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Improved *piggyBac* Transformation with Capped Transposase mRNA in Pest Insects

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Abstract: Creating transgenic insects is a key technology in insect genetics and molecular biology. A widely used instrument in insect transgenesis is the *piggyBac* transposase, resulting in essentially random genomic integrations. In contrast, site-specific recombinases allow the targeted integration of the transgene construct into a specific genomic target site. Both strategies, however, often face limitations due to low transgenesis rates. We aimed to enhance transgenesis rates by utilizing a capped mRNA as a source of transposase or recombinase instead of a helper plasmid. A systematic comparison of transgenesis rates in *Aedes* mosquitoes, as models for hard to transform insects, showed that suppling *piggyBac* transposase as mRNA increased the average transformation efficiency in *Aedes aegypti* from less than 5% with the plasmid source to about 50% with mRNA. Similar high transformation activity was observed in *Ae. albopictus* with *pBac* mRNA. No efficiency differences between plasmid and mRNA were observed in recombination experiments. Furthermore, a codon-optimized version of *piggyBac* transposase delivered as plasmid didn't improve the transformation efficiency in *Ae. aegypti* or the agricultural pest *D. suzukii*. We believe that the use of mRNA has strong potential for enhancing *pBac* transformation efficiencies in other mosquitoes and important agricultural pests such as tephritids.

Keywords: insect transgenesis; *Aedes*; *Drosophila suzukii*; tephritids; transformation efficiency; recombination efficiency; *piggyBac* transposase; helper plasmid; capped mRNA

1. Introduction

Creating transgenic insects is a rapidly advancing technology with various applications in insect genetics and molecular biology. For example, insect transgenesis can address fundamental research questions like uncovering the functions of specific genes and developing genetically modified strains with unique features that can be used in agricultural pest or vector control applications. These applications have been accelerated and become more widespread due to the discovery of CRISPR genome editing and its successful application to many insect species. However, the efficiency of integrating DNA constructs via CRISPR-HDR is often low and requires a sequenced genome of the target species. Therefore, transposon-based transformation still is a standard and widely used method to create transgenic insects because it is not dependent on the availability of a high-quality genome sequence. Many different class II transposable elements (DNA transposons) have been investigated for their suitability as a tool for insect transformation since their initial use more than 40 years ago [1-5]. The *piggyBac* (*pBac*) transposase has proven to be particularly important as a universal tool for insect transgenesis. It has been applied to a wide variety of insect orders and species [6-11], including *Aedes* and *Anopheles* mosquitoes [12-17]. Class II transposable elements naturally comprise the transposase gene flanked by inverted repeats. The transposase enzyme recognizes the inverted repeats to catalyze the excision of the gene from the genome and insert the gene at a new position (a

cut-and-paste mechanism). Insect transformation by transposable elements traditionally uses a bipartite system consisting of a helper plasmid encoding the transposase gene and a donor plasmid encoding the transgene construct flanked by the inverted repeat sequences of the transposable element. As a result, only the transgene construct is inserted in the genome. In contrast, the nucleic acid sequence encoding the transposase gene on the plasmid is not inserted into the genome. It gets lost after a few cell divisions in the developing embryo, ensuring the stability of the genomic integration.

Both plasmids are injected into the posterior pole of preblastoderm insect embryos to achieve transformation in the germline. Transposases commonly have very short recognition sequences (for example, TTAA for *pBac* transposase), which leads to essentially random integration of the encoded transgene construct into the target genome. This characteristic has proven a versatile tool to uncover gene functions due to insertional mutagenesis effects or enhancer traps [18, 19]. However, insertional mutagenesis can reduce the transformed lines' fitness, thereby reducing their utility. In contrast, site-specific recombination (SSR) systems can avoid this problem by targeting the integration of the transgene construct into a previously characterized "landing site" or "docking site" by a mechanism called recombinase-mediated cassette exchange (RMCE). RMCE landing-sites lines are usually created by transposon-mediated random genomic integration of a landing-site construct, typically consisting of a fluorescent marker flanked by recombination sites for an appropriate recombinase enzyme. The lines are then characterized for their viability and the integration site, and the best line(s) can be used for SSR experiments. SSR systems used in insects so far include the phiC31 integrase from the phage phiC31 [20], the Cre recombinase from the *Escherichia coli* phage *P1* [21], and the Flipase from the yeast *Saccharomyces cerevisiae* [22]. Similar to *pBac*, these recombinases can be provided as a helper plasmid into the early embryo, together with a donor plasmid encoding the gene construct of interest flanked by the recombination sequences compatible with the respective sequences at the genomic landing site.

In most cases of insect transgenic experiments, the transgenic insect is the basic product needed for the downstream experiments or a whole research project. Two measures of how efficiently a transgenic insect can be created are the transposition and recombination efficiencies. These efficiencies are ideally calculated as the number of observed independent transgenic events divided by the number of fertile G_0 adults resulting from the injection. If the latter is unavailable due to experiment design, the minimal efficiency can be calculated as the number of independent transgenic events divided by the total number of G_0 adults. In *Drosophila melanogaster* and some other species, the transposition and recombination efficiencies are usually sufficiently high that the transgenesis step does not represent a substantial barrier to progress [23-26]. However, in other insect species, transgenesis rates are often low [7]. For example, transgenesis rates mostly range between 0% and less than 10% in mosquitoes [7, 12-14, 27-31]. Low efficiencies of 1-5 % were also routinely obtained in our laboratory for *pBac*-mediated transposition and Cre- and phiC31-RMCE in the yellow fever mosquito, *Aedes aegypti*, leading to a low number of transgenic lines per injection. No line was often obtained despite hundreds or thousands of injected embryos and tens of thousands of offspring screened for the presence of the transgene construct. Thus, creating transgenic lines can become a significant bottleneck, especially in non-model organisms.

Higher transposition and recombination efficiencies in *Ae. aegypti* and other mosquito species would facilitate the application of transgenic work in this taxon of medically important insects. A widely used transgenesis tool in mosquitoes is the helper plasmid for *pBac*-mediated transformation driving transposase expression via the *D. melanogaster* heat shock promoter 70 (hsp70) [9]. The same promoter drives recombinase expression in Cre and phiC31 helper plasmids used in our lab [24]. One cause for the low efficiencies observed with these helper plasmids could be a low transcriptional activity of the exogenous *D. melanogaster* hsp70 promoter in *Aedes*. Additionally, a low activity of the enzyme itself could be responsible. Transcription-related effects could be solved by injection of capped mRNA instead of the helper plasmid. To test this hypothesis, we systematically compared the efficiency of hsp70 helper plasmids to that of *in vitro* transcribed, capped mRNA for *pBac* transposase, phiC31 integrase, and Cre recombinase in *Ae. aegypti*, and we also tested *pBac* and Cre

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mRNA efficiencies in the congeneric species *Ae. albopictus*. For *pBac* transposase, an alternative solution could also be using a hyperactive version of the *pBac* enzyme (IPB7) selected in a *S. cerevisiae* mutagenesis screen [32]. In the initial study, the hyperactive version of *pBac* did not improve the transposition rate in *Ae. aegypti* and *D. melanogaster* in genetic transformations [33]. However, a systematic comparison between the original *pBac* transposase and IPB7 under the control of the *D. melanogaster* hsp70 promoter achieved up to 15-fold higher transformation rates with IPB7 in *Tribolium castaneum*, *D. melanogaster*, and *Ceratitis capitata* ([34]; this study uses IPB7 under the name ihyPBase). We therefore decided to test this hyperactive *pBac* transposase again in *Ae. aegypti* and additionally in *Drosophila suzukii* (Spotted Wing *Drosophila*, SWD) embryos by performing comparative injections with both helper plasmids.

The data presented here confirm the results by Wright et al. [33] regarding the efficiency of the hyperactive *pBac* plasmid on the transposition rate in *Ae. aegypti*. IPB7 also doesn't improve the transposition rates in *D. suzukii*. The effect of mRNA on the transformation efficiency seems to be enzyme-dependent. While using Cre or phiC31 mRNA didn't improve the recombination efficiencies in the tested mosquito species, using *pBac* mRNA resulted in an average more than 30-fold increase in transposition efficiency compared to the helper plasmid in *Ae. aegypti*. The results in *Ae. albopictus* indicated that *pBac* mRNA activity is similarly high as in *Ae. aegypti*. We attribute these differences in mRNA efficiency between *pBac*-mediated transposition and Cre- or phiC31-mediated recombination to the completely different reaction dynamics. For *pBac* transposition, there are potentially millions of possible genomic integration sites. On the contrary, for RMCE, only one recombination site exists in the genome.

The substantial improvement of transposition efficiencies observed with *pBac* mRNA in the two *Aedes* species makes it a promising strategy to apply to other mosquito species for which low transformation rates with *pBac* plasmid were observed. Moreover, injections of mRNA as transposase source could be considered for other challenging to transform insect species, and also for other transposable elements like Minos, Hobo, or Hermes.

2. Results

2.1. ihypBase helper plasmid doesn't increase the transposition efficiencies in *Ae. aegypti* and D. *suzukii* embryos

To test if the original hyperactive version of the *pBac* transposase with the insect codon usage under the *D. melanogaster* hsp70 promoter (ihyPBase) [34] results in higher transposition efficiencies in the yellow fever mosquito, *Ae. aegypti*, embryos of the Higgs White Eye (HWE) strain were injected with varying concentrations of the phsp-ihyPBase helper plasmid in combination with two different donor plasmids, including a variation of helper-donor ratios. In parallel, the same injections were performed with the standard *phsp-pBac* plasmid [9]. Moreover, the results were compared to the transposition efficiencies obtained with the *phsp-pBac* plasmid in our laboratory over the years, which had as well been performed at various helper concentrations and helper/donor ratios in the attempt to optimize *phsp-pBac*-mediated transformation efficiencies (Table 1, Table S1). ihyPBase helper concentrations were chosen lower than the *phsp-pBac* concentrations typically used in expectance of higher transformation rates as observed in *T. castaneum*, *D. melanogaster*, and *C. capitata* [34].

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Table 1. Transformation efficiencies in *Ae. aegypti* using the standard *piggyBac* helper or ihypBase helper plasmids.

Exp. no	helper template	[helper/ donor] (ng/µl)	donor construct	donor plasmid (insert) size (bp)	no. injected embryos	larval hatch rate (%)	adult eclosion rate (%)	total no. G_0 families	% fertile G_0 families	no. transg. G_0 families	total no. G_1 screened	no. transg. events	transf. eff. (%)
1	*pBac*	300/150	AH452 *	(5267) 8649	623	29.21	78.57	8	100.00	4	n.d.	4	2.80
2	*pBac*	200/500	V3	(3545) 6911	234	31.20	86.30	25	52.00	1	8830	1	1.25
3	*pBac*	400/600	V285	(5180) 8682	705	25.82	64.29	18	88.89	2	14966	2	1.71
4	***pBac***	**300/500**	**V286**	**(5600) 9102**	**680**	**13.38**	**41.76**	**15**	**60.00**	**0**	**8772**	**0**	**0.00**
5	***pBac***	**160/185**	**V258**	**(6190) 9690**	**922**	**10.41**	**37.50**	**18**	**83.33**	**0**	**6631**	**0**	**0.00**
6	***pBac***	**400/600; 228/342**	**V258**	**(6190) 9690**	**599**	**24.21**	**34.48**	**11**	**100.00**	**0**	**18721**	**0**	**0.00**
7	*pBac*	300/150	V257	(7088) 10587	1113	42.59	76.37	26	100.00	2	93998	2	0.55
8	*pBac*	200/500	V19	(3630) 7163	257	18.29	63.83	9	100.00	2	5185	2	6.67
9	*pBac*	300/500	V19	(3630) 7163	192	33.85	61.54	11	63.64	0	2679	0	0.00
10	*pBac*	200/500	V19	(3630) 7163	399	13.53	61.11	13	46.15	0	1313	0	0.00
11	*pBac*	200/500	V19	(3630) 7163	462	14.72	48.53	7	100.00	2	1389	2	6.06

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12	*pBac*	300/300	V368	(5835) 11097	257	5.84	60.00	7	42.86	0	502	0	0.00
13	*pBac*	100/300	V368	(5835) 11097	148	5.41	62.50	4	75.00	0	382	0	0.00
												avg	**1.46**
14	**HypB**	**100/200**	**V286**	**(5600) 9102**	**594**	**8.08**	**75.00**	**12**	**75.00**	**0**	**9382**	**0**	**0.00**
15	**HypB**	**200/200**	**V286**	**(5600) 9102**	**730**	**8.63**	**79.37**	**17**	**70.59**	**0**	**7604**	**0**	**0.00**
16	**HypB**	**200/200**	**V258**	**(6190) 9690**	**895**	**6.82**	**65.57**	**13**	**69.23**	**0**	**5199**	**0**	**0.00**
												avg	**0.00**

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In the three injections with the ihyPBase plasmid, the larval hatch rate was about 7-8% lower than primarily observed in *phsp-pBac* injections (between 10-24%) (Table 1). However, this difference was insignificant (p = 0.081, single factor ANOVA). No noticeable difference could be observed for the adult emergence rate, and also the fertility of the G_0 families was comparable with both helper plasmids (Table 1 and Figure S1a - d; p = 0.175, and p = 0.647, single factor ANOVA). The average transformation efficiency in our laboratory with the *phsp-pBac* plasmid over the years was 1.46% (13 experiments in total, including seven experiments with no transgenic event). In this set of parallel injections with the ihyPBase plasmid and *phsp-pBac*, no transgenic event was recovered (a total of more than 2200 injected embryos each, and 23000 screened G_1 for ihyPBase, and 34000 screened G_1 for *phsp-pBac*).

Shown are the injection data using the standard *phsp-pBac piggyBac* helper plasmid (*pBac*) collected over six years and injections performed using the phsp-HypB plasmid (HypB) containing the insect codon optimized hyperactive *pBac*. Injections 4, 14, and 15, as well as 5, 6, and 16 (printed in bold) were performed in parallel with the same donor plasmid and eggs from the same female cohort. "Injected embryos" represents the number of black embryos 24 h post injection; "hatch rate" = no. larvae / no. black eggs (%); "eclosion rate" = no. adults / no. larvae (%); "no. transg. events" is the number of independent transposition events observed; "transf. eff." = the minimal transformation efficiency, calculated as: number of independent transpositions events/ total number of fertile G_0 adults. The actual transformation efficiency can be higher, because in group backcrosses of G_0 individuals, the number of infertile G_0 isn't known. Therefore, all G_0 are included in the calculation; n.a. = not applicable; n.d. = not determined; avg = average; * this data has been published before in Häcker et al. 2017 [35].

The same ihyPBase helper plasmid was tested for transposition efficiency in the agricultural pest *D. suzukii* and compared to previous injections with the standard *phsp-pBac* helper. In *D. suzukii*, the standard injection mix concentration for the *phsp-pBac* helper over the years was predominantly 200 ng/µl helper and 500 ng/µl donor plasmid. These concentrations were also used for ihyPBase injections in two experiments. Moreover, both helper plasmids were mixed at equal concentrations in three independent injection experiments (Table 2). As in *Ae. aegypti*, the ihyPBase didn't improve the transformation efficiency compared to the standard *phsp-pBac* plasmid. We also did not observe differences in development of injected embryos between *phsp-pBac* and ihyPBase injections (Table 2, Figure S1e - g).

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Table 2. Transformation efficiencies in *D. suzukii* with the standard *piggyBac* helper or ihypBase helper plasmids.

Exp. no.	helper template	[helper/ donor] (ng/µl)	donor construct	donor plasmid (insert) size (bp)	no. injected eggs	no. hatched larvae	hatch rate (%)	no. fertile adults	fertile eclosion rate (%)	no. transg. lines	transf. eff. (%)
Ds 1	*pBac*	200/500	AH443	(9191) 12576	75	*n. d.*	*n. d.*	25	*n.d.*	4	16.00
Ds 2	*pBac*	200/500	V220	(7865) 11249	1601	231	14.43	65	28.14	1	1.54
Ds 3	*pBac*	200/500	V221	(7867) 11252	443	102	23.02	9	8.82	0	0.00
Ds 4	*pBac*	200/500	V146	(7118) 10503	481	173	35.97	20	11.56	2	10.00
Ds 5	*pBac*	200/500	V183	(7583) 10968	753	305	40.50	43	14.10	5	11.63
Ds 6	*pBac*	200/500	V184	(9438) 12823	640	167	26.09	17	10.18	0	0.00
Ds 7	*pBac*	200/500	V185	(8493) 11878	802	346	43.14	53	15.32	6	11.32
Ds 8	*pBac*	200/500	V188	(10347) 13732	631	285	45.17	55	19.30	1	1.82
Ds 9	*pBac*	200/500	V213	(10059) 13443	538	107	19.89	27	25.23	0	0.00
Ds 10	*pBac*	200/500	V215	(8204) 11589	626	173	27.64	29	16.76	1	3.45
Ds 11	*pBac*	200/500	V226	(8163) 12054	410	51	12.44	9	17.65	1	11.11
Ds 12	*pBac*	200/500	V227	(10018) 13909	493	122	24.75	12	9.84	1	8.33
Ds 13	*pBac*	200/500	V228	(8607) 12498	310	97	31.29	13	13.40	0	0.00
Ds 14	*pBac*	200/500	V250	(9072) 12963	378	129	34.13	28	21.71	0	0.00
Ds 15	*pBac*	200/500	V229	(10434) 14325	339	92	27.14	26	28.26	1	3.85
Ds 16	*pBac*	200/500	V251	(10927) 14818	413	70	16.95	10	14.29	0	0.00
Ds 17	***pBac***	**200/500**	**V265**	**(9952) 13337**	**520**	**124**	**23.85**	**32**	**25.81**	**0**	**0.00**
										avg	**4.65**
Ds 18	***pBac***	**300/700**	**V265**	**(9952) 13337**	**582**	**238**	**40.89**	**77**	**32.35**	**5**	**6.49**
	pBac + hyPB	200+200/500	V221	(7867) 11252	1457	271	18.60	42	15.50	1	2.38

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Ds 19											
Ds 20	*pBac* + hyPB	200+200/500	V222	(7861) 11246	1898	401	21.13	34	8.48	0	0.00
Ds 21	*pBac* + hyPB	200+200/500	V223	(7865) 11249	488	82	16.80	30	36.59	2	6.67
										avg	**3.02**
Ds 22	hyPB	200/500	V209	(5538) 8922	1081	252	23.31	37	14.68	2	5.41
Ds 23	**hyPB**	**200/500**	**V265**	**(9952) 13337**	**98**	**32**	**32.65**	**8**	**25.00**	**0**	**0.00**
										avg	**2.70**

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Shown are the injection data using the standard *phsp-pBac* helper plasmid (*pBac*) (exp. Ds 1-18), and injections performed using phsp-HypB plasmid (hyPB) with insect codon optimized hyperactive *pBac* helper (Ds 22, 23) or a combination of both (Ds 19-21). The injections for AH443 [24], V220-223 [36], V146, V183-V185, V188, V213, V215, V226-229, V250-251 [37], V209 [38], and V265 [39], were performed across more than 10 years. All injected eggs were counted. "hatch rate" = no. larvae / no. injected eggs (%); "fertile eclosion rate" = no. fertile G_0 adults/ no. hatched larvae (%); "no. transg. lines" is the number of independent transgenic lines obtained (all G_0 were backcrossed individually, except injection Ds 18; here independent lines were identified from each family and confirmed by segregation analysis); "transf. eff." = the transformation efficiency, calculated as: number of independent transgenic events / total number of fertile G_0 adults; n.d. = not determined; avg = average.

2.2. *pBac* mRNA boosts the transposition rate in *Ae. aegypti*

To assess the efficiency of *in vitro* transcribed, capped mRNA as a *pBac* helper source, *pBac* mRNA injections were performed with six different donor plasmids (V19, V96, V97, V368, V369, V370) ranging from 7 to almost 12 kb in plasmid size and 3.6 to 6.5 kb in insert size. The minimal transformation efficiencies of these injections were compared to the *phsp-pBac* helper plasmid injections performed over the years in our laboratory with various donor plasmids (Table 3 and Table S2). Of the six donor plasmids used in *pBac* mRNA injections, V19 had previously been injected with the *pBac* helper plasmid. Moreover, the V370 donor plasmid used in mRNA injections is identical to the V258 donor injected with *pBac* helper plasmid except for an additional *attB* recombination site in V370. As all the plasmid helper and mRNA helper experiments were conducted at different time points, with different HWE female cohorts, by different experimenters, and some in a different laboratory (V369, V370), we also performed side-by-side injections with the *phsp-pBac* plasmid or *pBac* mRNA together with the V368 donor plasmid into embryos collected from the same batch of HWE females.

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Table 3. Transformation data in *Ae. aegypti* using the *phsp-pBac* helper plasmid or capped *pBac* mRNA as *piggyBac* source.

Exp. no	helper template	[helper/ donor] (ng/µl)	donor construct	donor plasmid (insert) size (bp)	no. injected embryos	hatch rate (%)	eclosion rate (%)	total no. G_0 families	no. fertile G_0 families	no. transg. G_0 families	total no. G_1 screened	no. transg. events *	no. transg. events/ G_0	transf. eff. (%)
1	plasmid	300/150	AH452 ***	(5267) 8649	623	29.21	78.57	8	8	4	*n.d.*	4	1	2.80
2	plasmid	200/500	V3	(3545) 6911	234	31.20	86.30	25	13	1	8830	1	1	1.59
3	plasmid	400/600	V285	(5180) 8682	705	25.82	64.29	18	16	2	14966	2	1	1.71
4	plasmid	300/500	V286	(5600) 9102	680	13.38	41.76	15	9	0	8772	0	*n.a.*	0.00
5	plasmid	160/185	V258	(6190) 9690	922	10.41	67.71	18	15	0	6631	0	*n.a.*	0.00
6	plasmid	400/600; 228/342	V258	(6190) 9690	599	24.21	58.62	11	11	0	18721	0	*n.a.*	0.00
7	plasmid	300/150	V257	(7088) 10587	1113	42.59	76.37	26	26	2	93998	2	*n.d.*	0.55
8	plasmid	200/500	V19	(3630) 7163	257	18.29	63.83	9	9	2	5185	2	1	6.67
9	plasmid	300/500	V19	(3630) 7163	192	33.85	61.54	11	7	0	2679	0	*n.a.*	0.00
10	plasmid	200/500	V19	(3630) 7163	399	13.53	61.11	13	6	0	1313	0	*n.a.*	0.00

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11	plasmid	200/500	V19	(3630) 7163	462	14.72	48.53	7	7	2	1389	2	1	6.06
12	**plasmid**	**300/300**	**V368**	**(5835) 11097**	**257**	**5.84**	**60.00**	**7**	**3**	**0**	**502**	**0**	***n.a.***	**0.00**
13	**plasmid**	**100/300**	**V368**	**(5835) 11097**	**148**	**5.41**	**62.50**	**4**	**3**	**0**	**382**	**0**	***n.a.***	**0.00**
													avg	1.49
14	mRNA	182/300	V96	(5278) 8813	113	11.50	46.15	6	3	1	370	4	4	66.67
15	mRNA	182/300	V97	(4307) 7841	298	5.03	73.33	11	4	2	514	7	≥ 2 - ≥5	63.64
16	mRNA	300/300	V370	(5870) 11131	576	31.25	86.67	12	12	11	4388	> 19	≥1	12.18
17	mRNA	300/300	V369	(6420) 11684	520	16.35	80.00	6	6	5	2173	> 8	≥1	11.76
18	mRNA	300/300	V19	(3630) 7163	527	8.92	48.94	28	17	10	1813	≥ 24	1 - ≥5	104.35 **
19	mRNA	100/300	V19	(3630) 7163	310	5.48	70.59	9	6	2	1195	≥ 7	≥3 - ≥4	58.33
20	mRNA	100/300	V19	(3630) 7163	646	1.61	80.00	7	7	3	1030	4	1 - 2	50.00
21	**mRNA**	**300/300**	**V368**	**(5835) 11097**	**449**	**7.35**	**66.67**	**18**	**10**	**5**	**1014**	**≥ 12**	**≥2 - 4**	**54.55**
22	**mRNA**	**100/300**	**V368**	**(5835) 11097**	**281**	**4.98**	**85.71**	**7**	**6**	**5**	**870**	**≥ 9**	**1 - ≥3**	**75.00**
													avg	49.02

Injection numbers across all experiments ranged from 150 to more than 1000 injected embryos per experiment for the helper plasmid, and 110 to less than 600 embryos for the helper mRNA (Table 3 and Table S2). In helper plasmid injections, *pBac* and donor concentrations varied between 160 and 400 ng/µl, and 150 and 600 ng/µl, respectively. In mRNA injections, the donor concentration was kept constant at 300 ng/µl, and the mRNA concentration varied between 100 and 300 ng/µl. G_0 injection survivors were backcrossed individually or in groups of up to 15 individuals, depending on the number of G_0 survivors and the current insectary capacity, and the offspring (G_1) screened for fluorescent marker expression. For all mRNA injections and three of the *pBac* plasmid injections (exp. 2, 3, 9), a subset of positive G_1 of each G_0 family was analyzed for the transgene copy number in the genome via droplet digital PCR (ddPCR). In case of single integration events identified by ddPCR, inverse PCR was performed to determine and distinguish between genomic integration sites. Some individuals were sacrificed for the transformation event analysis only after individual backcrossing and successful mating. The minimum number of independent genomic integration events per G_0 family was determined by summing up the number of G_1 with a different copy number or integration sites. A copy number greater than one was counted as one independent event in this analysis. The complete set of data for the integration event analysis is summarized in Table S3.

Data from 13 injection experiments using the *phsp-pBac* helper plasmid over six years and nine injection experiments using *pBac* capped mRNA over five years are displayed. Exp. 1-13 are identical to the ones shown in Table 1. Exp. 12, 13, 21, and 22 (printed in bold) were performed in parallel with eggs from the same female cohort; Injections 14 and 15 were performed in the WT Orlando laboratory strain, and all other injections in the Higgs White Eye strain; "Injected embryos" represents the number of black embryos 24 h post injection; "hatch rate" = no. larvae / no. black eggs (%); "eclosion rate" = no. adults / no. larvae (%); "no. transg. events" is the number of independent transposition events observed (multiple genomic integrations in one individual were counted as one event); "no. of transg. events / G_0" = the maximum number of independent transposition events detected in a G_0 founder individual as determined by ddPCR and iPCR analysis of positive G_1. If the number is given as '≥ number', then only a subset of positive G_1 was molecularly analyzed, and additional independent events might not have been detected; "transf. eff." = the minimal transformation efficiency, calculated as: number of independent transgenic events/ total number of G_0 adults. The transformation efficiency in several families is assumed to be higher (see text); n.a. = not applicable; n.d. = not determined; avg = average; * detailed numbers of all transgenic events identified in each G_0 family and calculations see Tables S2 and S3; ** transformation efficiency of > 100% results from single G_0 founder individuals producing more than one independent transposition event (as determined by ddPCR and iPCR); *** this data has been published before in Häcker et al. 2017 [35].

Across all injections, we observed a lower but not significantly different larval hatch rate in the mRNA helper injections (Figure 1a; p = 0.06619, one-factor ANOVA), which seemed independent of the size of the injected donor construct. There was no difference in the G_0 adult eclosion rate and in the fertility of the G_0 families between plasmid and mRNA helper (Figure 1b, c; p = 0.2340 and p = 0.6204, respectively, one-factor ANOVA). However, using the mRNA helper, the transposition efficiency increased from an average of 1,49% with the helper plasmid to more than 50% on average (Figure 1g, p =1.646E-06). These numbers refer to the calculated minimal transformation efficiency, i.e., the number of obtained independent transgenic events divided by the total number of adult G_0 survivors of each injection. Different independent events within one G_0 family as determined by digital PCR and inverse PCR were counted separately. The actual transformation efficiency, however, in several cases might have been even higher than the numbers reported here for the following reasons: i) G_0 injection survivors were not always backcrossed individually (Table S2). Thus, in some cases, more than one positive G_0 could have been in a positive pool, resulting in more than one independent transgenic event, which ddPCR and inverse PCR wouldn't have necessarily identified as only small numbers of G_1 individuals per family were assessed. This applies especially to the V369 and V370 injections (exp. 16, 17), the only mRNA injection experiments where the G_0 were exclusively backcrossed in large groups (12 individuals per cage, see Table S3). Moreover, of several G_0 families, the offspring was not molecularly characterized for copy number and integration sites. ii) All

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emerged G_0 adults were counted for the calculation, including sterile individuals, which on average, were about 40% (Table S4).

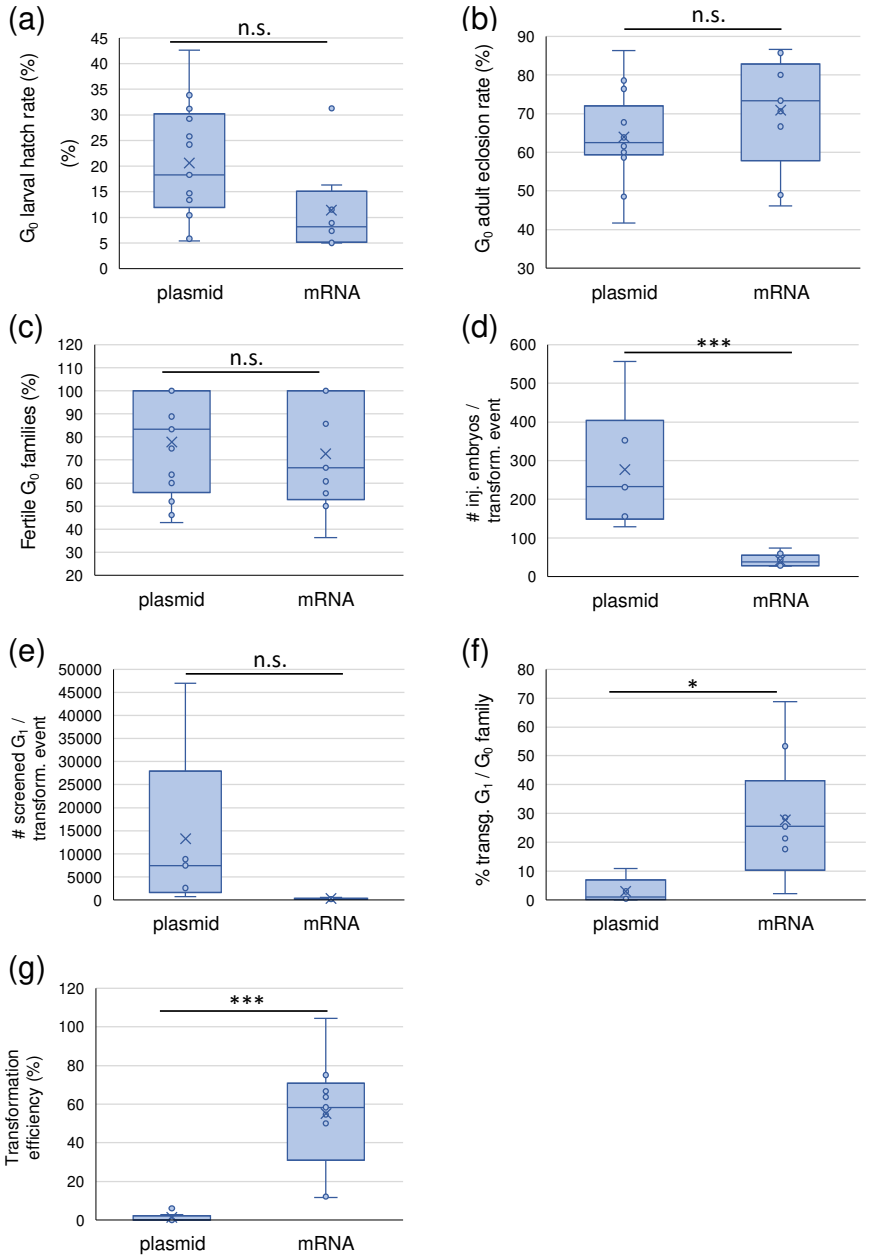

Figure 1. Effect of *pBac* helper plasmid and *pBac* mRNA injections in *Ae. aegypti* on larval hatch rate (**a**), G_0 adult eclosion rate (**b**), G_0 adult fertility (based on individually backcrossed G_0 across all *pBac* injection experiments (Table S4 for numbers) (**c**), the number of injected embryos per transposition event (**d**), the number of screened G_1 per transposition event (**e**), the percentage of transgenic G_1 per positive G_0 family (**f**), and the minimal transformation efficiency (%) (**g**). Box and whisker plots (exclusive median) are displayed based on the data presented in Table 3 and Table S2. The horizontal line represents the median; the cross (x) represents the mean; ns = no significant difference, $p > 0.05$; * = significant difference, $p \leq 0.05$; *** = significant difference, $p \leq 0.001$.

A frequent observation with *pBac* mRNA injections were multiple transgene construct integrations in one individual, ranging from two to 18 integrations at the higher mRNA

concentration, and two to 14 integrations at the lower concentration in the G_1 animals analyzed by ddPCR. Different integration frequencies were observed between the offspring of different G_0 founders and within the offspring of single-founder G_0 families, including individuals with single integrations at different genomic locations as determined by inverse PCR. On the contrary, from all *phsp-pBac* plasmid injections, only one individual with two integrations of a transgene construct was obtained. For the detailed results, see Tables S2 and S3.

2.3. Preliminary data indicate high transposition rates with *pBac* mRNA in *Ae. albopictus*

The efficiency of capped *pBac* mRNA was also assessed for *Ae. albopictus*. In this experiment, no control injections with *pBac* helper plasmid were performed, as the primary purpose was to create landing site lines for RMCE. G_0 survivors were exclusively backcrossed in groups of 10-15 individuals, and the positive G_1 offspring was not further analyzed molecularly to identify different transposition events within a G_0 family. Nevertheless, this experiment indicated a similarly high transposition rate in *Ae. albopictus* as in *Ae. aegypti*, as 15 of the 19 G_0 families produced positive G_1 (Table 4). This percentage of positive families is comparable to the *Ae. aegypti* mRNA injection experiments #16 and 17, where G_0 were backcrossed in a similar scheme (mostly groups of 12). These group backcrosses produced a similar rate of positive families, i.e., 11 of 12 (exp. 16) and five of six families (exp. 17) (Table 3, Table S2). If only the number of positive families is counted as independent transposition events in these experiments, this results in a transformation efficiency of 7%, i.e., in the same range as observed for the *Ae. albopictus* mRNA injection. We, therefore, assume that the actual transformation efficiency in *Ae. albopictus* was much higher.

Displayed are data from one injection experiment using *pBac* capped mRNA in *Ae. albopictus*. "no. injected embryos" total number of injected embryos; "hatch rate" = no. larvae / total no of injected eggs (%); "eclosion rate" = no. adults / no. larvae (%); "no. transg. events" is the number of independent transposition events observed; "transf. eff." = the minimal transformation efficiency, calculated as: number of independent transgenic events/ total number of G_0 adults. The actual transformation efficiency might be higher (see text). For more details in *Ae. albopictus* transformation data, please see Table S5. * In this plasmid, the *D. melanogaster* PUbCFP cassette was replaced with an *Ae. aegypti* PUbeCFP cassette from pSL1180-HR-PUbeCFP (Addgene plasmid 47917).

2.2. Capped mRNA doesn't improve Cre- or phiC31-RMCE efficiencies in *Aedes.*

Based on the strong positive effect of capped mRNA on *pBac* transformation efficiencies, we subsequently tested the efficiency of capped mRNA in phiC31- and Cre-RMCE experiments in direct comparison to the respective helper plasmid.

For phiC31-RMCE, comparative injections were performed at two different helper/donor concentrations, 150/300 ng/µl and 300/500 ng/µl in *Ae. aegypti*. Concentrations were chosen to be in the same range as concentrations used in phiC31-RMCE experiments in two different landing site lines previously performed in our laboratory (Table S6). They were also comparable to the *pBac* mRNA concentrations. Five independent injections were performed for the lower concentration, two with the phiC31 helper plasmid and three with the phiC31 mRNA. For the higher concentration, two replicates each were performed (Table 5). No significant differences in the larval hatch rate, adult eclosion rate, or G_0 fertility were observed between helper plasmid and mRNA injections ($p = 0.109$, $p = 0.181$, $p = 0.456$, respectively, statistics done across all concentrations; see also Fig. S2;). Based on the numbers obtained with *pBac* mRNA, one transgenic event would have been expected for about every 50th injected embryo, assuming a similar efficiency. However, the overall recombination efficiency was very low. Only in one of the three replicates with mRNA at the lower helper/donor concentration, we observed one RMCE event. In comparison, we identified one donor plasmid integration event in one of the two replicates with helper plasmid at the higher helper/donor concentration (Table 5). The corresponding recombination efficiencies within the replicates were 10 and 6.45%, respectively, and 3.34 and 3.23% across the replicates.

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Table 4. Transformation data in *Ae. albopictus* using capped mRNA as *piggyBac* source.

Exp. no	helper template	[helper/ donor] (ng/µl)	donor construct	donor plasmid (insert) size (bp	no. injected embryos	hatch rate (%)	eclosion rate (%)	total no. G_0 families	no. fertile G_0 families	no. transg. G_0 families	total G_1 screened	no. transg events	no. transg. events/ G_0	transf. eff. (%)
1	mRNA	300/150	AH452 modified *	(5205) 7845	721	39.53	83.51	19	19	15	7049	≥ 15	≥ 1	6.3 %

Table 5. phiC31-RMCE injection data in *Ae. aegypti* using phsp-phiC31 helper plasmid or capped phiC31 mRNA.

Exp. no	helper template	[helper/ donor] (ng/µl)	donor construct	donor plasmid (insert) size (bp	no. injected embryos	hatch rate (%)	eclosion rate (%)	total no. G_0 families	no. fertile G_0 families	no. transg. G_0 families	total G_1 screened	no. transg. events	recomb. eff. (%)
1	*phsp*-phiC31	150/300	V101	(4404) 8216	510	3.14	75.00	9	6	0	1690	0	0
2	*phsp*-phiC31	150/300	V101	(4404) 8216	237	7.59	61.11	5	5	0	2851	0	0
3	mRNA	150/300	V101	(4404) 8216	502	4.38	59.09	11	4	0	534	0	0
4	mRNA	150/300	V101	(4404) 8216	335	3.28	54.55	4	2	0	458	0	0
5	mRNA	150/300	V101	(4404) 8216	361	3.32	83.33	7	4	1 *	318	1	10
6	*phsp*-phiC31	300/500	V101	(4404) 8216	443	2.71	91.67	9	4	0	1059	0	0
7	*phsp*-phiC31	300/500	V101	(4404) 8216	302	13.25	77.50	11	9	2	6068	2	6.45
8	mRNA	300/500	V101	(4404) 8216	412	2.18	100.00	9	9	0	6250	0	0
9	mRNA	300/500	V101	(4404) 8216	248	13.71	73.53	10	6	0 **	3650	0	0

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The efficiencies obtained with the helper plasmid were in the same range as those observed in the previous experiments, performed with the same or different donor constructs into different landing site lines (Table S6, exp. I - V).

Data from comparative injection experiments are displayed at two different helper/donor concentrations into the same landing site line; "no. injected embryos" represents the number of black embryos 24 h post injection; "hatch rate" = no. larvae / no. black eggs (%); "eclosion rate" = no. adults / no. larvae (%), "no. transg. events" is the number of independent recombination events observed; "recomb. eff." is the minimal recombination efficiency, calculated as: number of transgenic events/ total number of G_0 adults. The recombination efficiency can be higher, as in group backcrosses of G_0, the number of infertile G_0 is unknown. All positive individuals obtained showed the RMCE phenotype. * one additional family with transient donor phenotype; parental phenotype in next generation; ** one family with transient donor phenotype; parental phenotype in next generation

In phiC31-RMCE injections, the positive effect of the mRNA could not be observed when mRNA concentrations similar to the *pBac* transformations were used. Thus, concentrations were increased for Cre-RMCE injections to 450 ng/µl helper and 350 ng/µl donor. Helper plasmid and mRNA injections were performed in three independent replicates each. Similar to the phiC31 experiments, only one plasmid or mRNA replicates produced recombination events. In one of the plasmid helper injections, one family produced an RMCE phenotype, and another one offspring with a donor plasmid integration phenotype, corresponding to an overall minimum recombination efficiency of 2.44% (0.8% across all replicates, Table 6). In the mRNA injections, a single family produced offspring with an integration phenotype, corresponding to a minimum recombination efficiency of 3% (1% across all replicates). For complete injection, crossing, and screening data, see Table S7.

Data from comparative injection experiments are displayed at two different helper/donor concentrations into the same landing site line; "no. injected embryos" represents the number of black embryos 24 h post injection; "hatch rate" = no. larvae / no. black eggs (%); "eclosion rate" = no. adults / no. larvae (%); "no. transg. events" is the number of independent recombination events observed; "recomb. eff." is the minimal recombination efficiency, calculated as: number of transgenic events/ total number of G_0 adults. The recombination efficiency can be higher, as in group backcrosses of G_0, the number of infertile G_0 is unknown. * 1 integration, one RMCE phenotype; ** integration phenotype.

We also performed Cre-RMCE injections in *Ae. albopictus* into two different landing site lines. In one line (17A1), we tested injecting with either 150 ng/µl phsp CRE or 190 ng/µl helper mRNA. From 34 fertile families of G_0 founders injected with helper plasmid, one showed expression of the donor transgene in addition to the original landing site marker, indicating an integration event. This translates into a recombination efficiency with Cre helper plasmid of 2.27 % (Table S8). None of the 46 fertile families made of G_0 founders injected with helper mRNA showed expression of the transgene.

In a second line (1A3), the landing site integrated 1.3 Mb upstream of the Nix locus as determined via inverse PCR, and marker fluorescence was only observed in males. Thus, all injected embryos were heterozygous for the landing site. Notably, in this line, the *Ae. aegypti* PUbeCFP expression was not visible, but PCR could confirm the presence of the sequence. This line was only injected with 150 ng/µl phsp-CRE, and one showed expression of the donor plasmid fluorescence marker from a total of six fertile families. PCR of the animals that showed transgene expression suggested both excision of the original PUbeCFP sequence and multiple, potentially tandem, integrations of the plasmid, i.e., non-canonical recombination events.

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Table 6. Cre-RMCE injection data in *Ae. aegypti* using phsp-Cre helper plasmid or capped Cre mRNA.

Exp. no	helper template	[helper/ donor] (ng/µl)	donor construct	donor plasmid (insert) size (bp	no. injected embryos	hatch rate (%)	eclosion rate (%)	total no. G_0 families	no. fertile G_0 families	no. transg. G_0 families	total G_1 screened	no. transg. events	recomb. eff. (%)
1	*phsp*-Cre	450/350	V20	(1297) 4997	765	18.69	57.34	13	12	2 *	9283	2	2.44
2	*phsp*-Cre	450/350	V20	(1297) 4997	165	6.67	72.73	4	1	0	390	0	0
3	*phsp*-Cre	450/350	V20	(1297) 4997	397	1.26	80.00	3	1	0	137	0	0
4	mRNA	450/350	V20	(1297) 4997	413	10.90	73.33	4	1	1 **	2419	1	3.03
5	mRNA	450/350	V20	(1297) 4997	230	5.22	58.33	3	3	0	996	0	0
6	mRNA	450/350	V20	(1297) 4997	348	5.75	80.00	12	9	0	4926	0	0

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3. Discussion

Producing transgenic insects can be time-consuming because transgenesis efficiency can be very low in many insect orders and species. This is particularly true for *Aedes* mosquitoes using *piggyBac* transformations and recombination in cassette exchange experiments (RMCE). In many experiments published to date, the *phsp-pBac* helper plasmid [9] was used as a source of *pBac* transposase, with the *D. melanogaster hsp70* promoter to drive transposase expression. Minimum transposition rates in these publications are reported to be between 0 and 4 % [12, 30, 31, 35]. This transposition rate doesn't improve when the embryos are heat-shocked shortly after injection [30]. A large meta-analysis of *pBac* transformation efficiencies reports an average of 7% for *Ae. aegypti* (46 experiments, 3314 total G_0 adults, 239 independent transgenic lines) and 1.6% for *Ae. albopictus* (10 experiments, 5339 G_0 adults, 89 independent lines).

The hyperactive version (ihyPBase) of *pBac* transposase helper plasmid didn't yield transgenic lines in *Ae. aegypti* in three independent experiments with two different constructs and more than 170 G_0 adults. These injections used similar injection numbers and a similar size of the transgene constructs as in Eckermann et al., but at lower donor and helper plasmid concentrations than the ones used by these authors (300 ng/µl and 500 ng/µl, respectively) [34]. Moreover, Eckermann et al. performed individual backcrosses of all G_0, while we did group backcrosses. This might have masked the presence of transgenic G_0 in case those were less competitive than the non-transgenic G_0. On the other hand, at similar efficiencies as the ones observed by Eckermann et al. a few transgenic events should still have occurred. Instead, our results confirm the experiments by Wright et al., who also didn't obtain transposition events with ihyPBase in *Ae. aegypti*. However, we did not observe the G_0 sterility reported by Wright et al. for both, *Ae. aegypti* and *D. melanogaster* [33]. This might be attributed to the approximately two-fold lower ihyPBase helper concentrations or the absence of a heat shock treatment of the injected embryos [33] in our study. ihyPBase seemed to result in lower larval hatch rates than the standard phsp-*pBac* plasmid in our injections. However, the three injections performed with ihyPBase are not enough to assess if there is an actual effect. In *D. suzukii*, the ihyPBase helper plasmid yielded similar transposition efficiencies as the standard *pBac* helper plasmid. There were also no differences in the other life parameters between the ihyPBase and standard *pBac* helper plasmid injections in *D. suzukii*.

We hypothesized that the *D. melanogaster hsp70* promoter does not function well in *Aedes* mosquitoes, causing low transposase levels. Therefore, we performed *pBac*-mediated transformations using *in vitro* transcribed, capped mRNA. Comparing the results of 13 injections with the *pBac* helper plasmid and nine injections with capped mRNA in *Ae. aegypti*, the transposition efficiencies using capped mRNA on average went up more than 30-fold, from 0-5% to around 50%, some even higher. Thus, while hundreds of embryos had to be injected to obtain a transgenic line using the *pBac* helper plasmid, with the mRNA on average, a few dozen embryo injections were sufficient. These data are based on the results obtained with two different *Ae. aegypti* strains, Higgs White Eye, and Orlando, six different donor plasmids, three different mRNA concentrations, and different injection personnel. It is important to note that the transposition efficiencies reported in this work are minimal efficiencies, as the original purpose of the experiments was not to determine transposition efficiencies. Therefore, G_0 adults were backcrossed in groups to a certain extent in each injection. The actual efficiencies will consequently be higher, especially in mRNA injections # 16 and 17 (V370 and V369 donor, respectively), where all G_0 individuals were backcrossed in large groups, and only a fraction of the positive offspring was analyzed molecularly. All other mRNA injections provide a more accurate transposition efficiency estimate because most G_0 were backcrossed individually or in small groups. However, for all efficiency calculations, the total number of G_0 was considered, not the number of fertile G_0. Taking all individually backcrossed G_0 from all *pBac* injections and assessing their fertility, it can be estimated that the average G_0 fertility was about 57% for both mRNA helper and plasmid helper injections. The observed G_0 infertility rate of mostly 35-45% matches well with published numbers for *Ae. aegypti* [30, 31].

The results of the capped *pBac* mRNA injections in *Ae. albopictus* strongly suggest that capped mRNA has similarly high activity in *Ae. albopictus* as in *Ae. aegypti*. Even though the experiment didn't include individual G_0 backcrosses and the positive G_1 were not further assessed for integration copy number and integration sites, the high percentage of positive families is similar to that obtained in the *Ae. aegypti* injections #16 and 17, where we used a very similar G_0 group backcrossing scheme (mostly 12 G_0 per cage). Such a high percentage of positive families was not observed in any *pBac* helper plasmid injection in *Ae. aegypti*. If only the number of positive families is considered as the number of independent events for the transformation efficiency calculation of the *Ae. aegypti* injections #16 and 17, the transformation efficiency would be between 6 and 7%, i.e., identical to the one obtained for *Ae. albopictus* using this calculation.

One important effect of the mRNA-mediated transformation was that many G_1 individuals contained more than one genomic copy of the transgene construct, as determined by ddPCR. Therefore, with *pBac* mRNA injections, it is recommended to do single backcrosses of at least some of the positive G_1. Analyzing the copy number after line establishment is necessary to identify lines with a single integration event. One approach to minimize the frequency of multiple genomic insertions would be to titrate the mRNA concentration used in injections to optimize the fraction of G_1 carrying only one genomic integration. We did not observe an apparent correlation between the mRNA concentration and the max. number of integrations per individual or the relative frequency of G_1 with more than one integration. However, a precise analysis of a possible correlation was not possible, as in mRNA injections #16 and 17, the positive individuals were group backcrossed until G_2. Molecular analysis of integration events was only performed with generation G_3. The two generations of backcrossing integrations might have segregated, consistent with the overall low number of integration events per individual observed in these two injections compared to the other experiments.

Transformation efficiencies with mRNA observed in our hands were substantially higher than the numbers reported for *Aedes* mosquitoes in the literature [7, 14, 27]. Specifically, in one large-scale experiment (6000 embryos), Labbe et al. injected a *pBac* helper plasmid and helper mRNA mix (200 ng/µl and 300 ng/µl, respectively) into *Ae. albopictus*, resulting in a transformation efficiency of 2.2-3.6% [14]. This estimate of transformation efficiency incorporates a correction for an assumed 30-50% G_0 infertility. Haghighat-Khah et al. injected 700 ng/µl helper mRNA in *Ae. aegypti* embryos, resulting in only 1% transposition efficiency (two experiments totaling 3000 embryos) [27]. Only one in nine independent lines resulting from their injections showed more than one transgene integration. We can only speculate about the reason for the differences in transposition efficiencies between published work and our study. Based on our observations with 100 and 300 ng/µl of *pBac* mRNA in the injection mix, it is possible that at 700 ng/µl, barely any germline cells with only one or very few integrations arose. Instead, most transformed germ cells had so many integrations that the resulting zygotes were not viable. Additionally, the strain's genetic background and the number of generations the strain had been cultivated in the lab could influence the transformation efficiency. While we injected into HWE and Orlando laboratory strains, the experiments by Labbe et al. and Haghighat-Khah et al. were performed in strains of Malaysian origin [14, 27].

Injections using phiC31 capped mRNA for RMCE in *Ae. aegypti* have been published before. In three different landing site line injections, using 730 ng/µl mRNA helper in the injection mix, the authors obtained no recombination event, once obtained only a donor plasmid integration event, and once obtained an RMCE event. Both of the latter events occurred at an efficiency between 4 and 5 % [27]. Our study included parallel helper plasmid injections into embryos from the same female cohorts to directly compare the recombination efficiencies with plasmid helper and mRNA helper. Based on the high transposition efficiencies observed with *pBac* mRNA, it was unexpected that efficiencies for RMCE experiments were not increased using mRNA as a recombinase source. This result occurred for phiC31 and Cre recombinase, where even higher mRNA concentrations were used (Table 5, 6). The phiC31-RMCE efficiency of 10% achieved in one of the injections is in the same range as the published efficiencies [27], although at about 5-fold lower mRNA concentration.

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The reasons for the lack of an mRNA effect in RMCE experiments in *Ae. albopictus* and *Ae. aegypti* are currently not known. We can exclude the inactivity of the injected mRNA due to degradation, because for every injected slide of embryos, the leftover injection mix from the needle was recovered and run on a gel to confirm mRNA integrity. One big difference between *pBac*-mediated transposition and Cre- or phiC31-mediated recombination is the lower frequency of sites in the genome at which recombination events can occur. *pBac* transposase uses TTAA sequences in the genome for insertion, which are predicted to occur every 256 base-pairs. In contrast, the recombination sites for Cre and phiC31 don't occur naturally in *Aedes* genomes. Thus, the genomes of our landing site lines contain exactly one position where recombination can take place, and the rate-limiting step might be the likelihood of the enzyme and donor plasmid being present at this position simultaneously. This likelihood can be increased by increasing the amount of injected mRNA and donor plasmid. However, this would be in the range of a maximum two- to three-fold increase as the viscosity of the nucleic acid solution limits the injectability.

Moreover, experiments published previously by us and others show that in case of Cre recombinase, the amount of Cre enzyme in the commonly used injection concentrations is not limiting. In genomic excision experiments, excision rates between 25% and 100% were obtained when both recombination sites were close [27, 35, 40]. Furthermore, the high efficiency of the excisions also excludes the possibility that heterochromatic factors silence the landing site constructs. A reason for the missing effect of the mRNA in RMCE injections might be the limited likelihood of co-localizing all three components for RMCE (landing site, enzyme, donor plasmid) at the same time at a single site in the genome. On the other hand, the same situation applies to other insect species, such as *D. melanogaster*, *D. suzukii*, and *Anastrepha suspensa*, in which RMCE efficiencies between 10 and 20% were achieved (single experiments, [24, 26, 41, 42]).

Nevertheless, for Cre-RMCE, we obtained the first one-step RMCE published so far in *Aedes aegypti*. In previous experiments, we only achieved two-step Cre-RMCE, obtaining an integration line first, from which, by injection of only Cre helper plasmid, the complete RMCE event was obtained [35].

4. Materials and Methods

4.1. Insect rearing

Aedes aegypti wild-type strains and transgenic lines were reared in an insectary at constant conditions of 27°C, 70% RH, and a 12:12 hrs light:dark cycle. Larvae were fed on Tetra TabiMin fish food pellets. The adult mosquito diet was sterile-filtered 10% (w/v) sucrose solution. Moreover, adult females were fed once per week with pig blood purchased from a butcher shop. *Aedes aegypti* laboratory strains used in the experiments were the Orlando wild type strain and the Higgs White Eye (HWE) strain (a spontaneous white eye mutant strain of the Rexville D strain from Puerto Rico [43]).

A lab colony of *Aedes albopictus* was established with pupae and larvae collected from an auto-salvage yard in Manassas, Virginia, in 2018. Animals were reared under standard laboratory conditions at 21-26°C, 80% RH, 16 h light:8 h dark for three generations prior to injections for *pBac*-mediated transformation [44]. Larvae were fed on a MWF schedule with 1 ml of a larval food slurry consisting of 1 liter DI water, 120 g dog food (Nutro Ultra Small Breed Puppy, Nutro Products Inc., Franklin, TN, USA), and 40 g frozen brine shrimp (Sally's Frozen Brine Shrimp, San Francisco Bay Brand, Newark, CA, USA) [45]. Adult females were provisioned with organic raisins (Newman's Own, Westport, CT, USA) to allow ad libitum sugar feeding. They were allowed to blood feed on a human host for egg production. The Georgetown University IRB has determined that mosquito blood feeding is not human research and does not require IRB approval; however, the blood feeding protocol has been approved by the Georgetown University OHS office.

The wild-type *D. suzukii* USA strain and transgenic lines were maintained at 25°C and 55–60% humidity with a 12 h photoperiod. Flies were briefly anesthetized with CO_2 for screening and to set up crosses.

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4.2. *In vitro* transcription (IVT) of *pBac*, phiC31, and Cre mRNA for injections

4.2.1. Production of the IVT templates

pBac IVT template was obtained by PCR on the *phsp-pBac* plasmid [9], using the forward primer P1269 (5'GAAACTAATACGACTCACTATAGGGAGAGCCGCCACatgggtagttctttagacgatg; upper case letters represent T7 initiation sequence and linker) and the reverse primer P1270 (5'cttattagtcagtcagaaacaac). The PCR reaction contained 2 ng plasmid DNA, 500 nM of each primer, 200 µM of each dNTP, 1x Q5 reaction buffer, and 1 µl Q5 Polymerase (NEB) in a final volume of 100 µl. The reaction was run in a Biorad C1000 Touch Thermal Cycler (initial denaturation at 98°C for 30 sec, 30 x [98°C for 10 sec; 51°C for 20 sec; 72°C for 1 min] followed by the final elongation at 72°C for 2 min).

Cre and phiC31 IVT templates were obtained by PCR on plasmids AH445 (phsp-Cre) and AH444 (phsp-phiC31), respectively. Primers were P2203 (5'GAAACTAATACGACTCACTATAGGGAGAGCCGCCACatgtccaatttactgaccgtacacc) and P2204 (5'gctaatcgccatcttccagcag) for Cre, and P1630 (5'GAAACTAATACGACTCACTATAGGGAGAGCCGCCACatggacacgtatgccggtgcttac) and P1631 (5'ctaggccgctacgtcttcggtgc) for phiC31. The PCR reaction contained 10 ng plasmid DNA, 500 nM of each primer, 100 µM of each dNTP, 1x Platinum Taq reaction buffer, 1.25 mM MgCl2, and 1 µl Platinum Taq DNA Polymerase (Life Technologies) in a final volume of 50 µl. The reaction was run in a Biorad C1000 Touch Thermal Cycler (initial denaturation at 95°C for 2 min, 35 x [94°C for 30 sec; 59°C for 30 sec; 72°C for 2 min] followed by the final elongation at 72°C for 10 min).

The PCR products were analyzed and purified by 1% agarose gel electrophoresis and gel extracted with the ZymoClean Gel DNA Recovery kit according to the manufacturer's instructions.

4.2.2. In vitro transcription reaction and mRNA purification

IVT was performed using the NEB HiScribe T7 Arca mRNA kit (#2060S) according to the manufacturer's instructions, using 800 - 1000 ng IVT template. mRNA was purified using the MegaClear Transcription clean-up kit (AM1908) according to the manufacturer's instructions choosing elution option 1 (50 µl elution solution on the column, incubate at 65°C for 5 min) performed twice, and including the optional Ammonium Acetate precipitation. mRNA quality was analyzed by agarose gel electrophoresis, and mRNA was stored in 5 µl aliquots at -80°C until use.

4.3. Preparation of injection mixes

Transposase, recombinase-encoding helper plasmids, or in vitro transcribed, capped mRNA were mixed with the corresponding donor plasmids at the final concentrations specified in Tables 1-5 in RNAse-free 1x embryonic injection buffer (5 mM KCl, 0.1 mM NaPO4, pH 6.8). To remove particles and dust that could clog the injection needles, the injection mixes were centrifuged at 13000 rpm for 30 min at 4°C. The supernatant was taken carefully without disturbing a possible pellet and stored in 5 µl aliquots at -80°C until further use.

4.4. Embryonic microinjections of *Ae. aegypti*

Injections for *pBac*-mediated transformation: *Ae. aegypti* transgenic lines were created by injecting preblastodermal embryos of the HWE strain with the *phsp-pBac* helper plasmid [9] or *pBac in vitro* transcribed, capped mRNA and a donor plasmid at varying concentrations (see Table 1 and 3), in 1x embryonic injection buffer (EIB; 5 mM KCl, 0.1 mM NaPO4, pH 6.8). Injected embryos were kept moist for two days to allow completion of embryonic development before transferring to water with some TabiMin fish food for hatching. Survivors were sexed in the pupal stage and backcrossed to HWE in small groups or individually. G_1 offspring was collected for 3-6 gonotrophic cycles and screened for the presence of the transgenic marker (DsRed or eGFP fluorescent protein) at the larval or pupal stage. Positive G_1 were again backcrossed individually or in groups to establish transgenic lines.

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Injections for recombinase-mediated cassette exchange: preblastoderm embryos of the *Ae. aegypti* landing site lines for phiC31-RMCE (lines V19-M2M1, V19-M26M3m2; V19 landing site construct = attP_3xP3-eGFP_attPrev) or Cre-RMCE (line V3-M30M1; V3 landing site construct = FRT_3xP3-DsRed_FRT3_loxN_3xP3-FRT5_AmCyan-lox2272-loxP_attP220rev) were injected with the recombinase helper plasmid or in vitro transcribed, capped recombinase mRNA and a donor plasmid at varying concentrations (see Tables 4 and 5), in 1x EIB. Further rearing and crossing were identical to *pBac* injections described above.

4.5. Embryonic microinjections of *Ae. albopictus*

4.5.1. Preparation of donor plasmid

To generate the modified AH452 construct (pXL-BACII_FRT_3xP3DsRed_FRT3_loxN-PUbeCFP-lox2272), the *D. melanogaster* PUbCFP cassette from AH452 [27, 35, 40] was replaced with an *Ae. aegypti* PUb-eCFP cassette from pSL1180-HR-PUbeCFP (Addgene plasmid 47917). One fragment from the AH452 plasmid was amplified using primers Vector_F (5′ CTAAATTGTAAGCGTTAATATTTTG 3′) and Vector_R (5′ATAACTTCGTATAAGGTATACT 3 ′), and a second fragment was amplified using primers SG_frag_2_Forward (5′ CTGGATCATAACTTCGTATAGGATACTTTATACGAAGTTATAACTCGAC 3′) and SG_frag_2_Reverse (5′ ACGCTTACAATTTAGGTGGCACTTTTCGGGGAAATGT 3′). A third fragment containing the *Ae. aegypti* PUb-eCFP cassette was amplified from pSL1180-HR-PUbeCFP using primers SG_frag_1_Forward (5′ CTTATACGAAGTTATTATCTTTACATGTAGCTTGTGCATTGAATCCAA 3′) and SG_frag_1_Reverse (5′ GAAGTTATGATCCAGACATGATAAGATACATTGATGAGTTTGG 3′). All PCR bands were gel excised and purified using a QIAquick Gel Extraction Kit (QIAGEN, Germantown, MD, USA). Bold sequences in SG_frag_1_Forward and Vector_R, in SG_frag_1_Reverse and SG_frag_1_Forward, in Vector_R and SG_frag_1_Reverse, and Vector_F and SG_frag_2_Reverse represent complementary sequences. The complementary ends of each fragment were then annealed in a single reaction using an In-Fusion Cloning Kit (Takara, Ann Arbor, MI, USA).

4.5.2. Injections for *pBac*-mediated transformation

Aedes albopictus transgenic lines were created by injecting preblastodermal embryos with *pBac in vitro* transcribed, capped mRNA (300 ng/µl) and the donor plasmid pXL-BACII_FRT_3xP3DsRed_FRT3_loxN-PUbeCFP-lox2272 (150 ng/µl) in 1x EIB. Injected embryos were kept moist for two days to allow completion of embryonic development before transferring to water with three droplets of larval food slurry (described in *Ae. albopictus* insect rearing above). Survivors were sexed at the pupal stage and backcrossed to WT individuals in small groups of 10-15. G_1 offspring were collected for 2-3 gonotrophic cycles and screened for the presence of the transgenic marker (DsRed or eCFP fluorescent protein) at the larval or pupal stage. Positive G_1 were again backcrossed individually to establish transgenic lines.

4.5.3. Injections for recombinase-mediated cassette exchange

Pre-blastoderm embryos of an *Ae. albopictus* landing site line for Cre-RMCE (line 17A1; landing site construct = pXL-BACII_FRT_3xP3DsRed_FRT3_loxN-PUbeCFP-lox2272) were injected with either the recombinase helper plasmid (150 ng/µl) or with *in vitro* transcribed, capped recombinase mRNA (190 ng/µl) and a donor plasmid (250 ng/µl) in 1x EIB. Survivors were sexed at the pupal stage and backcrossed to WT individuals (see the Manassas, VA collected population described above) individually or in small groups of 1-3. G_1 offspring were collected for 2-3 gonotrophic cycles and screened for the presence of the transgenic marker (3xP3AmCyan) at the larval or pupal stage. Positive G_1 individuals were backcrossed individually.

An additional *Ae. albopictus* transgenic line (1A3) with a sex-linked landing site (the landing-site integrated 1.3 Mb upstream of the *Nix* locus as determined via inverse PCR and was only observed in males) was injected with recombinase helper plasmid (150 ng/µl) and a donor plasmid (250 ng/µl)

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in 1x EIB. Survivors were sexed at the pupal stage and backcrossed to WT individuals individually or in small groups of 4-12. G_1 offspring were collected for two gonotrophic cycles and screened for the transgenic marker (3xP3AmCyan) at the larval or pupal stage. Positive G_1 were again backcrossed individually.

4.6. Embryonic microinjections of *D. suzukii*

Germline transformation with *piggyBac* constructs was carried out as previously described [11]. A mixture of the *piggyBac* donor construct (500 or 700 ng/µl) and the phsp-*pBac* or the phsp-HypB transposase helper (200 or 300 ng/µl) was injected into WT embryos. In a third series of experiments, the two helper plasmids were combined at 200 ng/µl each with 500 ng/µl donor plasmid. G_0 adults were individually (unless otherwise stated) crossed to WT flies, and G_1 flies were screened for fluorescence. Segregation tests were conducted by outcrossing the transformants to WT flies, and transgenic lines were established from single G_1-positive adults.

4.7. Copy number variation (CNV) and linkage analysis of transgene integrations by droplet digital PCR

The number of genomic integrations of the transgene cassettes in *Ae. aegypti* was analyzed by ddPCR probing for the eGFP or DsRed marker genes. The reference gene was mEF1, a one-copy gene in the *Ae. aegypti* genome. ddPCR was performed with the Biorad QX200 and Auto-DG System. 20 µl CNV reactions contained 20-100 ng *Ae. aegypti* genomic DNA, 1x ddPCR Supermix for probes (Biorad #1863010), 1x primer-probe mix target gene (FAM-labeled), 1x primer-probe mix reference gene (HEX-labeled), and 1U EcoRI (NEB: EcoRI cuts within the transgene constructs but not within the PCR amplicons). Droplets were generated with the Automated Droplet Generator. PCR cycling conditions (deep well block) were: 95°C for 10 min, 40x [94°C for 30 sec, 60°C for 1 min], 98°C for 10 min, 4°C hold. The ramp rate was 2°C /sec. Primer and probes were prepared and stored as a 20x primer-probe mix consisting of 18 µM each of forward and reverse primer and 5 µM probe (the final concentration of primers and probe in the reaction was 900 nM and 250 nM, respectively). Primers and probe used for target gene EGFP were: EGFP-for (P106) = 5'caaagaccccaacgagaagc, EGFP-rev (P108) = 5'gtccatgccgagagtgatcc, EGFP-probe = 5' FAM-cgatcacatggtcctgctgg-BHQ1. Primers and probe used for target gene DsRed were: DsRed-for (P49) = 5'gatccacaaggccctgaagc, DsRed-rev (P50) = 5'gctccacgatggtgtagtcc, DsRed-probe = 5' FAM-tcgttgtgggaggtgatgtc-BHQ1. Primers and probe used for reference gene mEF1 were: mEF-for (P63) = 5'tccggtttgcctacgatacc, mEF-rev (P64) = 5'actgggcagttgtactcacg, mEF-probe = 5' HEX-tcgggaatgggtgaattgca-BHQ1.

The distribution of positive and negative droplets in each well was analyzed individually and the threshold corrected manually if necessary.

For linkage analysis, the CNV experiment was once conducted with restriction digest and once without and the results were compared. If two transgene cassettes are linked on the same chromosome, the copy number obtained from the undigested reaction is approximately ½ of the digested reaction. Linkage analysis requires cautious preparation of genomic DNA to avoid shearing forces. Depending on the extent of shearing and the distance of the two integration sites, the value obtained from undigested DNA can converge towards the value of the digested sample.

4.8. Analysis of genomic integration sites by inverse PCR

Genomic locations of the transgene constructs were determined by inverse plus nested PCR according to the following protocol: genomic DNA (600 ng) was digested with 4 U MspI in a 20 µl reaction for 1 hr at 37°C. Digested DNA was immediately precipitated, pelleted, and re-dissolved in 50 µl TE buffer. The complete amount of MspI-digested DNA (50 µl) was used for the subsequent self-ligation reaction in 350 µl total volume containing 1x T4 Ligation Buffer and 2 µl T4 DNA ligase (NEB, 400,000 U/ml) overnight at 16°C. Ligated DNA was precipitated and dissolved in 50 µl. 3 µl DNA was PCR amplified in 20 µl containing 1x Phusion Flash High-Fidelity Polymerase Mastermix (Thermo Scientific F548S) and 500 nM of each primer. Cycling conditions for touchdown inverse PCR

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(iPCR) were: 98°C for 10 sec, 5x [98°C for 1 sec; Tm + 5°C for 5 sec, reduced by 2°C per cycle; 72°C for 1 min], 30x [98°C for 1 sec; Tm + 5°C for 5 sec; 72°C for 1 min], 72°C for 1 min, 12°C hold. The annealing temperature was adjusted for each primer pair.

The iPCR reaction was either directly purified by agarose gel electrophoresis and sent to sequencing, or diluted 1:100, and 1 µl used for the (semi-) nested PCR. (Semi-) nested PCR reactions were identical to the iPCR reactions. Cycling conditions for (semi-) nested PCR (nPCR) were: 98°C for 10 sec, 30x [98°C for 1 sec, Tm minus 5°C for 5 sec, 72°C for 1 min], 72°C for 1 min, 12°C hold. Primers for probing the 3' *piggyBac* integration site were mfs12 (5'CCTCGATATACAGACCGATAAAACAC)/P139 (5'CTTTTATCGAATTCCTGCAGC) (iPCR) and mfs34 (5'CGTACGTCACAATATGATTATCTTTCTAGG)/P139 (nPCR), and for probing the 5' *piggyBac* integration site mfs10 (5'ACGACCGCGTGAGTCAAAATGACG)/ mfs11 (5'ATCAGTGACACTTACCGCATTGACA) (iPCR) and mfs10/mfs31 (5'CGACTGAGATGTCCTAAATGCACAG) (nPCR).

4.9. Transformation efficiency calculation

Minimal transformation efficiency was calculated as the number of obtained independent transgenic lines divided by the total number of adult G_0 survivors of each injection. The actual transformation efficiency in several cases was higher than the numbers reported here for several reasons. All emerged G_0 adults were counted for efficiency calculation, including sterile individuals who are often excluded from such calculations; this was necessary to avoid bias in efficiency calculation between the individual backcross and the group backcross experiments, where sterile individuals would not have been detected. Moreover, not all potentially present independent integration events were detected because i) only the individuals with CN = 1 were analyzed for their genomic integration site by inverse PCR. Thus, potentially different integration events in individuals with CN > 1 were not detected; ii) only a subset of positive G_1 offspring of most G_0 families was individually backcrossed and analyzed, while the rest was group backcrossed and kept as backup; iii) in some experiments with lots of positive G_0 families not all families were further characterized (V19, V369, V370); iv) some G_1 found dead in cages did not produced sufficient quality DNA for digital and inverse PCR. Finally, it has to be noted that individual backcrossing in V369 and V370 injections was only started at the G_3 generation after two generations of group backcrosses of positive individuals. Not all positive G_1 were used for backcrossing. Some integration events might have been lost.

4.10. Statistics

Differences in fitness parameters and transgenesis efficiencies between phsp-*pBac* and phsp-HypB injections or helper plasmid and helper mRNA injections were analyzed with single factor ANOVA, at alpha = 0.05.

5. Conclusions

piggyBac mRNA strongly increased the *piggyBac* transformation efficiencies in *Ae. aegypti* and *Ae. albopictus*. The use of mRNA probably circumvents inefficient transcription from *pBac* helper plasmids. In contrast, RMCE efficiencies could not be improved in the two tested *Aedes* species. Here, the limiting factor might not be the recombinase availability in the first place but rather the more complex reaction dynamics.

Our results show that using *pBac* mRNA could be a promising approach to improve transformation efficiencies in other insects, especially if low efficiency is suspected to be caused by low transposase expression from the helper plasmid. Moreover, it could also be applied to other transposases such as Hermes, Minos, or Hobo. Therefore, the use of transposase mRNA has the potential to make a change in the field of insect transformation.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. **Table S2: Comparative *pBac* helper plasmid and *pBac* mRNA injection data in**

Ae. aegypti. Data from 13 injection experiments using the *phsp-pBac* helper plasmid over six years and nine injection experiments using capped *pBac* mRNA over five years are displayed. Exp. 1-13 are identical to the ones shown in Suppl. Table S1. Exp. 12, 13, 21, and 22 were performed in parallel with eggs from the same female cohort. Injections 14 and 15 were performed in the WT Orlando laboratory strain and all other injections in the Higgs White Eye strain. Both V368 plasmid helper injections (# 12, 13) yielded just 14 adult survivors versus 34 from the mRNA helper injections. Therefore, a larger experiment might have produced transgenic offspring from the plasmid injections. It can still be said with confidence, however, that the transposition efficiency is much lower than that using mRNA, as with an average of one transgenic event per three G_0 adults, at a similar efficiency to the mRNA injections, the plasmid injections should have resulted in five to seven independent transgenic events for these 14 G_0. "no. injected embryos" represents the number of black embryos 24 h post injection; "hatch rate" = no. larvae / no. black eggs (%); "eclosion rate" = no. adults / no. larvae (%), "no. single/group crossed" shows the number of G_0 backcrossed individually or in groups, respectively. "group size" refers to the number of G_0 individuals per family; "no. transgenic events" refers to the number of independent transposition events observed, which can be higher than the number of transgenic families if more than one founder per family was positive or/and if more than one independent event occurred in a G_0 individual. In the case of the mRNA injections, these numbers are given as minimal numbers, as in none of the families all positive G_1 offspring were analyzed molecularly. Therefore, additional independent transformation events might have been missed. This is especially the case for the V369 and V370 injections, where G_0 were backcrossed mostly in large groups, and only a small subset of G_3 of some of the positive families analyzed molecularly; "total number G_1 of transg. fam." refers to the total number of G_1 screened across all positive families; "no. pos. G_1 of transg. fam." refers to the number of positive G_1 across all positive families within one injection experiment; "G_1 with multiple integrations" = given is the number of G_1 individuals with more than one donor construct integration out of the total number of G_1 analyzed molecularly; "max. no. integr. / individual" = max. number of donor construct integrations detected within one individual by ddPCR; "transf. eff." is the minimal transformation efficiency, calculated as: number of independent transgenic events/ total number of G_0 adults. The transformation efficiency in several families is assumed to be higher (see text). n.a. = not applicable; n.d. = not determined; avg = average; M = male; F = female. **Table S3: Integration event analysis in *pBac* mRNA injections in *Ae. aegypti*.** The detailed backcrossing schemes and integration event analysis are shown for the positive G_1, G_2, and G_3 generations of the *pBac* mRNA injections. The integration copy number was determined by ddPCR. Subsequently, the genomic integration position for individuals with only one integration was determined by inverse PCR. Individuals with more than one integration (by ddPCR) were not further investigated by iPCR for the genomic integration site. For the injections of the donor plasmids V369 and V370, the individual backcrossing of positive individuals was started at the G_3 generation due to limited rearing capacity in the G_1 and G_2 generations. The positive G_1 and G_2 individuals were backcrossed to the parental WT line (HWE) as male or female groups. This most likely is the reason why in these two injections, only one to two copies of the donor plasmid per individual were counted by ddPCR, as integrations located on different chromosomes or with sufficient distance on the same chromosome will have segregated in the G_1 and G_2 generation backcrosses. **Table S4: Determination of the fertility rate of microinjected *Ae. aegypti* G_0 adults.** Across all injections with *pBac* helper plasmid or mRNA, the experiments with single G_0 backcrosses were identified. If three or more G_0 were individually backcrossed in one experiment, it was included in this table to determine the G_0 fertility in the individual backcrosses. The information regarding the total number of individually backcrossed G_0, the number of fertile individual G_0 founders, and the percentage of fertile individual founders is boxed. The percentage of fertile G_0 individuals was calculated as no. of fertile single founder families / no. indiv. backcrossed G_0 adults * 100. **Table S5: *pBac* mRNA injection data in *Ae. albopictus*.** Displayed are detailed data from an injection experiment using capped *pBac* mRNA. "no. injected embryos" represents the number of black embryos 24 h post injection; "hatch rate" = no. larvae / no. black eggs (%); "eclosion rate" = no. adults / no. larvae (%), "no. single/group crossed" shows the number of G_0 backcrossed individually or in groups, respectively. "group size" refers to the number of G_0 individuals per family; "no. transgenic events" refers to the number of independent transposition events observed, which can be higher than the number of transgenic families if more than one founder per family was positive or/and if more than one independent event occurred in a G_0 individual. These numbers are given as minimal numbers, as the positive G_1 offspring were not analyzed molecularly. Therefore, additional independent transformation events were likely missed, because G_0 individuals were backcrossed in large groups; "total number G_1 of transg. fam." refers to the total number of G_1 screened across all positive families; "no. pos. G_1 of transg. fam." refers to the number of positive G_1 across all positive families within one injection experiment; "G_1 with multiple integrations" = given is the number of G_1 individuals with more than one donor construct integration out of the total number of G_1 analyzed molecularly; "max. no. integr. / individual" = max. number of donor construct integrations detected within one individual by ddPCR; "transf. eff." is the minimal transformation efficiency, calculated as: number of independent transgenic events/ total

number of G_0 adults. The transformation efficiency in several families is assumed to be higher (see above). n.a. = not applicable; n.d. = not determined; avg = average; M = male; F = female. **Table S6: Comparison of *phiC31*-RMCE injection data in *Ae. aegypti* using *phsp-phiC31* helper plasmid or capped *phiC31* mRNA.** Displayed are data from comparative injection experiments at two different helper/donor concentrations into the same landing site line (LSL) (exp. 1 -9); previous *phiC31*-RMCE injections with the helper plasmid (exp I – V) had been performed in different landing site lines and at different helper/donor concentrations (400/600 and 200/300 ng/µl). The landing site line used in exp. I – III turned out to have two linked, non-segregating landing site construct integrations, as determined by ddPCR after the injections had been performed. Therefore, in some individuals, we found an RMCE event at one landing site and an integration event at the other landing site. These were counted here only as one recombination event per individual. The LSL used for exp. IV and V had only one genomic integration site; "no. injected embryos" represents the number of black embryos 24 h post injection; "hatch rate" = no. larvae / no. black eggs (%); "eclosion rate" = no. adults / no. larvae (%), "no. transg. events" is the number of independent recombination events observed; "recomb. eff." is the minimal recombination efficiency, calculated as: number of transgenic events/ total number of G_0 adults. The recombination efficiency can be higher, as in group backcrosses of G_0, the number of infertile G_0 is unknown. M = male; F = female; a) RMCE phenotype; one additional family with transient donor phenotype that showed parental phenotype in next generation; b) both integration events; c) one family with transient donor phenotype that showed parental phenotype in next generation; d) all injected eggs counted; e) mixed larvae either from the 400/600 or 200/300 injection; f) 2 RMCE and 2 integration events; g) 1 RMCE and 1 integration event. **Table S7:** Comparison of *Cre*-RMCE injection data in *Ae. aegypti* using *phsp-Cre* helper plasmid or capped *Cre* mRNA. Data from comparative injection experiments are displayed at two different helper/donor concentrations into the same landing site line; "no. injected embryos" represents the number of black embryos 24 h post injection; "hatch rate" = no. larvae / no. black eggs (%); "eclosion rate" = no. adults / no. larvae (%); "no. transg. events" is the number of independent recombination events observed; "recomb. eff." is the minimal recombination efficiency, calculated as: number of transgenic events/ total number of G_0 adults. The actual recombination efficiency can be higher, as in group backcrosses of G_0, the number of infertile G_0 isn't known; M = male; F = female. **Table S8: Comparison of *Cre*-RMCE injection data in *Ae. albopictus* using *phsp*-Cre helper plasmid or capped *Cre* mRNA.** Displayed are data from comparative injection experiments. Plasmid helper injections were performed into two different landing site lines. Exp. 1 was performed with LSL line 1A3, where the landing-site construct integrated 1.3 Mbp upstream of the *Nix* locus. Thus, all injected embryos were heterozygous for the landing site. Exp. 2 was performed with LSL line 17A1; "no. injected embryos" represents the number of black embryos 24 h post-injection; "hatch rate" = no. larvae / no. black eggs (%); "eclosion rate" = no. adults / no. larvae (%); "total number G_1 of transg. fam." refers to the total number of G_1 screened across all positive families; "no. pos. G_1 of transg. fam." refers to the number of positive G_1 across all positive families within one injection experiment; "no. transg. events" is the number of independent recombination events observed; "recomb. eff." is the minimal recombination efficiency, calculated as: number of transgenic events/ total number of G_0 adults. The recombination efficiency can be higher, as in group backcrosses of G_0, the number of infertile G_0 isn't known; M = male; F = female. **Figure S1: Effect of *phsp-pBac* helper plasmid and *phsp*-hyPB helper plasmid in *Ae. aegypti* and *D. suzukii*.** Box and whisker plots (exclusive median) are displayed on the data presented in Tables 1 and 2 for *Ae. aegypti* larval hatch rate (a), G_0 adult eclosion rate (b), G_0 fertility (percentage of fertile G_0 families in each experiment) (c), and the minimal transformation efficiency (%) (d), as well as *D. suzukii* larval hatch rate (e), G_0 adult eclosion rate (f),) and the minimal transformation efficiency (%) (g). The horizontal line represents the median, the cross (x) represents the mean; n.s. = no significant difference ($p > 0.05$, single factor ANOVA). **Figure S2: Effect of *phsp-phiC31* helper plasmid and *phiC31* mRNA injections in *Ae. aegypti*** on larval hatch rate (a), G_0 adult eclosion rate (b), G_0 fertility (c), and the minimal transformation efficiency (%) (d). Results from injections at low (150/300 ng/µl, exp. 1-5, Table 5) and high (300/500 ng/µl, exp. 6-9, Table 5) helper/donor concentrations were combined for data analysis. Box and whisker plots (exclusive median) are displayed in the data presented in Table 4. The horizontal line represents the median, the cross represents the mean; n.s. = no significant difference ($p > 0.05$, single factor ANOVA). .

Author Contributions: IH and MFS designed the study in *Ae. aegypti*, MH and PA designed the study in *Ae. albopictus*, YY designed the study in *D. suzukii*, IH and TR performed the *pBac* transformation experiments in *Ae. aegypti*, IH, TR, DMC, and HS performed RMCE experiments in *Ae. aegypti*, MH performed *pBac* transformation and RMCE experiments in *Ae. albopictus*, YY performed *pBac* transformation experiments in *D. suzukii*; IH, TR, MH, YY, DMC, and HS analyzed and interpreted the data; IH prepared the original draft of the manuscript, and all authors reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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