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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 $pIE\text{-}Dmhid^{Ala5}$ 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-PUbD-sRed-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-PUbD-sRed-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

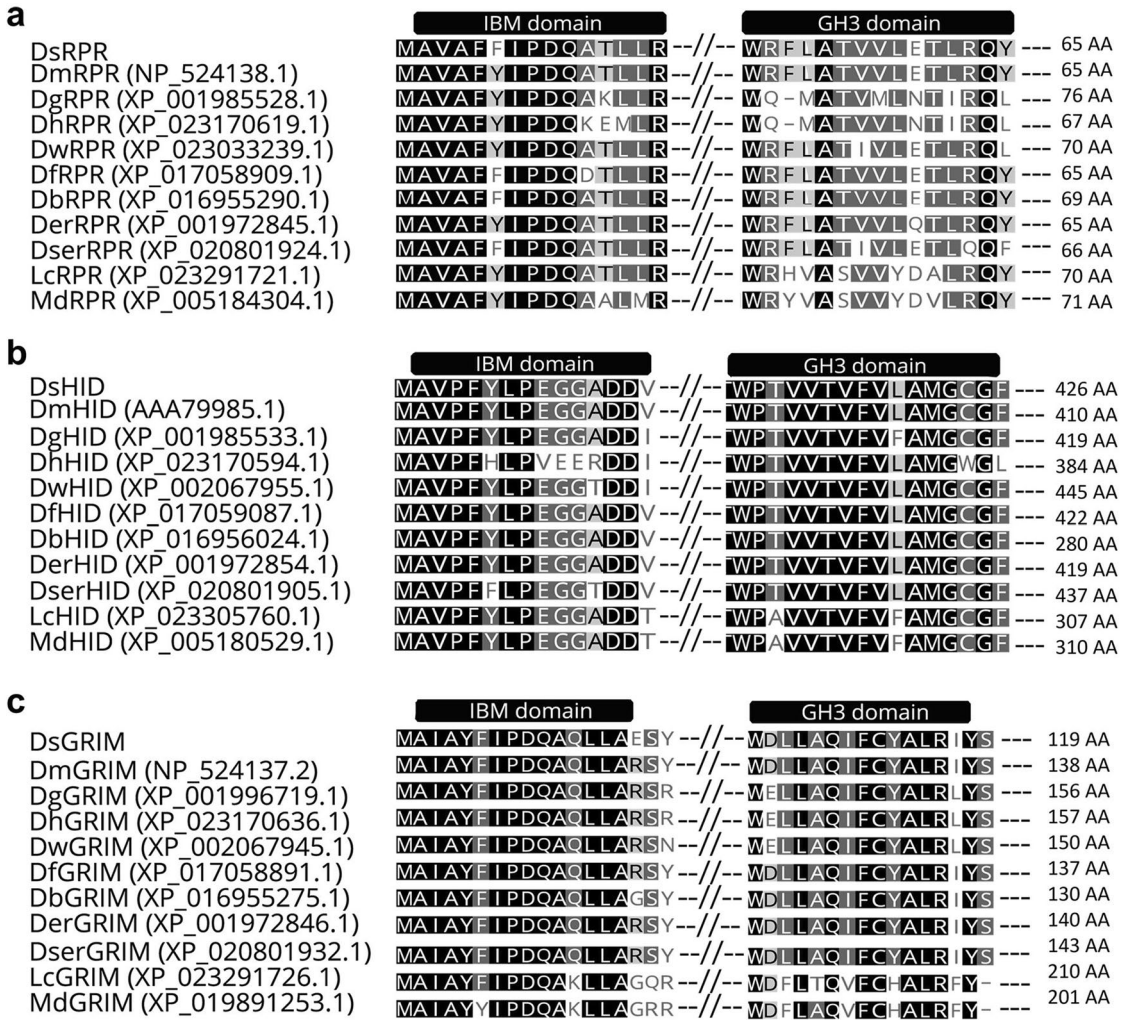

Fig. 1 Alignment of the *D. suzukii* RHG proteins with orthologs in other dipterans: **a** REAPER (RPR), **b** HEAD INVOLUTION DEFECTIVE (HID) and **c** GRIM. In each case, the protein from *D. suzukii* (Ds) is aligned with orthologs from *D. melanogaster* (Dm), *D. grimshawi* (Dg), *D. hydei* (Dh), *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

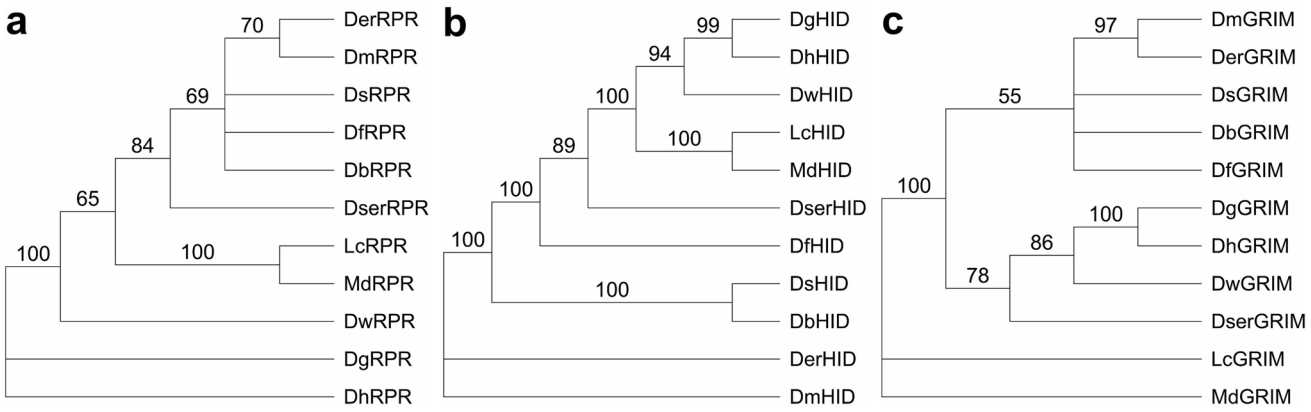

Fig. 2 Phylogenetic analysis of dipteran RPR, HID and GRIM proteins. Unrooted neighbor-joining trees were constructed with **a** RPR, **b** HID and **c** GRIM amino acid sequences. Bootstrap values (1000 replicates) are shown on the nodes of the trees. Species abbreviations are the same as in Fig. 1

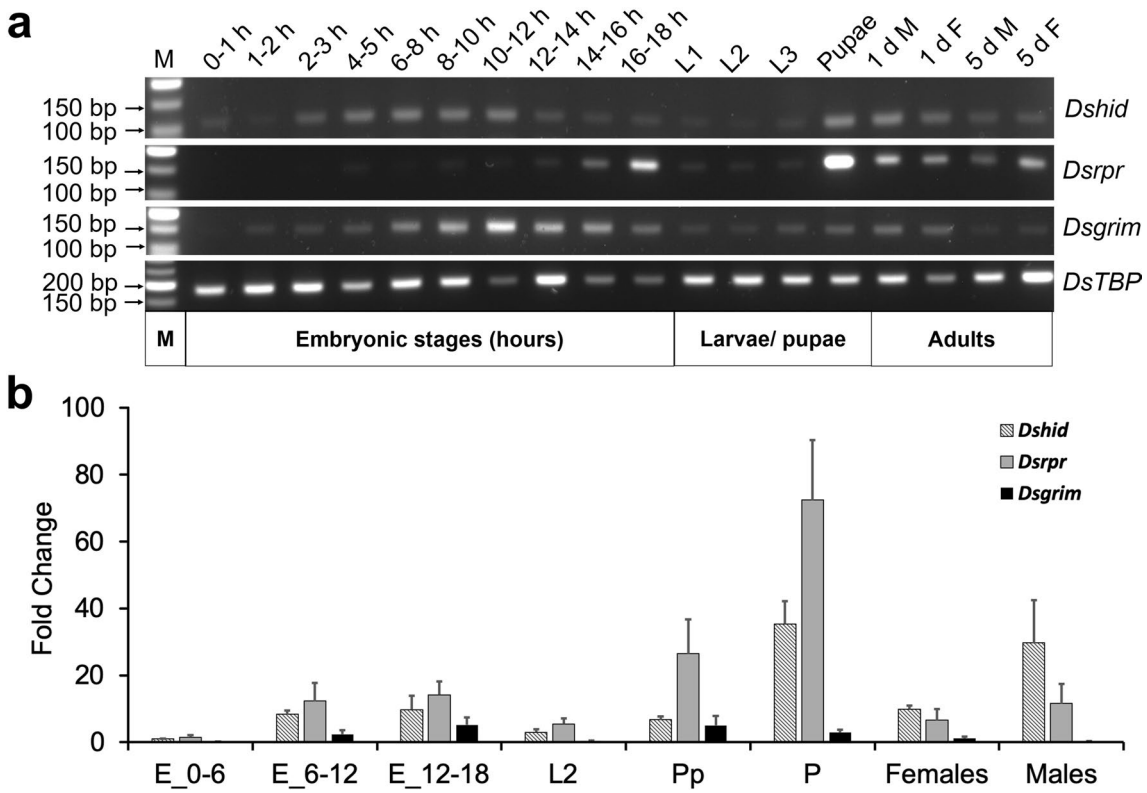

Fig. 3 Gene expression profiles of *Dshid*, *Dsrpr* and *Dgrim* throughout the development of *Drosophila suzukii.* **a** Reverse transcriptase-PCR analysis of *Dshid, Dsrpr* and *Dsgrim.* RNAs from embryos collected at different time points after egg laying (in hours), larvae, pre-pupae 24 h, pupae, 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

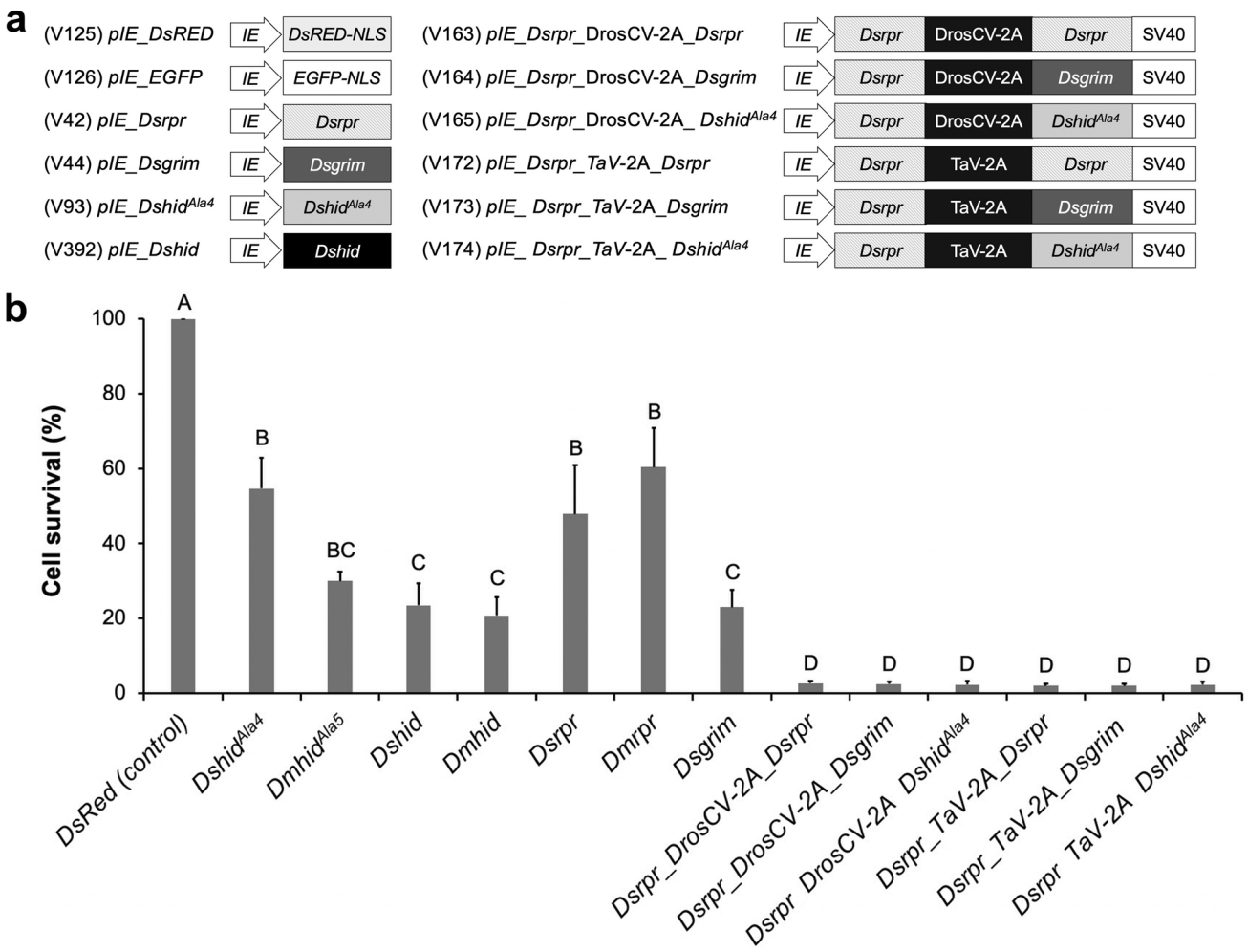

Fig. 4 Functional activity of *Drosophila suzukii* pro-apoptotic genes. **a** Schematic map of the *pIE4* test plasmids. **b** S2 cells were co-transfected with *pIE4-EGFP* plasmid and one of the *pIE* expression plasmids or *pIE4-DsRed* control plasmid to visualize the successfully transfected cells 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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