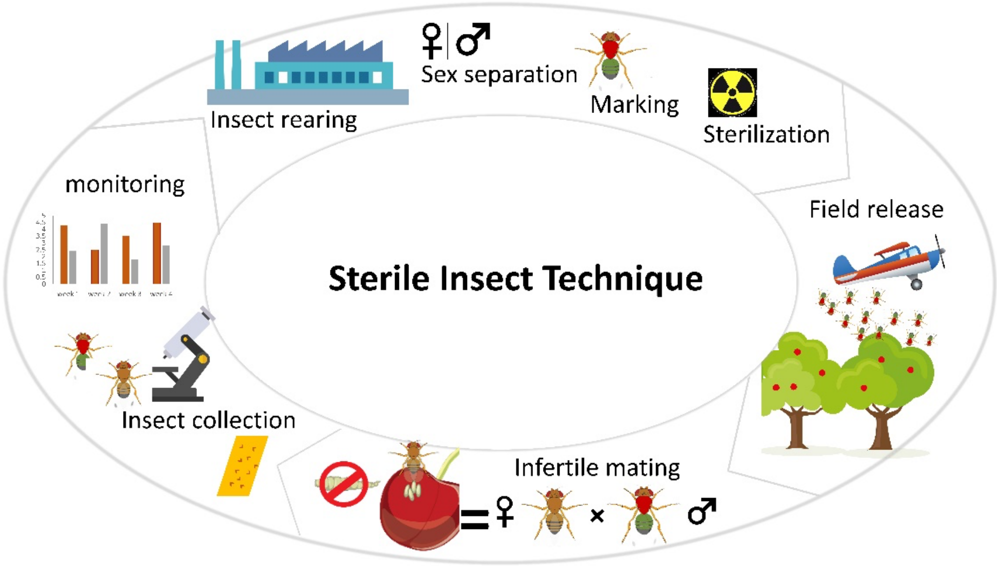

Figure 2*:* Improved Sterile Insect Technique (SIT).
This figure illustrates the key steps in the SIT process: (1) Mass rearing of target insect species under controlled, industrial conditions; (2) Separation of sexes within the rearing facility, eliminating females prior to irradiation; (3) Marking and sterilization of male insects using ionizing radiation; (4) Release of sterilized males into the target area, where they compete with wild-type males to mate with wild-type females; (5) Mating leads to nonviable offspring, resulting in population suppression; and (6) Periodic sampling and monitoring of the released population to assess program effectiveness. This process minimizes non-target effects and supports environmentally sustainable pest management. Elements such as arrows, color codes, and labeled steps correspond to the flow of events depicted in the diagram.

The genetic modifications for SIT may face regulatory challenges and potential public resistance. Ultimately, the decision about application of genetic SIT through classical or modern methods is influenced by the specific pest species, legal framework, resource limitation, and the peculiarity of pest control objectives.

1.2.2 Need for conditional expression systems

The need for conditional expression systems in pest control has become increasingly apparent as traditional pest control methods face challenges such as non-target effects, environmental persistence, and resistance development.

Conditional expression systems offer a more refined approach, allowing precise temporal and spatial control over the expression of pest control agents (Xia et al., 2017). Such systems include effector genes and regulatory sequences, allowing the gene expression at a desired time only.

In a recent field study, the effectiveness of the sterile insect technique (SIT) against *D. suzukii* was demonstrated using male-only releases in a strawberry field in Kent, UK. The researchers achieved this by ‘manually’ sorting males from females using fine paint brushes under CO_2 anesthesia before subjecting only the males to X-ray sterilization. Over the course of the growing season, between 9,000 and 60,000 sterile males were released weekly, resulting in up to 91% suppression of the wild SWD female population compared to untreated controls. This manual sex sorting was crucial to the success of the SIT program, as it prevented the release of potentially fruit-damaging sterile females and maximized the mating disruption effect of released sterile males (Hawes et al., 2022). However, the manual sex sorting of male flies is labor intensive and prone to have a female contamination as well.

To address the sex separation issue in rearing facilities, various genetic techniques have been developed focusing on creating strains where the expression of lethal genes can driven with female-specific introns or where a combination of promoter and reporter genes have been studied for embryonic lethality strains (Thomas et al., 2000; Handler, 2016; McGuire et al., 2004). A commonly used conditional expression system is a “tetracycline-controlled expression system” (stated as tTA system/Tet system in the following sections). In this system, the insects are reared with tetracycline or doxycycline. The lethal gene is activated upon feeding insects tetracycline-free food, resulting in no offspring. The Tet-Off system has been successfully tested in the model organism *D. melanogaster* (Heinrich & Scott, 2000; Thomas et al., 2000; Horn & Wimmer, 2003). This system has been one of the effective methods of insect population control, as evidenced by a study on the breakdown of a Tet-Off conditional lethality genetic system (Zhao & Schetelig, 2020). Some other examples are, female-specific yolk protein *yp3* and the oncogene *ras64B,* which are used to eliminate females without tetracycline (Thomas et al., 2000). Embryonic genes *sry-α* and *nullo* are also being used in the Tet-Off system to induce embryonic lethality, and such strains are called transgenic embryonic sexing strains (TESS) (Horn & Wimmer, 2003). Genetically improved SIT has the potential to bring about significant cost savings by explicitly targeting female insects while they are in their embryonic phase (Schetelig & Handler, 2012; Yan & Scott, 2015; Gong et al., 2005; Schetelig et al., 2009; Ogaugwu et al., 2013; Lewandoski, 2001; Meza et al., 2018; Schetelig et al., 2016). A comparative system to Tet-Off is called the Tet-On system, the reverse of Tet-Off. One can add tetracycline or doxycycline to activate gene activity (Gitzinger et al., 2009). Large amounts of antibiotics are needed in the factories to maintain the rearing facilities for such a system. This can decrease fly fecundity, it is costly, and waste disposal is critical (Schetelig & Handler, 2012). Another study suggests that the high dose of antibiotics (tetracycline and doxycycline) in the food leaves negative impacts on fly development (Yan et al., 2023). In addition to that, recent studies have reported that single conditional Tet-Off expression systems, that are based on antibiotic regulation, carries the risk of a genetic breakdown due to primary site mutations and second-site mutations (explained in section 1.3.3) (Zhao et al., 2020; Knudsen et al., 2020). Therefore, the need for a backup conditional expression system is increasing. It is also suggested that two or more independent conditional expression systems be used in an insect as a backup to benefit the tight control of lethal gene expression. Several potential expression systems (Fig. 3) have been discussed in detail, highlighting their suitability for developing robust and combinatorial systems (Jaffri et al., 2020).

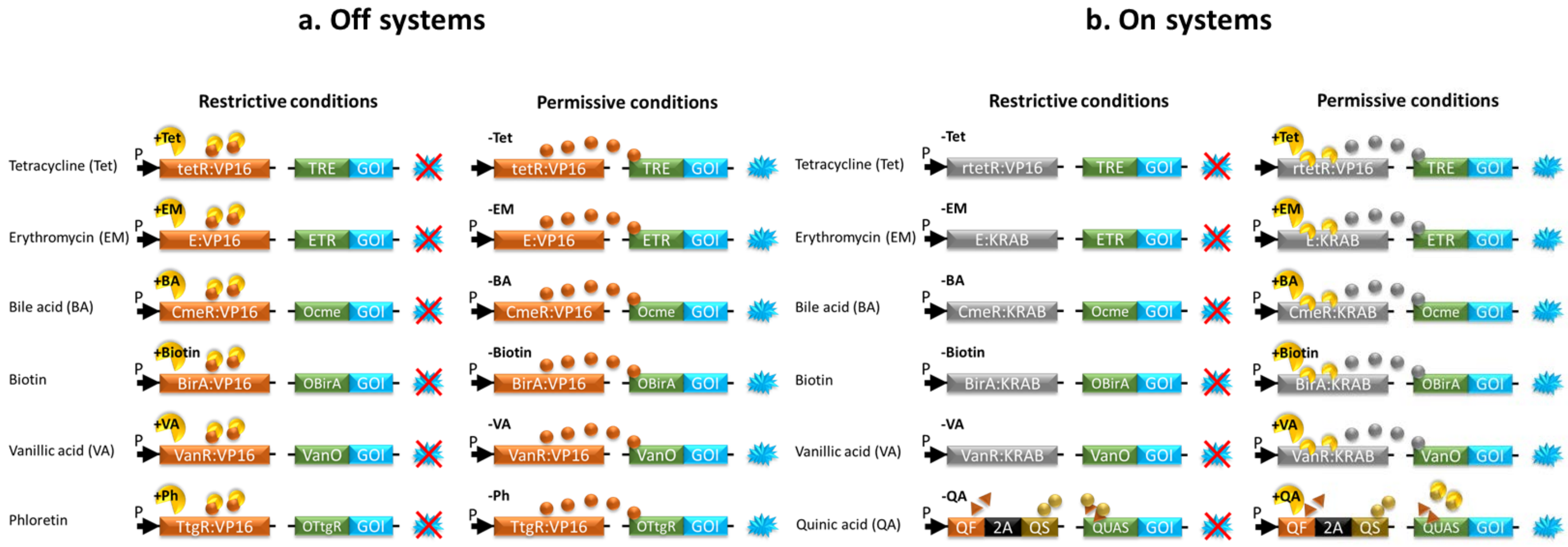

Figure 3: A few examples of drug inducible/ conditional expression systems.
The conditional expression systems that can be controlled by several drugs, tetracycline (Tet), erythromycin (EM), bile acid (BA), biotin, vanillic acid (VA), phloretin, and quinic acid (QA), are displayed. (a) Off systems: Gene expression is activated in the absence of the respective control molecule. (b) On systems: In the absence of control molecules, the transcriptional repressor does not allow the expression of the gene of interest (GOI). The control molecules bind to the transcriptional repressor and inhibit binding to its operator, thereby activating gene expression. For the Q system, the activation factor (QF) and suppressor (QS) can be combined with 2A peptides —a short sequence that allows both proteins to be produced separately from a single genetic construct. In the absence of QA, QS binds to QF, inhibiting gene expression. In the presence of QA it inhibits QS, and QF is released to activate the expression of the GOI (Jaffri et al., 2020).

1.3 Quinic acid system (Q system)

Like the Tet-Off system (discussed in section 1.2.2), the Q system is also a binary expression system that provides tight regulation and high inducibility for the gene of interest. It is derived from the quinic acid (QA) utilization gene cluster of *Neurospora crassa* and has emerged as a powerful tool for conditional gene expression in molecular biology research (Riabinina & Potter, 2016). In *D. melanogaster*, the Q system has shown promising results in minimizing background expression compared to Tet-based systems (Potter et al., 2010). The Q system could be adapted for pest control like the Tet systems by placing a lethal gene under the control of the Q system activator QF, and its expression could be triggered by QA leading to targeted female elimination (Fig 4b).

1.3.1 Origin and components

The Q System functions through a series of molecular interactions. The four main components of the Q system are QF (transcriptional activator), QUAS (QF binding sequence), QS (transcriptional repressor), and QA (external regulator). In its primary state, QF binds to QUAS sequences and activates downstream gene transcription. However, when the QS repressor is present, it binds with QF and inhibits its ability to transcribe the gene of interest (Fig. 4a). Upon addition of quinic acid (QA) the QS–QF interaction is disrupted, restoring the transcriptional activation function of QF (Fölsz et al., 2022). This mechanism enables precise control of gene expression.

1. **QF:** is a part of the qa-1F gene and acts as the transcriptional activation unit. It is composed of three functional modules: a DNA binding and dimerization domain (DBD), a middle domain (MD), and a transcriptional activation domain (AD) (Riabinina et al., 2015). DBD binds QUAS sites as it has a Zn2-Cys6 module, and AD-controlled transcription compounds engage the QS repressive elements (Riabinina & Potter, 2017). The AD recruits other proteins, such as RNA polymerase and transcription factors, to initiate transcription. The MD likely plays a role in protein-protein interactions, facilitating the transcriptional complex assembly.

2. **QUAS:** QUAS typically contains five tandem repeats of a 16-base pair sequence called QF binding sites (Potter et al., 2010). These sites are located immediately upstream of a minimal promoter (Fölsz et al., 2022). A minimal promoter contains the core elements necessary for transcription initiation, such as the TATA box, but lacks the upstream regulatory sequences that would normally control gene expression. This arrangement allows for low basal expression in the absence of QF and high QF-induced expression (Potter et al., 2010). Due to the modular nature of QUAS the expression levels can be optimized to reduce background expression by modifying the number and arrangement of QF binding sites (Riabinina et al., 2015). For example, increasing the number of QUAS (QF binding sites) can enhance the system's responsiveness to QF, leading to higher levels of gene expression. In the absence of QF, the QUAS-controlled gene remains unexpressed (Potter et al., 2010).
3. **QS**: adapted from the qa-1S gene, QS acts as a repressor. It prevents transcription activation when it binds to QF (Fig 4). QS functions by sterically hindering QF's interaction with the transcriptional machinery. This particular interaction between QS and QF ensures the Q System remains tightly regulated (Potter CJ., 2010).
4. **QA**, a small molecule, can reverse QS's repressive action and results in temporal control of gene expression (Patel et al., 1981). Quinic acid (QA) binds to QS, causing a conformational change to reduce its affinity for QF. The QF dissociates from QS and bind to QUAS, initiating gene expression. This interplay between QF, QUAS, QS, and QA forms the basis of the Q System's functionality in genetic research (Riabinina & Potter, 2022) (Fig. 4). The Q system has been successfully implemented in a variety of organisms, including fungi, insects, and mammals, demonstrating its versatility and broad applicability.

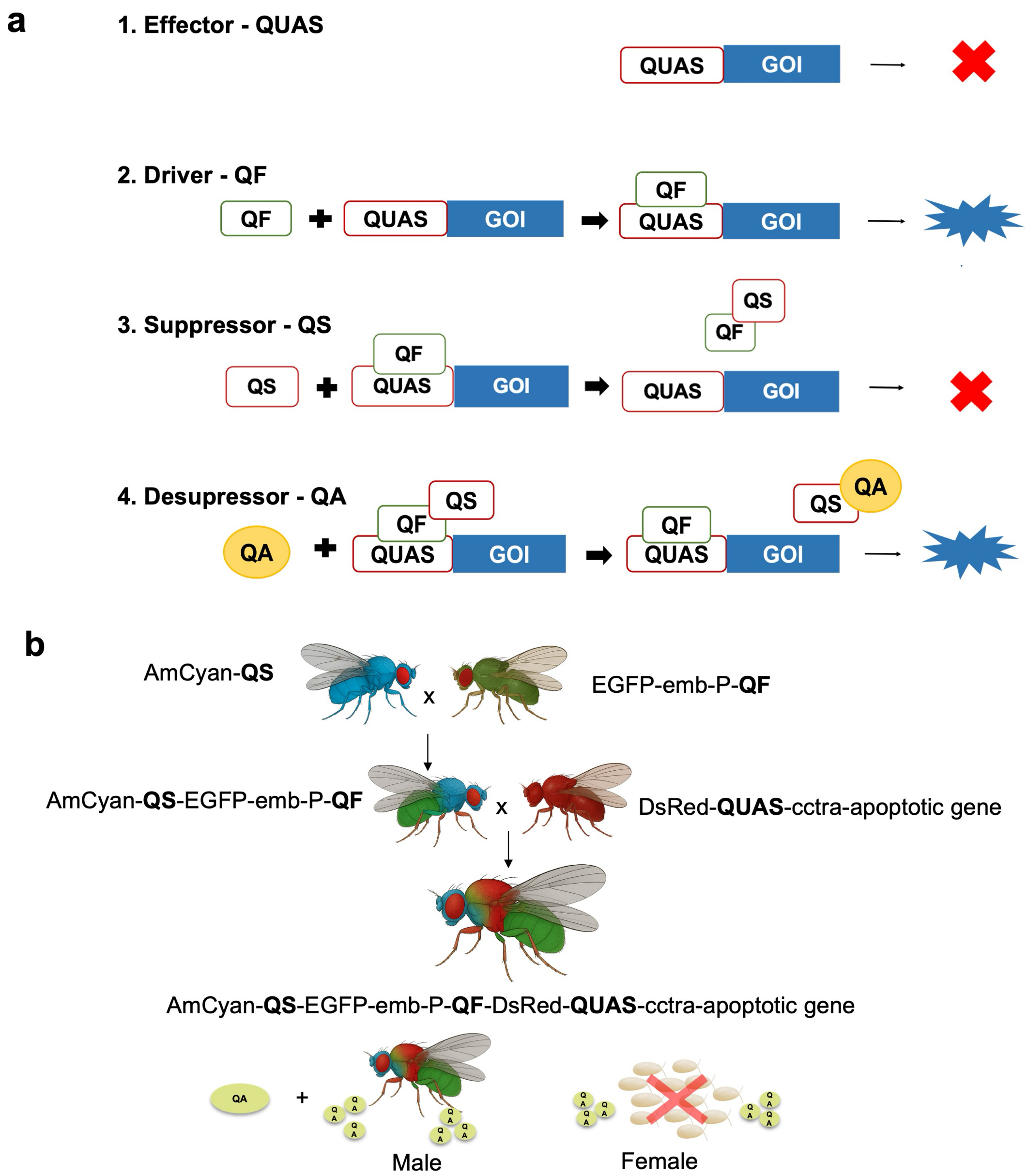

Figure 4: Quinic acid system (Q system) and its application in pest control.

The Q system consists of three genetic components. a1) The QUAS, as an effector, cannot initiate gene expression. a2) The gene expression is activated in the presence of the activation agent QF. (a3) the third genetic component is the suppressor, QS. In the presence of QS, QF is eliminated from QUAS, repressing the gene expression. (a4) The fourth component is QA, the appropriate amount of QA added to the system, elevates the QS, allowing QF to drive the gene expression.

b) Schematic representation of sex-specific lethality system using the Q-system in *D. suzukii*. In this strategy, flies carrying AmCyan-QS will be crossed with flies expressing EGFP-emb-P-QF to generate progeny with both QS and QF components. These flies will then be crossed with a line carrying the DsRed-QUAS-cctra-apoptotic gene. The resulting offspring are expected to carry all transgenes: AmCyan-QS, EGFP-emb-P-QF, and DsRed-QUAS-cctra-apoptotic gene. Upon supplementation with quinic acid (QA), which inactivates the QS repressor, QF will drive expression of the apoptotic gene via QUAS elements. Due to the cctra promoter's female-specific activity, lethality will be induced only in females, allowing selective survival of males under QA treatment (illustrations for (b) were generated via ChatGPT and assambled on PowerPoint).

To implement the Q system for pest control, pest species could be engineered to express lethal genes under the control of the Q system's regulatory elements. The expression of these genes could then be induced by adding QA. This approach would offer several benefits such as specificity (to target specific pest species, minimizing off-target effects), temporal control (gene expression could be precisely timed to critical stages in the pest's life cycle), and environmental safety (Quinic acid, is a naturally occurring compound, potentially reducing environmental concerns).

1.3.2 Applications of the Q system in living organisms

The Q system has been successfully used in *D. melanogaster* (Potter & Luo, 2011). Beyond *Drosophila*, the Q system has been used in mammalian cells to expand its potential applications to biomedical research (Potter et al., 2010), *C. elegans,* providing a new tool for genetic manipulation in this nematode model (Wei et al., 2012); Silkworm (*Bombyx mori*) as an economically significant insect model (Duan et al., 2013); Zebrafish, allowing for spatiotemporal control of gene expression in this important vertebrate model organism (Subedi et al., 2014); mosquitoes, to study gene function and develop potential genetic control strategies (Riabinina et al., 2016), and plants, for providing new opportunities for plant biotechnology and research (Persad et al., 2020). Like any other gene control system, QF expression can also be manipulated with strong or weak promoters; therefore, it is suitable for the establishment of transgenic sexing strains (TESS).

1.3.3 Benefits of the Q system over the well-established Tet-Off system

The Q system provides several key benefits in developing TESS to control pests. The widely used Tet-Off system is based on the antibiotic tetracycline, which is prone to build resistance in genetically modified pest populations because of primary and secondary site mutations (Knudsen et al., 2020; Zhao et al., 2020).

The primary site mutations can result from DNA replication errors, damage caused by mutagens, or insertion/deletion of nucleotides directly in the targeted genetic elements, such as the tetracycline-responsive promoter, the tetracycline transactivator (tTA) gene, or the tetracycline operator sequences. Secondary site mutations occur outside the primary target but still affect the system's function.

These may involve genes related to tetracycline uptake or metabolism, regulatory elements influencing system component expression, or genes interacting with system components. Both primary and secondary mutations can be caused by naturally occurring replication or repair errors in DNA, exposure to physical or chemical mutagens, additions or losses of portions of DNA, slippage in repetitive sequences, or single nucleotide polymorphisms (SNPs). As these mutations build up gradually, pest populations will develop resistance, which compromises the effectiveness of the Tet-off system in controlling genetically pest populations (Thomas et al., 2000; Horn & Wimmer, 2003; Schetelig et al., 2009).

Contrary to the Tet system, the Q system utilizes QA. This non-antibiotic inducer significantly reduces the risk of resistance development, making it a more sustainable and effective system for establishing TESS lines. It has been studied that *Drosophila* larvae are more receptive to QA in their diet compared to adult flies (Riabinina et al., 2015). This makes the Q system to be advantageous for the control of sex-specific embryonic lethality. The QA could help suppress the lethal gene's expression during rearing, enabling both sexes to survive. Upon changing the *Drosophila* diet to the QA-free diet, the lethal gene could be expressed only in females; hence, the selective sex elimination of a specific genome was possible for the pest.

Strategies such as using multiple binary expression systems could be considered to minimize the risk of resistance development. Some examples of combined binary systems that have been developed are the Q system and tTA system combination, which were successfully demonstrated in mammalian cells and could be adapted for insect pest control (Mao et al., 2019; Eckermann et al., 2014). Q system and GAL4 system integration demonstrated the use of these systems to label and manipulate specific subsets of neurons in *Drosophila* (Potter et al., 2010). Li and Stavropoulos (2016) also showcased using the Q system alongside the GAL4 system to manipulate sleep and memory genes in *Drosophila* independently.

For pest management, it is possible to express a lethal gene under QUAS in specific embryonic tissues and then drive this expression with tissue-specific QF drivers to induce the expression of the lethal gene at a specific point when adding QA to the medium at specific developmental stages. While using a combination of two systems, it is also possible to simultaneously induce lethality in one cell population while labeling or manipulating another, providing insights into cell-cell interactions during embryonic development (Potter et al., 2010). The Q system's non-antibiotic nature, efficacy in early developmental stages, compatibility with other expression systems, and fine-tuned control potential make it an excellent choice for developing advanced transgenic sexing strains.

2. Research objectives

The main goal of this study was to develop transgenic embryonic sexing strains (TESS) of *D. suzukii* carrying Q system components. For this purpose, the efficacy of endogenous pro-apoptotic genes and the promoters of embryonic genes were studied. The most effective genes and embryonic promoters were then cloned with the genetic elements of the Q system to be introduced into *D. suzukii*.

2.1 Isolation or evaluation of embryonic promoters and apoptotic genes for the development of TESS with Q system

To evaluate components of the Q system, the pro-apoptotic genes (*rpr, hid,* and *grim*) and embryonic genes (*nullo, slam, bnk* and *sry-α*) were isolated from *D. suzukii* US strain for the potential use in a Q system-based Transgenic embryonic sexing system (TESS). Secondly, the developmental expression profiles of isolated genes should be analyzed, and the efficacy of isolated genes and previously isolated embryonic promoters in S2 cells should be tested. The pro-apoptotic genes and embryonic promoters were integrated into RMCE plasmids for in-vitro gene expression analysis in *D. suzukii*.

2.2 Analysis of controlled expression of pro-apoptotic genes in S2 cells with Q system

The second goal of this thesis was to build Q system components with these promoters and apoptotic genes fused with fluorescent protein and integrate them into *piggyBac* vectors for Germline transformation. However, these vectors were tested in S2 cells before initiating germline transformation to ensure the functional performance of pro-apoptotic genes under fluorescent tags with Q system driving genetic elements QF, QUAS, QS, and quinic acid.

2.3 Generation of transgenic strains for conditional lethality in *D. suzukii* based on the Q system

After successful analysis of the Q system with pro-apoptotic genes and testing efficient embryonic promoters for TESS, *piggyBac* constructs carrying Q system driver, effector, and suppressor components were microinjected to obtain the transgenic lines.

3. Methods

3.1 *D. suzukii* rearing and sample collection

D. suzukii wild type USA strain was maintained at 25 °C and 60% humidity with a 12-h photoperiod. The samples were collected from *D. suzukii* food vials at desired time points.

3.1.1 Preparation of *Drosophila* food

The rearing food was prepared by adding 500 ml of tap water, 500 ml of VE water, 8 g agar, 108 g flour mix (10 g Soy flour, 18 g Brewer's yeast, and 80 g corn flour), 80 g malt (Malzin), 22 g molasses, 10 ml Nipagin and 5.25 ml of Propionic acid in 1 L food preparation. Food was cooked in a Mediaclave (INTEGRA bioscience Deutschland GmbH) under a program that cooks at 121 °C for 20 min and then cools until 50 °C. Nipagin and propionic acid were added to the food once it was cool. The food was dispensed to the vials and stored to cool down overnight. The food vials were plugged in the next day to avoid humidity in the vials.

Table 3.1: Recipe for *Drosophila suzukii* food preparation.

Material	1L	3L	5L	8L	10L
Tap Water	450 ml	1350 ml	2250 ml	3600 ml	4500 ml
VE Water	450 ml	1350 ml	2250 ml	3600 ml	4500 ml
Agar (Fadenagar)	8 g	24 g	40 g	64 g	80 g
Flour mix	108 g	324 g	540 g	864 g	1080 g
Malt (Malzin)	80 g	240 g	400 g	640 g	800 g
Molasses (Zuckerrübensirup)	22 g	66 g	110 g	176 g	220 g
Add Nipagin and propionic after 20 min of sterilization (food should be at 50 °C)					
Nipagin (Ethyl 4-Hydroxybenzoate):	2 g	6 g	10 g	16 g	20 g
Proprionic Acid	6.25 ml	18.75 ml	31.25 ml	50 ml	62.5 ml

Table 3.2: Flour Mix

Cooking volume	Soy Flour	Brewer's Yeast	Corn Flour
10 L	100 g	180 g	800 g
8 L	80 g	144 g	640 g

3.1.1.1 Preparation of *D. suzukii* food plates

The food prepared following protocol (3.1.1) was poured into the Petri dishes and cooled until solidified. A night before injection, flies were transferred into the cage covered with these plates.

3.1.1.2 Preparation of Grape juice Agar plates

Water was boiled for grape juice agar plates, and then agar was slowly added to the boiling water. The mixture was allowed to cook and stir for 5 minutes and removed from the heated stir plate. Once it was cooled down to 55 °C, grape juice was added. The mixture was then poured under the sterile bench into Petri dishes and allowed to cool until the liquid solidified.

Table 3.3: Materials used for grape juice plates for egg collection.

Materials	**Volume**
H_2O	70 ml
Grape juice	30 ml
Agar	1 g
Total volume	100 ml

3.2 tBLAST search for gene sequences

As a starting point, the coding sequences of the *D. melanogaster* genes *Dmrpr* (FBgn0011706), *Dmhid* (FBgn0003997), *Dmgrim* (FBgn0015946), *Dmsry-α* (FBgn0003510), *Dmnullo* (FBgn0004143), *Dmbnk* (FBgn0004389) and *Dmslam* (FBgn0043854) were obtained from FlyBase (http://flybase.org/) and used as tBLASTx search queries against SWDbase. Based on the top hits recovered for each blast search, primers were designed to amplify the full-length coding sequences of the three orthologs from *D. suzukii*.

3.2.1 Protein sequence alignments from other species for pro-apoptotic genes

Orthologues of RHG proteins from *D. grimshawi* (Dg), *D. hydei* (Dh), *D. willistoni* (Dw), *D. ficusphila* (Df), *D. biarmipes* (Db), *D. erecta* (Der), *D. melanogaster* (Dm), *D. serrata* (Dser), *Lucilia cuprina* (Lc) and *Musca domestica* (Md) were downloaded from NCBI as following:

Table 3.4: Accession numbers of the RHG Proteins, that were used to study phylogenetic analysis

Species	Proteins	Accession number
Drosophila grimshawi	DgRPR	XP_001985528.1
	DgHID	XP_001985533.1
	DgGRIM	XP_001996719.1
Drosophila hydei	DhRPR	XP_023170619.1
	DhHID	XP_023170594.1
	DhGRIM	XP_023170636.1
Drosophila willistoni	DwRPR	XP_023033239.1
	DwHID	XP_002067955.1
	DwGRIM	XP_002067945.1
Drosophila ficusphila	DfRPR	XP_017058909.1
	DfHID	XP_017059087.1
	DfGRIM	XP_017058891.1
Drosophila biarmipes	DbRPR	XP_016955290.1
	DbHID	XP_016956024.1
	DbGRIM	XP_016955275.1
Drosophila erecta	DerRPR	XP_001972845.1
	DerHID	XP_001972854.1
	DerGRIM	XP_001972846.1
Drosophila melanogaster	DmRPR	NP_524138.1
	DmHID	AAA79985.1
	DmGRIM	NP_524137.2
Drosophila serrata	DserRPR	XP_020801924.1
	DserHID	XP_020801905.1
	DserGRIM	XP_020801932.1
Lucilia cuprina	LcRPR	XP_023291721.1
	LcHID	XP_023305760.1
	LcGRIM	XP_023291726.1
Musca domestica	MdRPR	XP_005184304.1
	MdHID	XP_005180529.1
	MdGRIM	XP_019891253.1

3.2.2 Protein sequence alignments from other species for embryonic genes

Orthologues of embryonic genes from different species were downloaded from NCBI as following:

Table 3.5: *Drosophila suzukii* NULLO (DsNULLO) was aligned with orthologs from other *Drosophila* specie.

Specie names	Protein name	Accession number
Drosophila hydei	DhNULLO	XP_023161247.1
Drosophila kikkawai	DkNULLO	XP_017022675.1
Drosophila miranda	DmirNULLO	XP_017156218.1
Drosophila navojoa	DnNULLO	XP_017964581.1
Drosophila obscura	DoNULLO	XP_022210380.1
Drosophila willistoni	DwNULLO	XP_002071148.1
Drosophila biarmipes	DbNULLO	XP_016948821.1
Drosophila melanogaster	DmNULLO	NP_511067.3
Drosophila rhopaloa	DrNULLO	XP_016982709.1
Drosophila serrata	DserNULLO	XP_020805612.1

Table 3.6: *Drosophila suzukii* SERENDIPITY-α (DsSRY-α) was aligned with orthologs from

Specie names	Protein name	Accession number
Drosophila grimshawi	DgSRY-α	XP_001995347.1
Drosophila hydei	DhSRY-α	XP_023168708.1
Drosophila melanogaster	DmSRY-α	NP_524580.1
Drosophila virilis	DvSRY-α	XP_002056142.1

Table 3.7: *Drosophila suzukii* BOTTLENECK (DsBNK) was aligned with orthologs from

Specie names	Protein name	Accession number
Drosophila melanogaster	DmBNK	NP_524604.2
Drosophila miranda	DmirBNK	XP_017140437.1
Drosophila obscura	DoBNK	XP_022213695.1
Drosophila kikkawai	DkBNK	XP_017034088.1
Drosophila willistoni	DwBNK	XP_002072000.1
Drosophila hydei	DhBNK	XP_023168586
Drosophila virilis	DvBNK	XP_002055918.1
Drosophila grimshawi	DgBNK	EDV90810.1

Table 3.8: ***Drosophila suzukii*** **SLOW-AS-MOLASSES (DsSLAM) was aligned with orthologs from**

Specie names	**Protein name**	**Accession number**
Drosophila grimshawi	DgSLAM	XP_001988112.1
Drosophila pseudoobscura	DpSLAM	XP_001355861.2
Drosophila melanogaster	DmSLAM	NP_001285668.1
Drosophila busckii	DbuSLAM	ALC38995.1

3.3 Molecular Methods

3.3.1 Insect DNA Extraction

The genome of *D. suzukii* embryos, larvae, pupae, and adults was extracted using the Quick-DNA Tissue/Insect DNA Miniprep™ kit (Zymo Research). The samples were secured in the vials containing beads and smashed with a homogenizer (precellys® from Bertin Technologies). These tubes were microcentrifuges at 10,000 x *g* for 1 min. Then 400 µl supernatant was transferred to a Zymospin III-F filter in a collection tube and centrifuged at 8,000 x *g* for 1 min. 1200 µl of genomic lysis buffer was added to the filtrate in the collection tube. 800 µl of the mixture was then transferred to the Zymo-Spin IICR column in a collection tube and centrifuged at 10,000 x *g* for 1 min. The flowthrough was discarded, and 200 µl DNA pre-wash buffer was added to the column and centrifuged again for 1 min. 500 µl g-DNA Wash buffer was added to the column and centrifuged again for 1 min. The column was then transferred to the 1.5 ml microcentrifuge tube, and DNA was eluted in 50 µl of DNA elution buffer by centrifuging at 10,000 x *g* for 30 sec.

3.3.2 Insect RNA extraction

The Quick-RNA Tissue/Insect Microprep Kit (Zymo Research) was used to extract the RNA of *D. suzukii*. As stated above, samples were homogenized. After homogenization, samples were briefly centrifuged to get rid of foam. The supernatant was transferred to a microfuge tube. An equal volume of RNA lysis buffer was added and vortexed briefly. Then, 800 µl of the sample was transferred to the gDNA removal column and collection tube. After spinning it for 30 sec, the column was discarded. An equal volume of 95% ethanol was added to flow through and appropriately mixed by pipetting. This mixture was then transferred to an RNA purification column fitted with a collection tube. 500 µl of RNA priming buffer was added and spun for 30 sec. Flowthrough was discarded. To wash and bind RNA, 500 µl RNA wash buffer was added and spun for 30 sec. Another 500 µl of RNA wash buffer was added and spun for 2 min. The column was further transferred to an RNAase-free microfuge tube. 100 µl nuclease-free water was then used to elute RNA from the column. RNA was further stored at -80 °C for long-term storage.

3.3.3 cDNA synthesis

To synthesise the cDNA, 500 -1000 ng of total RNA per reaction was used using the QuantiTect® Reverse Transcription Kit (Qiagen). Residual gDNA was removed by a ‘gDNA-elimination step”, and cDNA was synthesized using an RT-primer mix at 42 °C for 30 min. The enzyme was later inactivated at 95 °C for 3 min. Both steps were performed according to the manufacturer’s protocol, and cDNA was stored at -20 °C until used as a PCR template. A dilution of the respective cDNA 1:10 was used for further experiments.

3.3.4 Polymerase Chain Reaction

Following the protocol (3.3.4.1), polymerase chain reactions were performed to isolate the pro-apoptotic and embryonic genes and clone them into pIE expression plasmids or *piggyBac* transformation plasmids. All PCR reactions for in vitro DNA amplification are performed using the Bio-Rad thermal cycler C1000 (Germany).

3.3.4.1 Phusion Flash Polymerase Master Mix

Components	20 µl	50 µl	Final conc.
2x Phusion flash PCR master mix	10 µl	25 µl	1x
Primer F	1 µl	2 µl	0.5 µM
Primer R	1 µl	2 µl	
Template DNA	1 µl	2 µl	
H_2O	7 µl	19 µl	

Cycling conditions

Cycling	3 step protocol		
	Temp	**Time**	**Cycles**
Initial denaturation	98 °C	10 sec	1
Denaturation	98 °C	1 sec	30
Annealing	__ °C	5 sec	
Extension	72 °C	15 sec /kb	
Final extension	72 °C	1 min	1
Hold	4 °C	∞	

3.3.4.2 PCR Platinum Taq DNA Polymerase (Invitrogen)

Components	Concentration	25 µl	50 µl
10x PCR Buffer-Mg	1 x	2.5 µl	5 µl
dNTPs Mix (2mM each)	0.2 mM	1 µl	2 µl
50 mM $MgCl_2$		0.5 µl	1 µl
Primer F	10 µM	1 µl	2 µl
Primer R	10 µM	1 µl	2 µl
Platinum Taq	5 U/ µl	0.2 µl	0.4 µl
Template		1 µl	2 µl
H_2O		17.8 µl	35.6 µl

Cycling conditions

Cycling	3 step protocol		
	Temp	**Time**	**Cycles**
Initial denaturation	95 °C	2 min	
Denaturation	95 °C	30 sec	35
Annealing	__ °C	30 sec	
Extension	72 °C	1 min /kb	
Final extension	72 °C	5 min	1
Hold	4 °C	∞	

3.3.4.3 PCR Q5 high fidelity Polymerase

Components	Concentration	25 µl	50 µl
5X Q5 Reaction Buffer	1x	5 µl	10 µl
dNTPs Mix	10 mM	0.5 µl	1 µl
Primer F	10 µM	1.25 µl	2.5 µl
Primer R	10 µM	1.25 µl	2.5 µl
High GC enhancer	1x	0.25 µl	0.5 µl
Q5 DNA polymerase	0.02 U/ µl	0.25 µl	0.5 µl
Template	<100 ng	0.5 µl	1 µl
H_2O		16.25 µl	32.5 µl

Cycling conditions

Step	Temp	Time	
Initial denaturation	98 °C	30 sec	
	98 °C	5-10 sec	30 cycles
	50-72 °C	10-30 sec	
	72 °C	20-30 s/kb	
Final extension	72 °C	2 min	
	4 °C	∞	

3.3.5 Purification of PCR Products and double-digested DNA Fragments

The excised gel bands containing PCR products or double-digested DNA fragments were transferred into 1.5 ml Eppendorf tubes. As the supplier recommended, the purification was performed with the ZymoResearch DNA purification kit or ZymoResearch Gel purification kit™. The DNA was eluted into 10-20 µl water.

3.3.6 Agarose gel electrophoresis

Agarose gel electrophoresis of nucleic acids was performed to analyze polymerase chain reactions (PCR). Agarose gels contained 1 % (w/v) agarose and 0.4 µg/ml SYBR green in 1x TAE buffer. Samples were supplemented with DNA loading dye. The nucleic acids were separated horizontally at 100-120 V in 1x TAE buffer for 30-40 min. VersaDoc, Quantity One Software (4.6.9) was used to visualize PCR fragments using the filter setting on SYBR Green.

3.3.7 TOPO ligation

TOPO TA cloning was performed to clone amplified PCR products using the TOPO TA cloning kit™ (Thermo Fischer). PCR product 1 µl was inserted into 1 µl of TOPO vector, 1 µl salt solution, and 2 µl H_2O was added to the reaction was incubated at 25 °C for one hour.

3.3.8 Double Digestion

Double digestion was also carried out for sticky ends as well as blunt end ligation of elongated PCR products and the plasmids. The digestion was performed as the enzyme supplier recommended with 1 µl enzyme per 1 µg DNA. An agarose gel electrophoresis was performed to separate the double-digested DNA. The DNA fragments in the size of interest were excised and purified.

3.3.9 Ligation

Ligation was performed to create a circular plasmid containing the gene of interest. The ligation solution was 5 µl in total volume and contained three ratios of double-digested DNA, one ratio of double-digested plasmid DNA, 1 µl T4 DNA ligase, and 1 µl T4 DNA ligase buffer. The solution was incubated at room temperature for one to two hours and then transformed into *E. coli* cells.

3.3.10 Restriction enzyme analysis

Restriction enzyme-based analysis was performed to ensure that ligation and transformation were successful. The plasmid was checked using a restriction enzyme, which cuts inside the new ligated gene fragment and creates distinct fragment sizes. The digestion was performed as recommended by the enzyme supplier (NEB, Thermo Fisher Scientific). The digestion results were checked by agarose gel electrophoresis, and the fragment sizes were compared to the calculated sizes.

3.3.11 DNA transformation

An aliquot of competent *E. coli* cells was gently thawed on ice for 10 min. Then, 2 µl of ligation mixture, or 200 ng plasmid DNA, was added to the cells and incubated on ice for 30 min. The cells were then subjected to a heat shock at 42 °C for 45 sec and placed directly back on ice for 3 min. 200 µl SOC medium (room temperature) was added to the cells containing no antibiotics. The cells were incubated for 60 min at 37 °C at 220 rpm. Then, the cells were plated on LB agar plates containing the appropriate antibiotic. The plates were incubated at 37 °C overnight.

3.3.12 Colony PCR

Colony PCR was performed to determine the presence of DNA insert. For this, primers (Appendix 3) of the corresponding plasmid were used to run PCR using the following protocol.

3.3.12.1 Dream Taq Polymerase

Components	**Concentration**	**15 µl**
Dream Buffer	10 x	1.5 µl
dNTPs	2 mM	1.5 µl
Primer F	10 µM	0.25 µl
Primer R	10 µM	0.25 µl
Taq polymerase	5 U/ µl	0.15 µl
Template	colony	1 µl
H_2O		11.45 µl

Cycling	**3 step protocol**		
	Temp	Time	Cycles
Initial denaturation	95 °C	2 min	
Denaturation	95 °C	30 sec	34
Annealing	__ °C	30 sec	
Extension	72 °C	1 min /kb*	
Final extension	72 °C	5 min	1
Hold	4 °C	∞	

*The extension time is 1 min for PCR products up to 2 kb. For longer products, it should be prolonged by 1 min/kb.

3.3.13 DNA isolation

A positive colony was inoculated into 4 ml of LB media at 37 °C overnight to isolate DNA for further cloning, sequencing, or Maxiprep for injections. This 4 ml culture was reinoculated into 300 ml LB media overnight for maxiprep. QIAGEN® Plasmid Mini and Maxi Kits were used.

3.3.14 Determination of DNA concentration

The DNA concentration of plasmids, purified PCR products and double-digested DNA fragments was determined photometrically. For the quantification, 1 µl reference (H_2O or elution buffer, depending on the eluent of the sample) and 2 µl sample were pipetted on a plate reader (BioTek). The absorbance was measured at 260 nm. The purity was determined using the ratio of A260 nm/A280 nm.

3.3.15 DNA /RNA precipitation

DNA was precipitated a few times to improve DNA concentration. In 100 µl DNA, 10 µl salt solution (NaAc) and 250 µl 100% ethanol were added. The solution was incubated at -20 °C overnight for at least 1 hour or better. It was then centrifuged at 13000 rpm for 10 min at 4 °C. Later; it was rinsed with Rnase-free 500 µl of 70% ethanol and air dried. The pellet was then dissolved in 20 µl of RNAse-free water.

3.4 Sequencing

The plasmid constructs were sequenced by sending samples to Eurofins (https://eurofinsgenomics.eu/). The results were analyzed using Geneious Prime software. A maximum of 100 ng plasmid concentration was sent for sequencing.

3.5 Construction of PCR4-TOPO plasmids

3.5.1 Embryonic genes

The Platinum Taq DNA polymerase™ (Invitrogen) (Table 3.3.4.2) was used to amplify *Dsbnk*, *Dsslam, Dssryα* and *Dsnullo* genes. In 25 µl reaction mix, annealing at 52 °C for 30 sec and extension at 72 °C for 1 min (*Dsnullo* 642 bp and *Dsbnk* 903 bp) or 2 min (*Dsslam* 3414 bp) with primers P1370/P1371 (refer to appendix 3 for all primer sequences), P1374/P1375 and P1372/P1373 respectively. *Dssryα* (1593 bp) was amplified by using Phusion Flash High-Fidelity DNA Polymerase™ (Thermo Fisher Scientific), annealing at 52 °C for 5 sec and extension at 72 °C for 30 sec with primers P1376/P1377. The PCR products were transferred to the vector pCR4 by TOPO cloning.

The promoters to be used for this project were isolated previously by a colleague (Jonas Schwirz).

3.5.2 Pro-apoptotic genes

The *Dsrpr* and *Dsgrim* were also isolated previously by a colleague (Jonas Schwirz).

Dshid was also amplified with Phusion Flash High-Fidelity PCR Master mix™ (Thermo Fisher Scientific, USA) with annealing at 55 °C for 5 sec and extension at 72 °C for 30 sec. The low abundance of *Dshid* mRNA was compensated by adopting a two-step amplification strategy. Exons 1 and 2 were amplified using primers P81/P41, and exons 3 and 4 were amplified using primers P165/P42. Later, these exons were combined by reamplifying them with the external primer pair P41/P42. The 1281 bp *Dshid* product was transferred to the vector pCR4 by TOPO cloning, using primers P41/P42, resulting in construct V381.

To prevent *Dshid* inactivation by phosphorylation (Bergmann et al., 1998; Schetelig et al., 2011), four potential MAPK phosphorylation sites were identified in the *Dshid* sequence, and the acceptor residues were replaced with Alanine to generate *Dshid*^{Ala4}. This constitutively active *Dshid* gene was synthesized by Eurofins (Germany), resulting in construct V45. The integrity of all vectors was confirmed by restriction digestion and sequencing using primers M13/M14, and sequences were analyzed using the Geneious Prime software.

3.6 Construction of pIE expression plasmids for pro-apoptotic genes

The expression vectors for *Dsrpr* (V42) and *Dsgrim* (V44) were constructed by Jonas Schwirz earlier. The *Dshid* sequence was reamplified from vector V381 using primers P1654/P1655 containing restriction sites for SacII and NotI, respectively. The PCR product was transferred to vector *pIE4*, resulting in expression vector V392. To prepare vector (V93) *pIE4Dshid*^{Ala4}, (V45) *pEX*-K4-*Dshid*^{Ala4} was digested with SacII and NotI, and the insert was transferred to *pIE4* plasmid, prepared using the same enzymes.

3.7 Construction of 2A peptide plasmids for cell culture

The 2A peptide plasmids were constructed by amplifying the *Dsrpr* sequence from construct (V12) using primers P213/P150, the *Dsgrim* sequence from construct (V44) using primers P313/P162, and the *Dshid*^{Ala4} sequence from construct (V45) using primers P314/P315. Pairs of genes were transferred to construct (V142) using the restriction enzymes ApaI and NotI to join them via the DrosCV2A peptide or to construct (V145) using the same restriction enzymes to join them via the TaV2A peptide (Schwirz et al. 2020).

3.8 Construction of RMCE plasmids

The RMCE plasmids were cloned by amplifying *Dsrpr*, *DshidAla*^{4}, and *Dsgrim* from the plasmids (V163) pIE4_*Dsrpr*_DrosCV-2A_*Dsrpr*_SV40, (V165) pIE4_*Dsrpr*_DrosCV-2A_*Dsrpr*_SV40, and (V164) pIE4_*Dsrpr*_DrosCV-2A_*Dsgrim*_SV40 by using primers P1068/P1071, P1070/P1071, and P1069/P1071 respectively. PCR fragments were then inserted into (AH448) pSL_loxN-3xP3-PUbDsRed-lox2272 (Schetelig et al., 2018) by SmaI and SalI restriction sites to generate (V388) pSL_loxN-3xP3-*Dsrpr*_SV40-PUbDsRed-lox2272, (V350) pSL_loxN-3xP3-*Dshid*^{Ala4}_SV40-PUbDsRed-lox2272, and (V337) pSL_loxN-3xP3-*Dsgrim*_SV40-PUbDsRed-lox2272, respectively.

3.9 Construction of *piggyBac* transformation plasmids for cellularization genes

The upstream flanking sequences of the cellularization genes in both *D. melanogaster* and *D. suzukii* were acquired from FlyBase and SWDbase, respectively. Subsequently, these sequences were subjected to alignment using the Geneious software, and a search was conducted to identify conserved motifs, including TAGteam motifs (Bosch et al., 2006) as well as the TATA box. High-molecular-weight genomic DNA was extracted from adult *D. suzukii* specimens using DNAzol Reagent from Thermo Fisher Scientific. Promoter fragments were then generated through PCR amplification of the genomic DNA. After undergoing confirmation of their nucleotide sequences, these promoter fragments were further amplified and utilized to replace the *D. melanogaster* polyubiquitin (DmPUb) promoter within the *piggyBac* vector denoted as pBacXLII-attP-PUbAmCyan_DmPUb_DsRed-NLS-SV40 this replacement was executed at the Bsu36I and MluI restriction sites, resulting in the creation of the test vectors designated as V205 to V208.

3.10 Construction of *piggyBac* plasmids for Q system

3.10.1 Cloning of transcriptional activators (QF)

Two plasmids (V277) pCaSpeR-act5cB-QF#7-ActinpolyA and (V278) pCaSpeR4-tubulin-QF#7m1 were purchased from Addgene (Riabinina et al. 2015). QF was further cloned into *piggyBac* plasmids for germline transformation of *D. suzukii.* ActinpolyA-ApaI-AscI (972 bp) was amplified using P829/P811 from pCaSpeR-act5cB-QF#7-ActinpolyA (V277). ActinpolyA was replaced in V277 using restriction enzymes NotI and PstI, adding ApaI-AscI restriction sites to the 3’ ActinpolyA (V305 M3814). The act5cB-QF#7-ActinpolyA-ApaI-AscI fragment was excised and inserted into (V92) pBacXLII-attP-PUbEGFP-SV40 using BglII and AscI to obtain an unconditional constitutive driver: (V291) pBacXLII-attP_PUbEGFP_act5cB-QF#7-actinpolyA.

For another driver (V321) pBacXLII-attP_PUbEGFP_act5cB-QF#7-actinpolyA, NotI-XbaI-SV40-AscI-PstI (239 bp) was amplified using P830/P831 from V92, then replace the ActinpolyA in the pCaSpeR-act5cB-QF#7-ActinpolyA using NotI and PstI, to obtain V306_pCaSpeR-act5cB-QF#7-SV40-AscI. The act5cB-QF#7-ActinpolyA-ApaI-AscI fragment was excised and inserted into (V92) pBacXLII-attP-PUbEGFP-SV40 using BglII and AscI.

Driver (V406), pBacXIII-attP_Pub-EGFP_tubulin-QF7m1-hsp70, was generated by amplifying tubulin-QF7m1-hsp70 from the plasmid V278 using primers P1828/P1826. MluI and ApaI restriction enzymes were used to digest the PCR product, and V92 was used to ligate the product into the plasmid.

Driver (V407) pBacXLII-attP_Pub-EGFP-Dmhsp70-QF7m1-hsp70 was generated by amplifying Dmhsp70 (412 bp) with Phusion flash polymerase (Table 3.3.4.1), was amplified from plasmid V265 by using P1775/P1776 and inserted into V278 with enzymes EcoRI and KpnI, this intermediate construct was stored in a glycerol stock as G5795. Dmhsp70-QF7#m1 cassette was amplified from this intermediate plasmid with primers P1825/P1826 and digested with enzymes ApaI to insert into V291.

Driver (V408) pBacXIII-attP_Pub-EGFP_Pdsbnk-QF7m1-hsp70, *Dsbnk* (1607 bp) was generated by Phusion flash polymerase (Table 3.3.4.1) using primers P1773/1774 and inserted into V278 by EcoRI and KpnI; the intermediate was stored for further cloning in glycerol stock M5793.

3.10.2 Cloning of effector (pQUAST)

To clone the effector plasmid (V288) pBacXIII-attP-PUbDsRedT3-QUAST-Dmh70-AmCyan-SV40, the cassette EcoRI-Amcyan-SV40-SacII-AscI (957 bp) was amplified using P835/P832 from (V94) p3xP3_FRT_AmCyan_att and inserted into pQUAST (V280) using EcoRI and SacII, to create an intermediate plasmid. Then QUAST-Dmh70-AmCyan-SV40 fragment was excised and inserted into V85 using MluI and AscI to obtain a fluorescent effector.

The lethality effectors were constructed as follows.

1. (V289) pBacXIII-attP-PUbDsRedT3-QUAST-Dmh70- Dshid^{Ala4}-SV40 was cloned by amplifying Dshid^{Ala4}-SV40 (1533bp), from V158 (M2575) *pIE4-Dsrpr*-Dshid^{Ala4}-SV40 using P834 /P832 and inserted into pQUAST (V280) using EcoRI and SacII.
2. (V290) pBacXIII-attP_PUbDsRedT3_QUAST-Dmh70-Dshid^{Ala4}-*Cctra*-SV40 (a female-specific lethality effector) was created by amplifying Dshid-*Cctra* using P885 /P832 from V251 and inserting into pQUAST using BglII and KpnI to obtain an intermediate plasmid. Then Dmh70-*Cctra*-Dshid^{Ala4}-SV40 was moved into V85 using MluI and AscI.
3. (V388) pBacXIII-attP_PUbDsRedT3_QUAST-Dmh70-*Dsrpr*-SV40 was developed by amplifying *Dsrpr* (244 bp) from V160 (M2571) with Primers P1501/P1502, that gives three additional restriction sites around *Dsrpr* for future cloning's. The PCR product was inserted into V309 (M3779) with enzymes EcoRI and NotI. The intermediate plasmid and V85 were further restricted and ligated with MluI and AscI to obtain the final construct.
4. For (V402) pBacXLII_attP-PUbDsRedT3_QUAST-Dmhsp70-Dsgrim-SV40, *Dsgrim* (380 bp) was amplified with primers P1779/1780 from (V159) *pIE4_Dsrpr*-DrosCV2A-*Dsgrim*_SV40. The fragment was then inserted into V388 with restriction enzymes AgeI and NheI.
5. For (V389), the *Dsrpr*_DrosCV-2A_*Dsrpr* (473 bp) was amplified from V163_*pIE4_Dsrpr*_DrosCV-2A_*Dsrpr*_SV40 with P1781/P1783, The fragment was then inserted into V388 with restriction enzymes AgeI and NheI.
6. For (V400) pBacXLII_attP-PUbDsRedT3-QUAST-Dmhsp70-Dsrpr-DrosCV2A-Dshidala4-SV40, *Dsrpr*_DrosCV-2A_*Dshid*^{Ala4} (1546 bp) was amplified from V165_ pIE4_*Dsrpr*_DrosCV-2A_*Dshid*^{Ala4}_SV40 by using primers P1781/P1782. The fragment was then inserted into V388 with restriction enzymes AgeI and NheI.
7. For (V401), *Dsrpr*_DrosCV-2A_*Dsgrim* (641 bp) was amplified with primers P1781/P1780 from V164_pIE4_*Dsrpr*_DrosCV-2A_*Dsgrim*_SV40; the fragment was then inserted into V388 with restriction enzymes AgeI and NheI.

3.10.3 Cloning of suppressor (QS)

Promoter tubP (2774 bp) was amplified from (V278) pCaSpeR4-tubulin-QF#7m1 with primers P1075/P873, then replaced the tubP in tubP-QS (V279) using EcoRI and KpnI restriction sites, which give an additional Bsu36I site to the 5' of tubP. Then, the Bsu36I-tubP-QS-SV40 fragment was excised and inserted into V85 using Bsu36I and ApaI to obtain a constitutive repressor (V295) pBacXIII-attP_PUbAmCyan_ptubP-QS-SV40.

For (V296) pBacXIII-attP_PUbAmCyan_PUb-QS-SV40, tubP was amplified with primers P1075/P874 from V279_tubP-QS, then replaced the tubP in tubP-QS using EcoRI and KpnI, which give an additional MluI site to the 3' of tubP. Then, the QS-SV40 was excised and inserted into V195 using MluI and ApaI.

A repressor (V354) pBacXIII-attP-PUbDsRedT3-ptubP-QS-SV40 (M4715) was cloned by digesting V85 (M2155) for DsRed and V295 to take out AmCyan from it and ligated together.

3.10.4 Cloning of 2 in 1- Driver-2A-Suppressor (QF-2A-QS)

TaV-2A (109 bp) was amplified from V172_pIE4-*Dsrpr*-TaV-2A-*Dsrpr*-SV40 using P827/P828, and QS (2676 bp) was amplified from tubP-QS (V279, M3614) using V825/V826, then insert into pCaSpeR- ActinP-QF#7m-actinpolyA (V305, M3615) or pCaSpeR-Dsb2Tubulin-QF#7-SV40 by Gibson assembled using BamHI, to obtain pActinP-QS-TaV-2A-QF#7-actinpolyA (V390)

3.11 Quantitative Real-Time PCR

All primers used for qPCR were designed using Geneious Prime software. The amplicon selected was ranged between 140 and 180 bp. To determine the efficiency of qPCR primers, the standard curves were generated by using a dilution series of the cDNA as a template. The primers with above 95% efficiency were selected for qPCR. Standard curve analyses in qPCR assays was used to set the reaction threshold as well that would determine the Ct values for each run. The relative quantification was evaluated using the Ct-method according to the $2^{-\Delta\Delta Ct}$ formula .

$$\Delta\, Ct = Ct\ target\ gene - Ct\ endogenous\ control\ (reference)$$

$$\Delta\,\Delta\, Ct = Ct\ sample - Ct\ calibrator$$

$$n - fold\ expression = 2 - \Delta\,\Delta\, Ct$$

In the given formula, 'sample' is defined as cDNA from dsRNA of *D. suzukiii* samples used.

All qPCRs are performed using "SYBR Green Super Mix" accordingly.

Component	**Concentration**	**volume**
cDNA-template		2 µl
SYBR Green Mix		5 µl
F primer	500 nM	0.5 µl
R primer	500 nM	0.5 µl
PCR-H_2O		2 µl

The RT-PCR was performed using platinum Taq polymerase as described above., annealing at 55 °C (*His3*, *TBP*, *nullo*, *bnk* and *slam*) or 60 °C (*Dshid, Dsrpr, Dsgrim, AK, GADPH, α-Tub*, *sry-α*) in the cycle conditions described below.

Cycling	**3 step protocol**		
	Temp	Time	Cycles
Initial denaturation	94 °C	4 min	
Denaturation	94 °C	30 sec	35
Annealing	__ °C	30 sec	
Extension	72 °C	1 min	
Final extension	72 °C	4 min	1
Hold	4 °C	∞	

3.12 *Drosophila* S2 cell culture

3.12.1 Preparing Schneider media

Transient cell transfection experiments were performed multiple times with *D. melanogaster* Schneider 2 (S2) cells (Schneider, 1972). Cells were grown in Schneider's medium at 25 °C with 10% heat-inactivated fetal bovine serum (Hi-FBS) and 1% penicillin/streptomycin in closed-capped flasks without CO_2.

3.12.2 Thawing and expanding cells

The S2 cells vial was taken from liquid nitrogen and placed in a water bath at 25 ^{o}C. Once cells were thawed, 5 ml fresh S2 media was added to the cells, and cells were resuspended gently. The suspension was transferred to a 15 ml falcon and centrifuged at 1000 rpm for 3 min to remove DMSO. After that, the supernatant was discarded, and cells were resuspended into 5 ml complete FBS media and transferred to 25 ml T-25 Flask (CELLSTAR) for adhesive cells. Viable cells were counted by trypan blue staining (section 3.12.3) using cell counter TC20™ (Bio-Rad). Cells were passaged into new flasks with fresh media every 2-3 days to keep cell density less than 6 x 10^6 cells/ml.

3.12.3 Trypan blue staining

Trypan blue staining was used to measure cell viability in the TC20 cell counter. 5 µl of trypan blue (Thermo Fischer) was mixed with 5 µl cell suspension and poured into cell counter slides TC20.

3.12.4 Transfection

On the day of transfection, cell viability was calculated. Cells were transfected only if cell viability was more than 95%. Cells were further diluted to reach a maximum concentration of 1 x 10^6 cells/ml. The tissue culture slides (Sarstedt, Germany) were placed into the wells of 24-well plates. 500-1000 µL of cell suspension (in Complete Schneider media) were seeded to the wells. Cells were allowed to settle down for 3 h. The transfection mix was prepared for transfection as follows:

Table 3.9: Standard protocol for transfection for 1µg Plasmid.

Components	**15 µl**
Plasmid DNA	1 µg
Xfect	0.3 µl
Xfectin buffer (stored at -80°C or 4 °C)	Upto 30 µl
+270 µl Schneider media without FBS	

The transfection mix was incubated for 10 min at room temperature. Cell culture media was replaced with the transfection mix in the 24-well plate. Transfection was incubated for 4 h and media was replaced after 4 h with complete schneider media. Cells were incubated to grow for 16 h at 25 °C.

3.12.5 Fix cells

A 24-well plate containing transfected cells were washed with 1x PBS. After washing, cells were covered with 4% paraformaldehyde (2 g paraformaldehyde powder in 50 ml 1 x PBS; 1M NaOH to adjust the pH) solution and incubated for 15 min. Paraformaldehyde was removed, and cells were washed twice with 1x PBS.

1x PBS

For 1L

Sodium chloride (NaCl)	8.0 g
Potasium chloride (KCl)	0.2 g
Disodiumydrogenphosphat (Na_2HPO_4)	1.42 g

3.12.6 Transfection of cells for pro-apoptotic and cellularization gene constructs

For analysis of cell viability through fluorescent cell count, 0.5 mg *pIE4-EGFP* plasmid was co-transfected with one of the *pIE* expression plasmids (0.5 mg) or with *pIE4-DsRED* control vector (0.5 mg) to visualize the successfully transfected cells. *Drosophila* S2 cells were transfected with constructs V205–V208 (1mg each) for the embryonic promoter's efficiency analysis. For pro-apoptotic genes, the cells were co-transfected similarly with constructs V93, V42, V44, V392, V163, V164, V165, V172, V173 and V174.

3.12.7 Transfection of cells with Q system carrying constructs

To examine the Q system in S2 cells, up to three constructs were co-transfected with QF, QUAS, and QS. In some wells, QS was co-transfected as a single, triple, or five copy. The needed concentration of plasmids was calculated using this online tool (www.molbiotools.com/dnacalculator). To make a total DNA concertation not more than 2.2 mg/reaction, 0.4 µg QUAS was transfected with 0.25 mg QF and 0.29 mg 1x QS, 0.87 mg 3x QS, and 1458 mg 5x QS.

3.12.8 Freezing cells

Drosophila S2 cells were borrowed from THM and frozen for future use using the following method. Cells were cultures in Schneider medium and passed 3-4 times in 25 ml flask to get a stable line with confluency up to $1x10^7$ cell/ml (confluent). Cells were resuspended by gently pipetting up and down and transferred to a 15 ml falcon tube. Cells were centrifuged at 1000 rpm, and the supernatant was discarded. Cells were resuspended in 10 ml freezing media and aliquoted in 5x 2 ml tubes with screw-lid.

Tubes were incubated in an isopropanol freezing container at -80 overnight. The next day, the cells were transferred to the liquid nitrogen tank.

Freezing Media Preparation

For 100ml freezing media:

Hi-FBS from Gibco (Life Technologies), (Heat Inactivated)	50 ml
DMSO (10 %)	10 ml
Schneider-media	40 ml

3.12.9 Cell Imaging and counting

Transfected cells were imaged for morphology using an inverted microscope (Leica DM IL LED, Leica Microsystems, Wetzlar, Germany). Furthermore, the Leica M205 fluorescent microscope was equipped with DsRed, EGFP, YFP, and CFP filters. The exposure time and magnification were kept the same for each replicate. Using the automated cell count function, fluorescent cells were counted in Image J (Fiji). For that, raw images were converted to an 8-bit standardized format; a threshold of 30 was set for each image, and inverted and watershed were applied to separate confluent cells (Fig. 5).

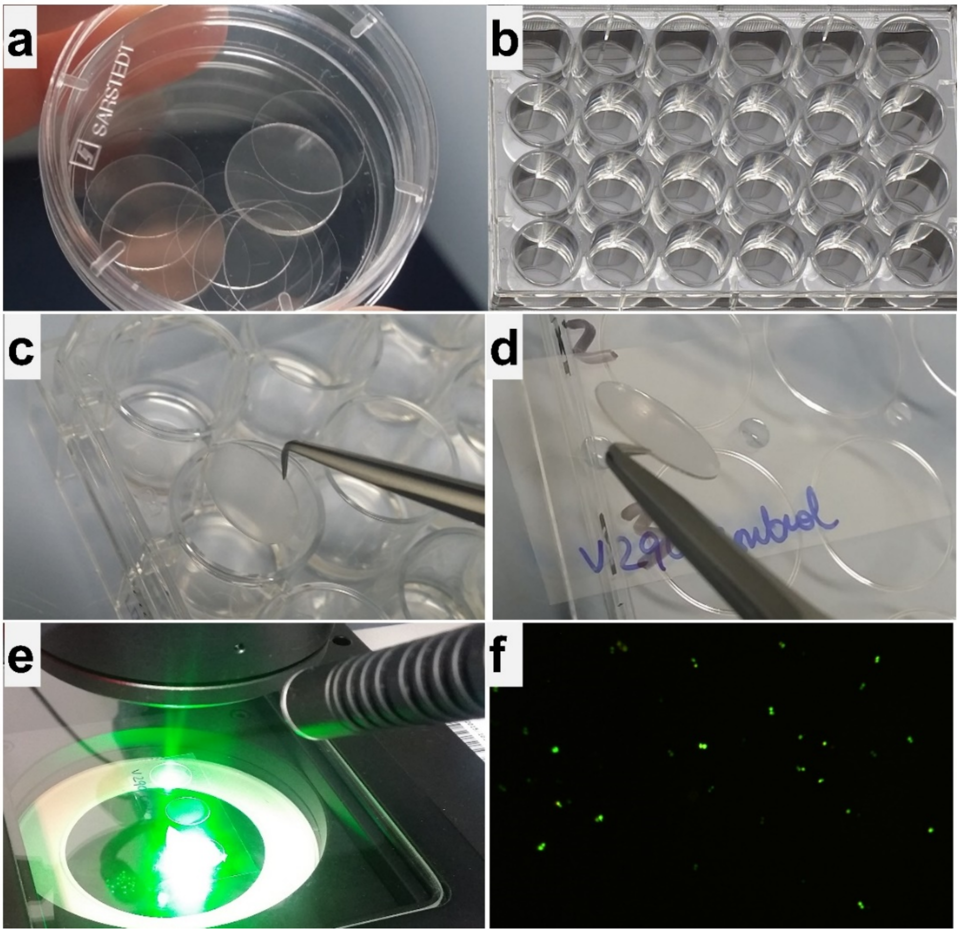

Figure 5: Cell transfection procedure.
(a) The tissue culture (TC) slides were placed in the wells of (b) 24-well plate, and S2 cells were seeded into each well. (c) after transfection, the TC slides were carefully removed from the wells with the help of sharp-headed forceps. (d) The TC slide was placed onto the coverslip over a drop of TE buffer to prevent the cells from drying quickly. (e) the coverslip was then placed under the fluorescent microscope. Fluorescent cell images were then captured at 100x under the microscope.

3.13 Embryonic microinjection protocol

3.13.1 Injection Mix

To generate transgenic strains for *D. suzukii*, *piggyBac* plasmids were used along with helper plasmids: either (AH286) phs-Dmhsp70-pBactransposase-pBac3UTR, its mRNA, or a hyper helper (V315) pSLaf_hsp70P-iPB7-hs3UTR_af (insect codon-optimized version). Maxi prepped EF plasmids were prepared for injections. To minimize the toxicity of unwanted compounds in the maxiprep, the plasmids were filtered before creating the injection mix.

For the filtration process, 10 µl salt solution (NaAc or NH_3Ac) and 250 µl of 100% ethanol were added to 100 µl DNA. The mixture was gently inverted a few times and incubated at -20°C for 1 hour. After incubation, the tubes were centrifuged at 14000 rpm for 10-15 min at 4 °C. The resulting pallet was washed with 500 µl of 70% ethanol and centrifuged at 14000 rpm for 10 min at 4 °C. The pellet was airdried and then dissolved in 50 µl of EF-H_2O. The DNA concertation was measured to prepare the injection mix.

Each injection mix, with a total volume of 20 µl, contained 500-700 ng/µl of plasmid with 200-300 ng/ µl of helped plasmid (DNA or mRNA), 2 µl HOBO injection buffer, and ddH_2O.

3.13.2 mRNA of *piggyBac* transposase AH286

The piggyBac transposase was amplified using primers P1269/P1270 and the template plasmid AH286. A PCR reaction was performed using Q5 polymerase (see table 3.3.4.3) at an annealing temperature of 51 °C and 60 sec of extension time. The PCR product was verified on 1 % agarose gel and subsequently purified using a zymogen PCR purification kit.

An *in vitro* transcription (IVT) reaction was performed for capping and tailing with NEB HiScribe T7 ARCA kit. For capping, 720 ng of purified PCR product (4 µl) was combined with 2x ARCA mix (10 µl), T7 pol mix (2 µl), and ddH2O to a final volume of 20 µl. The reaction mix was incubated at 37 °C for 30 min, then the DNAse I (2 µl) was added following the incubation for another 15 min at 37 °C. 1 µl of this reaction was taken out to run on a gel for integrity check.

For tailing, a 20 µl of IVT reaction was prepared with 5 µl of Poly (A) polymerase and 10 µl ddH_2O. The reaction mixture was incubated at 37 °C for 30 min. 2 µl of this reaction was taken out to run on gel for integrity check. In the end, mRNA cleanup was carried out using the MEGAclear kit ambion by life technologies protocol.

3.13.3 Preparation of flies

D. suzukii (WT) strain were collected on the first day of emergence and maintained on standard *Drosophila* food for 2 to 3 weeks prior to injection. The night before injection, flies were transferred to a fly cage (Fig. 6a) and incubated in a rearing incubator overnight. The cages were covered with regular *Drosophila* food carrying petri dishes. On the day of injection, the food plates were replaced with grape juice agar plates for egg collection. Eggs were collected on grape juice agar plates every 30 to 40 min.

3.13.4 Needles

High-quality needles are one of the most important things for a smooth injection. 1.0 mm OD borosilicate capillaries were pulled by Sutter instrument P-2000 from Science Products GmbH at heat: 730, FIL: 4, DEL:125, and PUL:130. The needle was mounted onto the needle holder of the injection microscope, and the injection pressure and constant pressure were adjusted to suitable conditions (Pi: 800 Pc: 500 in most cases). A needle was filled with a 2 µl injection mix, and the tip was broken with the help of sharp forceps under the injection microscope.

3.13.5 Embryo collection and dechorionizing

Under a dissection microscope, eggs were collected from grape juice agar plates with forceps under the microscope on the collection slides (coverslips with double-sided tape). To ensure that embryo injections to be carried out before cellularization initiates in the embryos and to maximize the chances of injected construct reaching the germline cells, embryos were collected every 30-40 mins. The temperature of the injection room was maintained at 18-20 °C to slow down embryo development. Embryos were carefully collected from the grape juice agar plates onto a collection slide with sticky tape using sharp forceps.

The embryos were dechorionated by sliding them on the collection slides from left to right using forceps. The chorion was removed as the embryos adhered to the sticky tape. Then, the embryos were carefully transferred onto rowing slides (a slide with a stripe of double-sided tape) with their posterior ends aligned in the same direction. The embryos were allowed to dry for a maximum of 3-4 minutes before being covered with Halocarbon oil 700 (Sigma Life Sciences) to avoid them drying completely.

3.13.6 Embryo injections

The slide with rowed embryos was placed under the injection microscope (Olympus SZX16) so that the posterior side of the embryos could face the injection needle. The needle was positioned right in the center, ensuring the tip of the needle and the posterior side of the embryos were visible under the 20x objective. To inject the injection mix, the needle was carefully inserted inside the embryo with the help of a movable desk of the microscope. Once the needle was in the posterior end of the embryo, the injection mix was dispensed by pressing the pressure pedal once. It was ensured that the injection drop was not too big to burst the embryo. Then, the needle was withdrawn and aligned against the next embryo in the row. In this process, embryos that had already formed pole cells were skipped and destroyed on the slide.

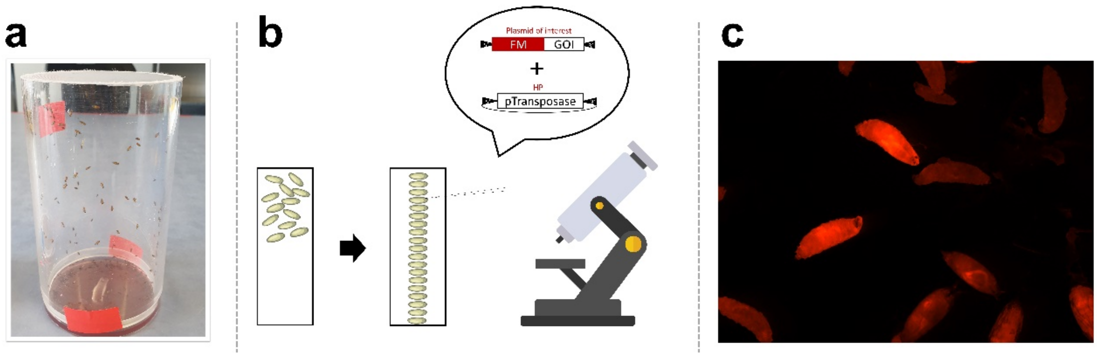

Figure 6*:* **Generation of transgenic strains of *D. suzukii.***
(a) *D. suzukii*, 3-4 weeks old, were collected in the cage, covered with a grape juice agar plate. The flies lay eggs on the plate. (b) the eggs are collected from the agar plates on the double-tapped slides; the embryos are dechorionized, then aligned on another slide, and injected under the microscope with a gene of interest and helper constructs. (c) The transient larvae are screened under the fluorescent microscope after 48 hours of injections.

3.13.7 Post-injection embryo handling

After injections, coverslips with injected embryos were placed in a petri dish with wet black filter paper. These Petri dishes were then transferred to an oxygen chamber containing wet tissue papers to maintain a humid atmosphere and incubated at 25 °C in a rearing incubator. After 16-18 and 48 h of injections, injected embryos were examined under a microscope, and hatched larvae were collected with a brush. These larvae were then screened for transient fluorescent expression and transferred to standard *Drosophila* food vials (section 3.1).

Injected embryos exhibited relatively slow growth, starting to emerge 10-14 days post-injection. Each emerged adult male (G0) was individually backcrossed with 3-4 wild-type virgin females, while each emerged adult female (G0) was backcrossed with 3-4 wild-type males. The injections and backcrosses were thoroughly documented. Depending on the injected construct, the G1 embryos and adults were then screened for transgenic gene expression of *DsRed*, *EGFP*, or *AmCyan*.

4. Results

4.1 Isolation or characterization of pro-apoptotic genes for lethality-inducing construct

To identify the pro-apoptotic genes of *D. suzukii,* specifically, *reaper*, *hid* and *grim,* which could be used to design Q system to induce lethality in *D. suzukii*, the orthologs of *D. melanogaster* were used from the SWDbase. These genes are well known to induce apoptosis in *D. melanogaster* through activation of caspases. Our investigation yielded the coding sequence for *Dsrpr* (DS10_00012288), which consists of 198 base pairs (bp) and encodes a 66 amino acid protein. Similarly, the coding sequence for *Dshid* (DS10_00012680) comprises of 1281 bp, which corresponding to a 427 amino acid protein. The coding sequence for *Dsgrim* (DS10_00013088) was also identified, covering 360 bp and encoding a 120 amino acid protein.

4.1.1 Structural characterization of *D. suzukii* pro-apoptotic genes

Phylogenetic relationships were assessed using the neighbor-joining method. It revealed that all three pro-apoptotic genes were clustered with their orthologs from several other *Drosophila* species. DsRPR protein (Fig. 7a) was linked to the ortholog of *D. melanogaster* with a 96.9% similarity and 92.3% identity, the DsHID protein (Fig. 7b) exhibited a closer relationship with its counterpart in *D. biarmipes*, showcasing a 96.5% similarity and 94.6% identity, in contrast to its ortholog in *D. melanogaster* with a 91.8% similarity and 86.7% identity. Additionally, the DsGRIM protein (Fig. 7c) is closely related to the ortholog in *D. melanogaster*, depicting a 93.4% similarity and 86.8% identity. The identification of crucial functional domains in pro-apoptotic proteins was a noteworthy outcome. These high identity percentages suggest that these genes are well conserved in the *Drosophila* species. BLAST identity is calculated as the number of matching bases over the total number of alignment columns, including gaps. The high identities suggest that these proteins maintain similar structures and functions across species (Li, 2018). In all three RHG proteins (Fig. 8), suggesting their pro-apoptotic functions are likely to be preserved.

The high sequence identities suggest these proteins retain their pro-apoptotic functions in *D. suzukii*. This conservation is crucial for designing and developing effective genetic control strategies based on these genes. The high identities and conservation of functional domains (IBM and GH3) prove that these proteins maintain similar structures and interaction capabilities with regulatory partners. The similarity to *D. melanogaster* orthologs (particularly for DsRPR and DsGRIM) indicates that genetic constructs or techniques developed for *D. melanogaster* might be adaptable *to D. suzukii.* (Li et al., 2021).

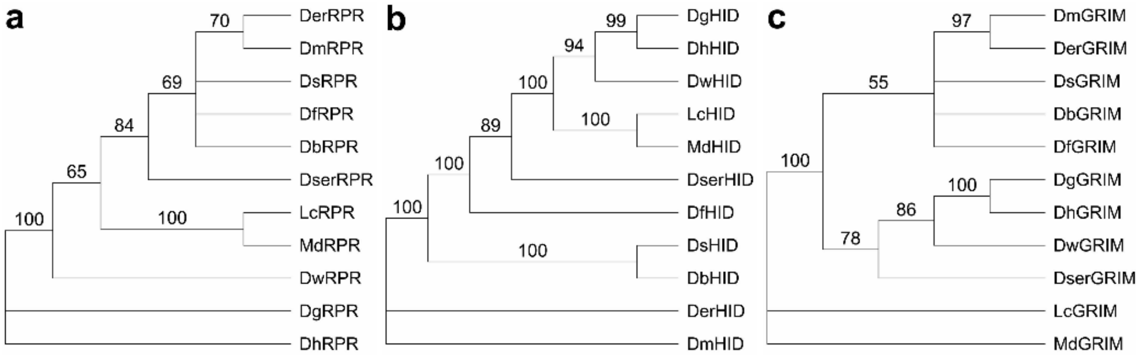

Figure 7*:* Phylogenetic analysis for the *Drosophila suzukii* pro-apoptotic genes with other species.
Unrooted neighbor-joining trees were constructed with (a) RPR, (b) HID, and (c) GRIM amino acid sequences. Bootstrap values (1000 replicates) are shown on the nodes of the trees. (Jaffri et al., 2020)

The IAP-Binding Motif (IBM) is a crucial functional domain found in all three pro-apoptotic proteins (RPR, HID, and GRIM) of *D. suzukii*, as shown in Figure 8. This domain is well-characterized, and its presence in *D. suzukii* is consistent with previous findings of other *Drosophila* species. It is located at the N-terminus of the proteins, typically consists of the first 4 amino acids, and is highly conserved across species. The IBM interacts directly with the BIR (Baculovirus IAP Repeat) domains of IAP (Inhibitor of Apoptosis) proteins. This interaction releases active caspases from IAP inhibition and promotes ubiquitination and degradation of IAPs (Chai et al., 2000; Wu et al., 2001). The conservation of the IBM in *D. suzukii* suggests that the fundamental mechanism of apoptosis initiation through IAP antagonism is preserved in this species. The GH3 (Grim Helix 3) domain is another important functional motif identified in the *D. suzukii* pro-apoptotic proteins, as depicted in Figure 8. This domain was previously known in other *Drosophila* species, but its identification in *D. suzukii* confirms its conservation across species (Clavería et al., 2002).

The GH3 (Grim Helix 3) domain, located downstream of the IBM, spans approximately 20-30 amino acids and forms an amphipathic α-helix (Clavería et al., 2002). Its functions include mitochondrial localization, targeting proteins to mitochondria, and independent pro-apoptotic activity, inducing cell death even in the absence of the IBM, albeit less efficiently (Freel et al., 2008). When present with the IBM, it enhances overall pro-apoptotic activity (Clavería et al., 2002). The exact mechanism of GH3-induced apoptosis is not fully resolved, but it may involve inducing cytochrome c release from mitochondria and potentially interacting with mitochondrial membranes (Olson et al., 2003; Freel et al., 2008). The pro-apoptotic genes' protein sequences from *D. suzukii* (Fig. 8) coincide with those from other species. The highly conserved nature of both the IBM and GH3 functional domains is shown in this image. The crucial role of these domains in regulating apoptosis is highlighted by their conservation among species (Zhou, 2005). The identification of these domains in *D. suzukii* is novel, extending our understanding of apoptotic machinery in this pest species. The high degree of conservation observed in Figure 8 suggests that the pro-apoptotic mechanisms are likely very similar between *D. suzukii* and other *Drosophila* species.

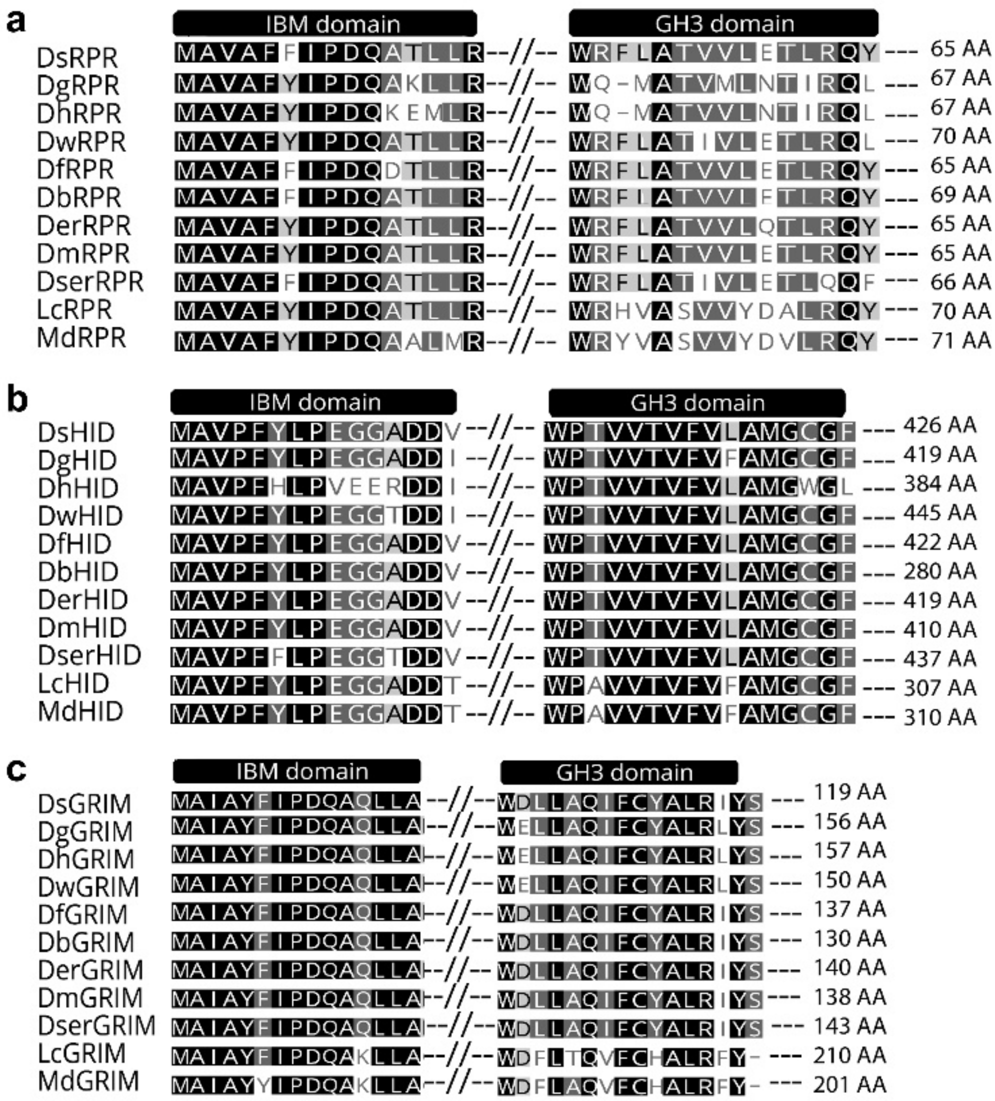

Figure 8: The alignment of pro-apoptotic genes in *D. suzukii* with those of other species is depicted through protein comparisons.

(a) REAPER (RPR), (b) HEAD INVOLUTION DEFECTIVE (HID), and (c) GRIM. For each case, the protein from *D. suzukii* (Ds) is aligned with orthologs from *D. melanogaster* (Dm), *D. grimshawi* (Dg), *D. hydei* (Dh), *D. willistoni* (Dw), *D. ficusphila* (Df), *D. biarmipes* (Db), *D. erecta* (Der), *D. serrata* (Dser), *Lucilia cuprina* (Lc), and *Musca domestica* (Md). Black shading indicates identical amino acids, while grey highlights signify conservative changes. The IBM and GH3 domains are visually presented (Jaffri et al., 2020).

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4.1.2 Expression profiles of pro-apoptotic genes verified by RT-qPCR

A quantitative expression analysis using RT-qPCR was performed to study how pro-apoptotic genes express throughout the life span of *D. suzukii*. The onset of expression for these pro-apoptotic genes occurs early in embryonic development. *Dshid* and *Dsrpr* expressed genes in the first 6 hours (Fig. 9) post-egg laying, and their levels continue to increase throughout the developmental process. This early activation indicates an essential role for these genes in the initial phases of embryonic development. Various expression patterns emerge as development progresses into the larval and pupal stages. In pre-pupae, *Dsrpr* expression is approximately 20 times stronger than *Dshid*. *Dsrpr* achieves maximum expression during the late pupal stage, exhibiting roughly 70-fold upregulation, parallel to *Dshid*, which shows about 35-fold enhancement, suggesting a possibly preeminent function for *Dsrpr* in apoptotic mechanisms at this phase.

The transition from pupae to adult induces another change in expression patterns. As pupae develop into adult males, *Dsrpr* expression decreases drastically from 70-fold to 20-fold, allowing *Dshid* to exceed expression by around 10-fold. The significant dysregulation of *Dsrpr* in adult males indicates a sex-specific function for this gene in apoptotic mechanisms, potentially associated with male-specific developmental occurrences or physiological functions. It is worth noticing that *Dshid, Dsrpr*, and *Dsgrim* showed 20, 4, and almost 2-fold lower expression of these genes in adult females compared to adult males' expression profiles. In all the developmental stages, *Dsgrim* is consistently expressed the least relative to the other two pro-apoptotic genes studied. Although its expression commences 6-12 hours into embryogenesis, it remains significantly lower than other pro-apoptotic genes.

The varying levels of expression at different developmental stages and the sex-specific differences observed in adults underscore the intricate control of programmed cell death processes. These findings provide valuable insights into the potential roles of *Dsrpr, Dshid*, and *Dsgrim* in regulating apoptosis throughout key developmental stages, from early embryogenesis to adulthood.

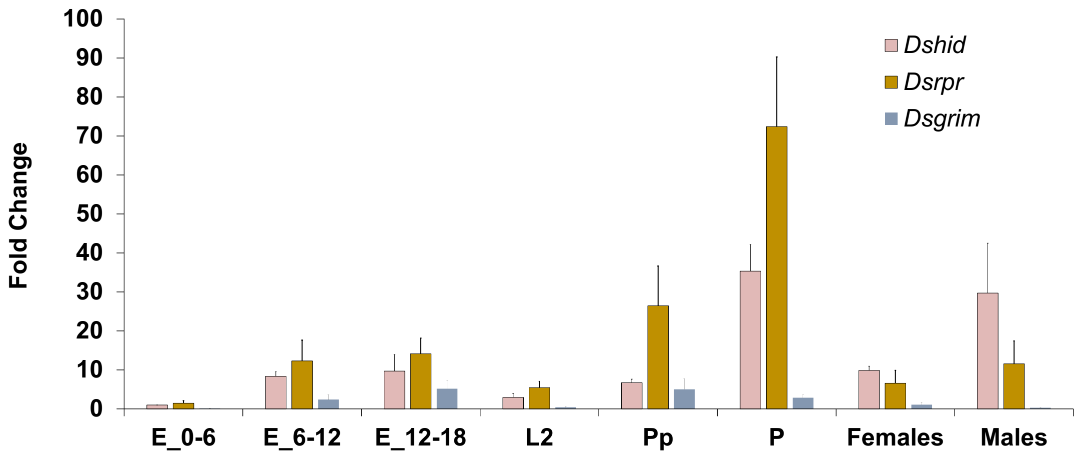


Figure 9: Relative gene expression profiles of RHG pro-apoptotic genes.
The relative expression levels of pro-apoptotic genes were assessed using RT-qPCR at various time points post-egg laying (in hours), including larvae, pre-pupae at 24 hours, pupae, adult females, and males (1 and 5 days old). The normalization was conducted with respect to *DsTBP*, the reference gene, and further normalized to *Dshid* 0-6 h. The bars represent the mean and standard error derived from three replicate experiments (Jaffri et al., 2020).

4.1.3 Functional assessment of *D. suzukii* pro-apoptotic genes in S2 cells

The functionality of pro-apoptotic genes was assessed through the transfection of each respective gene into S2 cells. The gene expression constructs V42, V44, V392, and V93 represented *Dsrpr*, *Dsgrim*, *Dshid*, and the mutant *Dshid*^{Ala4}, respectively. Additionally, bicistronic constructs were designed by combining *Dsrpr* with *Dsrpr* (V163, V172), *Dsrpr* with *Dsgrim* (V164, V173), and *Dsrpr* with *Dshid*^{Ala4} (V165, V174), as illustrated in Figure 10a to evaluate whether increasing the gene dosage enhances apoptotic activity, and to serve as a control for assessing synergistic interactions observed in constructs combining *Dsrpr* with other pro-apoptotic genes. Following a 16-hour post-transfection period of S2 cells with pro-apoptotic genes sourced from *D. suzukii,* a striking alteration in cell morphology was observed, as visually represented in Figure 11. Specifically, these transfected cells displayed a reduced confluency and underwent substantial changes in their cellular structure, often exhibiting ruptured cell membranes (Elmore, 2007). The observation of ruptured cell membranes in transfected cells is particularly significant. While apoptosis is typically associated with maintained membrane integrity until late stages, the presence of ruptured membranes suggests that the cells may have progressed to secondary necrosis, a process that occurs when apoptotic cells are not cleared by phagocytes (Silva, 2010). This progression from apoptosis to secondary necrosis is often observed in invitro systems where phagocytes are absent, and it is characterized by the loss of membrane integrity and the release of cellular contents (Rogers et al., 2017). The bicistronic constructs, especially those incorporating *Dsrpr* with *Dsgrim* or *Dshid*^{Ala4}, demonstrated enhanced lethality compared to single gene constructs, indicating a synergistic effect. This observation corresponds with the established cooperative roles of pro-apoptotic genes in *Drosophila*, wherein combinations of these genes frequently yield more pronounced apoptotic responses (Steller, 2008). The morphological modifications are signs of cellular stress and compromise, indicating that each pro-apoptotic gene's expression significantly affected the analyzed cells' overall fitness and structural integrity.

This notable observation highlights the potential of these pro-apoptotic genes in inducing cellular changes consistent with programmed cell death processes. The changes observed in cell morphhology are consistent with the expected impact of pro-apoptotic gene expression. These findings contribute valuable insights into the functional attributes of these genes and underscore their potential applicability in building a conditional expression system for SIT. Furthermore, these results emphasize their capacity to diminish cell fitness and viability, further highlighting their potential utility in various biological applications.

Another experiment was conducted to gain a more comprehensive understanding of the impact of these genes on cell viability. In this experiment, the gene constructs were co-transfected with an additional plasmid carrying the pIE4-EGFP gene as an expression marker. This approach facilitated a direct comparison of the effects of pro-apoptotic genes from *D. suzukii* (*Dsrpr*, *Dshid*, *Dshid*^{Ala4}, and *Dsgrim*) with those from *D. melanogaster* (*Dmhid*, *Dmhid*^{Ala5}, and *Dmrpr*). This enabled an assessment across the function among both species.

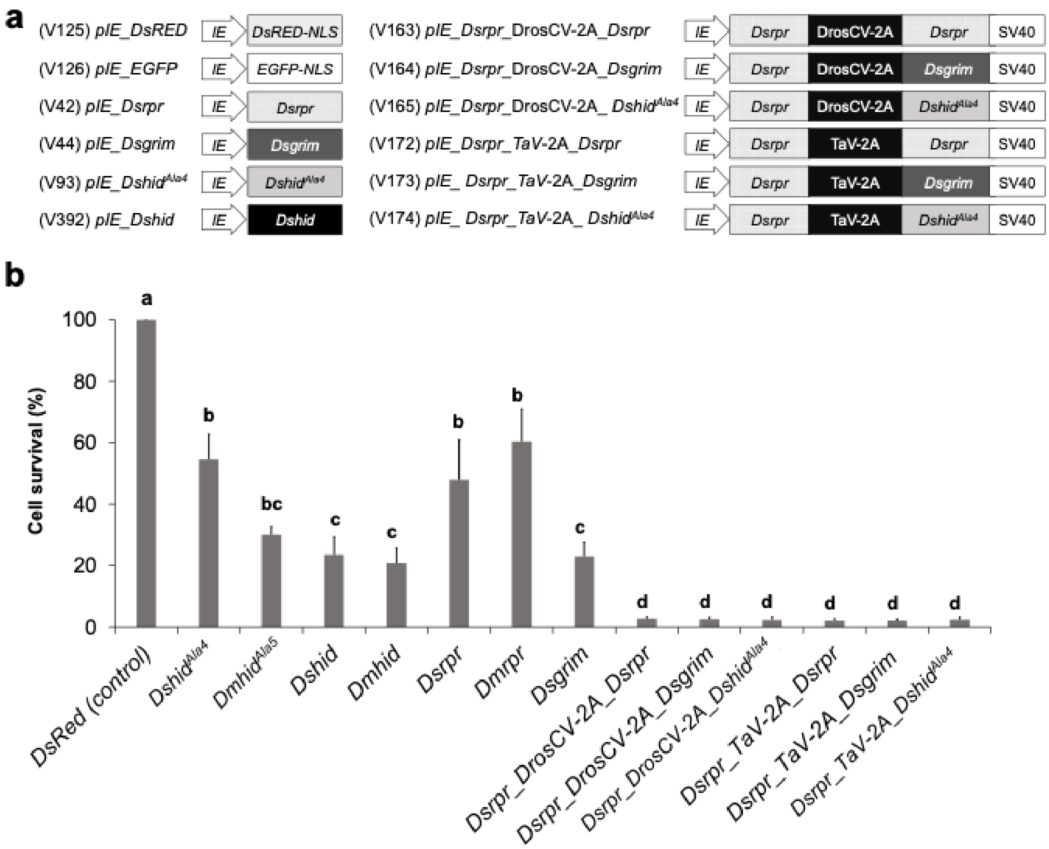


Figure 10: Transfection of S2 cells with pro-apoptotic genes.
The S2 cells were transfected with pIE4 plasmids carrying pro-apoptotic genes. (a) Schematic map of the pIE4 pro-apoptotic genes carrying plasmids. (b) S2 cells co-transfected with pro-apoptotic genes carrying plasmids and pIE4-EGFP plasmid or pIE4-DsRed control plasmid to visualize the transfected cells: EGFP positive cells were counted using Image J (Fiji) as survived cells. The experiment was carried out in three replicates. Mean and standard errors are shown in the figure, bars with different uppercase letters are significantly different at $P < 0.050$ (one-way ANOVA, Holm-Sidak method for pairwise multiple comparison, Appendix 10a) (Jaffri et al., 2020).

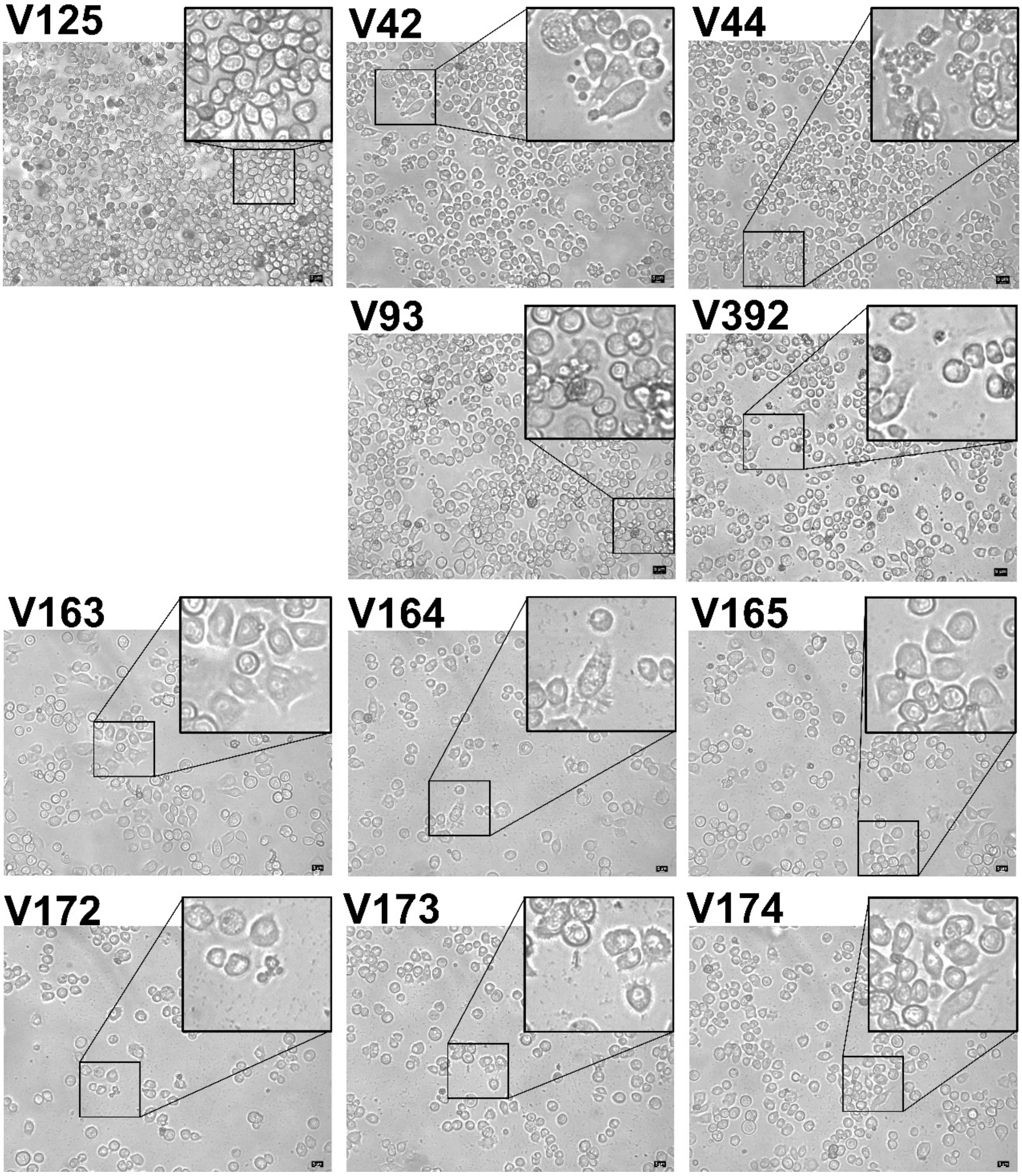


Figure 11: Morphological test of S2 cells induced with pro-apoptotic genes.
The S2 cells were transfected with plasmids carrying pro-apoptotic genes, as in Fig. 10. V125, the control, shows healthy and confluent cells. Cell morphology was compared with the control. The cells displayed reduced confluency and cell membrane rupture. The constructs V42, V44, V93, and V392, which carry single pro-apoptotic genes, show less confluency and ruptured cell walls. V163, V164, V165, V172, V173, and V174 carry two pro-apoptotic genes that appear to cause more cell death than single pro-apoptotic genes. In these wells, cells are much less confluent, which tells that genes present in cell culture did not let the cells grow (scalebar = 5 µm) (Jaffri et al., 2020).

Cell viability was assessed by quantifying the number of cells displaying green fluorescence, indicative of EGFP expression (indicate living cells). The results of this quantitative analysis revealed that introducing single constructs featuring *Dsrpr*, *Dshid*, *Dshid^{Ala4}*, and *Dsgrim* led to a significant reduction in the viability compared to the control group, as illustrated in Figure 10b. A similar trend was observed when single constructs carrying *Dmhid*, *Dmhid^{Ala5}*, and *Dmrpr* were employed, signifying a substantial decrease in cell viability relative to the control. The comparative analysis between the RHG (*reaper, hid, grim*) pro-apoptotic genes of *D. suzukii* and their *D. melanogaster* counterparts revealed intriguing findings. Specifically, no statistically significant differences in cell viability were observed between *DshidAla^4/DmhidAla^5*, *Dshid/Dmhid*, and *Dsrpr/Dmrpr*. These results strongly support the earlier assertion regarding the conservation of apoptotic activity due to conserved functional domains within these genes.

Furthermore, the introduction of binary constructs, combining two pro-apoptotic genes, resulted in a substantially higher degree of lethality compared to single expression constructs, as demonstrated by statistical significance ($P < 0.001$, as determined by One-way ANOVA) (see Fig. 4b). This observation underscores the potential for synergistic effects when these genes are used in combination, highlighting their collective potency in inducing cellular demise.

To validate the comparative analysis of single-gene constructs, a follow-up experiment was conducted several months later using a distinct set of S2 cells sourced from the THM repository (Fig. 12). This subsequent analysis yielded findings consistent with the previously reported results (as depicted in Fig. 11), thereby reinforcing the conclusion that all apoptotic genes tested exhibited statistically significant differences in lethality induction when compared to the control group. This repetition was important to confirm the reproducibility and robustness of the initial findings, and eliminate any discrepencies or batch effects that might result from differences in cell line passages, experimental procedures, or environmental factors that could otherwise compromise the validity of these results. The subsequent analysis produced outcomes consistent with earlier data (Fig. 11), further supporting the conclusion that all tested pro-apoptotic genes induced statistically significant differences in lethality relative to the control group. Specifically, no significant distinctions were evident in the number of viable cells between *Dshid* and *Dmhid*, *Dsrpr* and *Dmrpr*, and *Dshid*^{Ala4} and *Dmhid*^{Ala5}.

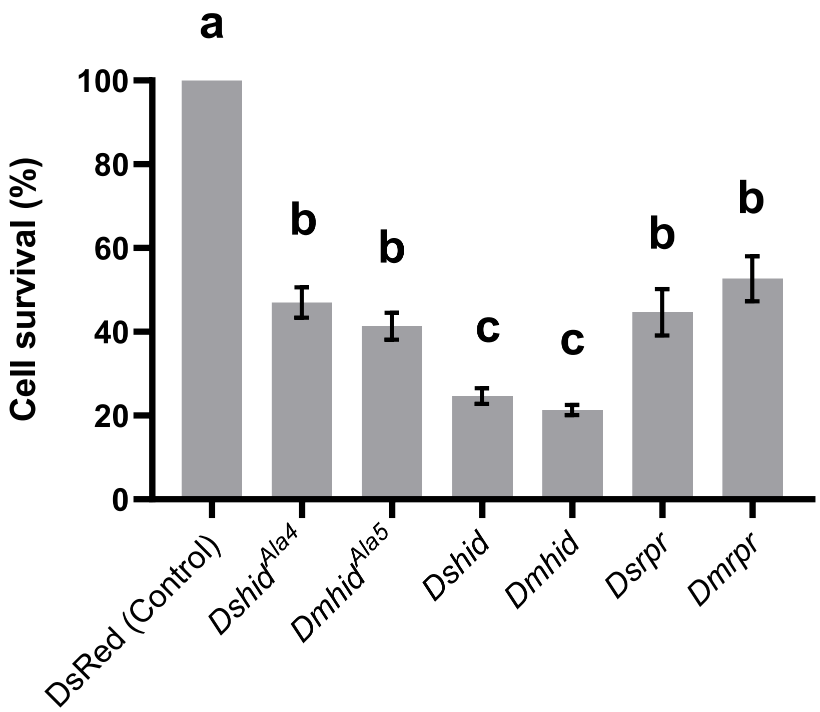


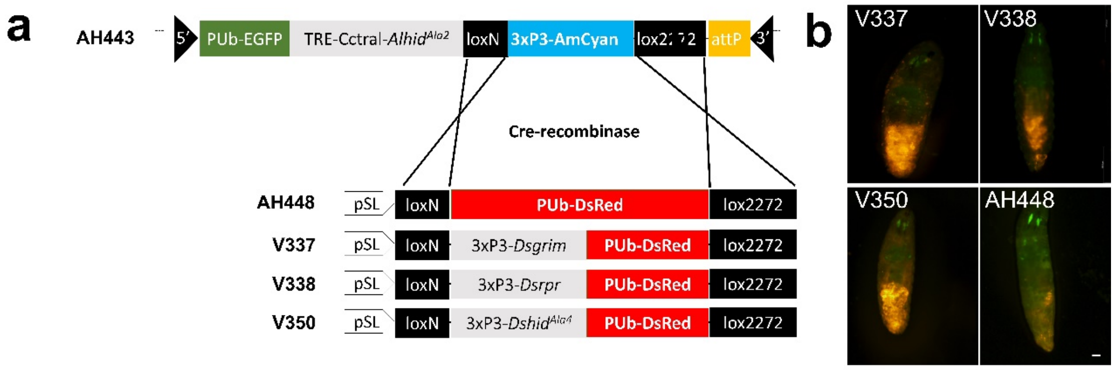
Figure 12: Additional Transfection of S2 cells with pro-apoptotic genes.
Another set of S2 cells (from THM) were transfected with pIE4 plasmids carrying pro-apoptotic genes to compare and confirm the gene functions. S2 cells co-transfected with pro-apoptotic genes carrying plasmids and pIE4-EGFP plasmid or pIE4-DsRed control plasmid to visualize the transfected cells: EGFP positive cells were counted using Image J (Fiji) as survived cells. The experiment was carried out in three replicates. Mean and standard errors are shown in the figure; bars with different uppercase letters are significantly different at $P < 0.050$ (one-way ANOVA, Holm-Sidak method for pairwise multiple comparisons (Appendix 10b).

4.1.4 Functional characterization of pro-apoptotic genes in *D. suzukii* embryos

The study involved assessing the functionality of pro-apoptotic genes by introducing them into *D. suzukii* embryos through injection. Specifically, the pro-apoptotic genes *Dsrpr*, *Dshid*, and *Dsgrim* were incorporated into RMCE donor constructs, designated as V338, V350, and V337, respectively. These constructs were then introduced into the embryos of a transgenic *D. suzukii* line designed for Recombinase-mediated cassette exchange (RMCE), known as AH443_PUbEGFP-TRE-*Cctral-AlhidAla*^{2}-loxN-3xP3-*AmCyan*-lox2272 (Fig. 13) and the construct AH448 was injected as a control (Schetelig et al., 2018). The injected constructs were expected to replace the gene cassette between loxN and lox2272, resulting in the expression of the *AmCyan* gene in the ocelli of *D. suzukii*, inducing apoptosis.

After 48 hours of injections, the hatched larvae were collected, screened, and counted to assess their transient expression of either the *DsRed* or *AmCyan* genes. It was observed that the survival rate of injected embryos was significantly lower in those with RMCE donor constructs containing pro-apoptotic genes (Fig. 13) compared to the control group. None of the larvae with transient expression survived to adulthood; they all died before reaching that stage. In contrast, 20% of the larvae from the control batch survived to adulthood. The low survival rate in embryos with the RMCE donor constructs containing pro-apoptotic genes can be attributed to the fact that these genes were under the control of the 3xP3 promoter, which likely led to the expression of pro-apoptotic genes in the larval nervous system (Thomas et al., 2002), causing lethality in transient larvae.

In short, the introduction of pro-apoptotic genes into *D. suzukii* embryos through RMCE constructs resulted in a substantial decrease in survival rates among the injected larvae. These findings underscore the potential of these pro-apoptotic genes to induce lethality, particularly when directed by the 3xP3 promoter, even within the larval nervous system. This research provides valuable insights into the functional characteristics of these genes and their potential utility in genetic manipulation strategies aimed at controlling *D. suzukii* populations.



c ***In vivo*** **expression analysis of pro-apoptotic genes in *D. suzukii***

Construct	Expression cassette	Injected eggs	Hatch rate	Transient DsRed expression in L1 Larvae		Emerged adult	Survival rate (From larvae to adult)
V337	Dsgrim + DsRed	315	2.5% (8/315)	Yes	5	0	0%
				No	3	3	100%
V338	Dsrpr + DsRed	335	3.5% (12/335)	Yes	6	0	0%
				No	6	3	50%
V350	Dshid^{ala4} + DsRed	305	2.6% (8/305)	Yes	5	0	0%
				No	3	3	100%
AH448	DsRed	312	11.2% (35/312)	Yes	25	5	20%
				No	10	10	100%

Figure 12: Microinjection of RMCE constructs carrying pro-apoptotic genes to *D. suzukii* embryos.
Transient expression of pro-apoptotic genes was observed in the embryos from a transgenic *D. suzukii* line carrying landing sites for RMCE (a) Schematic map of the constructs. (b) Images representing transient larvae within 48 h after injection clearly show transient expression of donor plasmids (scale bar = 100 µm). (c) Following the injection experiments, the survival of larvae and adults was recorded. Data indicate the effects of pro-apoptotic gene expression under 3xP3 promoter on injection survival rates (Jaffri et al., 2020).

The results comprehensively understand the isolation, structural characterization, and functional activity of pro-apoptotic genes in *D. suzukii.* All experiments, including RT-qPCR, cell morphology, viable cell count, and embryonic injections, do not suggest a single expression gene showing a high expression level in all those experiments. Interestingly, *Dsrpr* exhibits a high expression profile with the pupal stage of *D. suzukii*, and *Dshid* expresses great potency in cell death induction. However, in the Cell culture test, *Dsgrim* and *Dshid* tend to achieve the same level of apoptosis in S2 cells. This shows a robust competition between *Dsrpr* and *Dshid*. The results provide insights to enhance our knowledge of apoptotic processes in this species and to hold promise for developing novel lethality-inducing genetic constructs for pest control strategies.

4.2 Isolation and characterization of *D. suzukii* embryonic genes

The cellularization genes of *D. suzukii* were also identified on the SWDbase by using orthologs of *D. melanogaster.* The *Dsnullo* (DS10_00005287) is an intron-less gene like its ortholog from *D. melanogaster* (*Dmnullo*). The CDS is 642 bp, encoding a putative protein with 213 amino acids. The *Dssry-α* (DS10_00012897) consists of three exons. The CDS is 1593-bp, encoding a 530 amino acid protein. *Dsbnk* (DS10_00007356) is an intron-less gene like *Dsnullo*. The 903-bp *Dsbnk* CDS encodes a protein with 303 amino acids. Finally, *Dsslam* (DS10_00010822) has two exons. The 3414 bp *Dsslam* CDS encodes the largest of the four cellularization proteins, with 1137 amino acids.

4.2.1 Structural characterization of *D. suzukii* embryonic genes

DsNULLO is the most closely related to its ortholog in *D. biarmipes* (99% similarity, 96% identity), but also like its orthologs in *D. melanogaster* (94%). The N-terminus carries a myristoylation site as well as a cluster of positively charged amino acids to target the protein to the plasma membrane (Resh, 1999). There are five other conserved segments (Appendix 10a) that are required for NULLO proteins to stabilize the basal junction components in the nascent cleavage furrows of blastoderm cells (Hunter et al., 2002).

DsSRY-α: most closely related to its ortholog in *D. melanogaster* (94% similarity, 87% identity; Fig. 14). The N-terminus of DsSRY-α features a cysteine-rich motif, possibly a transmembrane segment (Appendix 10b). A conserved C-terminal region shows high similarity to Ezrin, Radixin, and Moesin (ERM) proteins, which facilitate actin–membrane interactions (Tsukita et al., 1997), suggesting DsSRY-α fulfills a similar role in the reorganization of microfilaments during cellularization (Ibnsouda et al., 1998).

DsBNK is most closely related to its ortholog in *D. melanogaster* (85% similarity, 80% identity; (Appendix 10c)

DsSLAM is most closely related to its ortholog in *D. melanogaster* (88% similarity, 74.6% identity; (Appendix 10d).

The phylogenetic analysis of all four proteins using a neighbor-joining algorithm revealed that DsSRY-α, DsBNK, and DsSLAM clustered with their *D. melanogaster* orthologs, whereas DsNULLO clustered with DbNULLO and DmNULLO (Fig. 8).

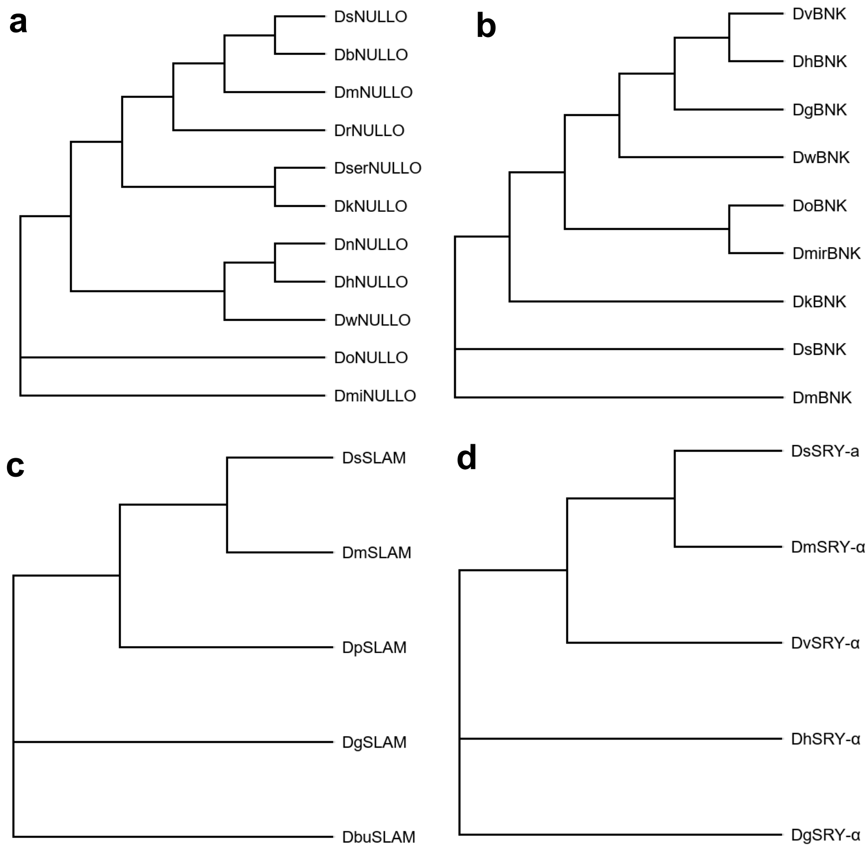


Figure 13: Phylogenetic analysis of *Drosophila* species.
NULLO, SRY-α, BNK and SLAM proteins. Unrooted neighbor-joining trees were constructed with amino acid sequences from (a) NULLO, (b) SRY α, (c) BNK, and (d) SLAM. Bootstrap values (1000 replicates) are shown on the nodes of the trees (Yan et al., 2020).

4.2.2 Expression profiles of embryonic genes verified by RT-qPCR

The RT-qPCR was conducted to examine the expression patterns of embryonic genes. The findings indicated that the expression of these embryonic genes commences during the 0-6 hours following egg laying and subsequently diminishes in later developmental stages. Notably, *Dsbnk* exhibited its highest gene expression during the 0–6-hour timeframe and displayed no expression thereafter (Fig. 15). *Dsnullo*, conversely, displayed consistently low gene expression throughout the developmental stages, particularly within the 0-6-hour window and in contrast to *Dsbnk, Dssry-α,* and *Dsslam* demonstrated relatively lower expression levels. Two reference genes, *TBP* and *AK,* were used to normalize this data. The transcripts of *Dssry-α* were 123-fold more abundant, *Dsnullo* was 1040-fold more abundant, *Dsbnk* was 13,469-fold more abundant, and *Dsslam* exhibited a 56-fold increase in abundance in 0–6-hour embryos compared to 6-12-hour embryos. Additionally, it showed remarkable differences, being 116-fold, 153-fold, 2433-fold, and 49-fold more abundant in 0–6-hour embryos compared to adult females at different stages, respectively.

A comparative analysis shows that in 0–6-hour embryos, the transcripts of *Dssry-α, Dsbnk*, and *Dsslam* were respectively 17.5-fold, 53-fold, and 21-fold more abundant than *Dsnullo* mRNA. It is worth noting that the relatively large variations (as indicated by the error bars) in gene expression among 0-6 hour embryos may be attributed to differences in the ratios of eggs collected during the early blastoderm stages (2-3 hours) compared to eggs from other time periods within the 0-6-hour window across three replicates. Nevertheless, it's important to highlight that the expression levels of all four genes significantly decreased at later developmental stages.

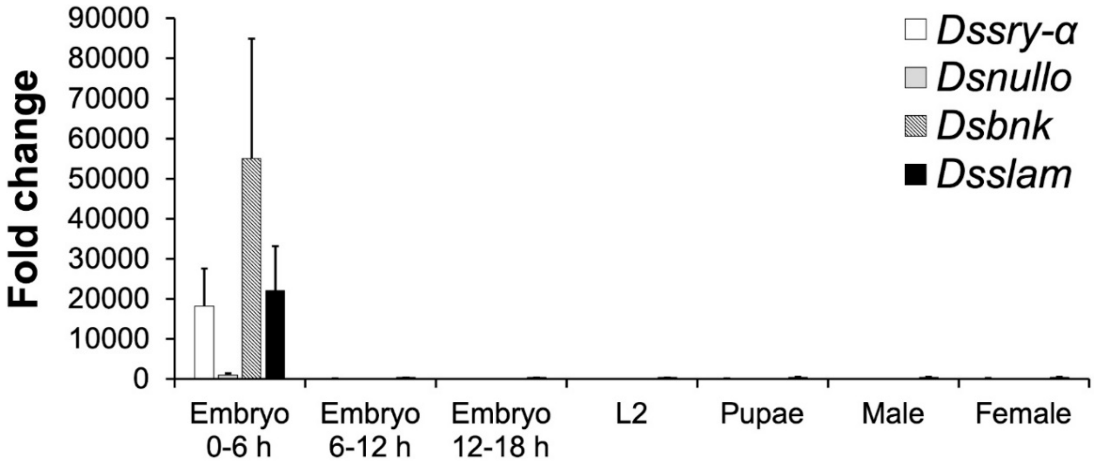


Figure 14: Quantitative real-time-PCR to determine the relative expression of cellularization genes.
Analysis was conducted on three distinct embryonic stages (0-6 h, 6-12 h, and 12-18 h after egg laying), as well as second instar larvae (L2), pupae, and males and females aged 5 days. The normalization of gene expression was carried out using the reference genes *TBP* and *AK*, and the results were relative to the expression level of *Dsnullo* at 6-12 h embryos. The displayed values represent the mean and standard error obtained from three replicate experiments (Yan et al., 2020).

4.2.3 Testing the promoter regions of embryonic genes in S2 cells

To characterize and compare the functional capacity of embryonic promoters, the *piggyBac* test plasmids were generated where embryonic promoters *Dsryα, Dsnullo, Dsslam,* and *Dsbnk* were linked to the DsRed fluorescent reporter gene (Fig. 16a). These plasmids had *DmPUb-AmCyan* gene cassette as well that confirms the gene transfection. The S2 cells were transfected with each of the four constructs. After 16 h, the fluorescent cells with *AmCyan* expression and *DsRed* expression were counted and analyzed. The ratio of red to blue cells presented the actual number of cells with gene expression under embryonic promoters. It was observed that the *Dsnullo* (construct V206) was able to generate the most robust *DsRed* signal (Fig. 16b and c), and the highest DsRed: AmCyan ratio (Fig. 16c) was observed, suggesting the peak activity as a gene promoter (*P* =0.001 in comparison to V205 and V208, *P* =0.002 in comparison to V207, according to one-way ANOVA).

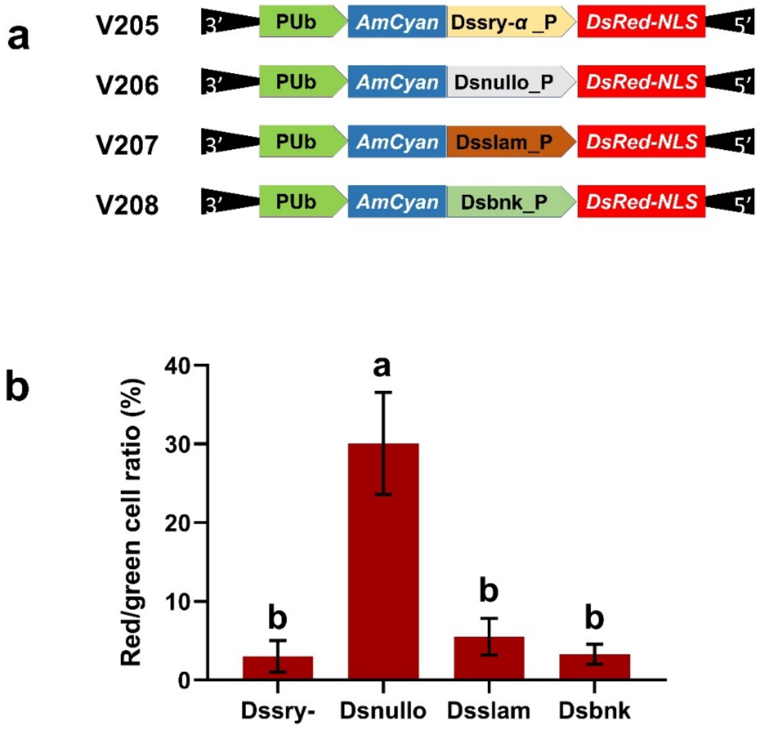


Figure 15: Functional characterization of *D. suzukii* cellularization gene promoters.
(a) Plasmids V205, V206, V207, and V208 were employed, utilizing the promoters *Dssry-α, Dsnullo, Dsslam,* and *Dsbnk* to drive *DsRed* expression. All experimental vectors included an AmCyan marker gene regulated by the constitutive *D. melanogaster* polyubiquitin (DmPUb) promoter (b) *Drosophila* S2 cells underwent transfection with *piggyBac* vectors, and the count of cells exhibiting red and blue fluorescence was conducted using Image J. Subsequently, the ratio of red to blue cells was calculated. Each bar represents the mean ± SE of n = 3 experiments.

Bars labelled with different letters indicate significant differences at $P < 0.05$ (one-way ANOVA and Holm-Sidak's multiple comparisons test, see Appendix 10c) (Yan et al., 2020).

In conclusion, the investigation into the expression patterns of embryonic genes using quantitative real-time PCR has provided valuable insights into their temporal dynamics during early *D. suzukii* development. Notably, *Dsbnk* as an embryonic gene, displayed its peak expression during the 0-6 hours window, followed by a complete absence of expression thereafter, while *Dsnullo* exhibited consistently low expression levels, particularly within the 0-6-hour timeframe. In contrast, *Dssry-α* and *Dsslam* displayed relatively lower expression levels. The results from transfection experiments in S2 cells demonstrated that the *Dsnullo* promoter (construct V206) exhibited the strongest DsRed signal and the highest DsRed:AmCyan ratio, indicating its superior activity as a gene promoter. These suggest that the *Dsnullo* promoter holds promise as a candidate for inducing embryonic lethality in a genetic control system like TESS.

4.3 Q system

4.3.1 in vitro analysis for the *piggyBac* plasmids carrying Q system elements.

The central objective of this research has revolved around the development of a Q system-based conditional expression system in *D. suzukii*. To assess the effectiveness of the Q system in *Drosophila*, S2 cells were initially subjected to transient transfection for analysis.

Plasmid generation: A transcriptional factor, QF was designed to be regulated by the ubiquitously active promoter act5cB. This vector, denoted as (V291) pBacXLII-attP_Pub-EGFP_act5cB-QF#7-actinpolyA, was incorporated with an actin polyA tail and, a PUb-EGFP expression cassette to facilitate the visualization of transfected cells exhibiting green fluorescent protein expression (Fig. 17a,b). In parallel, an effector construct named QUAS (V288) pBacXLII_attP-PUbDsRedT3-QUAST-Dmhsp70-AmCyan-SV40) was designed to drive the expression of the *AmCyan* as a reporter gene under the control of the QUAS element. This effector construct also contained the *DsRed* marker, ensuring the identification of cells containing the plasmid (Fig. 17a,c). Similarly, a third genetic component (the suppressor), QS (V354) pBacXLII-attP-PUbDsRedT3_PUb-QS-SV40), was developed with the ubiquitous promoter tubP and SV40 polyA tail. The presence of QS constructs within cells is to be indicated by the red fluorescence, again ensuring the identification of cells containing the plasmid (Fig. 17a).

Transfection: The S2 cells were subjected to transient transfection in a 24-well plate configuration as described in (section 3.12.4), and fluorescence was captured after 16 hours of transfection. Since the Q system operates through the interplay of its Driver (QF), Suppressor (QS), and Effector (QUAS). The QF binds to the QUAS promoter sequence to initiate gene expression of downstream elements, such as *AmCyan* (Huiet & Giles, 1986; Potter et al., 2010). QS, inhibits QF activity by directly binding to it and preventing its interaction with QUAS and eventually suppressing gene expression (Giles et al., 1991; Potter et al., 2010). This regulatory mechanism enables precise control of target gene expression, which can be further modulated by the addition of QA. QA disrupts QS-QF binding, releasing QF to activate QUAS-driven gene expression (Riabinina et al., 2015; Potter et al., 2010)

This experiment was divided into two parts. First to see, whether the *piggyBac* transposon could effectively deliver and maintain Q system components (QUAS, QF, and QS) for stable gene expression in S2 cells. Second to quantify the performance of Q system in the *Drosophila* system by using florescent cell count method. The quantitative efficacy of the Q system was defined as its capacity to suppress or activate *AmCyan* expression in a dose-dependent manner.

4.3.1.1. Qualitative assessment of the Q system in S2 cells:

This experiment focuses on evaluating whether *piggyBac* can serve as an effective expression vector to deliver Q system components in S2 cells. For this pupose fluorescent imaging and cell count method was used to observe the effects of Effector_QUAS (E), Driver_QF (D), and Suppressor_QS (S) elements on the expression of a gene of interest (*AmCyan*), as well as reduction of *AmCyan* expressing cells upon the addition of QA at different concentrations.

1. In three replicates of a 24-well plate, S2 cells were transfected with the Effector (E) (V288) *DsRed*-QUAS_*AmCyan,* alone (Fig. 17a-b). This transfection resulted in the expression of *DsRed* fluorescence but not blue (*AmCyan*), It is important to note that *DsRed* is under the control of a constitutive promoter, allowing its expression to occur independently of the Q system. In contrast, *AmCyan* is under the control of the QUAS, which requires activation by the Driver (QF) for its expression. Therefore, the observed lack of blue cells confirms that the Effector alone cannot activate QUAS-driven gene expression without the Driver element.

2. In the next three replicates, S2 cells were co-transfected with the Effector (E) V288 and Driver (D) V291 *EGFP*-QF. In this case, the *AmCyan* expression was clearly observed along with the *DsRED* expression. This implies that the Driver QF triggered *AmCyan's* expression.

3. S2 cells were further co-transfected (in three replicates) with the Effector (E) V288 and Driver (D) V291 and the Suppressor x5 (S) V354 *DsRed*-QS. Despite the presence of the Suppressor, *AmCyan* expression can still be observed, indicating that the (S) could not completely inhibit the expression of

AmCyan (Fig. 18). However, the number of *AmCyan*-expressing cells was significantly reduced.

4. Another combination of Effector (E) V288, Driver (D) V291, and the Suppressor x5 (S) V354 were further treated with different concentrations of QA (10 µg/ml, 500 µg/ml, and 5 mg/ml, respectively) to ensure that QA releases gene suppression. Figure 17 a-b indicates that in all cases (10 µg/ml, 500 µg/ml, and 5 mg/ml), *AmCyan* expression can be seen. However, in the cells with QA concentration of 5 mg/ml, blue cells were more than the ones before. This suggests that the presence of QA did not enhance the expression of *AmCyan* to the level as it was in the absence of QS, but still, the cells expressing *AmCyan* were more than those without QA (Fig. 17b).

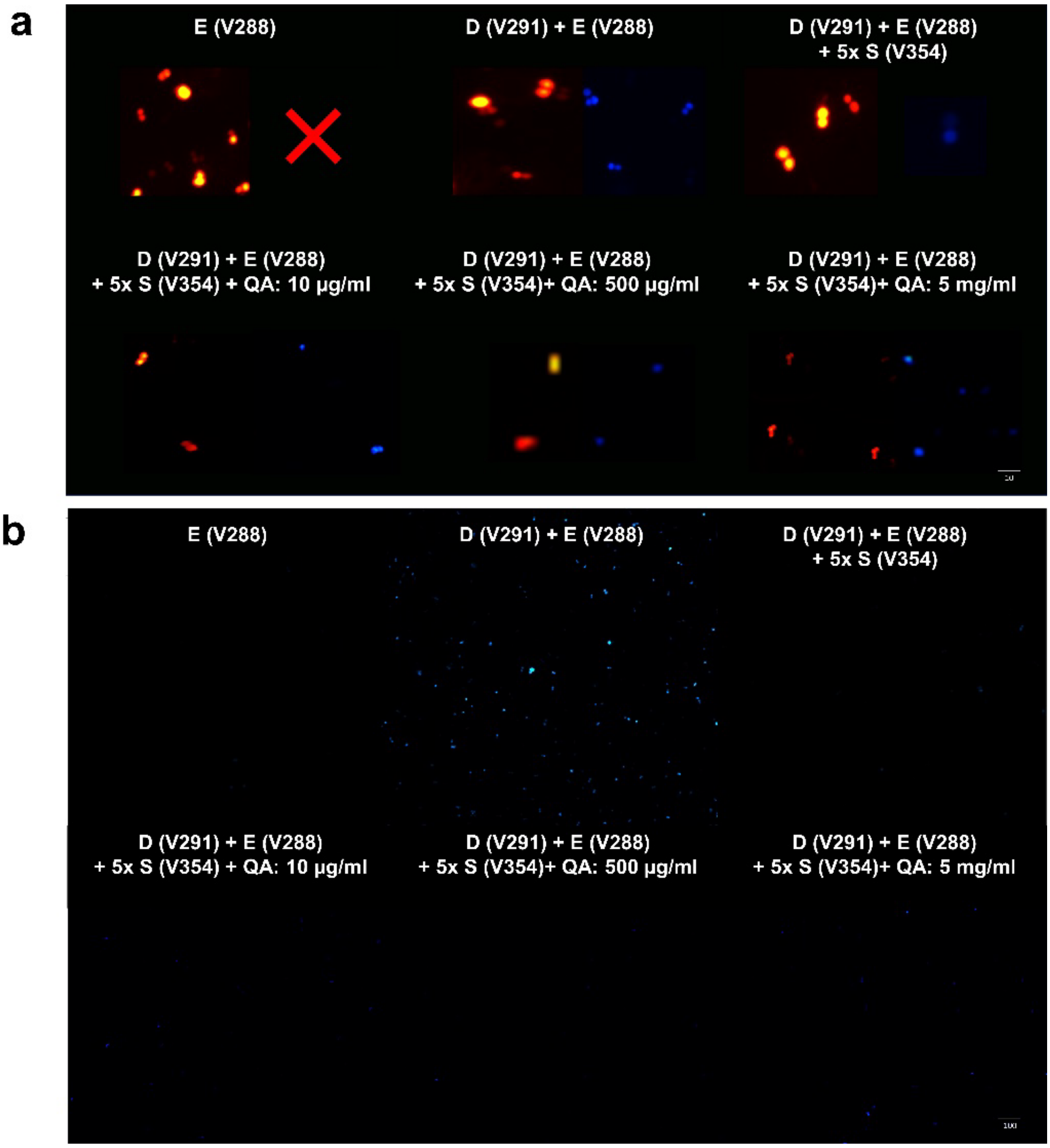


Figure 16: Fluorescence microscopy images demonstrating that *AmCyan* expression is controlled by QF in S2 cells.

(a) shows S2 cells transfected with the E (V288): Cells transfected with the effector construct (V288_DsRed-QUAS_AmCyan) alone show only DsRed fluorescence, as *AmCyan* expression requires activation by the driver (QF). D (V291) + E (V288): Co-transfection with the driver construct (V291_EGFP-QF) results in both *DsRed* and *AmCyan* fluorescence, confirming QF-mediated activation of the QUAS-controlled *AmCyan* reporter. D (V291) + E (V288) + 5× S (V354): Addition of the suppressor construct (V354_QS) reduces *AmCyan* fluorescence due to QS-mediated repression of QF activity. D (V291) + E (V288) + 5× S (V354) + QA: Treatment with increasing concentrations of quinic acid (QA; 10 µg/mL, 500 µg/mL, 5 mg/mL) progressively relieves QS-mediated repression, restoring *AmCyan* fluorescence in a dose-dependent manner. (b) Images showing *AmCyan* fluorescence alone under identical conditions as in panel (a). E (V288): No AmCyan fluorescence is observed due to the absence of QF. D (V291) + E (V288): Strong AmCyan fluorescence is visible, indicating successful activation by QF. D (V291) + E (V288) + 5× S (V354): Suppressed *AmCyan* fluorescence due to QS activity. D (V291) + E (V288) + 5× S (V354) + QA: Increasing QA concentrations restore *AmCyan* fluorescence in a dose-dependent manner (scale bar = 100 µm).

4.3.1.2. Quantitative analysis of Q system efficacy in S2 cells:

This section examines the Q system's ability to modulate gene expression levels quantitatively, focusing on suppression efficiency (via QS dosage) and activation (via QA modulation). The efficacy of Q system was measured by analyzing cell counts of *AmCyan*-expressing S2 cells using Image J (Fiji), with results normalized to total transfected cells. Dose-dependent effects were assessed by transfecting increasing suppressor (QS) copies (1x, 3x, 5x). In this experiment, the S2 cells were transfected as above. The analysis indicated an inverse relationship between the copies of QS and gene expression levels – higher QS doses correlated with reduced gene expression. To ensure the reliability and reproducibility of the results, the experiment was carried out in three separate replicates.

The results (Fig. 18b) suggested that the higher the number of suppressors, the lower the gene expression will be. Notably, the *AmCyan* expression could not be completely eliminated even when QS was transfected in 5x concentration, suggesting to test for more copy numbers of QS to acheive 100% suppression in S2 cells. Additionally, to systematically explore the QA-mediated modulation of gene expression, three distinct concentrations of QA were strategically employed: 5 µg/ml, 500 µg/ml, and 5 mg/ml. A noticeable response was seen when cells were treated with 5 µg/ml of QA. Interestingly, even with 5 µg/ml QA, the gene's activity could be increased by reducing the effect of gene suppression element QS. However, the most substantial effect was achieved with 5 mg/ml of QA, effectively restoring *AmCyan* expression levels. These findings show that the system can respond to food additive QA signals in the *Drosophila* system.

Statistical analysis (Appendix 10d) revealed significant differences (P =0.0002) between the effector construct alone and the effector construct co-transfected with the driver. Similarly, a notable distinction was observed between the effector-driver co-transfection with the suppressor (P =0.38). However, the transfection results of 1x QS, 3x QS, and 5x QS were not significantly different from each other. This denotes further investigation on which dose of QS would be needed to suppress complete gene expression. Further, treatment of S2 cells with lower QA concentrations was not significantly different to 1x QS, 3x QS, and 5x QS, but 5 mg/ml (P =0.07). QA data shows no significant difference with 1x

QS, 3x QS (*P* =0.99), and 5x QS (*P* =0.28) to the level that it has no significant difference with E+D as well, suggesting the gene expression was reduced but not completely. This quantitative approach signifies the Q system's capacity for controlled gene regulation. Furthermore, QA's dose-responsive activity highlights its potential as a dietary or environmental trigger in *Drosophila* models, though incomplete suppression at 5x QS doses underscores the need for system optimization (to test further at 7x QS or 10x QS) to eliminate residual leaky expression.

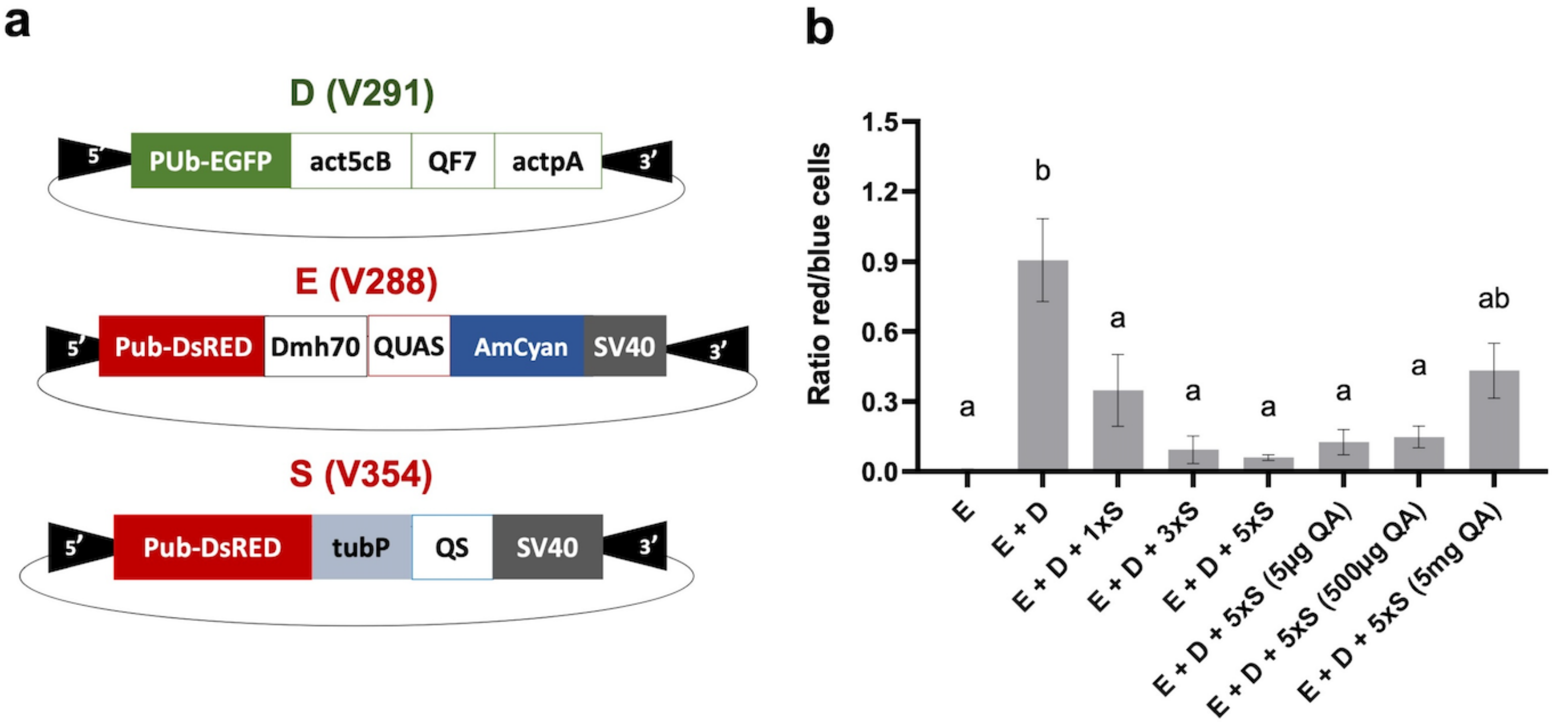


Figure 17: Q system components and their effects on gene expression in transfected S2 cells.
(a) gene constructs of the Q system components, Driver (V291), Effector (V288), and Suppressor (V354) have been cloned in *piggyBac* vectors. (b) T The graph represents the ratio of cells showing red/blue fluorescence. Cells were counted using Image J (Fiji) as survived cells. The experiment was replicated three times. Transfection of S2 cells with the Effector V288-DsRed-QUAS alone shows absence of *AmCyan* expression. Transfection of S2 cells with both the Effector DsRed-QUAS and the Driver QF, resulting in evident *AmCyan* expression that indicates successful gene activation through QF. Transfection of S2 cells with the Effector DsRed-QUAS, the Driver QF, and the Suppressor 5x QS, showing reduced *AmCyan* expression and suggesting partial suppression of *AmCyan.*) Transfection of S2 cells with the Effector DsRed-QUAS, Driver QF, Suppressor 5x QS, and QA at concentrations of 10 µg/ml, 500 µg/ml, and 5 mg/ml, respectively. In all three cases, the *AmCyan* expression is observable. The figure displays the mean and standard errors, and bars labeled with distinct uppercase letters indicate significant differences at $P < 0.050$ (one-way ANOVA and the Holm-Sidak method for pairwise multiple comparisons).

In summary, these experiments demonstrate how the Q system components can be used to regulate gene expression in *Drosophila*. The presence of the Driver QF effectively drove the expression of the gene of interest (*AmCyan*), and the Suppressor (QS) did inhibit *AmCyan* expression but reduced the blue cells suggesting to use higher copy numbers of QS to completely suppress *AmCyan*. Furthermore, adding QA at various concentrations did allow the *AmCyan* expression again. These results convinced us to carry out a quantitative analysis further. This analysis is based on fluorescence microscopy images based on (i) cell count that shows red and blue fluorescence using Image J (Fiji) software. (ii) the ratio of cells showing red/blue fluorescence. This ratio is an indirect measure of gene expression controlled by the Q system component. (iii) The experiment was replicated three times, indicating that the cell counting and ratio calculation were performed on multiple sets of images. (iv) The data were analyzed using one-way ANOVA and the Holm-Sidak method for pairwise multiple comparisons. This statistical approach helps determine if there are significant differences between the various experimental conditions.

4.3.2 Pro-apoptotic genes expressed in S2 cells with the Q system.

As per the steps discussed earlier, the Effector construct QUAS carrying pro-apoptotic genes (*DshidAla^4*, *Dsrpr*, and *Dsgrim* under the *DsRed* marker) were transfected individually on its own and did not cause any cell death in the culture. This setup also served as a baseline control in the experiment to evaluate the effect of Q system-induced gene expression. The red fluorescent cells serve as an indicator of viable cells after transfection within the cell culture (Fig. 19a-c), suggesting that the pro-apoptotic genes in QUAS construct did not trigger cell death in this system without QF (activation factor) confirming that no basal or leaky expression of pro-apoptotic genes was observed. The effectiveness of Q system was measured by how well the cell death is observed (low number of fluorescent cells) to QF efficiency and high number of fluorescent cells in prescence of QS, then again low number of fluorescent cells when QA is added. Number of flurescent cells here count for living cells.

The introduction of driver V291_QF significantly reduced the number of surviving cells (Fig. 19a-c), indicating that the driver successfully activates the expression of pro-apoptotic genes, likely through the Q system's transcriptional activation mechanism. The resulting cell death was achieved at the level of 40% viable cells for *Dshid*^{Ala4} (Fig. 19a), 10% for *Dsgrim* (Fig. 19b) and 18% for *Dsrpr* (Fig. 19c).

The suppressor (S) V295_QS was added from 1x to 3x concentrations, which reduced cell viability. Typically, a suppressor is expected to inhibit gene expression, leading to increased cell viability when dealing with pro-apoptotic genes. However, in this case, the suppressor strangely led to an even more significant reduction in cell viability, especially for *Dshid*^{Ala4} and *Dsrpr.* This outcome is counterintuitive and challenges our conventional understanding of suppressor function. For *Dsgrim*, the suppressor tends to enhance cell viability from 10% to 30 %. However, at a 5x concentration, the suppressor was found to decrease gene expression and enhance the number of surviving cells to 50%, 56%, and 78% in *Dshid*^{Ala4} (Fig. 19a), *Dsgrim* (Fig. 19b) and *Dsrpr* (Fig. 19c) respectively. The suppressor's behavior at higher doses (5x) corresponds more accurately with anticipated outcomes. The suppressor decreased gene expression and increased the number of viable cells on a 5x dose, aligning with a suppressor's conventional function in gene regulatory systems (Potter et al., 2010).

Similarly, when cells were exposed to 5 µg/ml of QA, it was evident that this concentration did not contribute to the initiation of gene expression. However, a 5 mg/ml of QA was sufficient to decrease cell survival significantly, resulting in 5% viable cells for *Dshid*^{Ala4} (Fig. 19a), 19% for *Dsgrim* (Fig. 19b), and 10% for *Dsrpr* (Fig. 19c). The dose-dependent effect noted here for QS and QA, is common in biological systems and highlights the necessity for extensive characterization of regulatory elements across various doses. It also highlights the complexity of gene regulation and the potential for non-linear responses in synthetic biological systems (Elowitz & Leibler, 2000).

This experiment further proves that the Q system functions with excellent control over the expression of pro-apoptotic genes. No significant cell death was observed without the QF driver, indicating minimal leaky expression (Potter et al., 2010). The introduction of the QF driver resulted in excessive apoptosis of cells, indicating the capability of the system to overexpress the desired target genes when needed effectively. The dose-dependent response to QA tells the capability of the Q system for fine-tuned regulation. The Q system demonstrates tunable control over gene expression, as shown by its ability to regulate pro-apoptotic genes and induce significant cell death when activated. However, the observed partial suppression and unexpected effects at lower suppressor doses highlight some limitations. While the system is robust and versatile under specific conditions, these results emphasize the importance of further optimization with increased QS copy numbers as well as increased QA concentrations to fully understand if tight gene regulation is possible.

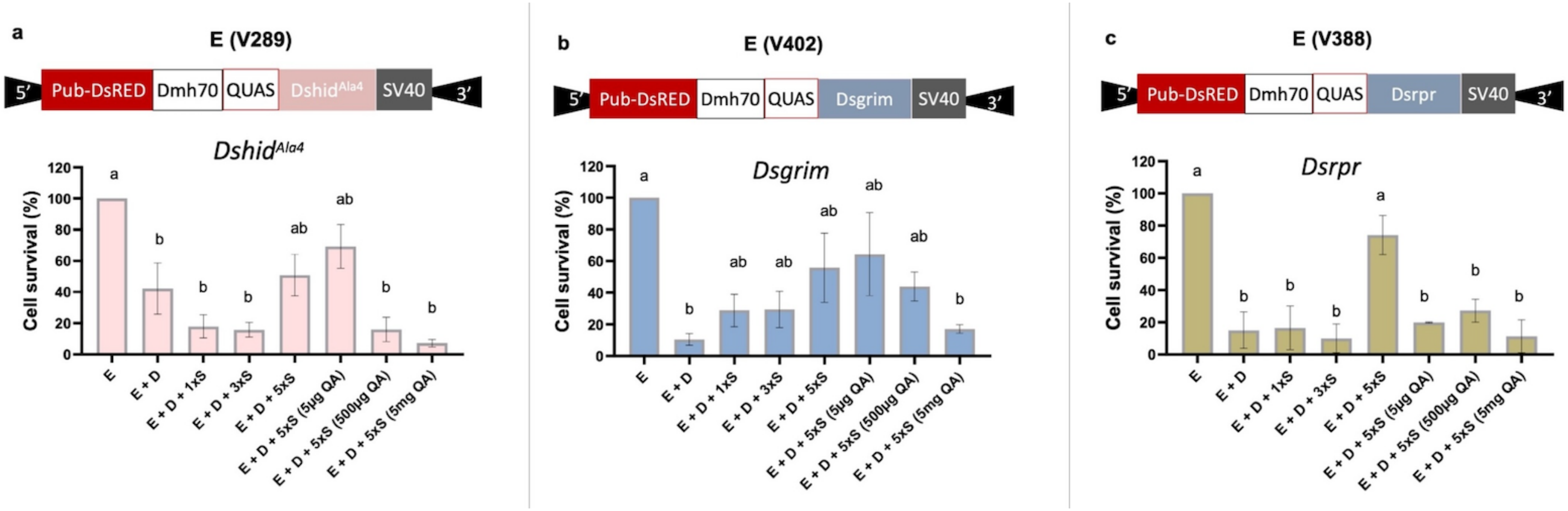


Figure 18: Inducing the expression of pro-apoptotic genes in S2 cells using the Q system.

(a) The Effector construct E (V289) contains PUb-DsRED, Dmh70, QUAS-controlled *Dshid*^{Ala4}, and SV40 terminator. Cell survival was assessed under different conditions (mean ± SEM): E alone (100%), E+D (~40%), E+D+1×S (~20%), E+D+3×S (~15%), E+D+5×S (~50%), and E+D+5×S with varying concentrations of QA: 5µg/mL (~70%), 500µg/mL (~15%), and 5mg/mL (~5%). Significant differences in survival rates are indicated by different letters (a, b, ab), with treatments sharing the same letter not significantly different (p>0.05). (b) The Effector construct E (V402) contains PUb-DsRED, Dmh70, QUAS-controlled *Dsgrim*, and SV40 terminator. Cell survival was measured under the same conditions as in (a): E alone (100%), E+D (~10%), E+D+1×S (~30%), E+D+3×S (~30%), E+D+5×S (~55%), and E+D+5×S with QA: 5µg/mL (~65%), 500µg/mL (~45%), and 5mg/mL (~15%). Statistical significance is indicated as in (a). (c) The Effector construct E (V388) contains PUb-DsRED, Dmh70, QUAS-controlled *Dsrpr*, and SV40 terminator. Cell survival was quantified under identical conditions: E alone (100%), E+D (~15%), E+D+1×S (~15%), E+D+3×S (~10%), E+D+5×S (~75%), and E+D+5×S with QA: 5µg/mL (~20%), 500µg/mL (~25%), and 5mg/mL (~10%). Statistical significance is shown as in previous panels. Cells were counted using Image J (Fiji) as survived cells. The experiment was carried out in three replicates. Mean and standard errors are shown in the figure; bars with different uppercase letters are significantly different at P < 0.050 (one-way ANOVA, Holm-Sidak method for pairwise multiple comparison, Statistical data in appendix 10-e, f and g).

Like the morphological observation in (Fig. 10), additional morphological tests were conducted using control V288_QUAS-*AmCyan*, and the test constructs were V289_QUAS-*Hid*^{Ala4} and V290_QUAS-*Cctra-Hid*^{Ala4}. A remarkable change in cell confluency appeared after a 16-hour post-transfection period of S2 cells, as depicted visually in Figure 20. The cells transfected with control (E) V288_QUAS-*AmCyan* appeared healthy and confluent. Their co-transfection with driver (D) V291_QF also did not trigger cell rupture. Neither was there a morphological change when both (E) and (D) were co-transfected with (S) V295_QS. However, an obvious morphological difference can be spotted in cell confluency of cells targeted with V289_QUAS-*Dshid*^{Ala4} and V290_QUAS-*Cctra-Dshid*^{Ala4}. All Effectors (E) transfected alone showed a healthy and confluent cell morphology. The cell membranes were ruptured upon adding V291_QF with V289 and V290, showing the QF successfully derived gene expression in S2 cells. Moreover, upon transfection of V295_QS, cell confluency appeared to be healthy. These changes in cell shape are strong indicators of cellular stress and vulnerability, suggesting that each pro-apoptotic gene's expression had a noticeable impact on the analyzed cells' overall health and structural integrity.

The observed disruptions in cell morphology align with the expected outcomes of pro-apoptotic gene expression, which are known to trigger and propel programmed cell death pathways. These findings provide valuable insights into the functional properties of these genes under the control of the Q system and underscore their potential usefulness in genetic manipulation strategies. Furthermore, these results underscore their ability to reduce cell fitness and viability, further emphasizing their potential value in the generation of Q system-based TESS in *D. suzukii*.

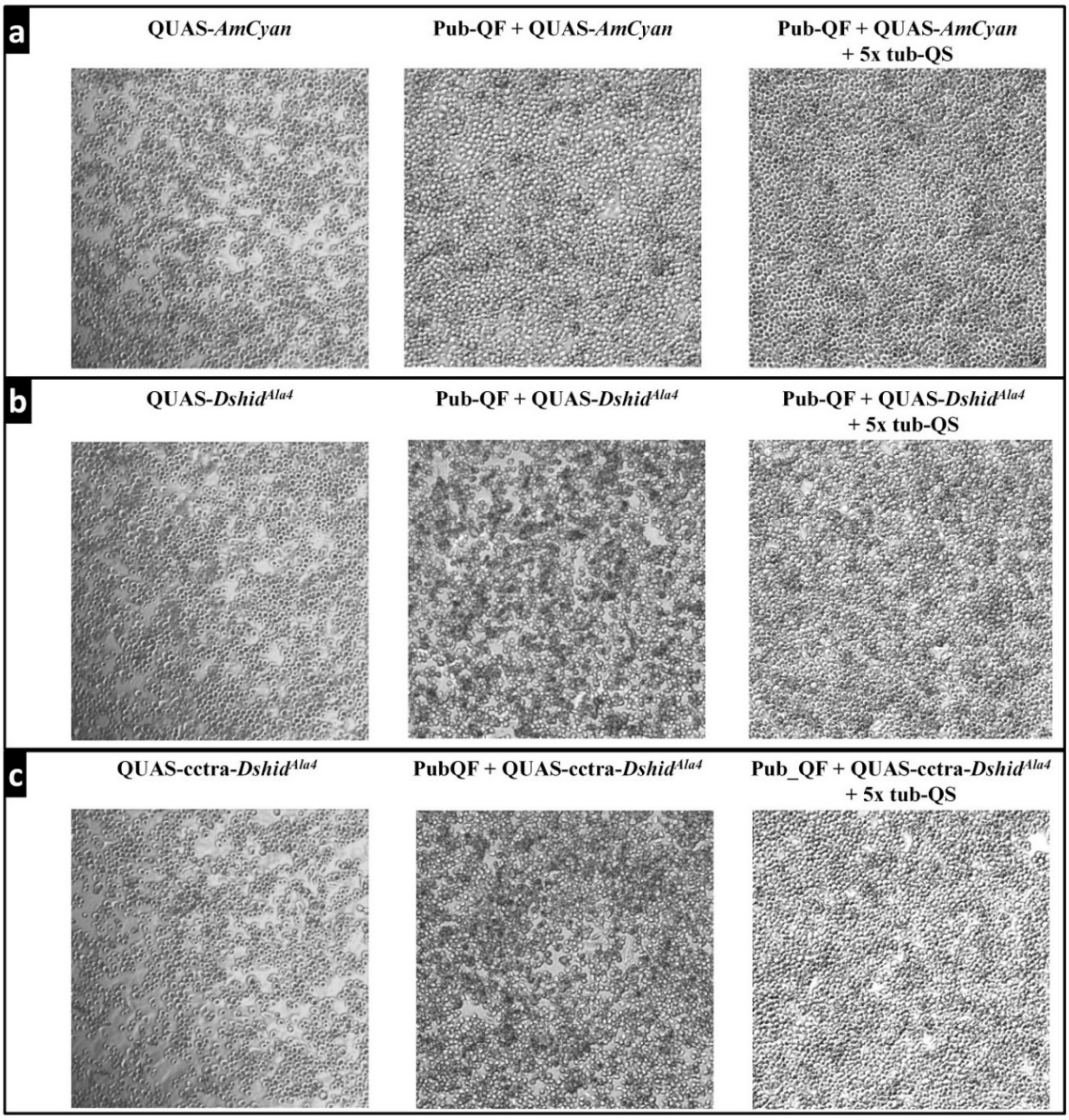


Figure 19: Q system-mediated induction and repression of cell death in Drosophila S2 cells.

(a) Control and QF/QUAS-driven expression of *AmCyan*. Left: S2 cells transfected with QUAS-*AmCyan* alone show normal cell morphology. Middle: Co-transfection with Pub-QF and QUAS-*AmCyan* results in robust *AmCyan* expression, with no apparent effect on cell viability. Right: Addition of 5x tub-QS, a repressor of QF activity, suppresses *AmCyan* expression, restoring morphology to that of the control. (b) Induction and repression of cell death by *Dshid*^{Ala4}. Left: S2 cells transfected with QUAS-*Dshid*^{Ala4} alone display normal morphology. Middle: Co-transfection with Pub-QF and QUAS-*Dshid*^{Ala4} leads to extensive cell death, as evidenced by the presence of rounded, shrunken, and fragmented cells. Right: Co-expression of 5x tub-QS with Pub-QF and QUAS-*Dshid*Ala4 suppresses cell death, resulting in cell morphology similar to the control. (c) Induction and repression of cell death by *Cctra-DshidAla4*. Left: S2 cells transfected with QUAS-*Cctra-Dshid*^{Ala4} alone show normal morphology. Middle: Co-transfection with Pub-QF and QUAS-*Cctra-Dshid*^{Ala4} induces significant cell death, as indicated by the presence of apoptotic cell bodies. Right: Addition of 5x tub-QS represses QF activity, preventing cell death and restoring normal cell morphology.

4.3.3 Germline transformation of *D. suzukii* to develop transgenic strains carrying Q system

4.3.3.1 Transgenic lines for Effector QUAS

In the process of developing a gene expression system mediated by QA in *D. suzukii*, the cloned constructs were introduced into embryos of wild-type (WT) *D. suzukii* by microinjection.

V288: One of the effector constructs, V288 (PUb-DsRed_Dmhsp70-QUAS-PUb-*AmCyan*), was initially injected to produce a transgenic line. 500 ng/µl Plasmid was co-injected with 200 ng/µl phsp-pBac transposase helper of AH286. For V288, a total of 351 embryos were subjected to injection. Among these, only 35 embryos successfully developed into adult flies, resulting in a 15% hatch rate. These adult flies were then crossbred with WT flies, resulting in the screening of 21 independent transgenic G1 lines (both males and females) for *DsRed* fluorescence, resulting in 67% of Larvae to adult survival. It's important to note that while 21 independent lines were obtained, this does not necessarily mean 21 unique integration events. Multiple lines could potentially arise from the same integration event in the G0 germline. It is also possible to have multiple integrations within one injected individual (Potter et al., 2010). Southern blot or PCR analyses would be required to confirm the number and locations of integration events. It is worth mentioning that *AmCyan* expression was not observed in any of these flies, confirming the integrity of QUAS as an effector; that gene of interest would not be expressed without QF. The difference in *DsRed* brightness between larvae depicted in Fig 21 (a) and (b) can be attributed to several factors, including differences in transgene integration sites and copy numbers. Despite these differences, the observation of *DsRed* fluorescence in both cases confirms the successful genomic integration and expression, consequently confirming the success of the transformation process in *D. suzukii*.

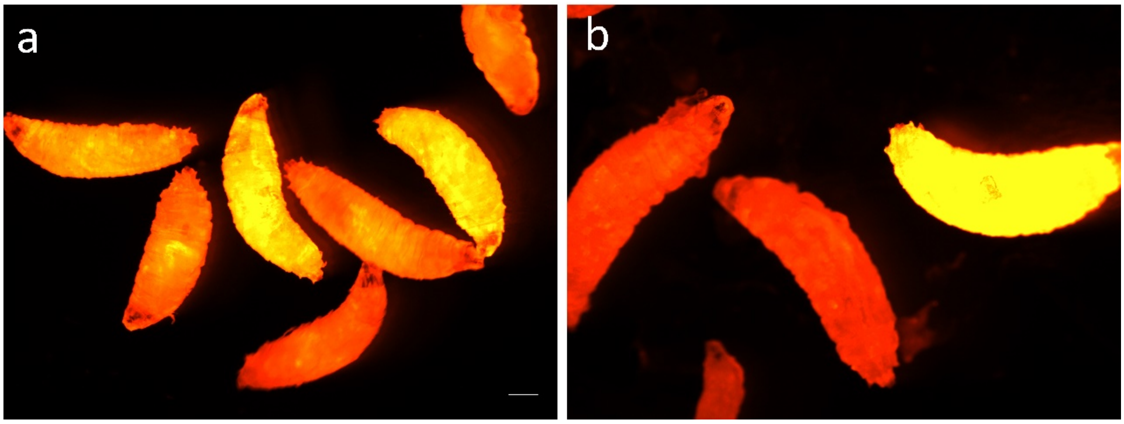


Figure 20: Fluorescent images of *Drosophila suzukii* transgenic larvae.
a) V288 (QUAS-*AmCyan*) has been integrated into the *D. suzukii* genome and shows *DsRed* expression under a fluorescent microscope. The *DsRed* signal appears uniformly distributed throughout the larval body. b) V289 (QUAS-*Dshid*^{Ala4}) has been integrated into the *D. suzukii* genome and shows *DsRed* expression under a fluorescent microscope. The fluorescence intensity appears slightly lower, which may be due to differences in integration site, and copy number (scalebar = 10 µm).

V289: Similarly, V289 was injected twice, first with AH286 as a helper construct and then with phsp-pBac transposase helper of AH286 as a helper. The construct-to-helper ratio was kept like that of V288, which is 500/200 ng/µl. In the first attempt, 286 embryos were injected. They yielded a 12% hatch rate and a 25% larvae-to-adult survival rate. Injection did not yield any integration events. In the second injection attempt, 500 ng/µl plasmid was co-injected with 200 ng/µl phsp-pBac transposase helper of AH286. A total of 435 embryos were injected, yielding an 8% hatch rate. Larvae to adult survival were as good as 82%.18 injected Go individuals were subsequently group-backcrossed with WT *D. suzukii*. Only one integration event occurred, leading to the establishment of 4 transgenic lines originating from the same event, as detailed in Table 4.1 and Figure 23. Achieving another strain from a single injection attempt with phsp-pBac transposase helper confirms its efficiency in transgenesis.

The successful generation of transgenic lines using the phsp-pBac transposase helper demonstrates its efficiency in *D. suzukii* transgenesis. However, to accurately determine the number of unique integration events and rule out multiple insertions, molecular analyses such as Southern blotting or inverse PCR should be performed on each line.

Table 4.1: Summary of microinjection experiments using effector (QUAS) plasmids in *Drosophila suzukii* embryos for transgenic line generation.

The “Effector plasmids” V288 and V289 were microinjected into the *D. suzukii* embryos to generate transgenic flies carrying effector constructs. The table presents details on plasmid combinations, concentrations, number of injected embryos, hatch and survival rates, number of emerged adults, G0 crosses, G1 transgenics, and observed transmission rates. Transgenic lines were identified based on the presence of fluorescent markers in the G1 generation..

Plasmid	Helper plasmid	plasmid/HP (ng/µl)	Heatshock	injected emryos	Htched larvae	Hatch rate	Avg	Emerged adults*	Larvae to adult survival	Avg	Go Crosses	G1 transgenic	Transgenic Line	Transmission rate %
V288_control	phsp-pBac	500/200	-	351	(52/351)	14,80%	14,80%	35	67%	67%	35	21	M_1f_1 , M_2f_1, F_5f_1, F_2m_1	60
V289_QUAS	AH286	500/200	-	286	(35/286)	12,20%	10,10%	9	25%	54%	2	0	-	0
V289_QUAS	phsp-pBac	500/200	-	435	(35/435)	8%		29	82%		18	1	M_1f_1,M_1f_1, M_1m_1, M_1m_2	6

*The difference in number of emerged adults and Go crosses is because all energed constructs were not crossed to avoid massive rearing in the incubators. Therefore transmission rate has been calculated as Go cross/G1 transgenic x 100.

4.3.3.2 Transgenic lines for Driver QF

In the pursuit of developing the transcriptional factor (driver QF) line, multiple injections of the **V291**_pBacXLII_attP-PUbEGFP-act5cB-QF#7-actinpolyA, were carried out. Unfortunately, despite several injections, no transgenic line was successfully generated. It was also noticed that QF7 had lethal effects, as evidenced by a low injection survival rate, which predominantly ranged from 3% to 10% on average 6.5%, and the larvae to adult survival at 13% on average. Unfortunately, for most injections, hatched larvae were found dead on the food vials (Fig. 22).

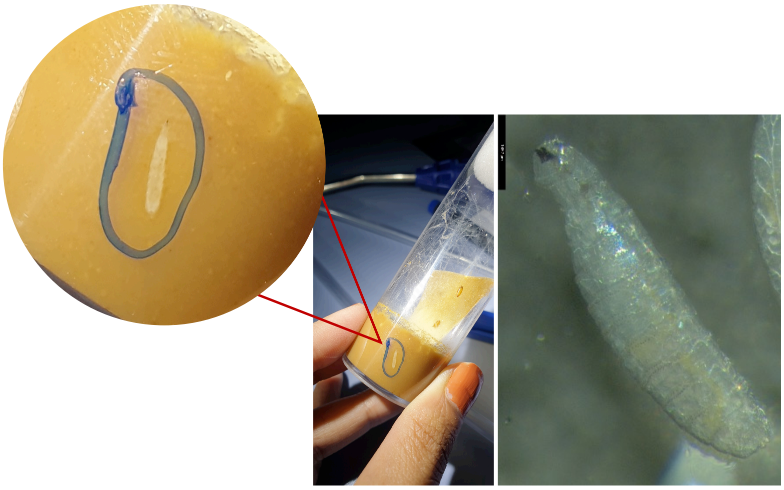


Figure 21: Screening of post-injection hatched larvae injected with QF driver.
The larvae injected with QF were mostly found dead inside the food vial. No other contamination in the food vials could cause this death.

To evaluate its viability, another genetic driver, **V321**_pBacXLII-attP-PUbEGFP-act5cB-QF#7-SV40-driver, was introduced, resulting in a hatch rate of 3% and a larvae-to-survival rate of 34%. It was observed that the QF7 driver exhibited some toxicity when introduced into the embryos. Consequently, an alternative variant of the QF driver denoted as QF7m1, was employed for subsequent injections. It is worth noting that QF7m1 has been previously recognized for its reduced toxicity and successfully generates strains when injected into *D. melanogaster* embryos, as compared to QF7 (Riabinina et al. 2015).

Two constructs, specifically V406_pBacXLII_attP-PUb-EGFP_tubP-QF7m1-hsp70 and V407_pBacXLII _attP-PUb-EGFP_Dmhsp70-QF7m1-hsp70, were introduced through injections following a similar protocol. **V406** underwent two rounds of injections, initially with an AH286 helper, resulting in a hatch rate of 19% and a larvae-to-adult survival rate of 25%. Subsequently, it was injected with the phsp-pBac transposase helper, yielding a hatch rate of 17% and a larvae-to-adult survival rate of 26%. As for **V407** underwent multiple injection attempts, Fig. 23. On average, these attempts yielded a hatch rate of 13% and a larvae-to-adult survival rate of 13%. Regrettably, no transgenic lines could be successfully established from these efforts, as indicated in Table 4.3.

4.3.3.3 Transgenic lines for Suppressor QS

Several attempts were undertaken to establish transgenic lines for the suppressor constructs. Two distinct plasmids were employed, each carrying different polyA tails following the QS suppressor. These plasmids were denoted as V295_pBacXLII -attP-PUbAmCyan-rev_tubP-QS-SV40 and V296_pBacXLII-attP-PUbAmCyan-rev_PUb-QS-SV40. They were introduced into wild-type (WT*)* *D. suzukii* embryos via injection.

The average hatch rate observed for V295 was 10%, while for V296, it was a modest 5.6%. Regarding larvae-to-adult survival, V295 exhibited an average of 45%, whereas V296 showed 44%. It is noteworthy that despite these injection attempts, no integration events were observed, as detailed in Table 4.2. Several constructs were injected to obtain transgenic lines of QF, QUAS, and QS to test the Q system *in vivo.* The graph shows each construct's hatch rate and larvae-to-adult survival rate.

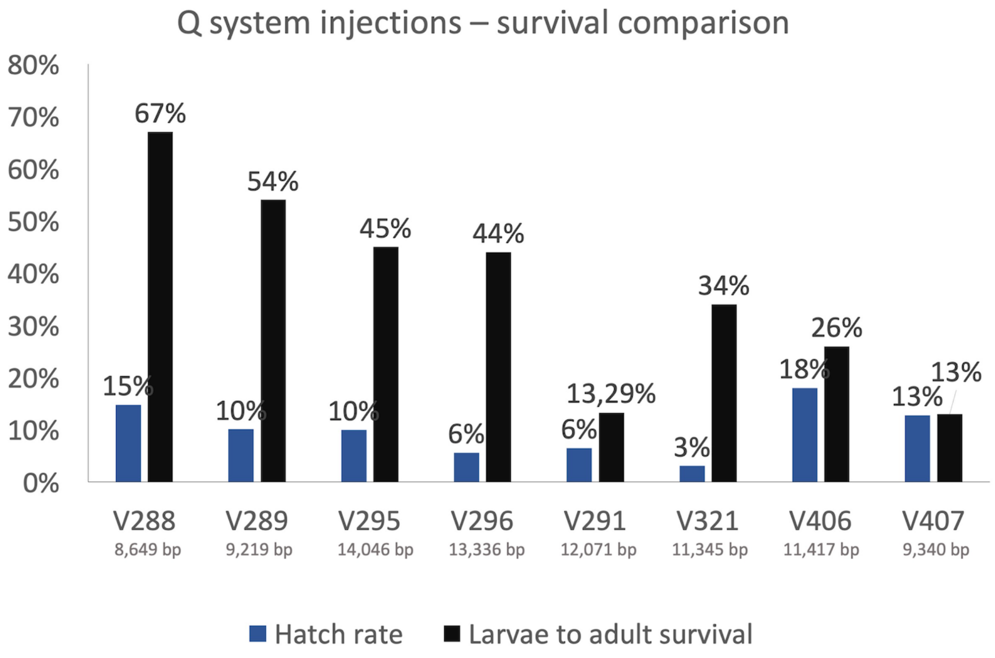


Figure 22: Comparison of injected Q system constructs.

The control construct V288 (8,649 bp) demonstrates larvae-to-adult survival (67%) despite a moderate hatch rate (15%), suggesting high developmental viability once hatched. QUAS construct V289 (9,219 bp) shows the larvae-to-adult survival (54%) with a 10% hatch rate. QS variants V295 (14,046 bp) and V296 (13,336 bp) exhibit similar performance with 45% and 44% larvae-to-adult survival respectively, though V296 shows a lower initial hatch rate (6% versus 10%). Among QF variants, performance varies considerably—V291 (12,071 bp) and V407 (9,340 bp) show the poorest larvae-to-adult survival (13.29% and 13% respectively), while V321 (11,345 bp) achieves 34% survival despite having the lowest hatch rate (3%). Notably, V406 (11,417 bp) demonstrates the highest hatch rate (18%) among all constructs but relatively modest larvae-to-adult survival (26%). These results indicate that construct size and type significantly influence both embryo viability and developmental progression.

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Table 4.2: Summary of microinjection experiments using driver (QF) plasmids in *Drosophila suzukii* embryos for transgenic line generation.

Q system "Driver plasmids" microinjected into the *D. suzukii* embryos to generate transgenic flies carrying effector constructs. The table presents details on plasmid combinations, concentrations, number of injected embryos, hatch and survival rates, number of emerged adults, G0 crosses, G1 transgenics, and observed transmission rates. Transgenic lines were identified based on the presence of fluorescent markers in the G1 generation.

Plasmid	Helper plasmid	plasmid/HP (ng/µl)	Heatshock	injected emryos	Htched larvae	Hatch rate	Avg	Emerged adults*	Larvae to adult survival	Avg	Go Crosses	G1 transgenic	Transgenic Line	Transmission rate %
V288_control	phsp-pBac	500/200	-	351	(52/351)	14,80%	14,80%	35	67%	67%	35	21	M_1f_1 , M_2f_1, F_5f_1, F_2m_1	60
V291_QF	V315	500/300	-	149	(4/149)	2,70%		4 (dead)	0		0	0	0	0
V291_QF	phsp-pBac	150/150	-	650	(17/650)	2,60%		4	23%		4	0	0	0
V291_QF	phsp-pBac	150/150	-	501	(18/501)	3,60%		1	5,50%		1	0	0	0
V291_QF	V315	250/250	-	549	(51/549)	9,30%		26	33%		17	0	0	0
V291_QF	AH286	250/150	60 min	387	(11/387)	2,80%		9	0		0	0	0	0
V291_QF	AH286	400/200	-	472	(46/472)	9,70%	6,49%	28	15%	13,29%	7	0	0	0
V291_QF	V315	400/250	-	725	(59/725)	8,10%		17	10%		6	0	0	0
V291_QF	V315	400/250	60 min	538	(30/538)	5,60%		16	16%		5	0	0	0
V291_QF	V315	400/250	-	441	(43/441)	9,80%		11	25%		11	0	0	0
V291_QF	AH286	400/250	60 min	455	(39/455)	8,60%		13	0		0	0	0	0
V291_QF	AH286	500/250	-	371	(32/371)	8,60%		12	18,70%		6	0	0	0
V321_QF	phsp-pBac	700/300	-	946	(29/946)	3,10%	3,10%	17	34%	34%	10	0	0	0
V406_QF	AH286	500/200	-	696	(134/696)	19,30%	18,05%	57	25%	26%	34	0	0	0
V406_QF	phsp-pBac	500/200	-	642	(108/642)	16,80%		44	26%		28	0	0	0
V407_QF	phsp-pBac	500/200	-	179	(15/179)	8,40%		-	0		0	0	0	0
V407_QF	phsp-pBac	500/200	-	259	(27/259)	10,40%		10	0		0	0	0	0
V407_QF	AH286	500/200	-	587	(48/587)	8,20%	12,82%	10	0	13%	0	0	0	0
V407_QF	V315	500/200	-	783	(159/783)	20,30%		77	46%		74	0	0	0
V407_QF	AH286	500/300	-	642	(108/642)	16,80%		44	18%		20	0	0	0

*The difference in number of emerged adults and Go crosses is because all energed constructs were not crossed to avoid massive rearing in the incubators. Therefore transmission rate has been calculated as Go cross/G1 transgenic x 100.

Table 4.3: Summary of microinjection experiments using suppressor (QS) plasmids in *Drosophila suzukii* embryos for transgenic line generation.

Q system "Suppressor plasmids" V295 and V296 were microinjected into the *D. suzukii* embryos to generate transgenic flies carrying effector constructs. The table presents details on plasmid combinations, concentrations, number of injected embryos, hatch and survival rates, number of emerged adults, G0 crosses, G1 transgenics, and observed transmission rates. Transgenic lines were identified based on the presence of fluorescent markers in the G1 generation.

Plasmid	Helper plasmid	plasmid/HP (ng/µl)	Heatshock	injected emryos	Htched larvae	Hatch rate	Avg	Emerged adults*	Larvae to adult survival	Avg	Go Crosses	G1 transgenic	Transgenic Line	Transmission rate %
V288_control	phsp-pBac	500/200	-	351	(52/351)	14,80%	14,80%	35	67%	67%	35	21	M_1f_1 , M_2f_1, F_5f_1, F_2m_1	60
V295_QS	phsp-pBac	500/200	-	269	(23/269)	8,60%	10,13%	15	52%	45%	12	0	0	0
V295_QS	phsp-pBac	500/200	-	336	(25/336)	7,40%		11	36%		9	0	0	0
V295_QS	V315	500/200	60 min	834	(120/834)	14,40%		82	47%		57	0	0	0
V296_QS	phsp-pBac	500/200	-	692	(31/336)	4,54%	5,62%	21	58%	44%	18	0	0	0
V296_QS	V315	500/200	60 min	687	(46/687)	6,69%		17	30%		14	0	0	0

*The difference in number of emerged adults and Go crosses is because all energed constructs were not crossed to avoid massive rearing in the incubators. Therefore transmission rate has been calculated as Go cross/G1 transgenic x 100.

The experimental findings presented in this section confirm the Q system's integrity in S2 cells and shed light on the functionality and potential applications of the Q system in genetic manipulation strategies. The morphological analysis conducted on the S2 cells transfected with pro-apoptotic genes under the influence of the Q system highlighted significant changes in cell confluency and morphology. These observations aligned with the expected outcomes of pro-apoptotic gene expression, further confirming their potential utility in programmed cell death processes. However, to strengthen the validity and reliability of these findings, it is crucial to emphasize the need for robust statistical evidence to support the results and comparisons made. Future studies should include cell count data, apoptosis assays, and appropriate statistical comparisons to evaluate the system's effects more rigorously.

In the subsequent section on the germline transformation of *D. suzukii* to develop transgenic strains carrying the Q system, several effector constructs (V288 and V289) were introduced into embryos via microinjection. While the hatch rates were 10-15%, the establishment of transgenic lines for V288 underscored the potential success of the system in generating transgenic strains, emphasizing the importance of the phsp-pBac transposase helper. In contrast, the development of the transcriptional factor (driver QF) line faced challenges, with no successful transgenic lines being generated. This was attributed to the observed toxicity of the QF7 driver and was partially mitigated by the introduction of QF7m1. Quantitative measurements of toxicity effects and statistical comparisons between QF7 and QF7m1 are needed to substantiate these observations. The attempts to establish transgenic lines for QF drivers (V406 and V407) demonstrated variability in hatch rates and larvae-to-adult survival rates, with no transgenic lines being successfully established, emphasizing the complexity of generating driver lines in the Q system.

The endeavors to establish transgenic lines for the suppressor construct presented mixed results. Despite 6-10% hatch rates and approximately 45% larvae-to-adult survival rates, no integration events were observed for the suppressor constructs V295 and V296, indicating potential challenges in using suppressors in the Q system. The reported survival percentages for QUAS (51%), QS (45%), and QF (22%) suggest differences in construct viability. However, these averages should be supported by statistical tests (e.g., ANOVA) to confirm if the differences are statistically significant.

In conclusion, while the experimental findings provide valuable insights into the Q system's potential in *D. suzukii*, there is a critical need to inject more constructs for comprehensive statistical analyses to support the observed results and comparisons. Future studies should incorporate appropriate statistical methods, including significance tests, confidence intervals, and effect size calculations, to enhance the robustness and reliability of the findings. This approach will provide a stronger foundation for understanding the Q system's functionality and potential applications in genetic pest management strategies.

5. Discussion

5.1. Pro-apoptotic genes: a promising approach for sustainable pest management in *D. suzukii*

Apoptosis is one of the fundamental and highly regulated processes in living organisms. It contributes to the development and maintains balance within the body. Apoptosis can be triggered by internal or external factors in response to stress signals. The pathway involves several pro-apoptotic genes that facilitate cellular changes, i.e., cytoplasmic shrinkage or nuclear fragmentation (Goyal et al., 2000). The essential pro-apoptotic genes from the RHG family are *reaper, head involution defective (hid),* and *grim.* It has been reported that RHG genes consist of two functional domains: IBM and GH3. IBM binds with Diap1 to release apoptotic caspases, and GH3 induces the mitochondrial death pathway in a caspase-independent manner (Olson et al., 2003). Schetelig group studied pro-apoptotic genes in Caribbean fruit flies before and concluded that the removal of one of these domains results in blockage of the pro-apoptotic activity, hence proving the importance of the existence of both domains (Schetelig et al., 2011). In this study, after the isolation of three pro-apoptotic genes, their protein sequences were analyzed to confirm their IBM and GH3 regions since they are responsible for initiating apoptosis (Sandu et al., 2010; Wang & Clem, 2011). Both motifs appear to be identical to other Drosophilids (Fig. 7).

The gene expression trend (RT-qPCR) from the studies of pro-apoptotic genes in *D. melanogaster* tells *Dmhid* is expressed highest, *Dmrpr* in moderate levels in pupae, and *Dmgrim* at low levels throughout development (Graveley et al., 2011). This study observed a similar pattern with RT-qPCR analysis (Fig. 8), suggesting the conserved fundamental roles of pro-apoptotic genes between two species. The *D. melanogaster* pro-apoptotic genes have been used to tightly regulate ectopic expression in other species i. e., *A. suspensa* (Schetelig & Handler, 2012) and mammalian cells (Tait et al., 2004). Some studies suggest that endogenous genes work more efficiently than homologous genes from closely related species (Li et al., 2014).

When these three genes were transfected in S2 cells independently, *Dsrpr* was observed to have less pro-apoptotic efficiency than *Dsgrim* and *Dshid*. In this perspective, *Dsgrim* and *Dshid* seem to be better candidate genes for TESS development in *D. suzukii*. From the analysis in S2 cells, it is observed that the functions of *Dshid* and *Dsgrim*, *Dmhid,* and *Dmhid*^{Ala5} are almost equally efficient in inducing cell death. Furthermore, a weak expression from *Dmrpr* has already been observed before (Edman et al., 2015; Schetelig et al., 2011). The mutated form of the hid gene was used in this study, where mutations in MAPK phosphorylation sites prevent the downregulation of HID by Ras signaling pathways (Bergmann et al., 1998). The unexpected results from the mutated version of *Dshid*^{Ala4} are challenging to understand because the mutated version of *Dmhid*^{Ala5} has been the most efficient in induced cell death. Previous studies showed that the *A. suspensa* and *L. cuprina* TESS strains using the phospho-mutated version of *hid* were more effective at causing cell death (Schetelig & Handler, 2012; Yan & Scott, 2015). However, our S2 cell culture analysis has shown different results. The only interpretation of this could be that the cell death-inducing ability of *Dshid*^{Ala4} might be different in S2 cells than in an analysis conducted on *in vivo* cell networks. This discrepancy may have arisen because S2 cells might exhibit different activity *in vivo* due to changes in biological components, signaling pathways, or protein interactions unique to this cell line. S2 cells exhibit a unique array of apoptotic regulators, such as DIAP1 and DIAP2, which may interact differently with the mutant *Dshid*^{Ala4} than they do in other cell types or animals (Muro et al., 2006). These findings highlight the necessity of accounting for cell type-specific responses and the constraints of generalizing results from *in vitro* systems to *in vivo* environments.

Recent studies show the Tet-Off system for *D. suzukii* has been established with the pro-apoptotic gene *Dmhid*^{Ala5} to induce female-specific lethality. Schetelig et al. (2021) developed a transgenic female-killing system using the nullo promoter to drive tTA expression and a sex-specifically spliced hid gene as the lethal effector. This technique achieved effective female mortality without tetracycline, with the female survival rates as low as 0.8% in certain transgenic lines.

Given this successful establishment of various efficient transgenic strains using *Dmhid*^{Ala5} that functions efficiently, a transgenic line with pro-apoptotic genes fused to an RMCE construct was injected. The *in vivo* experiment, which was conducted on a limited scale (Fig. 11), led to premature mortality, which prevented a definitive outcome.

5.2. Two pro-apoptotic genes together can induce maximum lethality.

It has been previously suggested from the results of Figure 9 that *Dsgrim* and *Dshid* are strong genes that induce lethality and can be generated to achieve an effective TESS for the SIT of *D. suzukii*. However, for field control use, we need to make sure that the lethality induced is 100%, leaving no chance of contamination of released insects with females.

Dsrpr and *Dshid* induced around 80% cell death in our experiment (Fig. 9); this degree of efficacy may be inadequate for practical field usage. The potential survival of even a minor fraction of females could undermine the efficacy of a SIT program. Previous studies suggested using a combination of pro-apoptotic genes causing higher level lethality than using a single pro-apoptotic gene (Schetelig et al., 2011). To ease up the process of development of a sexing strain, it would be better if the combination is inserted in a single construct to ensure the same insertion position. An efficient co-expression system has been published (Schwirz et al., 2020). In the case of *D. suzukii*, it has been reported to concurrently express two fluorescent genes using a short coding sequence incorporating picornaviral self-cleaving 2A peptides. This mechanism allows for the utilization of two or more genes in a proportional ratio through ribosomal skipping (Donnelly et al., 2001). Employing this system enables the independent expression of two or more copies of genes, facilitating the achievement of a high level of lethality.

In our S2 cell culture setup, *Dsrpr* demonstrated limited lethality when expressed alone, whereas it exhibited approximately 100% lethality when transfected in combination with another *Dsrpr*. A strange observation is that *Dsrpr,* which presents to be a weak lethality-inducing gene, as well as *Dshid*^{Ala4} when combined with 2A peptide, had the strong lethal expression, enough to cause almost 0% viability and similarly with *Dsgrim*. Thus, any of these combinations with conditional expression can be developed to build a TESS system for *D. suzukii*. However, TESS should be tested carefully to avoid any leaky expression of the construct to avoid rearing problems in the TESS system (Yan et al., 2020; Yan et al., 2017; Schetelig et al., 2016).

5.3. *Dsnullo* is a stronger embryonic promoter that induces gene expression in embryos.

For the genetic pest-control system with the above-mentioned pro-apoptotic genes. It is important to avoid unspecified gene expression that can induce undesired lethality in females, as reported in ovarian development, before reducing the productivity of those strains (Schetelig et al., 2016; Yan et al., 2017; Yan et al., 2020) and therefore early-stage gene expression is essential to allow lethality at early developmental stages of an embryo. These four cellularization genes were observed to be conserved in *D. suzukii* in their sequences and transcription levels in early blastoderm stages. The functional features of cellularization genes are the regulation of actin filaments, microfilaments, and membrane polarization (Mazumdar & Mazumdar, 2002). *Drosophila* embryogenesis has 17 stages, where the cellular blastoderm arises after 2 hours and lasts for around 50 minutes after the egg is laid (Campos-Ortega and Hartenstein 1985). The cellularization genes (*Dsnullo, Dsbnk, Dsslam,* and *Dssry-α*) have been studied to ensure early and stage-specific expression (Schetelig & Handler, 2012; Schetelig et al., 2009; Yan & Scott, 2015). Our results from RT-qPCR in this study show high expression of these genes in embryonic stages at 0-6 h max, and then expression reduces. qPCR analysis shows that *Dsslam* and *Dsbnk* expressed 400 to 12000-fold, suggesting them to be the most suitable candidates for a TESS. However, *Dssry-α* and *Dsnullo* can also be used as moderate promoters for later stages. This study primarily aims to analyze one or more appropriate early expression promoters, as multiple promoters may facilitate the development of independent systems within an insect, serving as a backup to establish a stable line that mitigates mutations and associated challenges in the released insects (Zhao et al., 2020).

This study observed that all four embryonic genes had an expression to coordinate the cellularization process: low expression for *Dsnullo* and *Dssry-α,* moderate expression for *slam,* and high expression for *Dsbnk*. In previous studies, the cellularization gene expression was observed at 0.5 h of the post-egg laying stage (Rose & Wieschaus, 1992; Edman et al., 2015; Yan & Scott, 2015). *Dssry-α* promoters have been tested to induce embryonic lethality in transgenic flies of *A. suspensa* (Schetelig & Handler, 2012). These promoters were further tested in S2 cells for functional analysis in the *Drosophila* system. Even though *Dsnullo* showed the lowest level of expression as compared to *Dsslam, Dssrya,* and *Dsbnk* at 0–6 h embryonic stage, its promoter successfully derived the most observable expression of *DsRed* in the cell culture tests (Yan et al., 2020). This observation suggests that *Dsnullo* mediates higher protein production in embryonic cells compared to other early promoters. That makes *Dsnullo* a prominent embryonic promoter to be used in TESS for *D. suzukii*. In comparative studies, the *Dsnullo* promoter regularly outperformed other cellularization gene promoters, including *Dssry-α, Dsbnk*, and *Dsslam*, in facilitating the expression of transgenic genes during early embryogenesis. The strong promoter activity was also confirmed in transgenic *D. suzukii* lines, where *Dsnullo*-driven constructs displayed significant and prompt expression of effector genes at the crucial early blastoderm stage (Schetelig et al., 2021). Moreover, the *Dsnullo* promoter has been modulating the expression of the tetracycline transactivator (tTA) in Tet-Off systems, allowing precise regulation of transgenic expression via antibiotic supplementation (Yan et al., 2023).

5.4. The efficiency of the Q system in S2 cells suggests it can be used to build TESS strains of *D. suzukii*

Q system is a highly sophisticated tool for manipulating gene expression as a binary expression system. The Q system is often referred to as a "binary expression system" due to its core components: the transcriptional activator QF and the QUAS binding site (Potter et al., 2010). However, its QS, the repressor, plays a critical role as a third component in modulating QF–QUAS interactions. QS suppresses QF activity and prevents gene expression, while QA can relieve this repression by inhibiting QS (Elowitz & Leibler, 2000).

The main goal of this study has been to develop Q system-based conditional expression system that could control the expression of lethal genes in *D. suzukii.* Q system was previously developed successfully in *D. melanogaster* (Potter & Luo, 2011). However, we have incorporated the Q system components into *piggyBac* vector bones for germline transformation. For the initial tests, to confirm whether *piggyBac* incorporated Q system genetic elements would work in the *Drosophila* system, the initial experiments with *AmCyan* as a gene of interest have suggested the system works efficiently in the *Drosophila* system. A similar test was performed in another study by (Potter et al., 2010), where luciferase (*luc2*) activity was expressed in S2 cells under the same ubiquitous promoters, *actin5c* was used to drive QF and QS (regulatory genetic elements of the Q system). Transfecting S2 cells with QF and QUAS-*luc2* led to a remarkable 3300-fold increase in gene expression, with QF alone showing no capability of inducing gene expression.

In our investigation, considering *AmCyan* as the gene of interest and *DsRed* as a marker gene for confirming transient transfection, we needed to establish a ratio of cells expressing both *DsRed* and *AmCyan*. Our results also had a similar expression pattern as of (Potter et al., 2010), where QUAS alone failed to give any gene expression, and co-transfection of QF with QUAS gave highly significant *AmCyan* expression. However, suppression of expressed genes is subject to several copies of QS added to the system. 1 to 3 copies of QS had suppressed gene expression to 50%. However, five copies of QS showed a significantly higher level of gene suppression. Similar is the case with the addition of QA to reverse QS activity. The addition of 5 µg/ml, 50 µg/ml, and 5 mg/ml QA resulted in the re-expression of AmCyan. However, 5 mg/ml QA resulted in more *AmCyan* expressing cells compared to lower concentrations. It is interpreted that the higher the concentration, the more the QS reversion. A similar trend was observed with luciferase activity (Potter et al., 2010) when different concentrations of QA were used to revert QF-QUAS-QS-induced gene suppression; there, 250 µg/ml and 5 mg/ml QA concentration had reduced luciferase activity from 1,000-fold to more or less100-fold, which is also highly significant. The cell culture studies were limited to a maximum QA concentration of 5 mg/ml, as they were conducted only to assess whether the system and gene combinations are applicable to *Drosophila* systems.

The efficiency of the Q system in S2 cells indeed suggests promising potential for developing strains of *D. suzukii*. QF has been studied to induce expression by approximately 3,300-fold compared to QUAS alone (Potter et al., 2010). Such high expression is important for efficiently driving deadly genes in TESS strains. The Q system also displayed dose-dependent inhibition by QS and inducibility with QA (Riabinina et al., 2016). This fine control over gene expression is advantageous for TESS applications, allowing suppression during rearing and activation when needed to eliminate female insects. When used with another expression system GAL4/UAS system, QF showed minimal cross-activation of UAS (~1,500-fold less than QUAS activation), and GAL4 exhibited low cross-activation of QUAS (~200-fold less than UAS activation), showing high specificity for using multiple binary systems in a single organism (Potter et al., 2010).

Structural analyses reveal that QUAS, like UAS, contains a core consensus motif (CGG-N11-CCG) critical for transcription factor binding (Riabinina et al., 2015). In a previous *Drosophila* study using 10xUAS or 20xUAS constructs resulted in high expression levels in specific contexts, such as optogenetic tools (Guglielmi et al., 2015). Since QUAS shares similar mechanism as of UAS and both employ modular transcription factors (QF for QUAS, GAL4 for UAS) that bind to tandem repeats of their respective cis-acting sequences to recruit RNA polymerase II (Potter et al., 2010) it can be expected that by increasing the number of QUAS repeats may directly improve transcriptional activity as it would provide additional binding sites for the QF activator. Additionally, the terminator efficiency can also critically impact transcriptional fidelity.

The long-term stability of transgenic constructs is crucial for the sustained effectiveness of TESS strains. Although Potter et al. (2010) reported that Q system remained stable across multiple generations in *D. melanogaster* this needs to be thoroughly verified in *D. suzukii* because genomic context and epigenetic factors can influence transgene stability across species (Wimmer, 2003). Additionally, potential effects of Q system components and QA on *D. suzukii* fitness need to be thoroughly assessed for comprehensive fitness assays, including life history traits, reproductive capacity, and competitive ability, to ensure that TESS strains remain competitive in field conditions (Leftwich et al., 2016). Another important aspect to study is the kinetics of gene induction and suppression using the Q system in *D. suzukii* to ensure precise temporal control of lethal gene expression for TESS applications (Zhu & Handler, 2012). Future research should prioritize organism-specific fine-tuning, particularly in non-model systems where endogenous transcriptional machinery may differ (Nguyen, 2024).

5.4.1. *piggyBac* can be a good expression vector for Q system elements.

The *piggyBac* system is an effective transposon vector that has found great application in harboring the genes of interest for functional analyses. It offers several advantages, its 'cut and paste' mechanism ensures that any –genes are precisely inserted within chromosomal sites flanked by the TTAA sequence (Agren & Clark, 2018). the *piggyBac* transposon system has been widely used as an expression vector in insects (Schetelig & Handler, 2013). However, the expression of any gene depends highly on the location of transgene integration (Sarkar et al., 2006).The notable feature of *piggyBac* lies in its exceptional ability to integrate Q system elements into the host genome efficiently (Chen et al., 2020). One of *piggyBac's* benefit is its functional flexibility in various hosts, such as bacteria, yeast, insects, and mammalian cells.

One issue with *piggyBac* mediated transformation is that the expression of transgenes can be impacted by the site of integration. If integrated near a tissue-specific transcription enhancer, transgene expression may occur in unintended tissues and stages. Integration into a heterochromatic region can result in low levels of transgene expression. These positional effects present challenges for regulated gene expression systems, such as the tetracycline-dependent transactivator (tTA) (Wimmer, 2003). The *piggyBac-based* transformation has been used in *Drosophila* to successfully build a dependent expression system (Schetelig & Handler 2013).

5.4.2. Pro-apoptotic genes are compatible with the Q system to induce lethality.

After confirming the Q system efficiency by the AmCyan reporter gene, further effector constructs were built using pro-apoptotic genes *Dsrpr*, *Dshid*^{Ala4,} and *Dsgrim* as reporter genes with QUAS. The purpose of this study was to build

TESS to control lethal gene expression. Like the results from *AmCyan* expression, the absence of gene expression with only effector QUAS shows in the target of QUAS and no leakiness of the construct. Similarly, co-transfection of the QUAS-*rpr*, QUAS-*hid*^{Ala4,} and QUAS-*grim* with QF resulted in a reduced number of viable cells, suggesting lethality has been induced in the cells. Since pro-apoptotic genes have been transfected into S2 cells before (Fig. 9), their transfection under QUAS did not induce any lethality, showing the integrity of QUAS as soon as the constructs with co-transfected with QF (the activator of Q system) a significant cell death was observed.

5.4.3. sex-specific spliced intron can be used for Q system.

A potential strategy for implementing the Q system in the sterile insect technique is to utilize a sex-specific spliced intron to introduce expression of female-specific lethality. Schetelig and Handler (2012) demonstrated the use of sex-specific introns to create conditional female-specific lethality in *Anastrepha suspensa*. They utilized the transformer (*tra*) gene's sex-specific splicing to control the expression of a lethal gene, resulting in female-specific mortality. This approach has been further adapted and applied in *Drosophila suzukii* as well where the sex-specific splicing of the transformer gene has been used to develop genetic sexing strain (Wimmer et al., 2014). The use of a sex-specific spliced intron in the Q system (Fig 4b) could offer several advantages, particularly in the context of the sterile insect technique (SIT):

- **Female-specific lethality:** Female-specific lethality is acheived by incorporating a sex-specific spliced intron, into the contstruct. A sex-specific intron can induce lethality in female flies by exploiting sex-specific alternative splicing to restrict functional expression of a lethal gene to females. Several systems are using the transformer (*tra*) intron, such as the tetracycline-transactivator (tTA) system in *C. hominivorax* (screwworm) or *D. suzukii.* In these systems the intron is retained in males but spliced out in females due to sex-specific spliceosomal recognition. One example is the *Chtra* intron in *C. hominivorax* is inserted into the tTA coding sequence. In females, spliceosomal machinery recognizes the intron’s polypyrimidine tract and 3′ splice site, excising it to produce functional tTA mRNA. The resulting tTA protein binds tetracycline operator (tetO) sequences, that induces lethality through transcriptional squelching (Concha et al., 2020; Scott et al., 2022). This approach produces a lethal gene expression only in females, resulting in effective reduction of the female population while allowing males to be released. This can significantly impact the reproduction rate of the pest species (Ray et al., 2023). By incorporating a sex-specific intron into the Q system for TESS, the female targets can be eliminated in early development stage reducing the rearing cost and ensuring that only male insects are released into the field. This allows for a more controlled release of sterile insects and minimizes the potential harm to non-target populations and reduces the cost associated with raising and sterilizing both male and female insects (Schetelig & Handler, 2012; Ogaugwu et al., 2013).

- **Reduction in non-target effects:** Utilizing sex-specific splicing minimizes the impact on non-target species. Since the lethality is restricted to females of the target species, it reduces the risk of affecting other organisms in the ecosystem. By utilizing a sex-specific spliced intron in the Q system for the sterile insect technique, precise and efficient control over the population of pest insects can be achieved without the need for physical sex separation or the use of harmful chemical pesticides. Sex-specific splicing occurs early in embryonic development in many insects (Ray et al., 2021). By utilizing these early-acting mechanisms, the Q system could achieve precise temporal control over gene expression, potentially eliminating females at very early developmental stages (Concha et al., 2022)**.**
- **Cost-effectiveness:** Implementing a system that selectively targets females can reduce the costs associated with rearing and sterilizing both sexes. Focusing resources only on the production and release of sterile males also improves the overall efficacy and cost-effectiveness of SIT programs. Schetelig et al. (2009) demonstrated this through a transgenic system in *C. capitata* (medfly), where female-specific mortality during embryogenesis eliminated the costs associated with rearing females through larval, pupal, and adult stages. In another study, Yan et al. (2021) highlighted that feeding larvae constitutes a significant portion of operational costs due to the exponential increase in dietary requirements as larvae develop. By removing females before this phase, facilities can reduce larval biomass by approximately 50%, leading to proportional savings in diet costs. The timing of female lethality remains as a critical determinant of cost efficiency. Conditional systems that induce mortality at later developmental stages, such as during the pupal phase, fail to fully capitalize on potential savings. For example, Thomas et al. (2000) compared medfly strains with pupal-stage versus embryonic female lethality and found that the former required 25–30% more dietary yeast due to the extended rearing period. This aligns with Leftwich et al. (2016), who estimated that shifting female lethality from the pupal to embryonic

stage in New World screwworm (*Cochliomyia hominivorax*) programs could reduce larval diet expenditures by up to 40%. Similarly, Champer et al. (2024) designed a CRISPR-Cas9-mediated system in *C. capitata* that combined sex conversion and embryonic female lethality. This system demonstrated a 37% reduction in rearing costs compared to conventional methods. The progressive increase in resource consumption during larval development means that early female removal impacts cost reduction. Furthermore, embryonic lethality systems mitigate risks associated with accidental female releases, which could otherwise lead to unintended population persistence in the field (Champer et al., 2024).

- **Ensuring female-free release batches:** Manual or mechanical sorting errors can compromise the male-only releases required for effective population suppression, while the infrastructure and labor for these processes further inflate costs. In contrast, transgenic embryonic sexing systems (TESS) integrate molecular mechanisms, such as tetracycline-regulated lethal genes, to ensure that only males survive beyond the egg stage (Yan et al., 2021; Leftwich et al., 2016). A notable example is the TESS developed *for Lucilia cuprina* (Australian sheep blowfly), which achieved 100% female lethality during embryogenesis (Yan & Scott, 2021). These findings emphasize on the importance of operational advantages of implementing embryonic female lethality in SIT. By integrating these molecular tools to achieve sex-specific lethality at the earliest viable stage, SIT program can be optimized for minimum labor-intensive processes, and enhance the overall scalability of insect-rearing operations.

In this study when S2 cells were transfected with the QUAS with female-specific intron (*Cctra*), and in parallel transfected without female-specific intron (*Cctra*) (Fig. 20), the lack of observable difference between transfected *Drosophila* S2 cells could be because the S2 cells are derived from late stage *Drosophila* embryos and do not maintain sex-specific regulation systems (Schneider I., 1972; Ray et al., 2023). Therefore, S2 cells may not express the required splicing factors to process the *Cctra* intron correctly. The transformer (*tra*) gene involved in sex-specific splicing may not be active in these cells (Sturtevant, 2023).

Another reason could be that the *Cctra* intron is from *C. capitata* (medfly), while S2 cells are from *D. melanogaster*. Despite the conservation of sex determination mechanisms, there may be species-specific differences in splicing regulation (Salvemini et al., 2014). Therefore, Further investigation into the specific splicing regulatory elements might be required for *Cctra* function in different contexts to provide valuable insights for developing more robust genetic sexing systems.

5.4.4. Trouble shoots of microinjections.

After several failed attempts to generate a transgenic line of QF, it can be concluded that the QF might be toxic to flies. The data from extensive microinjection experiments (Figure 23 and Table 4.2) provide quantitative estimation for the toxicity of QF. Control injections V288 demonstrated substantially higher hatch rates (15%) and larvae-to-adult survival (67%) compared to QF-containing constructs. Among QF variants, V321 showed the most severe toxicity (3% hatch rate), while V406 exhibited the best survival ratio (18% hatch rate, 26% larvae-to-adult survival). The injection data demonstrates that while V406 and V407 has a marginally higher hatch rate (18% vs. 15% for control), it displays reduced larvae-to-adult survival (26% compared to 67% for the control) suggesting that QF7m1's toxicity might have manifested during post-embryonic development rather than during embryogenesis. The improvement in survival ratios from QF7 to QF7m1 illustrates the benefit of structural modifications (MD deletion) and terminator optimizations (hsp70 terminator). However, the persistent inability to establish transgenic lines with any variant suggests that residual toxicity remains sufficient to prevent stable transformation. The injection results from this study also suggest that *D. suzukii* may be more sensitive to QF toxicity than *D. melanogaster*, where Riabinina et al. (2015) successfully generated stable lines with QF2w (QF7m1 equivalent).

For future experiments, it is recommended to prioritize in vivo testing of reported embryonic promoters, especially *Dsbnk* (a comparatively weak promoter), to inject QF constructs (V403, see appendix. 7) and validate their potential in the Transgenic Embryonic Sexing System (TESS). Continued efforts to optimize the Q system-based conditional expression system will contribute to its broader applicability. Generating all three transgenics carrying QF, QUAS, and

QS either individually and then crossing them over to obtain an All-in-one strain for testing with QA or cloning All-in-one construct with QF-QUAS-QS by using bicistronic express on (Schwirz et al., 2020) can be pursued. However, achieving transformation with such a long construct poses significant challenges.

5.4.5. Reducing toxicity of QF

The QF activation factor consists of three structural domains: a DNA binding and dimerization domain DBD, a middle domain MD, and a transcriptional activation domain AD. These domains and their functionality have been studied in detail since QF appeared to be toxic in the *Drosophila* system (Riabinina et al., 2015). In a previous study (Potter et al., 2010), the toxicity of QF was observed while generating *D. melanogaster* lines. When using the SV40 terminator, which enhances mRNA stability and prolongs transcript half-life, researchers observed lethal levels of QF protein accumulation. This overexpression prevented the establishment of stable transgenic lines, as ubiquitous QF expression under strong promoters (e.g., tubulin or actin) caused developmental lethality or severe fitness defects in surviving adults. But they overcame this toxicity when the SV40 terminator was replaced by the hsp70 terminator a weaker transcriptional termination signal. This modification reduced mRNA stability, leading to lower steady-state QF protein levels. The adjusted expression brought QF concentrations below the toxic threshold while maintaining sufficient activity to drive QUAS-regulated transgenes.

Middle Domain Deletion: The native QF protein possessed a middle domain in the QF protein, a residue that was established as a significant contributor to toxicity. This domain was shown to be non-essential for achieving the transcriptional activation effect of QF. Riabinina et al. (2015) demonstrated QF toxicity through systematic domain analysis and transgenic experiments in *D. melanogaster*. Through their experimental analysis, they discovered that constructs containing the middle domain of QF either failed to produce transgenic animals or resulted in extremely unhealthy flies (Riabinina et al., 2015). This finding implicated the QF middle domain as the major source of toxicity. Based on this dentification of the middle domain as the source of toxicity, they created two improved variants of QF with reduced toxicity but maintained functionality. The first variant, QF2, was generated by completely removing the middle domain, resulting in a direct fusion of the QF DNA binding domain to the QF activation domain. This modified transcription factor retained high activity levels and remained repressible by QS, similar to the original QF, but without the associated toxicity. QF2 thus became the preferred choice when high transcriptional activity was required (Riabinina et al., 2015). The QF7 (Addgene #46127) used in this study represent the QF2 variant.

C-Terminal Modification: The second variant, $QF2^w$, differs from QF2 as the last two amino acids (glutamic acid and glutamine) on the C-terminus of QF2 were replaced by four lysine residues in $QF2^w$. This mutation changed the charge on the C-terminus from negative (E-Q) to positive (K+K+K+K+). This alteration in charge reduces the transcriptional activity of $QF2^w$ compared to QF2, making QF2w relatively a weak transcriptional activator and also reduced the potential for toxicity while still allowing for effective gene expression control (Riabinina et al., 2015; Riabinina & Potter, 2016). The QF7m1 construct (Addgene #46126) used in this study contains the key C-terminal modification characteristic of $QF2^w$ as described in Riabinina et al. (2015)

The injections data of this study provide evidence supporting the QF toxicity. The QF7 variant (corresponding to QF2 in Riabinina et al., 2015) was designed to reduce toxicity through deletion of the middle domain. However, our results with construct V291_pBacXLII-attP-PUbEGFP-act5cB-QF#7-actinpolyA and V321_pBacXLII-attPPUbEGFP-act5cB-QF#7-SV40 demonstrate that despite this modification, toxicity persists at levels sufficient to result in failed establishment of transgenic lines. Our injection data revealed severely compromised survival rates (for V291: average 6.5% hatch rate, 13% larvae-to-adult survival and for V321: average 3% hatch rate, 35% larvae-to-adult survival), suggesting that MD removal alone does not completely eliminate QF toxicity in *D. suzukii*. The QF7m1 variant (equivalent to QF2w in Riabinina et al., 2015) incorporates additional C-terminal modifications, replacing the terminal glutamic acid and glutamine residues with four lysines. This alteration changes the charge from negative to positive, reportedly resulting in reduced toxicity. Our experiments with constructs V406_pBacXLII-PUb-EGFP_tubPQF7m1-hsp70 and V407_pBacXLII-PUb-EGFP_Dmhsp70-QF7m1-hsp70 showed improvement in hatch rates (18% and 13%, respectively) compared to QF7, but still failed to generate viable transgenic lines. This suggests that while QF7m1's modifications reduce toxicity, they remain insufficient to enable stable transgenesis in our experimental system.

Since both these versions still appeared to be toxic in *D. suzukii*. These results suggest the need for further mutated versions of QF for lethality reduction Possible future enhancements include targeting further refinements to the MD of QF for the successful transformation in *D. suzukii.* Since the MD was identified as the major source of QF toxicity, further studies could focus on identifying specific regions within the MD that contribute most to toxicity. It is needed to target more deletions or modifications in MD, that would maintain functionality while further reducing toxicity. While the original activation domain (AD) was not the primary source of toxicity, experimenting with different ADs or creating chimeric ADs could potentially yield variants with even lower toxicity and optimal activity levels. These proposed improvements could be tested individually or in combination to develop next-generation QF variants with minimal toxicity and optimal functionality for various applications, including pest control strategies in *D. suzukii*.

Additionally, further studies on the long-term effects and stability of these variants in different organisms would be beneficial to understand their potential in practical applications fully. In general, this alteration has helped to neither overstimulate nor undercut the transcriptional activity of QF and growing lines in *D. melanogaster*. However, further research or modifications are needed for its implementation in *D. suzukii.*

Use of terminator: The experimental evidence demonstrates that regulatory elements significantly influence QF toxicity. Potter et al. (2010) reported that replacing the SV40 terminator with the hsp70 terminator reduced QF toxicity, enabling the generation of viable transgenic lines. This terminator substitution likely functions by reducing mRNA stability and consequently lowering steady-state QF protein levels below the toxic threshold. Our comparative analysis of V321_pBacXLII-attPPUbEGFP-act5cB-QF#7-SV40-driver (3% hatch rate) versus hsp70-terminated constructs (13-18% hatch rate) supports this finding, showing improved embryonic survival with the hsp70 terminator.

Use of Promoter: Promoter strength can also emerge as a critical determinant of QF toxicity. Our experimental design incorporated the strong ubiquitous promoters. It is recommended to use relatively weak embryonic promoters (that has been isolated already) i.e., Dsbnk to drive QF expression, following the rationale that weaker promoters might reduce QF7/QF7m1 expression below toxic levels. The choice of promoter needs to be optimized to meet the desired gene expression, which consequently impacts experimental results. The expression levels of QF can be optimized by using a combination of different promoters and enhancers to regulate its expression (Stojanov et al., 2020). For example, the minimal hsp70 promoter, consisting of a 250 bp core sequence, has been shown to drive low basal expression while retaining inducibility. In *D. melanogaster*, this promoter has been observed to exhibit approximately 10- to 100-fold lower baseline activity compared to the full-length hsp70 promoter (Riabinina et al., 2015). Alternatively, endogenous promoters such as *bnk* or *slam* in *D. suzukii* have demonstrated weaker transcriptional activity (Yan et al., 2020). Future work should prioritize the testing of such weak promoters, particularly in combination with QF2 or QF2w variants, to reduce toxicity while ensuring adequate transcriptional activation of lethal effectors for SIT-based applications.

5.4.6. other reasons for not having a transgenic line.

Creating transgenic lines through microinjections is a common technique used in genetic engineering to introduce exogenous genes into organisms. Despite the marginally higher hatch rate of V406 and V407, no transgenic lines were established from any QF7m1 injections. Successful transgenesis requires not only initial embryonic survival but also germline integration and transmission, processes that appear severely compromised by even the modified QF variants. However, there can be several reasons for not successfully generating a transgenic line using this method.

- **Low efficiency:** Microinjection techniques are often inefficient, and only a small percentage of injected embryos successfully incorporate the transgene into their genome. One explanation is that the introduction of recombinant DNA occurs in the cell cytoplasm, and only a minor portion successfully reaches the nucleus (Chenuet et al., 2009).

- **Embryo loss:** The process of microinjection can be stressful for embryos eventually leading to a high rate of embryo loss. depending on several stress levels caused by microinjections (injection pressure, needle shape and size of hole, climate of injection room, mishandling of embryos etc) this process can lead to chances of successful transgenic integration. Automated injection systems (aided by computers and microprocessors) have enabled consistent and reproducible high injection rates, facilitating quantitative microinjection (Jinturkar et al., 2011).
- **Off-target effects:** Unintended integration into genomic regions can be caused by micro injection of exogenous genes. It can lead to unpredictable effects on the development and phenotype of the organism. Identification of permissive sites for transgene expression and utilizing them for the efficient introduction of single-copy transgenes through homologous recombination can reduce off-target effects(Wallace et al., 2000).
- **Helper plasmid selection:** Helper plasmids can also emerge as a critical factor, with V407/V315 combinations achieving a 20.3% hatch rate versus only 10.4% with phsp-pBac. This pattern aligns with Handler and Harrell's (2001) observation that transposase source and expression dynamics can dramatically affect integration efficiency. For example, <u>Different helper plasmids</u> were used across experiments. Table 4.2 shows that V406 was injected with either AH286 or phsp-pBac helpers, while V288 utilized only phsp-pBac. These different helpers may influence early embryonic survival through varying transposase expression kinetics, as demonstrated by Häcker et al. (2023) and Yan and Schetelig (2024) in their systematic comparisons of helper plasmid performance. Häcker et al. (2023) directly compared phsp-pBac plasmid helpers with synthetic capped mRNA transposase sources in *D. suzukii*. They observed that embryos injected with mRNA helpers exhibited 2.5x higher transformation efficiency than those using plasmid helpers, but hatch rates dropped by 60% at mRNA concentrations exceeding 500 ng/µl due to cytotoxicity. In contrast, *phsp-pBac* plasmid helpers produced stable hatch rates (~40–50%) but lower transformation success (<10%), likely

due to delayed transposase expression from heat-shock promoters. Similarly, Yan and Schetelig (2024) emphasized that helper plasmid concentration and embryo desiccation time critically affect survival. Their optimized protocol for *D. suzukii* microinjection recommends 200 ng/µl mRNA combined with a 90-minute embryo desiccation period, balancing hatch rates (55–60%) and transgenesis efficiency (25–30%). These findings underscore that helper selection is not only a technical variable but a determinant of developmental viability, particularly in *D. suzukii*.

- **DNA concentration optimization:** Our experiments utilized standard concentrations of donor and helper plasmids (500/200 ng/µl) for microinjection, which aligns with recent protocols for *D. suzukii* transformation (Yan & Schetelig, 2024). However, Yan and Schetelig (2024) demonstrated that increasing concentrations to 700 ng/µl (donor) and 300 ng/µl (helper) improved transformation efficiency for some constructs, albeit with reduced hatch rates. This trade-off is evident in our results with V406, which achieved the highest hatch rate (18%) among QF constructs but failed to produce transgenic lines. The data from our V288 control injections showed consistent hatch rates around 14.8% across experiments, suggesting that the injection technique remained stable. However, varying DNA concentrations for the QF constructs (from 150/150 to 500/300 ng/µl) produced inconsistent results, with no clear pattern of improved survival at lower concentrations. This indicates that the nature of the genetic cargo, rather than simply DNA concentration, influenced embryonic development and transformation success. While higher DNA concentrations can enhance transformation potential, they simultaneously cause cytotoxicity. Yan and Schetelig (2024) recommend intermediate concentrations (~250-400 ng/µl) to balance these effects, which aligns with our most successful injections.
- **Construct size and complexity:** The successful QUAS constructs contain smaller functional elements than the QF and QS constructs, aligning with established inverse relationships between insert size and transposition efficiency (Fraser et al., 1995). Large constructs face multiple disadvantages, i.e., reduced packaging efficiency during embryonic cell division, increased distance between terminal inverted repeats, and

potentially disruptive secondary structures that interfere with transposase accessibility (Handler & O'Brochta, 2012). The consistent failure of all larger constructs (QF/QS) despite varying technical parameters strongly supports size/complexity as a limiting factor. The most striking pattern observed in this study is the inverse relationship between construct size and transformation success. Only the smallest constructs (V288 at 8,649 bp and V289 at 9,219 bp) showed any detectable germline transmission (60% and 6% respectively), while all larger constructs (>9.3 kb) failed to produce transgenic offspring despite reasonable hatch rates. This size-dependent effect aligns with published observations in insect transformation. Handler and Harrell (1999) reported that *piggyBac* integration efficiency decreases logarithmically with increasing insert size, with optimal efficiency achieved with constructs under 10 kb. Literature suggests that while *piggyBac* can technically accommodate larger inserts, transformation efficiency significantly diminishes beyond 10-13 kb (Eckermann et al., 2023). The reduced efficiency with larger constructs may be attributed to multiple factors including decreased mobility of the transposase-DNA complex, conformational constraints affecting transposition, and potential toxicity of the larger genetic cargo when expressed in the host.

- **Microinjection parameters** such as needle position, volume delivered, and embryo handling can significantly impact survival. As noted by Potter et al. (2010), variations in injection volume particularly affect sensitive constructs, potentially explaining why V406 (with the less toxic QF7m1) might better tolerate minor technical variations in injection procedures. Manual microinjection introduces variability in delivery volume and positioning within embryos, which leads to inconsistent results across experiments (Yan and Schetelig, 2024). This variability is evident in our data, where hatch rates for similar constructs ranged considerably (e.g., V291_QF injections ranged from 2.6% to 9.8%). Recent advances in automated microinjection systems, as described by Alegria et al. (2024), could substantially improve consistency in future experiments by standardizing injection parameters and reducing operator variability.

- **Batch Effects:** The experimental data shows considerable variation even within the same construct injections. For instance, V406_QF with the AH286 helper showed a 19.3% hatch rate in one experiment, while another injection with phsp-pBac showed 16.8%. Such variability suggests that batch effects may account for some of the observed differences.
- **Species-specific biological constraints:** The divergent outcomes between *D. suzukii* and published reports in *D. melanogaster* highlight potential species-specific constraints. While QF7m1 (equivalent to QF2w) reportedly functions in *D. melanogaster* (Riabinina et al., 2015), our data suggests *D. suzukii* may have lower tolerance thresholds for foreign transcription factors. This species variation aligns with observations by Schetelig et al. (2009), who noted significant differences in transgenic construct performance between closely related tephritid species.

To improve transformation efficiency in future experiments, several strategies could be considered including miniml construct size by implementing a modular approach, separating Q-system components across multiple constructs. Optimising microinjection parameters specifically for *D. suzukii* embryos, including needle design, injection pressure, and embryo handling. Exploring alternative QF variants with reduced toxicity, such as QF2 which lacks the middle domain identified as the toxic element (Riabinina et al., 2015). Consider site-specific integration systems like phiC31 for larger constructs, which may overcome size limitations of transposon-based approaches. Implement optimal DNA concentrations (250-400 ng/µl) as recommended by Yan et al. (2024) to balance transformation efficiency and embryonic survival. Or with robotic microinjection systems that have recently been developed to increase throughput and reproducibility in embryo transformation experiments. These systems utilize machine vision and precision robotics to inject thousands of embryos per day, achieving higher consistency and reduced operator error compared to manual methods (Qin et al., 2024). The adoption of such technology has been shown to significantly improve the efficiency of transgenesis workflows in *Drosophila* and other model organisms (Qin et al., 2024).

6. Conclusion and Outlook

This thesis has significantly advanced in understanding of apoptosis in *D. suzukii* and the potential of genetic manipulation strategies for pest control. The crucial findings of this study include identification of Dsnullo promoter as a compelling candidate for inducing gene expression at embryonic stage due to its high activity during S2 cell transfection assays. The adverse effect of the *Dsrpr* and *Dshid* in the induction of apoptosis has raised additional questions on how apoptosis can be controlled in this organism, and the need of bicistronic apoptotic gene expression to attain complete lethality for TESS systems.

Successful development of transgenic *D. suzukii* strains carrying the QUAS component of the Q system provides a foundation for future genetic control strategies. However, the inability to generate a transgenic line for the driver QF leads to main concerns about the process of generating TESS. This study lacks robust statistical analyses in the experiments where QF injections were attempted. Challenges in developing certain transgenic lines need further research due to potential toxicity issues.

Future research directions should maintain the conduct of replicated injections for QF construction. Optimization of construct designs is essential to minimize toxicity issues and improve transgenic line establishment success rates with combinations of weak or strong promoters. Refined microinjection techniques and exploring alternative methods for transgenic line creation will be crucial for practically implementing these findings in insect pest control strategies. these injection attempts must be analyzed statistically to validate and extend the current results, particularly in evaluating Q system components' efficiency and potential toxicity. Furthermore, transgenic strains' long-term stability and effectiveness in laboratory and field conditions must be analyzed.

This comprehensive approach will ensure that the promising results observed in S2 cells translate into practical and sustainable pest management solutions. The successful implementation of the Q system in *D. suzukii* could significantly advance our ability to control this agricultural pest, potentially offering a more efficient and environmentally friendly alternative to current pest management strategies.

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Appendix

1. Abbreviations

AD	activation domain
AmCyan	Anemonia majano cyan fluorescent protein
BLAST	Basic Local alignment search tool
BNK	bottle neck protein
bp	base pair
cDNA	complementary DNA
CDS	coding sequence
Conc.	concentration
ct	cycle
Ct Value	cycle threshold value
DBD	DNA-binding and dimerization domain
Dmbnk	*Drosophila melanogaster* bottle neck gene
Dmhid	*Drosophila melanogaster* head involution defective gene
Dmrpr	*Drosophila melanogaster* reaper gene
Dmslam	*Drosophila melanogaster* slow as molasses gene
DMSO	dimethyl sulfoxide
Dmsry-α	*Drosophila melanogaster* serendipity alpha
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
Ds	*Drosophila suzukii*
DsREnt	Discosoma species red fluorescent protein
dsRNA	double stranded RNA
E. coli	*Escherichia coli*
EGFP	enhanced green fluorescent protein
fsRIDL	female specific release of insects carrying a dominant lethal
g	gram
GAL4-UAS	yeast transcription activator protein Gal4- upstream activating sequences
gDNA	Genomic DNA
h	hour
HID	Head involution defective (protein)
Hi-FBS	heat inactivated fetal bovine serum
kb	kilobase
L	liter
LB	lysogeny broth
MAPK	mitogen-activate protein kinase
MD	transcriptional activation domain
mg	milligram
min	minutes

ml	milliliter
mM	millimolar
mRNA	messenger RNA
NCBI	National center for biotechnology information
ng	nanogram
°C	degree celsius
PBS	phosphate buffer saline
PCR	Polymerase chain reaction
Q system	quinic acid system
QA	quinic acid
QF	quinic acid system activation factor
qRT-PCR	Quantitative real time PCR
QS	quinic acid system gene suppressor
QUAS	quinic acid system gene regulator
RNA	ribonucleic acid
rpm	rotations per minute
RT	reverse transcription
S2 cells	Schneider cells
sec	Seconds
SOC	Super Optimal broth with Catabolite repression
SIT	Sterile Insect Technique
SWFBase	Spotted Wing Fly Base
Temp	temperature
TESS	transgenic embryonic sexing strain
USA	United states of America
VE water	vollentsalztes Wasser (De-salinated water)
WT	Wild type
µg	microgram
µl	microliter
µM	micromolar

2. qPCR primers list

Gene name	Primer numbers	Primer Name	Sequence
Sry-α	P1543	Dssrya_qF	ATCGGACAAGATGGCTCTGAC
	P1544	Dssrya_qR	GCATGGCCTCGTTTAAGAAGGA
Nullo	P1547	Dsnullo_qF	GCTGAAAATGTGAAGAGCGGAG
	P1548	Dsnullo_qR	CTTTTGCTTCCTGGCCGAAATG
Bnk	P1551	Dsbnk_qF1	CTGACCAACACCTTTGAGTCG
	P1552	Dsbnk_qR1	TGGTGGTGGCTATGTTCTGG
Slam	P1562	Dsslam_qF	CACGCTGCAGATATCAAGGC
	P1563	Dsslam_qR	GGATACACTTTGCCTCCAGTTCC
Hid	P1362	Dshid_qF1	GATGAGCGCGAGTACCAG
	P1363	Dshid_qR1	GGATGCGTCCATTGAACTC
Reaper	P1545	Dsrpr_qF	GAGCAGAAGGAGCAGCAGAT
	P1546	Dsrpr_qR	TATTTGCCGGACTTTCTGCC
Grim	P1549	Dsgrim_qF	CTAGGAAGTCAGCAGGGATCG
	P1550	Dsgrim_qR	CGCTGCTGATTTCGAAGGAT
TBP	P740	DsTBP_F	CCACGGTGAATCTGTGCT
	P741	DsTBP_R	GGAGTCGTCCTCGCTCTT
AK	P1234	DsAK_qF	ACGGTGAACCCAATGGCACCGC
	P1235	DsAK_qR	CAACAGCGACTTGGAGTCGGAGGC
His3	P1360	DsHis3_qF1	GAACGGTTGCCCTGCGTG
	P1361	DsHis3_qR1	AGCTCTGGAATCGCAGGTCAG

3. Primers for cloning

Primer number	Primer Name	Sequence
P9	Dsrpr-ORF-f	AAGAGAAAGTCATTGAGTCACCGG
P10	Dsrpr-ORF-f	TGTACTCTGGATTTTGGACTGGAC
P41	Dshid-F	ATGGCCGTGCCCTTTTATTTGC
P42	Dshid-R	TCATCGCGCCGCAAAGAAG
P81	Dshid-R	TCATCGCGCCGCAAAGAAGCCACAG
P135	Dsgrim-F	ATGGCCATTGCCTACTTCATACCCGAC
P136	Dsgrim-R	TTAACTGCCGCTGCTGATTTCGAAGG
P149	SacII-Dsrpr	AGTCCCGCGGATGGCAGTGGCATTTTTCATAC
P150	NotI-Dsrpr	AGTCGCGGCCGCTCACTGCGATGGCTTGCG
P161	SacII-Dsgrim	AGTCCCGCGGATGGCCATTGCCTACTTC
P162	NotI-Dsgrim	AGTCGCGGCCGCTTAACTGCCGCTGCTGAT
P165	Dshid-IBM	ATGGCCGTGCCCTTTTATTTGCCCGAG
P213	attP-Bsp119I-R	TCATTCGAACTGTACTAGTCGCGCTC
P313	ApaI-Dsgrim	ACATGGGCCCATGGCCATTGCCTACTTCATACCCGAC
P314	ApaI-Dshid	ACATGGGCCCATGGCCGTGCCCTTTTATTTGCC
P315	Dshid-NotI	AGTCGCGGCCGCTCATCGCGCCGCAAAGAAGC
P811	ActinpolyA-ApaI-AscI-PstI-R	ACTCTGCAGGCGCGCCGGGCCCCCAAGTGTGAGTGTGTGTGGGTTA
P825	Actin-QS-Gib-F	CGTCTAATCCAGAGACCCCGATGAACACCATCCCGGCACGCCA
P826	QS-Gibson-R	TCAAGCGACGAGGTGCTCGCCGCCCGGTGAACAGT
P827	QS-TaV2A-Gib-F	GCGAGCACCTCGTCGCTTGAGCGATCGCGCGTGCTGAAGGT
P828	Tav2A-QF-Gib-R	CTTGGGTGGCATGTTGGATCGGGCCCCGGATTTTCCTCAACA
P829	ActinpA-NotI-F	ACTGCGGCCGCGATCCGTCGACCATGAAGATCAA

P830	SV40-NotI-XbaI-F	AAAGCGGCCGCGACTCTAGATCATAATCAGCCAT
P831	SV40-AscI-PstI-R	ACTCTGCAGGCGCGCCGATACATTGATGAGTTTGGACA
P832	SV40-AscI-SacII-R	ACTCCGCGGCGCGCCGATACATTGATGAGTTTGGACA
P834	DshidAla4-EcoRI-F	ACTGAATTCATGGCCGTGCCCTTTTATTTGCCCGA
P885	Aae-btub-dsR	CCCTTTAATACGACTCACTATAGGGAGAACACGGTACTGTTGCGATCC
P1068	Dsrpr-SmaI-F	ATTCCCGGGATGGCAGTGGCATTTTTCATA
P1069	Dsgrm-SmaI-F	ATTCCCGGGATGGCCATTGCCTACTTCAT
P1070	DshidAla4-SmaI-F	ATTCCCGGGATGGCCGTGCCCTTTTATTTGC
P1071	SV40-BgIII-SaII-R	ACTGTCGACAGATCTGATACATTGATGAGTTTGGA
P1269	pBac-mRNA-F	GAAACTAATACGACTCACTATAGGGAGAGCCGCCACATGGGTAGTTCTTTAGACGATG
P1270	pBac-mRNA-R	CTTATTAGTCAGTCAGAAACAAC
P1370	Dsbnk-ORF-F	ATGAGCATCAGCACTTTCAACTTCCAG
P1371	Dsbnk-ORF-R	TTAGGCACTCATTGAGATGCGTTGC
P1372	Dsnullo-ORF-F	ATGGGCAGCACTCATTCCGCTG
P1373	Dsnullo-ORF-R	CTAGATCTTCACCAGTCGTTCGCGG
P1374	Dsslam-ORF-F	ATGGTTGTAAACACCGCAGCCATG
P1375	Dsslam-ORF-R	CTATACCTCCACGGCCCTTCGG
P1376	Dssrya-ORF-F	ATGGAGTCGTTGTTGGTTCAGT

P1377	Dssrya-ORF-R	TCAATCTAATCTAAGAATGTCGGTGATC
P1501	Ds-rpr-EcoRI-PacI-AgeI-F	GGCGAATTCTTAATTAAACCGGTATGGCAGTGGCAT
P1502	Ds-rpr-NotI- NheI-BsaI	TAAGCGGCCGCGCTAGCGGTCTCTCACTGCGATGGC
P1654	SacII-DsHID-F	AGTCCCGCGGATGGCCGTGCCCTT
P1655	NotI-DsHID-R	AGTCGCGGCCGCTCATCGCGCCGCAA
P1773	Bnk-EcoRI-F	ACGTAGAATTCAGGAGGTGGAGCAATGAG
P1774	Bnk-KpnI-R	ACGTAGGTACCGTTGAAGGCTGATAAACG
P1775	Hsp-EcoRI-F	ACGTAGAATTCCTCGAGAAATTTCTCT
P1776	Hsp-KpnI-R	ACGTAGGTACCATCGATCAGATCCCCCAG
P1779	AgeI-Dsgrim F	AGTCACCGGTATGGCCATTGCCTACTTC
P1780	NheI-Dsgrim R	AGTCGCTAGCTTAACTGCCGCTGCTGAT
P1781	AgeI-rpr-F	AGTCACCGGTATGGCAGTGGCATTTTTC
P1782	NheI-hid-R	AGTCGCTAGCTCATCGCGCCGCAAAGA
P1783	NheI-rpr-R	AGTCGCTAGCTCACTGCGATGGCTTGCG
P1825	Dmhsp-apaI-F	ACGTAGGGCCCAATTCCTCGAGAAATTTC
P1826	Hsp7-apaI-R	ACGTAGGGCCCGGATCTAAACGAGTT
P1828	Tub-mluI-F	ACGTAACGCGTAATTCGATATCAAGCTTG

4. Equipment

Mediaclave 10/30	INTEGRA bioscience, Germany
Precellys® Homogenizer	Bertin instruments, Germany
Thermal cycler C1000	Bio-Rad, Germany
qPCR Thermocycler CFX96	Bio-Rad, Germany
VersaDoc Molecular Imager	Bio-Rad, Germany
Plate reader	BioTek, USA
TC20™ Automated cell counter	Bio-Rad, Germany
Leica DM IL LED	Leica Microsystems, Germany
Olympus SZX16	Olympus, Germany
Leica M205	Leica Microsystems, Germany
Sutter instrument P-2000	Science products GmbH

5. Websites and Software

tBLASTx	https://blast.ncbi.nlm.nih.gov/Blast.cgi
FlyBase	http://flybase.org/
NCBI	http://www.ncbi.nlm.nih.gov/
NCBI-blast	http://blast.ncbi.nlm.nih.gov/Blast.cgi
DNA calculator	www.molbiotools.com/dnacalculator
Image J	https://imagej.nih.gov/ij/
Graphpad Prism (Version 8.0)	https://www.graphpad.com/scientific-software/prism
Geneious Prime	https://www.geneious.com/prime/
LASX	https://www.leica-microsystems.com/products/microscope-software/p/leica-las-x-ls/
VersaDoc, Quantity One Software (4.6.9)	https://www.bio-rad.com/de-de/product/quantity-one-1-d-analysis-software?ID=1de9eb3a-1eb5-4edb-82d2-68b91bf360fb

6. Kits

Product name	Cat #	Company
ZR Tissue & Insect RNA MicroPrep™ kit	R2030	Zymo Research, USA
ZR Tissue and Insect DNA Miniprep™ kit	D6016	Zymo Research, USA
Turbo DNase™	AM2239	Thermo Fisher Scientific, USA
iScript cDNA Synthesis™ Kit	1708891	Bio-Rad, USA
QuantiTect Reverse Transcription Kit	205311	QIAGEN, Germany
DNA Clean and Concentrator kit (DCC)	D4013	Zymo Research, USA
Zymoclean Gel DNA Recovery Kit (capped columns)	D4007	Zymo Research, USA
QIAGEN Plasmid Mini Kit	12123	QIAGEN, Germany
QIAGEN Plasmid Midi Kit	12143	QIAGEN, Germany
QIAGEN Plasmid Maxi Kit	12162	QIAGEN, Germany
TOPO TA cloning Kit	K4575J10	Thermo Fisher Scientific, USA
Xfect Transfection Reagent	631318	TaKaRa Bio, USA
SsoAdvanced SYBR Green Supermix		Biorad
T4 DNA ligase		
Platinum Taq DNA polymerase™	10966-018	Invitrogen, USA
Phusion Flash High-Fidelity PCR Master mix™		Thermo Fisher Scientific, USA
Dream Taq Polymerase	EP0701	Thermo Scientific
Mix & Go! E. coli Transformation Kit and Buffer Set	T3001	Zymo Research
Zero Blunt TOPO PCR Cloning Kit	450031	Invitrogen

7. Constructs

Project	Vector number	Glycerol stock	Vector name
Embyonic promoters	V207	M4411	pBacXLII-attP-PUbAmCyan_Dsslam_DsRed-NLS-SV40
	V208	M4410	pBacXLII-attP-PUbAmCyan_Dsbnk_DsRed-NLS-SV40
RMCE constructs	V337	M4407	pSL_loxN-3xP3-Dz-grim_SV40-PUbDsRed-lox2272
	V338	M4406	pSL_loxN-3xP3-Dz-rpr_SV40-PUbDsRed-lox2272
	v339	M4408	pSL_loxN-3xP3-Dz-grim_SV40-PUbDsRed-lox2272
	v340	M4405	pSL_loxN-3xP3-Dz-rpr_SV40-PUbDsRed-lox2272
	v350	M4419	pSL_loxN-3xP3-BglII-PUbDsred-lox2272
	v351	M4421	pSL_loxN-3xP3-PUbAmcyan-lox2272
	V363	M4653	pSL_loxN-3xP3-DshidAla4-PUbDsRed-SV40-lox2272
	V364	M4654	pSL_loxN-Bsu361-3xp3-BglII-PUbAmCyan-lox2272
	V365	M4711	pSL_loxN-DssryαP-DsRed-PUbAmCyan-lox2272
	V366	M4713	pSL_loxN-DsnulloP-DsRed-PUbAmCyan-lox2272
embryonic genes for functional analysis	V373	M5110	pCR4_ Dsslam Exon 2
	V375	M5133	pCR4_Dsslam Exon 1
	V376	M5108	pCR4_Dssryα
		M4717	pCR4_Dsbnk
		M4718	pCR4_Dsnullo
Apoptotic gene	V381	M5272	pCR4_DsHid Ex1-3
	V382	M5274	pCR4_DsHid Ex3-4

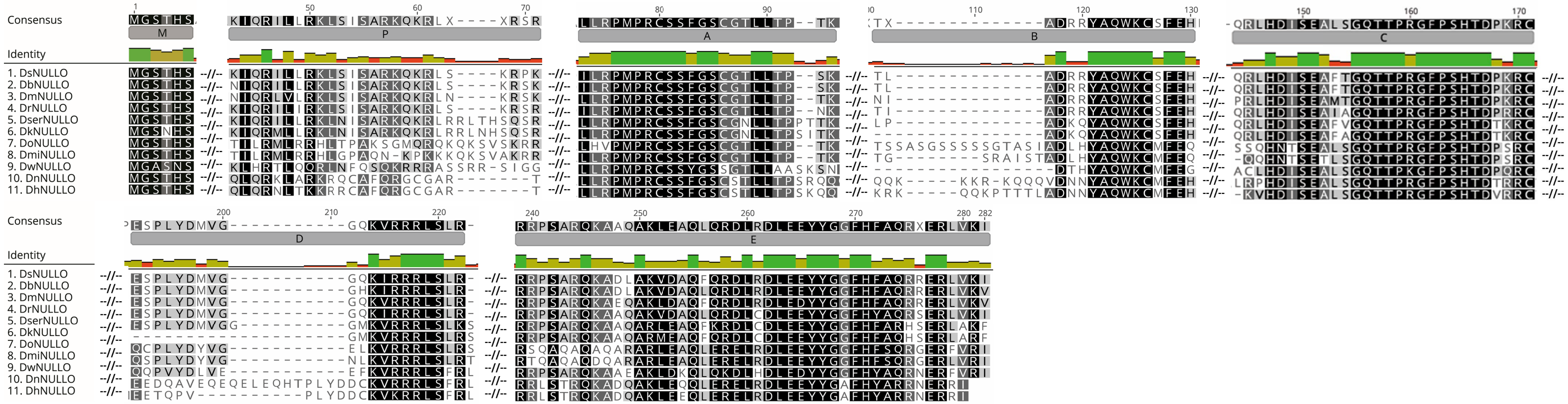
	v392	M5707	pIE4_Dshid
Q system	V295	M4270	pBacXLII-attP-PUbAmCyan-rev_tubP-QS-SV40
	V296	M4499	pBacXLII-attP-PUbAmCyan-rev_PUb-QS-SV40
	V354	M4568	pBacXLII-attP-PUbDsRedT3_PUb-QS-SV40
	v388	M5430	pBacXLII_attP-PUbDsRedT3-QUAST-Dmhsp70-Dsrpr-SV40
	v389	M5330	pCasper pQUAST-Dmhsp70-Dsrpr-SV40
	v391	M5699	pCasper_ActinP-QS-TaV-2A-QF7-actinpolyA
	v400	m5808	pBacXLII_attP-PUbDsRedT3-QUAST-Dmhsp70-Dsrpr-DrosCV2A-Dshidala4-SV40
	v401	M5799	pBacXLII_attP-PUbDsRedT3-QUAST-Dmhsp70-Dsrpr-DrosCV2A-Dsgrim-SV40
	v402	M5802	pBacXLII_attP-PUbDsRedT3_QUAST-Dmhsp70-Dsgrim-SV40
	v403	M5793	pCasper_DsbnkP-QF7m1
	v405	M5795	pCasper_Dmhsp70-QF7m1
	v406	M5821	pBacXLII_attP-PUb-EGFP_tubP-QF7m1-hsp70
	v407	M5815	pBacXLII_attP-PUb-EGFP_Dmhsp70-QF7m1-hsp70

8. Plasmids for Cell culture

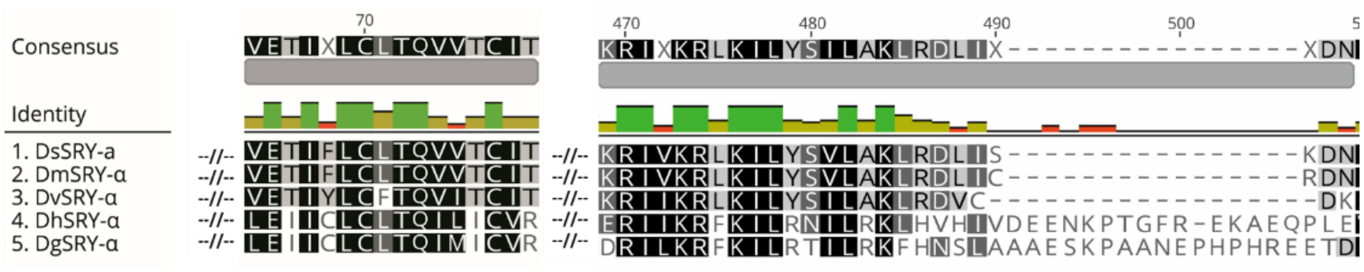
Vector number	Created by	Glycerol stock	Vector name
v42	Jonas	M1910	pIE4_Dz-reaper
v44	Jonas	M2075	pIE4_Dz-grim
v93	Jonas	M2192	pIE4_Dz-hidAla4
v125	Jonas	M2260	pIE4_DsRed-NLS
v126	Jonas	M2259	pIE4_EGFP-NLS
v163	Jonas	M2588	pIE4_Dz-rpr_DrosCV-2A_Dz-rpr_SV40
v164	Jonas	M2589	pIE4_Dz-rpr_DrosCV-2A_Dz-grim_SV40
v165	Jonas	M2590	pIE4_Dz-rpr_DrosCV-2A_Dz-hidAla4_SV40
v172	Jonas	M2597	pIE4_Dz-rpr_TaV-2A_Dz-rpr_SV40
v173	Jonas	M2598	pIE4_Dz-rpr_TaV-2A_Dz-grim_SV40
v174	Jonas	M2628	pIE4_Dz-rpr_TaV-2A_Dz-hidAla4_SV40
v205	Jonas	M3108	pBacXLII_attP-PUbAmCyan_Dz-sryα_DsRed-NLS-SV40
v206	Jonas	M3110	pBacXLII_attP-PUbAmCyan_Dz-nullo_DsRed-NLS-SV40
v207	Azka	M4591	pBacXLII_attP-PUbAmCyan_Dz-slam_DsRed-NLS-SV40
v208	Azka	M4110	pBacXLII_attP-PUbAmCyan_Dz-bnk_DsRed-NLS-SV40
v209	Jonas	M3112	pBacXLII_attP-PUbAmCyan_Dz-b2T_DsRed-NLS-SV40
V288	Ying	M4227	pBacXLII_attP-PUbDsRedT3-QUAST-Dmhsp70-AmCyan-SV40
V289	Ying	M3813	pBacXLII_attP-PUbDsRedT3-QUAST-Dmhsp70-Dz-hidAla4-SV40
V290	Ying	M4228	pBacXLII_attP-PUbDsRedT3-QUAST-Dmhsp70-*Cctra*-Dz-hidAla4-SV40
V291	Ying	M4229	pBacXLII_attP-PUbEGFP-act5cB-QF#7-actinpolyA
V295	azka	M4270	pBacXLII-attP-PUbAmCyan-rev_tubP-QS-SV40
V354	azka	M4568	pBacXLII-attP-PUbDsRedT3_PUb-QS-SV40

v392	azka	M5707	pIE4_DsHid
v402	azka	M5802	pBacXLII_attP-PUbDsRedT3-QUAST-Dmhsp70-Dsgrim-SV40
v388	azka	M5430	pBacXLII_attP-PUbDsRedT3-QUAST-Dmhsp70-Dsrpr-SV40
	Marc	#1450	pIE4_Dmhidala5
	Marc	M131	pIE4_Dmhid
	Marc	M133	pIE4_Dmrpr

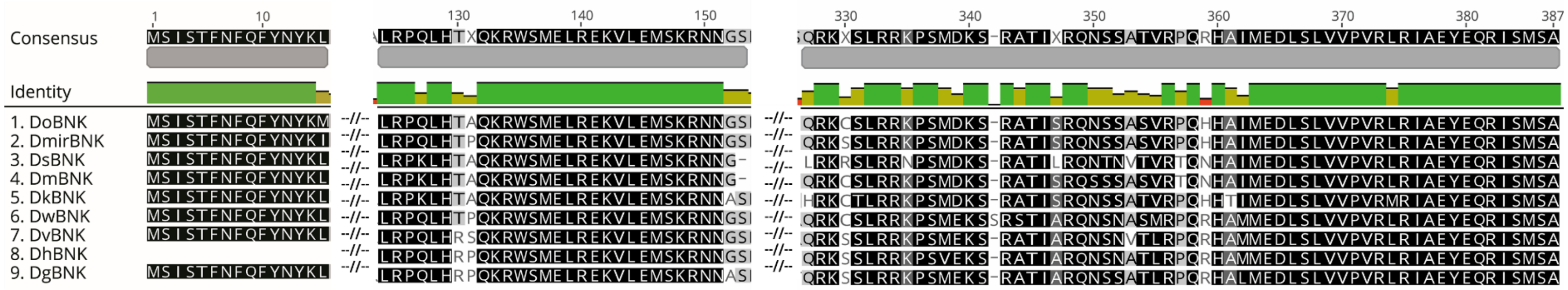
9. Conserved regions of embryonic genes



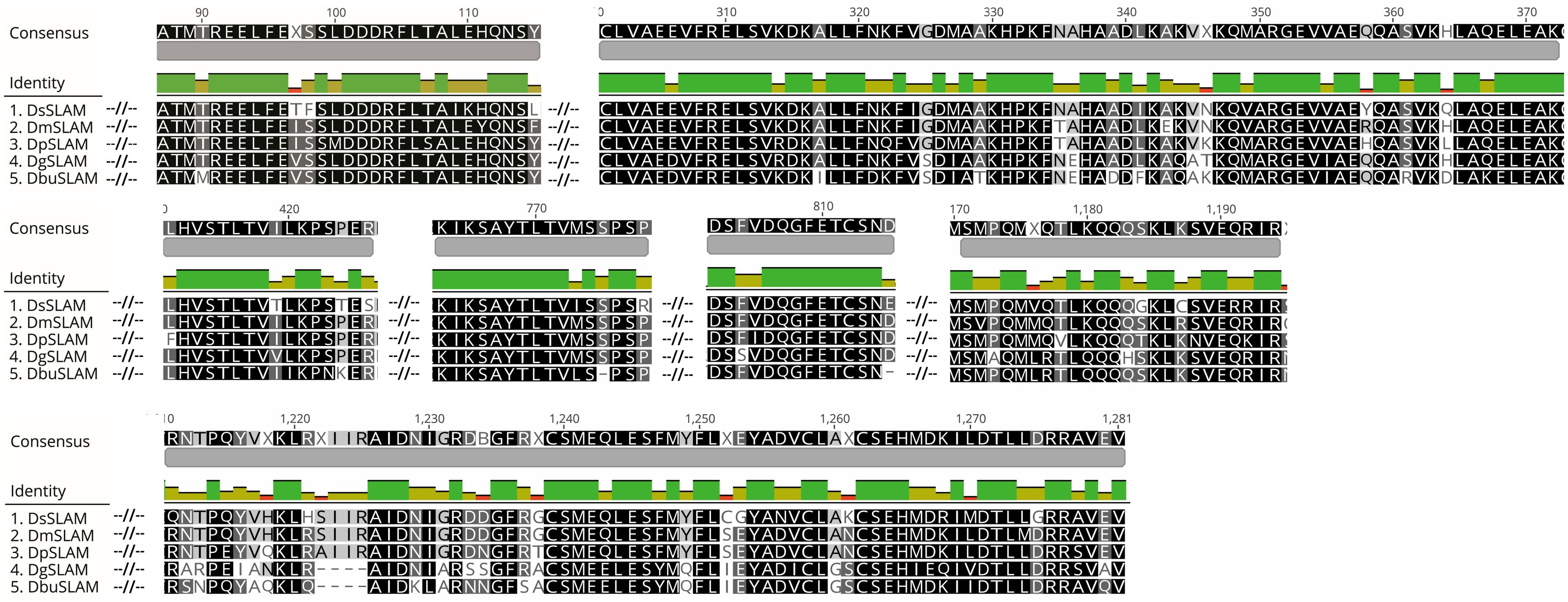
Appendix 9a: *Drosophila suzukii* NULLO (DsNULLO) is aligned with orthologs from other *Drosophila* species described above.



Appendix 9b: ***Drosophila suzukii*** **SERENDIPITY-α (DsSRY-α) is aligned with orthologs from** ***Drosophila*** **species.**



Appendix 9c: ***Drosophila suzukii*** **BOTTLENECK (DsBNK) is aligned with orthologs from** ***Drosophila*** **species.**



Appendix 9d: *Drosophila suzukii* SLOW-AS-MOLASSES (DsSLAM) is aligned with orthologs from other *Drosophila* species.

10. Statistics

Appendix 10a. Statistical analysis for figure 10b was carried out running one-way ANOVA, Holm-Sidak method for pairwise multiple comparisons using SigmaPlot.

Comparison	Diff of Means	t	P	P<0.050
CONTROL vs. Dmhid	5,492	11,271	<0,001	Yes
CONTROL vs. $Dmhid^{Ala5}$	4,529	9,294	<0,001	Yes
CONTROL vs. Dmrpr	2,264	4,645	0,002	Yes
CONTROL vs. Dsgrim	5,233	10,738	<0,001	Yes
CONTROL vs. Dshid	5,218	10,707	<0,001	Yes
CONTROL vs. $Dshid^{Ala4}$	2,632	5,401	<0,001	Yes
CONTROL vs. Dsrpr	3,136	6,435	<0,001	Yes
CONTROL vs. Dsrpr_DrosCV-2A_Dshid	8,512	17,467	<0,001	Yes
CONTROL vs. Dsrpr_DrosCV-2A_Dsrpr	8,480	17,402	<0,001	Yes
CONTROL vs. Dsrpr_DrosCV-2A_Dsgrim	8,374	17,184	<0,001	Yes
CONTROL vs. Dsrpr_TaV-2A_Dsrpr	8,618	17,685	<0,001	Yes
CONTROL vs. Dsrpr_TaV-2A_Dshid	8,618	17,685	<0,001	Yes
CONTROL vs. Dsrpr_TaV-2A_Dsgrim	8,618	17,685	<0,001	Yes
Dmhid vs. Dsrpr_DROSCV-2A_$Dshid^{Ala4}$	3,020	6,197	<0,001	Yes
Dmhid vs. Dsrpr_DROSCV-2A_Dsrpr	2,988	6,131	<0,001	Yes
Dmhid vs. Dsrpr_DrosCV-2A_Dsgrim	2,882	5,914	<0,001	Yes
Dmhid vs. Dsrpr_TaV-2A_$Dshid^{Ala4}$	3,126	6,414	<0,001	Yes
Dmhid vs. Dsrpr_TaV-2A_Dsrpr	3,126	6,414	<0,001	Yes
Dmhid vs. Dsrpr_TaV-2A_Dsgrim	3,126	6,414	<0,001	Yes
$Dmhid^{Ala5}$ vs. Dmhid	0,963	1,976	0,762	No
$Dmhid^{Ala5}$ vs. Dsgrim	0,703	1,443	0,978	No
$Dmhid^{Ala5}$ vs. Dshid	0,688	1,413	0,979	No
$Dmhid^{Ala5}$ vs. Dsrpr_DrosCV-2A_Dsgrim	3,845	7,890	<0,001	Yes
$Dmhid^{Ala5}$ vs. Dsrpr_DrosCV-2A_$Dshid^{Ala4}$	3,983	8,173	<0,001	Yes
$Dmhid^{Ala5}$ vs. Dsrpr_DrosCV-2A_Dsrpr	3,951	8,107	<0,001	Yes
$Dmhid^{Ala5}$ vs. Dsrpr_TaV-2A_Dsgrim	4,089	8,390	<0,001	Yes
$Dmhid^{Ala5}$ vs. Dsrpr_TaV-2A_$Dshid^{Ala4}$	4,089	8,390	<0,001	Yes
$Dmhid^{Ala5}$ vs. Dsrpr_TaV-2A_Dsrpr	4,089	8,390	<0,001	Yes
Dmrpr vs. Dmhid	3,229	6,625	<0,001	Yes
Dmrpr vs. $Dmhid^{Ala5}$	2,266	4,649	0,002	Yes
Dmrpr vs. Dsgrim	2,969	6,092	<0,001	Yes
Dmrpr vs. Dshid	2,954	6,062	<0,001	Yes
Dmrpr vs. $Dshid^{Ala4}$	0,368	0,755	1,000	No
Dmrpr vs. Dsrpr	0,872	1,790	0,868	No
Dmrpr vs. Dsrpr_DrosCV-2A_Dsgrim	6,110	12,539	<0,001	Yes
Dmrpr vs. Dsrpr_DrosCV-2A_$Dshid^{Ala4}$	6,248	12,822	<0,001	Yes
Dmrpr vs. Dsrpr_DrosCV-2A_Dsrpr	6,216	12,756	<0,001	Yes
Dmrpr vs. Dsrpr_TaV-2A_Dsgrim	6,354	13,040	<0,001	Yes
Dmrpr vs. Dsrpr_TaV-2A_$Dshid^{Ala4}$	6,354	13,040	<0,001	Yes
Dmrpr vs. Dsrpr_TaV-2A_Dsrpr	6,354	13,040	<0,001	Yes
Dsgrim vs. Dmhid	0,260	0,533	1,000	No
Dsgrim vs. Dsrpr_TaV-2A_Dsgrim	3,385	6,947	<0,001	Yes
Dsgrim vs. Dsrpr_TaV-2A_$Dshid^{Ala4}$	3,385	6,947	<0,001	Yes
Dsgrim vs. Dsrpr_TaV-2A_Dsrpr	3,385	6,947	<0,001	Yes
Dsgrim vs. Dsrpr_DrosCV-2A_$Dshid^{Ala4}$	3,279	6,730	<0,001	Yes
Dsgrim vs. Dsrpr_DrosCV-2A_Dsrpr	3,247	6,664	<0,001	Yes
Dsgrim vs. Dsrpr_DrosCV-2A_Dsgrim	3,141	6,446	<0,001	Yes
Dshid vs. Dsgrim	0,0149	0,0307	1,000	No

Dshid vs. Dmhid	0,275	0,564	1,000	No
Dshid vs. Dsrpr_TaV-2A_Dshid^{Ala4}	3,400	6,978	<0,001	Yes
Dshid vs. Dsrpr_TaV-2A_Dsgrim	3,400	6,978	<0,001	Yes
Dshid vs. Dsrpr_DrosCV-2A_Dsrpr	3,262	6,695	<0,001	Yes
Dshid vs. Dsrpr_DrosCV-2A_Dshid^{Ala4}	3,294	6,760	<0,001	Yes
Dshid vs. Dsrpr_DrosCV-2A_Dsgrim	3,156	6,477	<0,001	Yes
Dshid^{Ala4} vs. Dsrpr	0,504	1,034	0,999	No
Dshid^{Ala4} vs. Dsrpr_TaV-2A_Dsgrim	5,986	12,284	<0,001	Yes
Dshid^{Ala4} vs. Dsrpr_TaV-2A_Dshid	5,986	12,284	<0,001	Yes
Dshid^{Ala4} vs. Dsrpr_TaV-2A_Dsrpr	5,986	12,284	<0,001	Yes
Dshid^{Ala4} vs. Dsrpr_DrosCV-2A_Dshid^{Ala4}	5,880	12,067	<0,001	Yes
Dshid^{Ala4} vs. Dsrpr_DrosCV-2A_Dsrpr	5,848	12,001	<0,001	Yes
Dshid^{Ala4} vs. Dsrpr_DrosCV-2A_Dsgrim	5,742	11,783	<0,001	Yes
Dshid^{Ala4} vs. Dmhid	2,860	5,870	<0,001	Yes
Dshid^{Ala4} vs. Dsgrim	2,601	5,337	<0,001	Yes
Dshid^{Ala4} vs. Dshid	2,586	5,306	<0,001	Yes
Dshid^{Ala4} vs. Dmhid^{Ala5}	1,897	3,894	0,014	Yes
Dsrpr vs. Dsgrim	2,097	4,303	0,005	Yes
Dsrpr vs. Dmhid	2,356	4,836	0,001	Yes
Dsrpr vs. Dshid	2,082	4,272	0,005	Yes
Dsrpr vs. Dmhid^{Ala5}	1,393	2,860	0,180	No
Dsrpr vs. Dsrpr_TaV-2A_Dsgrim	5,482	11,250	<0,001	Yes
Dsrpr vs. Dsrpr_TaV-2A_Dshid^{Ala4}	5,482	11,250	<0,001	Yes
Dsrpr vs. Dsrpr_TaV-2A_Dsrpr	5,482	11,250	<0,001	Yes
Dsrpr vs. Dsrpr_DrosCV-2A_Dshid^{Ala4}	5,376	11,033	<0,001	Yes
Dsrpr vs. Dsrpr_DrosCV-2A_Dsrpr	5,344	10,967	<0,001	Yes
Dsrpr vs. Dsrpr_DrosCV-2A_Dsgrim	5,238	10,749	<0,001	Yes
Dsrpr_DrosCV-2A_Dsgrim vs. Dsrpr_TaV-2A_Dsgrim	0,244	0,501	1,000	No
Dsrpr_DrosCV-2A_Dsgrim vs. Dsrpr_TaV-2A_Dsrpr	0,244	0,501	1,000	No
Dsrpr_DrosCV-2A_Dsgrim vs. Dsrpr_TaV-2A_Dshid^{Ala4}	0,244	0,501	1,000	No
Dsrpr_DrosCV-2A_Dsgrim vs. Dsrpr_DrosCV-2A_Dshid^{Ala4}	0,138	0,283	1,000	No
Dsrpr_DrosCV-2A_Dsgrim vs. Dsrpr_DrosCV-2A_Dsrpr	0,106	0,217	1,000	No
Dsrpr_DrosCV-2A_Dsrpr vs. Dsrpr_TaV-2A_Dsgrim	0,138	0,283	1,000	No
Dsrpr_DrosCV-2A_Dsrpr vs. Dsrpr_TaV-2A_Dshid^{Ala4}	0,138	0,283	1,000	No
Dsrpr_DrosCV-2A_Dsrpr vs. Dsrpr_TaV-2A_Dsrpr	0,138	0,283	1,000	No
Dsrpr_DrosCV-2A_Dsrpr vs. Dsrpr_DrosCV-2A_Dshid^{Ala4}	0,0321	0,0659	1,000	No
Dsrpr_DrosCV-2A_Dshid^{Ala4} vs. Dsrpr_TaV-2A_Dsrpr	0,106	0,217	1,000	No
Dsrpr_DrosCV-2A_Dshid^{Ala4} vs. Dsrpr_TaV-2A_Dsgrim	0,106	0,217	1,000	No
Dsrpr_DrosCV-2A_Dshid^{Ala4} vs. Dsrpr_TaV-2A_Dshid	0,106	0,217	1,000	No
Dsrpr_TaV-2A_Dsgrim vs. Dsrpr_TaV-2A_Dshid^{Ala4}	0,000	0,000	1,000	No
Dsrpr_TaV-2A_Dsrpr vs. Dsrpr_TaV-2A_Dshid^{Ala4}	0,000	0,000	1,000	No

Dsrpr_TaV-2A_Dsrpr vs. Dsrpr_TaV-2A_Dsgrim	0,000	0,000	1,000	No

Appendix 10b: Statistical analysis for figure 12 was carried out running one-way ANOVA, Holm-Sidak method for pairwise multiple comparisons using SigmaPlot.

Comparison	Diff of Means	t	P	P<0.050
CONTROL vs. Dmhid	78.700	17.048	<0.001	Yes
CONTROL vs. Dshid	75.400	16.333	<0.001	Yes
CONTROL vs. Dmhid^{Ala5}	58.700	12.715	<0.001	Yes
CONTROL vs.Dsrpr	55.400	12.001	<0.001	Yes
CONTROL vs. Dshid^{Ala4}	55.400	12.001	<0.001	Yes
CONTROL vs. Dmrpr	47.400	10.268	<0.001	Yes
Dmrpr vs. Dmhid	31.300	6.780	<0.001	Yes
Dmrpr vs. Dshid	28.000	6.065	<0.001	Yes
Dmrpr vs. Dmhid^{Ala5}	3.300	0.715	0.865	No
Dmrpr vs. Dshid^{Ala4}	8.000	1.733	0.486	No
Dmrpr vs. Dsrpr	8.000	1.733	0.426	No
Dmhid^{Ala5} vs. Dmhid	20.000	4.332	0.008	Yes
Dmhid^{Ala5} vs. Dshid	16.700	3.618	0.022	Yes
Dsrpr vs. Dmhid	23.300	5.047	0.002	Yes
Dsrpr vs. Dshid	20.000	4.332	0.006	Yes
Dsrpr vs. Dmhid^{Ala5}	3.300	0.715	0.930	No
Dshid vs. Dmhid	3.300	0.715	0.736	No
Dshid^{Ala4} vs. Dmhid	23.300	5.047	0.002	Yes
Dshid^{Ala4} vs. Dmhid^{Ala5}	3.300	0.715	0.865	No
Dshid^{Ala4} vs. Dsrpr	0.000	0.000	1.000	No
Dshid^{Ala4} vs. Dshid	20.000	4.332	0.007	Yes

Appendix 10c: Statistical analysis for figure 16 was carried out running one-way ANOVA, Holm-Sidak method for pairwise multiple comparisons using GraphpadPrism.

Holm-Sidak's multiple comparisons test	Mean Diff.	P Value	Summary
V205 vs. V206	-0.2707	0.0047	**
V205 vs. V207	-0.025	0.9539	ns
V205 vs. V208	-0.00267	0.9601	ns
V206 vs. V207	0.2457	0.0057	**
V206 vs. V208	0.268	0.0047	**
V207 vs. V208	0.02233	0.9539	ns

Appendix 10d: Statistical analysis figure 17 was carried out running one-way ANOVA, Holm-Sidak method for pairwise multiple comparisons using GraphpadPrism.

Holm-Sidak's multiple comparisons test	**Mean Diff.**	**P Value**	**Summary**
E vs. E + D	-0.8998	0.0002	***
E vs. E + D + 1xS	-0.3428	0.3889	ns
E vs. E + D + 3xS	-0.0883	0.999	ns
E vs. E + D + 5xS	-0.05482	0.999	ns
E vs. E + D + 5xS **(5µg QA)**	-0.1207	0.9938	ns
E vs. E + D + 5xS **(500µg QA)**	-0.1429	0.9857	ns
E vs. E + D + 5xS **(5mg QA)**	-0.427	0.1442	ns
E + D vs. E + D + 1xS	0.5571	0.0235	*
E + D vs. E + D + 3xS	0.8115	0.0007	***
E + D vs. E + D + 5xS	0.845	0.0004	***
E + D vs. E + D + 5xS **(5µg QA)**	0.7791	0.001	**
E + D vs. E + D + 5xS **(500µg QA)**	0.757	0.0013	**
E + D vs. E + D + 5xS **(5mg QA)**	0.4729	0.0782	ns
E + D + 1xS vs. E + D + 3xS	0.2545	0.7177	ns
E + D + 1xS vs. E + D + 5xS	0.2879	0.5972	ns
E + D + 1xS vs. E + D + 5xS **(5µg QA)**	0.222	0.8375	ns
E + D + 1xS vs. E + D + 5xS **(500µg QA)**	0.1999	0.8937	ns
E + D + 1xS vs. E + D + 5xS **(5mg QA)**	-0.0842	0.999	ns
E + D + 3xS vs. E + D + 5xS	0.03348	0.999	ns
E + D + 3xS vs. E + D + 5xS **(5µg QA)**	-0.03245	0.999	ns
E + D + 3xS vs. E + D + 5xS **(500µg QA)**	-0.05455	0.999	ns
E + D + 3xS vs. E + D + 5xS **(5mg QA)**	-0.3387	0.3906	ns
E + D + 5xS vs. E + D + 5xS **(5µg QA)**	-0.06593	0.999	ns
E + D + 5xS vs. E + D + 5xS **(500µg QA)**	-0.08803	0.999	ns
E + D + 5xS vs. E + D + 5xS **(5mg QA)**	-0.3721	0.2859	ns
E + D + 5xS **(5µg QA)** vs. E + D + 5xS **(500µg QA)**	-0.0221	0.999	ns
E + D + 5xS **(5µg QA)** vs. E + D + 5xS **(5mg QA)**	-0.3062	0.5259	ns
E + D + 5xS **(500µg QA)** vs. E + D + 5xS **(5mg QA)**	-0.2841	0.5972	ns

Appendix 10e: Statistical analysis for figure 19a was carried out running one-way ANOVA, Holm-Sidak method for pairwise multiple comparisons using Graphpad Prism.

Holm-Sidak's multiple comparisons test	**Mean Diff.**	**P Value**	**Summary**
E vs. E + D	57.76	0.0189	*
E vs. E + D + 1xS	82.02	0.0006	***
E vs. E + D + 3xS	84.12	0.0005	***
E vs. E + D + 5xS	49.13	0.0558	ns
E vs. E + D + 5xS **(5µg QA)**	30.72	0.4446	ns
E vs. E + D + 5xS **(500µg QA)**	83.96	0.0005	***

E vs. E + D + 5xS **(5mg QA)**	92.7	0.0002	***
E + D vs. E + D + 1xS	24.26	0.6276	ns
E + D vs. E + D + 3xS	26.36	0.5973	ns
E + D vs. E + D + 5xS	-8.626	0.9909	ns
E + D vs. E + D + 5xS **(5µg QA)**	-27.03	0.5957	ns
E + D vs. E + D + 5xS **(500µg QA)**	26.21	0.5973	ns
E + D vs. E + D + 5xS **(5mg QA)**	34.94	0.3419	ns
E + D + 1xS vs. E + D + 3xS	2.104	0.9984	ns
E + D + 1xS vs. E + D + 5xS	-32.89	0.3726	ns
E + D + 1xS vs. E + D + 5xS **(5µg QA)**	-51.29	0.0427	*
E + D + 1xS vs. E + D + 5xS **(500µg QA)**	1.948	0.9984	ns
E + D + 1xS vs. E + D + 5xS **(5mg QA)**	10.68	0.9864	ns
E + D + 3xS vs. E + D + 5xS	-34.99	0.3419	ns
E + D + 3xS vs. E + D + 5xS **(5µg QA)**	-53.4	0.0344	*
E + D + 3xS vs. E + D + 5xS **(500µg QA)**	-0.1561	0.9984	ns
E + D + 3xS vs. E + D + 5xS **(5mg QA)**	8.578	0.9909	ns
E + D + 5xS vs. E + D + 5xS **(5µg QA)**	-18.41	0.8472	ns
E + D + 5xS vs. E + D + 5xS **(500µg QA)**	34.83	0.3419	ns
E + D + 5xS vs. E + D + 5xS **(5mg QA)**	43.57	0.1177	ns
E + D + 5xS **(5µg QA)** vs. E + D + 5xS **(500µg QA)**	53.24	0.0344	*
E + D + 5xS **(5µg QA)** vs. E + D + 5xS **(5mg QA)**	61.97	0.0106	*
E + D + 5xS **(500µg QA)** vs. E + D + 5xS **(5mg QA)**	8.734	0.9909	ns

Appendix 10f: Statistical analysis for figure 19c was carried out running one-way ANOVA, Holm-Sidak method for pairwise multiple comparisons using Graphpad Prism.

Holm-Sidak's multiple comparisons test	**Mean Diff.**	**P Value**	**Summary**
E vs. E + D	84.9	0.005	**
E vs. E + D + 1xS	83.46	0.0054	**
E vs. E + D + 3xS	90	0.0036	**
E vs. E + D + 5xS	25.83	0.7567	ns
E vs. E + D + 5xS **(5µg QA)**	80.07	0.0069	**
E vs. E + D + 5xS **(500µg QA)**	72.77	0.0124	*
E vs. E + D + 5xS **(5mg QA)**	88.68	0.0039	**
E + D vs. E + D + 1xS	-1.444	0.9998	ns
E + D vs. E + D + 3xS	5.097	0.9998	ns
E + D vs. E + D + 5xS	-59.07	0.0392	*
E + D vs. E + D + 5xS **(5µg QA)**	-4.832	0.9998	ns
E + D vs. E + D + 5xS **(500µg QA)**	-12.13	0.9981	ns
E + D vs. E + D + 5xS **(5mg QA)**	3.776	0.9998	ns
E + D + 1xS vs. E + D + 3xS	6.542	0.9997	ns
E + D + 1xS vs. E + D + 5xS	-57.63	0.043	*
E + D + 1xS vs. E + D + 5xS **(5µg QA)**	-3.388	0.9998	ns
E + D + 1xS vs. E + D + 5xS **(500µg QA)**	-10.68	0.999	ns
E + D + 1xS vs. E + D + 5xS **(5mg QA)**	5.22	0.9998	ns
E + D + 3xS vs. E + D + 5xS	-64.17	0.0262	*
E + D + 3xS vs. E + D + 5xS **(5µg QA)**	-9.93	0.9991	ns
E + D + 3xS vs. E + D + 5xS **(500µg QA)**	-17.23	0.9784	ns

E + D + 3xS vs. E + D + 5xS **(5mg QA)**	-1.321	0.9998	ns
E + D + 5xS vs. E + D + 5xS **(5µg QA)**	54.24	0.0574	ns
E + D + 5xS vs. E + D + 5xS **(500µg QA)**	46.94	0.1158	ns
E + D + 5xS vs. E + D + 5xS **(5mg QA)**	62.85	0.0284	*
E + D + 5xS **(5µg QA)** vs. E + D + 5xS **(500µg QA)**	-7.296	0.9997	ns
E + D + 5xS **(5µg QA)** vs. E + D + 5xS **(5mg QA)**	8.608	0.9995	ns
E + D + 5xS **(500µg QA)** vs. E + D + 5xS **(5mg QA)**	15.9	0.9852	ns

Appendix 10g: Statistical analysis for figure 19b was carried out running one-way ANOVA, Holm-Sidak method for pairwise multiple comparisons using Graphpad Prism.

Holm-Sidak's multiple comparisons test	Mean Diff.	P Value	Summary
E vs. E + D	89.44	0.0081	**
E vs. E + D + 1xS	71.12	0.0529	ns
E vs. E + D + 3xS	70.53	0.0542	ns
E vs. E + D + 5xS	44.13	0.5298	ns
E vs. E + D + 5xS **(5µg QA)**	35.63	0.7976	ns
E vs. E + D + 5xS **(500µg QA)**	56.08	0.2265	ns
E vs. E + D + 5xS **(5mg QA)**	82.85	0.0157	*
E + D vs. E + D + 1xS	-18.32	0.9853	ns
E + D vs. E + D + 3xS	-18.91	0.9853	ns
E + D vs. E + D + 5xS	-45.31	0.5042	ns
E + D vs. E + D + 5xS **(5µg QA)**	-53.81	0.2694	ns
E + D vs. E + D + 5xS **(500µg QA)**	-33.36	0.8099	ns
E + D vs. E + D + 5xS **(5mg QA)**	-6.598	0.9916	ns
E + D + 1xS vs. E + D + 3xS	-0.5903	0.9916	ns
E + D + 1xS vs. E + D + 5xS	-26.99	0.9412	ns
E + D + 1xS vs. E + D + 5xS **(5µg QA)**	-35.49	0.7976	ns
E + D + 1xS vs. E + D + 5xS **(500µg QA)**	-15.04	0.9916	ns
E + D + 1xS vs. E + D + 5xS **(5mg QA)**	11.72	0.9916	ns
E + D + 3xS vs. E + D + 5xS	-26.4	0.9412	ns
E + D + 3xS vs. E + D + 5xS **(5µg QA)**	-34.9	0.7976	ns
E + D + 3xS vs. E + D + 5xS **(500µg QA)**	-14.45	0.9916	ns
E + D + 3xS vs. E + D + 5xS **(5mg QA)**	12.31	0.9916	ns
E + D + 5xS vs. E + D + 5xS **(5µg QA)**	-8.502	0.9916	ns
E + D + 5xS vs. E + D + 5xS **(500µg QA)**	11.95	0.9916	ns
E + D + 5xS vs. E + D + 5xS **(5mg QA)**	38.71	0.7114	ns
E + D + 5xS **(5µg QA)** vs. E + D + 5xS **(500µg QA)**	20.45	0.9824	ns
E + D + 5xS **(5µg QA)** vs. E + D + 5xS **(5mg QA)**	47.22	0.4528	ns
E + D + 5xS **(500µg QA)** vs. E + D + 5xS **(5mg QA)**	26.76	0.9412	ns

Curriculum Vitae

Publications

Yan Y, **Jaffri SA,** Schwirz J, Stein C, Schetelig MF (2020) Identification and characterization of four *Drosophila suzukii* cellularization genes and their promoters. BMC Genetics
https://doi.org/10.1186/s12863-020-00939-y

Jaffri SA, Yan Y, Schetelig MF (2020) Functional characterization of the *Drosophila suzukii* pro-apoptotic genes reaper, head involution defective and grim, Apoptosis
https://doi.org/10.1007/s10495-020-01640-2

Jaffri SA, Yan Y, Scott M, Schetelig MF (2020) conditional expression systems for the pest management of *D. suzukii* (Publisher: Springer)
https://doi.org/10.1007/978-3-030-62692-1_10

Apoptosis
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Functional characterization of the *Drosophila suzukii* pro-apoptotic genes *reaper*, *head involution defective* and *grim*

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Abstract
Apoptosis is a fundamental process for the elimination of damaged or unwanted cells, and is a key aspect of development. It is triggered by pro-apoptotic genes responding to the intrinsic pathway that senses cell stress or the extrinsic pathway that responds to signals from other cells. The disruption of these genes can therefore lead to developmental defects and disease. Pro-apoptotic genes have been studied in detail in the fruit fly *Drosophila melanogaster*, a widely-used developmental model. However, little is known about the corresponding genes in its relative *D. suzukii*, a pest of soft fruit crops that originates from Asia but is now an invasive species in many other regions. The characterization of *D. suzukii* pro-apoptotic genes could lead to the development of transgenic sexing strains for pest management. Here, we describe the isolation and characterization of the pro-apoptotic genes *reaper* (*Dsrpr*), *head involution defective* (*Dshid*) and *grim* (*Dsgrim*) from a laboratory strain of *D. suzukii*. We determined their expression profiles during development, revealing that all three genes are expressed throughout development but *Dsrpr* is expressed most strongly, especially at the pupal stage. Functional analysis was carried out by expressing single genes or pairs (linked by a 2A peptide) in S2 cell death assays, indicating that *Dsgrim* and *Dshid* are more potent pro-apoptotic genes than *Dsrpr*, and the lethality can be significantly enhanced by co-expression of two genes. Therefore, the binary or multiple expression of different pro-apoptotic genes can be considered to build an efficient transgenic sexing system in *D. suzukii*.

Keywords 2A peptide · RHG proteins · Sterile insect technique

Introduction

Apoptosis (a form of programmed cell death) is an evolutionarily conserved process that eliminates unwanted cells during development as well as cells damaged by stress [1]. Apoptotic cells are characterized by plasma membrane blebbing, cytoplasmic condensation and shrinkage, progressive chromatin degradation, and nuclear fragmentation, before the apoptotic bodies are engulfed by cells of the immune system. The process can be triggered by intracellular stress-response signals via the mitochondria (*intrinsic pathway*) or ligands released by other cells (*extrinsic pathway*). These converge on a small group of pro-apoptotic genes, which in *Drosophila melanogaster* include the closely linked loci *reaper* (*rpr*), *head involution defective* (*hid*), *grim* and *sickle* (*skl*) on chromosome 3 [2, 3]. The corresponding proteins contain an N-terminal RHG motif and an internal GH3 domain, which activate different but synergistic downstream pathways [4]. The RHG motif binds to inhibitor of apoptosis proteins (IAPs) such as Diap1, which normally

The GenBank accession numbers are as follows: *Dshid* mRNA: MN982930; *Dsgrim* mRNA: MN982931; and *Dsrpr* mRNA: MN982932.

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sequesters caspases in an inactive complex. The binding of RHG proteins to Diap1 displaces the caspases, including three (Dronc, Dcp-1 and DrICE) that trigger apoptosis [5]. The conserved RHG motif is therefore also described as an IAP-binding motif (IBM) [6, 7].

Although pro-apoptotic genes have been studied in detail in *D. melanogaster*, this is not the case for the related species *D. suzukii*, a pest of soft-skinned fruits. *D. suzukii* is native to Southeast Asia but has spread throughout much of North America and Europe [8–11]. Adult females pierce the soft skin of fruits with their ovipositor, laying eggs which hatch into larvae that feed on the fruit pulp, causing large-scale damage [12, 13]. *D. suzukii* has a short generation time and a population can thus grow rapidly [14]. Insecticides have been developed and can control *D. suzukii* efficiently [15] but species-specific and sustainable ones have not been reported. Thus, several approaches have been followed to environment friendly control methods for *D. suzukii* [14, 16]. The sterile insect technique (SIT) is one of those alternative and environmentally friendly strategies that can be used as a targeted biocontrol measure. For traditional SIT program, large numbers of flies sterilized with gamma radiation are released into the field to mate with their wildtype counterpart, and lead to no viable offspring therefore reducing the population size [17, 18]. The SIT has already been used to successfully control the Mediterranean fruit fly, *Ceratitis capitata* [19], new world screwworm *Cochliomyia hominivorax,* and other tephritid fruit flies, tsetse flies and various lepidopteran pests [20–22]. Efficient SIT strategies require the production of genetic sexing strains to facilitate the mass separation of sterilized males and females so that only males are released in the field [23, 24]. In *C. capitata,* this was achieved through classical genetics and sex-linked markers generating a heat-inducible female-specific lethal system [19]. This system could not be transferred to other species yet, because the phenotypic markers identified are genetically unknown so far. Therefore, other approaches were followed to produce transgenic embryonic sexing systems (TESS) in which active pro-apoptotic genes containing a sex-specific intron are expressed during early embryonic development to kill females [25, 26]. In addition, redundant or multi-lethal systems were recommended to improve strain stability under mass-rearing conditions and reduce the risk of resistance in the field if fertile males were to be released [27, 28]. It was also reported that endogenous genes are more efficient than exogenous ones when generating transgenic sexing strains [29, 30]. Therefore, we wanted to isolate the endogenous genes from *D. suzukii* for the development of efficient TESS.

Here, we isolated the pro-apoptotic genes *Dsrpr*, *Dshid,* and *Dsgrim* from *D. suzukii* that are described as apoptosis inducing in *D. melanogaster* and identified their conserved functional motifs by comparing the orthologs of different insect species. The fourth member, *sickle* (*skl*) [31, 32], that enhances but doesn't induce apoptosis, was not isolated. The expression profiles of the genes were verified by Reverse-Transcriptase (RT) and quantitative Real-Time (qRT) PCR, confirming similar patterns to those from *D. melanogaster*. We further tested the activity of each gene in S2 cell death assays, as well as the co-expression of pairs of apoptotic genes using a 2A peptide containing vector [33]. These experiments allowed us to select appropriate candidate genes for the development of TESS strategies for the control of *D. suzukii* in the future.

Methods

Insect rearing and sample collection

Wild-type *D. suzukii* flies (USA strain) were maintained at 25 °C and 60% humidity with a 12-h photoperiod. Embryos were collected over a duration of 60 min, and were allowed to develop on grape juice agar plates (1% agar, 30% grape juice) as previously described [34]. The larvae and pupae were collected from stock vials at the desired age. Adult males and females were isolated immediately after they emerged, and were sampled 1 or 5 d later.

Gene sequence isolation and analysis

A high-quality *D. suzukii* reference genome sequence [35] is available at SWDbase (https://spottedwingflybase.org/). The coding sequences of the *D. melanogaster* genes *Dmrpr* (FBgn0011706), *Dmhid* (FBgn0003997) and *Dmgrim* (FBgn0015946) were obtained from FlyBase (https://flybase.org/) and used as tBLASTx search queries against SWDbase. Based on the hits recovered for each search, primers were designed to amplify the full-length coding sequences of the three orthologs from *D. suzukii*. Total RNA was isolated from adult flies (5 days old) using the ZR Tissue & Insect RNA MicroPrep kit (Zymo Research, USA) and treated with Turbo DNase (Thermo Fisher Scientific, USA). The iScript cDNA Synthesis Kit (Bio-Rad, USA) was used to synthesise cDNA from 0.5 µg of DNA-free total RNA. cDNA was diluted to 1:10 according to the protocol for further use.

The *Dsrpr* and *Dsgrim* cDNAs were amplified in 25 µl reactions comprising 0.1 µl Platinum Taq DNA polymerase (Invitrogen, USA), 2.5 µl 10×PCR buffer, 1 µl 50 mM $MgCl_2$, 2.5 µl 10 mM dNTPs, 0.75 µl 10 µM of each primer, and 1 µl diluted cDNA. The cycling conditions were 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 60 s, and a final extension step at 72 °C for 5 min. The 198-bp *Dsrpr* amplicon was transferred to the vector pCR4 by TOPO™ TA Cloning™ Kit cloning (Invitrogen GmbH) using primers P9/P10 (all primers are listed in Online Resource 1), resulting in construct V12. The 360-bp *Dsgrim* product was transferred similarly using primers P135/P136, resulting in construct V34.

The *Dshid* cDNA was amplified in a 20 µl reaction comprising 10 µl Phusion Flash High-Fidelity PCR Master mix (Thermo Fisher Scientific), 1 µl 10 µM of each primer and 1 µl diluted cDNA. The cycling conditions were 98 °C for 10 s, followed by 30 cycles of 98 °C for 30 s, 55 °C for 5 s and 72 °C for 30 s, and a final extension step at 72 °C for 2 min. The low abundance of *Dshid* mRNA was overcome by adopting a two-step procedure in which exons 1 and 2 were amplified using primers P81/P41 and exons 3 and 4 by using primers P165/P42 before combining the products and reamplifying with the external primers P41/P42. The 1281 bp *Dshid* product was transferred to the vector pCR4 by TOPO™ TA Cloning™ Kit cloning (Invitrogen GmbH), using primers P41/P42, resulting in construct V392.

To generate *Dshid*^{Ala4}, four potential MAPK phosphorylation sites were identified in the *Dshid* sequence (Online Resource 2), and the acceptor residues were replaced with alanine to prevent inactivation by phosphorylation [30, 36]. This constitutively active *Dshid* gene was synthesized by Eurofins (Germany), resulting in construct V45. The integrity of all vectors was confirmed by restriction digestion and sequencing using primers M13/M14, and sequences were analyzed using the Geneious Prime software [37].

Isolation and analysis of the Dshid 3′UTR

A 2260 bp region of the 3′UTR from *Dshid* was amplified from an embryonic cDNA (2–4 h) pool from *D. suzukii* by using primers P82/P84 (all primers are listed in Online Resource 1) and in-silico predictions on the *Dshid* gene. The amplified region was cloned into the pCR4 vector using the TOPO™ TA Cloning™ Kit cloning (Invitrogen), sequenced and resulting sequences analyzed for previously predicted *bantam* miRNA binding sites [38] (Online Resource 3) using the Geneious Prime software.

Protein sequence alignments from other species

Orthologues of RHG proteins from *D. grimshawi* (Dg), *D. hydei* (Dh), *D. willistoni* (Dw), *D. ficusphila* (Df), *D. biarmipes* (Db), *D. erecta* (Der), *D. melanogaster* (Dm), *D. serrata* (Dser), *Lucilia cuprina* (Lc) and *Musca domestica* (Md) were downloaded from NCBI as following: DgRPR (XP_001985528.1), DhRPR (XP_023170619.1), DwRPR (XP_023033239.1), DfRPR (XP_017058909.1), DbRPR (XP_016955290.1), DerRPR (XP_001972845.1), DmRPR (NP_524138.1), DserRPR (XP_020801924.1), LcRPR (XP_023291721.1), MdRPR (XP_005184304.1), DgHID (XP_001985533.1), DhHID (XP_023170594.1), DwHID (XP_002067955.1), DfHID (XP_017059087.1), DbHID (XP_016956024.1), DerHID (XP_001972854.1), DmHID (AAA79985.1), DserHID (XP_020801905.1), LcHID (XP_023305760.1), MdHID (XP_005180529.1). DgGRIM (XP_001996719.1), DhGRIM (XP_023170636.1), DwGRIM (XP_002067945.1), DfGRIM (XP_017058891.1), DbGRIM (XP_016955275.1), DerGRIM (XP_001972846.1), DmGRIM (NP_524137.2), Dser (XP_020801932.1), LcGRIM (XP_023291726.1), MdGRIM (XP_019891253.1).

Construction of pIE expression plasmids

The *Dsrpr* sequence was reamplified from construct V12 (see above) in a 25-µl reaction comprising 0.1 µl Platinum Taq DNA polymerase, 2.5 µl 10×PCR buffer, 1 µl 50 mM $MgCl_2$, 2.5 µl 10 mM dNTPs, 0.75 µl 10 µM of each primer P150/P149 (all primers are listed in Online Resource 1) and 1 µl 100 ng template DNA. The cycling conditions were 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 52 °C for 30 s and 72 °C for 60 s, and a final extension step at 72 °C for 5 min. The product was digested with NotI and SacII (New England Biolabs, USA) and was transferred to vector *pIE4* prepared with the same enzymes, resulting in expression vector V42. The *Dsgrim* sequence was reamplified in the same manner, except the template was vector V34 (see above). The product was transferred to vector *pIE4* as above, resulting in expression vector V44. The *Dshid* sequence was reamplified from vector V381 using primers P1654/P1655 containing restriction sites for SacII and NotI, respectively. The 25-µl reaction comprised 0.2 µl Platinum Taq DNA polymerase, 2.5 µl 10×PCR buffer, 0.75 µl 50 mM $MgCl_2$, 1.0 µl 10 mM dNTPs, 1 µM of each primer and 0.5 µl V381. The cycling conditions were 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 52 °C for 30 s and 72 °C for 2 min, then a final extension step at 72 °C for 5 min. The PCR product was transferred to vector *pIE4* as above, resulting in expression vector V392. To prepare vector V93_*pIE4_Dshid*^{Ala4}, V45_*pEX*-K4-*Dshid*^{Ala4} was digested with SacII and NotI and the insert was transferred to *pIE4*, prepared using the same enzymes. The previously reported overexpression plasmids *pIE-Dmrpr*, *pIE-Dmhid* and *pIE-Dmhid*^{Ala5} harboring *D. melanogaster rpr* and *hid* genes [30] were also included in the assays.

Construction of 2A peptide expression plasmids

The 2A peptide plasmids (Fig. 4a) were constructed by amplifying the *Dsrpr* sequence from construct V12 using primers P213/P150 (all primers are listed in Online Resource 1), the *Dsgrim* sequence from construct V44 using primers P313/P162 and the *Dshid*^{Ala4} sequence from construct V45 using primers P314/P315. Pairs of genes were transferred to construct V142 using the restriction enzymes ApaI and

NotI to join them via the DrosCV2A peptide, or to construct V145 using the same restriction enzymes to join them via the TaV2A peptide [33].

Construction of RMCE plasmids

Dsrpr, Dshid^{Ala4}, and *Dsgrim* were amplified by PCR in a 25-µl reaction comprising 0.1 µl Platinum Taq DNA polymerase, 2.5 µl 10×PCR buffer, 1 µl 50 mM $MgCl_2$, 2.5 µl 10 mM dNTPs, 0.75 µl 10 µM of each primer and 1 µl 100 ng template DNA. The cycling conditions were 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s, and a final extension step at 72 °C for 5 min; on plasmids V163_pIE4_*Dsrpr*_DrosCV-2A_*Dsrpr*_SV40, V165_pIE4_*Dsrpr*_DrosCV-2A_*Dsrpr*_SV40, and V164_pIE4_Dsrpr_DrosCV-2A_*Dsgrim*_SV40 by using primers P1068/P1071, P1070/P1071, and P1069/P1071, respectively (all primers are listed in Online Resource 1). PCR fragments were then inserted into AH448_ pSL_loxN-3xP3-PUbDsRed-lox2272 [39] by SmaI and SalI restriction sites to generate V388_pSL_loxN-3xP3-*Dsrpr*_SV40-PUbDsRed-lox2272, V350_pSL_loxN-3xP3-*Dshid^{Ala4}*_SV40-PUbDsRed-lox2272, and V337_pSL_loxN-3xP3-*Dsgrim*_SV40-PUbDsRed-lox2272, respectively.

Quantitative real-time PCR

SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) was used for qPCR with 100 ng cDNA in a CFX96 Touch Real-Time time PCR Detection System (Bio-Rad). The cycling conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 65 °C for 5 s, and 95 °C for 0.5 s. Reactions were carried out on three biological replicates each comprising three technical replicates. Samples for all developmental stages were collected for each biological replicate from the same culture. The $2^{-\Delta\Delta Ct}$ method was used for all samples, and the data were normalized to the control gene *TBP* [40].

Cell culture

We used *D. melanogaster* Schneider 2 (S2) cells [41] grown in Schneider's medium at 25 °C with 10% heat-inactivated fetal bovine serum (Hi-FBS) and 1% penicillin/streptomycin in closed capped flasks without CO_2. Cells were passaged every 2–3 days unless ≥ 90% viability was achieved. Transient transfection was carried out using Xfectin reagent (Takara, Japan) according to the manufacturer's instructions. To monitor the cell damage, we seeded 24-well plates, each well lined with a 13-mm TC coverslip (Sarstedt, Germany), with 5×10^5 cells (live cell count) in a volume of 500 µl medium and allowed the cells to settle for 3 h. The cells were then transfected with 1 µg plasmid DNA using 0.3 µl Xfectin and 27.4 µl Xfectin buffer in 270 µl serum-free Schneider's medium for 4 h. Transfection was stopped by removing the reagents and replenishing the cells with 500 µl Schneider's medium containing Hi-FBS and penicillin/streptomycin as above. The cells were incubated for a further 16 h at 25 °C before fixing in 4% paraformaldehyde for 15 min and washing twice with 1×PBS. Morphological images were taken with an inverted microscope (DM IL LED, Leica Microsystems, Wetzlar, Germany). For cell counting, 0.5 µg *pIE4-EGFP* plasmid was co-transfected with one of the *pIE* expression plasmids (0.5 µg) or with *pIE4-DsRed* control vector (0.5 µg) to visualize the successfully transfected cells. Cells expressing EGFP were imaged using M205FA MZ FLIII microscopes (Leica Microsystems, Germany) with EGFP filter sets ($\lambda_{excitation} = 500/20$; $\lambda_{emission} = 535/30$) using consistent settings. TC coverslips containing adhesive fluorescent S2 cells were placed on a slide over a drop of Hi-FBS. We captured 25 images per TC coverslip, and cells were counted using Image J (Fiji) by first converting to 8-bit (threshold 30) inverted images, and then applying the watershed and automated cell count functions. For the comparison of *Dmhid* to *Dshid, Dmrpr* to *Dsrpr,* and *Dmhid^{Ala5}* to *Dshid^{Ala4},* we captured ten images per TC coverslip and fluorescent S2 cells were counted as described before.

Statistical analysis

Statistical analysis was carried out using SigmaPlot v14 for the differences in viability of S2 cells after post transfection of different *pIE4* vectors. Data were square root transformed to pass the normality test, and analyzed by one-way ANOVA, and means were separated using the Holm–Sidak method. In total, two transfection experiments were performed, one for constructs expressing the single pro-apoptotic genes *Dsrpr, Dshid*, *Dshid^{Ala4}*, *Dmrpr*, *Dmhid,* and *Dmhid^{Ala5}* and a second, with *Dsgrim*, and all 2A peptide constructs expressing two pro-apoptotic genes. Each experiment was normalized to its control and individual percentages calculated. All data was then statistically compared to generate Fig. 4b. Detailed statistics is provided as Online Resource 4.

Results

D. suzukii pro-apoptotic genes can be isolated by homology-based screening

Three *D. suzukii* pro-apoptotic genes were identified by searching the SWDbase using orthologs from *D. melanogaster.* The coding sequence of *Dsrpr* (DS10_00012288) was found to be 198 bp in length, encoding a protein of 66 amino acids. DsRPR (Fig. 1a) was most closely related to its ortholog in *D. melanogaster* (96.9% similarity, 92.3% identity). The CDS of *Dshid* (DS10_00012680) was found to be 1281 bp in length, encoding a protein of 427 amino acids. The DsHID protein (Fig. 1b) was more closely related to its ortholog in *D. biarmipes* (96.5% similarity, 94.6% identity) than its ortholog in *D. melanogaster* (91.8% similarity and 86.7% identity). The coding sequence of *Dsgrim* (DS10_00013088) was found to be 360 bp in length, encoding a protein of 120 amino acids. DsGRIM (Fig. 1c) was also most closely related to its ortholog in *D. melanogaster* (93.4% similarity, 86.8% identity). Phylogenetic analysis using a neighbor-joining algorithm revealed that all three pro-apoptotic genes clustered with their orthologs from several other *Drosophila* species, notably *D. biarmipes* (Fig. 2). Canonical RHG/IBM and GH3 domains were identified in all three proteins, suggesting their pro-apoptotic functions are likely to be retained.

D. suzukii pro-apoptotic genes are expressed throughout development

Apoptosis is initiated during embryonic stage 11 in insects and thereafter becomes widespread and dominant during embryonic and post-embryonic development, as previously reported in *D. melanogaster* [42]. We analyzed the mRNA profiles of the three *D. suzukii* genes by RT-PCR and found that *Dsgrim* and *Dshid* expression commenced within the first hour of embryogenesis, whereas *Dsrpr* expression commenced after 4 h. *Dsrpr* expression levels were highest during late embryogenesis and the pupal stage

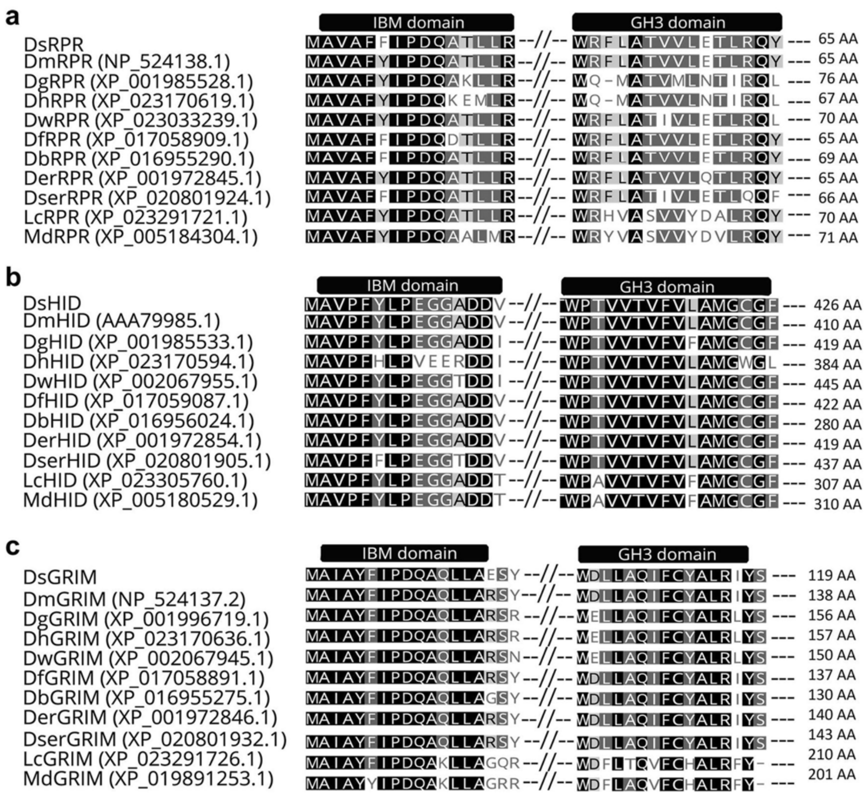

Fig. 1 Alignment of the *D. suzukii* RHG proteins with orthologs in other dipterans: **a** REAPER (RPR), **b** HEAD INVOLUTION DEFECTIVE (HID) and **c** GRIM. In each case, the protein from *D. suzukii* (Ds) is aligned with orthologs from *D. melanogaster* (Dm), *D. grimshawi* (Dg), *D. hydei* (Dh), *D. willistoni* (Dw), *D. ficusphila* (Df), *D. biarmipes* (Db), *D. erecta* (Der), *D. serrata* (Dser), *Lucilia cuprina* (Lc) and *Musca domestica* (Md). Identical amino acids are shaded in black and conservative changes in gray. The IBM and GH3 domains are shown

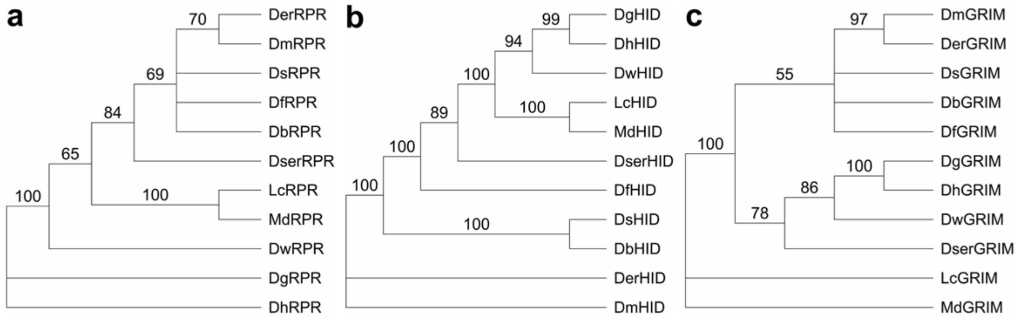

Fig. 2 Phylogenetic analysis of dipteran RPR, HID and GRIM proteins. Unrooted neighbor-joining trees were constructed with **a** RPR, **b** HID and **c** GRIM amino acid sequences. Bootstrap values (1000 replicates) are shown on the nodes of the trees. Species abbreviations are the same as in Fig. 1

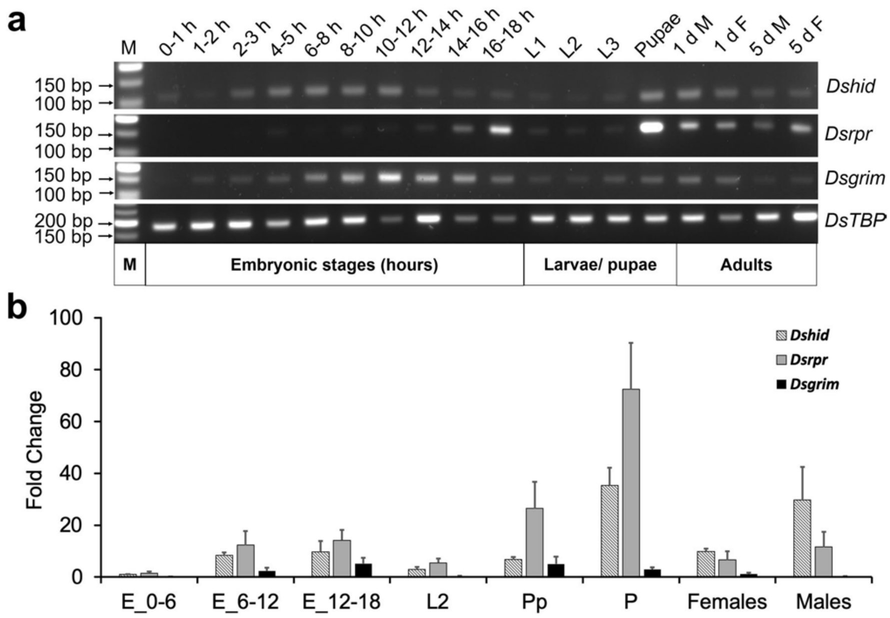

Fig. 3 Gene expression profiles of *Dshid*, *Dsrpr* and *Dgrim* throughout the development of *Drosophila suzukii*. **a** Reverse transcriptase-PCR analysis of *Dshid, Dsrpr* and *Dsgrim*. RNAs from embryos collected at different time points after egg laying (in hours), larvae, pre-pupae 24 h, pupae, adult females and males (1 and 5 days old). *DsTBP*, which is expressed at all stages, is the loading control. M is the molecular weight ladder. The PCR product sizes are 109 bp for *Dshid,* 146 bp for *Dsgrim,* 147 bp for *Dsrpr,* and 182 bp for *DsTBP.* **b** Relative expression levels of pro-apoptotic genes at different time points after egg laying (in hours), larvae, pre-pupae 24 h, pupae, adult females, and males (1 and 5 days old) as determined by qRT-PCR. Expression levels were normalized to *DsTBP* (reference gene), which is expressed at all stages. In addition to that, expression was further normalized to *Dshid* 0–6 h. The mean and standard error from three replicate experiments are shown

(Fig. 3a). Similar profiles were revealed by qPCR when the data were normalized to the 0–6 h embryonic stage expression of *Dshid*. We found that *Dshid* and *Dsrpr* expression commences at the embryonic stage, increases throughout development and peaks in late pupae. Comparative analysis indicated that *Dsrpr* is expressed at a higher level than the

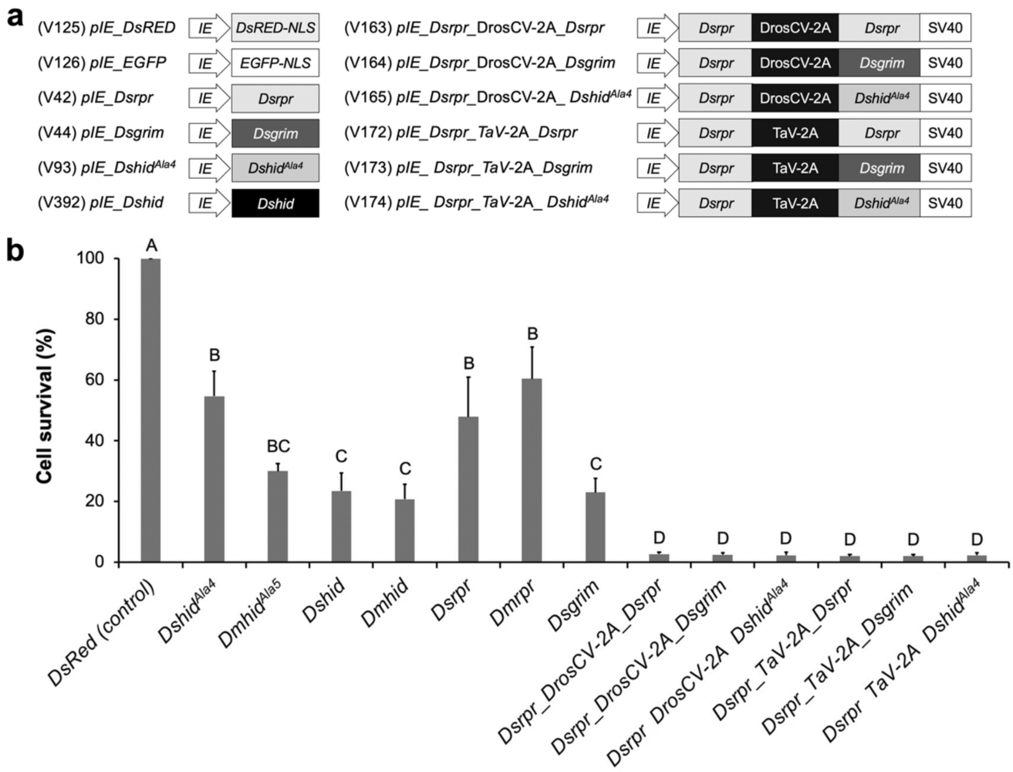

Fig. 4 Functional activity of *Drosophila suzukii* pro-apoptotic genes. **a** Schematic map of the *pIE4* test plasmids. **b** S2 cells were co-transfected with *pIE4-EGFP* plasmid and one of the *pIE* expression plasmids or *pIE4-DsRed* control plasmid to visualize the successfully transfected cells using M205FA MZ FLIII microscope (Leica Microsystems). Number of EGFP positive cells as survived cells, were counted using Image J (Fiji). The experiment was carried out in three replicates. Mean and standard errors are shown in the figure, bars with different uppercase letters are significantly different at $P<0.050$ (one-way ANOVA, Holm–Sidak method for pairwise multiple comparison)

other two genes, reaching almost 20-fold higher than *Dshid* in the pupae, whereas *Dsgrim* is expressed at the lowest level (Fig. 3b).

Activity of *D. suzukii* pro-apoptotic genes in S2 cells

To characterize each *D. suzukii* pro-apoptotic gene, we generated the single expression constructs V42, V44, V392 and V93, representing the wild-type *Dsrpr*, *Dsgrim* and *Dshid* genes and the constitutive *Dshid* mutant $Dshid^{Ala4}$, respectively (Fig. 4a). The binary constructs in which *Dsrpr* was paired with another copy of itself (V163, V172), with *Dsgrim* (V164, V173) or with $Dshid^{Ala4}$ (V165, V174), were also developed to test the pro-apoptotic activity from combinations (Fig. 4a). The two distinct constructs representing each pairing correspond to the use of two different picornaviral 2A peptides [33], namely DrosCV-2A (V163, V164, V165) and TaV-2A (V172, V173, V174). After 16 h, the transfected cells from single expression constructs had lost confluence, there was a change in cell shape, and in some cases there was evidence of membrane rupture, and such phenomena was not observed in the cells that transfected with *pIE4-DsRed* control vector (V125) (Online Resource 2), suggesting expression of each pro-apoptotic gene was able to reduce the fitness of the cells, but not trigger widespread apoptosis. Quantitative analysis by cell counting suggested that single constructs using *Dsrpr, Dshid,* $Dshid^{Ala4}$, and *Dsgrim* ($P<0.001$, One-way ANOVA) significantly reduced the cell number compared to the control (Fig. 4b). In addition, all binary constructs killed more cells compared to single expression constructs ($P < 0.001$, One-way ANOVA), confirming that the combinations of pro-apoptotic genes were most efficient in reducing the cell viability (Fig. 4b). We also compared cell death activity of *Dshid, Dshid*^{Ala4}, and *Dsrpr* to the previously reported *Dmhid, Dmhid*^{Ala5}, and *Dmrpr* genes [30] (Fig. 4b). No significant difference was observed in the activity of *Dmrpr* versus *Dsrpr* ($P = 0.868$, One-way ANOVA), *Dmhid* versus *Dshid* ($P = 1.000$, One-way ANOVA), and *Dshid*^{Ala4} versus *Dmhid*^{Ala5} ($P = 0.014$; One-way ANOVA, see Online Resource 4). The comparison was repeated in another set of cells (Online Resource 6) in triplicates to ensure the results from Fig. 4b.

Activity of *D. suzukii* pro-apoptotic genes in larvae-pupae.

To characterize in vivo activity of pro-apoptotic genes in *D. suzukii,* we microinjected pro-apoptotic genes *Dsrpr, Dshid* and *Dsgrim* incorporated into RMCE donor constructs as V338, V350 and V337, respectively, and AH448 as a control [39] into the embryos of the previously established transgenic *D. suzukii* landing site line carrying the construct AH443_PUbEGFP-TRE-*CctraI-Alhid*^{Ala2}-loxN-3xP3-AmCyan-lox2272 (Online Resource 7) [39]. 48 h after injections, hatched larvae were counted and screened for transient expression (DsRed or AmCyan). We observed low survival rate for embryos injected with the RHG containing donor constructs (Online Resource 7) compared to the control. There were also no adult survivors for all larvae transiently expressing the RHG containing constructs while 20% of the control larvae survived to adulthood. Because apoptotic genes were driven by the 3xP3 promoter, it can be speculated that 3xP3 lead the expression of apoptotic gene already in larval tissues [43, 44], causing lethality in transient larvae.

Discussion

Apoptosis is a highly regulated mechanism for the targeted and programmed destruction of cells that are damaged or unwanted. In contrast to the events that occur during necrosis, apoptosis is a controlled and beneficial process, with essential roles in development and homeostasis. It can be initiated intrinsically in response to cell stress or extrinsically by extracellular ligands. Still, the pathways converge on a small number of pro-apoptotic genes that directly mediate the cellular-level biochemical and cellular changes involved in programmed cell death, such as cytoplasmic shrinkage, chromatin condensation and nuclear fragmentation [1]. The most important pro-apoptotic genes include the RHG family, named after the founder members *rpr*, *hid* and *grim*. These genes were initially identified in *D. melanogaster,* and the corresponding proteins are characterized by an N-terminal IBM that interacts with Diap1 to release pro-apoptotic caspases and an internal GH3 domain that induces the mitochondrial death pathway in a caspase-independent manner [7]. Removing either IBM or GH3 motifs from the protein partially or entirely blocked the pro-apoptotic activity of the responsive genes from the Caribbean fruit fly *Anastrepha suspensa* [30], the Scuttle Fly *Megaselia scalaris* [45], the primary malaria vector *Anopheles gambiae* [46], and the silkworm *Bombyx mori* [47]. Thus, IBM and GH3 motifs are critical features responsible for functional pro-apoptotic genes [48, 49]. The IBM and GH3 motifs in HID and GRIM are identical among *D. suzukii*, *D. melanogaster* and several other *Drosophila* species (Fig. 1a, b). In RPR homologs, the IBM motif is nearly identical among these species, but the GH3 domain is less well conserved (Fig. 1c). It was suggested that the amino acid changes in the GH3 domain, which is potentially a HID and GRIM binding motif, reduces the effectiveness of RPR [48, 50]. Indeed, we observed less pro-apoptotic activity from *Dsrpr* compared to these from *Dshid* and *Dsgrim* in the cell death assays (Fig. 4b), suggesting that *Dshid* and *Dsgrim* are better candidate genes for the development of TESS in *D. suzukii.* In addition to GH3 and IBM domains, we also searched for *bantam* miRNA binding sites in the *Dshid* 3′UTR that have been reported in the *Dmhid* 3′UTR homologous sequence [38]. All five regions are conserved and could also play a role in *Dshid* regulation (Online Resource 3).

Both *D. suzukii* and *D. biarmipes* have similar spots on the wings and are closely related to each other [35, 51], and it was suggested that *D. suzukii* diverged from *D. biarmipes* approximately 6 to 9 million years ago [52]. The phylogenetic analysis here also confirmed that the DsHID, DsGRIM and DsRPR are closely related to their orthologs from *D. biarmipes* (Fig. 2). In *D. melanogaster*, *hid* and *rpr* genes are expressed at moderate (*hid*) or high (*rpr*) levels in pupae, moderate levels in embryos and low levels in larvae and adults. In contrast, *grim* is expressed at low levels through development [53]. Similar patterns were observed for the expression of *Dsrpr*, *Dshid* and *Dsgrim* (Fig. 3), suggesting that also the functional roles and regulation pathways of these genes could be conserved between the two species. In fact, the activity of certain pro-apoptotic genes is well conserved across different species. For example, ectopic expression of pro-apoptotic genes from the European green blow fly *L. sericata* [50] and *M. scalaris* [45] caused tightly-regulated cell death in *D. melanogaster,* and *D. melanogaster rpr* could efficiently induce apoptosis in *A. suspensa* [54] and mammalian cells [55]. However, other studies showed that endogenous genes work more efficiently and can be tightly controlled compared to homologous genes from closely related species [29]. Using *D. melanogaster* S2 cells as a reporter system, we identified that the *Dshid* and *Dsgrim* as well as *Dmhid* and *Dmhid*^{Ala5} are equally effective to reduce the cell viability while *rpr* homologs and the *Dshid*^{Ala4} are less effective to confer cell death (Fig. 4b). A weaker activity of *rpr* was reported before [30, 50], but the result from *Dshid*^{Ala4} is unexpected because the mutations in the MAPK phosphorylation sites should avoid downregulation of HID by the Ras signaling pathways [36]. Previous studies showed that the *A. suspensa* and *L. cuprina* TESS strains using the phospho-mutated version of *hid* were more effective at causing cell death [25, 54]. The Ras1/MAPK pathway may affect the cell death-inducing ability of *Dshid*^{Ala4} differently in S2 cells than in analysis conducted on in vivo cell networks. Indeed, injecting a transgenic line with cell death promoting constructs led to a reduction of progeny in a small-scale transient in vivo assay (Online Resource 7).

The efficacy of an SIT program relies on mass-rearing and releasing a large number of insects [17, 22]. For example, more than 15 million sterile *C. hominivorax* are released per week for efficient containment of this species [21]. It was demonstrated that the spontaneous mutations occur in the pro-apoptotic gene of a TESS strain with a 1 in a million frequency [56]. Uncontrolled breakdown of the TESS during mass rearing due to the loss-of-function mutation could lead to the release of females. Consequently, employing multiple lethal genes or the development of redundant lethal systems would be important for the efficiency and stability of TESS strains [27, 28]. Previous reports also showed that combinations of pro-apoptotic genes caused a higher level of lethality than a single gene [30]. An efficient co-expression system was recently described in *D. suzukii* using picornaviral self-cleaving 2A peptides [33], which can express two or more genes in a stoichiometric ratio by ribosomal skipping [57]. Thus, two copies of the same gene should express and be translated independently and confer higher lethality levels compared single copy expression systems. The difference in lethality between single versus multiple copies of a pro-apoptotic genes has functionally been shown in *D. melanogaster, C. capitata*, *L. cuprina* and *A. suspensa* flies. There, lethality tests with heterozygous and homozygous transgenic individuals showed that lethality was most effective in homozygous individuals, carrying the double amount of copies [58, 59]. Similarly, while single DsRPR was conferring only low lethality numbers in our setup, the double amount of DsRPR co-expressed by a 2A peptide might have reached the required dosage for cell death. Previous reports showed that the 2A peptide is always cleaved with the upstream protein, not the downstream protein [33, 57, 60]. However, this doesn't hinder the correct translation of the upstream or downstream proteins [33, 57, 60–63]. Here, co-expression of *rpr* and *rpr, hid* or *grim* with the help of 2A peptides confirmed the essential interaction of pro-apoptotic cell death genes to confer lethality that was reported with different expression strategies in *D. melanogaster* before [3, 48]. Our studies verified *Dsrpr* as an always expressed apoptotic gene in embryonic, larval and pupal stages (Fig. 3b) showing its role as a global regulator of apoptosis. The apoptotic effects were more pronounced when cells were transfected with the bicistronic constructs, with a clear impact on cell growth as well as morphology resulting in 97–98% cell death (Fig. 4b; Online Resource 2). Thus, different pro-apoptotic genes in combination with conditional systems like the tetracycline-controllable Tet-Off system can be developed into TESS strains in *D. suzukii.* With several copies of apoptotic genes on one construct, the desired effect could be enhanced, and the system could cope with the loss of individual genes due to missense mutations, ensuring that the emergence of resistance would be delayed. However, a strong cell death effector is not preferred for TESS if the lethal effect is leaky [64–66]. Consequently, the performance of pro-apoptotic genes needs to be carefully evaluated in the TESS strains for an efficient, safe, and sustainable control program of *D. suzukii*.

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Author contributions SAJ, YY and JS performed the research. YY and MFS conceived the study. SAJ, YY and MFS analyzed data and wrote the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest We confirm that no author has any conflict of interest to disclose, all authors have approved the version submitted for publication, the work in this article is original and has not been published previously, and the article is not under consideration by any other journal.

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Identification and characterization of four *Drosophila suzukii* cellularization genes and their promoters

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Abstract

Background: The spotted-wing Drosophila (*Drosophila suzukii*) is a widespread invasive pest that causes severe economic damage to fruit crops. The early development of *D. suzukii* is similar to that of other Drosophilids, but the roles of individual genes must be confirmed experimentally. Cellularization genes coordinate the onset of cell division as soon as the invagination of membranes starts around the nuclei in the syncytial blastoderm. The promoters of these genes have been used in genetic pest-control systems to express transgenes that confer embryonic lethality. Such systems could be helpful in sterile insect technique applications to ensure that sterility (bi-sex embryonic lethality) or sexing (female-specific embryonic lethality) can be achieved during mass rearing. The activity of cellularization gene promoters during embryogenesis controls the timing and dose of the lethal gene product.

Results: Here, we report the isolation of the *D. suzukii* cellularization genes *nullo*, *serendipity-α*, *bottleneck* and *slow-as-molasses* from a laboratory strain. Conserved motifs were identified by comparing the encoded proteins with orthologs from other Drosophilids. Expression profiling confirmed that all four are zygotic genes that are strongly expressed at the early blastoderm stage. The 5′ flanking regions from these cellularization genes were isolated, incorporated into *piggyBac* vectors and compared in vitro for the promoter activities. The *Dsnullo* promoter showed the highest activity in the cell culture assays using *D. melanogaster* S2 cells.

Conclusions: The similarities in the gene coding and 5′ flanking sequence as well as in the expression pattern of the four cellularization genes between *D. melanogaster* and *D. suzukii*, suggest that conserved functions may be involved in both species. The high expression level at the early blastoderm stage of the four cellularization genes were confirmed, thus their promoters can be considered in embryonic lethality systems. While the *Dsnullo* promoter could be a suitable candidate, all reported promoters here are subject to further in vivo analyses before constructing potential pest control systems.

Keywords: Spotted-wing Drosophila, Sterile insect technique, Transgenic embryonic sexing system , *D. melanogaster* S2 cells, Tetracycline-off system

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Background

The spotted-wing Drosophila (*Drosophila suzukii* Matsumura) is native to eastern Asia but has become a widespread invasive pest of fruit crops [1, 2]. The sterile insect technique (SIT) is a bio-control strategy that works by releasing a large number of typically radiation-sterilized males in the environment so they can compete with wild-type males. Mating between sterilized males and wildtype females produces no offspring, so the repeated release of sterile males leads to population suppression or eradication [3–5]. SIT has been used to control tephritid fruit flies, notably the Mediterranean fruit fly (*Ceratitis capitata*), with great success [4]. Consequently, it is also proposed as a cost-effective and environment-friendly control strategy for *D. suzukii* [6, 7]. Since sterilized females would compete with wild-type females for sterilized males and would still cause damage to fruit, *C. capitata* genetic sexing strains (GSS) have been developed to achieve male-only release [8], which is far more efficient and cost-effective than bisexual releases in the field [9, 10]. These GSSs use recessive pupal color or temperature sensitive lethal (tsl) mutations and Y:autosome translocations thus sexing can be achieved based on the pupal color or temperature-controlled female lethality [8]. However, it is difficult to transfer genetic sexing systems to other pest species because suitable recessive mutations and chromosome rearrangements are required in the target species.

Transgenic embryonic sexing systems (TESS) have been developed in several agricultural pests to enable male-only releases by killing all females at the embryonic stage [11–14]. The general mechanism involves the incorporation of binary tetracycline-off (Tet-off) modules into transposon vectors for germ-line transformation. Typically, the promoters of endogenous cellularization genes are used to express the *tetracycline transactivator* (*tTA*) gene (driver). In the absence of tetracycline, tTA binds to the tetracycline response element (TRE) and induces the TRE-linked pro-apoptotic gene (effector) only in females due to the presence of a sex-specific intron. Eliminating females before the feeding stage could reduce the insect diet cost during mass rearing thus lead to a more cost-effective SIT program.

A cellularization gene promoter is a key component of an efficient TESS because it controls *tTA* expression and determines the timing and dose of effector gene expression [13, 15]. There are four zygotic genes required for cellularization of the syncytial blastoderm in *D. melanogaster*: *nullo*, *serendipity-α* (*sry-α*), *bottleneck* (*bnk*) and *slow-as molasses* (*slam*) [16]. A 95-bp 5′ flanking sequence containing four conserved motifs is both necessary and sufficient for the blastoderm-specific expression of *Dm_sry-α* [17, 18]. The promoter activity at pre-blastoderm stage in *D. melanogaster* may also be controlled by TAG-team motifs, a series of 7-bp sequences commonly found in the 5′ flanking region of *D. melanogaster* genes expressed during early development [19]. The *nullo* and *sry-α* promoters have been used successfully to drive *tTA* and induce embryonic lethality in *D. melanogaster* [20]. In tephritid fruit flies, the *sry-α* promoter has been used most widely, and TESS systems incorporating this promoter have been reported in *Anastrepha suspensa* [11], *C. capitata* [12] and *Anastrepha ludens* [21]. The *bnk* and *nullo* promoters were used in the calliphorid blowfly *Lucilia cuprina* [13, 14]. Thus far, the *slam* promoter has not been used to develop functional TESS strains , possibly due to its low activity at the embryonic stage [12, 22]. However, a TESS promoter must not be too active, if the Tet-Off system is used, because high levels of *tTA* expression are also deleterious [23]. Therefore, systematic evaluation of cellularization genes and their promoters could facilitate the development of optimal TESS systems.

Here we identified four *D. suzukii* cellularization genes (*nullo*, *sry-α*, *bnk* and *slam*) based on the reference genome sequence in the Spotted Wing Drosophila database, SWDbase [24]. We isolated the coding sequence (CDS) of each gene from our laboratory strain (*D. suzukii* USA) and confirmed the presence of conserved motifs by comparing the encoded proteins with orthologs in other *Drosophila* species. The expression profile of each gene during development was verified by reverse transcription (RT)-PCR and quantitative real-time (qRT) PCR. We then isolated the 5′ flanking sequences from the four genes and incorporated them into *piggyBac* vectors for in vitro evaluation. Based on the promoter activity of these genes in insect cells, we discuss their potential applications in biocontrol strategies for *D. suzukii*.

Results

D. suzukii cellularization genes

Four *D. suzukii* cellularization genes were identified by homology searches in SWDbase and were compared in silico to known sequences. *Ds_nullo* (DS10_00005287) was identified as an intron-less gene like its *D. melanogaster* ortholog (*Dm_nullo*). The full-length 642-bp *Ds_nullo* CDS encodes a putative protein with 213 amino acids, most closely related to its ortholog in *D. biarmipes* (99% similarity, 96% identity), but also similar to its orthologs in *D. melanogaster* (94%) and *D. rhopaloa* (92%), with lower similarity (57–77%) in other Drosophilds (Fig. 1a; Additional file 2 Fig. S1). The N-terminus of DsNULLO features a myristoylation site and a cluster of positively charged amino acids, which may target the protein to the plasma membrane [25]. The remainder of the polypeptide features five conserved segments (Additional file 2 Fig. S1) required for NULLO proteins to stabilize the basal junction components in the nascent cleavage furrows of blastoderm cells [26].

A

	DsNULLO	DbNULLO	DmNULLO	DrNULLO	DserNULLO
DsNULLO		99%	94%	92%	77%
DbNULLO	99%		95%	92%	77%
DmNULLO	94%	95%		90%	77%
DrNULLO	92%	92%	90%		76%
DserNULLO	77%	77%	77%	76%	

B

	DsSRY-α	DmSRY-α	DvSRY-α	DhSRY-α	DgSRY-α
DsSRY-α		94%	68%	51%	54%
DmSRY-α	94%		67%	51%	53%
DvSRY-α	68%	67%		51%	54%
DhSRY-α	51%	51%	51%		74%
DgSRY-α	54%	53%	54%	74%	

C

	DsBNK	DmBNK	DmirBNK	DkBNK	DoBNK
DsBNK		85%	70%	71%	66%
DmBNK	85%		70%	73%	67%
DmirBNK	70%	70%		72%	88%
DkBNK	71%	73%	72%		69%
DoBNK	66%	67%	88%	69%	

D

	DsSLAM	DmSLAM	DpSLAM	DgSLAM	DbuSLAM
DsSLAM		84%	77%	70%	69%
DmSLAM	84%		85%	74%	73%
DpSLAM	77%	85%		75%	72%
DgSLAM	70%	74%	75%		79%
DbuSLAM	69%	73%	72%	79%	

Fig. 1 Similarities in the NULLO, SRY-α, BNK and SLAM proteins from *Drosophila spp.* **a** *Drosophila suzukii* NULLO (DsNULLO) is aligned with orthologs from *Drosophila biarmipes* [DbNULLO] (GenBank: XP_016948821.1), *Drosophila melanogaster* [DmNULLO] (GenBank: NP_511067.3), *Drosophila rhopaloa* [DrNULLO] (GenBank: XP_016982709.1), and *Drosophila serrata* [DserNULLO] (GenBank: XP_020805612.1). **b** *Drosophila suzukii* SERENDIPITY-α (DsSRY-α) is aligned with orthologs from *Drosophila grimshawi* [DgSRY-α] (GenBank: XP_001995347.1), *Drosophila hydei* [DhSRY-α] (GenBank: XP_023168708.1), *Drosophila melanogaster* [DmSRY-α] (GenBank: NP_524580.1) and *Drosophila virilis* [DvSRY-α] (GenBank: XP_002056142.1). **c** *Drosophila suzukii* BOTTLENECK (DsBNK) is aligned with orthologs from *Drosophila melanogaster* [DmBNK] (GenBank: NP_524604.2), *Drosophila miranda* [DmirBNK] (GenBank: XP_017140437.1), *Drosophila obscura* [DoBNK] (GenBank: XP_022213695.1), and *Drosophila kikkawai* [DkBNK] (GenBank: XP_017034088.1). **d** *Drosophila suzukii* SLOW-AS-MOLASSES (DsSLAM) is aligned with orthologs from *Drosophila grimshawi* [DgSLAM] (GenBank: XP_001988112.1), *Drosophila pseudoobscura* [DpSLAM] (GenBank: XP_001355861.2), *Drosophila melanogaster* [DmSLAM] (GenBank: NP_001285668.1), and *Drosophila busckii* [DbuSLAM] (GenBank: ALC38995.1). The protein alignment was performed in Geneious Prime program and similarities (Blosum45, threshold 1) are shown

Ds_sry-α (DS10_00012897) has three exons. The full-length 1593-bp *Ds_sry-α* CDS encodes a putative protein with 530 amino acids, most closely related to its ortholog in *D. melanogaster* (94% similarity, 87% identity; Fig. 1b). The N-terminus of DsSRY-α features a cysteine-rich motif, possibly a transmembrane segment (Additional file 2 Fig. S2). A conserved C-terminal region shows high similarity to Ezrin, Radixin and Moesin (ERM) proteins which facilitate actin–membrane interactions [27], suggesting DsSRY-α fulfils a similar role in the reorganization of microfilaments during cellularization [28].

Ds_bnk (DS10_00007356), like *Ds_nullo*, is an intronless gene. The 903-bp *Ds_bnk* CDS encodes a putative protein with 303 amino acids, most closely related to its ortholog in *D. melanogaster* (85% similarity, 80% identity; Fig. 1c). Finally, *Ds_slam* (DS10_00010822) has two exons. The 3414-bp *Ds_slam* CDS encodes the largest of the four cellularization proteins, with 1137 amino acids. DsSLAM is most closely related to its ortholog in *D. melanogaster* (88% similarity, 74.6% identity; Fig. 1d). Previous studies have not identified any functional motifs in DmBNK [29] or DmSLAM [30]. However, several highly conserved regions were identified in DsBNK (Additional file 2 Fig. S3) and DsSLAM (Additional file 2 Fig. S4). The phylogenetic analysis of all four proteins using a neighbor-joining algorithm revealed that DsSRY-α, DsBNK and DsSLAM clustered with their *D. melanogaster* orthologs, whereas DsNULLO clustered with DbNULLO and DmNULLO (Fig. 2).

D. suzukii cellularization genes are strongly expressed during embryogenesis

The RT-PCR results suggest that mRNA levels of all four cellularization genes were undetectable during the 0–0.5 h time window, reached a sharp peak around the onset of cellularization (2–3 h after egg laying) and then declined, but nevertheless persisted to the adult stage (Additional file 2 Fig. S5). *Ds_sry α* expression peaked slightly later than *Ds_nullo*, as previously reported for the *D. melanogaster* orthologs [31]. The *Ds_bnk* and *Ds_slam* transcripts were particularly abundant at the 1–3 h stage, but declined sharply thereafter (Additional file 2 Fig. S5). Primers for the reference genes *TATA binding protein* (*TBP*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *arginine kinase* (*AK*), *a-Tubulin* (*α-tub*) and *Histone H3* (*His3*) were also evaluated for their performance using RT-PCR analysis [32, 33]. The primers that amplified products from most or all stages (Additional file 2 Fig. S5) were further evaluated for efficiency using a serial dilution of cDNA as templates. The primer

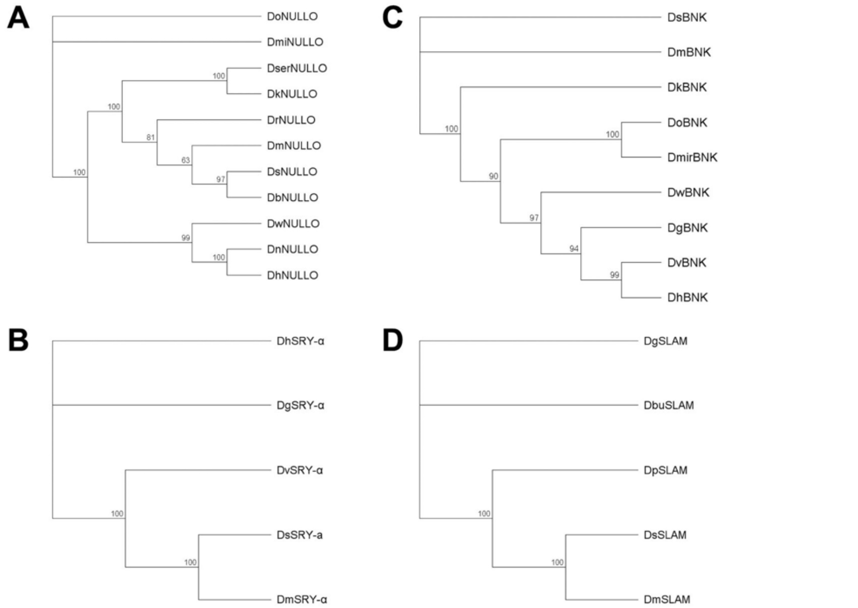

Fig. 2 Phylogenetic analysis of *Drosophila* spp. NULLO, SRY-α, BNK and SLAM proteins. Unrooted neighbor-joining trees were constructed with amino acid sequences from **a** NULLO, **b** SRY-α, **c** BNK, and **d** SLAM. Bootstrap values (1000 replicates) are shown on the nodes of the trees. In addition to the orthologs in Fig. 1a, *Drosophila hydei* [DhNULLO] (GenBank: XP_023161247.1), *Drosophila kikkawai* [DkNULLO] (GenBank: XP_017022675.1), *Drosophila miranda* [DmirNULLO] (GenBank: XP_017156218.1), *Drosophila navojoa* [DnNULLO] (GenBank: XP_017964581.1), *Drosophila obscura* [DoNULLO] (GenBank: XP_022210380.1), and *Drosophila willistoni* [DwNULLO] (GenBank: XP_002071148.1) are used for the alignment. In addition to the orthologs in Fig. 1c, *Drosophila grimshawi* [DgBNK] (GenBank: EDV90810.1), *Drosophila hydei* [DhBNK] (GenBank: XP_023168586), *Drosophila virilis* [DvirBNK] (GenBank: XP_002055918.1) and *Drosophila willistoni* [DwBNK] (GenBank: XP_002072000.1) are used for the alignment. Other species abbreviations are the same as in Fig. 1

efficiency was calculated as 99.4% for *Ds_TBP*, 93.6% for *Ds_GAPDH*, 99.5% for *Ds_AK*, and 89.6% for *Ds_His3*. Consequently, primers for *Ds_TBP* and *Ds_AK* that showed high efficiency were used in the qRT-PCR experiments. The qRT-PCR data were consistent with our semi-quantitative RT-PCR analysis, with all four cellularization genes expressed at the highest level during the early blastoderm stages (Fig. 3). When normalized with *Ds_TBP* and *Ds_AK*, the *Ds_sry-α*, *Ds_nullo*, *Ds_bnk* and *Ds_slam* transcripts were 123-, 1040-, 13,469- and 56-

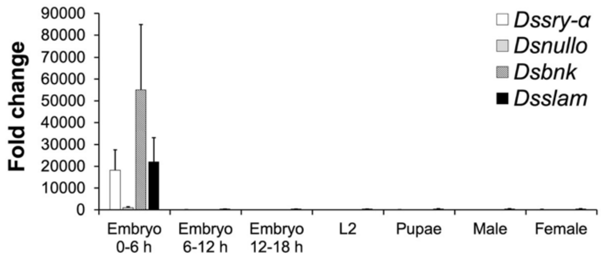

Fig. 3 Quantitative real-time-PCR to determine relative expression of cellularization genes. Three different embryonic stages (0–6 h, 6–12 h and 12–18 h after egg laying), the second instar larvae (L2), pupae and 5 d old males and females were analyzed. The gene expression was normalized to the reference genes *TBP* and *AK*, and related to the *Dsnullo* expression level at 6–12 h embryos. The mean and standard error from three replicate experiments are shown

fold more abundant in 0–6 h embryos than 6–12 h embryos, and 116-, 153-, 2433- and 49-fold more abundant in 0–6 h embryos than adult female, respectively (Fig. 3). Furthermore, in 0–6 h embryos the *Dssry-α*, *Dsbnk* and *Dsslam* transcripts were respectively 17.5-fold, 52.9-fold and 21.2-fold more abundant than *Dsnullo* mRNA. The relatively large variations (error bars) in gene expression from 0 to 6 h embryos are possibly due to the different ratios of eggs collected at early blastoderm stages (2–3 h) to eggs from other time period within 0–6 h from three replicates. Nevertheless, the expression levels of all four genes were much lower at later developmental stages (Fig. 3).

5′ flanking region of *D. suzukii* cellularization genes

The 5′ flanking region of *Ds_sry-α* lacked motif IV but contained two TAGteam motifs, as well as a TATA box (TATATAAA) 23 bp upstream of the putative transcription start site (TSS) (Fig. 4A; Additional file 2 Fig. S6). The 5′ flanking region of *Ds_nullo* contained one TAGteam motif and a TATA box (TATATAT) 24 bp upstream of the predicted TSS (Fig. 4A; Additional file 2 Fig. S7). The 5′ flanking region of *Ds_bnk* contained three TAGteam motifs and a TATA box (TATATAAA) 25 bp upstream of the TSS (Fig. 4A; Additional file 2 Fig. S8). However, neither of 5′ flanking sequences of *Ds_slam* and *Dm_slam* contained TAGteam motifs; there was also no TATA box in these regions identified (Fig. 4A; Additional file 2 Fig. S9). We prepared four *piggyBac* test vectors (V205–V208) in which the 5′ flanking sequence of *Ds_sry-α*, *Ds_nullo*, *Ds_slam* or *Ds_bnk* was linked to the *DsRed* reporter gene, respectively (Fig. 4B). All vectors also contained a *DmPUb-AmCyan* marker, allowing us to use an EGFP filter to determine the efficiency of transfection. We transfected *D. melanogaster* S2 cells with each of the four constructs, fixed the cells 18 h post-transfection and counted the numbers of blue and red fluorescent cells. The ratio of red to blue cells therefore provided an internally-consistent readout of regulation activity from the 5′ flanking sequence. We found that the 5′ flanking sequence of *Ds_nullo* (construct V206) generated the strongest DsRed signal (Fig. 4C) and the highest DsRed:AmCyan ratio (Fig. 4D) indicating the highest activity as a gene promoter ($P = 0.001$ when compared to V205 and V208, $P = 0.002$ when compared to V207, one-way ANOVA).

Discussion

Early development in many insect species involves a syncytial blastoderm stage followed by cellularization after a certain number of nuclear divisions, and this process is orchestrated by a small number of zygotic genes [16]. Four major cellularization genes have been identified in

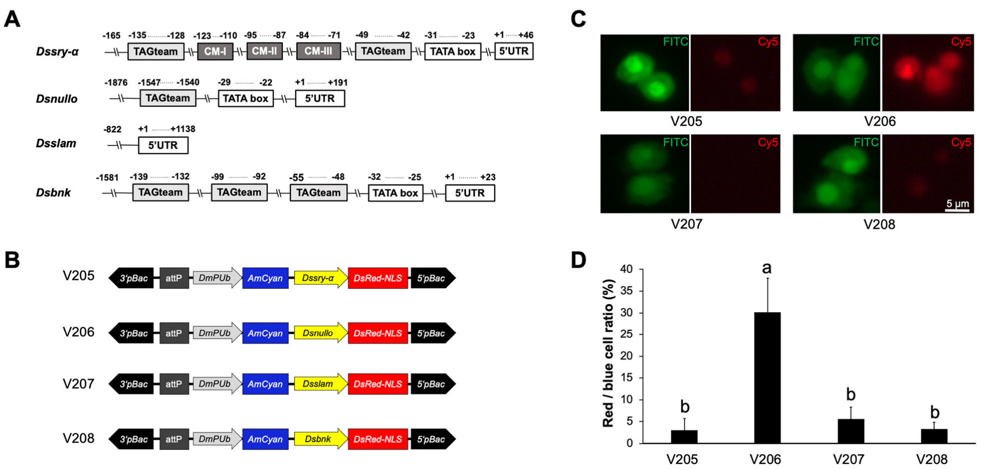

Fig. 4 Functional characterization of *Drosophila suzukii* cellularization gene promoters. **(a)** Schematic map of the 5′ flanking sequences from four cellularization genes (*Dssry-α, Dsnullo, Dsslam* and *Dsbnk*). The positions of TAGteam motifs, conserved motifs (CM), TATA box and 5′ UTR were indicated. **(b)** *piggyBac* test vectors V205, V206, V207 and V208, in which the *Dssry-α, Dsnullo, Dsslam* and *Dsbnk* promoters, are used to drive the expression of DsRed with a nuclear localization signal (NLS), respectively. All test vectors contain an AmCyan marker gene regulated by the constitutive *D. melanogaster polyubiquitin* (*DmPUb*) promoter and an *attP* recombination site. **(c)** *Drosophila* S2 cells were transfected with *piggyBac* vectors and images taken under epifluorescent light conditions using the FITC filter for AmCyan or CY5 filter for DsRed. **(d)** The number of cells that show red and blue fluorescence was counted using Image J, and the ratio of red to blue cells was calculated. Each bar presents the mean ± SE of $n = 3$ experiments. The bars with different letters are significantly different at $P < 0.05$ (one-way ANOVA, Duncan's multiple range test)

the developmental model organism *D. melanogaster* (*nullo*, *sry-α*, *bnk* and *slam*), and the conserved sequences of the orthologs in *D. suzukii* (Additional file 2 Fig. S1–4) together with the peak of expression at the early blastoderm stage (Fig. 3) suggest that similar functions may be involved in both species, including the regulation of actin filaments, microfilaments and membrane polarization [16]. *D. melanogaster* embryogenesis has been divided into 17 stages, with the cellular blastoderm arising during stage 5, 2 h 10 min and 2 h 50 min after egg laying [34]. During this stage, *nullo*, *sry-α*, *bnk*, and *slam* are expressed to coordinate the cellularization process, but expression declines thereafter, to low levels for *nullo* and *bnk*, and to moderate levels for *sry-α* and *slam* [35]. In many fly species, the expression of all four cellularization genes is typically detected as early as 1–2 h after egg laying [13, 22, 31]. However, the corresponding transcripts were already present in *D. suzukii* embryos collected 0–1 h after egg laying, suggesting that gene expression commences at the start of embryogenesis (Additional file 2 Fig. S5). This apparent early expression may reflect the facultative ovoviviparity of *D. suzukii* females, allowing them to retain fertilized eggs, which can therefore develop to a certain extent before laying [36]. However, the transcripts of all four cellularization genes were undetectable during the 0–0.5 h time window (Additional file 2 Fig. S5), confirming the absence of maternal gene expression and that egg retention was not responsible for the early transcripts – instead, the early zygotic gene expression was a genuine phenomenon.

The early and stage-specific expression is important for the development of genetic pest-control systems because it allows us to focus the effect of lethal effector transgenes at the correct developmental stage [11, 13, 37]. Specifically, constitutive and unconditional effector gene expression later in development could lead to undesired female sterility or lethality in a control strain and has been reported previously in interfering with ovarian development and reducing productivity of those strains [14, 15, 21]. Our qRT-PCR data showed that the four *D. suzukii* cellularization genes were expressed with a similar profile to their orthologs in *D. melanogaster* (Fig. 3). The considerably higher gene expression in 0–6 h embryos than 6–12 h embryos for *Ds_nullo* and *Ds_bnk* (1040-fold and 13,469-fold, respectively), suggesting that their promoters are likely early active and would therefore be useful candidates for the development of TESS systems in *D. suzukii*, whereas the *Ds_sry-α* and *Ds_slam* promoter may allow moderate but constitutive expression at later developmental stages. Therefore, those early-embryonic promoters could be used to establish *D. suzukii* sexing systems for SIT programs in the future. Multiple promoters and independent systems will also be important in dealing with natural resistance processes [38]. Those backup systems would help establish stable lines that overcome primary-site mutations during rearing processes and quality control of the mass-reared strains, and help strains to cope with second-site maternal-effect suppressors [38].

The 5′ flanking region of the *D. suzukii* cellularization genes contained several TAGteam motifs, which are often found in *D. melanogaster* genes expressed early in development, and act as binding sites for the transcriptional activator Zelda [19, 39]. The 5′ flanking region of *Ds_bnk* contains three TAGteam motifs 91–139 bp upstream of the predicted TSS, whereas that of *Ds_sry-α* contains two such motifs 93–135 bp upstream of the predicted TSS (Fig. 4A). In contrast, the 5′ flanking region of *Ds_nullo* features only one TAGteam motif, which is much further away from the TSS: 1576 bp upstream (Fig. 4A). Such differences in the number and location of TAGteam motifs may contribute to differences in gene expression at the 0–6 h embryonic stage, since *Ds_bnk* and *Ds_sry-α* mRNA were more than 10-fold more abundant than *Ds_nullo* mRNA (Fig. 3). Interestingly, neither the *Ds_slam* nor the *Dm_slam* 5′ flanking region feature any TAGteam motifs (Fig. 4A; Additional file 2 Fig. S9) but *Ds_slam* was nevertheless expressed at relatively high levels during the 0–6 h embryonic stage. This suggests that the transcriptional activation of *Ds_slam* may be less dependent on Zelda compared to the other genes.

piggyBac-based vectors have been used to evaluate the embryonic activity of the promoters using the *A. suspensa* cell line UFENY-AsE01, an embryonic cell line derived originally from 20-h old embryos [40]. Previously, *sry-a* promoters from *C. capitata* and *A. suspensa* were able to mediate cell death in these AsE01 cells as well as embryonic lethality in transgenic flies [11, 37, 41]. In addition, the *D. melanogaster* S2 cells are also derived from late embryonic stages [42], and have been one of the few *D. melanogaster* cell lines that can be used for heterologous gene expression [43]. Often high levels of heterologous protein expression in S2 cells are due to strong promoters in the expression vectors [44, 45]. Although *Ds_nullo* was expressed at the lowest level among the four genes at the 0–6 h embryonic stage, its promoter achieved the strongest DsRed expression in the cell culture tests (Fig. 4C, D). Such observation suggested that the *Ds_nullo* promoter mediated higher protein production than other tested promoters in the embryonic S2 cells. The weaker activity of *Ds_sry-α* promoter may be due to the absence of motif IV, which was suggested to function as a positive *cis*-acting regulatory element. Previous reports verified that the deletion of motif IV in the *Dm_sry-α* promoter resulted in a more than four-fold loss of activity at the blastoderm stage [18]. Weak transgene expression was also observed for the *slam* promoters from *C. capitata* [12] and *L. cuprina* [22], which do not have TATA box motifs. Similarly, both *Dm_slam* and *Ds_slam* promoter sequences lack TATA box motifs (Additional file 2 Fig. S9). Such TATA-less genes often show multiple TSSs [46], and are more flexibly regulated compared to TATA-containing genes [47]. Thus, *slam* in these species may involve multiple elements for its correct regulation. Computational and functional studies in other species revealed an importance for transcriptional activity based on motifs in the core promoter sequence [48] and a number of relevant transcription factors involved have been studied [47]. But ultimately, the reported promoters have to be functionally tested to evaluate their potential for pest control applications.

Conclusions

For *D. melanogaster*, the gene networks that regulate early development were intensively studied to understand the basic biological processes such as cellularization, sex determination and patterning [16, 19, 39, 48]. Here we have verified the similarities and differences in the promoter and CDS sequences of four cellularization genes between *D. melanogaster* and *D. suzukii*. More functional studies are needed at transcription or protein level [17, 29–31] to understand the gene interaction and network connection during early development in *D. suzukii*. In addition, *D. melanogaster* S2 cells are potentially a good system to study early genes and their promoters due to its embryonic origin [42]. Our results indicated that the *Ds_nullo* promoter can be considered for driving embryonic lethality in a genetic control system such as a TESS. Nevertheless, all reported promoters here have to be further functionally analysed to evaluate their in vivo performance before constructing potential pest control systems.

Methods

Insect rearing and sample collection

Wild-type *D. suzukii* USA specimens were maintained at 25 °C and 60% humidity with a 12-h photoperiod. Eggs were collected over a 30-min or 1-h period on grape juice agar plates as previously described [49]. We allowed the eggs to develop to the desired developmental time point before freezing them in liquid nitrogen. The larvae and pupae were directly collected from stock vials at the desired stage. Adult males and females were isolated immediately after eclosion and sampled 1 or 5 days later.

Gene sequence isolation and analysis

The *gene query* function was not available in SWDbase [24] when this work was initiated in 2014. Therefore, the CDS of the *D. melanogaster* genes *Dm_sry-α* (FBgn0003510), *Dm_nullo* (FBgn0004143), *Dm_bnk* (FBgn0004389) and *Dm_slam* (FBgn0043854) were obtained from FlyBase (http://flybase.org/) and were used as tBLASTx queries against SWDbase. The *Ds_sry-α* query matched a 1593-bp CDS on scaffold 705. *Ds_nullo* matched a 642-bp CDS on scaffold 5. *Ds_bnk* matched a 903-bp CDS on scaffold 21. Finally, *Ds_slam* matched a 1677-bp CDS on scaffold 181. Based on these hits, primers were designed to amplify the full-length CDS of each gene from cDNA prepared from wild-type *D. suzukii* USA embryos (Additional File 1). Total RNA was isolated from embryos at the early blastoderm stage (3–4 h after egg laying) using the ZR Tissue & Insect RNA MicroPrep kit (Zymo Research) and residual DNA was removed with Turbo DNAse (Ambion). The iScript cDNA Synthesis Kit (Bio-Rad) was used to prepare cDNA from 1 μg DNA-free RNA. For *Ds_bnk*, *Ds_slam*, and *Ds_nullo*, each 25-μl reaction mix comprised 0.2 μL Platinum Taq DNA polymerase (Invitrogen), 2.5 μL 10x PCR buffer, 0.75 μL 50 mM $MgCl_2$, 1.0 μL 10 mM dNTP mix, 1.0 μL of each primer (0.4 μM), and 0.5 μL cDNA. An initial denaturation step at 95 °C for 2 min was followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s and extension at 72 °C for 1 min (*nullo* and *bnk*) or 2 min (*slam*), and a final extension at 72 °C for 5 min. For *Ds_sry-α*, multiple PCR attempts using the Platinum Taq DNA polymerase did not result in amplificates, thus the Phusion Flash High-Fidelity DNA Polymerase (Thermo Fisher Scientific) was used. PCR was carried out using 10 μL of the Phusion Flash High-Fidelity PCR Master mix with 1.0 μL of each primer and 1.0 μL cDNA (0.4 μM) in a 25-μl reaction. An initial denaturation step at 98 °C for 10 s was followed by 30 cycles of denaturation at 98 °C for 30 s, annealing at 52 °C for 5 s and extension at 72 °C for 30 s, a final extension at 72 °C for 1 min, and a 4 °C hold. The PCR products were separated by 1% agarose gel electrophoresis and extracted from the gels using the QIAquick Gel Extraction Kit (Qiagen) before cloning in vector pCR4-TOPO. The presence of inserts was confirmed by restriction digestion with EcoRI and sequencing using M13 primers. Sequence translation, polypeptide alignment and phylogenetic analysis were performed using the Geneious Prime software [50].

Gene expression analysis by RT-PCR and qRT-PCR

The RT-PCR was assembled using platinum Taq polymerase as described above. The reaction profile comprised an initial denaturation step at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C (*His3*, *TBP*, *nullo*, *bnk* and *slam*) or 60 °C (*AK, GADPH*, *α-Tub*, *sry-α*) for 30 s, and extension at 72 °C for 1 min, and a final extension at 72 °C for 4 min. We used SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) with 100 ng of template cDNA for the qRT-PCR experiments. The reactions were performed in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) and each comprised an initial denaturation step at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 20 s, extension at 65 °C for 5 s, and a machine related 95 °C for 0.5 s step. All experiments were carried out in three biological triplicates each with three technical replicates.

The $2^{-\Delta\Delta Ct}$ formula was used for all samples and values were normalized to the geometric mean of the reference genes *TBP* and *AK*. All primer sequences were listed in Additional file 1.

Promoter isolation and plasmid construction

The upstream flanking sequences of the *D. melanogaster* and *D. suzukii* cellularization genes were obtained from FlyBase and SWDbase, respectively. The sequences were aligned using Geneious and searched for conserved motifs, TAGteam motifs [19] and the TATA box. High-molecular-weight genomic DNA was prepared from *D. suzukii* adults using DNAzol Reagent (Thermo Fisher Scientific). Promoter fragments were amplified from genomic DNA using the primers listed in Additional file 1 and were transferred to pCR4-TOPO as described above. After confirming the nucleotide sequences, the promoter fragments were amplified and used to replace the *D. melanogaster polyubiquitin* (*DmPUb*) promoter in the *piggyBac* vector pXLBacII-attP-PUbAmCyan_DmPUb_DsRed-NLS-SV40 [51] at the Bsu36I and MluI restriction sites, to obtain the test vectors V205–V208.

Cell culture experiments

Drosophila Schneider 2 (S2) cells [42] were grown on Schneider's medium containing 10% heat-inactivated fetal bovine serum (Hi-FBS) and 1% penicillin/streptomycin in closed-capped flasks without CO_2 at 25 °C. Cells were passaged every 2 days until ≥90% viability was achieved. For transient transfection, we used Xfectin transfection reagent (Takara) according to the manufacturer's instructions. We placed a 13-mm TC coverslip (Sarstedt) into each well of a 24-well plate to facilitate imaging, then seeded each well with 5×10^5 cells in 500 μL of medium. After 3 h, the settled cells were transfected using 2 μg of plasmid DNA, 0.6 μL Xfectin, 27.4 μL Xfectin buffer and 270 μL serum-free Schneider's medium for 4 h. Transfection was stopped by refreshing the dishes with 500 μL Schneider's medium containing 10% Hi-FBS and 1% penicillin/streptomycin. The cells were incubated for ~ 18 h at 25 °C before fixing in 4% paraformaldehyde for 15 min and washing briefly with PBS prior to microscopy.

Cell imaging and counting

Drosophila S2 cells transfected with constructs V205–V208 were imaged using an inverted microscope equipped with a fluorescent slide containing the FITC (ex: 490/20; detecting also AmCyan expression) and CY5 (ex: 620/60) filters (Leica DM IL LED, Leica Microsystems, Wetzlar, Germany). All settings (e.g., exposure time, gain, magnification) were identical for each test so that the fluorescence intensity could be compared. Fluorescent cells were counted in Image J (Fiji) using the automated cell count function. Specifically, raw images were converted to an 8-bit standardized format and inverted before a watershed was applied to separate any cells in direct contact. The counting threshold was set to 30 for the DsRed filter. The differences in red:blue cell ratios (data were square root transformed) for different *piggyBac* vectors were tested by one-way analysis of variance (ANOVA) and means were separated using Duncan's multiple range test in SigmaPlot v12.5 (Systat Software).

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12863-020-00939-y.

Additional file 1. Primer sequences.

Additional file 2: Figure S1. *Drosophila suzukii* NULLO protein alignment. DsNULLO is aligned with orthologs from *Drosophila biarmipes* [DbNULLO] (GenBank: XP_016948821.1), *Drosophila hydei* [DhNULLO] (GenBank: XP_023161247.1), *Drosophila kikkawai* [DkNULLO] (GenBank: XP_017022675.1), *Drosophila melanogaster* [DmNULLO] (GenBank: NP_511067.3), *Drosophila miranda* [DmirNULLO] (GenBank: XP_017156218.1), *Drosophila navojoa* [DnNULLO] (GenBank: XP_017964581.1), *Drosophila obscura* [DoNULLO] (GenBank: XP_022210380.1), *Drosophila rhopaloa* [DrNULLO] (GenBank: XP_016982709.1), *Drosophila serrata* [DserNULLO] (GenBank: XP_020805612.1) and *Drosophila willistoni* [DwNULLO] (GenBank: XP_002071148.1). Identical amino acids are shaded black and conservative changes are indicated in gray. All proteins contain a consensus site for N-terminal myristoylation (M) followed by a positively charged cluster (P). The remainder of the protein contains five conserved regions of amino acids (A–E) separated by short non-conserved regions. **Figure S2.** *Drosophila suzukii* SERENDIPITY-α (SRY-α) protein alignment. DsSRY-α is aligned with orthologs from *Drosophila grimshawi* [DgSRY-α] (GenBank: XP_001995347.1), *Drosophila hydei* [DhSRY-α] (GenBank: XP_023168708.1), *Drosophila melanogaster* [DmSRY-α] (GenBank: NP_524580.1) and *Drosophila virilis* [DvSRY-α] (GenBank: XP_002056142.1). The putative transmembrane domain is underlined and the region of similarity with proteins of the ERM family is boxed. **Figure S3.** *Drosophila suzukii* BOTTLENECK (BNK) protein alignment. DsBNK is aligned with orthologs from *Drosophila grimshawi* [DgBNK] (GenBank: EDV90810.1), *Drosophila hydei* [DhBNK] (GenBank: XP_023168586), *Drosophila kikkawai* [DkBNK] (GenBank: XP_017034088.1), *Drosophila melanogaster* [DmBNK] (GenBank: NP_524604.2), *Drosophila miranda* [DmirBNK] (GenBank: XP_017140437.1), *Drosophila obscura* [DoBNK] (GenBank: XP_022213695.1), *Drosophila virilis* [DvirBNK] (GenBank: XP_002055918.1) and *Drosophila willistoni* [DwBNK] (GenBank: XP_002072000.1). Highly conserved regions are marked with red lines. **Figure S4.** *Drosophila suzukii* SLOW-AS-MOLASSES (SLAM) protein alignment. DsSLAM is aligned with orthologs from *Drosophila grimshawi* [DgSLAM] (GenBank: XP_001988112.1), *Drosophila pseudoobscura* [DpSLAM] (GenBank: XP_001355861.2), *Drosophila melanogaster* [DmSLAM] (GenBank: NP_001285668.1), and *Drosophila busckii* [DbuSLAM] (GenBank: ALC38995.1). Highly conserved regions are marked with red lines. **Figure S5.** Reverse-transcriptase (RT)-PCR to evaluate the reference **(a)** and cellularization **(b)** genes through the development of *Drosophila suzukii*. Primer sequences for reference genes *TATA binding protein* (*TBP*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *arginine kinase* (*AK*), *α-Tubulin* (*α-Tub*) and *Histone H3* (*His3*) (Zhai et al., 2014; Li and Handler, 2017), as well as for four cellularization genes can be found in "Additional file 1". Embryos collected at different time points after egg laying (in hours), larvae (first, second and third instar), pupae (2 days after prepupae), adult female and male (1 and 5 days old). Additional 0–0.5 h samples were used for cellularization genes. The PCR product sizes are 140 bp for *AK*, 130 bp for *GAPDH*, 189 bp for *α-Tub*, 129 bp for *His3*, and 182 bp for *TBP*, 161 bp for *sry-α*, 155 bp for *nullo*, 159 bp for *bnk*, and 149 bp for *slam*. M is the molecular weight ladder. **Figure S6**. Alignment of the *Ds_sry-α* and *Dm_sry-α* 5′ flanking sequences. The upstream flanking sequence from *Ds_sry-α* (165 bp, before it reaches the upstream gene

DS10_00012896, the ortholog of *D. melanogaster janus A*) and *Dm_sry-a* (167 bp) together with the 5'-UTR (annotated in green) are compared. The 5' flanking sequences of *Ds_sry-a* and *Dm_sry-a* contain three and four conserved motifs that confer blastoderm-specific expression, respectively. Both 5' flanking sequences contain two TAGteam motifs (annotated in yellow), and a TATA box (TATATAAA) 23 bp upstream of the putative transcription start site. The *Ds_sry-a* 5' flanking sequence was fused to DsRed-NLS in V205 to act as a gene promoter for the in vitro test. **Figure S7**. Alignment of the *Ds_nullo* and *Dm_nullo* 5' flanking sequences. The upstream flanking sequence from *Ds_nullo* (1876 bp) and *Dm_nullo* (1753 bp) together with the 5'-UTR (annotated in green) are compared. The 5' flanking sequences of *Ds_nullo* sequence contains one TAGteam motif (annotated in yellow), whereas that of *Dm_nullo* contains none. The TATA box (TATATAT) is 24 bp upstream of the putative transcription start site. The *Ds_nullo* 5' flanking sequence was fused to DsRed-NLS in V206 to act as a gene promoter for the in vitro test. **Figure S8**. Alignment of the *Ds_bnk* and *Dm_bnk* 5' flanking sequences. The upstream flanking sequence from *Ds_bnk* (1581 bp) and *Dm_bnk* (1523 bp) together with the 5' UTR (annotated in green) are compared. The 5' flanking sequences of *Ds_bnk* and *Dm_bnk* contain three and four TAGteam motifs (annotated in yellow), respectively, and the TATA box (TATATAAA) is 25 bp upstream of the putative transcription start site. The *Ds_bnk* 5' flanking sequence was fused to DsRed-NLS in V208 to act as a gene promoter for the in vitro test. **Figure S9**. Alignment of the *Ds_slam* and *Dm_slam* 5' flanking sequences. The upstream flanking sequence from *Ds_slam* (822 bp) and *Dm_slam* (887 bp) together with the 5' UTRs (annotated in green) are compared. Neither the *Ds_slam* nor *Dm_slam* 5' flanking sequences contain TAGteam motifs or a TATA box. The *Ds_slam* 5' flanking sequence was fused to DsRed-NLS in V207 to act as a gene promoter for the in vitro test.

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Authors' contributions
Y.Y., S.A.J., J.S., and C.S. performed the research. Y.Y. and M.F.S. conceived the study, analyzed data and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article [and its supplementary information files]. The GenBank accession numbers are as follows: *Ds_sry-a* mRNA: MK392555; *Ds_nullo* mRNA: MK392556; *Ds_bnk* mRNA: MK392557; *Ds_slam* mRNA: MK392558.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Publisher's Note

Chapter 10
Conditional Expression Systems for *Drosophila suzukii* Pest Control

Syeda A. Jaffri, Ying Yan, Maxwell J. Scott, and Marc F. Schetelig

Abstract The sterile insect technique (SIT) is an environmentally friendly pest control method involving sterilization of mass reared insects and their release into the field to suppress insect populations. It has been a valuable tool for insect pest management for several decades. Besides the classical genetic approaches in use, transgenic systems have been established that have or could be transferred to the agricultural pest species *Drosophila suzukii* to improve the efficiency of population suppression such as through the production and release of only male flies. For male-only strains, conditional gene expression systems are required to inhibit female lethality due to expression of a lethal gene. Practically, such a strain with an "off-system" can be reared in a bisexual way in the mass rearing facility with food supplement and create male-only populations for field release by activating female lethality through removal of the supplement. In this chapter, we discuss conditional expression systems that have been developed in the past and their potential for *D. suzukii* control. Methods include the Tet-Off and Tet-On, Erythromycin-Off, Biotin-On, Vanillic acid regulated, Phloretin-Off, Bile acid-Off, and the Quinic acid systems for expression control. In addition, systems that work on stimuli based on light and temperature are discussed.

Keywords Binary expression · Conditional lethality · Insect control · Tet-Off · E-Off · Q system · BA-Off · BA-On · Biotin system · VA-Off · VA-On systems

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10.1 Introduction

Agricultural pests are a global challenge as they contribute to damage the crops. *Drosophila suzukii* Matsumura is now one global player of insect pests that has established around the world and is a devastating pest for the soft fruit industry (Mazzi et al. 2017; Yeh et al. 2020; Panel et al. 2018). The damage caused by *D. suzukii* females is due to oviposition into the soft fruits and their larvae feeding and destroying the fruit flesh. The negative impact of *D. suzukii* is further intensified due to the short generation time of *D. suzukii* that can complete several generations in a single season and is overwintering in many countries (Panel et al. 2018; Rendon et al. 2018). The presence of the alive *D. suzukii* larvae in fruit can cause an entire shipment to be rejected.

To control *D. suzukii* and its burden to economy and environment, several methods have been developed. A traditional control method for *D. suzukii* is the use of one or more chemical pesticides such as spinosyns, pyrethroids, or organophosphates (Cowles et al. 2015; Beers et al. 2011; Timmeren and Isaacs 2013). However, it should be highlighted that control methods based on chemical pesticides are weather-dependent and broad spectrum (i.e., not species-specific) and may cause negative effects on biodiversity (Geiger et al. 2010). It is anticipated that *D. suzukii* will develop resistance to one or more of the commonly used insecticides (Haviland and Beers 2012). However, the development of new chemical insecticides is costly and time-consuming (Borovok et al. 2008; Osei et al. 2003). Although insecticide resistance management helps to avoid the development of resistances, it is often overlooked or misused and can become infective under certain levels of resistance. Thus, there is an increased need to develop environmentally friendly strategies for pest control for *D. suzukii* that could offer a species-specific, sustainable, and cost-effective alternative for its control and is applicable in the framework of area-wide integrated pest management. The alternative strategies could be a supplement to current insecticide applications, but if so, it must be highly effective in the time period between the last (allowed) spray and the harvest. Common waiting periods are between 1 and 2 weeks with varying length depending on crops, formulation, dose, and chemicals used (Vijayasree et al. 2014).

The sterile insect technique (SIT) (Knipling 1955) is an efficient method to control insect pest populations. Classical SIT relies on the mass rearing of insect population, exposing them to irradiation to induce sterility and the mass release of the sterile insects into the field (Klassen 2005). Sterilized males mate with wild females in the field, which as a consequence do not produce any viable offspring that eventually cause the intended population reduction (Fig. 10.1) (Vreysen et al. 2007). The SIT has been implemented to control various insect species in large control programs including the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), the New World screwworm, *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae), several other tephritids, tsetse flies, and Lepidopteran pests (Ingaramo and Beckett 2009; Robinson 2002b; Scott et al. 2017; Klassen and Curtis 2005; Dupont-Filliard et al. 2001). In the early days of the SIT

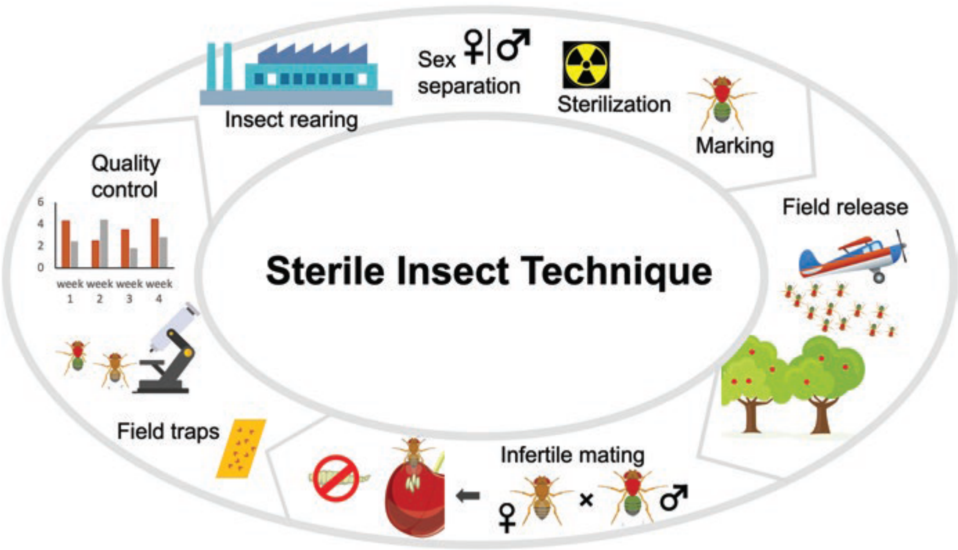

Fig. 10.1 The sterile insect technique (SIT). The SIT consists of mass rearing of insects in a rearing facility (sex sorting, elimination of females, marking and sterilization of males). The sterilized male-only populations are then released to the field area. Wild females that mate with sterile males produce no progeny. This technique helps to suppress the insect population. Targeted field area is continuously monitored by insect collection and analysis

and in the current *C. hominivorax* program (Concha et al. 2016), the release of insects from both sexes was used and resulted in a population reduction, but the release of male-only populations has proven to be most effective in the years and decades thereafter (Rendon et al. 2004). The release of only male fruitflies reduces the rearing costs and avoids additional fruit damage caused by egg deposition from released females. Therefore, to achieve maximum efficiency of the technique, it is important to consider male-only release and remove females from the population during early development within the rearing process. Consequently, for multiple invasive insects, the so-called genetic sexing strains have been established, which produce only males under certain rearing conditions (Franz 2005).

Strategies to enable SIT for novel invasive pest species include the generation of transgenic embryonic sexing strains (TESS) or unisex sterility that, like classical sexing or sterilization, induce lethality in the insects at the embryonic level. This can be achieved by expressing lethal factors in insect pests with the help of conditional expression systems (Schetelig and Handler 2012b; Yan and Scott 2015; Gong et al. 2005; Schetelig et al. 2009a; Ogaugwu et al. 2013; Lewandoski 2001; Meza et al. 2018; Schetelig et al. 2016). Functional systems in drosophilids include lethality and sexing system in *Drosophila melanogaster* (Meigen) (Heinrich and Scott 2000; Thomas et al. 2000; Horn and Wimmer 2003), while the establishment of sexing strains in *D. suzukii* has several constraints. The commonly used expression system for TESS is the Tet-Off system but to date this system has not been established in *D. suzukii*. Studies in *D. melanogaster* also suggest there are resistance constraints that need to be considered for tight control of any genetic trait. A single conditional control system will develop resistance in the insects based on different mechanisms and mutations in the flies (Zhao et al. 2020; Knudsen et al. 2020). Therefore, the use of two or more independent conditional expression systems would benefit a tight control and serve as a backup and mitigation for resistance development. In this chapter, we have compared potential expression systems that can be considered to develop effective stacked systems that could serve the sterile insect technique for *D. suzukii*. For practical use, any system needs to tightly control gene expression with a flexibility in the time window to switch expression On or Off. The focus of this article is on conditional expression systems that have a potential to be used in *D. suzukii*. Two broader groups of conditional expression systems have been investigated: (1) drug-inducible conditional expression systems (Table 10.1), and (2) conditional expression systems regulated by external stimuli.

10.2 Drug-Inducible Conditional Expression Systems

10.2.1 Gene Expression Systems Inducible by Antibiotics

10.2.1.1 Tetracycline-Controlled Gene Expression Systems

The tetracycline-based binary expression systems (Tet systems) originated from *Escherichia coli* and were developed by Gossen and Bujard in 1992. The Tet systems are comprised of three components: first, a tetracycline responsive element (TRE) that has a target gene under the control of a minimal or core promoter and multiple copies of the binding site of the tetracycline repressor (TetR) from the tet operon (tetO). The core promoter contains transcription initiation site and a polymerase binding site. Second, a tetracycline controllable transactivator (tTA) has been composed by fusing the TetR (Gossen and Bujard 1992) to the transcription activation domain from the herpes simplex virus protein VP16 (Triezenberg et al. 1988). Third, the antibiotics of the tetracycline family can be used as control molecules as binding of tetracycline to TetR inhibits binding to tetO (Fig. 10.2a). In the Tet-Off system, in the absence of tetracycline or its derivates, i.e., doxycycline, tTA binds to TRE and activates the minimal promotor that initiates transcription of an effector gene. When tetracycline is present and binds to tTA, the complex cannot bind to the TRE, and transcription of the targeted gene is terminated (Gossen and Bujard 1992). Another variant of a tetracycline-based system is the Tet-On (rtTA) system (Fig. 10.2b). Here, an engineered form of tTA, the rtTA has been created that can only bind to the TRE in the presence of tetracycline or doxycycline (Gossen et al. 1995).

Tet-Off systems have been established in *D. melanogaster* (Handler 2016; McGuire et al. 2004; Heinrich and Scott 2000; Thomas et al. 2000; Horn and Wimmer 2003). Combinations of promoter and reporter genes have been studied to develop female-specific lethal strains. For example, the female-specific yolk protein

Table 10.1 Overview of possible drug-inducible gene expression systems. Conditional gene expression systems are listed with their driver, operator, and repressor components as well as other known parameters

System	Origin	Regulator	Operator (Effector)	Complexity	Drug element	Drug source	Potential effect on insects	Stability of drug (Half-life)	Drug costs[a]	Conc for cells test	Tested in	In vivo test
Tet-On	*Escherichia coli*	rtTA rtetR: VP16	TRE (7x tetO)	Two genetic elements	Tetracycline/ Doxycycline	Antibiotic	Might affect microbiota	11 h	5 g Tet 27 € 5 g Dox 67 €	Tet: 1 μg/mL Dox: 0.1 μg/mL	Mammalian cells, insect cells	Mice, rats, mosquitoes, *L. cuprina*, *A. suspensa*, C. capitata, A. ludens, *D. melanogaster*
Tet-Off	*Escherichia coli*	tetR: VP16										
E-On	*Escherichia coli*	E:KRAB	ETR (8x operator 35 bp each)	Two genetic elements	Erythromycin	Antibiotic	Might affect microbiota	2 h	25 g 172 €	1 μg/mL	Mammalian cells	Mice
E-Off	*Escherichia coli*	E:VP16										
VA-On	*Caulobacter crescentus*	VanR: KRAB	VanO (8x operator, 12 bp each)	Two genetic elements	Vanillic acid	Plants, vanilla beans	No harmful effect known	N/A	25 g 27 €	16.8 μg/mL	Mammalian cells	Mice
VA-oOff	*Caulobacter crescentus*	VanR: VP16	VanO (2x operators)								Mammalian cells	
Ph-oOff	*Pseudomonas putida*	PTtgA-VP16	O_{tTgR} (2x operators)	Two genetic elements	Phloretine	Flavonoids in apples	No harmful effect known	70 h	25 mg 28 €	0.13 μg/mL	Mammalian cells	Mice

(continued)

Table 10.1 (continued)

System	Origin	Regulator	Operator (Effector)	Complexity	Drug element	Drug source	Potential effect on insects	Stability of drug (Half-life)	Drug costs[a]	Conc for cells test	Tested in	In vivo test
BA-On	*Campylo - bacter jejuni*	CmeR-KRAB	Ocme (14x operators)	Two genetic elements	Cholic acid/ bile acid	Cholesterol in liver	No harmful effect known	38.5 h	25 g 20 €	102 μg/mL	Mammalian cells	Mice
BA-Off		CmeR-VP16	Ocme (8x operators)			Cholesterol in liver					Mammalian cells	
Biotin-On	*Escherichia coli*	BirA:VP16	OBirA (3x operators)	Two genetic elements	Biotin	Vitamin H	No harmful effect known	2 h	5 g 170 €	0.002 μg/mL	Mammalian cells and mice	Mice
Q system	*Neurospora crassa*	QF and QS 816 and 918 aa	QUAS (5x UAS units of the qa cluster)	Three genetic elements	Quinic acid	Soft fruits	No harmful effect known	N/A	5 g 14 €	5 mg/mL	Mammalian cells, plants, and insects	*Drosophila melanogaster, C. elegans,* mosquitoes and plants

[a]Drug costs (2020) are taken from www.alfa.com

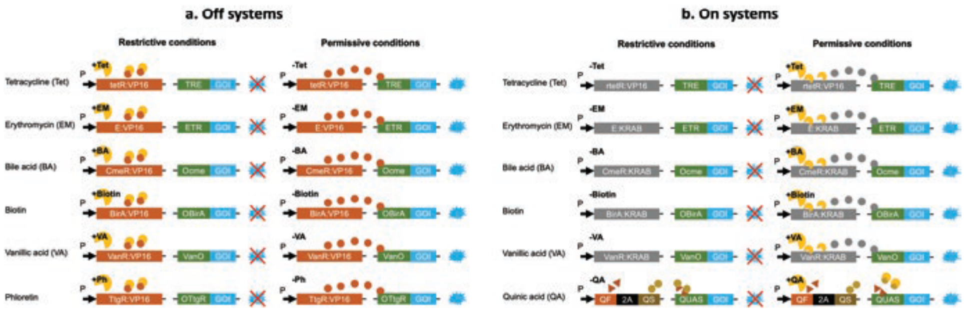

Fig. 10.2 Drug-inducible conditional expression systems. Mechanism of conditional gene expression systems controllable by tetracycline (Tet), erythromycin (E), bile acid (BA), biotin, vanillic acid (VA), phloretin, and quinic acid (QA) are displayed. (**a**) Off systems: all systems harbor a promoter (P) that drives the expression of a transcriptional factor consisting of a DNA binding domain fused to the VP16 transcription activation domain. The control molecules bind to the transcriptional factor and repress binding to its operator, resulting in no expression of the gene of interest (GOI). Gene expression is activated in the absence of the respective control molecule. (**b**) On systems: all systems harbor a promoter (P) that drives the expression of a transcriptional repressor consisting of a DNA binding domain fused to the KRAB transcription repression domain. In the absence of control molecules, the transcriptional repressor does not allow the expression of the gene of interest (GOI). The control molecules bind to the transcriptional repressor and inhibit binding to its operator and thus gene expression is activated. For the quinic acid (QA) system, the activation factor (QF) and suppressor (QS) can be combined with 2A peptides in a single construct. In the absence of quinic acid, QS binds to QF inhibiting gene expression. In presence of quinic acid (QA), QA inhibits QS and QF is released to activate the expression of the GOI

enhancer *yp3* has been used to drive tTA and activate the oncogene *Ras64B* resulting in female elimination in the absence of tetracycline (Thomas et al. 2000). Similarly, the *Yp1* promoter has been used to express tTA and activate the pro-apoptotic *hid* gene, inducing 100% female-specific lethality when reared on tetracycline-free food (Heinrich and Scott 2000). To induce embryo-specific lethality, Horn and Wimmer developed a transgene-based embryonic lethality system by using the cellularization gene promoters *sry-α* and *nullo*, the Tet-Off system, and the phosphor-mutated hid^{Ala5} gene (Horn and Wimmer 2003). For all systems, maternal contribution of tetracycline allowed the suppression of lethality by restricting the Tet-Off systems. A transfer of such systems from *D. melanogaster* to *D. suzukii* and the development of conditional genetic control strains and strategies should be possible. Tet systems have also been established in many other organisms including mice, rats (Zhu et al. 2002; Lewandoski 2001), mosquito species including *Aedes aegypti* (Linnaeus) (Fu et al. 2010) and *Anopheles stephensi* Liston (Diptera: Culicidae) (Lycett et al. 2004), and other insects, including *Lucilia cuprina* (Wiedemann) (Yan and Scott 2015), *C. hominivorax* (Concha et al. 2016) (Diptera: Calliphoridae), *Anastrepha suspensa* (Loew) (Schetelig and Handler 2012a), *C. capitata* (Ogaugwu et al. 2013), and *A. ludens* (Loew) (Diptera: Tephritidae) (Schetelig et al. 2016).

10.2.1.2 Erythromycin-Controlled Gene Expression Systems

A macrolide-based transgene control system has been characterized and cloned from *E. coli* (Noguchi et al. 1995, 2000). Two systems have been developed from erythromycin-responsive gene regulation elements. In the E-Off system, the Erythromycin (EM)-dependent transactivator (ET1) has been developed by fusing the erythromycin resistance gene repressor (E) also known as MphR(A) (Noguchi et al. 2000) to the VP16 transactivator (Triezenberg et al. 1988). In addition, an ET-dependent macrolide-responsive promoter (P_{ETR}) was assembled from the E. coli MphR(A)-specific operator (ETR) and a minimal promoter derived from the human cytomegalovirus promoter $P_{hCMVmin}$ (Fig. 10.2a). The system functions when ET1 binds and activates the transcription of P_{ETR} in the absence of EM. In the presence of EM, binding of ET1 to ETR is inhibited, P_{ETR} is not activated, and gene expression is stopped. In the E-On system, the repressor (E) has been fused to the trans-silencing domain (KRAB) to generate repressor transrepressor (ET4) (Moosmann et al. 1997) (Fig. 10.2b). ET4 represses P_{ETR}ON in the absence of EM. In the presence of EM, ET4 is released and gene expression is driven by the promoter.

Erythromycin-inducible expression systems have functional compatibility to tetracycline expression systems that make them highly efficient for generating a stacked lethality control system for *D. suzukii* pest control. Combining the E-Off and Tet-Off systems would enable further control of gene regulation (Weber et al. 2002).

10.2.1.3 Geneticin and Puromycin-Based System

A novel drug-inducible sex separation technique has been developed by Kandul et al. (2020) that is based on antibiotic-resistance genes rather than small-molecule-regulated DNA binding transcription factors. The two antibiotics geneticin and puromycin were lethal to *D. melanogaster* when added to the diet. Transgenic strains carrying constitutively expressed resistance genes, *NeoR* and *PuroR*, were viable. To create sex-specific systems, the *NeoR* and *PuroR* genes were combined with sex-specifically spliced introns from the *transformer* and *doublesex* genes. Here, males and females can be positively selected by rearing populations on either geneticin or puromycin (Kandul et al. 2020).

10.2.1.4 Disadvantages of Antibiotic-Based Systems

The addition of high concentration of antibiotics to the mass reared insect diet could reduce the fitness of male insects. It has been reported that tetracycline can impair fertility and male courtship, possibly through disruption of mitochondrial function (Zeh et al. 2012; Ballard and Melvin 2007; Moullan et al. 2015). A further problem is the constant use of antibiotics in insect diets, which could lead to antibiotic-resistant strains of gut microbiota or the shift of the insect microbiome due to the antibiotic pressure. For a mass rearing operation, the cost of the antibiotic is also a consideration. Geneticin and puromycin are in general more expensive than tetracycline.

10.2.2 Gene Expression Systems Inducible by Non-antibiotic Molecules

10.2.2.1 Quinic Acid-Controlled Gene Expression System

The Q system is a repressible binary expression system based on qa-gene cluster from the bread fungus, *Neurospora crassa*. The regulatory genes allow the fungus to use quinic acid as a carbon source (Giles et al. 1985). The Q system offers appealing features for a transgene expression as it can provide temporal control of gene expression. The synthetic Q system consists of four components: (1) a gene-regulator or effector QUAS, (2) a driver QF, (3) a suppressor QS, and (4) a food element quinic acid (QA). Quinic acid is a naturally occurring nontoxic compound, with antioxidative properties.

The driver component QF (also known as activation factor) of the Q system binds to the upstream region of effector component QUAS. The QUAS consists of five structural and enzymatic genes (Patel et al. 1981; Baum et al. 1987). In absence of quinic acid QA, the QS suppressor binds to QF (driver) and prevents activation of gene expression. In the presence of QA, QA binds to QS and releases QF, which can bind to QUAS and activate the expression of downstream genes (Giles et al. 1991) (Fig. 10.2b). The original QF consists of three structural domains, DBD (DNA binding and dimerization domain), MD (middle domain), and the transcriptional activation domain (AD). However, QF was found toxic in the *Drosophila* system (Riabinina et al. 2015). Two variants of QF have been designed to avoid toxicity and maintain the functional activity of QF—the QF2 and QF2w. QF2 was designed by deleting the middle domain (MD) and was still fully capable of driving gene expression in *D. melanogaster* (Riabinina et al. 2015). QF2w was further designed by changing the last two amino acids (glutamic acid and glutamine) of QF2 to four lysine(s) that change the charge on the C-terminus from negative to positive. This makes QF2w a weaker transcriptional activator but also less toxic. In addition, it can be more efficiently suppressed by QS than QF2. The Q system has been successfully used in *D. melanogaster* (Potter and Luo 2011), mammalian cells (Potter et al. 2010), *C. elegans* (Maupas) (Wei et al. 2012), *Danio rerio* (Hamilton) (Subedi et al. 2014), mosquitoes (Riabinina et al. 2016), and plants (Persad et al. 2020). Like any other gene control systems, QF expression can also be manipulated with strong or weak promoters.

QA temporal gene control can be achieved by the amount of QA fed to the flies, and duration of exposure. *Drosophila* larvae are more receptive to QA in the food than adult flies. That makes it a better control agent for embryonic lethality control (Riabinina et al. 2015). The Q system can be combined with other expression systems to induce tightly controlled, specific and multi-gene expression. For example, it can be used together with the widely established tTA (Mao et al. 2019; Eckermann et al. 2014) and GAL4 systems (Potter et al. 2010; Li and Stavropoulos 2016).

10.2.2.2 Biotin-Controlled Gene Expression System

The novel biotin-inducible gene expression system can be considered as an ideal control strategy for transgene expression due to nontoxic characters of biotin as a naturally occurring Vitamin H. Weber et al. in 2007 developed a synthetic model on *E. coli* biotin BirA (Chapman-Smith et al. 2001) (a bifunctional protein) that activates biotin by coupling to AMP (biotinyl-5′-AMP) and a transfer of the biotin group to the biotin carboxyl carrier protein subunit of acetyl-CoA-carboxylase which represses the biotin biosynthesis operon. The synthetic system consists of a biotin-dependent transactivator. BIT (BirA fused to the herpes simplex transactivation domain VP16 (Triezenberg et al. 1988) (Fig. 10.2a) that binds to a synthetic target promoter. BIT–Promoter interaction enables adjustable and reversible transgene expression. An initial test in mammalian cell lines with the biotin-inducible control system suggested that 10 nM (0.002 μg/mL) biotin is sufficient to activate gene expression. A downside of biotin-inducible expression systems can be the natural presence of biotin in insect diets that could lead to unexpected gene expression.

10.2.2.3 Vanillic Acid-Controlled Gene Expression System

Vanillic acid (VA)-controlled gene expression system is based on gene regulation elements from *Caulobacter crescentus* which is a freshwater bacterium. It can utilize VA as carbon source to convert metabolic energy in the citric acid cycle (Thanbichler et al. 2007; Harwood and Parales 1996). The system consists of a transcriptional repressor (Van R), an operator VanO, and a gene that is expressed with operator and repressor in VanAB. In the absence of VA, transcriptional repressor (VanR) binds to operator (VanO) upstream of the promoter region of VanAB gene cluster and inhibits VanAB gene expression. In the presence of VA, VanR binds to VA instead of VanO which derepresses the metabolic pathway (Thanbichler et al. 2007). VA is the oxidized form of vanillin and found at high concentrations in vanilla beans and has been used as a food additive (Sinha et al. 2008). VA was reported to be a suppressor of apoptosis in Neuro-2A cells (Huang et al. 2008) and also acts against snake venom (Dhananjaya et al. 2006) and cell carcinogenesis (Vetrano et al. 2005). Due to its nontoxic nature, VA can be used as a safe inducer molecule for controlling gene expression.

The synthetic VA-based systems have been designed as VA-Off and VA-On systems, respectively, which respond exclusively to the food additive VA. The VA-Off system (Fig. 10.2a) was designed by fusing the VanR DNA binding domain to the domain VP16 activation domain (Triezenberg et al. 1988) to generate a transcription factor (VanA1) that binds to VanO-operator sequences upstream of a core promoter. VA triggers the release of VanA1, and thus switches off gene expression. For the VAN_{ON} system (Fig. 10.2b), VanR was fused to the KRAB domain (Moosmann et al. 1997) to generate a trans-silencer (VanA4). Multiple copies of the VanO-operator sequence were placed between the gene of interest (GOI) and a constitutive promoter. Binding of VanA4 to Van-O sequences inhibits transcription of the GOI. VA triggers the release of VanA4, which derepresses expression and switches on gene expression. The VA-Off system has been tested in mammalian cells and mice, and suggested to be more efficient than VA-On system as it shows maximum gene regulation without epigenetic imprinting compared to the KRAB-containing VAN_{ON} design (Ayyanathan et al. 2003; Peng et al. 2009). In cell culture tests using VA-Off and VA-On systems, 100 μM (16.8 μg/mL) VA turned out to be sufficient for gene expression induction in VA-On and gene suppression in VA-Off system. However, use of Vanillic acid system in *D. suzukii* could be compromised by the presence of vanillic acid 2.8–16.1 mg/100 g in unripe to ripe strawberries (Mahmood et al. 2012), approx. 110 mg/kg in blue berries, and 45 mg/kg in black berries (Zadernowski et al. 2005). This could be problematic for future field releases of fertile transgenic males carrying a vanilla-regulated female lethal system.

10.2.2.4 Phloretin-Controlled Gene Expression System

Phloretin-controlled gene expression system is based on gene regulation elements from *Pseudomonas putida*—a soil bacterium from the habitat of plant rhizosphere. The bacterium has an evolved RND (resistance/nodulation/division) family transporter T_{tgABC} which is controlled by its repressor TtgR, binding to the specific operator O_{TtgR} in the T_{tgR} promoter (P_{TtgR}). Phloretin binds to O_{TtgR} operator to release TtgR repressor that results in the production of T_{tgABC}. (Teran et al. 2003). Phloretin is mainly found in apples and the root barks of the apple trees, and acts as an antibacterial plant defense metabolite (Teran et al. 2006). It protects skin from UV light (Oresajo et al. 2008) and has been used as a chemopreventive agent for cancer treatment (Wu et al. 2009) or a penetration enhancer for skin-based drug delivery (Valenta et al. 2001). A synthetic mammalian phloretin-adjustable control element (PEACE) has been designed by fusing TtgR repressor to VP16 (Fig. 10.2a) to generate a mammalian transactivator TtgA1, which can bind to the O_{TtgR} operator and activates the expression of downstream gene. In the presence of phloretin, gene expression is suppressed due to the intercept of binding between TtgA1 and O_{TtgR}. The phloretin system has been tested in mammalian cell culture and other derivatives, suggesting phloretin as ideal inducer molecule for complete gene repression with maximum concentration of 50 μM (0.13 μg/mL) (Gitzinger et al. 2009).

10.2.2.5 Bile Acid-Controlled Gene Expression System

Bile acid (BA)-controlled gene expression system is from gene regulation elements of *Campylobacter jejuni*—a bacteria that causes food poisoning (Klancnik et al. 2012). *C. jejuni* has a three-gene operon CmeABC that encodes for an efflux system to promote resistance to antimicrobial compounds. In *C. jejuni*, the CmeABC expression is controlled by the repressor CmeR, which is a member of tetR family and predicted to be involved in recognizing inducer molecule (Routh et al. 2009). CmeR binds to the operator O_{cme} that is controlled by a promoter P_{cmeABC} and represses the transcription of CmeABC (Lin et al. 2005). Bile acids can bind to the CmeR repressor and inactivate gene expression in a dose-dependent manner. Bile acids are known to improve digestion of lipids and fat-soluble vitamins in mammalian intestines and are synthesized from cholesterol in the liver (Hofmann 2009).

The bile acid-controlled gene expression elements have been used to develop the BA-Off and BA-On systems. The BA-Off system (Fig. 10.2a) is comprised of a bile acid-dependent transactivator CmeA1, in which the CmeR repressor has been fused to VP16 transactivator domain of Herpes simplex virus (Rossger et al. 2014). In the absence of bile acid, the transactivator CmeA1 binds to the operator Ocme sequences that are upstream of a core promoter and activates gene expression. While in the presence of bile acid, CmeA1 is prevented from binding to Ocme, and gene expression is not activated. On the other hand, the transsuppressor CmeA2 has been designed for the BEAR-on system (Fig. 10.2b) by fusing the CmeR repressor to the transsilencer human KRAB (Moosmann et al. 1997). Specifically, CmeA2 binds to Ocme and represses the gene expression when bile acid is absent, and CmeA is released from Ocme, thus the gene expression is activated when bile acid is present. This system has been successfully tested in mammalian cells (Rossger et al. 2014). Due to the presence of bile salts in fatal calf serum (FCS) of cell growth media, the BEAR-On system showed a basal level of gene expression. In mammalian cells, both BEAR-Off and BEAR-On systems were responsive to bile acid derivatives with max concentration of 250 μM (102 μg/mL). Meanwhile, other cholic acid derivatives have also been tested for BEAR-On system, and the results suggested that 100 mg/kg cholic acid, 30 mg/kg deoxycholic acid, and 30 mg/kg chenodeoxycholic acid are sufficient to trigger gene expression in mice (Rossger et al. 2014).

10.3 External Stimuli-Inducible Conditional Expression Systems

Classical genetic sexing strains (GSS) in *C. capitata* use elevated temperature to achieve sex separation. GSS females are homozygous for a *temperature-sensitive lethal (tsl)* mutation, while males have the same *tsl* mutation but in addition carry a wild-type copy of the *tsl* gene (unknown) translocated to the Y chromosome (Franz 2005). Incubation of embryos at 33–36 °C causes female-specific lethality (Robinson

2002a). Since GSS have been successfully used to produce billions of male *C. capitata* for field release, it is attractive to consider temperature-regulated transgenic systems for controlling insect viability or fertility. Heat-shock promoters have been studied and used to induce heat-activated temporal and spatial gene expression in *Drosophila* (Monsma et al. 1988). Similarly, a variety of heat-shock promoters have been used to regulate conditional expression of genes in insect species. For example, the heat-shock gene promoters *hsp26* and *hsp70* in *D. melanogaster* (Hara et al. 2008; Thomas et al. 2000), *hsp23*, *hsp70*, and *hsp90* in the blow fly *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) (Tachibana et al. 2005), and *hsp70* in *C. capitata* (Kalosaka et al. 2009). In addition to heat-shock promoters, temperature-sensitive proteins such as the β2 proteasome subunit gene (*Prosβ2'*) of *D. melanogaster* (Smyth and Belote 1999) have been explored as a means for achieving environmental control of insect viability. *Prosβ2*' causes pupal lethality at 29 °C, but allows survival to adulthood at 25 °C and has also been tested in the tephritid *A. suspensa* (Nirmala et al. 2009). An interesting alternative is to use temperature-sensitive versions of the conserved sex determination gene *transformer 2*, which is essential for female development. Early studies in *D. melanogaster* found that the mutant Tra2 proteins appear to function normally at 16 °C but not 26 °C (Belote and Baker 1982). *D. suzukii* strains carrying the same temperature-sensitive mutations in the *tra2* gene were made using CRISPR/Cas9 technology (Li and Handler 2017). While the results were promising, the full potential could not be evaluated due to the low survival of *D. suzukii* above 26 °C in the laboratory strains.

In addition to temperature, light-inducible systems offer an alternative conditional means for controlling insect viability. Ramos et al. have generated a laser-inducible heat-shock-mediated ectopic gene expression in the butterfly, *Bicyclus anynana* (Butler) (Lepidoptera: Nymphalidae), using the *Drosophila hsp70* gene promoter (Ramos et al. 2006). In *C. elegans*, single cell expression of genes in a variety of cell types of endodermal, mesodermal, or ectodermal origin have been achieved by using *hsp70*-induced gene expression after pulsing with a laser (Stringham and Candido 1993). In addition, optogenetic switches like Cry2-CIB1 have been integrated into existing strategies to develop a robust light-controllable Tet system and accurately manipulate gene expression by light stimulation (Yamada et al. 2018). Light induction has the advantage that it can be fine-dosed and together with varying Dox concentrations, a fine-tuned and tight gene expression can be achieved (Yamada et al. 2020).

Such systems are promising technologies because the use of heat and light can open possibilities to artificially control gene expression in every possible developmental stage.

10.4 Summary

10.4.1 Comparison of Different Systems

Complexity of conditional systems plays an important role in the generation of transgenic insect strains. Most of the conditional expression systems consists of two gene components. A driver element that regulates and acts on an effector element. In addition, a molecule that can be added for conditional regulation of the system is needed. Such systems can and have been generated by establishing either two independent transgenic lines carrying the driver and effector constructs or as so-called all-in-one system that implements both into one transgenic strain. These transgenic lines are further crossed or inbred to generate double homozygous lines carrying driver and effector components (Hara et al. 2008). However, other tools like the quinic acid-based system involve and require triple homozygous strains (Potter and Luo 2011). Those systems could be simplified by using a bicistronic expression cassette carrying driver QF and QS (Eckermann et al. 2014; Schwirz et al. 2020). In that context, all the abovementioned systems could be established also in *D. suzukii*. The gene constructs can be integrated into the *D. suzukii* genome using either transposon-mediated germline transformation for elements up to 15 kb (Schetelig and Handler 2013), recombinase-mediated cassette exchange transformation and P[acman] system for large genetic elements of more than 100 kb (Haecker et al. 2017; Schetelig et al. 2009b; Venken et al. 2006) or CRISPR-mediated HDR for short genetic elements (Li and Handler 2017).

Toxicity of driver elements and small molecules: In several studies the overexpression of tTA was found to be toxic (Knudsen et al. 2020), and the transcriptional activator (QF) of the quinic acid system is also toxic (Riabinina et al. 2015). The expression of such toxic drivers under constitutive promoters would make it difficult to keep TESS strains for sexing and could interfere with fitness parameters even at low expression levels. This would affect the efficiency of insect strains in SIT programs, in which fitness and especially competitiveness of the released males are of greatest importance to the success of the program. For *D. suzukii*, to date the potential toxic effects of the drivers have not been reported.

Drug and cost efficacy: The induction ability of a system is defined by the minimum drug quantity required to promote or switch off gene expression. For Tet-Off systems, doxycycline has demonstrated better induction ability compared to other antibiotics from the tetracycline family, though the costs of doxycycline are usually higher than tetracycline based on small-scale price comparisons. Bulk quantities of these chemicals could be cheaper. Comparing the induction ability of the other systems, biotin and cholic acid (BA systems) require small amount (10 nM) of drugs to induce gene expression in mammalian cells. The biotin system is also challenging to use as the half-life of biotin is only 2 h, and it is the most expensive drug available among all (see Table 10.1). Cholic acid in comparison has a half-life of 38.5 h and requires only 10 nM to regulate gene expression. In the case of the VA system, the stability of vanillic acid is not known but has a good induction ability at 100 μM concentrations. A maximum of 50 μM concentration for phloretin with a half-life of 70 h can also be a most economical system considering price and stability of the compound.

10.4.2 Building Stacked Systems

An ideal approach to tightly regulate a transgene expression can be by building a stacked system with two or more completely independent conditional systems to induce female lethality or reproductive sterility as independent factors or to build two independent female-specific lethal strains with different apoptotic genes as a backup mechanism. A combination of Q and Tet-Off systems, where the Tet-Off system confers sex-specific lethality and the Q system sperm-specific lethality has been proposed but not yet demonstrated (Eckermann et al. 2014). The VA-Off system has been tested in parallel to the Tet-Off (Gossen and Bujard 1992) and E-Off systems (Weber et al. 2002) in mammalian cells. They present an interference-free independent gene regulation, suggesting these three systems can be used parallel a relatively tight gene regulation control (Gitzinger et al. 2012; Weber et al. 2002). Furthermore, these expression systems can be improved by using a diverse number of operators, transactivation domains, different distances between operator site and minimal promoter (Gitzinger et al. 2012).

However, to generate lethality strains to be used for SIT, the use of "On" systems is an economically suitable option because the effector molecule drug is not needed for constant maintenance of the population. Lethality can be induced by adding the respective molecules when sex separation and sterility are needed before release. Nevertheless, some developmental non-feeding stages (embryos and pupae) cannot be treated, which limits the flexibility of on systems.

Resistance in a genetically modified pest population can occur due to the possibility of primary and secondary site mutations as described recently by Knudsen et al. (2020) and Zhao et al. (2020). These mutations can be avoided by the establishment of dual redundant lethality systems that do not share functional components and function through different pathways or simply harbor redundant systems. Combinations of two or multiple systems are less likely to accumulate resistant mutations. Gene regulation with independent systems can ensure effective sex separation and reduce the chances of a genetic breakdown of conditional lethal expression systems. However, while using any substance for the control of conditional systems, it is important to consider the negative effects of them. Constant exposure to the substances may cause unwanted fitness effects, physiological changes, or changes in microbiota of the insects (Chatzispyrou et al. 2015; Wang et al. 2015; Zeh et al. 2012). In addition, mass rearing facilities and large-scale productions must calculate the efficiency of all economic factors included in SIT programs, when conditional systems are used. This has to consider regular mass rearing parameters and the fitness and stability of molecularly engineered strains as well as the waste management of insect food that includes additional effector molecules. In this respect, some insect diets might need special treatment like heat to decontaminate foods before disposal. At the same time, other substances could directly be disposed of and do not create extra costs and handling efforts during the production process.

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I hereby confirm that this thesis and the work presented in it are entirely my own. Where I have consulted the work of others, this is always clearly stated. All statements taken literally from other writings or referred to by analogy are marked, and the source is always given.

To word-proof and add linguistic coherence to my dissertation, I utilized several digital tools, including Grammarly, ChatGPT, and Perplexity. No AI-generated text was included without revision or verification, and all scientific reasoning, analysis, and interpretation were entirely my own. Although the use of AI tools was limited to barely a few paragraphs, Grammarly was extensively used throughout the dissertation for clarity, proofing, and simplifying complex sentences. The prefix setting of ‘Academic writing' in Grammarly was used.

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