Improvement on the genetic engineering of an invasive agricultural pest insect, the cherry vinegar fly, Drosophila suzukii

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Abstract

Background: The invasive fly Drosophila suzukii has become an established fruit pest in Europe, the USA, and South America with no effective and safe pest management. Genetic engineering enables the development of transgene-based novel genetic control strategies against insect pests and disease vectors. This, however, requires the establishment of reliable germline transformation techniques. Previous studies have shown that D. suzukii is amenable to transgenesis using the transposon-based vectors piggyBac and Minos, site-specific recombination (lox/Cre), and CRISPR/Cas9 genome editing.

Results: We experienced differences in the usability of piggyBac-based germline transformation in different strains of D. suzukii: we obtained no transgenic lines in a US strain, a single rare transgenic line in an Italian strain, but observed a reliable transformation rate of 2.5 to 11% in a strain from the French Alps. This difference in efficiency was confirmed by comparative examination of these three strains. In addition, we used an attP landing site line to successfully established φC31-integrase-mediated plasmid integration at a rate of 10% and generated landing site lines with two attP sequences to effectively perform φC31-Recombinase Mediated Cassette Exchange (φC31-RMCE) with 11% efficiency. Moreover, we isolated and used the endogenous regulatory regions of Ds nanos to express φC31 integrase maternally to generate self-docking lines for φC31-RMCE. Besides, we isolated the promoter/enhancer of Ds serendipity α to drive the heterologous tetracycline-controlled transactivator (tTA) during early embryonic development and generated a testes-specific tTA driver line using the endogenous beta-2-tubulin (β2t) promoter/enhancer.

Conclusion: Our results provide evidence that the D. suzukii strain AM derived from the French Alps is more suitable for piggyBac germline transformation than other strains. We demonstrated the feasibility of using φC31-RMCE in the cherry vinegar fly and generated a set of lines that can be used for highly efficient integration of larger constructs. The φC31-based integration will facilitate modification and stabilization of previously generated transgenic lines that carry at least one attP site in the transgene construction. An early embryo-specific and a spermatogenesis-specific driver line were generated for future use of the binary expression system tet-off to engineer tissue- and stage-specific effector gene expression for genetic pest control strategies.

Keywords: Binary expression system, Enhancer/promoter, Insect transgenesis, Molecular entomology, Pest management, Spotted wing Drosophila, Sterile insect technique

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Background

The invasive pest *Drosophila suzukii* commonly referred to as the cherry vinegar fly or the spotted wing *Drosophila* (SWD) originated from East Asia [1, 2]. It was described for the first time in Japan in 1916. In 2008, the fly has concomitantly been reported in Europe (Spain and Italy) and the USA (California), where the SWD presents a major threat to the soft and stone fruit industry [1–3]. The fly is armed with a prominent serrated ovipositor that enables it to lay eggs inside ripening intact fruits. The larvae eat and develop inside the fruits and lead to a crop loss of up to 100% [4]. Several insecticides have been used to control the fly with limited degrees of success [5, 6]. A genetic control method, the Sterile Insect Technique (SIT), might provide the most promising pest management strategy. SIT was proposed more than 75 years ago as biological control method to fight agricultural pests and diseases vectors. It is a species-specific birth control strategy, which makes it safe for pollinators and natural enemies and is thus environmentally friendly [7]. The SIT consists of mass rearing of the target pest in large numbers, sexing, sterilization of the males and successive inundative releases in the target area. Genetic engineering offers different approaches for improvement of SIT [8–13]. For example, a transgene-based conditional embryonic lethality system was developed as a way to induce reproductive sterility, which can replace the need for ionizing radiation and ensure production of competent males [9, 10]. A transgenic female-specific embryonic lethality system developed for several dipterans, notably tephritid fruit flies, serves a method to eliminate females during early embryonic development and facilitates the production of only males for SIT releases [11–14].

The ability to genetically manipulate biological systems from mammalian and insect cell lines to insects and mouse has been revolutionized by the discovery and utilization of the most versatile transposon, *piggyBac* [15–17]. It belongs to the class II DNA transposons, which work by a cut and paste mechanisms [18]. *piggyBac*-based vectors were generated to insert cargo sequences at a **TTAA** recognition sequence in the genome of the target species. *piggyBac*-based germline transformation has been successfully established for many model and non-model organisms including *Drosophila melanogaster* [19–21], *Ceratitis capitata* [22, 23], *Anastrepha suspensa* [24], *Drosophila suzukii* [25], *Anopheles gambiae* [26], *Aedes aegypti* [27], *Musca domestica* [28], among others. The increase in the efficiency of germline transformation due to the use of a hyperactive version of the *piggyBac* transposase was demonstrated in several insects [23]. An inherent characteristic of transposon vectors using *piggyBac* is the random integration in the genome which makes them a useful tool for mutagenesis screens, enhancer traps, and exon traps [19, 29–31]. Also, in cases, when no clear target sequence can be identified, the random integration might result in a set of insertions, from which to choose the most suitable ones. However, this randomness is considered a drawback, when different transgenes were to be compared in the same genomic context [32, 33].

Site-specific recombinases (SSR) offer a more precise approach for genetic engineering of biological systems [34, 35]. In the presence of the respective recombinase, recombination takes place between two identical sequences in case of Flp/FRT and Cre/lox [36, 37] or non-identical sequences in case of φC31 attP/attB [38]. The use of SSR necessitates the generation of landing site lines by integrating at least a single landing site (FRT, lox or attP) into the genome of the target species. This is routinely done by including the sequence within a transposon vector and integrate it randomly in the genome. Once generated, these landing sites can be used repeatedly to integrate different transgenes. In case a single landing site is integrated, the transgene of interest has to be delivered in a plasmid vector that has the respective recombinase recognition sequence which leads to integration of the whole plasmid including the antibiotic resistance gene. To avoid this, two landing sites can be placed close to each other into the genome ideally separated by a marker. The transgene to be inserted has to be flanked by two recombinase recognition sequences, which facilitate double recombination events leading to a recombinase mediated cassette exchange (RMCE). The φC31-based integration and RMCE have been established in many insects for either modification and or stabilization of previously generated transgenes [39] or for site-specific germline transformation, which allows examination of different transgenes in the same genomic context [40]. Furthermore, the use of the φC31 system allows for large transgenes to be integrated. In fact, BAC constructs of up to 133 kb were integrated using this system [41]. Moreover, in *Drosophila* and mosquitoes the φC31 system has been used to generate self-docking strains that expresses the integrase from the enhancer/promoter of the maternal effect gene *nanos*. This has remarkably improved the efficiency of site-directed germline transformation [42, 43].

To generate transgene-based reproductive sterility or sexing strains, food supplement-controlled binary expression systems have widely been used for conditional and tissue- or stage-specific gene expression [8–14]. The UAS/Gal4 system has intensively been used in *D. melanogaster* to drive tissue-specific expression of dsRNA to knockdown genes and study their function [44, 45]. The tet system has initially been developed to be used in human cell culture and has since been engineered into tet-off and tet-on systems [46–48]. In insect biotechnology, the tet-off system was used e.g. to control the expression
of effector molecules such as the proapoptotic gene head involution defective (hid), which leads to apoptotic cell death [8]. To drive the heterologous transactivator of such a binary expression system to cause effective reproductive sterility [9, 10] or female-specific killing [11, 13, 14, 49] based on early embryonic lethality, the promoter/enhancers of cellularization-specific genes need to be identified and isolated. Moreover, to direct sperm-specific expression for transgenic marking [50–52] or the development of multifactorial reproductive sterility [53], the use of promoters/enhancers active during spermatogenesis are of interest.

Here we show that D. suzukii strains originated from different locations can be transformed using piggyBac germline transformation with varying efficiency. In addition, we demonstrate the successful use of φC31-based site-specific germline transformation both by integration in one attP site or by RMCE. Moreover, we provide a set of D. suzukii self-docking lines expressing φC31 integrase maternally during oogenesis. Furthermore, we provide an early embryo-specific and a spermatogenesis-specific driver line for using the tet-off binary expression system to drive tissue-specific expression of effector genes.

Results

Comparison of piggyBac germline transformation in different D. suzukii strains

Transposon-based vectors have been intensively used for genetic manipulation from cell culture to mouse. The vector piggyBac has gained particular attention due to its versatility and usability in different systems. When we started to use piggyBac for germline transformation of an Italian strain of D. suzukii, we had only poor success and retrieved a rare transgenic line (06_F5M2) carrying construct HMMA006 [52], which mediates early embryonic expression of tTA (Fig. 1), with a transformation rate of 1.6% (300 embryos injected, 200 survived, 60 fertile, 1 transgenic line). However, several previous attempts with the same construct and additional attempts with five other constructs were unsuccessful. Changing to a US strain did not improve our approach, since trying the same five different constructs in this strain did not yield any transgenic lines. Only once we changed to a US strain did not improve our approach, since trying the same five different constructs in this strain did not yield any transgenic lines. Only once we changed to the strain Alpes Maritimes (AM) isolated from the French Alps [54], we started to get reliable piggyBac germline transformation to work. In this strain, we regularly obtained transgenic lines for three different constructs with transformation rates between 2.5 and 11% (Additional file 1): The testes-specific driver construct HMMA389, which is designed to be also useable for φC31-mediated RMCE and mediates spermatogenesis-specific expression of tTA (Fig. 2); the DsRed-marked construct HMMA185 containing two attP sites for φC31-mediated RMCE (Fig. 3); as well as the construct HMMA223 to generate self-docking lines for φC31-mediated RMCE (Fig. 4). Additional file 2 provides a list of the obtained lines.

To examine the suitability of the three different strains for piggyBac germline transformation in a truly comparative manner, we injected construct HMMA223 to generate more self-docking lines for φC31-mediated RMCE (Fig. 2c) into similar amounts of embryos on the same day and with the same injection needle to minimize variations in the injection procedure. Table 1 shows that no transgenic lines were obtained with the US or Italian strains, but were successfully obtained with the French AM strain with a transformation rate of 4.2%. This demonstrates the higher usability of the AM strain for piggyBac germline transformation.

Isolation of an enhancer/promoter region active during early embryonic development

To direct gene expression specifically at early embryonic development, we identified the serenipity a (sry a) gene by homology search in the D. suzukii genome database (www.spottedwingflybase.org) using the Dm_sry a sequence as query. The open reading frame of the Ds_sry a gene from the translation start codon to the stop codon is 1593 bp without introns. The gene has a 5’UTR of 49 bp, which demarcates the transcription start site (Fig. 1a). The Ds_sry a coding sequence encodes a putative protein of 530 amino acids, which shares 86% identity to Dm_Sry a protein.

To validate the cellularization-specific expression of the isolated Ds_sry a gene, we performed whole mount in situ hybridization on different stage wild type embryos using a DIG-labelled antisense probe against the whole Ds_sry a ORF plus the 5’ UTR. These in situ hybridizations detected expression only during blastoderm cellularization with no expression at earlier or later embryonic stages (Fig. 1c-e).

To identify the necessary upstream and downstream regulatory elements driving cellularization-specific gene expression, we compared the Ds_sry a sequence with the characterized counterpart in D. melanogaster [9]. To examine, whether the 300 bp upstream regulatory element plus the 49 bp 5’UTR drive cellularization-specific gene expression, we fused this 349 bp enhancer/promoter fragment of the Ds_sry a gene to tTA (Fig. 1b) and generated D. suzukii line 06_F5M2 [52] by piggyBac-based germline transformation. Embryos from this line were then tested by whole mount in situ hybridization for expression of tTA, which revealed the respective cellularization-specific expression pattern of Ds_sry a (Fig. 1f-h) indicating that the isolated promoter/enhancer element is suitable for stage-specific gene expression during early embryonic development.
Spermatogenesis-specific driver for binary tet-off expression system

Since direct expression of effector molecules potentially causing harm obstructs the generation of transgenic lines, we aim to establish the tet-off binary system in *D. suzukii* to develop transgenic improvements for SIT approaches. To examine this binary expression system, we used the *Ds_β2t* enhancer/promoter [52] to generate construct HMMA389 (Fig. 2a). By piggyBac-based germline transformation, we obtained the spermatogenesis-specific driver line 389_F25M1 that expresses tTA in the testes. The spermatogenesis-specific expression was confirmed by in situ hybridization and compared to the endogenous expression of *Ds_β2t* (Fig. 2b-d).

φC31-mediated site-specific germline transformation

Modification and/or stabilization of transgenes generated by transposon-based vectors by site-specific recombination have been demonstrated in *D. melanogaster* and *Ceratitis capitata* [39, 55]. To establish φC31-based site-specific germline transformation by integration of a transgene construct into a single *attP* site, we injected
donor plasmid HMMA182 carrying an EGFP transformation marker and the bacterial attachment sequence attB along with helper plasmid HMMA098 expressing φC31 integrase under the promoter of the Ds-hsp70 gene into pre-blastoderm embryos of the DsRed-marked transgenic embryonic driver line 06_F5M2 (attP#1). This line was generated with construct HMMA006 [52], which harbours in addition to the early embryonic tTA-driver also an attP site (Figs. 1b, 3A). Out of 250 injected embryos 110 hatched and 40 fertile G₀ crosses gave rise to four independent integrations (Additional file2), which were identified by showing both red and green fluorescent markers (Fig. 3A ′-A ″), resulting in a site-specific transformation efficiency of 10%.

φC31-mediated recombination mediated cassette exchange

To examine a docking line with two attP sites in opposite orientation of a DsRed-based transformation marker for establishment of RMCE in D. suzukii, we used the docking line 185_F3F1 (RMCE#1), which resulted from piggyBac-mediated integration of vector HMMA185 (Fig. 3B) into the AM strain (Additional files 1 and 2). In this line, we confirmed the presence of the two attP sites by sequencing. To see whether the φC31-based RMCE works in D. suzukii, we co-injected into this line plasmid HMMA336 having two attB recombination sites in opposite orientation flanking an EGFP-based transformation marker and the transgene of interest (an effector to drive Cas9 expression under the control of the binary expression system tet-off) along with the helper plasmid HMMA098 (Fig. 3B). We obtained 71 G₀ fertile crosses, of which eight gave rise to F₁ progeny that showed EGFP and absence of DsRed fluorescence (Fig. 3B ′-B ″′) indicating an RMCE rate of 11.3%. RMCE line 336_F3F2 was then used to verify the faithful double recombination event by PCR and sequencing of the resulting hybrid attL and attR sites (Fig. 3B).

Isolation of an enhancer/promoter region active during oogenesis and in the germline to generate self-docking lines for φC31-mediated RMCE

To improve φC31-mediated RMCE further, we wanted to establish self-docking lines (Fig. 4) that express φC31 integrase maternally in addition to carrying two attP recombination sites. In this respect, we identified the Ds_nanos gene by homology search in the D. suzukii genome database (www.spottedwingflybase.org) using the Dm_nanos sequence as query. The open reading frame of the Ds_nanos gene from the translation start codon to the stop codon is 2433 bp, which is interrupted by three introns. The gene has a 5’UTR of 236 bp, which demarcates the transcription start site and a 3’UTR of 878 bp (Fig. 4a). To validate the oogenesis- and germline-specific gene expression of the isolated Ds_nanos gene, we performed whole mount in situ hybridization on ovaries using DIG-labelled antisense probes against the Ds_nanos 3’UTR and 103 bp of exon IV. These in situ hybridizations detected expression in ovarian nurse cells (Fig. 4b).
To identify the necessary upstream and downstream regulatory elements driving oogenesis-specific gene expression, we compared the \textit{Ds\_nanos} sequence with the characterized counterpart in \textit{D. melanogaster}. To examine whether the 2 Kb enhancer/promoter region including the 5'UTR drives oogenesis-specific gene expression, we fused this 2 Kb enhancer/promoter fragment of the \textit{Ds\_nanos} gene to the coding region of \textit{\phi C31 integrase} (Fig. 4c) and generated \textit{D. suzukii} lines 223\_F7M1 and 223\_M3M2 by \textit{piggyBac}\-based germline transformation of the AM strain (Additional files 1 and 2). In addition, two more self-docking lines were generated in the comparative approach to evaluate the different \textit{D. suzukii} strains (Table 1). Ovaries from line 223\_M3M2 were then tested by whole mount in situ hybridization for expression of \textit{\phi C31 integrase}, which revealed the respective nurse cell-specific expression in the ovaries (Fig. 4d) resembling \textit{Ds\_nanos} expression, which indicates that the isolated promoter/enhancer element is suitable for maternal gene expression.

**Discussion**

The discovery that exogenous DNA can be stably introduced into the germline of living organisms which can then be stably inherited by the offspring has tremendously contributed to the advancement of biological and biomedical research and in particular functional genetic studies [15, 19, 30, 31]. The road for insect genetic engineering has been well paved by geneticists working with the model organism \textit{D. melanogaster}. Genetic screens in \textit{D. melanogaster} using \textit{P-element} based transformation vectors to perform insertional mutagenesis, enhancer- and gene-traps, as well as ectopic or overexpression studies provided an enormous contribution to our understanding of gene function [56–58]. Unfortunately, the \textit{P-element} is not functional in other organisms due to the requirement of host-specific factors [59]. Transformation vectors based on the lepidopteran transposable element \textit{piggyBac} have been used to engineer many insects [20, 22, 25, 26] and encouraged the establishment of new insect model systems such as \textit{Tribolium castaneum} [60, 61].
Fig. 4 D. suzukii nanos and the use of its promoter/enhancer for directed expression. a Schematic representation of the D. suzukii gene nanos. b Whole mount in situ hybridisation to detect nanos expression in wildtype D. suzukii ovaries. c piggyBac-based transgenic construct HMMA223 to generate φC31 integrase RMCE self-docking lines. RMCE in a self-docking line, which provides both the recombination target sequences attP as well as the φC31 integrase driven by the nanos promoter/enhancer providing maternal expression, will result in marker exchange as well as cargo gene (GOI) integration and removal of the integrase source. d Whole mount in situ hybridisation to detect φC31 integrase expression in transgenic D. suzukii ovaries carrying construct HMMA223. Expression of nanos or φC31 integrase, respectively, is detected in the nurse cells of the ovaries.

Table 1 Comparative piggyBac transformation efficiency in different D. suzukii strains

| Origin of D. suzukii strain | No. of injected embryos | Hatched larvae | Fertile crosses | No. of transgenics | Transformation rate in % |
|-----------------------------|------------------------|----------------|-----------------|-------------------|--------------------------|
| Italy                       | 400                    | 190            | 35              | 0                 | –                        |
| France (AM)                 | 450                    | 210            | 47              | 2                 | 4.2                      |
| USA                         | 430                    | 240            | 50              | 0                 | –                        |
The invasive fruit pest, *D. suzukii*, had been successfully transformed using piggyBac-based vectors [25, 54, 62]. We have used three different lab strains of *D. suzukii* from Italy, USA, and France. After many attempts to generate transgenic *D. suzukii* using piggyBac germline transformation with different constructs by microinjection into the Italian strain, we obtained only one transgenic line, 06_FSM2, with a low transformation efficiency of 1.6% and failed to obtain any transgenic flies from the US strain. When we obtained the French strain AM (which was kindly provided to us by N. Gompel, Munich), we managed to get reliably transgenics with varying efficiency. Based on these observations and the comparative examination of these three strains (Table 1), we recommend the AM strain for piggyBac germline transformation.

Due to the random integration of transposon-based transformation vectors and the limited size of cargo they can carry, we decided to extend the toolkit for *D. suzukii* transformation by firmly establishing a site-specific transformation technology. Recombinase-based site-specific germline transformation such as (Cre/lox, flp/FRT and φC31 attP/attB) had been established in many model and non-model insects and shown to overcome the shortcomings of transposon-based germline transformation [40]. The Cre/lox Recombinase Mediated Cassette Exchange has recently been established for the cherry vinegar fly *D. suzukii* [63]. In this study, we demonstrate the feasibility of using the φC31 integrase system to integrate a construct in a single attP landing site. This approach had previously been established for *D. melanogaster* and the Mediterranean fruit fly *Ceratitis capitata*, where it was used to modify transgenic lines generated by random transposon-based vectors and to stabilize the transgene by subsequent deletion of one of the inverted repeats required for transposition [39, 55]. In addition, we have generated a docking line with two attP sites in opposite orientations and show that φC31-mediated RMCE works in *D. suzukii*. The use of an endogenous source of φC31 integrase by expression from a germline specific enhancer/promoter had been shown to increase the efficiency of φC31-mediated integration and RMCE [42, 43]. In this regard, we set to generate self-docking lines that express φC31 integrase maternally. We isolated the endogenous *Ds-nanos* gene (Fig. 4) in order to use the upstream enhancer/promoter and the downstream 3′UTR for directing the expression of φC31 integrase to the nurse cells for maternal contribution to the early embryo. By random piggyBac germline transformation, we generated four transgenic lines with a *DsRed* body marker and the φC31 integrase cassette flanked by attP sites.

To be able to conditionally drive expression of effector genes in a tissue- or stage-specific manner, a suppressible or inducible binary expression system is required. This has been successfully exploited to develop biotechnological pest control strategies such as early embryonic lethality or female-specific embryonic lethality [9-14]. To develop such transgenic pest control strategies for the invasive pest *D. suzukii*, we identified a gene that is active during early embryonic stages (*Ds_sry α*) and a gene that is spermatogenesis-specific (*Ds_β2t*) [52]. 350 bp upstream regulatory sequence of the *Ds_sry α* gene were identified to be sufficient to drive the expression of tTA specifically during cellularization similar to the endogenous gene. This driver line will be usable to generate reproductive sterility or sexing lines by driving expression of pro-apoptotic genes as previously described for several tephritid fruit pests [10-12, 14]. Such systems will be very important to establish SIT programs for the control of this invasive pest species. In addition, we were able to generate a spermatogenesis specific driver line using the promoter of the *Ds_β2t* gene described previously [52].

**Conclusion**

By comparing different *D. suzukii* strains for their usability for piggyBac-based germline transformation, we could clearly identify the AM strain derived from the French Alps as the most suitable one. In addition, we demonstrated that φC31-based site-specific integration and RMCE can be used routinely in the cherry vinegar fly, *D. suzukii*, and generated four self-docking lines for RMCE. The φC31-based integration will facilitate efficient integration of larger transgenic constructs and allow for the modification and stabilization of previously generated transgenic lines that carry at least one attP site in the transgene construction.

**Methods**

*Drosophila suzukii* strains

All fly experiments were performed in our well-equipped safety level one (S1) laboratory, which is certified for generating and using genetically modified insects. Wild type *D. suzukii* from Italy, USA (both kindly provided by Prof. Marc F. Schetelig), and French Alps (Prof. Dr. Nicolas Gompel) as well as the generated transgenic flies were reared on standard *Drosophila* food and kept at 25°C throughout this study.

**Nucleic acid isolation**

Genomic DNA isolation was done from a mix of adult males and females using NucleoSpin® DNA Insect (Macherey-Nagel) according to the manufacturer instructions. Total RNA was isolated from 0 to 24 h embryos enriched for 0–4 h stages using ZR Tissue & Insect RNA MicroPrep (Zymo Research Europe, 79110 Freiburg) according to manufacturer instructions.

All PCR amplifications during the course of this study were performed using Phusion DNA polymerase and Phusion-HF buffer (New England Biolabs GmbH, D-
65926 Frankfurt am Main). A list of the used primers is provided in Additional file 3. Plasmid min-preps and PCR products were purified using NucleoSpin Plasmid and NucleoSpin Gel and PCR Clean-up kits (Macherey-Nagel GmbH & Co., 52355 Düren, Germany), respectively. NucleoSpin Plasmid Transfection-grade (Macherey-Nagel) or QIAGEN Plasmid Plus Midi Kit (QIAGEN GmbH, 40724 Hilden, Germany) were used to prepare plasmids for germline transformation.

Amplification of cDNA ends

To isolate the 5’UTR and the 3’UTR of the early embryonic gene Ds

syr

a and the maternal effect gene Ds

n

anos, total RNA from 0 to 24 h old (enriched for 0-4 h) D. suzukii embryos was isolated and 1.3 μg were used to generate 5’ RACE-ready cDNA or 3’ RACE-ready cDNA using SMARTer® RACE cDNA amplification kit (Takara Bio Europe SAS, 78100 Saint-Germain-en-Laye, France) according to manufacturer instructions.

The 5’UTR of Ds

syr

a and Ds

n

anos were recovered by RACE PCR using gene specific primers HM#34 and HM#76, respectively, along with the universal primer (UPM) provided with the kit using Advantage2 DNA polymerase (Takara) with the following program: 94°C 2 min, (94°C 30 s, 72°C 3 min) 5X, (94°C 30 s, 70°C 30 s, 72°C 3 min) 5X, (94°C 30 s, 68°C 30 s, 72°C 3 min) 30X. A single prominent band for each gene was recovered, purified, cloned into pCRII (Thermo Fisher Scientific) to generate pCRII_sry

a (HMMA001) and pCRII_nos_5UTR (HMMA002), and sequenced using standard M13 primer.

To recover the 3’UTR of Ds

syr

a and Ds

nanos, the gene specific primers HM#42 and HM#77, respectively, along with UPM provided with the kit using Advantage2 DNA polymerase (Takara) were used with the following program: 94°C 2 min, (94°C 30 s, 72°C 3 min) 5X, (94°C 30 s, 70°C 30 s, 72°C 3 min) 5X, (94°C 30 s, 68°C 30 s, 72°C 3 min) 30X. A single prominent band for each gene was recovered, purified, cloned into pCRII (Thermo Fisher Scientific) to generate pCRII_syr

a_3UTR (HMMA002) and pCRII_nos_3UTR (HMMA003), and sequenced using standard M13 primer.

Plasmids construction

The plasmid HMMA020 was generated by PCR amplification of the coding sequence of D. suzukii Ds

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2t gene plus the 5’UTR using primer pair HM#16/HM#17 and advantage 2 DNA polymerase (Invitrogen) with program 98°C 3’ followed by [98°C 30 ″ , 55°C 30 ″ , 72°C 2 ″ ]35X and cloned into the pCRII vector (Invitrogen).

To generate plasmid HMMA021 for its use in vitro synthesis of RNA probes, the tTA coding sequence was excised from mfs#1215 [10] using EcoRV/BamHI and cloning into pCRII vector digested by the same enzymes.

To generate plasmid HMMA339 for in vitro synthesis of RNA probe against φC31 integrase mRNA, 800 bp of the coding sequence was digested out from plasmid HMMA98 using Smal/NcoI and cloned into pCRII plasmid digested by EcoRV/NotI.

The plasmid FCNH01 was generated by PCR amplification of 800 bp of Cas9 coding sequence using primers pair HM#560/HM#561 with program 98°C 3’ followed by [98°C 30 ″ , 64°C 30 ″ , 72°C 30 ″ ]5X [98°C 30 ″ , 72°C 1 ″ ]35X, digested by and cloned into XhoI/BamHI sites of pCRII vector.

To generate piggyBac transformation vector HMMA185 and HMMA186, first plasmid HMMA006 [52] was digested by AseI to remove syr

a-tTA, and the backbone was ligated to give rise to HMMA007. attP220 was PCR amplified from HMMA007 using primer pair HM#368/HM#369 and program 98°C 3’ followed by [98°C 30 ″ , 58°C 30 ″ , 72°C 20 ″ ]5X [98°C 30 ″ , 72°C 1 ″ ]35X and cloned into EcoRV cut site of HMMA007 to give rise to HMMA185. To generate HMMA186 the EcoRI/Hpal fragment Plb:nsEGFP from mfs#1213 [51] was cloned into the EcoRI/Hpal sites of HMMA185.

For the generation of piggyBac transformation vectors HMMA330 and HMMA331, first Gibson assembly was performed to clone EGFP/PSV40 and the 3XP3 promoter into the piggyBac backbone of HMMA007 digested by EcoRI to give rise to HMMA227, in which the EGFP gene was then replaced by DsRed.T3 from HMMA007 by AgeI/NotI to give rise to HMMA228. Then the attP220 was PCR amplified from HMMA007 using primer pair HM#131/HM#117 with PCR program 98°C 3’ followed by [98°C 30 ″ , 60°C 30 ″ , 72°C 20 ″ ]35X and cloned into EcoRI site of HMMA227 and HMMA228 giving rise to HMMA304 and HMMA305, respectively. Finally, the AseI/AgeI fragments from mfs#1213 and mfs#1214 [51] containing the Plb promoter were cloned into AseI/AgeI sites of HMMA304 and HMMA305 to give rise to HMMA330 and HMMA331, respectively.

To generate the spermatogenesis specific driver construct HMMA389, 1 kb upstream region of D. suzukii Ds

β

2t gene including the 5’UTR was PCR amplified from genomic DNA of the wild type Italian strain using primer pair HM#35/HM#36 with program 98°C 3’ [98°C 30 ″ , 61°C 30 ″ , 72°C 30 ″ ]5X [98°C 30 ″ , 67°C 30 ″ , 72°C 30 ″ ]35X and cloned in NeoI/XbaI sites of mfs#1215 [10] giving rise to HMMA015. The Dm

β

2t 3UTR was then PCR amplified from gDNA of wild type D. melanogaster strain OreR using primer pair HM#706/HM#707 with program 98°C 3’ [98°C 30 ″ , 63°C 30 ″ , 72°C 20 ″ ]5X [98°C 30 ″ , 70°C 30 ″ , 72°C 20 ″ ]35X and cloned into HMMA015 to give rise to HMMA253. Finally, the AseI fragment from HMMA253 was cloned into the AseI site of the transformation vector HMMA331.
To generate *attB* integration vector HMMA182 which can be used to integrate a plasmid into single *attP* site, the 5- *piggyBac* region was PCR amplified from plasmid HMMA006 using primer pair T7/mfs#370, with program 98 °C 3′ [98°C 30 ′, 51°C 30 ′, 72°C 20 ′] 40X digested by EcoRV and cloned into the blunted BamHI site of HMMA172, giving rise to HMMA181. Then the EcoRI/ Apal fragment containing the P*:lsEGFP* was excised mfs#1213 [51] and cloned into EcoRI/Apal of HMMA181.

To generate the helper plasmid HMMA098, the coding sequence of *φC31* was PCR amplified from plasmid mfs#1289 [39] using primers pair MK153/HM#123 with program 98 °C 3′ [98°C 30 ′, 72°C 1 ′ 20 ′] 35X. The reverse primer introduces the *SV40 nuclear localization sequence* at the C-terminus, which can improve the efficiency of *φC31* integrase [64]. A second round of PCR using primer pair MK153/HM#203 was used to amplify *φC31*ins using 1 μl of the first PCR reaction as a template with program 98 °C 3′ [98°C 30 ′, 67°C 30 ′, 72°C 1 ′] 5X [98°C 30 ′, 72°C 1 ′ 20 ′] 35X and cloned into HMMA051 NotI/ NotI replacing the *piggyBac* transposase coding sequence and giving rise to HMMA098. *piggyBac* helper HMMA051 was generated by cloning the *SV40* 3UTR digested from CH#705 by HindIII/NotI into HMMA050 HindIII/NotI sites. The latter was made by PCR amplification of *Ds-hsp70* promoter [52] from gDNA using primer pair HM#73/HM#74 and program 98°C 3′ followed by [98°C 30 ′, 58°C 30 ′, 72°C 30 ′] 5X [98°C 30 ′, 66°C 30 ′, 72°C 30 ′] 35X and cloning into EcoRI site of HMMA049, which was generated by cloning the *piggyBac* transposase coding sequence excised from MK004 [23] by EcoRI/NotI into the shuttle vector pSLaf1180af [65].

To generate *φC31* integrase based RMCE donor plasmids, HMMA253 and HMMA254, the annealed oligos HM#101/HM#337 generating the bacterial attachment site *attP* were cloned into SpeI site of pCRII vector (Invitrogen) giving rise to HMMA172. The gypsy insulators were digested out using SpeI/EcoRI from a fragment amplified from mfs#1213 [51] using primer pair HM#469/HM#470 with program 98°C 3′ followed by [98°C 30 ′, 70°C 30 ′, 72°C 2 ′] 35X and cloned into the cut site of HMMA172 to give rise to HMMA189. The EcoRI/NotI fragments P*:lsEGFP* and P*:DsRed.T3 were excised from HMMA186 and HMMA185, respectively, and cloned into HMMA189 to give rise to HMMA190 and HMMA191, respectively. Finally, *SV40* was PCR amplified from HMMA007 using primer pair HM#179/HM#124 and program 98°C 3′ followed by [98°C 30 ′, 62°C 30 ′, 72°C 20 ′] 5X [98°C 30 ′, 68°C 30 ′, 72°C 20 ′] 35X and cloned along with annealed oligos HM#101/HM#108 into HMMA190 and HMMA191 NotI/XbaI-blunted.

To generate HMMA336, for *φC31* RMCE, the tetracycline responsive element TRE along with the *P-element* basal promoter was PCR amplified from CH 727 [9] using primers pair HM#584/ CH6R [9] with PCR program 98°C 3′ followed by [98°C 30 ′, 69°C 30 ′, 72°C 30 ′] 35X and cloned into EcoRI/Clai sites of HMMA56 [52] replacing the hsp70 promoter giving rise to HMMA317 then the Ascl fragment containing *Cas9* fused to the T*Rep* and the *SV40* 3UTR was cloned into Ascl site of HMMA253.

To generate self-docking transformation plasmid HMMA223 the Ascl fragment containing nosE/P-φC31-nos was excised from the shuttle vector HMMA221 and cloned into Ascl site of HMMA185. HMMA221 was generated by replacement of *Cas9* coding sequence in plasmid HMMA167 by *φC31* integrase CDS. To make HMMA167, first the 3UTR of *Ds-nanos* was PCR amplified from HMMA013 using primer pair HM#94/HM#95 with program 98°C 3′ followed by [98°C 30 ′, 66°C 30 ′, 72°C 30 ′] 5X [98°C 30 ′, 72°C 1 ′] 35X and cloned into the shuttle vector pSLaf1180af [65] XbaI/AvfI sites giving rise to HMMA062. Then *Cas9* CDS was excised from HMMA056 [52] and cloned into Clai/XbaI sites of HMMA062 giving rise to HMMA165. Then the palindromic (self-complementary) oligo HM#102 was annealed to itself to introduce the 2X BbsI recognition site and cloned into the Clai site of HMMA165 to give rise to HMMA166. Finally, a 2 Kb upstream regulatory region of *Ds-nanos* gene including the 5′UTR was PCR amplified from gDNA using primer pair HM#345/ HM#113 and program 98°C 3′ followed by [98°C 30 ′, 72°C 1 ′ 30 ′] 35X and cloned into HMMA166 BbsI site by golden gate resulting in HMMA167.

**Germline transformation**

All *piggyBac* germline transformation experiments were performed using transformation vector and helper plasmid MK006 [23] at a final concentration of 500 ng/μL and 200 ng/μL respectively. For *φC31*-mediated site-specific transformation and *φC31*-mediated RMCE, the donor vectors were injected along with the helper plasmid HMMA098 at a concentration of 500 ng/μL and 300 ng/μL, respectively. The materials and the procedure of germline transformation were as described previously [23, 52]. Emerged *G₀* flies were crossed individually to three wild type flies of the opposite sex.

**Generation of RNA probes**

To generate DIG-labelled antisense RNA probes for in situ hybridization against *Ds_sry*, *Ds_nanos*, tTA, *Cas9*, or *φC31* integrase, DNA templates for in vitro transcription were prepared by restriction enzyme linearization of pCRII vectors containing either the whole gene pCRII_ *Ds_sry* (HMMA020), the 3′RACE fragment pCRII_ *Ds-nanos* 3′UTR (HMMA013), the coding sequence pCRII_ tTA (HMMA021), or 800 bp of the coding sequence of
in case of pCRII_Cas9 (FCMH01) and pCRII_φC31 (HMMA399) using XhoI, BamHI, NotI, NotI, or EcoRI, respectively. The antisense RNA labelling reaction was done using the DIG-labelling kit (Thermo Fisher Scientific) according to manufacturer instructions using 1 μg of DNA as template in a total reaction mix of 20 μL. The reaction was allowed to proceed for 3 h at 37 °C followed by Turbo DNasel treatment (Thermo Fisher Scientific) for 30 min to remove template DNA. Two microliter of 0.2 M EDTA were used to inactivate the reaction. The probes were then ethanol precipitated and resuspended in 100 μL RNA resuspension buffer (5:3:2 H2O: 20X SSC: formaldehyde) and stored at −80 °C.

Testes, ovary, and embryo whole mount in situ hybridization
Testes from 3 to 5 days old males from wild type D. suzukii, spermatogenesis specific driver line 389_M25M1, or progeny of the cross of the driver 389_M25M1 to the responder line 366_F3F1 were dissected in ice cold 1X phosphate buffered saline (PBS). Fixation and in situ hybridization were performed according to protocol by Lecuyer [66]. Anti-sense DIG labelled RNA probe against tTA was used to detect the expression driven by the Ds-β2t E/P. The Cas9 anti-sense RNA probe was used to detect the expression of Cas9 in the progenies arising from the cross testing the tet-off system. Anti-sense and sense probes previously described [52] were used as control.

To confirm the expression of the isolated Ds-nanos gene and the φC31 integrase driven by the regulatory regions of Ds-nanos in the ovaries of D. suzukii wild type flies and the transgenic self-docking line 223_F7M1, respectively, we collected 3–5 days old female flies and dissected the ovaries in ice-cold 1X PBS. The fixation and the in situ hybridization were performed as described [66].

To confirm the endogenous cellularization-specific expression of Ds_srya in wild type embryos, and whether the 349 bp of its upstream regulatory region including the 5′UTR are enough to drive expression of tTA in the transgenic driver line 06_F5M2 in a similar pattern, we performed embryo whole mount in situ hybridization using respective anti-sense DIG-labelled RNA probes in 0–24 h old embryos. Fixation and in situ hybridization were performed according to Lecuyer [66].

Microscopy
To observe and image testes, ovaries, and embryos, Zeiss Imager.Z2 equipped with two cameras, Axiocam 506 mono and Axiocam 305 colour (Zeiss, 73447 Oberkochen, Germany) was used. Images were taken using Axiocam 305 with bright field or DIC settings.

Screening for transgenic flies and fluorescence imaging were performed using Leica M205 FA fluorescence stereomicroscope equipped with camera Q imaging Microublisher 5.0 RTV (Leica Mikrosysteme Vertrieb Gmb, Wetzel, 35578 Germany). Transgenic flies were screened using filter sets RFP (excitation: ET546/10, emission: ET605/70) or GFP-LP (excitation: ET480/40, emission: ET510 LP), respectively, and imaged using cold light (Fig. 3A″,B″), filter sets RFP (Fig. 3A‴,B‴), or EYFP (excitation: ET500/20, emission: ET535/30; Fig. 3A‴‴,B‴‴).

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12863-020-00940-5.

Additional file 1: Supplementary Table 1. piggyBac transformation rates in D. suzukii AM strain.

Additional file 2: Supplementary Table 2. List of transgenic lines.

Additional file 3: Supplementary Table 3. List of primers used.

Abbreviations
φC31-RMCE: φC31-Recombinease Mediated Cassette Exchange; Ds-nanos: Drosophila suzukii nanos; tTA: Tetraacycline-controlled transactivator; β2t: Betat-2-tubulin; SRO: The spotted wing Drosophila; SIT: Sterile Insect Technique; SSR: Site-specific recombinases; BAC: Bacterial artificial chromosome; dsRNA: Double strand RNA; hid: Head involution defective; AM: Alpes Maritimes; sryα: Serendipity α; Dm_sryα: Drosophila melanogaster serendipity α; Ds_sryα: Drosophila suzukii serendipity α; DsRed: Discosoma Red; EGFP: Enhanced green fluorescent protein; Cas9: CRISPR associated protein 9; Dm_nanos: Drosophila melanogaster nanos; Dm: Digigovenigen 3′ UTR: 3′ untranslated region; 5UTR: 5′ untranslated region; cDNA: Complementary DNA; RACE: Rapid amplification of cDNA ends; PBS: Phosphat buffered saline; DIC: Differential interference contrast

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Authors’ contributions
E.A.W and H.M.M.A. conceived and designed the study; H.M.M.A. isolated the genes, designed the constructs, and performed in situ hybridizations; H.M.M.A. and F.H. generated the transgenic lines; E.A.W and H.M.M.A. wrote the manuscript; H.M.M.A. prepared the figures; all authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analysed during this study are included in this published article and its supplementary information files.
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