CriMCE: A method to introduce and isolate precise marker-less edits via CRISPR-mediated cassette exchange

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Running head:

CRISPR-based method for marker-less editing

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Abstract

The introduction of small, unmarked edits to the genome of insects is essential to study the molecular underpinnings of important biological traits, such as resistance to insecticides and genetic control strategies. Advances in CRISPR genome engineering have made this possible, but prohibitively laborious for most laboratories due to low rates of editing and the lack of a selectable marker. To facilitate the generation and isolation of precise marker-less edits we have developed a two-step method based upon CRISPR-mediated cassette exchange (CriMCE) of a marked placeholder for a variant of interest. This strategy can be used to introduce a wider range of potential edits compared to previous approaches whilst consolidating the workflow. We present proof-of-principle that CriMCE is a powerful tool by engineering three SNP variants into the genome of *Anopheles gambiae*, with 5-41x higher rates of editing than homology-directed repair or prime editing.

Introduction

Small genetic changes, such as single nucleotide polymorphisms (SNPs), can give rise to prominent phenotypes. For example, they are responsible for most genetic diseases in humans,[1] important agronomic traits in plants,[2] and insecticide resistance in insect vectors of disease.[3]

To study their molecular underpinning, it is essential to engineer small precise edits like these in the laboratory,[4] whilst excluding any transformation markers or gene editing debris that could interfere with the observed phenotype. The introduction of such marker-less edits has been facilitated by the discovery and expansion of CRISPR (clustered regularly interspersed short palindromic repeats) technologies.

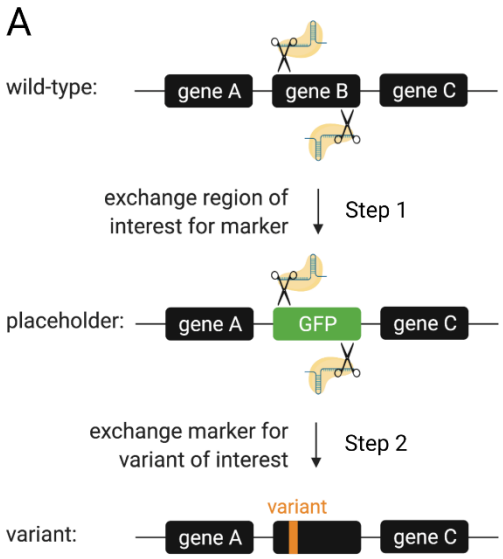
In its most common form, CRISPR genome editing comprises a Cas endonuclease, able to catalyse a DNA double-stranded break (DSB); and a guide RNA (gRNA) that directs the Cas protein to its target sequence.[5] Simple and complex edits can be introduced with precision at a CRISPR-induced break by presenting a modified DNA template for homology directed repair (HDR). Recently developed base editing and prime editing methods are less versatile but work independently of the HDR pathway and can raise the efficiency of editing in species where HDR is naturally low.[6–10] Base editing can induce transition point mutations through a Cas-deaminase fusion, whilst prime editing can introduce any point mutation or small indel by employing a Cas-reverse transcriptase fusion and a prime editing gRNA (pegRNA) that functions as a template for repair.[11] Neither have been widely tested in insects, however initial trials in *Drosophila* suggest that prime editing is no more efficient than HDR,[12] whilst base editing is effective but inherently imprecise.[13]

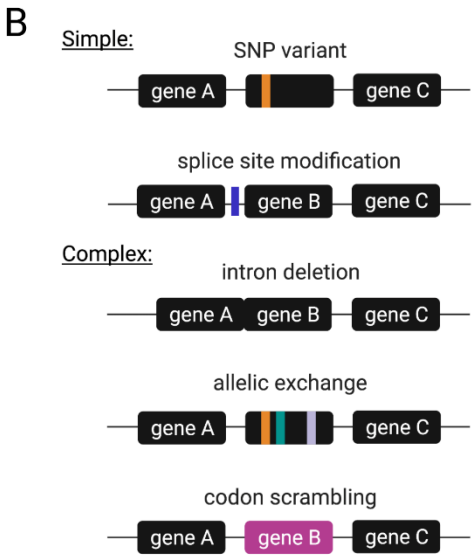
In insects, independent of the chosen technology, engineering small marker-less edits remains inefficient, with transformation rates rarely exceeding 5%.[12,14,15] The lack of a molecular marker further hinders the process of identifying and isolating rare transformants, which becomes prohibitively laborious, relying upon large numbers of single crosses and molecular identification of variants. Although there has been an expansion in the methods to engineer marker-less edits, this has not been met with a similar level of expansion in methods to isolate rare transformants.

We devised a two-step method to generate and facilitate the detection and isolation of precise marker-less edits, based upon CRISPR-mediated cassette exchange (CriMCE) of a marked placeholder for a variant of interest (**Figure 1A**). CriMCE relies upon the visual detection of an edit, through the loss of a marker (**Figure 1A**), which serves to enrich the pool of molecularly queried individuals for rare transformants, to reduce the labour and time required to isolate them (**Figure 1C**).

We demonstrate the value of CriMCE by deliberately introducing three SNP variants into the genome of the malaria mosquito, *Anopheles gambiae*, at the target site of a synthetic gene drive in the *doublesex* gene.[16,17] Gene drives are engineered selfish genetic elements that show promise in controlling disease vector populations,[16,18–20] but are susceptible to resistant mutations arising at the gene drive target site, in the form of SNPs or small indels.[21–23] For vector control strategies, including insecticides and gene drive, it is becoming increasingly important to anticipate the emergence of resistance and pre-emptively design contingency plans. The SNP variant strains generated in this study will be useful in studying the potential for resistance to gene drives targeting a highly conserved site on *doublesex* and will inform implementation strategies.

We show that CriMCE is more efficient than methods previously employed to introduce small, unmarked edits,[12,14,15] whilst retaining versatility that would allow the engineering of more complex modifications as well (**Figure 1B**).





C

	Visual identification	Scarless strain	Type of modification	Transgenesis steps	Generations to homozygosis (N)	Rearing effort	Generations sequenced
CriMCE	✓	✓	Simple & complex	2	3	medium	1-2
RMCE	✓	✗	Simple & Complex	2	3	medium	1-2
HDR	✗	✓	Simple & Complex	1	4	very high	3-4
base/prime editing	✗	✓	Simple	1	4	very high	3-4

Figure 1. CRISPR-mediated cassette exchange (CriMCE) is a two-step method for engineering the detection and isolation of marker-less edits via CRISPR-mediated homology-directed repair. (A) Step 1: To generate a marked placeholder strain, the region of interest (gene B) is replaced by a marker (GFP, green). Step 2: The marker is replaced by the native sequence containing the variant of interest (orange), through CRISPR-mediated cassette exchange (CriMCE), to obtain a marker-less strain carrying the variant. **(B)** Examples of the types of simple and complex genetic modifications that can be obtained using CriMCE. **(C)** Comparison of CriMCE to other methods used to make precise genomic edits, including recombinase mediated cassette exchange (RMCE), direct homology-directed repair (HDR) of a wild-type sequence and base or prime editing

Results

We tested the efficiency of CriMCE and demonstrated proof of principle by using it to engineer and isolate mutations that potentially confer resistance to a gene drive, previously developed against the *doublesex* (*dsx*) gene in the malaria mosquito, *Anopheles gambiae*.[16]

First, we generated a placeholder strain by inserting a GFP cassette in place of the entire female-specific exon (exon 5) of *dsx* via CRISPR-mediated HDR (**Figure 2A**). This strain was isolated based on GFP fluorescence, and displayed an intersex phenotype in homozygous females, consistent with the null mutation.[16]

We then performed CRISPR-mediated cassette exchange (CriMCE) of the placeholder for the marker-less SNP of interest (G→A, C→T or G→T), by injecting placeholder homozygotes and heterozygotes with a plasmid expressing Cas9 and gRNAs targeted to the placeholder, and a template for repair encoding the variant of interest (**Supplementary Figure 1D-E, 3B**). To maximise the recovery of editing events, we selected only the fraction of injected mosquitoes that showed transient RFP fluorescence as clear evidence of having taken up the CRISPR expression vector (**Supplementary Figure 1D-E**) and mated these to wild-type (**Figure 3**).

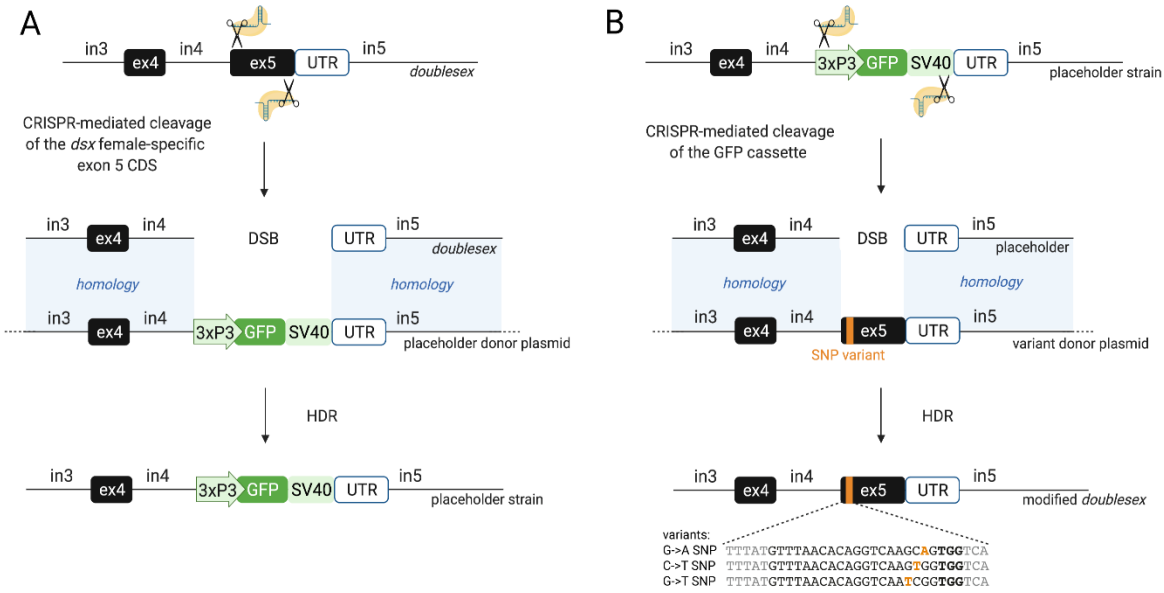


Figure 2. CriMCE relies upon the generation of a marked placeholder strain, and the subsequent exchange of the placeholder for the variant of interest through CRISPR-mediated HDR. (A) To generate the marked placeholder strain, the entirety of the exon 5 coding sequence (CDS) was removed via two CRISPR-mediated double-stranded breaks (DSBs) and replaced with a *3xP3::GFP::SV40* marker cassette (green) from a donor plasmid that served as a template for HDR. **(B)** To generate a strain carrying the variant of choice (G→A, C→T or G→T SNPs at exon 5) the marker cassette was removed via two CRISPR-mediated cleavages and exchanged for the exon 5 CDS containing the variant of interest (orange) from a donor plasmid, through HDR.

CriMCE-induced editing was evidenced by loss of GFP (<100% GFP inheritance) among the offspring of placeholder homozygotes, or by significant deviation below the Mendelian expectation of 50% GFP inheritance among the offspring of placeholder heterozygotes (**Figure 3**). We saw rates of precise editing up to 39% for the G→A SNP (evidenced by 61% GFP inheritance in the offspring of placeholder homozygotes) (**Figure 3A**), up to 100% for the C→T SNP, and up to 92% for the G→T SNP variant (evidenced by 0% and 4% GFP inheritance in the offspring of placeholder heterozygotes, respectively) (**Figure 3B**).

Incorporation of the SNPs of interest was confirmed by Sanger sequencing (**Supplementary Figure 2**). Notably, we did not detect any end-joining (EJ) events (N=55). Owing to the high rates of editing by CriMCE, G1 transformants that showed low levels of GFP inheritance can be immediately crossed to the placeholder strain that will act as a balancer, for rapid characterisation of each marker-less edit.

In two G1 clutches with altered GFP inheritance we also detected variant donor plasmid integration, evidenced by RFP at 2% and 18% amongst GFP negatives (with a median of 0% taken across all modified clutches) (**Supplementary Figure 1**). These were not considered as true transformants in our analysis (**Table 1**, **Figure 4**).

To compare our method to previously developed strategies employing HDR and prime editing to introduce and isolate marker-less edits,[12,14,15] we calculated three measures of transformation efficiency: the percentage of G0 founders that gave G1 transformants, the G1

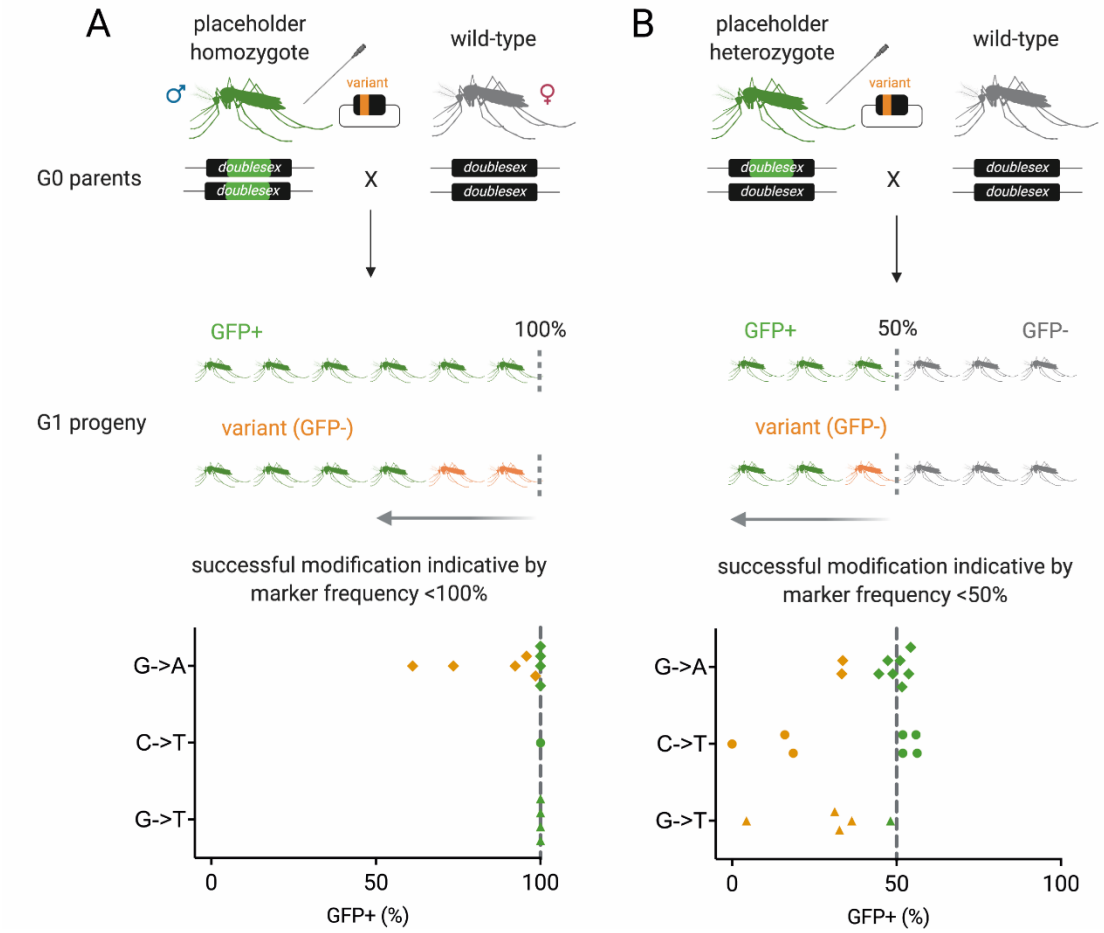


Figure 3. The introduction of a marker-less variant using CriMCE is evidenced by reduced rates of marker inheritance in the progeny of microinjected individuals of the placeholder strain. Marked placeholder male homozygotes (**A**) and heterozygotes of both sexes (**B**), were microinjected with a CRISPR helper plasmid and a variant donor plasmid to facilitate CriMCE of the placeholder for one of the variants of interest (G→A, C→T, G→T). G0 parent injected mosquitoes (green) were individually crossed to wild-type (grey) and their G1 progeny screened for GFP fluorescence. Successful introduction of each marker-less variant via CriMCE, was evidenced by a marker frequency of less than 100% in the progeny of placeholder homozygotes, and a marker frequency of less than 50% in the progeny of placeholder heterozygotes (orange). Lack of modification was evidenced by a marker frequency equal to 100% in the progeny of placeholder homozygotes and a marker frequency normally distributed around 50% in the progeny of placeholder heterozygotes (green).

transformant to G0 injected survivor ratio, and the G1 transformant percentage out of all G1 screened (**Table 1**). If the G1 transformant to G0 injected survivor ratio is high, then a high number of transformants can be obtained from a smaller number of injected survivors; whilst having a high percentage of G1 transformants out of total G1 screened, implies a reduced requirement for screening, whether this is done visually, like in the present study (less laborious), or by PCR and sequencing analysis, like in previous studies (more laborious). As a reference, we also show the efficiency of locus-specific marked transgene insertion through RMCE and HDR (**Table 1**).

In total, we detected visible editing in the progeny of 7/18 (38.9%) G0 micro-injected individuals with the G→A construct, 3/8 (37.5%) G0 micro-injected individuals with the C→T construct, and 4/9 (44.4%) G0 micro-injected individuals with the G→T construct (**Figure 3, Table 1**).

Table 1. Comparison of CriMCE to different transgenesis methods for the introduction of small precise marker-less edits or marked transgenes. Efficiency of each method is measured through

Transgenesis method and study		Organism	Eggs injected N	G0 Injected survivors N	G1 transformants N	Total G1 screened N	G0 Founders N (%)	G1 transformant to G0 injected survivor ratio	G1 transformants per G1 screened %
Introduction of precise marker-less edit									
CriMCE present study	G->A	*Anopheles gambiae*	380	18* (59)	111✦†	1716	7/18 (38.9)	6.17	6.47
	C->T	*Anopheles gambiae*	1025	21	166✦†	953	3/8 (37.5)	7.90	17.42
	G->T	*Anopheles gambiae*	963	23	74✦†	97	4/9 (44.4)	3.22	7.62
HDR	Kistler et al. (2015)	*Aedes aegypti*	636	61**	4✦✦	620	N/A	0.07	0.65
	Grigoraki et al. (2021)	*Anopheles gambiae*	338	19	4✦✦	290	1/19 (5.0)	0.21	1.38
Prime Editing Bosch et al. (2021)***	Plasmid pegRNA	*Drosophila melanogaster*	50	18	3✦✦	1767	1/18 (5.6)	0.17	0.17
	Plasmid pegRNA+ sgRNA	*Drosophila melanogaster*	50	15	28✦✦	1594	6/15 (40.0)	1.20	1.76
	Synthetic pegRNA	*Drosophila melanogaster*	50	11	20✦✦	866	4/9 (44.4)	1.82	2.31
Introduction of marked transgene									
RMCE Hammond et al. (2016)	7280	*Anopheles gambiae*	540	56**	15✦	4000	N/A	N/A	0.38
	11377	*Anopheles gambiae*	500	21**	4✦	2990	N/A	N/A	0.13
	5958	*Anopheles gambiae*	400	49**	2✦	4000	N/A	N/A	0.05
HDR	Gratz et al. (2014)	*Drosophila melanogaster*	N/A	50	599✦	7657	9/50 (18.0)	11.98	7.82
	Gantz et al. (2015)††	*Anopheles stephensi*	680	251**	2✦	25,712	N/A	0.01	0.01
	Hammond et al. (2016) 7280	*Anopheles gambiae*	350	48	278✦	1536	9/48 (18.8)	5.79	18.10
	Hammond et al. (2016) 5958	*Anopheles gambiae*	760	26	51✦	3184	3/26 (11.5)	1.96	1.60
	Adolfi et al. (2020)	*Anopheles stephensi*	504	184**	96✦	25,293	N/A	0.52	0.38
	Ang et al. (2022) *190-perfect*	*Aedes aegypti*	N/A	271**	350	9,774	13/13 (100.0)°	1.29	3.6
	Ang et al. (2022) *64+234-perfect*	*Aedes aegypti*	N/A	355**	207	22,158	8/17 (47.1)°	0.58	0.93

the G1 transformant to G0 injected survivor ratio and the % of G1 transformants isolated from screened G1 progeny.

*Only 18 out of 59 G0 injected survivors were kept and crossed to obtain G1 transgenics, due to Covid-19 restrictions in April 2020.
**In most studies G0 injected survivors are not being distinguished from non-injected survivors through transient expression of a fluorescent marker. The Kistler et al. (2015), Gantz et al. (2015), Hammond et al. (2016), Adolfi et al. (2020) and Ang et al. (2022) studies did not use such a method to distinguish injected survivors, or used all injected survivors (whether or not they showed signs of injection) to obtain transgenics.
***Showing the set of injections with greater success for each method of prime editing: (a) using pegRNA expressed from a plasmid to provide cleavage and a template for repair, (b) using plasmid pegRNA together with an sgRNA to provide cleavage, (c) injecting a synthetic pegRNA straight away.
✦Identified visually.
✦✦Identified through sequencing.
‡The number of transformants is equal to the number of individuals lacking a fluorescent marker in the progeny of placeholder homozygotes. The number of transformant in the progeny of placeholder heterozygotes it was estimated using this formula: (Total G1)/2 - GFP^+ - RFP^+.
‡‡Note that the transgene integrated by HDR in the Gantz et al. (2015) study was significantly larger in size compared to all other studies, which could have reduced efficiency of integration.
ºThe number of G0 founder pools that gave G1 transformants out of total G0 survivor pools is shown

CriMCE offers a marked improvement in transformation efficiency when compared to other approaches employed to introduce marker-less edits (**Figure 4**). Specifically, CriMCE shows a mean G1 transformant to G0 injected survivor ratio of 5.76 (±2.37 s.d.), compared to 0.14 (±0.10 s.d.) for direct HDR (Welch's t-test p=0.031) and 1.06 (±0.83 s.d.) for prime editing; and a mean G1 transformant per G1 screened percentage of 10.5% (±6.0% s.d.), compared to 1.0% (±0.5% s.d.) for direct HDR and 1.4% (±1.1% s.d.) for prime editing (Welch's t-test p=0.058) (**Figure 4**).

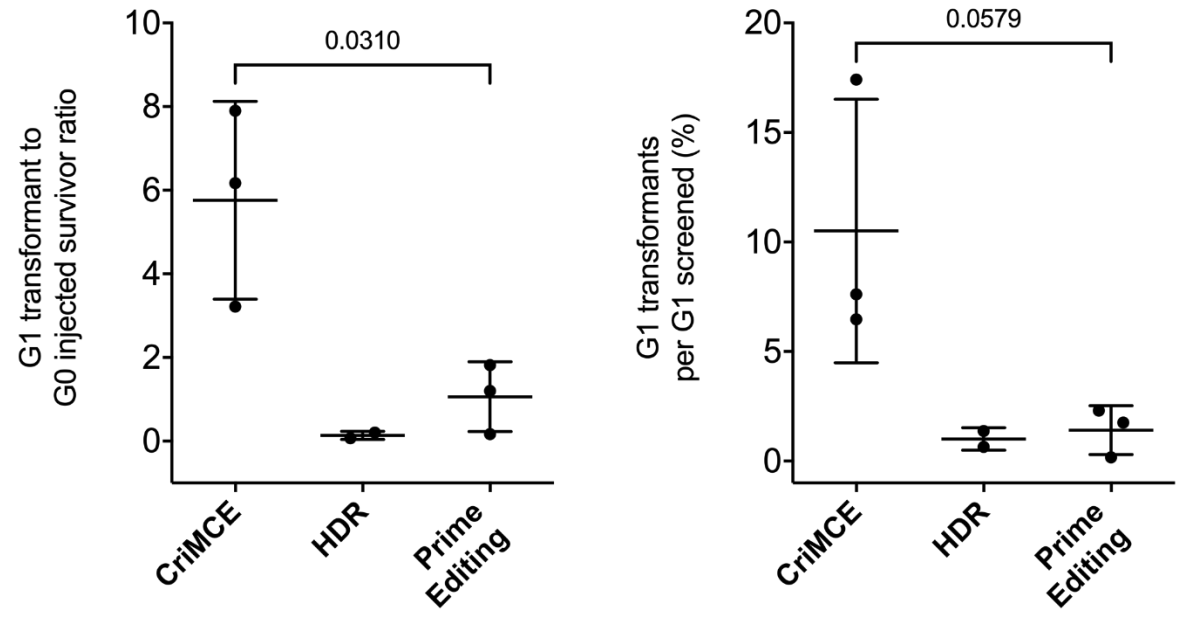


Figure 4. Comparison of CriMCE to different transgenesis methods for the introduction of small precise marker-less edits. Welch's t-test p-values of statistical comparisons between CriMCE and prime editing are shown on top of each graph. HDR could not be statistically compared due to its small sample size.

Discussion

To address the difficulty in engineering and isolating marker-less edits in insects, we have developed a strategy based upon CRISPR-mediated cassette exchange (CriMCE) of a marked placeholder for a variant of interest, allowing visual detection of transformation.

Unlike other two-step methods for marked cassette exchange or removal, like recombinase-mediated cassette exchange (RMCE) and Cre-Lox recombination, CriMCE relies upon HDR. This allows for comparatively high efficiency (when compared to RMCE) (**Table 1**), and uniquely traceless editing such that any phenotypic change can be attributed to the intended edit rather than ruminant attachment sites (**Figure 1**). Co-conversion of a target locus together with a gene that produces a visual phenotype is another HDR-based strategy that has been used to improve isolation of marker-less edits.[26] This filters individuals showing CRISPR activity, however it does not distinguish HDR events that incorporate the desired edit, from EJ events carrying unwanted indels.[26]

Increasing the relative frequency of HDR over error-prone EJ repair remains difficult. Our strategy leverages loss of a marked placeholder (GFP+) to indicate precise editing by HDR. By targeting CRISPR to non-coding regions of the placeholder, undesirable EJ events are screened out as they are unlikely to affect GFP expression. Furthermore, we express Cas9 under the control of *zpg* regulatory elements that are spatiotemporally restricted to enhance HDR.[27] Indeed, no EJ mutations were detected in GFP- negative transformants. This focuses molecular identification by PCR and sequencing on individuals carrying the desired edit, therefore reducing the rearing effort required to enrich the frequency of marker-less variants (**Supplementary Figure 3**).

Somewhat surprisingly, rates of HDR-induced editing are relatively high when marked mutations are introduced (**Table 1**),[23,28–30] but drop substantially when SNPs are directly inserted into a wild-type genomic locus, in *Aedes aegypti* and *An. gambiae* (**Table 1, Figure 4**).[14,15] Using CriMCE in *An. gambiae* we achieved high rates of HDR editing consistent with those for marked edit insertion in *An. gambiae* and *D. melanogaster* (**Table 1**, **Figure 4**).[28,29] In both cases, repair templates differ significantly from their target regions: transgenes introduced via HDR do not resemble their genomic target, while in the present study the wild-type target is replaced by a placeholder, which serves to differentiate it from the desired edit (**Figure 2**). Conversely, when direct HDR is used to induce small marker-less edits the repair template is almost identical to that of the wild-type target. It is still unclear why sequence dissimilarity between the exogenous repair template and its target should boost the efficiency of editing, but perhaps it functions to shift repair away from using the unmodified homologous chromosome as a template. Non-plasmid-based templates could also be used in a CriMCE strategy, such as single-stranded oligodeoxynucleotide (ssODN) that are simpler to produce and might further increase the rates of editing.[31]

CriMCE might be less efficient in species with inherently low rates of HDR, such as *An. stephensi* (**Table 1**),[20,23] and alternatives not reliant upon HDR, like base and prime editing,[11] have not yet been tested in non-model insects. In these species, CriMCE can be optimised by injecting placeholder homozygotes, so that rare events are distinguished by visual inspection alone (**Figure 3A**).

The CriMCE method can also mitigate against the risk of using previously untested and potentially inefficient gRNAs/pegRNAs that would otherwise expend undue effort on genetic crosses and molecular genotyping. Generating a marked placeholder prior to precise editing ensures that rare transgenesis using novel gRNA/pegRNAs is easily identifiable by a fluorescent marker. Previously tested guides can then be used to target the placeholder, inducing CriMCE. In this study we validate the use of two gRNAs that target a universal placeholder which is designed to function across insect species.

CriMCE is particularly powerful for experiments aimed at introducing a range of modifications to a single locus of interest, as a single placeholder strain can be exchanged for any number of variants. Indeed, a similar approach, based upon exchange of a marked allele for engineering of *kdr* pyrethroid resistance mutations was employed in *Drosophila*,[32] and could be further extended to incorporate newly discovered insecticide resistant SNPs.[33]

Moreover, CriMCE allows for complex mutations that are not possible using prime editing since the entire region ablated by the placeholder can be replaced with a region bearing any number of desired edits. This strategy, which we term allelic exchange (**Figure 1C**), could allow multiple linked SNPs to be introduced across a wide genetic locus. This would be useful in assessing how various resistant SNPs interact with each other to produce complex insecticide resistance phenotypes.[4] Other complex edits are also possible such as the introduction, modification or deletion of introns and splice site, or complete codon scrambling by which a coding sequence is modified without affecting the encoded amino acid sequence (**Figure 1C**). The latter strategy could serve to engineer synthetic alleles that are resistant to gene drive elements as a mechanism for gene drive recall.[34]

Finally, we describe how CriMCE can be used to target haploinsufficient genes, which by their nature, would be unable to tolerate a disruption from the placeholder, even if the desired edit is anticipated to be viable. In this case, integrating the placeholder within proximal intronic or neutral regions should permit editing (**Supplementary Figure 4**).

Conclusions

CriMCE is an efficient method to introduce and isolate precise and potentially complex marker-less edits by exchange of a visually marked intermediate. Our proof-of-principle experiments in *Anopheles gambiae* suggest that CriMCE is 5-41x more efficient than other strategies based on HDR or prime editing, whilst enabling an expanded range of potential edits and consolidating the workflow. In our experience the use of a placeholder strain does not prolong isolation of the desired edit and can be used as an important control or balancer in assessing its phenotype. We believe this strategy will be important in linking small genetic changes with a biologically relevant outcome across a range of insect species, with particular applications in the study of resistance to insecticides and gene drive technologies.

Materials and Methods

Molecular cloning of CRISPR plasmids

We used Golden Gate cloning to insert a dual gRNA expression cassette into the p174 master vector,[16] to generate CRISPR vectors p174102 and p17404 needed to catalyse genomic cleavage for the insertion of a placeholder cassette and the variant of interest, respectively. We first amplified a gRNA scaffold-U6 terminator-U6 promoter sequence, from plasmid p131 using primers containing *Bsa*I sites (underlined), and gRNA sequences (capitals): BsaI-T1-U6-F (gagggtctcatgctGTTTAACACAGGTCAAGCGGgttttagagctagaaatagcaagt) and BsaI-T3-U6-R (gagggtctcaaaacCTCTGACGGGTGGTATTGCagcagagagcaactccatttcat), to add *doublesex* targeting gRNAs onto p174 and BsaI-G1-U6-F (gagggtctcatgctGGTTAATTCGAGCTCGCCCGgttttagagctagaaatagcaagt) and BsaI-G2-U6-R (gagggtctcaaaacCAACTAGAATGCAGTGAAACagcagagagcaactccatttcat) to add placeholder targeting gRNAs. The PCR products were inserted into p174, through GoldenGate cloning, to create CRISPR vectors p174102 and p17404, containing a *zpg::hCas9*, a *3xP3::DsRed::SV40* marker and U6-expressed *doublesex*-targeting gRNAs (T1 and T3) or placeholder-targeting gRNAs (G1 and G3), respectively.

Molecular cloning of placeholder donor plasmid

A *3xP3::GFP::SV40* marker cassette was amplified from plasmid pK101,[16] using primers SgsI-3xP3-F (GGCGCGCCCCACAATGGTTAATTCGAGC) and SgsI-SV40-R (GGCGCGCCAAGATACATTGATGAGTTTGGAC). Genomic DNA regions ~1.8 kb upstream and downstream of the *doublesex* intron 4-exon 5 splice junction were amplified using primer pairs: 4050-KI-Gib1 (GCTCGAATTAACCATTGTGGACCGGTCTTGTGTTTAGCAGGCAGGGGA) with 4050-KI-Gib31 (TCCAAACTCATCAATGTATCTTGGCGCGCCATAAATGAATGGAAAGGTAAGGC), and 4050-KI-Gib32 (GAGCTCGAATTAACCATTGTGGGGCGCGCCGTATCTTTGTATGTGGGTGTGTG) with 4050-KI-Gib4 (TCCACCTCACCCATGGGACCCACGCGTGGTGCGGGTCACCGAGATGTTC), to make up the right and left homology arms, respectively, of the donor plasmid. To generate the placeholder donor plasmid pHolder-dsx the three PCR products were combined with a digested vector backbone containing a *3xP3::DsRed::SV40* marker cassette in a four-fragment Gibson assembly, so that the *dsx* homology arms flank the GFP placeholder cassette.

Molecular cloning of variant donor plasmids

An intermediate plasmid (pVar-dsx) was Gibson assembled to contain the same vector backbone and homology arms as for pHolder, and a sequence containing *BsaI* cloning sites, flanking the region of interest of an otherwise intact exon 5 (**Supplementary Figure 1A-C**). This allowed the Golden Gate cloning of annealed oligos containing three different *doublesex* exon 5 variants: a G→A SNP (GTTTAACACAGGTCAAGC**A**GTGGT, chromosome 2, position 47,997,665), a C→T SNP (GTTTAACACAGGTCAAG**T**GGTGGT, chromosome 2, position 47,997,666) and a G→T SNP (GTTTAACACAGGTCAA**T**CGGTGG, chromosome 2, position 47,997,667). The same plasmid, pVar-dsx, can be used to clone and study more variants at the same target site in the future.

Embryo microinjections

Anopheles gambiae G3 strain mosquitoes were reared at 26±2ºC and 65±10% relative humidity and blood-fed on cow blood using Hemotek membrane feeders.[18] Microinjections

were performed on freshly laid embryos as previously described.[24] Each microinjected plasmid was present in solution at 300 ng/µl.

To generate the placeholder strain, wild-type embryos were microinjected with the p174102 CRISPR plasmid and pHolder donor plasmid (**Supplementary Figure 1D-E**). In transformants, this caused the excision of the coding sequence (CDS) of the female-specific exon 5 of the *doublesex* gene and its replacement with a GFP marker cassette. All microinjection survivors (G0) were crossed to wild-type mosquitoes and positive transformants (G1) were identified through fluorescence microscopy, as GFP+.

To generate the SNP variant strains, placeholder homozygote males were crossed to placeholder heterozygote females, distinguished using the COPAS fluorescence-based larval sorter.[25] Their progeny was microinjected with the p174104 CRISPR plasmid and each of the variant donor plasmids (pVar-dsxGA, pVar-dsxCT, pVar-dsxGT) (**Supplementary Figure 1D-E**). In successful transformants, this caused the CRISPR-mediated cassette exchange of the marked placeholder for the *doublesex* exon 5 variants. Injected survivors (G0) were distinguished from non-injected survivors (G0), as they exhibited red fluorescence in their posterior, due to successful injection of the p174104 CRISPR plasmid, containing a DsRed cassette in its backbone, which acted as a co-injection marker (**Supplementary Figure 1D-E**). All injected survivors (G0) were crossed to wild-type and females were deposited to lay eggs individually. A decreased inheritance of the marked placeholder (GFP+) in G1 progeny indicated CRISPR-mediated cassette exchange of the placeholder for the variant sequence (**Figure 2**).

Molecular genotyping

Genomic DNA was extracted from queried individuals after they gave offspring, in single samples, amplified using primers dsx-exon5-R4 (AACTTATCGGCATCAGTTGCG) and dsx-intron4-F1 (GTGAATTCCGTCAGCCAGCA) and sequenced using the dsx-exon5-R2 primer (TGAATTCGTTTCACCAAACACAC), to decipher their genotype.

Analysis

Figures were designed on Biorender (full licence) and Adobe Illustrator and graphs were plotted and statistically analysed on Graphpad Prism 9.

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Author contribution statement

The idea was conceived by I.M., A.C. and A.M.H. The experiments were designed by I.M. with input from T.N. and A.M.H. The experiments, data visualisation and analysis were performed by I.M. The original draft was written by I.M. and edited by A.M.H. The manuscript was reviewed by all authors.

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