The potential for a released autosomal X-shredder becoming a driving-Y chromosome and invasively suppressing wild populations of malaria mosquitoes

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Abstract

Synthetic sex-ratio distorters based on X-chromosome shredding are predicted to be more efficient than sterile males for population suppression of malaria mosquitoes using genetic control. X-chromosome shredding operates through the targeted elimination of X-chromosome-bearing gametes during male spermatogenesis, resulting in males that have a high fraction of male offspring. Strains harboring autosomal constructs containing a modified endonuclease I-*Ppo*I have now been developed in the malaria mosquito *Anopheles gambiae*, resulting in strong sex-ratio distortion towards males. Data are being gathered for these strains for submission of regulatory dossiers for contained use and subsequent field release in West Africa. Since autosomal X-shredders are transmitted in a Mendelian fashion and can be selected against their frequency in the population is expected to decline once releases are halted. However, any unintended transfer of the X-shredder to the Y-chromosome could theoretically change these dynamics: This could lead to 100% transmission of the newly Y-linked X-shredder to the predominant male-biased offspring and its insulation from negative selection in females, resulting in its potential spread in the population and ultimately to suppression. Here, we analyze plausible mechanisms whereby an autosomal X-shredder could become linked to the Y-chromosome after release and provide data regarding its potential for activity should it become linked to the Y-chromosome. Our results strongly suggest that Y-chromosome linkage through remobilization of the transposon used for the initial genetic transformation is unlikely, and that, in the unexpected event that the X-shredder becomes linked to the Y-chromosome, expression and activity of the X-shredder would likely be inhibited by meiotic sex chromosome inactivation. We conclude that a functioning X-shredding-based Y-drive resulting from a naturally induced transposition or translocation of the transgene onto the Y-chromosome is unlikely.

Introduction

Mosquito species of the *Anopheles gambiae* complex are the main vectors of human malaria and pose an enormous burden on global health and economies. Every year, 300–500 million people are infected with malaria and approximately half a million die as a result of these parasitic infections, 70% of these being children less than 5 years old [1]. The progressive spread of insecticide resistant insects [2,3], combined with the current lack of an efficient malaria vaccine, has prompted the development of new methods. One of the most promising is genetic control, based on the release of laboratory-modified insects into the environment; these individuals mate with wild individuals and transmit control traits that can suppress or modify the targeted population[4,5]. The most commonly used approach to genetically control insects has been the mass release of sterile males — the so-called Sterile Insect Technique (SIT) [6,7]. Through mating with released sterile males, wild females are effectively sterilized, if they only mate once during their lifetime, because offspring of sterile males are incapable of development. If sufficient numbers of sterile males are released over a long enough period, the wild population can be effectively suppressed or even eradicated. However, the economic costs associated with mass production of sterile males, the difficulty of covering very large areas, combined with the need to maintain sterile releases for continued suppression, have restricted the implementation of this method to date.

One way to improve the efficiency of such approaches is through the release of fertile males that are daughterless. Since male mosquitoes do not contribute to disease transmission, releasing males that have viable and fertile sons can help to temporarily maintain the control trait in the population. Two strategies based on such fertile males have been developed in mosquitoes: fs-RIDL (for female-specific Release of Insects carrying Dominant Lethals) and sex ratio distorters

66 based on X-chromosome shredding. fs-RIDL is based on a construct that is lethal to females that
67 inherit it, so that daughters of released transgenic males born in the field and inheriting the
68 transgene die before maturing, but sons survive and pass the transgene to their offspring [8,9]. Sex-
69 ratio distortion based on X-chromosome shredding instead relies on the expression of a sequence-
70 specific endonuclease during male spermatogenesis that recognizes and cleaves sequences that are
71 both specific and abundant on the X-chromosome [10,11]. As a result, X-chromosome-bearing
72 gametes are excluded from the fertilizing-sperm population, biasing offspring sex-ratios towards
73 males [12–14]. Mathematical models suggest that both approaches can be more efficient than SIT
74 in terms of the number of modified males that need to be released to achieve a similar level of
75 population suppression [15,16]. Despite being more efficient, both fs-RIDL and autosomal X-
76 shredders (where the transgene is located on an autosome) are self-limiting—i.e. the constructs
77 underlying the phenotype will not spread in the population—because they are inherited in a
78 Mendelian fashion and do not provide any fitness advantage over the wild type, unlike self-
79 sustaining approaches such as those incorporating gene drive constructs [17,18]. Of the two male
80 daughterless systems, autosomal X-shredders are predicted to be more efficient because daughters
81 are effectively replaced with male-forming gametes pre-zygotically. Therefore, on average, twice
82 the number of transgenic male descendants are produced per released male, increasing the effective
83 number of modified males compared to fs-RIDL males. X-shredding also has the potential to be
84 used for self-sustaining genetic control applications, in the form of Y-chromosome drive as
85 originally proposed by Hamilton [5]. This could be done by linking a functional X-shredder to the
86 Y-chromosome, in which case both the Y-chromosome and the X-shredder gain a transmission
87 advantage through preferential inheritance of male-forming gametes [19].

88 An autosomal X-shredding sex-distorter was first developed in *An. gambiae* by Galizi et
89 al. [11]. They used variants of the I-*Ppo*I endonuclease that specifically cut a specific DNA target
90 sequence within the 28S ribosomal DNA locus, which in *An. gambiae* is located exclusively on
91 the X chromosome in approximately 200-400 copies [20]. The I-PpoI protein was modified to
92 restrict its activity to spermatogenesis, since expression of the native I-*Ppo*I resulted in male
93 sterility through postcopulatory, embryonic carryover and consequent dominant embryonic arrest
94 due to the shredding of the maternally inherited X-chromosome [21]. These I-*Ppo*I variants were
95 fused to eGFP and driven by the *An. gambiae beta-2 tubulin* regulatory regions, which become
96 active in primary spermatocytes entering male meiosis [22]. The resulting transformation
97 constructs also included the *DsRed* transformation marker driven by the neuron-specific *3xP3*
98 promoter, and the entire cassette was flanked by piggyBac-specific left and right arms containing
99 the inverted terminal repeat sequences (ITRs) (Figure 1A). These ITRs are characteristic for
100 transposable elements and are required for recognition by the co-injected piggyBac (PB)
101 transposase that catalyzes integration of the construct in the *An. gambiae* genome at TTAA sites.
102 A number of transgenic strains carrying independent autosomal insertions of the I-*Ppo*I protein
103 variants were generated by micro-injecting embryos of the *An. gambiae* G3 laboratory strain. Of
104 all the transgenic strains examined, ^{gfp}124L-2, since renamed by Target Malaria as Ag(PMB)1 (for
105 *An. gambiae* Paternal Male Bias strain 1), showed high sex ratio distortion among progeny of
106 transgenic males (approximately 95% males), without significantly affecting male fertility and
107 fitness [11]. Ag(PMB)1 contains a transgene that encodes a variant of I-*Ppo*I with an amino acid
108 substitution at position 124 in its dimerization domain and which is fused to eGFP with the F2A
109 self-cleaving peptide (eGFP(F2A)I-*Ppo*I-124L). This integrated into chromosome 3R-36D in
110 position 47,762,151bp, from where the sex-distortion phenotype was stably inherited over

111 consecutive generations. In large cage experiments, weekly inoculative releases of transgenic
112 Ag(PMB)1 males led to a reduction in frequency of females and egg productivity of the population
113 over successive generations consistent with model predictions [23].

114 Since Ag(PMB)1 males are fertile their release could produce viable offspring in the field,
115 unlike sterile males. This would provide invaluable information about how transgenic, laboratory-
116 reared males of *An. gambiae* disperse spatially once released through detection of their offspring,
117 even from small-scale releases aimed at capacity-building and methodology development.
118 However, unlike fs-RIDL strains that were directly developed for deployment, the lack of a
119 conditional expression system to control activity of eGFP(F2A)I-*Ppo*I-124L makes this strain
120 unsuitable for large-scale programs aiming directly for population suppression. The Ag(PMB)1
121 transgene was not designed for gene drive in its current form - for example it is not able to home
122 into targeted sequences. It also does not display any fitness advantage over wild type mosquitoes.
123 Consistent with this, models have shown that the Ag(PMB)1 transgene would disappear over time
124 when releases are discontinued [16].

125 However, one feature that is unique to an autosomal X-shredder is the possibility that it
126 could move to the Y-chromosome after release. If active, a Y-chromosome linked X-shredder
127 could directly benefit from the increased transmission of the Y-chromosome, thus increasing in
128 frequency, persisting longer and dispersing further than initial planned. The sequential events
129 required for such a driving Y to occur can be mapped to pathways to harm using a problem
130 formulation approach adopted widely in environmental risk assessments (Supplementary Figure
131 1, Supplementary Figure 2). Three requirements must be fulfilled for a driving Y to occur: (1) the
132 autosomal X-shredder must first move from its original autosomal position and become physically
133 linked to the Y-chromosome; (2) the X-shredder would need to be expressed from its new position

134 on the Y-chromosome during late spermatogenesis in a spatiotemporal manner that is similar to
135 its original expression from the autosome; and (3) it should impart no significant cost to male
136 fertility or male viability as a result of its new Y-chromosome-linkage (Supplementary Figure 1,
137 Supplementary Figure 2).

138 With regard to requirement (1), there are two possible mechanisms that could result in an
139 autosomal transgene moving to the Y-chromosome: (i) a transposase-mediated transposition to the
140 Y-chromosome of the PB transposable element that was used to create the transgenic strain, or (ii)
141 a recombination-mediated reciprocal translocation resulting in large chromosomal rearrangements
142 between the autosome and the Y-chromosome. Of the two mechanisms, translocation is the less
143 likely route, because translocations between autosomal segments and the Y-chromosome occur
144 very rarely in nature (see Discussion). Furthermore, because these events are so rare, they are
145 unlikely to be detected in small laboratory reared populations. On the other hand, transposition
146 from the autosome to the Y through the remobilization of the PB transposon could be possible, if
147 the X-shredder transgene co-occurs in a genome containing an active PB transposase.

148 In this paper, we addressed the possibility of transgene remobilization by examining whole
149 genome sequence data generated from individuals of the Ag(PMB)1 strain, the wild type G3
150 laboratory colony, and field-derived wild-caught individuals from Burkina Faso for the presence
151 of the PB transposase. With regard to requirement (2) above – expression from the Y – we
152 generated two independent transgenic strains containing the eGFP(F2A)I-*Ppo*I-124L (the same in
153 Ag(PMB)1) construct on the Y-chromosome and evaluated the level of expression and sex-ratio
154 distortion. Finally, we discuss implications for male fertility depending on the route of movement
155 to the Y-chromosome.

156

157 **Results**

158 **Evaluating the remobilization potential of the autosomal Ag(PMB)1 X-shredder transgene**

159 The Ag(PMB)1 strain was generated by Galizi et al. [11] by micro-injecting *An. gambiae* G3
160 embryos with a mixture of the transformation plasmid (pBac[3xP3-DsRed]β2-eGFP(F2A)I-*Ppo*I-
161 124L) and a helper plasmid, containing the PB transposase expressed from the *vasa* regulatory
162 regions [24], which direct expression in germline tissues (Figure 1A) [25]. By providing the PB
163 transposase only in *trans* from a transiently co-injected helper plasmid, the transformation
164 construct itself becomes immobilized once it is integrated in the genome. This is because, unlike
165 the complete PB transposable element, the transgene lacks the complete transposase enzyme that
166 is required for remobilization. Therefore, integrated PB transgenic constructs can only be
167 remobilized in mosquito transgenic strains, if a PB transposase source is available [26] .

168 To assess the stability of the Ag(PMB)1 transgene, we first evaluated whether the original
169 insertion site, as described in Galizi et al. [11], has remained stable in chromosome 3R-36D at
170 position 47,762,151 bp, over the approximately 100 generations since its initial generation. We
171 designed PCR primers that span the PB transgene and genomic boundary as originally reported
172 and repeated the PCR using genomic DNA from four pools of five transgenic larvae each of the
173 Ag(PMB)1 strain. PCR amplicons were Sanger sequenced and blasted against the *An. gambiae*
174 genome assembly. Compared to the inverse PCR results from Galizi et al. [11], we found that the
175 genomic integration site has remained identical (Supplementary Figure 3). This suggests that the
176 Ag(PMB)1 transgene has not remobilized in the strain, indicating that PB transposase does not
177 occur naturally in the genome of the laboratory colony. It also suggests that none of the other
178 naturally occurring transposable elements present in this strain are able to remobilize the
179 Ag(PMB)1 transgene, in the absence of PB.

180 To confirm this result, we next tested whether we could detect the gene encoding PB
181 transposase in the genomes of the G3 or Ag(PMB)1 strains. This would exclude the possibility
182 that PB transposase gene is present but is either non-functional, e.g. through mutations in its open
183 reading frame, or suppressed by gene silencing by piRNAs [27,28]. To do this, we generated whole
184 genome sequence (WGS) libraries from genomic DNA extracted from 10 individuals (5 females,
185 5 males) of the Ag(PMB)1 strain and downloaded WGS libraries from 24 previously sequenced
186 G3 individuals from the same insectary colony (PRJNA397539). We mapped the WGS data to the
187 PB helper plasmid originally used to generate the Ag(PMB)1 transgenic strain, containing the PB
188 transposase driven by the 5' and 3' regulatory regions of *An. gambiae vasa* gene. Mapping WGS
189 reads against the helper plasmid ensured that the coding sequence evaluated is experimentally
190 verified to catalyze excision of PB transgenes, instead of a different transposable element that may
191 be related at the sequence level but is unable to excise PB transgenes. Conveniently, the helper
192 plasmid included internal positive controls, in the form of regulatory sequences from the
193 endogenous single-copy *vasa* gene and parts of the flanking PB left and right arms of the
194 Ag(PMB)1 transgene (Figure 1A). We observed a high number of mapping WGS reads from G3
195 samples against the *vasa*-derived regulatory sequences on the helper plasmid, but no continuous
196 mapping in the region corresponding to the PB transposase enzyme (Figure 1B). For the
197 Ag(PMB)1 stain, genomic reads mapped to both the endogenous *vasa* regulatory sequences and
198 to internal sequences of the PB ORF that correspond with the parts of PB left and right arms
199 included in the transformation construct used to generate Ag(PMB)1, as expected (Figure 1A). No
200 reads were detected on the PB coding sequence that is excluded in the transformation construct,
201 thus making it non-autonomous (Figure 1A and B). We then repeated the same analysis using
202 single-mosquito WGS data from 81 field-caught individuals collected in Burkina Faso in 2012

203 (PRJEB1670), which is the site of previous releases of genetically sterile male mosquitoes
204 (Supplementary Figure 4)[29] . Similar to the results from the G3 samples, no reads mapped to the
205 part of the helper plasmid encoding the PB transposase open reading frame with reads mapping
206 exclusively to the regions of the endogenous *vasa* gene. Together, these results suggest that the
207 PB transposase is unlikely to be in the same background as the autosomal Ag(PMB)1 transgene
208 that is being considered for release.
209

210 **X-shredder expression from the Y-chromosome during spermatogenesis**

211 The second requirement for the Ag(PMB)1 X-shredder to display gene drive and invasiveness,
212 assuming the transgene has first moved to the Y-chromosome, is that it is expressed in a correct
213 spatiotemporal manner from its new location. In the Ag(PMB)1 strain, X-shredding is achieved
214 through the expression of the eGFP(F2A)I-*Ppo*I-124L transgene from the *An. gambiae beta2-*
215 *tubulin* regulatory regions, which is active shortly before the first meiotic division in primary
216 spermatocytes and continues throughout the subsequent stages of spermatozoa differentiation [30].
217 In previous work, we have shown that transgenes driven from this promoter are strongly expressed
218 when located on *An. gambiae* autosomes, but when they are inserted on the X-chromosome
219 expression is undetectable [31]. This includes various X-chromosome-linked X-shredder variants,
220 where no significant expression or sex bias was observed [11]. Similar observations of X-linked
221 transgene transcriptional suppression around meiosis have been made in other species as well [32–
222 34]. This phenomenon, called meiotic sex chromosome inactivation (MSCI), is thought to be one
223 of the main driving forces leading to the observed paucity of sperm-specific genes on the X
224 chromosome, both in *An. gambiae* mosquitoes and in other species [35–37]. By comparison, much
225 less is known about transgene expression during spermatogenesis from the *An. gambiae* Y

226 chromosome, which is estimated to be around 26 Mbp long, approximately 10% of the mosquito
227 genome [38], and is composed nearly entirely of a few massively amplified, tandemly arrayed
228 repeats and five known genes [39].

229 To confirm whether MSCI has a similarly inhibitory effect on transgene expression during
230 spermatogenesis on the Y-chromosome as the X-chromosome, we generated two independent
231 transgenic strains harboring the Ag(PMB)1 transgene, eGFP(F2A)I-*Ppo*I-124L, on the *An.*
232 *gambiae* Y-chromosome. The first transgenic strain, called YpBac-β2-^{gfp}124L, was generated by
233 random PB integration. We sequenced the insertion site of the YpBac-β2-^{gfp}124L transgene by
234 inverse PCR on genomic data extracted from transgenic males and found that the construct had
235 inserted within the ubiquitous Y-chromosome-specific transposable element *zanzibar* [40] (Figure
236 2A). The second transgenic strain, called YattP- β2-^{gfp}124L, was obtained by secondary φC31 site-
237 specific integration into an AttP docking site we previously inserted on the Y-chromosome [41].
238 Similar to the YpBac-β2-^{gfp}124L strain, the AttP site is located in a region of the Y-chromosome
239 containing the *zanzibar* repeat, though it is not possible to estimate the distance between these two
240 insertions given the lack of a continuous Y-chromosome genome assembly [40].

241 As would be expected from Y-linked insertions, transgenic offspring from males of both
242 Y-linked strains and wild-type G3 females were exclusively male. Testes from transgenic males
243 of both strains displayed no detectable eGFP signal by fluorescence microscopy, which would be
244 expected if the eGFP(F2A)I-*Ppo*I-124L X-shredder transgene was expressed (Figure 2B).
245 Conversely, expression from the 3xP3-DsRed transformation markers in both strains was
246 phenotypically indistinguishable from autosomal insertions (data not shown), suggesting that Y-
247 chromosome linkage does not interfere with somatic expression of transgenes, at least from these
248 two positions [41]. We next analyzed the levels of eGFP(F2A)I-*Ppo*I-124L transcription in the

249 testes of the YattP-β2-^{gfp}124L and YpBac-β2-^{gfp}124L strains, compared to the Ag(PMB)1 strain.
250 As a control, we also evaluated expression in testes of two additional strains from the Galizi et al.
251 [11] study (^{gfp}124L-3 and ^{gfp}111A-2, called here Ag(PMB)2 and Ag(PMB)3, respectively) and
252 wild-type male. Results from the quantitative RT–PCRs show no significant levels of
253 eGFP(F2A)I-*Ppo*I-124-L expression in the testes from both Y-linked transgenic lines (Figure 2C).
254 Consistent with the lack of X-shredder expression, we did not detect any sex bias among progeny
255 when transgenic males from each Y-linked strain were crossed to wild type G3 females (Figure
256 2A). These results highlight that, as would be expected from MSCI, the Y-chromosome is not
257 permissive to transgene expression from the *beta2-tubulin* promoter during late spermatogenesis,
258 similarly to the X-chromosome.
259
260 **Discussion**
261 Genetic control strategies that aim to suppressing wild populations of mosquito disease vectors
262 have garnered significant interest, and field trials of a number of these systems, including classical
263 SIT, IIT (*Wolbachia*-based sterility), and RIDL are now underway [42,43]. Synthetic sex-ratio
264 distorters based on X-chromosome shredding have now been developed in *An. gambiae* [11,44]
265 and more recently in *Dr. melanogaster* [45]. This system has been shown both theoretically and
266 experimentally to be more efficient than classical SIT, in terms of the number of insects that need
267 to be released [11,15,16]. In their most basic form, autosomal X-shredder constructs are designed
268 to be self-limiting, and their release can potentially result in local and limited suppression if
269 sufficient males are released over a long enough period. Nonetheless, field releases of fertile
270 autosomal X-shredder males have not yet been conducted.

271 Here, we have evaluated the theoretical possibility whereby a released autosomal X-
272 shredder could convert into a self-sustaining, driving Y-chromosome after release. The
273 requirements for such an event to occur include: (1) movement of the X-shredder to the Y-
274 chromosome; (2) its subsequent expression from the Y during late stages of spermatogenesis; and,
275 (3) no cost to male fitness. We reason that there are two possible mechanisms that could result in
276 the linkage of the autosomal Ag(PMB)1 to the Y-chromosome: (1) a transposase-mediated
277 remobilization of the transgene and (2) a large chromosomal rearrangement resulting in the
278 reciprocal translocation between the region of chromosome 3R containing the transgene and the
279 Y-chromosome.

280 We have evaluated the potential of remobilization of the Ag(PMB)1 transgenic construct
281 through transposition, mediated by the intact PB inverted terminal repeats (ITRs) on either side of
282 the transgene cassettes, which were used for the initial generation of the strain. Their presence
283 makes it at least theoretically possible that the Ag(PMB)1 transgene could remobilize from its
284 position on chromosome 3R, if a source of the PB transposase occurs in *trans.* We therefore
285 evaluated whether the PB transposase is present in the genomes of the laboratory Ag(PMB)1 and
286 G3 colonies, and also in field-derived samples from Burkina Faso. We found no evidence of the
287 complete PB transposase coding sequence in any of the samples we sequenced, suggesting that PB
288 is not present at appreciable frequencies in *An. gambiae* mosquitoes sampled from nature. This
289 result is supported by the long-term stability of the Ag(PMB)1 insertion site over 100 generations
290 since its original construction, a result that suggests that other naturally occurring repetitive
291 elements in the genome of the Ag(PMB)1 are not functionally capable of PB transgene
292 remobilization.

293 We were not able to test the second possibility of remobilization by chromosomal
294 translocation, as these occur very rarely during meiosis. Under standard laboratory rearing of the
295 Ag(PMB)1 strain in over 5 years, translocations involving the autosomal transgene and the Y-
296 chromosome have never been detected – an event that would be immediately noticeable since
297 fluorescent transgenic individuals would only be male. This extends to scaled experimental
298 conditions, in which large numbers of Ag(PMB)1 individuals were screened by high-throughput
299 sorting of individuals using the COPAS sorter based on the 3X-P3-DsRed marker and then
300 subsequently separated by sex at the pupal stage [23]. This is, in part, expected because of how
301 rarely such events occur, meaning that experimentally verified rates for such events are not readily
302 available for the Ag(PMB)1 strain. When translocations between autosomes and the Y are desired
303 they can be artificially induced in the laboratory, for example with ionizing radiation, chemical
304 agents, or UV radiation. This is commonly done for insects to link selectable phenotypes to the Y-
305 chromosome, in so-called genetic sexing strains (GSSs). GSSs are developed so that males can be
306 separated from females on a large scale in insect bio-factories that produce animals for genetic
307 control programs, such as SIT [46]. This is done by linking selectable traits, for example insect
308 color or high temperature tolerance, to the Y-chromosome using induced reciprocal translocations
309 of mutant alleles located on autosomes. Once generated, these GSSs are then maintained in large
310 numbers, with billions of insects being produced weekly. The large colony size makes it possible
311 to detect rare events that result in the breakdown of linkage between maleness and selectable trait.
312 One of two ways this can happen is through a "reverse" reciprocal translocation involving the
313 previously modified Y-chromosome and an autosome (known as a type 2 recombination event)
314 [47]. Because such events lead to a breakdown of the genetic sexing system and restore male
315 fertility of the semi-sterile males (arising from the translocation itself) leading to their

316 accumulation, their occurrence is tightly monitored in large-scale rearing operations. In the only
317 report that quantified the rate of type-2 recombination and distinguished it from type-1 (which
318 does not involve the translocated Y- chromosome and is more common among the two) in a GSS
319 of the Mediterranean fruitfly *Ceratitis capitata*, the rate was estimated to be 10^{-5} or less, i.e.
320 occurring in less than 1 out of 100,000 male individuals [47]. In this case however, there are two
321 significant factors that would indicate that the rate of an initial, uninduced autosome-Y
322 translocation would be much lower. First, the rate of recombination in the GSS describes an event
323 reversing a previously induced autosome-Y translocation, which is likely to be largely mediated
324 by homologous sequences that are now present on the two translocated Y fragments; a homology
325 that does not normally exist between autosomes and the Y chromosome. Therefore, the expected
326 recombination rate resulting in a reciprocal translocation between an autosome and Y would be
327 lower. Second, the reversion of the previously translocated autosomal fragment on the Y restores
328 male fertility, that was first compromised by the translocation to the Y (because of gamete
329 chromosomal imbalance – discussed below) [47]. This means that type-2 recombinant males will
330 have more viable offspring increasing their rate of occurrence in the population. Together, these
331 factors suggests that the probability of a translocation event involving the autosomal Ag(PMB)1
332 transgene and the Y-chromosome in progeny of released males born in the field is much lower
333 than 10^{-5}. This rate would depend on the size of the Y-chromosome and the relative rate of
334 recombination in the male germline, which in *An. gambiae* is approximately 1.6 cM Mb^{-1} for
335 chromosome 3R [48] and similar between males and females [49]. Finally, it is worth noting that
336 since there is no recombination in *Drosophila melanogaster* males, data on autosome-Y
337 translocation frequencies do not exist in this insect model, to our knowledge.

338 In the unlikely event that the transgene was to move to the Y-chromosome, we provide
339 data regarding the expression of the X-shredder from this chromosome, and conclude that MSCI
340 during spermatogenesis does affect the Y-chromosome of *An. gambiae*. Our results from two
341 transgenic strains harboring the Ag(PMB)1 X-shredder transgene in two different positions on the
342 Y-chromosome, reveal transcriptional suppression during late spermatogenesis from the *beta2-*
343 *tubulin* promoter, complementing our previous work which confirmed this for the *An. gambiae* X-
344 chromosome [11,35,36]. We found no evidence of X-shredder expression by RT-PCR, nor by
345 fluorescence microscopy of transgenic testis. Offspring of transgenic males from both Y-linked
346 strains therefore had sex-ratios similar to wild-type males. Hence, even if the Ag(PMB)1 transgene
347 successfully moved to the Y-chromosome by transposition it is unlikely that the X-shedder would
348 be active. Since MSCI-factors regulating transcriptional suppression physically spread across the
349 sex chromosomes after becoming localized on their unsynapsed axes [50,51], it is also expected
350 that translocated autosomal fragments would become suppressed by MSCI during meiotic stages
351 of spermatogenesis. Therefore, the weight of evidence argues strongly against the likelihood of
352 movement of the Ag(PMB)1 transgene to the Y chromosome, particularly via transposition.
353 However, the equally necessary perquisite for a pathway to a driving Y, namely expression of the
354 X-shredder on the Y chromosome during male meiosis, seems highly implausible based on the
355 evidence presented here.

356 The final requirement for a Y-linked X-shredder to spread through populations is that its
357 movement to the Y-chromosome and subsequent expression from it would have no significant
358 fitness costs to males harboring it. Such fitness costs would counteract the theoretical advantage
359 gained by the Y-linked X-shredder from increased transmission through elimination of X-bearing
360 sperm. Among the factors determining these fitness costs, the largest contributors would likely be

361 the mechanism leading to Y-linkage and the outcomes of this movement on each chromosome.
362 Reciprocal translocations between an autosome, in this case 3R, and the Y-chromosome typically
363 result in significant male fertility costs [52]. Because of the simultaneous segregation of non-
364 homologous centromeres (adjacent-1 segregation) during meiosis, only 50% of the offspring
365 produced by males are genetically balanced, i.e. males are 50% sterile [53]. In certain cases, this
366 semi-sterility can be even higher, for example in an *An. arabiensis* GSS showing 73.3% male
367 sterility [53]. Therefore, a Y-linked X-shredder that arose through a translocation event would
368 likely display such high male fertility costs that it would rapidly disappear from the population.
369 For transposition-mediated Y-linkage male fitness costs cannot be predicted *a priori*, as gamete
370 balance and genic content would depend on both the excision event (i.e. how much of the
371 surrounding chromosome is excised) and on the integration position on the Y-chromosome (i.e.
372 subsequent knock-out of genes essential for male fitness such as the male-determining gene).

373 In summary, the findings of the current study support the low probability of Y-chromosome
374 linkage through remobilization of the transposon used for the initial genetic transformation.
375 Moreover, even if such as rare event occurred, where the X-shredder would become linked to Y-
376 chromosome, activity of the X-shredder at the required stage of spermatogenesis would likely be
377 impeded via chromosome wide suppression of gene expression on sex chromosomes. Prospects
378 for the systematic generation of an X-shredding-based Y-drive for mosquito control in the future,
379 will need to find ways to circumvent this transcriptional suppression, for example using alternative
380 germline specific promoters [37].

381 **Methods**

382

383 **Mosquito rearing**

384 Wild-type *An. gambiae* strain (G3) and transgenic mosquito strains were reared under standard
385 conditions at 28 °C and 80% relative humidity with access to fish food as larvae and 5% (wt/vol)
386 glucose solution as adults. For egg production, young adult mosquitoes (3–5 d after emergence)
387 were allowed to mate for at least 6 d and then fed on mice. Three days later, an egg bowl containing
388 rearing water (dH_2O supplemented with 0.1% pure salt) was placed in the cage. One to two days
389 after hatching, the larvae (L1 stage) were placed into rearing water containing trays.

390

391 **Assaying transgene chromosomal location**

392 Primers were designed to amplify across the 5' and 3' genomic insertion boundaries of the
393 Ag(PMB)1 transgene. DNA was extracted from 4 individual pools (containing 5 transgenic larvae
394 each) of Ag(PMB)1 mosquitoes following >100 generations of lab maintenance. PCR across the
395 5' and 3' transgene flanking regions was performed using: pB-5SEQ (5′-
396 CGCGCTATTTAGAAAGAGAGA) with genomic primer RH57 (5'-
397 GAAAACCTTACAAGCGTCTTCAA) to amplify the 5′ junction and pB-3SEQ (5′-
398 CGATAAAACACATGCGTCAATT) with RH53 (5'-GATATTATCGCGCTCGGTCC) to
399 amplify the 3′ junction. Each PCR amplicon was Sanger sequenced using both forward and reverse
400 primers and the resulting sequences were aligned to compare genomic flanking and transgenic
401 sequences to the Galizi 2014 data [11].

402

403 **Mosquito whole genome sequencing and read mapping**

404 *Anopheles gambiae* WGS reads from 81 individuals collected in Burkina Faso in 2012 were
405 downloaded from the European Nucleotide Archive (Study Accession: PRJEB1670;
406 Supplementary Table 1). WGS data from the G3 laboratory colony were downloaded from the
407 SRA (Bioproject Accession: PRJNA397539). Genomic DNA from 10 Ag(PMB)1 individuals was
408 extracted using the Blood and Tissue Kit (Qiagen). For each sample, 100ng of input gDNA was
409 sheared using Covaris for a 350bp insert size. Library preparation was performed using the
410 Illumina TruSeq Nano kit. Each sample was tagged with a unique barcode, followed by three
411 2×150bp High Output V2.5 paired-end sequencing runs on the Illumina NextSeq550 platform,
412 obtaining an average of 265M reads per sample. WGS data from the Ag(PBM)1 have been
413 deposited and will be made available for submission of this manuscript. Fastq reads were quality
414 checked with FastQC [54] and converted to fasta format. Reads were then mapped against the *vasa*
415 driven piggyBac plasmid [24] using blast blast-2.2.26/bin/blastall -i db.fa -d sample.fasta -p blastn
416 -F "m L" -U T -e 1-e4 -a 40 -v 5 -b 40000 -K 40000. Only alignments with 98% identity over the
417 entire read length were kept. Coverage was computed for each sample and normalized to the read
418 depth of the most deeply sequenced sample using the following formula $X_i = X_i/(X_i/X_{max})$. To
419 clarify plotting, read depth is reported every 10 bp.
420

421 **Generation of Y-chromosome linked X-shredder transgenic strains**

422 The YpBac-β2-^{gfp}124L transgenic strain was generated as described in Galizi et al. [11]. Briefly,
423 *An. gambiae* G3 embryos were injected with a mixture of 0.2 µg µl−1 of the pBac[3xP3-DsRed]β2-
424 eGFP(F2A)I-*Ppo*I-124L plasmid and 0.4 µg µl−1 of helper plasmid containing a *vasa*-driven
425 piggyBac transposase [24]. The hatched larvae were screened for transient expression of the
426 DsRed marker and positives (~54%) crossed to wild-type mosquitoes. F1 progeny were analysed

427 for DsRed fluorescence and positives were crossed individually with wild-type mosquitoes to
428 obtain transgenic lines. The transgene of one strain derived from a G0 male was identified that was
429 transmitted exclusively to F1 sons, indicating Y-chromosome integration. The stain, now called
430 YpBac-β2-^{gfp}124L was established and maintained by crossing to wild type females. The YattP-
431 β2-^{gfp}124L strain was generated by co-injecting the pBac[3xP3-DsRed]β2-eGFP(F2A)I-*Ppo*I-
432 124L construct with a *vasa*2-driven ΦC31 integrase helper plasmid [24] into eggs of a strain
433 containing a Y-chromosome AttP docking site [41]. Crosses and screening were performed as
434 above.
435
436 **Sex ratio and fertility assays**
437 To assay adult sex ratio, 10 transgenic males of each line were crossed to 10 wild-type females. In
438 all crosses, mosquitoes were allowed to mate for 3-5 days after the blood meal gravid females were
439 placed individually in oviposition cups. Larvae were reared to adulthood and sex was counted.
440 The number of eggs laid as well as the number of larvae hatching were also counted, but only for
441 the YattP- β2-^{gfp}124L to assay male fertility.
442
443 **qRT-PCR analysis**
444 qRT-PCRs were performed on mosquito total RNA as described in Galizi et al. [11]. Briefly, 10
445 pairs of testes from each transgenic strain were pooled to constitute a biological replicate for total
446 RNA and protein extraction using TRI reagent (Ambion). RNA was reverse-transcribed using
447 Superscript II (Invitrogen) after TURBO DNA-free (Ambion) treatment following the
448 manufacturer's instructions. Quantitative real-time–PCRs (qRT–PCR) analyses were performed
449 on cDNA using the Fast SYBR-Green master mix on a StepOnePlus system (Applied Biosystems).

450 Ribosomal protein Rpl19 gene was used for normalization. At least two independent biological
451 replicates from independent crosses were subjected to duplicate technical assays. We used primers
452 RPL19Fwd (5′-CCAACTCGCGACAAAACATTC-3′), RPL19Rev (5′-
453 ACCGGCTTCTTGATGATCAGA-3′), eGFP-F (5′-CGGCGTGCAGTGCTTCA-3′) and eGFP-R
454 (5′-CGGCGCGGGTCTTGT-3′).
455

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466

467 **Author Disclosure Statement**

468 No competing financial interests exist.

469 **Figure Legends**

470 **Figure 1. piggyBac transposase components in laboratory and wild type individuals. (A)**
471 Schematic of a wild type piggyBac (PB) transposon from *Trichoplusia ni* (middle; NCBI accession
472 DQ236240.1), the Ag(PMB)1 transformation construct (top) and the PB helper plasmid (bottom).
473 Shown are the regions of the endogenous PB locus present in the two microinjected plasmids
474 highlighting how both the transformation and helper plasmid lack the complete machinery required
475 for transposon mobility. pBacL (left) and pBacR (right) arms present in the pBac[3xP3-DsRed]β2-
476 eGFP(F2A)I-*Ppo*I-124L transformation construct contain the entire flanking inverted terminal
477 repeats (ITRs) and partial regions of the PB open reading frame (ORF). The helper PB plasmid
478 containing the complete PB ORF driven from the *vasa*2 regulatory regions lacks the flanking ITRs.
479 Sequences of the transformation construct are integrated in the genome are shown in solid lines,
480 whereas sequences present only transiently in injected individuals are shown in dashed lines. **(B)**
481 Mapping of whole genome sequencing reads from the G3 and Ag(PMB)1 controls (bottom in
482 negative y-axis; grey and red) and the 81 wild type individuals collected in Burkina Faso villages
483 (top; blue). The position of the *An. gambiae vasa* regulatory regions (yellow boxes) and the PB
484 transposase ORF (black box) is also shown. Reads are normalized by scaling counts to the number
485 of reads in the most abundant sample.

486

487 **Figure 2. Transcriptional suppression of Y-linked X-shredder constructs abolishes sex ratio**
488 **distortion. (A)** eGFP fluorescence from dissected wild type (WT), Ag(PMB)1 and YpBac-β2-
489 ^{gfp}124L testis. **(B)** Quantitative RT-PCR showing the relative expression of eGFP(F2A)I-*Ppo*I
490 variants in different X-shredder strains, compared to expression in the Ag(PMB)1 strain.
491 Expression of the X-shredder is undetectable in both Y-chromosome insertions. Expression levels

492 from two additional autosomal strains, Ag(PMB)2 and Ag(PMB)3, which also led to sex ratio
493 distortion are shown. **(C)** Progeny analysis of males from the two Y linked X-shredder strains
494 crossed to wild-type females. Shown is the average number of eggs laid per n females analyzed (±
495 represents the standard error of the mean; SEM). Average percentage of larvae hatching from the
496 eggs (±SEM), from n females analyzed. Average percentage of males in the progeny (±SEM) from
497 n females. The total number of eggs or individuals counted in each experiment is given in
498 parentheses. Sequences (20bp each side) flanking the PB integration site (TTAA) of the
499 transformation constructs are also shown.

500 **Supplementary Figure 1. Potential Pathways to harm by piggyBac transposition of**
501 **Ag(PMB)1 to the Y chromosome.**

502 **Supplementary Figure 2. Potential Pathways to harm by reciprocal translocation of the**
503 **autosomal Ag(PMB)1 to the Y chromosome.**

504 **Supplementary Figure 3. Analysis of Ag(PMB)1 transgene insertion.** Blue and violet filled
505 blocks highlight the 5' and 3' genomic sequences flanking the transgene based on sanger
506 sequencing of the genomic PCRs. Shown are the results from the Galizi et al.[11] inverse-PCR
507 analysis (below) and the sequencing of genomic PCRs from the present study (above). The location
508 of Ag(PBM1) transgene is shown in a red box and is located on chromosomal arm 3R upstream of
509 AGAP010019 gene (grey filled arrow). Base-pair positions on chromosome 3R are also shown.
510 The analysis confirms that the insertion site of Ag(PMB)1 strain has remained stable over 100
511 generations of maintenance in the laboratory.

512 **Supplementary Figure 4. Location of Burkina Faso individuals used in this study.** Longitude
513 and latitude of samples collected in 4 locations in Burkina Faso in 2012. Names of villages are
514 shown as well as the number of individuals from each site.

515 **Supplementary Table 1. Burkina Faso individuals Ag1000gs supplementary information**

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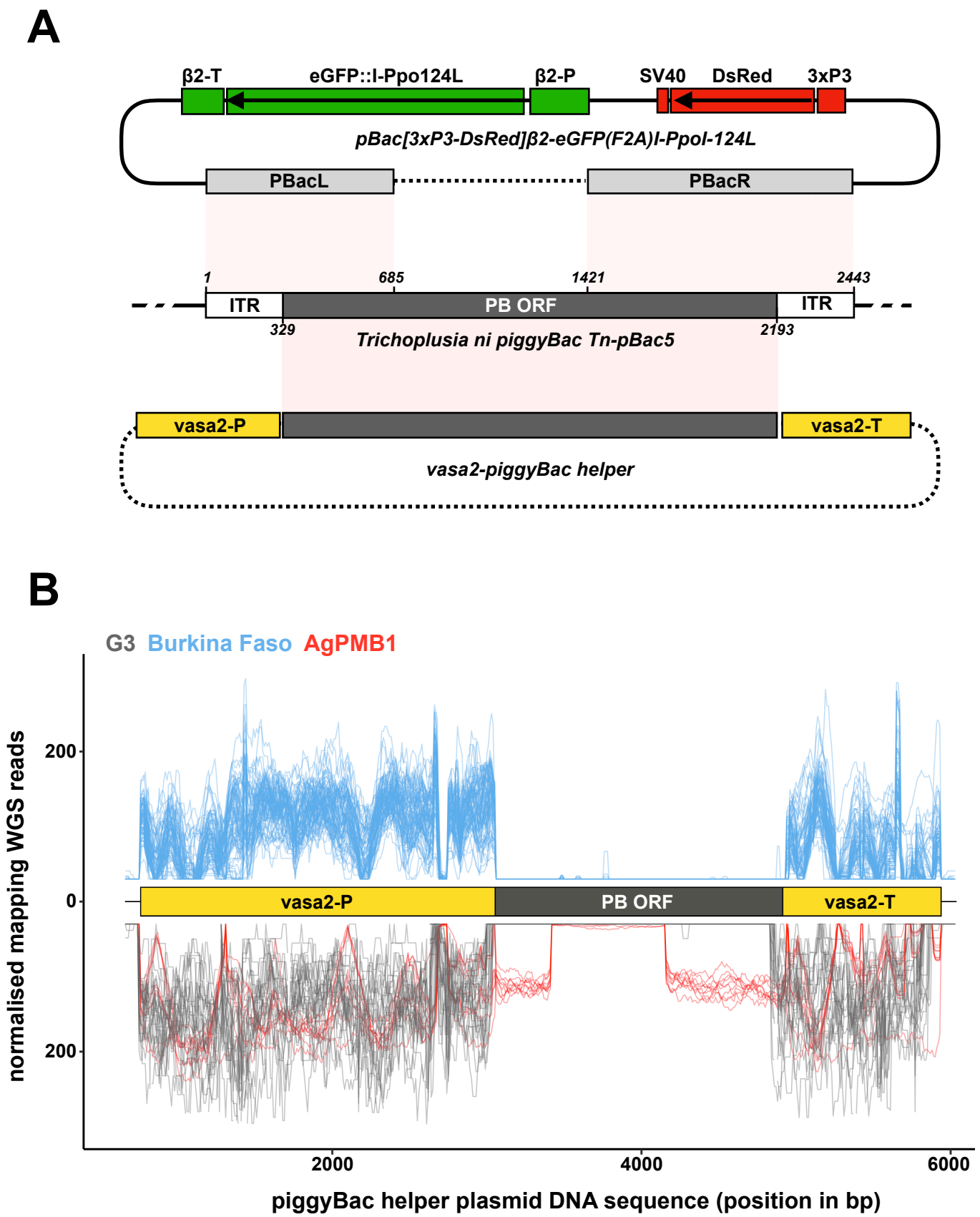
639

Figure 1


A
β2-T
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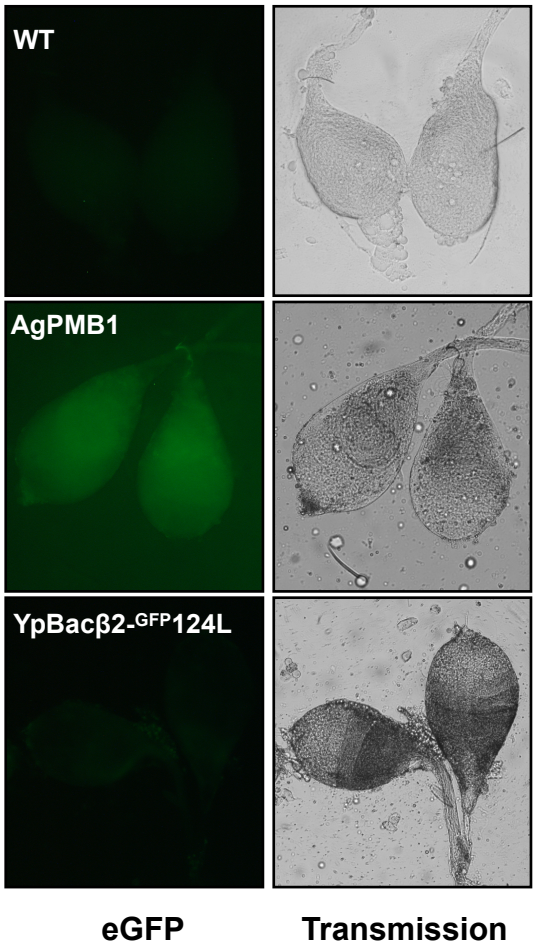
β2-P
SV40
DsRed
3xP3
pBac[3xP3-DsRed]β2-eGFP(F2A)I-PpoI-124L
PBacL
PBacR
1
685
1421
2443
ITR
PB ORF
ITR
329
2193
Trichoplusia ni piggyBac Tn-pBac5
vasa2-P
vasa2-T
vasa2-piggyBac helper
B
G3
Burkina Faso
AgPMB1
normalised mapping WGS reads
200
0
200
vasa2-P
PB ORF
vasa2-T
2000
4000
6000
piggyBac helper plasmid DNA sequence (position in bp)

Figure 2

A

Strain	Construct	Eggs per female	hatching %	% males	Insertion site
YpBac-β2-^{gfp}124L	pBac[3xP3-DsRed]β2-eGFP::I-PpoI-124L	N/A	N/A	50.6 ±2.9 n=7 (891)	TGCTGAATTCTAAAA GTTTG**TTAA**ACGTAT ATTTGTATCTAGTT
YattP- β2-^{gfp}124L	attB[eCFP]β2-eGFP::I-PpoI-124L	135.5 ±8.5 n=19 (2574)	81.2 ±3.4 n=19	53.4 ±1.2 n=19 (1850)	TCATTGCTAACATGA AGGCA**TTAA**AAACAA AGGATACCAAGTCA

B



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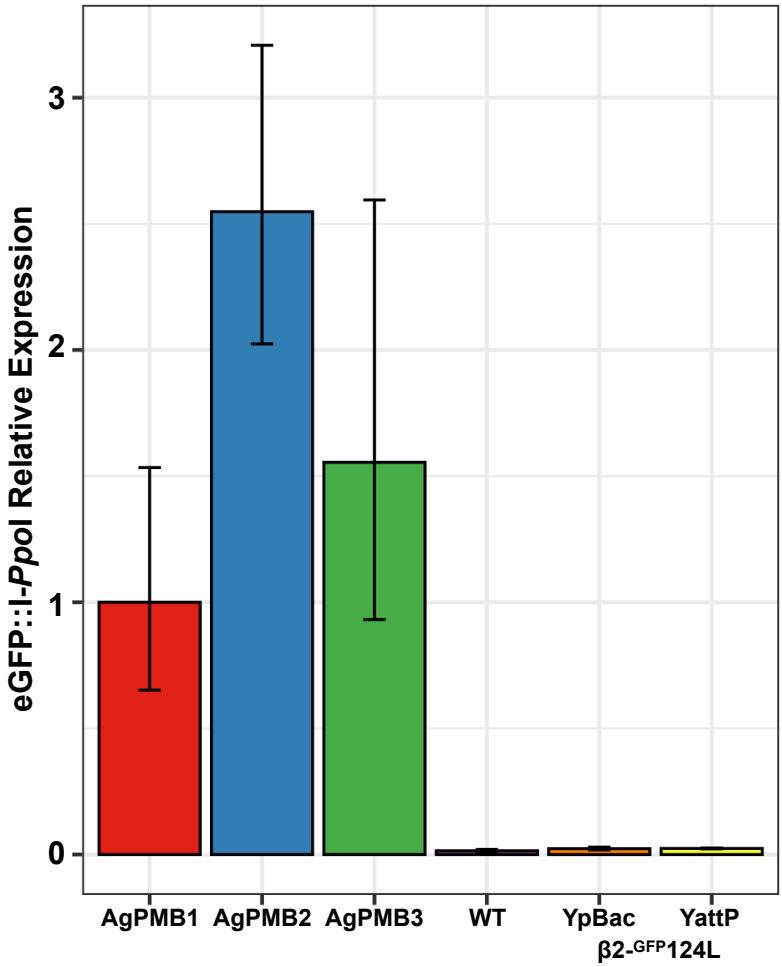
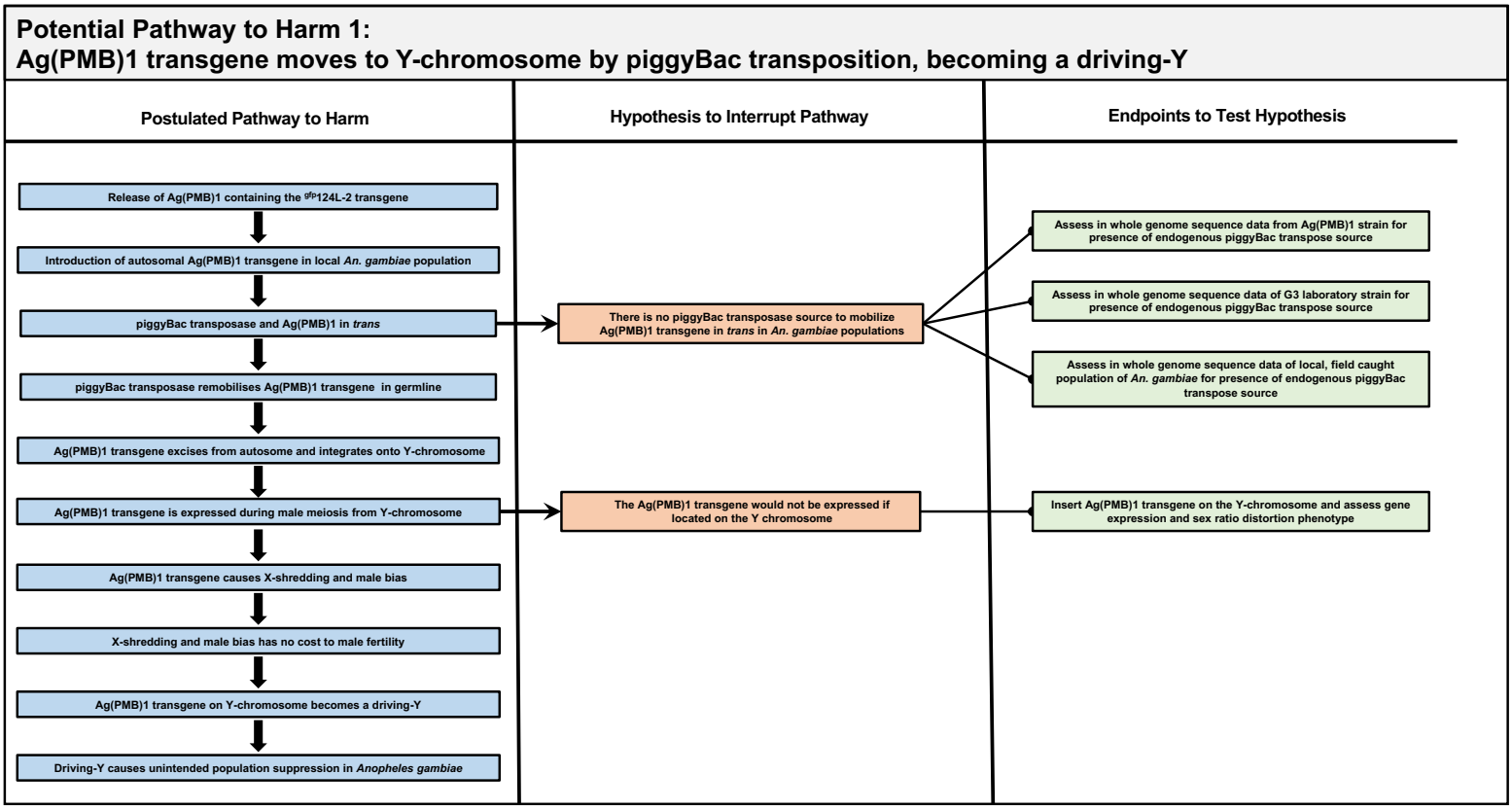


Supplementary Figure 1



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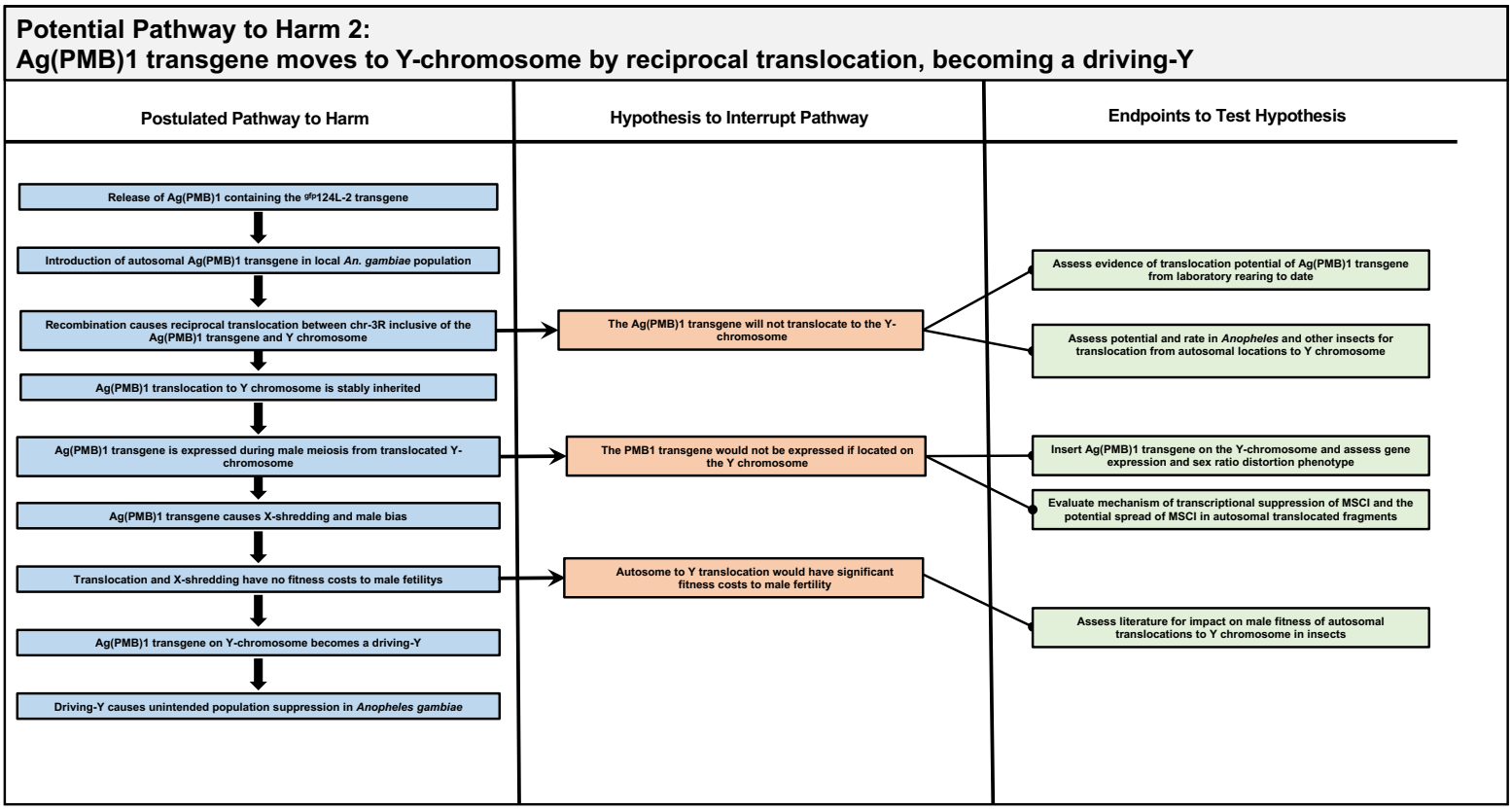
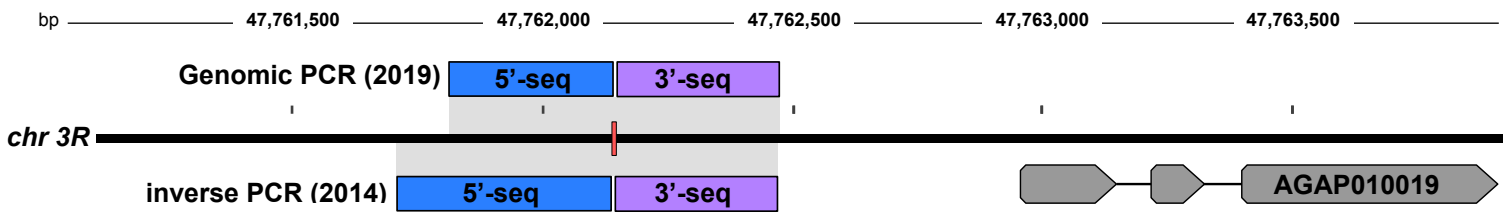
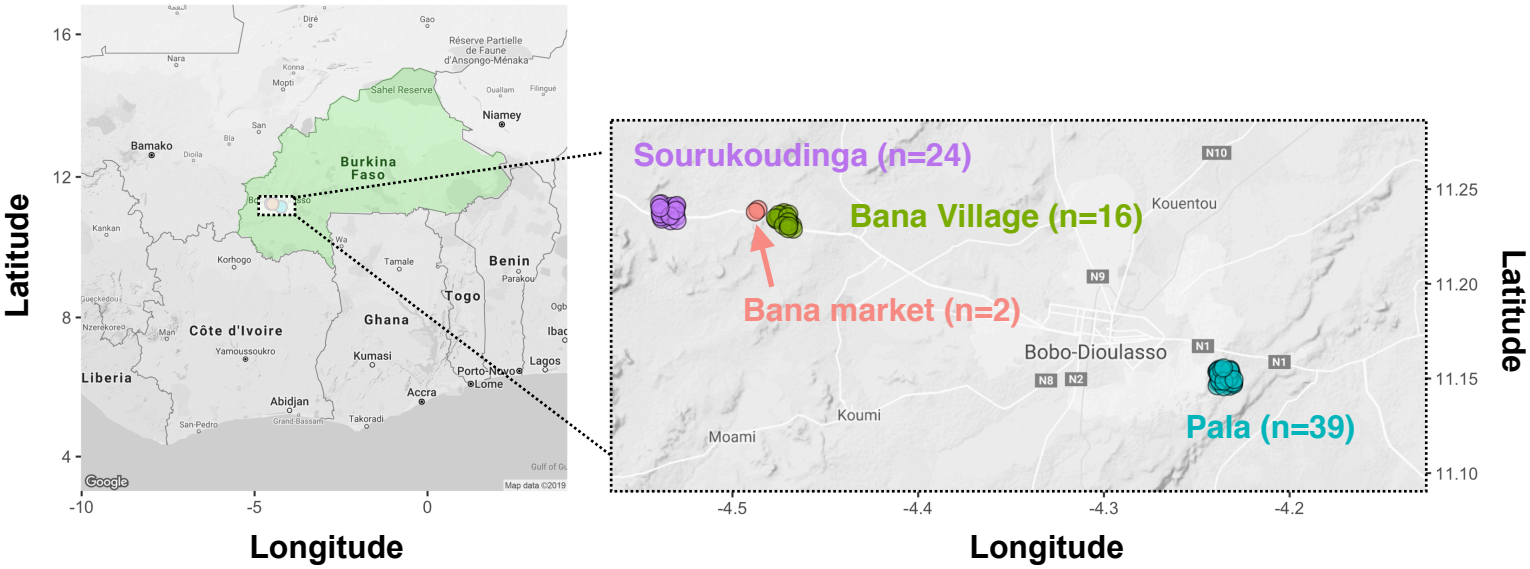


Supplementary Figure 3



Supplementary Figure 4