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SYNCAS: Efficient CRISPR/Cas9 gene-editing in difficult to transform arthropods

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ABSTRACT

The genome editing technique CRISPR/Cas9 has led to major advancements in many research fields and this state-of-the-art tool has proven its use in genetic studies for various arthropods. However, most transformation protocols rely on microinjection of CRISPR/Cas9 components into embryos, a method which is challenging for many species. Alternatively, injections can be performed on adult females, but transformation efficiencies can be very low as was shown for the two-spotted spider mite, *Tetranychus urticae*, a minute but important chelicerate pest on many crops. In this study, we explored different CRISPR/Cas9 formulations to optimize a maternal injection protocol for *T. urticae*. We observed a strong synergy between branched amphipathic peptide capsules and saponins, resulting in a significant increase of CRISPR/Cas9 knock-out efficiency, exceeding 20%. This CRISPR/Cas9 formulation, termed SYNCAS, was used to knock-out different *T. urticae* genes – *phytoene desaturase, CYP384A1* and *Antennapedia* – but also allowed to develop a co-CRISPR strategy and facilitated the generation of *T. urticae* knock-in mutants. In addition, SYNCAS was successfully applied to knock-out *white* and *white-like* genes in the western flower thrips, *Frankliniella occidentalis*. The SYNCAS method allows routine genome editing in these species and can be a game changer for genetic research in other hard to transform arthropods.

1. Introduction

The genome editing technology, CRISPR/Cas9, has revolutionized possibilities for treating diseases or developing new promising therapies, but its impact goes beyond the biomedical world. It has led to great advances in functional genetics research (Gudmunds et al., 2022) and offers new possibilities for the improvement of crops (Ahmad et al., 2021; Zaidi et al., 2020) and controlling harmful pests and disease vectors (Gantz et al., 2015; Hammond et al., 2016; Yadav et al., 2023). Despite the success in some organisms, many species remain whose genomes are very hard or even impossible to manipulate. Currently, the most common method for transforming arthropods is via microinjection of CRISPR/Cas9 components (Cas9 protein and sgRNA or plasmids encoding them) directly in to the eggs or embryos (Gratz et al., 2013; Gui et al., 2016; Hu et al., 2019; Kotwica-Rolinska et al., 2019; Li et al., 2022). Unfortunately, this technique is a huge barrier for the transformation of many species whose eggs do not survive the damage caused by the injection, whose eggs are hard to collect or who give live birth (vivipary) instead of laying eggs. Furthermore there are lots of parameters that require optimization such as penetration techniques, egg collection time, injection timing, injection position, the method for sealing eggs after injection and incubation conditions (Xu et al., 2019).

Alternative methods, relying on the injection of CRISPR/Cas9 components in the body cavity of adult females, can be used to circumvent this barrier. In 2018, a technology termed Receptor-Mediated Ovary Transduction of Cargo (ReMOT Control) was developed (Chaverra-Rodriguez et al., 2018). ReMOT Control relies on a conserved process of ovary and egg maturation called vitellogenesis in which yolk is accumulated in developing oocytes through receptor mediated endocytosis. It was shown that Cas9 ribonucleoprotein (RNP) uptake can be improved by attaching the yolk protein ligand that is recognized by the oocyte receptors to the Cas9 protein. Although this method increased the genome editing efficiency in *Aedes aegypti*, *Nasonia vitripennis* and *Ixodes scapularis* (Chaverra-Rodriguez et al., 2018, 2020; Sharma et al., 2022), it also has its limitations due to the requirement of a specific ligand that is recognized by the oocytes and hence the need for a specific recombinant Cas9 protein. A different approach to increase delivery of Cas9 RNPs to the oocytes was attempted in *N. vitripennis* by adding Branched Amphiphilic Peptide Capsules (BAPC) to the formed RNP. Although the injection of this mix in the ovary resulted in a tenfold increase in

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gene-editing efficiency (Chaverra-Rodriguez et al., 2020), the obtained CRISPR editing frequencies remained rather low with only 0.8% of the offspring being edited. In 2020, Dermauw et al. injected the micro-arthropod *Tetranychus urticae* with a CRISPR/Cas9 mix containing Cas9 protein and chloroquine as an endosomal escape reagent (EER), but genome editing efficiencies were very low (<0.2% edited offspring). This is in contrast with Shirai et al. (2022) who did obtain high genome editing efficiencies in *Tribolium castaneum* and *Blattella germanica* (up to 21.8% and 71.4% edited offspring respectively) using the same approach with or without chloroquine and termed this technique "direct parental CRISPR" or "DIPA-CRISPR". In those species it was demonstrated that the age of injected individuals is a critical parameter to obtain high transformation frequencies as the eggs have to be in the vitellogenic phase (Shirai et al., 2022). Recently, DIPA-CRISPR was performed in *A. aegypti* where timing of the injection was again a critical parameter (Shirai et al., 2023)

The two-spotted spider mite, *T. urticae,* is a highly polyphagous, cosmopolitan plant pest, able to feed on more than a 1,100 different plant species (Migeon and Dorkeld, 2022). Besides its extreme adaptability to different host plants, *T. urticae* also developed resistance to more than 90 acaricides (Mota-Sanchez and Wise, 2022). Some of the mechanisms behind resistance and host adaptation in this species have been unveiled thanks to the completely Sanger sequenced and annotated spider mite genome (Grbić et al., 2011; Wybouw et al., 2019a) and genetic tools such as bulked segregant analysis (BSA) and RNA-sequencing (De Beer et al., 2022; Fotoukkiaii et al., 2021; Snoeck et al., 2019; Villacis-Perez et al., 2022). However, in order to validate involvement of specific genes in acaricide resistance or host plant adaptation, a reliable gene editing tool is required (De Rouck et al., 2023). Not only is *T. urticae* a tremendous pest, it is also considered a model organism for Chelicerata, the second largest group of terrestrial animals comprising arthropods such as mites, ticks, scorpions and spiders (Grbic et al., 2007). Hence, further stressing the need for these gene editing tools as their impact can go beyond *T. urticae.*

Although the application of CRISPR/Cas9 *in vivo* was already demonstrated for zebrafish a decade ago (Chang et al., 2013; Hwang et al., 2013), species belonging to the subphylum of the Chelicerata could not be transformed until recently. A first proof of principle for the use of CRISPR/Cas9 in a chelicerate animal was provided for *T. urticae* by Dermauw et al. (2020). This was done by targeting *phytoene desaturase* (*PD, tetur01g11270;* https://bioinformatics.psb.ugent.be/orcae/overview/Tetur), a gene involved in the synthesis of body pigment. Disruption of this gene results in loss of red pigment in the eyes and legs (henceforth referred to as "albinism"), a phenotype which can be visually observed (Bryon et al., 2017). Knock-out (KO) mutants were obtained by injection of Cas9 RNPs in the body cavity without addition of a ligand or BAPC. Since *T. urticae* is an arrhenotokous species (unfertilized eggs develop in haploid males), injections were done in virgin females hence recessive KO mutations would always be visible in the phenotype of male offspring (Fig. 1). Although this study was considered as a break-through for genetic engineering in chelicerates, only a small fraction of the males (less than 0.2%) showed a CRSIPR/Cas9 mediated KO (Dermauw et al., 2020). These low gene-editing efficiencies impede routine applications of CRISPR/Cas9 as mutations without distinct phenotypes are hard to identify among offspring.

In this study, various CRISPR/Cas9 formulations were investigated to increase CRISPR/Cas9 delivery to the eggs upon maternal injection. Transfection agents such as lipofectamine, BAPC and endosomal escape reagents such as chloroquine and saponins were explored. Lipofectamine is a common lipid-based transfection reagent and has been used *in*

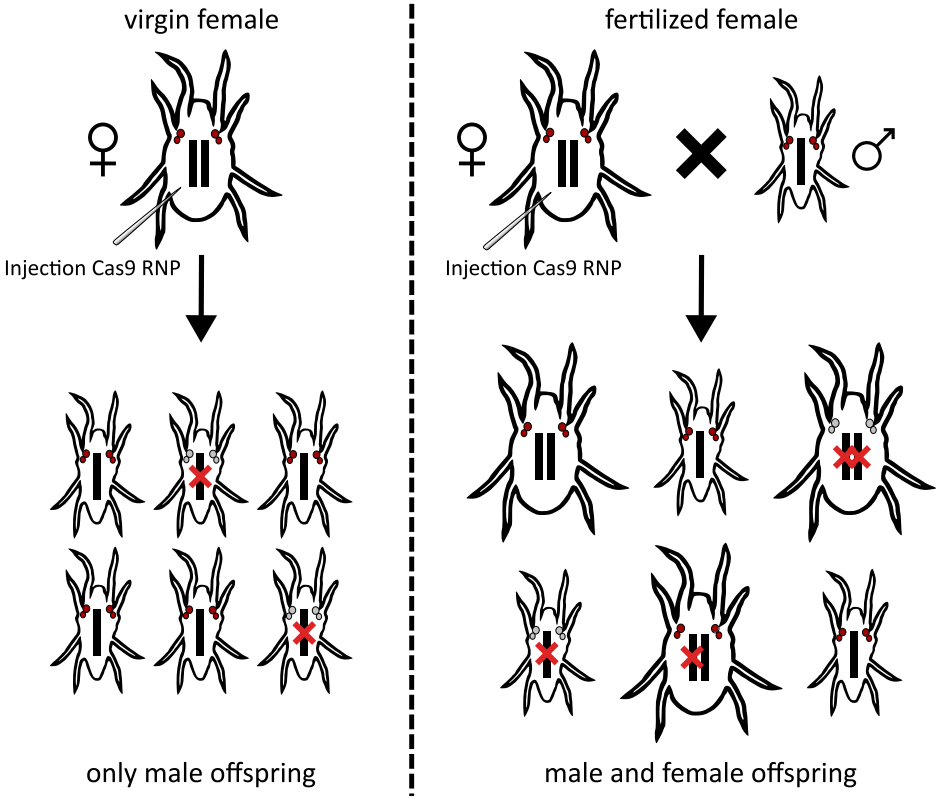

Fig. 1. Graphical comparison of CRISPR/Cas9 editing in *T. urticae* when injecting virgin females or fertilized females. Left: virgin females only lay haploid eggs which develop into male mites. Disruption of the PD gene (marked with a red cross) results in the loss of pigmentation (albinism), a recessive trait. Since males only possess one copy of PD, disruptions are always visible in the phenotype. Right: fertilized females will produce both male offspring (from eggs that remained unfertilized) and female offspring (from eggs that were successfully fertilized). In females the albino phenotype is only visible when both copies are disrupted, hence the observed % of albinos is an underestimate of the fraction of PD genes that were successfully knocked-out.

vivo for the delivery of CRISPR/Cas9 component in nematodes (Adams et al., 2019). The second transfection reagent, BAPC, is based on cationic nano-spheres comprised of self-assembling branched amphiphilic peptides which are very stable being resistant to disruption by chaotropes, proteases, and elevated temperatures (Kunte et al., 2022; Phoreus Biotech, 2023). Furthermore, these nanoparticles display low cytotoxicity and have been used for the delivery of DNA, dsRNA, mRNA and CRISPR/Cas9 RNPs (Avila et al., 2016, 2018; Chaverra-Rodriguez et al., 2020; Kunte et al., 2022). Next, chloroquine is an aminoquinolone derivative developed in the middle of previous century for the treatment of malaria and other conditions. Later it was widely investigated for its endosomal escape properties through its "proton sponge effect" leading to an influx of water molecules which in turn causes swelling and leakage from endosomal vesicles (Hajimolaali et al., 2021; Maxfield, 1982; Pandey and Harris, 2023). Saponins, on the other hand, are a diverse group of glycosidic compounds consisting of a triterpenoid or steroidal aglycones linked to an oligosaccharide moiety. They occur naturally in plants as secondary metabolites, are widely distributed in the plant kingdom and are well known for their foaming properties and amphipathic character (Moses et al., 2014; Vincken et al., 2007). Saponins have gained significant scientific interest due to their versatile biological activities, including anti-inflammatory properties (Passos et al., 2022). Saponins have also gained attention in agricultural applications for their plant protection properties as they have shown efficacy against several plant pathogens and pests, including fungi, nematodes, and insects (Desmedt et al., 2020; Singh and Kaur, 2018; Trdá et al., 2019). Interestingly, some saponins do exhibit strong endosomal escape activities (Cao et al., 2020; Sama et al., 2019), an important property for improved delivery of molecules such as CRISPR/Cas9 RNPs.

A strong synergism between BAPC and saponins was observed, and resulted in unparalleled CRISPR/Cas9 knock-out frequencies. This CRISPR/Ca9 formulation based on BAPC and saponins, termed SYNCAS, was also used to target other genes than *PD*, including *CYP384A1* (*tetur38g00650*) and *Antennapedia* (*Antp*, *tetur20g02430*). Furthermore, the formulation was tested to introduce a knock-in (KI) by introducing an etoxazole resistance mutation (Van Leeuwen et al., 2012) in *Chitin synthase 1* (*CHS1*, *tetur03g08510*). Last, SYNCAS was tested in another hard to transform organism, *Frankliniella occidentalis*, a species belonging to the insect order of Thysanoptera which, similar to *T. urticae*, is also arrhenotokous. The *white* and *white-like* gene were targeted, resulting in eye color morphs. To conclude, using this new protocol, CRISPR/Cas9 can be routinely applied in *T. urticae* and *F. occidentalis* and potentially many other hard to transform arthropods.

2. Materials and methods

2.1. Mite and insect rearing

The London strain of *T. urticae* is reference laboratory strain whose complete genome was Sanger sequenced in 2011 (Grbić et al., 2011). All knock-out injection experiments were performed with mites from this strain. The Alb-NL strain used in complementation tests was previously described (Bryon et al., 2017). For the knock-in of the etoxazole resistance mutation in *CHS1*, strain GSS (German susceptible strain) was used (Stumpf et al., 2001). All strains were maintained on *Phaseolus vulgaris cv*. "Prelude" at room temperature with 16:8 light:dark photoperiod. In order to have mites of similar age in the experiments, adult females were placed on a bean leaf and allowed to lay eggs for 24 h after which they were removed. The bean leaves were kept in climate chambers at 26 °C, 60% relative humidity (RH) and 16:8 light:dark photoperiod. After 8 days, deutonymph females were transferred to a new bean leaf without males (for most experiments requiring virgin females) or with males (for the experiment requiring fertilized females). Females were kept in the incubator until injection experiments were performed (3–4 days in adult stage). After experiments mites were kept in the incubators under the same conditions.

F. occidentalis was reared in containers with green bean pods in climate chambers at 25 °C, 65% R.H., and 16:8 light:dark photoperiod. Thrips were synchronized for experiments by allowing females to lay eggs for 24 h, after which they were removed. After 11 days, virgin females that hatched from these eggs, but that were still in the pupal stage, were collected and transferred to bean leaves placed on wet cotton until they reached the adult stage. Injections were performed on 16 day old females. After injecting, insects were again moved to bean leaves and kept in the climate chamber. Pollen (Nutrimite™, Biobest) was added as source of protein.

2.2. spCas9 and sgRNAs

Recombinant *Streptococcus pyogenes* Cas9 protein (Alt-R® S.p. Cas9 Nuclease V3) was purchased from Integrated DNA Technologies (Leuven, Belgium) at a custom concentration of 50 μg/μl. This Cas9 contains three nuclear localization sequences (NLS), one located on the N-terminus and two on the C-terminus (Vakulskas, 2022). Single guide RNAs (sgRNAs) were designed using the CRISPOR website (Concordet and Haeussler, 2018) and were ordered from Biolegio (Nijmegen, The Netherlands) consisting of the 20 nt guide sequence and 80-mer "Synthego scaffold" with 2′-O-methyl analogs and 3′ phosphorothioate internucleotide linkages at the first three 5′ and 3′ terminal RNA residues to increase stability. The sgRNAs were dissolved in provided 1x TE buffer and concentrations were validated using a Denovix DS-11 FX spectrophotometer. An overview of sgRNAs used in this study is shown in Table S1.

2.3. Injection protocol

All injections were done using a Nanoject III microinjector (Drummond Scientific) and needles made out of 3–000-203-G/X Glass Capillaries (Drummond Scientific). Needles were pulled using a P-1000 Micropipette Puller (Sutter Instruments) with following settings: "heat: 500, pull: 60, velocity: 70, delay: 200, pressure: 500, Ramp: 490" and were sharpened with a BV-10 Micropipette Beveler (Sutter Instruments) at a 15° angle. Injections were done under a Leica S8 APO microscope (Leica).

T. urticae females were aligned on Allura red stained 2% agarose gel platforms with their dorsal side in contact with the agar. These platforms were made by adding two glass microscope slides (26 × 76 mm, 1.1 mm thick), attached to each other by double-sided tape, into a Petri dish immediately after pouring the agar plates. After solidification, the gels were cut to form a staircase-like platform. Females were then injected ventrally in the body cavity between the third pair of legs with 3 nl of solution. *F. occidentalis* females were placed on double sided tape with their dorsal side and were injected in the middle of the ventral side of the abdomen with 5 nl of solution. After injection, females were placed on detached bean leaves (on wet cotton in Petri dishes) and allowed to lay eggs with a transfer to a new bean leaf every 24 h. Eggs were collected up to 72 h after injection.

2.4. CRISPR/Cas9 formulations

CRISPR/Cas9 Ribonucleoprotein particles (RNPs) targeting the *PD* gene (*tetur01g11270*) were prepared by mixing 1.5 μl of Cas9 nuclease (50 μg/μl) with 1.5 μl of sgPD1 and 1.5 μl of sgPD2 (both 4 μg/μl). The mixture was incubated for 10 min at room temperature. After incubation, about 0.5 μl of variable compounds were added (Table 1). An overview of all compounds used in this study and their supplier can be found in Table S2. The dose of saponin added was determined by preliminary experiments where a range of concentrations was injected. The highest concentration where females were still able to produce sufficient offspring was selected (data not shown). For the formulations that contain BAPC (either alone or in mixture), an incubation of 30 min on ice was done after adding all compounds. Finally, the injection mix was

Table 1
List of the various compounds added to the Cas9 RNP mixes used to target the *PD* gene of *T. urticae*.

Formulation	Composition
Unformulated	0.5 μl nuclease free water (NFW)
Chloroquine	0.5 μl chloroquine (25 μg/μl)
Saponins	0.5 μl saponins (3 μg/μl)
Lipofectamine (3%)	0.15 μl Lipofectamine (100 v/v%) + 0.35 μl NFW
Lipofectamine (10%)	0.5 μl Lipofectamine (100 v/v%)
BAPC	0.5 μl BAPC (10 μg/μl)
BAPC + chloroquine	0.5 μl BAPC (10 μg/μl) + 0.25 μl chloroquine (51 μg/μl)
BAPC + saponins	0.5 μl BAPC (10 μg/μl) + 0.2 μl saponins (7.5 μg/μl)
BAPC + lipofectamine (5%)	0.5 μl BAPC (10 μg/μl) + 0.25 μl Lipofectamine (100 v/v%)

centrifuged at 4 °C for 10 min at 20,000 g and kept on ice until used for injection. Between 300 and 400 virgin females were injected per replicate. All treatments were performed in three independent replicates. Offspring was screened for albinism, mutants were analyzed using complementation tests and Sanger sequencing (see 2.5) and KO frequencies were calculated. Based on the results of these first formulations, further optimization of the injection mix containing BAPC and saponins was done by increasing the sgRNA:Cas9 from 1:1 to 2:1, as this can further increase transformation efficiencies (Seki and Rutz, 2018) using only the most efficient sgRNA (sgPD2): 1.5 μl of Cas9 nuclease (50 μg/μl), 3 μl of sgRNA (10 μg/μl), 0.5 μl BAPC (10 μg/μl) and 0.2 μl saponins (7.5 μg/μl). This formulation will henceforth be referred to as the "optimized formulation".

2.5. Screening and analysis of mutants

Male haploid progeny of injected mothers was visually screened for albinism in all experiments. For the first experiments where both sgPD1 and sgPD2 were used, individuals showing a mutant phenotype were placed on individual leaf discs and crossed with virgin females of the ALB-NL line containing a known mutation in the *PD* gene. The female offspring of this cross was screened for albinism as it was known that the mutant alleles of these lines could not genetically complement each other (Dermauw et al., 2020). Two to four days after the cross to the ALB-NL strain, males that were still alive were individually crushed in 18 μl STE buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8) and 2 μl proteinase K (10 mg/ml). This crude extract was incubated at 37 °C for 30 min followed by proteinase K deactivation at 98 °C for 10 min. A PCR was performed on these raw DNA extracts amplifying a 535 bp region containing the two sgRNA targeting sites using the GoTaq® G2 DNA Polymerase and the "phytoene desaturase sg1+2" primer pair (Table S3). The temperature profile consisted out of a 2 min denaturation step at 92 °C followed by a touch-down of 5 cycles with denaturation at 92 °C for 30 s, annealing at 60 °C–1 °C/cycle for 30 s and elongation at 72 °C for 1 min. Next, 37 cycles of 30s at 92 °C, 30s at 55 °C and 1 min at 72 °C were performed. After a final elongation of 72 °C for 5 min, a quality check was performed by running 5 μl of PCR product in a 2% agarose gel for 30 min at 100 V. The remaining PCR product was purified using an EZNA® Cycle Pure Kit (Omega Bio-Tek). Purified PCR template was sent to LGC genomics for Sanger sequencing. For the experiment evaluating efficiency in diploid eggs, F2 sons emerging from F1 albino females were also analyzed using the PCR and Sanger sequencing protocol, but were not crossed to ALB-NL. Based on these results it was clear that the loss of eye coloration was a reliable phenotypic marker to score KO efficiencies. Hence, for the remaining *PD* KO experiments, mutants were no longer crossed to ALB-NL or sent for Sanger sequencing, but were considered KO mutants based on the albinism phenotype.

2.6. Other target genes in T. urticae: CYP384A1 and Antp

Two other target genes, *CYP384A1* (*tetur38g00650*) and *Antennapedia* (*Antp*, *tetur20g02430*), were selected to investigate whether high KO frequencies can also be obtained for genes other than *PD*. For each target gene, 100–200 virgin-females were injected per replicate using the optimized formulation with sgCYP for *CYP384A1* or sgAntp for the *Antp* gene. Offspring was screened for mutant phenotypes (yellow-colored mites for *CYP384A1* and deformed legs for *Antp*) and mutants were analyzed by Sanger sequencing as described earlier (see2.5) using primer pairs "*CYP384A1* target site" and "*Antp* target site" (Table S3) for *CYP384A1* and *Antp* mutant samples respectively. In an additional experimental set-up, females were injected with the optimized formulation, but containing a 1:1 mix of both sgPD and sgAntp (using 1.5 μl of each sgRNA) to evaluate the frequency of double KO events. In this latter experiment, offspring was scored for *Antp*, *PD* or *Antp* + *PD* KO phenotypes, but mutants were not further analyzed by Sanger sequencing.

2.7. Age and ploidy dependent knock-out efficiency

To evaluate whether the age of injected females has an effect on the KO frequency in the offspring, the optimized formulation was injected in virgin adult females who had reached adulthood for 1 day, 4 days and 7 days. The experiment was performed three times with 100 females of each age group injected in every replicate. Additionally, to see the effect of the formulation in diploid mites, 200–300 fertilized females were injected per replicate. As a control, 100 virgin females were also injected with the same mixture. Offspring was counted and the percentage KO was calculated based on screening for albinism. Each F1 albino daughter resulting from the injection of fertilized females was transferred to a single leaf disc at the deutonymph stage and allowed to reach the adult stage. About 2 weeks later 3–12 F2 males were collected per F1 albino mother and the *PD* gene was analyzed in each individual son via Sanger sequencing as described earlier (see 2.5).

2.8. Precise knock-in of chitin synthase resistance

In order to evaluate whether the SYNCAS method can also be used to introduce specific mutations by homology directed repair (HDR), attempts were made to introduce a single nucleotide polymorphism (SNP) in *chitin synthase 1* (*CHS1*, *tetur03g08510*). The targeted SNP is a replacement of adenine to thymine at position 3049 in the *CHS1* coding sequence resulting in the substitution of isoleucine by phenylalanine at position 1017 in the chitin synthase protein which confers etoxazole resistance (Van Leeuwen et al., 2012). A single-stranded oligo DNA nucleotide (ssODN) of 192 bp was designed containing this SNP along with several silent mutations that disrupted the target site of the sgRNA (File S1), preventing further Cas9 cleavage upon HDR of the *CHS1* gene. The silent mutations also served as a control for false positives as the SNP at position 3049 is a common mutation and might appear in the population by chance. Various approaches were examined to obtain efficient KI results (Table S4). In one approach, only the ssODN was added to the CRISPR/Cas9 mix while in other approaches a HDR enhancer (enhancer V2), which blocks the non-homologous end joining (NHEJ) pathway, was also added. However, adding the enhancer to the CRISPR/Cas9 mix seemed to cause aggregate formation, leading to difficulties when loading the mixture into the needle. Therefore, mites

were injected twice: once with the CRISPR/Cas9 mix without enhancer and once with the enhancer only or with enhancer and extra ssODN repair template. 400 virgin females were injected in each replicate of each treatment. After three days, eggs were sprayed with a diagnostic dose of 110 mg a.i./L etoxazole (Van Leeuwen et al., 2012). Five days later, the bean leaves were screened and survivors were analyzed by sequencing as described earlier, but using primer pair "*CHS1* target site" (Table S3).

2.9. Gene editing in F. occidentalis

To test whether the SYNCAS formulation could also be used in other arthropods, knock-out experiments were performed in *F. occidentalis*. The *white* gene (NCBI Gene ID: 113205575) and *white-like* gene (NCBI Gene ID: 113217734) were selected as target genes and were targeted using "sgwhite" and "sgwhite-like" respectively (see Table S1 for target sequences). These two target genes were selected based on orthology with the white protein of *D. melanogaster* (NCBI: NP_476787.1) using the *F. occidentalis* genome assembly, Focc_2.1 (Rotenberg et al., 2020). Specificity of the composition of CRISPR/Cas9 mix used is shown in Table S5. Injections were performed 3 times independently for *white* and once for *white-like*. Additionally, one injection was performed targeting both genes (using 1.5 μl of each sgRNA in the mix). For each replicate 100 to 200 virgin females were injected. Eight days after injecting, the male offspring was screened for a divergent phenotype. Analysis of mutants (DNA extraction, PCR, purification and sequencing) was performed as described for *T. urticae*, but using the "*F. occidentalis white*" and "*F. occidentalis white-like*" primer pair (Table S3) for injections targeting the *white* and *white-like* gene respectively.

2.10. Phylogenetic analysis of white and white-like in F. occidentalis

Protein sequences of white, white-like, scarlet and brown of thrips species for which a genome assembly is available were collected and manually curated (File S2). *Drosophila melanogaster*, *Bombyx mori* and *T. castaneum* white, scarlet and brown sequences were also included in the phylogenetic analysis, as well as two metazoan conserved ABCG proteins from *D. melanogaster* and *F. occidentalis,* serving as outgroup. Alignment of the sequences was performed with MAFFT version 7 (Katoh and Standley, 2013) with "auto" option. A maximum likelihood phylogenetic tree was obtained through IQ-TREE 1.6.12 ran with default parameters (Nguyen et al., 2015) The integrated ModelFinder function (Kalyaanamoorthy et al., 2017) indicated LG + F + R4 as the best-fit model. Ultrafast bootstrap (UFBoot) were set at 10,000 replicates (Hoang et al., 2018).

2.11. F. occidentalis knock-in

After establishing an efficient transformation protocol for *F. occidentalis*, attempts were made to restore the *white* gene in a thrips KO line. About 200 females were injected with 5 nl of injection mix (Table S5) using "sgWR" (Table S1). As was done for *T. urticae*, silent mutations were incorporated in the ssODN repair template (170bp) as a control for possible contamination with wild type (WT) individuals (File S1). Half of the injected thrips were injected a second time with 1 nl of enhancer V2 (100 μM). A PCR was performed on DNA from restored offspring (individuals displaying a full or partial WT phenotype) as described earlier using the "*F. occidentalis white*" primers (Table S3) and obtained PCR product was cloned in DH5-α cells using the CloneJET PCR Cloning Kit (Thermo Scientific™) following the manufacturer's instructions. Transformed cells were grown on LB-agar containing 100 μg/ml of carbenicillin and 96 single colonies were sent to LGC for sanger sequencing using the pJET1.2 forward and reverse sequencing primers from the kit.

3. Results

3.1. Evaluation of various CRISPR/Cas9 formulations on knock-out efficiency in T. urticae

Based on the experimental setup from Dermauw et al. (2020), different adjuvants such as chloroquine, saponins, lipofectamine and BAPC were added to the Cas9 RNP targeting the *PD* gene of *T. urticae* in an attempt to increase the low transformation efficiency obtained previously. After injecting the CRISPR/Cas9 mix in the body cavity of virgin female spider mites, the transformation efficiency was scored as the percentage of the total offspring showing an albino phenotype (Fig. 2). None of the single adjuvants were able to significantly increase the transformation efficiency (<1% offspring showing albinism). However, in one treatment a strong synergistic effect was observed between BAPC and saponins, reaching editing efficiencies of 5–6% in the eggs deposited within 48 h after injection (Fig. 2A, Table S6). All 66 albino males found in the three replicates of this treatment were crossed to virgin females of the ALB-NL strain. About 86% (54/66) of these crosses were successful and all of them produced albino daughters, indicating a disruption of the *PD* gene is responsible for the observed phenotype. In addition, for a total of 90 albino samples (over all treatments) Sanger sequencing data was obtained confirming KO events (deletions or insertions) at the target site (File S3). In this initial experiment both sgPD1 and sgPD2 were used. However, as just 2 out of 90 of CRISPR/Cas9 events were located in sgPD1 target region, only sgPD2 was used in all further experiments. Using the optimized formulation with exclusively sgPD2, KO efficiencies up to 24% were obtained whereas control mixes where either saponins or BAPC were replaced with water only resulted in efficiencies below 1% (Fig. 2B).

3.2. Knock-out of CYP384A1 and Antp

To validate whether high editing efficiencies could also be obtained for other genes using SYNCAS, two other target genes were selected. The first gene, *CYP384A1* (*tetur38g00650*), is also involved in the pigment synthesis pathway in spider mites. It likely acts as carotenoid ketolase and disruption of this gene results in a phenotype referred to as "lemon" due to the yellow color resulting from the lack of keto-carotenoids (Wybouw et al., 2019b). The second gene, *Antennapedia* (*Antp*, *tetur20g02430*), is a Hox gene whose homologue was shown to be involved in the leg development of the spider *Achaearanea tepidariorum* as RNAi silencing of the gene resulted in larvae containing 10 legs instead of 8 (Khadjeh et al., 2012), while in spider mites RNAi silencing of *Antp* results in loss of the fourth pair of legs (Luo et al., 2023). For these two target genes similar KO efficiencies as for the *PD* gene were obtained (Fig. 2B, Table S7). *CYP384A1* KO mutants exhibited the expected lemon phenotype (Fig. 2) while KO mutants of *Antp* resulted in six-legged spider mites or mites with a pair of last legs (L4) that were severely deformed (Fig. 2, Fig. S1, Video S1). Among the offspring of females injected with both sgPD2 and sgAntp, double KO mutants were observed having both the albinism and deformed leg phenotype (Fig. S1, Table 2, Video S1). Within three out of six groups up to 100% of the albino offspring also had the deformed leg phenotype resulting from *Antp* KO.

3.3. Effect of age and fertilization of mothers on knock-out efficiency

To evaluate whether the age of injected females has an effect on the KO frequency in the offspring, females whom had reached adulthood for 1 day, 4 days and 7 days were injected with the optimized formulation. No effect of age on KO efficiency could be observed (Fig. 3A, Table S8). The potential of SYNCAS to transform diploid eggs was also evaluated by injecting fertilized females. Although a lower KO frequency was observed in the female offspring, a moderate transformation rate of about 5% was still observed (Fig. 3B, Table S9). Peculiarly, the analysis

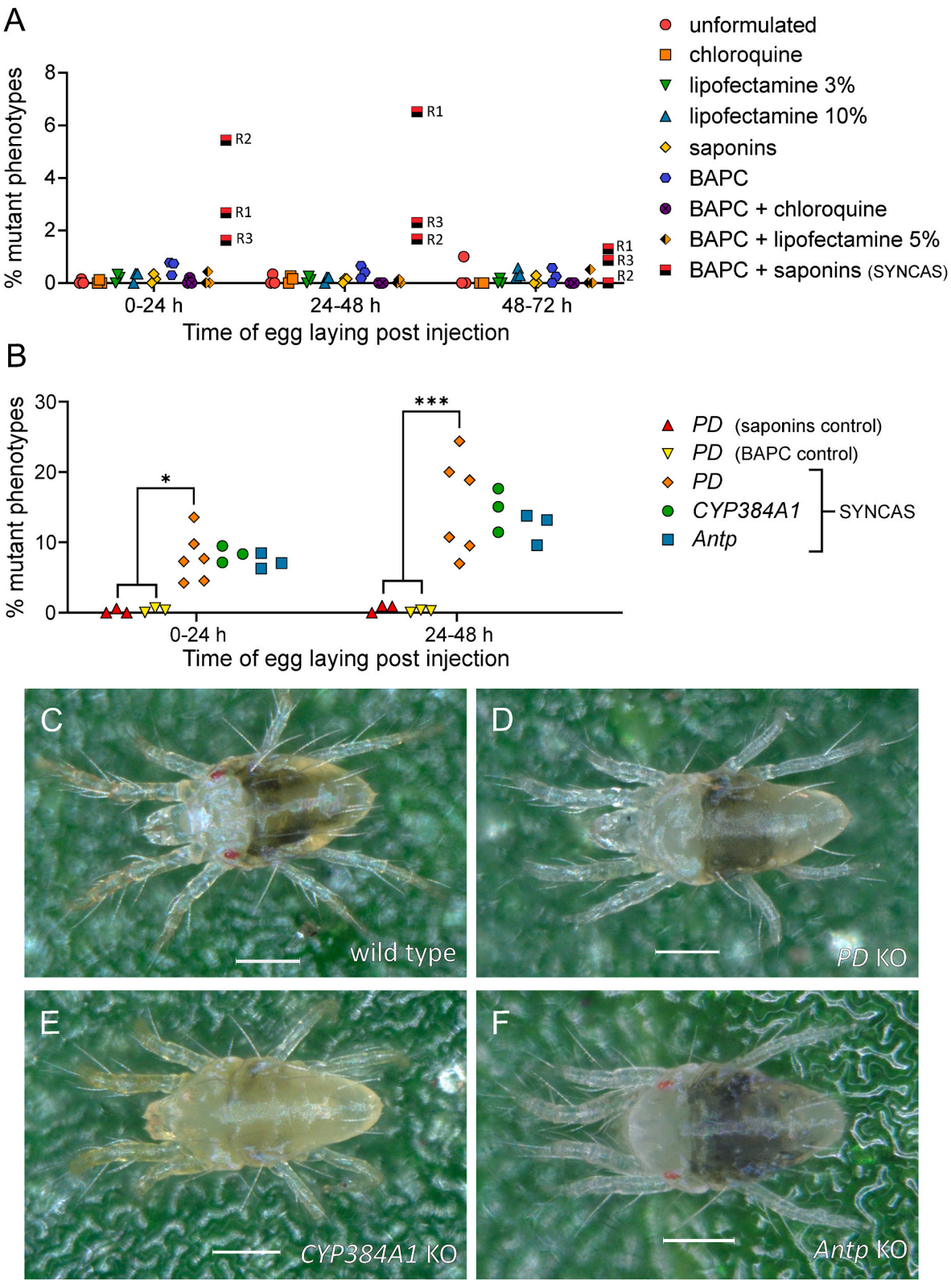

Fig. 2. Knock-out frequencies and phenotypes observed in *T. urticae* in haploid male offspring after injection of virgin females. A) Effect of various adjuvants on the percentage of offspring showing albinism resulting from CRISPR/Cas9 knock-out of PD using sgPD1 and sgPD2. R1-3 indicate the replicate numbers of the BAPC + saponins experiment. B) Obtained frequency of mutant phenotypes in offspring with the optimized BAPC + saponins formulation (SYNCAS) for three different target genes: *PD* (using only sgPD2), *CYP384A1* and *Antp*. For *PD*, two control treatments were performed in which one of the adjuvants used in the optimized formulation was replaced with water. In the "saponins control" and "BAPC control" BAPC and saponins were replaced with water respectively. A Two-Way ANOVA with Šidák correction for multiple testing was applied as statistical test comparing all PD groups. *: $p < 0.05$, **: $p < 0.01$, ***:$p < 0.001$. C) wild type phenotype of a *T. urticae* male. D–F) *PD*, *CYP384A1* and *Antp* mutant phenotypes in *T. urticae* males. Scalebar = 100 μM. Data used to generate graphs is shown in Table S6 & Table S7.

Table 2
CRISPR/Cas9 double KO efficiency targeting both *Antp* and *PD*. R: replicate number, TPI: Time Post Injection. #mothers represents the number of injected females that were still alive at the end of the corresponding TPI interval. "%Double KO/Albinism" represents the fraction of albino mutants in which the Antp gene was also knocked-out.

R	TPI (h)	#Mothers	#Offspring	#*Antp* KO	#*PD* KO	#*Antp*+*PD* KO	%Double KO/Albinism
1	0–24	64	127	14	0	4	100
	24–48	55	111	10	2	5	71.4
2	0–24	104	165	11	6	2	25
	24–48	74	152	11	3	2	40
3	0–24	65	13	1	0	1	100
	24–48	42	8	1	0	3	100

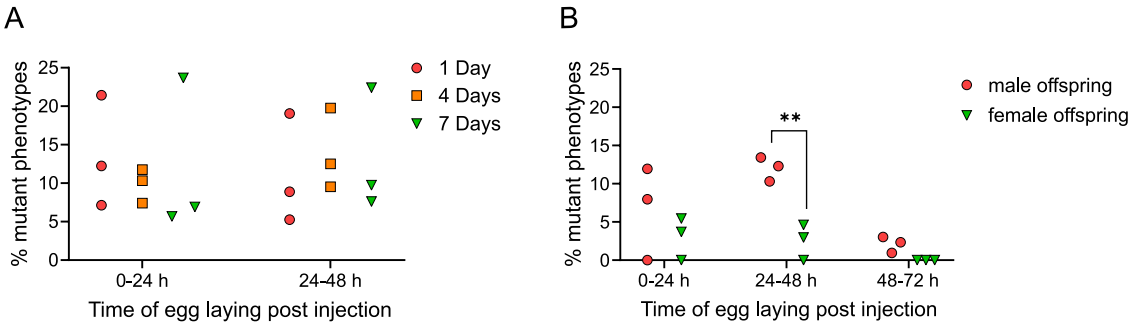

Fig. 3. Evaluation of the effect of two biological parameters on CRISPR KO efficiency in the offspring of *T. urticae* using the SYNCAS protocol targeting *PD*. A) Effect of age of injected mothers. B) Effect of fertilization: haploid males develop from eggs that remained unfertilized after mating while diploid females develop from eggs that were successfully fertilized after mating. A Two-Way ANOVA with Šidák correction for multiple testing was applied as statistical test for both datasets, comparing gene editing frequencies of the different groups within each time interval. **: $p < 0.01$. Data used to generate graphs is shown in Table S8 & Table S9.

of the F2 albino sons from these virgin albino mothers revealed that some females contained more than two different CRISPR events in the target site (Fig. S2).

3.4. *CHS1* knock-in formulations

In addition to mutations resulting from NHEJ upon DNA breaks, CRISPR/Cas9 can also be used to introduce specific mutations by using

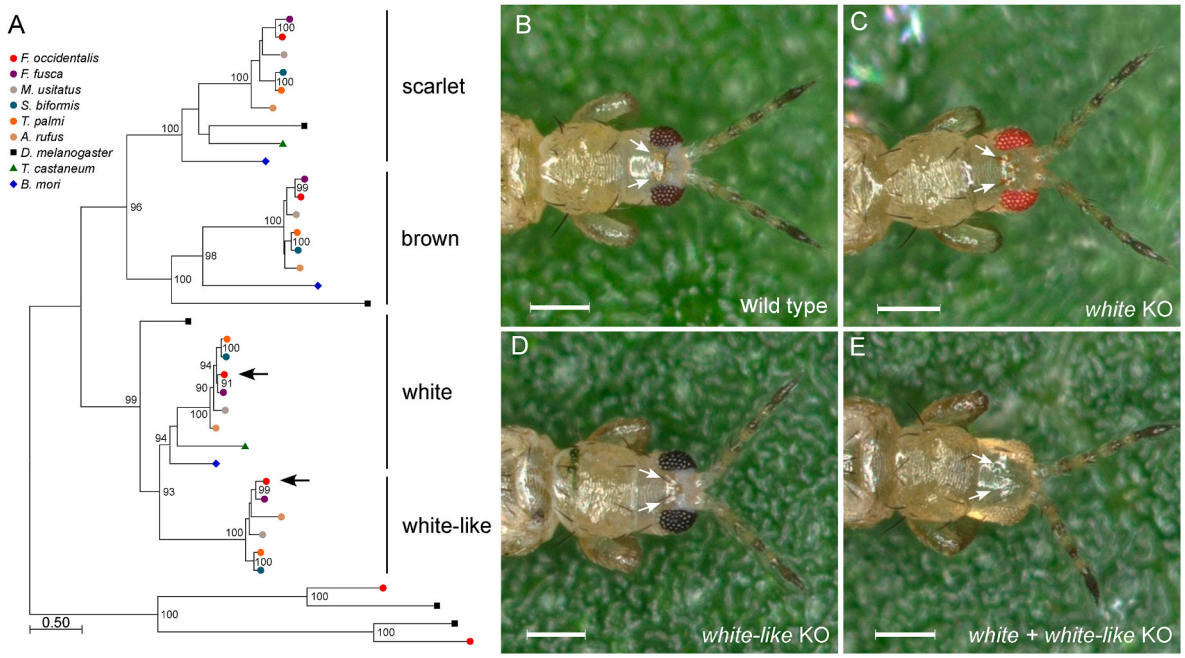

Fig. 4. Gene editing of *F. occidentalis white* and *white-like* A) Phylogenetic analysis of ABCG proteins white, white-like, scarlet and brown using ABCG protein sequences from various thrips species, *D. melanogaster*, *T. castaneum* and *B. mori*. Additionally, two metazoan conserved ABCG proteins from *D. melanogaster* and *F. occidentalis* were included in the analysis. Alignment of sequences was done using MAFFT, while the maximum likelihood phylogenetic tree was constructed using IQ-TREE. *F. occidentalis* white and white-like are indicated with a black arrow. B-E) CRISPR/Cas9 KO phenotypes obtained in *F. occidentalis* for two different target genes. B) wild type phenotype. C-D) Mutant phenotypes resulting from KO in *white* and *white-like*, respectively. E) Mutant phenotype obtained after double KO of both *white* and *white-like*. White arrows indicate ocelli. Scalebar = 100 μM.

the homology directed repair (HDR) pathway. We aimed to introduce an I1017F mutation in the *chitin synthase 1* gene (*CHS1*, tetur03g08510), a mutation known to cause high resistance levels against the acaricide etoxazole (Van Leeuwen et al., 2012). This was done using an ssODN repair template containing this SNP and some silent mutations disrupting the target site to prevent further Cas9 cleavage upon HDR of the *CHS1* gene (Fig. S3). In a first approach the CRISPR Cas9 formulation optimized for KO was adjusted by increasing the BAPC concentration and including the ssODN in the mix. In one replicate, two males were found that survived etoxazole spraying and in which the ssODN repair was correctly integrated whereas survivors could not be observed in the two remaining replicates. Therefore, the set-up was repeated using a HDR stimulating agent, enhancer V2, either by adding it to the CRISPR mix or by performing a second injection run with the compound. This last approach improved the KI efficiency as multiple males with the ssODN repair template could be found in all replicates, and overall, KI efficiencies (expressed as %EtoxR/mother) reached 2–14% (Table S10).

3.5. Gene editing and phylogenetic analysis of *F. occidentalis white* and *white-like*

In order to examine whether SYNCAS could also be used in other difficult to transform arthropods, a CRISPR/Cas9 mix with sgRNAs targeting the *white* or *white-like* gene and with an adjusted concentration of saponins (being the highest dose where sufficient offspring is still produced) was injected in *F. occidentalis* females. *white* mutants displayed bright red ommatidia while *white-like* mutants had solid black ommatidia (Fig. 4). Transformation efficiencies up to 30% and 32% were obtained for *white* and *white-like* respectively (Table 3). Sanger sequencing data was obtained for 57 *white* and 9 *white-like* mutants (File S3), all confirming KO deletions at the target site. Additionally, an injection was performed with a mix targeting both *white* and *white-like* where about 20% off the offspring showed a complete absence of eye pigmentation with transparent ommatidia and ocelli resulting from a combined KO in both genes although different forms of chimerism were also found among the offspring (Fig. 4, Table S11).

In *D. melanogaster* disruption of the *white* gene results in fruit flies with white colored ommatidia (Mackenzie et al., 1999). Although *white* and *white-like* were selected based on orthology with the *D. melanogaster* gene, disruption of either of these genes in thrips did not result in this phenotype. Therefore, a phylogenetic analysis was performed, which showed that ABCG proteins from each species clustered in their respective groups (white, white-like, scarlet and brown), except for *D. melanogaster* white (Fig. 4).

Attempts were also made to establish a protocol to introduce specific mutations by restoring the *white* gene in a thrips KO line generated from the KO experiments. Six individuals with partially restored (chimeric) eyes and one individual with fully restored eyes were identified among the offspring (Table S12). However, Sanger sequencing revealed that the DNA was not restored by HDR using the ssODN repair template, but that a new NHEJ event had occurred transforming the out-of-frame deletion to an in-frame deletion (Fig. 5). Since chimerism was observed in some cases, samples were further investigated for cells which might have correctly repaired the KO by using the repair template. This was done by cloning the originally obtained PCR product used for Sanger sequencing in DH5-α cells and sending individual colonies for Sanger sequencing. For two individuals (individual 5 and 7, both females), a few colonies indeed showed the correctly restored *white* gene indicating a fraction of their tissue contained DNA that was repaired by the HDR pathway (Fig. 5, Table S13).

3.6. Knock-out mutation types observed

Over the various KO experiments, sequencing data was collected for the different target genes. For each gene, the observed type of mutations was recorded (Fig. 6). Although many different insertion and deletion events were observed, specific types of mutation events seemed to preferentially occur within one target gene. In the *PD* gene of *T. urticae*, for example, deletions of three or six nucleotides arose noticeably more than other types of mutations. For *CYP384A1* single nucleotide deletions were most abundant while for *Antp* nearly all sequences contained two nucleotide deletions. Last, seven nucleotide deletions were the most frequently observed in the *white* gene of *F. occidentalis*.

4. Discussion

DNA serves as the genetic code and fundamental blueprint that provides the instructions necessary for growth, development, and functioning of all living organisms. Biotechnological approaches, such as CRISPR/Cas9, that allow to alter this code offer many opportunities ranging from functional genomics to gene therapy, crop improvement and pest management. The control of arthropod pests is crucial to protect crops and sustain animal and human health. CRISPR/Cas9 mediated gene editing can contribute to the understanding of important mechanisms such as pesticide resistance formation and help discover alternative safe products for the management of these detrimental pests. Moreover, CRISPR/Cas9 has the potential to be used as a pest control method itself by incorporating it in a gene drive system causing super-Mendelian inheritance of desired traits such as reduced fecundity and has been demonstrated successfully in the malaria vector *Anopheles gambiae* (Kyrou et al., 2018) and invasive fruit pest *Drosophila suzukii* (Yadav et al., 2023). Currently, the most applied delivery method of Cas9 RNP complexes is through injection in eggs or embryos of the target organism which is not always possible. In this study different formulations were tested to increase the low gene editing efficiency obtained in a pioneer CRISPR/Cas9 experiment with *T. urticae* where Cas9 components were injected in adult females (Dermauw et al., 2020).

Although both BAPC and saponins have the prospects to increase the delivery of CRISPR/Cas9 components in the egg cells when co-injected in female organisms, neither of them was able to improve gene editing frequencies when used individually. Only when both compounds were combined, an increase of transformation efficiency was observed. Why specifically only the combination of both compounds results in high transformation rates, remains enigmatic. One possible hypothesis might

Table 3
CRISPR/Cas9 KO efficiencies obtained in *F. occidentalis* targeting the *white* or *white-like* gene. R: replicate number, TPI: Time Post Injection. #mothers represents the number of injected females that were still alive at the end of the corresponding TPI interval. *White-like* chimeras could hardly be distinguished from the wild type phenotype and were therefore not scored.

Gene	R	TPI (h)	#Mothers	#Offspring	#Chimera	#Full mutants	%Chimeric mutant offspring	%Full mutant offspring	%Total mutant offspring
white	1	0–24	115	231	7	36	3.03	15.58	18.61
		24–48	106	322	0	0	0.00	0.00	0.00
	2	0–24	119	535	22	102	4.11	19.07	23.18
		24–48	119	382	0	0	0.00	0.00	0.00
	3	0–24	46	69	3	18	4.35	26.09	30.43
		24–48	37	80	0	0	0.00	0.00	0.00
white-like	1	0–24	67	78		25		32.05	32.05
		24–48	64	82		0		0.00	0.00

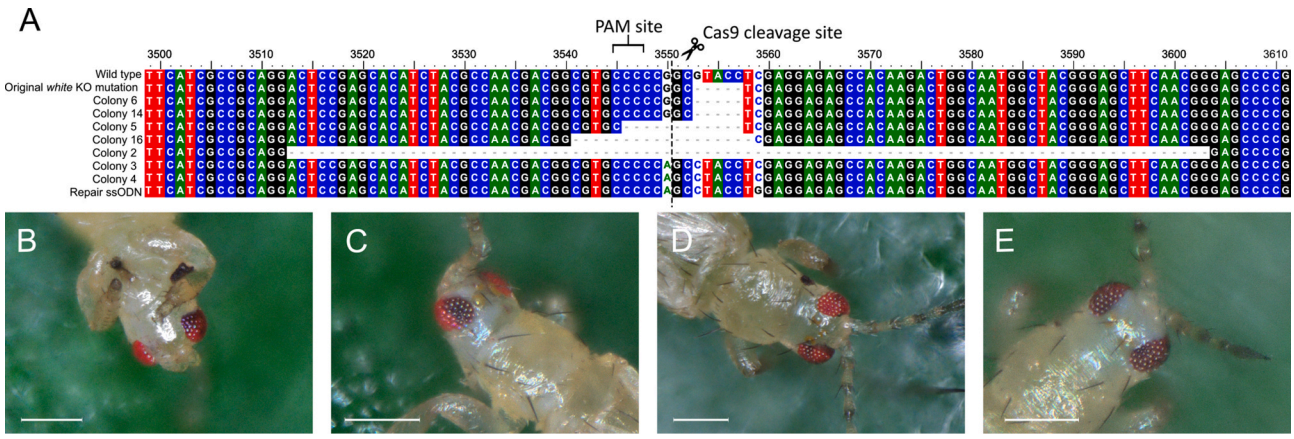

Fig. 5. Restoration of *white* KO mutants to wild type phenotype in *F. occidentalis*. A) Alignment of *white* sequences obtained from of a single individual with fully restored eyes (female 7, see Table S13). The sequences of colonies 2, 5 and 16 show a shift from the original out-of-frame deletion (5 nt deletion) to an in-frame deletion while the sequences of colonies 3 and 4 show the incorporation of the repair ssODN. Numbering above the alignment corresponds with the nucleotide position in the *white* gene. B-D) Individuals with partially restored (chimeric) eyes. E) Individual with fully restored eyes (female 7, see Table S13). Scalebar = 100 μM.

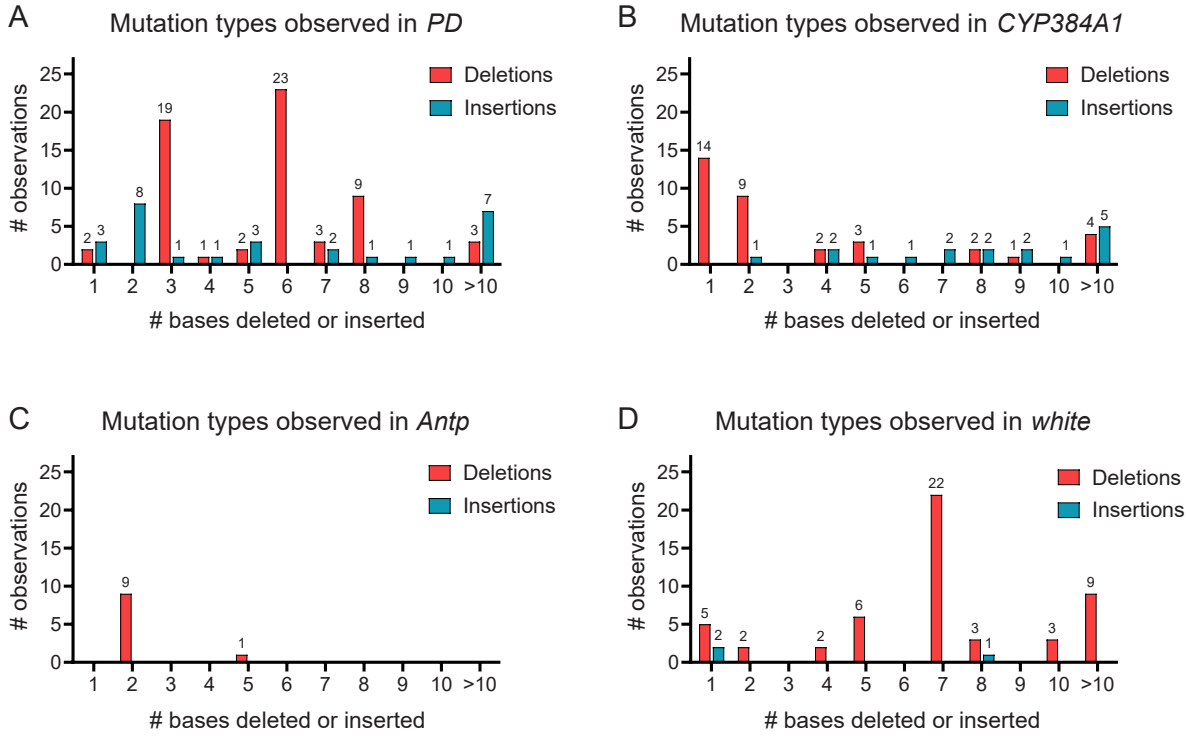

Fig. 6. Summary of the various mutations that were observed in the different genes that were targeted in this study. A-C) Target genes in *T. urticae*. For *PD*, mutation types are reported for sgPD2. D) Target gene in *F. occidentalis*.

be that the cationic BAPC increases cellular uptake via endocytosis while saponins provide subsequent endosomal escape. Should this be the case however, it remains peculiar as to why chloroquine, another commonly used endosomal escape reagent, did not exhibit any synergism with BAPC.

Using the SYNCAS formulation, KO efficiencies up to 24.4% were obtained in the offspring of *T. urticae* whereas previously a KO efficiency of less than 0.2% could be acquired (Dermauw et al., 2020). Injection of CRISPR/Cas9 components in female adult insects, mites and ticks, circumventing difficult embryo injections, has gained increasing interest in recent years and has led to high transformation efficiencies in some species. In the diploid species *B. germanica*, *T. castaneum* and *A. aegypti* for example, editing efficiencies reached up to 21.8%, 71.4% and 3.5% respectively (Shirai et al., 2022, 2023). In the species *A. aegypti, Culex pipiens* and *I. scapularis* editing efficiencies of 0.1, 0.4 and 4.2% were obtained respectively using ligand-fused Cas9 (Chaverra-Rodriguez et al., 2018; Li et al., 2021; Sharma et al., 2022). For *N. vitripennis* and *Bemisia tabaci*, which like *T. urticae* have a haplodiploid sex determination system, gene editing efficiencies up to 0.8% and 12.7% were obtained respectively (Chaverra-Rodriguez et al., 2020; Heu et al., 2020). However, comparing efficiencies obtained in these various publications is not straightforward due to differences in the type and concentrations of Cas9 protein and/or adjuvants that were used. Although in some of these examples, either saponins or BAPC were used as

adjuvants, none evaluated the combined effect of BAPC and saponins. To the best of our knowledge, this is the first report of the synergistic action between these two compounds in the delivery of small molecules such as CRISPR/Cas9 RNPs.

Further, it was demonstrated that *PD* can be used as a visual marker in a co-CRISPR strategy as in three out of six groups all albino males were also mutant in *Antp*. Such approach is particularly interesting when trying to obtain KO mutants in genes which do not result in clear phenotypic changes. For example, sgPD2 could be used in combination with another sgRNA targeting a gene of interest (GOI) and subsequently resulting albino offspring could be analyzed for mutations in the GOI. This co-CRISPR strategy has previously been demonstrated for the nematode, *C. elegans*, where *unc-22* was used as marker gene which results in a twitching phenotype upon disruption (Kim et al., 2014). Also in fruit flies and moths co-CRISPR has proven valuable in increasing gene editing efficiency (Han et al., 2023; Kane et al., 2016).

The observed phenotypes upon *Antp* (*tetur20g02430*) KO where L4 legs were severely deformed or absent were in line with the phenotype shown by Luo et al. (2023) upon RNAi silencing of this gene. This is in contrast to experiments with another member of the Arachnida, the spider *A. tepidariorum,* where RNAi silencing of the orthologues gene resulted in 10-legged spiders (Khadjeh et al., 2012). In both species *Antp* seems to be duplicated (Grbić et al., 2011; Schwager et al., 2017), but only *tetur20g02430* was targeted by CRISPR and RNAi in *T. urticae.* Further experiments, targeting the duplicated *Antp* gene (*tetur20g02440*), should be performed to provide more clarity regarding the role of *Antp* genes in leg development. In *T. kanzawai*, a minimal candidate region of 8.96 kb was linked to the lemon phenotype using bulked segregant analysis and fine-mapping (Wybouw et al., 2019b). This region harbored the *CYP384A1* gene and a 3′ end fragment of a neighboring gene (*tetur38g00660* in *T. urticae* annotation). A disruptive frameshift mutation was identified in exon 4 of *CYP384A1* and was designated as the most likely cause of the lemon phenotype. Here, additional evidence is provided for the involvement of *CYP384A1* in keto-carotenoid synthesis as a CRISPR/Cas9 KO of this gene in the closely related *T. urticae* resulted in the same phenotype as observed by Wybouw et al. (2019b).

Using the SYNCAS protocol to target *PD* in fertilized *T. urticae* females resulted in a lower fraction of daughters displaying albinism compared to the fraction of sons having this phenotype. This result was not unexpected as in diploids both copies of *PD* have to be knocked-out in order to obtain an albino phenotype (Fig. 1), as this trait inherits recessively (Bryon et al., 2017; Dermauw et al., 2020). Given that some of the obtained albino daughters gave birth to F2 albino sons, sequencing single males allowed characterization of CRISPR events in the original mother. Surprisingly, this revealed that up to three different mutations could be observed, implicating that Cas9 cleavage likely occurred after the first DNA replication during mitosis in the egg resulting in chimeric females. Since the first cell division only happens 1–2 h after egg laying (Dearden et al., 2002) and some of these chimeric daughters developed from eggs laid between 24 and 48 h post injection, the injected Cas9 RNPs likely remain stable in the mites for at least 24 h. Another biological parameter that was investigated was the age of the injected mothers, but this parameter did not seem to influence gene editing efficiency in *T. urticae*. This is in contrast to *B. germanica* and *T. castaneum* where age of injected females did influence transformation frequencies with the highest efficiencies being observed when injecting females 4–5 days after adult emergence (Shirai et al., 2022). This difference could be due to the periodic and synchronized development of eggs where CRISPR/Cas9 delivery relies on females having eggs undergoing vitellogenesis as demonstrated for *B. germanica*. In *T. urticae*, eggs are produced continuously once adulthood is reached, hence vitellogenesis is also expected to occur perpetually (Kawakami et al., 2009; Wrensch and Young, 1975).

The I1017F mutation is one of the target-site resistance mutations with the highest phenotypic strength, being able to increase etoxazole resistance levels to more than 50,000-fold (De Rouck et al., 2023; Riga et al., 2017). Hence, this mutation was selected to be introduced in an etoxazole susceptible strain as screening for successful KI could be easily performed by spraying offspring with etoxazole at a dose lethal for WT individuals. Although a few successful KI events were detected among the offspring of females injected without HDR stimulating agent, more etoxazole resistant mites were found in the offspring of females treated with enhancer V2. According to the producer, NHEJ pathway, hence stimulating HDR and has been used *in vivo* in zebrafish and mice where KI efficiency increased over two- and three-fold respectively (Sakai et al., 2023; Zhang et al., 2023).

Last but not least, the CRISPR/Cas9 formulation was also successfully applied in *F. occidentalis*, an insect species belonging to the Thysanoptera, for which gene editing through classical embryo injection or alternative approaches has not yet been reported. Embryo injections in Thysanoptera are not straightforward due to their behavior of depositing eggs inside plant tissue, although artificial oviposition setups have been developed (Jangra et al., 2020). In this study we used CRISPR/Cas9 to introduce deletions in *white* and *white-like*, the paralogous genes of *D. melanogaster white*. Disruption of either *white* or *white-like* did not result in white colored ommatidia as observed in *D. melanogaster* (Mackenzie et al., 1999; Morgan, 1910). In contrast, the ommatidia of KO *white* and KO *white-like* were bright red and solid black respectively, while the KO of both genes resulted in transparent ommatidia and ocelli. This suggests that *F. occidentalis* white, contrary to *D. melanogaster* white, does no longer have a dual function in eye pigment transport, i.e. interacting with scarlet to transport dark (tryptophane derived) pigments and interacting with brown to transport red (guanine derived) pigments (Mackenzie et al., 1999). Instead, *F. occidentalis* probably has evolved a *white-like* gene of which the encoding protein only interacts with brown, and has a white protein that only interacts with scarlet.

Upon establishing an efficient KO protocol for *F. occidentalis*, attempts were made to create KI mutations by restoring a disrupted *white* gene. Within the offspring of injected *white* KO females, individuals with partially (chimeric) and fully restored eyes were identified, but the majority did not incorporate the used repair template. Instead, new deletions occurred shifting the out-of-frame deletion to an in-frame deletion indicating the NHEJ pathway was still dominant despite the use of enhancer V2. Even though the obtained efficiencies were not sufficient to enable routine KI experiments in this species, it provides a basis for future optimizations.

Various types of mutations were recorded over the different KO experiments. For each target gene, specific types of mutation events seemed to occur noticeably more than others. These results seem to be in line with *in vitro* cell assays where the type of mutation introduced upon DNA cleavage depends on the flanking DNA sequence at the target site (Allen et al., 2018). In addition, as mutations from microhomology-mediated end joining (MMEJ) are more consistent (Ata et al., 2018), it might suggest that MMEJ is the dominant repair pathway in *T. urticae*. However, it is important to note that mutations which did not result in a different phenotype were missed during the screening and are hence not represented in the sequencing results. This is especially the case for the *white* gene of *F. occidentalis* where in-frame deletions or insertions were not detected since these probably do not result in a loss-of-function as demonstrated in the KI experiment where some individuals were restored to the WT phenotype by shifting from out-of-frame to in-frame mutations.

In conclusion, the newly developed CRISPR/Cas9 protocol allows efficient gene editing of *T. urticae*, *F. occidentalis* and potentially many other arthropods for which genetic engineering tools are currently lacking. As such, it might provide a boost for genetic research in previously hard to transform arthropods.

Declaration of interest

The authors declare that a priority claim for the above-described

method has been submitted to the European Patent Office.

CRediT authorship contribution statement

Sander De Rouck: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Antonio Mocchetti:** Visualization, Methodology, Investigation, Formal analysis. **Wannes Dermauw:** Writing – review & editing, Supervision, Investigation, Conceptualization, Funding acquisition. **Thomas Van Leeuwen:** Writing – review & editing, Supervision, Resources, Investigation, Funding acquisition, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors did not use generative AI or AI-assisted technologies in the writing process.

Data availability

File S1 (Original data) (Figshare),
File S2 (Original data) (Figshare) and
File S3 (Original data) (Figshare).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2023.104068.

References

Adams, S., Pathak, P., Shao, H., Lok, J.B., Pires-daSilva, A., 2019. Liposome-based transfection enhances RNAi and CRISPR-mediated mutagenesis in non-model nematode systems. Sci. Rep. 9, 483. https://doi.org/10.1038/s41598-018-37036-1.

Ahmad, S., Tang, L., Shahzad, R., Mawia, A.M., Rao, G.S., Jamil, S., Wei, C., Sheng, Z., Shao, G., Wei, X., Hu, P., Mahfouz, M.M., Hu, S., Tang, S., 2021. CRISPR-based crop improvements: a way forward to achieve zero hunger. J. Agric. Food Chem. 69, 8307–8323. https://doi.org/10.1021/acs.jafc.1c02653.

Allen, F., Crepaldi, L., Alsinet, C., Strong, A.J., Kleshchevnikov, V., De Angeli, P., Palenikova, P., Khodak, A., Kiselev, V., Kosicki, M., Bassett, A.R., Harding, H., Galanty, Y., Muñoz-Martínez, F., Metzakopian, E., Jackson, S.P., Parts, L., 2018. Predicting the mutations generated by repair of Cas9-induced double-strand breaks. Nat. Biotechnol. https://doi.org/10.1038/nbt.4317, 10.1038/nbt.4317.

Ata, H., Ekstrom, T.L., Martínez-Gálvez, G., Mann, C.M., Dvornikov, A.V., Schaefbauer, K.J., Ma, A.C., Dobbs, D., Clark, K.J., Ekker, S.C., 2018. Robust activation of microhomology-mediated end joining for precision gene editing applications. PLoS Genet. 14, e1007652 https://doi.org/10.1371/journal.pgen.1007652.

Avila, L.A., Aps, L.R.M.M., Ploscariu, N., Sukthankar, P., Guo, R., Wilkinson, K.E., Games, P., Szoszkiewicz, R., Alves, R.P.S., Diniz, M.O., Fang, Y., Ferreira, L.C.S., Tomich, J.M., 2016. Gene delivery and immunomodulatory effects of plasmid DNA associated with Branched Amphiphilic Peptide Capsules. J. Contr. Release 241, 15–24. https://doi.org/10.1016/j.jconrel.2016.08.042.

Avila, L.A., Chandrasekar, R., Wilkinson, K.E., Balthazor, J., Heerman, M., Bechard, J., Brown, S., Park, Y., Dhar, S., Reeck, G.R., Tomich, J.M., 2018. Delivery of lethal dsRNAs in insect diets by branched amphiphilic peptide capsules. J. Contr. Release 273, 139–146. https://doi.org/10.1016/j.jconrel.2018.01.010.

Biotech, Phoreus, 2023. BAPC®- (branched amphipathic peptide capsules) drug delivery [WWW Document]. Phoreus Biotech. URL. https://phoreusbiotech.com/technology/bapc/, 6.14.23.

Bryon, A., Kurlovs, A.H., Dermauw, W., Greenhalgh, R., Riga, M., Grbić, M., Tirry, L., Osakabe, M., Vontas, J., Clark, R.M., Van Leeuwen, T., 2017. Disruption of a horizontally transferred phytoene desaturase abolishes carotenoid accumulation and diapause in Tetranychus urticae. Proc. Natl. Acad. Sci. U.S.A. 114, E5871–E5880. https://doi.org/10.1073/pnas.1706865114.

Cao, X.-W., Wang, F.-J., Liew, O.-W., Lu, Y.-Z., Zhao, J., 2020. Analysis of triterpenoid saponins reveals insights into structural features associated with potent protein drug enhancement effects. Mol. Pharm. 17, 683–694. https://doi.org/10.1021/acs.molpharmaceut.9b01158.

Chang, N., Sun, C., Gao, L., Zhu, D., Xu, X., Zhu, X., Xiong, J.-W., Xi, J.J., 2013. Genome editing with RNA-guided Cas9 nuclease in Zebrafish embryos. Cell Res. 23, 465–472. https://doi.org/10.1038/cr.2013.45.

Chaverra-Rodriguez, D., Macias, V.M., Hughes, G.L., Pujhari, S., Suzuki, Y., Peterson, D. R., Kim, D., McKeand, S., Rasgon, J.L., 2018. Targeted delivery of CRISPR-Cas9 ribonucleoprotein into arthropod ovaries for heritable germline gene editing. Nat. Commun. 9, 3008. https://doi.org/10.1038/s41467-018-05425-9.

Chaverra-Rodriguez, D., Dalla Benetta, E., Heu, C.C., Rasgon, J.L., Ferree, P.M., Akbari, O.S., 2020. Germline mutagenesis of Nasonia vitripennis through ovarian delivery of CRISPR-Cas9 ribonucleoprotein. Insect Mol. Biol. 29, 569–577. https://doi.org/10.1111/imb.12663.

Concordet, J.-P., Haeussler, M., 2018. CRISPOR: intuitive guide selection for CRISPR/Cas9 genome editing experiments and screens. Nucleic Acids Res. 46, W242–W245. https://doi.org/10.1093/nar/gky354.

De Beer, B., Villacis-Perez, E., Khalighi, M., Saalwaechter, C., Vandenhole, M., Jonckheere, W., Ismaeil, I., Geibel, S., Van Leeuwen, T., Dermauw, W., 2022. QTL mapping suggests that both cytochrome P450-mediated detoxification and target-site resistance are involved in fenbutatin oxide resistance in Tetranychus urticae. Insect Biochem. Mol. Biol. 145 https://doi.org/10.1016/J.IBMB.2022.103757, 103757–103757.

De Rouck, S., İnak, E., Dermauw, W., Van Leeuwen, T., 2023. A review of the molecular mechanisms of acaricide resistance in mites and ticks. Insect Biochem. Mol. Biol. 159, 103981 https://doi.org/10.1016/j.ibmb.2023.103981.

Dearden, P.K., Donly, C., Grbić, M., 2002. Expression of pair-rule gene homologues in a chelicerate: early patterning of the two-spotted spider mite Tetranychus urticae. Development 129, 5461–5472. https://doi.org/10.1242/dev.00099.

Dermauw, W., Jonckheere, W., Riga, M., Livadaras, I., Vontas, J., Van Leeuwen, T., 2020. Targeted mutagenesis using CRISPR-Cas9 in the chelicerate herbivore Tetranychus urticae. Insect Biochem. Mol. Biol. 120 https://doi.org/10.1016/j.ibmb.2020.103347, 103347–103347.

Desmedt, W., Mangelinckx, S., Kyndt, T., Vanholme, B., 2020. A phytochemical perspective on plant defense against nematodes. Front. Plant Sci. 11.

Fotoukkiaii, S.M., Wybouw, N., Kurlovs, A.H., Tsakireli, D., Pergantis, S.A., Clark, R.M., Vontas, J., Van Leeuwen, T., 2021. High-resolution genetic mapping reveals cis-regulatory and copy number variation in loci associated with cytochrome P450-mediated detoxification in a generalist arthropod pest. PLoS Genet. 17, e1009422.

Gantz, V.M., Jasinskiene, N., Tatarenkova, O., Fazekas, A., Macias, V.M., Bier, E., James, A.A., 2015. Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito Anopheles stephensi. Proc. Natl. Acad. Sci. USA 112, E6736–E6743. https://doi.org/10.1073/pnas.1521077112.

Gratz, S.J., Cummings, A.M., Nguyen, J.N., Hamm, D.C., Donohue, L.K., Harrison, M.M., Wildonger, J., O'Connor-Giles, K.M., 2013. Genome engineering of Drosophila with the CRISPR RNA-guided Cas9 nuclease. Genetics 194, 1029–1035. https://doi.org/10.1534/genetics.113.152710.

Grbic, M., Khila, A., Lee, K.-Z., Bjelica, A., Grbic, V., Whistlecraft, J., Verdon, L., Navajas, M., Nagy, L., 2007. Mity model: Tetranychus urticae, a candidate for chelicerate model organism. Bioessays 29, 489–496. https://doi.org/10.1002/bies.20564.

Grbić, M., Van Leeuwen, T., Clark, R.M., Rombauts, S., Rouzé, P., Grbić, V., Osborne, E. J., Dermauw, W., Thi Ngoc, P.C., Ortego, F., Hernández-Crespo, P., Diaz, I., Martinez, M., Navajas, M., Sucena, É., Magalhães, S., Nagy, L., Pace, R.M., Djuranović, S., Smagghe, G., Iga, M., Christiaens, O., Veenstra, J.A., Ewer, J., Villalobos, R.M., Hutter, J.L., Hudson, S.D., Velez, M., Yi, S.V., Zeng, J., Pires-daSilva, A., Roch, F., Cazaux, M., Navarro, M., Zhurov, V., Acevedo, G., Bjelica, A., Fawcett, J.A., Bonnet, E., Martens, C., Baele, G., Wissler, L., Sanchez-Rodriguez, A., Tirry, L., Blais, C., Demeestere, K., Henz, S.R., Gregory, T.R., Mathieu, J., Verdon, L., Farinelli, L., Schmutz, J., Lindquist, E., Feyereisen, R., Van de Peer, Y., 2011. The genome of Tetranychus urticae reveals herbivorous pest adaptations. Nature 479, 487–492. https://doi.org/10.1038/nature10640.

Gudmunds, E., Wheat, C.W., Khila, A., Husby, A., 2022. Functional genomic tools for emerging model species. Trends Ecol. Evol. 37, 1104–1115. https://doi.org/10.1016/j.tree.2022.07.004.

Gui, T., Zhang, J., Song, F., Sun, Y., Xie, S., Yu, K., Xiang, J., 2016. CRISPR/Cas9-Mediated genome editing and mutagenesis of EcChi4 in exopalaemon carinicauda. G3 GenesGenomesGenetics 6, 3757–3764. https://doi.org/10.1534/g3.116.034082.

Hajimolaali, M., Mohammadian, H., Torabi, A., Shirini, A., Khalife Shal, M., Barazandeh Nezhad, H., Iranpour, S., Baradaran Eftekhari, R., Dorkoosh, F., 2021. Application of chloroquine as an endosomal escape enhancing agent: new frontiers for an old drug. Expet Opin. Drug Deliv. 18, 877–889. https://doi.org/10.1080/17425247.2021.1873272.

Hammond, A., Galizi, R., Kyrou, K., Simoni, A., Siniscalchi, C., Katsanos, D., Gribble, M., Baker, D., Marois, E., Russell, S., Burt, A., Windbichler, N., Crisanti, A., Nolan, T., 2016. A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector Anopheles gambiae. Nat. Biotechnol. 34, 78–83. https://doi.org/10.1038/nbt.3439.

Han, W.-K., Tang, F.-X., Gao, H.-L., Wang, Y., Yu, N., Jiang, J.-J., Liu, Z.-W., 2023. Co-CRISPR: a valuable toolkit for mutation enrichment in the gene editing of Spodoptera frugiperda. Insect Sci. 30, 625–636. https://doi.org/10.1111/1744-7917.13122.

Heu, C.C., McCullough, F.M., Luan, J., Rasgon, J.L., 2020. CRISPR-Cas9-Based genome editing in the silverleaf whitefly (Bemisia tabaci). CRISPR J. 3, 89–96. https://doi.org/10.1089/crispr.2019.0067.
Hoang, D.T., Chernomor, O., von Haeseler, A., Minh, B.Q., Vinh, L.S., 2018. UFBoot2: improving the ultrafast bootstrap approximation. Mol. Biol. Evol. 35, 518–522. https://doi.org/10.1093/molbev/msx281.
Hu, X.F., Zhang, B., Liao, C.H., Zeng, Z.J., 2019. High-efficiency CRISPR/Cas9-Mediated gene editing in honeybee (Apis mellifera) embryos. G3 GenesGenomesGenetics 9, 1759–1766. https://doi.org/10.1534/g3.119.400130.
Hwang, W.Y., Fu, Y., Reyon, D., Maeder, M.L., Tsai, S.Q., Sander, J.D., Peterson, R.T., Yeh, J.-R.J., Joung, J.K., 2013. Efficient genome editing in zebrafish using a CRISPR-Cas system. Nat. Biotechnol. 31, 227–229. https://doi.org/10.1038/nbt.2501.
Jangra, S., Dhall, H., Aggarwal, S., Mandal, B., Jain, R.K., Ghosh, A., 2020. An observation on the embryonic development in Thrips palmi (Thysanoptera: thripidae) eggs obtained by an artificial oviposition setup. J. Asia Pac. Entomol. 23, 492–497. https://doi.org/10.1016/j.aspen.2020.03.012.
Kalyaanamoorthy, S., Minh, B.Q., Wong, T.K.F., von Haeseler, A., Jermiin, L.S., 2017. ModelFinder: fast model selection for accurate phylogenetic estimates. Nat. Methods 14, 587–589. https://doi.org/10.1038/nmeth.4285.
Kane, N.S., Vora, M., Varre, K.J., Padgett, R.W., 2016. Efficient screening of CRISPR/Cas9-Induced events in Drosophila using a Co-CRISPR strategy. G3 GenesGenomesGenetics 7, 87–93. https://doi.org/10.1534/g3.116.036723.
Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30, 772–780. https://doi.org/10.1093/molbev/mst010.
Kawakami, Y., Goto, S.G., Ito, K., Numata, H., 2009. Suppression of ovarian development and vitellogenin gene expression in the adult diapause of the two-spotted spider mite Tetranychus urticae. J. Insect Physiol. 55, 70–77. https://doi.org/10.1016/j.jinsphys.2008.10.007.
Khadjeh, S., Turetzek, N., Pechmann, M., Schwager, E.E., Wimmer, E.A., Damen, W.G. M., Prpic, N.-M., 2012. Divergent role of the Hox gene Antennapedia in spiders is responsible for the convergent evolution of abdominal limb repression. Proc. Natl. Acad. Sci. USA 109, 4921–4926. https://doi.org/10.1073/pnas.1116421109.
Kim, H., Ishidate, T., Ghanta, K.S., Seth, M., Conte Jr., D., Shirayama, M., Mello, C.C., 2014. A Co-CRISPR strategy for efficient genome editing in Caenorhabditis elegans. Genetics 197, 1069–1080. https://doi.org/10.1534/genetics.114.166389.
Kotwica-Rolinska, J., Chodakova, L., Chvalova, D., Kristofova, L., Fenclova, I., Provaznik, J., Bertolutti, M., Wu, B.C.-H., Dolezel, D., 2019. CRISPR/Cas9 genome editing introduction and optimization in the non-model insect Pyrrhocoris apterus. Front. Physiol. 10.
Kunte, N., Westerfield, M., McGraw, E., Choi, J., Akinsipe, T., Whitaker, S.K., Brannen, A., Panizzi, P., Tomich, J.M., Avila, L.A., 2022. Evaluation of transfection efficacy, biodistribution, and toxicity of branched amphiphilic peptide capsules (BAPCs) associated with mRNA. Biomater. Sci. 10, 6980–6991. https://doi.org/10.1039/D2BM01314B.
Kyrou, K., Hammond, A.M., Galizi, R., Kranjc, N., Burt, A., Beaghton, A.K., Nolan, T., Crisanti, A., 2018. A CRISPR–Cas9 gene drive targeting doublesex causes complete population suppression in caged Anopheles gambiae mosquitoes. Nat. Biotechnol. 36, 1062–1066. https://doi.org/10.1038/nbt.4245.
Li, X., Xu, Y., Zhang, H., Yin, H., Zhou, D., Sun, Y., Ma, L., Shen, B., Zhu, C., 2021. ReMOT control delivery of CRISPR-cas9 ribonucleoprotein complex to induce germline mutagenesis in the disease vector mosquitoes Culex pipiens pallens (Diptera: Culicidae). J. Med. Entomol. 58, 1202–1209. https://doi.org/10.1093/jme/tjab016.
Li, R., Meng, Q., Qi, J., Hu, L., Huang, J., Zhang, Y., Yang, J., Sun, J., 2022. Microinjection-based CRISPR/Cas9 mutagenesis in the decapoda crustaceans Neocaridina heteropoda and Eriocheir sinensis. J. Exp. Biol. 225, jeb243702 https://doi.org/10.1242/jeb.243702.
Luo, X., Xu, Y.-Q., Jin, D.-C., Guo, J.-J., Yi, T.-C., 2023. Role of the Hox genes, sex combs reduced, fushi tarazu and Antennapedia, in leg development of the spider mite Tetranychus urticae. Int. J. Mol. Sci. 24, 10391 https://doi.org/10.3390/ijms241210391.
Mackenzie, S.M., Brooker, M.R., Gill, T.R., Cox, G.B., Howells, A.J., Ewart, G.D., 1999. Mutations in the white gene of Drosophila melanogaster affecting ABC transporters that determine eye colouration. Biochim. Biophys. Acta BBA - Biomembr. 1419, 173–185. https://doi.org/10.1016/S0005-2736(99)00064-4.
Maxfield, F.R., 1982. Weak bases and ionophores rapidly and reversibly raise the pH of endocytic vesicles in cultured mouse fibroblasts. J. Cell Biol. 95, 676–681. https://doi.org/10.1083/jcb.95.2.676.
Migeon, A., Dorkeld, F., 2022. Spider Mites Web: a Comprehensive Database for the Tetranychidae.
Morgan, T.H., 1910. Sex limited inheritance in Drosophila. Science 32, 120–122. https://doi.org/10.1126/science.32.812.120.
Moses, T., Papadopoulou, K.K., Osbourn, A., 2014. Metabolic and functional diversity of saponins, biosynthetic intermediates and semi-synthetic derivatives. Crit. Rev. Biochem. Mol. Biol. 49, 439–462. https://doi.org/10.3109/10409238.2014.953628.
Mota-Sanchez, D., Wise, J.C., 2022. Arthropod Pesticide Resistance Database (APRD).
Nguyen, L.-T., Schmidt, H.A., von Haeseler, A., Minh, B.Q., 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol. Biol. Evol. 32, 268–274. https://doi.org/10.1093/molbev/msu300.
Pandey, E., Harris, E.N., 2023. Chloroquine and cytosolic galectins affect endosomal escape of antisense oligonucleotides after Stabilin-mediated endocytosis. Mol. Ther. Nucleic Acids 33, 430–443. https://doi.org/10.1016/j.omtn.2023.07.019.
Passos, F.R.S., Araújo-Filho, H.G., Monteiro, B.S., Shanmugam, S., Araújo, A.A. de S., Almeida, J.R.G. da S., Thangaraj, P., Júnior, L.J.Q., Quintans, J. de S.S., 2022. Anti-inflammatory and modulatory effects of steroidal saponins and sapogenins on cytokines: a review of pre-clinical research. Phytomedicine 96, 153842. https://doi.org/10.1016/j.phymed.2021.153842.
Riga, M., Bajda, S., Themistokleous, C., Papadaki, S., Palzewicz, M., Dermauw, W., Vontas, J., Leeuwen, T.V., 2017. The relative contribution of target-site mutations in complex acaricide resistant phenotypes as assessed by marker assisted backcrossing in Tetranychus urticae. Sci. Rep. 7 https://doi.org/10.1038/s41598-017-09054-y, 9202–9202.
Rotenberg, D., Baumann, A.A., Ben-Mahmoud, S., Christiaens, O., Dermauw, W., Ioannidis, P., Jacobs, C.G.C., Vargas Jentzsch, I.M., Oliver, J.E., Poelchau, M.F., Rajarapu, S.P., Schneweis, D.J., Snoeck, S., Taning, C.N.T., Wei, D., Widana Gamage, S.M.K., Hughes, D.S.T., Murali, S.C., Bailey, S.T., Bejerman, N.E., Holmes, C.J., Jennings, E.C., Rosendale, A.J., Rosselot, A., Hervey, K., Schneweis, B. A., Cheng, S., Childers, C., Simão, F.A., Dietzgen, R.G., Chao, H., Dinh, H., Doddapaneni, H.V., Dugan, S., Han, Y., Lee, S.L., Muzny, D.M., Qu, J., Worley, K.C., Benoit, J.B., Friedrich, M., Jones, J.W., Panfilio, K.A., Park, Y., Robertson, H.M., Smagghe, G., Ullman, D.E., van der Zee, M., Van Leeuwen, T., Veenstra, J.A., Waterhouse, R.M., Weirauch, M.T., Werren, J.H., Whitfield, A.E., Zdobnov, E.M., Gibbs, R.A., Richards, S., 2020. Genome-enabled insights into the biology of thrips as crop pests. BMC Biol. 18, 142. https://doi.org/10.1186/s12915-020-00862-9.
Sakai, Y., Okabe, Y., Itai, G., Shiozawa, S., 2023. An efficient evaluation system for factors affecting the genome editing efficiency in mouse. Exp. Anim. advpub 23. https://doi.org/10.1538/expanim.23-0045. –0045.
Sama, S., Jerz, G., Thakur, M., Melzig, M.F., Weng, A., 2019. Structure-activity relationship of transfection-modulating saponins – a pursuit for the optimal gene trafficker. Planta Med. 85, 513–518. https://doi.org/10.1055/a-0863-4795.
Schwager, E.E., Sharma, P.P., Clarke, T., Leite, D.J., Wierschin, T., Pechmann, M., Akiyama-Oda, Y., Esposito, L., Bechsgaard, J., Bilde, T., Buffry, A.D., Chao, H., Dinh, H., Doddapaneni, H., Dugan, S., Eibner, C., Extavour, C.G., Funch, P., Garb, J., Gonzalez, L.B., Gonzalez, V.L., Griffiths-Jones, S., Han, Y., Hayashi, C., Hilbrant, M., Hughes, D.S.T., Janssen, R., Lee, S.L., Maeso, I., Murali, S.C., Muzny, D.M., Nunes da Fonseca, R., Paese, C.L.B., Qu, J., Ronshaugen, M., Schomburg, C., Schönauer, A., Stollewerk, A., Torres-Oliva, M., Turetzek, N., Vanthournout, B., Werren, J.H., Wolff, C., Worley, K.C., Bucher, G., Gibbs, R.A., Coddington, J., Oda, H., Stanke, M., Ayoub, N.A., Prpic, N.-M., Flot, J.-F., Posnien, N., Richards, S., McGregor, A.P., 2017. The house spider genome reveals an ancient whole-genome duplication during arachnid evolution. BMC Biol. 15, 62. https://doi.org/10.1186/s12915-017-0399-x.
Seki, A., Rutz, S., 2018. Optimized RNP transfection for highly efficient CRISPR/Cas9-mediated gene knockout in primary T cells. J. Exp. Med. 215, 985–997. https://doi.org/10.1084/jem.20171626.
Sharma, A., Pham, M.N., Reyes, J.B., Chana, R., Yim, W.C., Heu, C.C., Kim, D., Chaverra-Rodriguez, D., Rasgon, J.L., Harrell, R.A., Nuss, A.B., Gulia-Nuss, M., 2022. Cas9-mediated gene editing in the black-legged tick, Ixodes scapularis, by embryo injection and ReMOT Control. iScience 25. https://doi.org/10.1016/J.ISCI.2022.103781, 103781–103781.
Shirai, Y., Piulachs, M.-D., Belles, X., Daimon, T., 2022. DIPA-CRISPR is a simple and accessible method for insect gene editing. Cell Rep. Methods 2, 100215. https://doi.org/10.1016/j.crmeth.2022.100215.
Shirai, Y., Takahashi, M., Ote, M., Kanuka, H., Daimon, T., 2023. DIPA-CRISPR gene editing in the yellow fever mosquito Aedes aegypti (Diptera: Culicidae). Appl. Entomol. Zool. 58, 273–278. https://doi.org/10.1007/s13355-023-00831-y.
Singh, B., Kaur, A., 2018. Control of insect pests in crop plants and stored food grains using plant saponins: a review. LWT 87, 93–101. https://doi.org/10.1016/j.lwt.2017.08.077.
Snoeck, S., Kurlovs, A.H., Bajda, S., Feyereisen, R., Greenhalgh, R., Villacis-Perez, E., Kosterlitz, O., Dermauw, W., Clark, R.M., Van Leeuwen, T., 2019. High-resolution QTL mapping in Tetranychus urticae reveals acaricide-specific responses and common target-site resistance after selection by different METI-I acaricides. Insect Biochem. Mol. Biol. 110, 19–33. https://doi.org/10.1016/J.IBMB.2019.04.011.
Stumpf, N., Zebitz, C.P.W., Kraus, W., Moores, G.D., Nauen, R., 2001. Resistance to organophosphates and biochemical genotyping of acetylcholinesterases in Tetranychus urticae (Acari: tetranychidae). Pestic. Biochem. Physiol. 69, 131–142. https://doi.org/10.1006/pest.2000.2516.
Trdá, L., Janda, M., Macková, D., Pospíchalová, R., Dobrev, P.I., Burketová, L., Matušinsky, P., 2019. Dual mode of the saponin aescin in plant protection: antifungal agent and plant defense elicitor. Front. Plant Sci. 10.
Vakulskas, C.A., 2022. CAS9 Mutant Genes and Polypeptides Encoded by Same, 11427818.
Van Leeuwen, T., Demaeght, P., Osborne, E.J., Dermauw, W., Gohlke, S., Nauen, R., Grbić, M., Tirry, L., Merzendorfer, H., Clark, R.M., 2012. Population bulk segregant mapping uncovers resistance mutations and the mode of action of a chitin synthesis inhibitor in arthropods. Proc. Natl. Acad. Sci. USA 109, 4407–4412. https://doi.org/10.1073/pnas.1200068109.
Villacis-Perez, E., Xue, W., Vandenhole, M., De Beer, B., Dermauw, W., Van Leeuwen, T., 2022. Intraspecific diversity in the mechanisms underlying abamectin resistance in a cosmopolitan pest. bioRxiv 2022. https://doi.org/10.1101/2022.11.25.517948, 11.25.517948-2022.11.25.517948.
Vincken, J.-P., Heng, L., de Groot, A., Gruppen, H., 2007. Saponins, classification and occurrence in the plant kingdom. Phytochemistry 68, 275–297. https://doi.org/10.1016/j.phytochem.2006.10.008.
Wrensch, D.L., Young, S.S.Y., 1975. Effects of quality of resource and fertilization status on some fitness traits in the two-spotted spider mite, Tetranychus urticae koch. Oecologia 18, 259–267.
Wybouw, N., Kosterlitz, O., Kurlovs, A.H., Bajda, S., Greenhalgh, R., Snoeck, S., Bui, H., Bryon, A., Dermauw, W., Van Leeuwen, T., Clark, R.M., 2019a. Long-term population studies uncover the genome structure and genetic basis of xenobiotic and

host plant adaptation in the herbivore Tetranychus urticae. Genetics 211, 1409–1427. https://doi.org/10.1534/genetics.118.301803.
Wybouw, N., Kurlovs, A.H., Greenhalgh, R., Bryon, A., Kosterlitz, O., Manabe, Y., Osakabe, M., Vontas, J., Clark, R.M., Van Leeuwen, T., 2019b. Convergent evolution of cytochrome P450s underlies independent origins of keto-carotenoid pigmentation in animals. Proc. R. Soc. B Biol. Sci. 286, 20191039–20191039 https://doi.org/10.1098/rspb.2019.1039.
Xu, J., Xu, X., Zhan, S., Huang, Y., 2019. Genome editing in insects: current status and challenges. Natl. Sci. Rev. 6, 399–401. https://doi.org/10.1093/nsr/nwz008.
Yadav, A.K., Butler, C., Yamamoto, A., Patil, A.A., Lloyd, A.L., Scott, M.J., 2023. CRISPR/Cas9-based split homing gene drive targeting doublesex for population suppression of the global fruit pest Drosophila suzukii. Proc. Natl. Acad. Sci. USA 120, e2301525120. https://doi.org/10.1073/pnas.2301525120.
Zaidi, S.S.-A., Mahas, A., Vanderschuren, H., Mahfouz, M.M., 2020. Engineering crops of the future: CRISPR approaches to develop climate-resilient and disease-resistant plants. Genome Biol. 21, 289. https://doi.org/10.1186/s13059-020-02204-y.
Zhang, Y., Marshall-Phelps, K., de Almeida, R.G., 2023. Fast, precise and cloning-free knock-in of reporter sequences in vivo with high efficiency. Development 150, dev201323. https://doi.org/10.1242/dev.201323.