Defining transformation events for gene drive in species complexes

John B. Connolly (✉ john.connolly12@imperial.ac.uk)
Imperial College London https://orcid.org/0000-0002-7033-3463

Short Report

Keywords: backcrossing, engineered gene drive, genomic target locus, homing, homology directed repair, introgression, sibling species, species complex, transformation event, vertical gene drive transfer

Posted Date: April 13th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2806607/v1

Abstract

Engineered gene drives (EGDs) that allow the super-Mendelian inheritance of genetic traits could one day be used to reduce the vectorial capacity of *Anopheles* species that transmit human malaria in Africa. Many *Anopheles* species belong to complexes of closely related sibling species that can produce fertile interspecific hybrid females. In cases where the genomic target locus of the EGD is conserved amongst sibling species from the same complex as the released target species, it would therefore be plausible that the EGD could be vertically transmitted from the target species to sibling species by interspecific mating. To differentiate genetically modified organisms, the term 'transformation event' is used, based on the specific genomic location of the transgenic construct, as a result of random genomic integration. In contrast, an EGD is generated via its precise and reproducible insertion in its specific genomic target locus. These considerations pose two key questions for the use of EGD in species complexes: (i) what does the definition of "transformation event" mean in the context of vertical gene drive transfer of the EGD to sibling species in species complexes and (ii) does *de novo* transformation of an EGD into a sibling species constitute the same transformation event as introgression by backcrossing into a sibling species of an EGD that had been originally transformed in the target species? While definitions of the term transformation event that have been provided by national and intergovernmental organisations are somewhat ambiguous, they do provide scope for broad interpretation of vertical gene drive transfer of a specific EGD to different sibling species of the target species as the same transformation event. There also appears to be some consensus that definitions of transformation event support the notion that *de novo* transformations of an EGD in sibling species constitute the same transformation events as introgression by backcrossing into sibling species of an EGD that had been originally inserted in the target species.

Introduction

The World Health Organization (WHO) has estimated that in 2021 there were 234 million cases of malaria in Africa, resulting in 593,000 deaths (WHO 2022). In Africa, malaria is caused by parasites of the *Plasmodium* genus, typically *P. falciparum* (Haemosporida: Plasmodiidae) and vectored by mosquitoes of the *Anopheles* genus, the most significant species of which are **sibling species** from the **species complex**, *Anopheles gambiae* sensu lato (s.l.), namely *An. gambiae* sensu stricto (s.s.), *An. coluzzii* and *An. arabiensis*, as well as the species *An. funestus* s.s. from the *An. funestus* group (all Diptera: Culicidae; Coetzee et al., 2013).

In recent years, successive annual reductions in the incidence of malaria, which have largely been achieved using insecticide-treated bednets, indoor residual spraying and anti-*Plasmodium* chemotherapeutic and chemopreventative interventions, have dissipated. This has ushered in the need for additional, complementary approaches to address the outstanding malaria transmission, including the use of novel vector control agents based on genetic modification (GM), such as **engineered gene drives** (**EGDs**; see **Glossary**) (AUDA-NEPAD, 2018; WHO, 2020).

An EGD, which with specific reference to homing gene drives in this article, typically encodes the *clustered regularly interspaced short palindromic repeats* (CRISPR) Cas9 endonuclease under the control of a germline promoter and a ubiquitously and constitutively expressed guide RNA (gRNA) that together form the CRISPR Cas9/gRNA **ribonucleoprotein** complex to recognise and generate a double-stranded break in the **genomic target locus** (**GTL**), is itself inserted into a GTL that is recognised and cleaved by the ribonucleoprotein of the EGD. In a process known as **homing**, the EGD from the transgenic chromosome expresses the ribonucleoprotein, which causes a double stranded break in the GTL on the homologous chromosome. This chromosomal break is repaired by **homology directed repair**, which uses the transgenic chromosome as a template to repair the cleaved homologous chromosome and, in doing so, pastes the EGD into the homologous chromosome. This means that most gametes, and thus offspring, from the transgenic containing the EGD will also be transgenic. This therefore allows the EGD, and any linked genetic traits that could for example reduce the vectorial capacity of *Anopheles* species, to be inherited at **super-Mendelian** ratios, and ultimately lead to its potential fixation in mosquito target populations.

The species complex *An. gambiae* s.l. contains both vector and non-vector species. Moreover, this complex contains semi-permeable species boundaries, so that fertile **interspecific hybrids** can be obtained from the numerous combinations of sibling species that have been tested thus far in laboratory crosses, some of which have also been detected, albeit rarely, in the field (Davidson, 1964; White, 1971; Besansky et al., 2003; Epopa et al., 2019; Connolly et al., 2023). Therefore, should the gRNA expressed in an EGD recognise a GTL that is conserved in sibling species, it is plausible that its environmental release would lead to **vertical gene drive transfer** (**VGDT**) via mating between sibling species, potentially leading to spread of the EGD, and increase in its frequency in sibling species. Moreover, for a population suppression EGD, this could subsequently lead to suppression of sibling species.

Regulation Of “transformation Events.”

Effective risk and regulatory frameworks that have been developed for a range of applications of GM organisms (GMOs; or referred to as Living Modified Organisms, abbreviated as LMOs, in the Cartagena Protocol on Biosafety to the Convention on Biological Diversity), including GM mosquitoes (WHO, 1991, 2021; OECD, 1993; CBD, 2016, 2000; EFSA, 2010, 2013; USDA et al., 2017; Mitchell and Bartsch, 2019; OGTR, 2019) are also applicable to EGDs, although some adaptations and nuancing may be required to accommodate some of the unique features of EGDs, such as the capacity of low-threshold EGDs to increase in frequency and persist in target populations (Devos et al., 2020a, b, 2021 a, b; EFSA et al. 2020).

In most jurisdictions globally, GMOs are regulated as products of their “transformation event” (Holst-Jensen et al., 2006; Lezaun, 2006; OECD, 2006; Voigt and Münichsdorfer, 2019). The concept is based on transgenic technology that relies on random genomic integration of the transgene into the genome (**see Figure 1A**), whereby the same transgene is inserted at different genomic loci that can produce different phenotypes via insertional mutagenesis or differential levels of transgene expression, sometimes referred

to as “position effects” (Nolan et al., 2002; Alonso et al., 2003; Gong et al., 2005; Kim and Gelvin, 2007; Phuc et al., 2007).

Glossary

Engineered gene drive (EGD): transgenic genetic elements that cause biased inheritance; the term “gene drive” can also be used to describe the process or phenomenon leading to biased inheritance or management strategy to apply gene drive (Alphey et al., 2020).
Genomic target locus (GTL): specific sequence in the genome that is recognised by the specific gRNA used in an EGD, and when conserved in sibling species, could lead to VGDT or *de novo* transformation of the EGD into the homologous genomic region of the sibling species.
Homing: In the germline, the EGD expresses its ribonucleoprotein which cleaves the GTL on the homologous chromosome, which is subsequently repaired by homology directed repair using the transgenic chromosome, on which the EGD resides, as a template; thus, the germline effectively becomes homozygous for the EGD so that most gametes, and thus progeny, of the transgenic strain, will also be transgenic.
Homology directed repair: cellular mechanism using homologous recombination to reconstruct a stretch of DNA after a double-stranded break.
Interspecific hybrid: offspring resulting from interbreeding of parents from two different species.
Introgression: stable incorporation of genetic material from one species to another via hybridisation followed by backcrossing of hybrid to that other species.
Ribonucleoprotein: ribonucleic acid, e.g., gRNA, complexed with protein, e.g., Cas9.
Sibling species: species that are members of the same species complex.
Species complex: biological entity consisting of a group of closely related species with similar morphology and semipermeable reproductive boundaries (Besansky et al., 2003).
Super-Mendelian: above the circa 50 percent rate of transmission of a dominant allele from a parent to its offspring.
Vertical gene drive transfer (VGDT): transfer by hybridisation of gene drive from one sibling species to another, leading to gene drive in latter where GTL is conserved.

A transformation event therefore provides for identification of the specific transgene insertion in the GMO, by using a unique event-specific molecular detection methodology that typically targets the unique junction between the host genome and the transgenic cassette. This allows for the differentiation of one GMO from another, including ones with closely related transgene sequences. Should the transformation event be crossed into different genetic backgrounds from the same species that are adapted to the local conditions, it will still be considered, and regulated, as the same “event” or product. For example, event

OX513a was crossed into different genetic backgrounds of *Aedes aegypti* (Diptera: Culicidae; Harris et al., 2011) and, in March 2007, dozens of maize varieties with event MON810 (insect resistant) were inscribed in the Common EU Catalogue of Varieties of Agricultural Plant Species (Coll et al., 2009).

However, in the generation of a transgenics containing an EGD, random integration of the transgene does not occur. Instead, the EGD can be inserted at the conserved GTL in different sibling species via *de novo* transformation using genomic editing by CRISPR-Cas9 and homology directed repair (see **Figure 1B**; Hammond et al., 2016; Kyrou et al., 2018). Here, germline transformation of a plasmid, which contains 5' and 3' regions of homology to the GTL flanking either side of the EGD, occurs by its insertion via homology directed repair into a double stranded break in the GTL that has been generated by its own ribonucleoprotein supplied *in trans*. This approach produces genetic modifications whereby an EGD is inserted precisely and reproducibly in the conserved GTL, the outcome of which can subsequently be verified by whole genome sequencing.

What Does The Definition Of Transformation Event Mean In The Context Of Vgdt Of The Egd To Sibling Species In Species Complexes?

Definitions of the term "transformation event" in GMO regulations or guidance could inform interpretations of "transformation event" in the context of EGDs in species complexes. However, there seems to be no internationally agreed definition of the term "transformation event" in documents from numerous national or intergovernmental organisations which use this term. Despite searches of instruments from 13 organisations, only four bodies have attempted to provide a definition (see **Box 1** and references therein).

Box 1. Bodies which use the term "transformation event."

African Union Development Agency - New Partnership for Africa's Development (AUDA-NEPAD, 2018, 2020)
Australian Office of the Gene Technology Regulator (OGTR, 2022)
Biosafety Clearing House and Secretariat of the Cartagena Protocol on Biosafety to the Convention of Biological Diversity (CBD, 2000, 2005, 2016; BCH, 2022)
European Union (EC, 2001; EU, 2003, 2014)
European Food Safety Authority (EFSA, 2007, 2010, 2013, 2020)
Food and Agricultural Organization (FAO, 2022)
Organisation for Economic Co-operation and Development (OECD, 2006)
Secretariat of the International Plant Protection Convention (IPPC, 2022)
US Department of Agriculture Animal and Plant Health Inspection Service (USDA, 2022a, b)
US Environmental Protection Agency (abbreviated to EPA; USDA, FDA, EPA, 2017; EPA, 2022; USDA, 2022a)
US Food and Drug Administration (abbreviated to FDA; FDA, 2001; OIRA, 2003; USDA, FDA, EPA, 2017; FDA, 2022; USDA, 2022a)

The first of these, the Secretariat to the Cartagena Protocol on Biosafety of the Convention on Biological Diversity (CBD) defined, in its 2016 guidance on risk assessment, “transformation event” as “an LMO with a specific modification that is the result of the use of modern biotechnology according to Article 3(i)a of the Protocol,” with the term “modern biotechnology” being defined in this Article as “*in vitro* nucleic acid techniques, including recombinant deoxyribonucleic acid (DNA) and direct injection of nucleic acid into cells or organelles.” While the term LMO is not explicitly defined in this 2016 guidance, it is so in Article 3(g) of the Protocol as “any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology.” Moreover, Article 3 (h) of the Protocol states that LMO “means any biological entity capable of transferring or replicating genetic material, including sterile organisms, viruses and viroids.” This means that the 2016 guidance here has defined the term “transformation event” with circular references to LMOs and modern biotechnology that effectively translate as “a biological entity capable of transferring or replicating genetic material that possesses a novel combination of genetic material obtained through the use of modern biotechnology with a specific modification that is the result of the use of modern biotechnology.” Furthermore, without a definition of the term “specific modification” in the Protocol or the 2016 guidance, ambiguity surrounding the term “transformation event” is simply amplified via the term “specific modification.” For example, specific modification could refer to either a certain process or particular occurrence. Therefore, the guidance here merely reaffirms the definition of LMO, while apparently failing to define more precisely the term “transformation event.”

A second intergovernmental body, the European Union (EU), provides relevant definitions pertaining to “transformation event” in two separate instruments. The first document, as highlighted by Lezaun (2006), is a 2001 explanatory memorandum from the European Commission on proposals for the traceability and labelling of GMOs which stated that “A transformation event is where a conventional organism is 'transformed', through the introduction of modified DNA sequences, resulting in formation of a GMO.” (EC, 2001; Lezaun, 2006). The second instrument of the European Union is the 2014 implementing regulation amending rules around reference laboratories for GMOs which defines in Article 2(e) that “‘GMO containing a single transformation event’ means a GMO that has been obtained through a single transformation process.”

The third, national, body is the FDA, which in 2001 published a new Proposed Rule for implementation of regulation of bioengineered foods under the Food, Drug and Cosmetic Act via the introduction of Parts 192.1 (e) and 592.1 (e), both of which stated that “*Transformation event* means the introduction into an organism of genetic material that has been manipulated in vitro. For the purpose of this part, “organism” refers to plants.” (FDA, 2001). However, the Rule was withdrawn from publication following the scrutiny of the Office of Information and Regulatory Affairs, the US Government’s central authority for the review of Executive Branch regulations (OIRA, 2003).

The intergovernmental body AUDA-NEPAD provides a glossary which defines “event” as **“a** term used to describe a plant and its offspring that contain a specific insertion of DNA. Events are distinguishable from each other by their unique site of integration of the introduced DNA” (AUDA-NEPAD, 2020). Although the term “transformation” does not accompany “event,” the context is clear so that this definition appears to accommodate both random and precise genomic integration.

Thus, while definitions of the term “transformation event” that have been provided by national and intergovernmental organisations are somewhat ambiguous, they do provide scope for broad interpretation of VGDT of a specific EGD to different sibling species of the target species as the same transformation event. In that sense, VGDT of an EGD amongst sibling species is comparable with the dozens of varieties of MON810 GM maize that have been generated from a single transformation event, which therefore are regulated as a single entity.

Does De Novo Transformation Of An Egd Into A Sibling Species Constitute The Same Transformation Event As Introgression By Backcrossing Into A Sibling Species Of An Egd That Had Been Originally Transformed In The Target Species?

Another direct consequence of the use of EGDs in species complexes where the GTL is conserved in sibling species is on risk assessment, where it may be desirable or necessary ahead of proposed field releases to introduce the EGD into, and assess its impact on, both the target species and sibling species. This could be achieved using two different approaches: *de novo* transformation of the EGD into each species or **introgression** of the EDG from the target species by backcrossing into sibling species (see **Figure 2**).

In the former, the EGD can be inserted at the conserved GTL in sibling species via *de novo* transformation using homology directed repair-based approaches (Hammond et al., 2016; Kyrou et al., 2018). In introgression by backcrossing, the EGD is introduced into sibling species via interspecific fertile female hybrids and backcrossing to parental sibling species for several generations. This approach has been successfully used to transfer non-gene-drive transgenes from one species, *An. gambiae*, to another, *An. coluzzii* (Pollegioni et al., 2022), including for release in the field (Yao et al., 2022). Thus, the original genetic modification can be introduced from one sibling species that was the initial transformation event into another sibling species via backcrossing. Both *de novo* transformation and introgression by backcrossing should essentially produce the ***same result***: transgenic strains containing the same EGD at a conserved GTL in different sibling species. But does *de novo* transformation of an EGD into a sibling species constitute the ***same transformation event*** as introgression by backcrossing into a sibling species of an EGD that had been originally transformed in the target species?

In reference to the CBD definition of transformation event, reproducible insertion of the same EGD at a conserved GTL by *de novo* transformation into sibling species could be interpreted as a “specific modification”, as in a “certain process,” using “modern biotechnology” of a “biological entity capable of

transferring or replicating genetic material." Equally, introgression of the EGD by backcrossing to sibling species would involve the same transformation event. This would appear to qualify both the *de novo* transformation and introgression by backcrossing of the same EGD as equivalent. In the case of the 2004 EU definition, Lezaun (2006) argued this definition was itself ambiguous, for example leaving open the possibility that "where" could refer to the place where the transformation occurred. Indeed, the place "where" transformation occurs could be interpreted from a biological perspective as the conserved GTL into which the modified DNA is introduced, so that the reproducible insertion of an EGD into a conserved GTL could represent the same transformation event as the original insertion of the EGD into the target species, regardless of method (introgression versus *de novo* transformation) and species (target versus sibling).

Considering the EU term of a "single transformation process," single can be defined as "sole" meaning "being the only one," so that this definition could be interpreted as a "GMO containing a single transformation event" means a GMO obtained by only one process of transformation. Reproducible insertion of the same EGD at a conserved GTL by *de novo* transformation into sibling species could therefore be interpreted as occurring by a single transformation event, according to this definition. In the 2001 definition of transformation event attempted by the FDA, it refers to the process by which a GMO is made, rather than to a particular occurrence, so that in the context of use of EGD in species complexes, reproducible insertion of the same EGD at a conserved GTL by *de novo* transformation into sibling species could therefore be interpreted as meeting the definition of, and consequently, the same transformation event as the original insertion of the EGD in the target species. Moreover, based on the AUDA-NEPAD definition of "event," reproducible insertion of the same EGD at a conserved GTL by *de novo* transformation into sibling species could be interpreted as the same transformation event. Likewise, introgression by backcrossing to sibling species of the EGD that had been originally inserted in the target species would represent the same event. Therefore, there appears to be some consensus that the available definitions of transformation event support the notion that *de novo* transformations of an EGD into sibling species constitute the same transformation events as introgression by backcrossing into sibling species of an EGD that had been originally transformed in the target species.

Conclusions

GMOs are typically regulated as products of their transformation event, based on transgenic technology that results in random genomic integration of the transgene into the genome. By contrast, an EGD is generated via the precise and reproducible insertion in its GTL, the outcome of which can subsequently be verified by whole genome sequencing. Moreover, in cases where the GTL of the EGD is conserved amongst sibling species from the same complex as the target released species, it is plausible that the EGD could be vertically transmitted from the target species to sibling species by interspecific mating. These considerations pose two key questions for the use of EGD in species complexes: (i) what does the definition of "transformation event" mean in the context of VGDT of the EGD to sibling species in species complexes and (ii) does *de novo* transformation of an EGD into a sibling species constitute the same transformation event as introgression by backcrossing into a sibling species of an EGD that had

been originally transformed in the target species? While definitions of the term “transformation event” that have been provided by national and intergovernmental organisations are somewhat ambiguous, they do provide scope for broad interpretation of VGDT of a specific EGD to different sibling species of the target species as the ***same transformation event***. In that sense, VGDT of an EGD amongst sibling species is comparable with strains of *Ae. aegypti* OX513a with a different genetic background or the multiple varieties of MON810 maize that have been generated from a single transformation event. Another direct consequence of the use of EGDs in species complexes where the GTL is conserved in sibling species is on risk assessment, where it may be desirable or necessary ahead of proposed field releases to introduce the EGD into, and assess its impact on, both the target species and sibling species. This could be achieved using two different approaches: de novo transformation of the EGD into each species or introgression of the EDG from the target species by backcrossing into sibling species. There also appears to be some consensus from the available definitions of transformation event to support the notion that *de novo* transformations of an EGD into sibling species constitute the ***same transformation events*** as introgression by backcrossing into sibling species of an EGD that had been originally inserted in the target species.

Declarations

Acknowledgements

I thank Boet Glandorf, Brinda Dass, Stephanie James, Fil Randazzo, Camilla Beech, Geoff Turner, John Mumford, and Silke Fuchs for their comments on earlier drafts of this article. I am a member of the Target Malaria not-for-profit international research consortium (www.targetmalaria.com), which is supported by a grant from the Bill & Melinda Gates Foundation and Open Philanthropy.

References

Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., Stevenson, D. K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C. C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D. E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W. L., Berry, C. C. and Ecker, J. R. 2003. Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science 301: 653-657.

Alphey, L. S., Crisanti, A., Randazzo, F. F. and Akbari, OS. 2020. Opinion: standardizing the definition of gene drive. Proc. Natl. Acad. Sci. USA 117: 30864-30867.

AUDA-NEPAD 2018. Gene drives for malaria control and elimination in Africa. African Union Development Agency - New Partnership for Africa's Development. URL: https://www.nepad.org/publication/gene-drives-malaria-control-and-elimination-africa. Accessed 1 Feb. 2023.

AUDA-NEPAD 2020. Glossary. African Union Development Agency - New Partnership for Africa's Development. URL: https://www.nepad.org/content/glossary. Accessed 1 Feb. 2023.

Bateman, J. R., Lee, A. M.and Wu, C. T. 2006. Site-specific transformation of *Drosophila* via phiC31 integrase-mediated cassette exchange. Genetics 173: 769-777.

Besansky, N. J., Krzywinski, J., Lehmann, T., Simard, F., Kern, M., Mukabayire, O., Fontenille, D., Toure, Y.and Sagnon, N. 2003. Semipermeable species boundaries between *Anopheles gambiae* and *Anopheles arabiensis*: evidence from multilocus DNA sequence variation. Proc. Natl. Acad. Sci. USA 100: 10818-10823.

BCH 2022. Biosafety Clearning House of Cartagena Protocol on Biosafety to Convention of Biological Diverity. URL: https://bch.cbd.int/en/. Accessed 1 Feb. 2023.

CBD 2000. Cartagena Protocol on Biosafety to the Convention on Biological Diversity: text and annexes. URL: https://bch.cbd.int/protocol/text/. Accessed 1 Feb. 2023.

CBD 2005. Handbook of the Convention on Biological Diversity including its Cartagena Protocol on Biosafety. Secretariat of the Convention on Biological Diversity. 3rd edition., Montreal, Canada. URL: https://www.cbd.int/handbook/. Accessed 1 Feb. 2023.

CBD 2016. The Cartagena Protocol on Biosafety. Guidance on risk assessment of living modified organisms and monitoring in the context of risk assessment. URL: www.cbd.int/doc/meetings/bs/mop-08/official/bs-mop-08-08-add1-en.doc. Accessed 1 Feb. 2023.

Coetzee, M., Hunt, R. H., Wilkerson, R., Torre, A. D., Coulibaly, M. B. and Besansky, N. J. 2013. *Anopheles coluzzii* and *Anopheles amharicus*, new members of the *Anopheles gambiae* complex. Zootaxa 3619: 246-274.

Coll, A., Nadal, A., Collado, R., Capellades, G., Messeguer, J., Mele, E., Palaudelmas, M. and Pla, M. 2009. Gene expression profiles of MON810 and comparable non-GM maize varieties cultured in the field are more similar than are those of conventional lines. Transgenic Res. 18: 801-808.

Connolly, J. B., Romeis, J., Devos, Y., Glandorf, D. C. M., Turner, G. and Coulibaly, M. B. 2023. Gene drive in species complexes: defining target organisms. Trends Biotechnol. 41: 154-164.

Davidson, G. 1964. *Anopheles gambiae*, a complex of species. Bull. World Health Organ. 31: 625-634.

Devos, Y., Bonsall, M. B., Firbank, L. G., Mumford, J., Nogue, F. and Wimmer, E. A. 2021a. Gene drive-modified organisms: developing practical risk assessment guidance. Trends Biotechnol. 39: 853-856.

Devos, Y., Bonsall, M. B., Nogué, F., Paraskevopoulos, K., Wimmer, E. A. and Firbank, L. G. 2020a. Outcome of a public consultation on the draft adequacy and sufficiency evaluation of existing EFSA guidelines for the molecular characterisation, environmental risk assessment and post-market environmental

monitoring of genetically modified insects containing engineered gene drives. EFSA Supp. Publ. 17:EN-1939.

Devos, Y., Gallani, B. and Firbank, L. G. 2020b. Stakeholder workshop “Problem formulation for the environmental risk assessment of gene drive modified insects” (15 May 2019, Brussels). EFSA Supp. Publ. 2020: EN-1819.

Devos, Y., Mumford, J. D., Bonsall, M. B., Glandorf, D. C. M. and Quemada, H. D. 2021b. Risk management recommendations for environmental releases of gene drive modified insects. Biotechnol. Adv. 54: 107807.

EC 2001. Explantory memorandum. Proposal for a regulation of the European Parliament and of the Council concerning traceability and labelling of genetically modified organisms and traceability of food and feed products produced from genetically modified organisms and amending Directive 2001/18/EC /* COM/2001/0182 final - COD 2001/0180 */. European Commission. Official Journal of the European Union C 304E: 327-330.

EFSA 2007. Guidance document for the risk assessment of genetically modified plants containing stacked transformation events by the Scientific Panel on Genetically Modified Organisms (GMO). EFSA J. 5: 1-5.

EFSA 2010. Guidance on the environmental risk assessment of genetically modified plants. EFSA J. 8: 1879.

EFSA 2013. Guidance on the environmental risk assessment of genetically modified animals. EFSA J. 11: 3200.

EFSA, Naegeli, H., Bresson, J. L., Dalmay, T., Dewhurst, I. C., Epstein, M. M., Guerche, P., Hejatko, J., Moreno, F. J., Mullins, E., Nogué, F., Rostoks, N., Sánchez Serrano, J. J., Savoini, G., Veromann, E., Veronesi, F., Bonsall, M. B., Mumford, J., Wimmer, E. A., Devos, Y., Paraskevopoulos, K. and Firbank, L. G. 2020. Adequacy and sufficiency evaluation of existing EFSA guidelines for the molecular characterisation, environmental risk assessment and post-market environmental monitoring of genetically modified insects containing engineered gene drives. EFSA J. 18: 6297.

EPA 2022. Introduction to biotechnology regulation for pesticides. United States Environmental Protection Agency (EPA). URL: https://www.epa.gov/regulation-biotechnology-under-tsca-and-fifra/introduction-biotechnology-regulation-pesticides. Accessed 1 Feb. 2023.

Epopa, P. S., Collins, C. M., North, A., Millogo, A. A., Benedict, M. Q., Tripet, F. and Diabate, A. 2019. Seasonal malaria vector and transmission dynamics in western Burkina Faso. Malar. J. 18: 113.

EU 2003. Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. Official Journal of the European Union L 268: 1-23.

EU 2014. Commission implementing regulation (EU) No 120/2014 of 7 February 2014 amending Regulation (EC) No 1981/2006 on detailed rules for the implementation of Article 32 of Regulation (EC) No 1829/2003 of the European Parliament and the Council as regards the Community reference laboratory for genetically modified organism. Official Journal of the European Union L 368: 99.

FDA 2001. Premarket notice concerning bioengineered foods. A proposed rule by the Food and Drug Administration on 01/18/2001. Unitied States Food and Drug Administration (FDA). 66 FR 4706: 4706-4738. URL: https://www.federalregister.gov/documents/2001/01/18/01-1046/premarket-notice-concerning-bioengineered-foods. Accessed 1 feb. 2023.

FDA 2022. Food from new plant varieties. Unitied States Food and Drug Administration (FDA). URL: https://www.fda.gov/food/food-ingredients-packaging/food-new-plant-varieties. Accessed 1 Feb. 2023.

FAO 2022. FAO GM foods platform. Food and Agriculture Organization of the United Nations (FAO). URL: https://www.fao.org/food/food-safety-quality/gm-foods-platform/browse-information-by/oecd-unique-identifier/en/. Accessed 1 Feb. 2023.

Gong, P., Epton, M. J., Fu, G., Scaife, S., Hiscox, A., Condon, K. C., Condon, G. C., Morrison, N. I., Kelly, D. W., Dafa'alla, T., Coleman, P. G. and Alphey, L. 2005. A dominant lethal genetic system for autocidal control of the Mediterranean fruitfly. Nat. Biotechnol. 23: 453-456.

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

Harris, A. F., Nimmo, D., McKemey, A. R., Kelly, N., Scaife, S., Donnelly, C. A., Beech, C., Petrie, W. D. and Alphey, L. 2011. Field performance of engineered male mosquitoes. Nat. Biotechnol. 29: 1034-1037.

Holst-Jensen, A., De Loose, M. and Van den Eede, G. 2006. Coherence between legal requirements and approaches for detection of genetically modified organisms (GMOs) and their derived products. J. Agric. Food Chem. 54: 2799-2809.

IPPC 2022. Glossary of phytosanitary terms (as adopted by CPM-16, 2022). International Standard for Phytosanitary Measures No. 5. Rome. FAO on behalf of the Secretariat of the International Plant Protection Convention (IPPC). URL: https://www.ippc.int/en/publications/622/. Accessed 1 Feb. 2023.

Kim, S. I. and Gelvin, S. B. 2007. Genome-wide analysis of Agrobacterium T-DNA integration sites in the Arabidopsis genome generated under non-selective conditions. Plant J. 51: 779-791.

Kyrou, K., Hammond, A. M., Galizi, R., Kranjc, N., Burt, A., Beaghton, A. K., Nolan, T. and Crisanti, A. 2018. A CRISPR-Cas9 gene drive targeting doublesex causes complete population suppression in caged

Anopheles gambiae mosquitoes. Nat. Biotechnol. 36: 1062-1066.

Lezaun, J. 2006. Creating a new object of government: making genetically modified organisms traceable. Soc. Stud. Sci. 36: 499-531.

Mitchell, H. J. and Bartsch, D. 2019. Regulation of GM organisms for invasive species control. Front. Bioeng. Biotechnol. 7: 454.

Nolan, T., Bower, T. M., Brown, A. E., Crisanti, A. and Catteruccia, F. 2002. piggyBac-mediated germline transformation of the malaria mosquito *Anopheles stephensi* using the red fluorescent protein dsRED as a selectable marker. J. Biol. Chem. 277: 8759-8762.

OECD 1993. Safety considerations for biotechnology: scale-up of crop plants. Organization for Economic Cooperation and Development (OECD), Paris. URL: https://www.oecd.org/science/biotrack/1958527.pdf. Accessed 1 Feb. 2023.

OECD 2006. Revised 2006: OECD guidance for the designation of a unique identifier for transgenic plants. Organisation for Economic Cooperation and Development (OECD). URL: https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV-JM-MONO(2002)7/rev1&doclanguage=en. Accessed 1 Feb. 2023.

OGTR 2019. A generic framework and generic risk factors to inform the environmental risk assessment of organisms. Office of the Gene Technology Regulator (OGTR). URL: https://www.ogtr.gov.au/sites/default/files/files/2021-07/generic_risk_assessment_framework_for_organisms.pdf. Accessed 1 Feb. 2023.

OGTR 2022. Australian Government. Department of Health and Aged Care. Office of the Gene Technology Regulator (OGTR). https://www.ogtr.gov.au/. Accessed 1 Feb. 2023.

OIRA 2003. Premarket Notice Concerning Bioengineered Foods. RIN: 0910-AC15. United States Office of Information and Regulatory Affairs of the Office of Management and Budget within the Executive Office of the President (OIRA). URL: https://www.reginfo.gov/public/do/eAgendaViewRule?pubId=200304&RIN=0910-AC15. Accessed 1 Feb. 2023.

Phuc, H. K., Andreasen, M. H., Burton, R. S., Vass, C., Epton, M. J., Pape, G., Fu, G., Condon, K. C., Scaife, S., Donnelly, C. A., Coleman, P. G., White-Cooper, H. and Alphey, L. 2007. Late-acting dominant lethal genetic systems and mosquito control. BMC Biol. 5: 11.

Pollegioni, P., Persampieri, T., Minuz, R. L., Bucci, A., Trusso, A., Martino, S. D., Leo, C., Bruttini, M., Ciolfi, M., Waldvogel, A. M., Tripet, F., Simoni, A., Crisanti, A. and Muller, R. 2022. Introgression of a synthetic sex ratio distortion transgene into different genetic backgrounds of *Anopheles coluzzii*. Insect Mol. Biol. 9: 5158.

USDA 2022a. Regulation of Biotech Plants. URL: https://www.usda.gov/topics/biotechnology/how-federal-government-regulates-biotech-plants. Accessed 1 Feb. 2023.

USDA 2022b. United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service. Movement of certain genetically engineered organisms, 7 CFR Parts 330, 340, and 372 [Docket No. APHIS–2018–0034] RIN 0579–AE47. US Government. Federal Register 85: 96. May 18, 2020 / Rules and Regulations. URL: https://www.aphis.usda.gov/brs/fedregister/BRS_2020518.pdf. Accessed 1 Feb. 2023

USDA, FDA, EPA 2017. The unified website for biotechnology regulation. 2017 update to the coordinated framework for the regulation of biotechnology. URL: https://usbiotechnologyregulation.mrp.usda.gov/biotechnologygov/resources. Accessed 1 Feb. 2023.

Voigt, B. and Münichsdorfer, A. 2019. Regulation of genome editing in plant biotechnology: European Union. In: Dederer, H. G.. and Hamburger, D. (eds.) Regulation of Genome Editing in Plant Biotechnology. Springer, Cham.

White, G. B. 1971. Chromosomal evidence for natural interspecific hybridization by mosquitoes of the *Anopheles gambiae* complex. Nature 231: 184-185.

WHO 1991. Strategies for assessing the safety of foods produced by biotechnology: report of a joint FAO/WHO consultation. World Health Organization (WHO), Geneva. URL: https://apps.who.int/iris/handle/10665/41465. Accessed 1 Feb. 2023.

WHO 2020. Evaluation of genetically modified mosquitoes for the control of vector-borne diseases. World Health Organization (WHO), Geneva. URL: https://www.who.int/publications/i/item/9789240013155. Accessed 1 Feb. 2023.

WHO 2021. Guidance framework for testing genetically modified mosquitoes, second edition. World Health Organization (WHO), Geneva. URL: https://www.who.int/publications/i/item/9789240025233. Accessed 1 Feb. 2023.

WHO 2022. World malaria report 2022. World Health Organzation (WHO), Geneva. https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2022. Accessed 1 Feb. 2023.

Yao, F. A., Millogo, A. A., Epopa, P. S., North, A., Noulin, F., Dao, K., Drabo, M., Guissou, C., Kekele, S., Namountougou, M., Ouedraogo, R. K., Pare, L., Barry, N., Sanou, R., Wandaogo, H., Dabire, R. K., McKemey, A., Tripet, F. and Diabate, A. 2022. Mark-release-recapture experiment in Burkina Faso demonstrates reduced fitness and dispersal of genetically-modified sterile malaria mosquitoes. Nat. Commun. 13: 796.

Figures

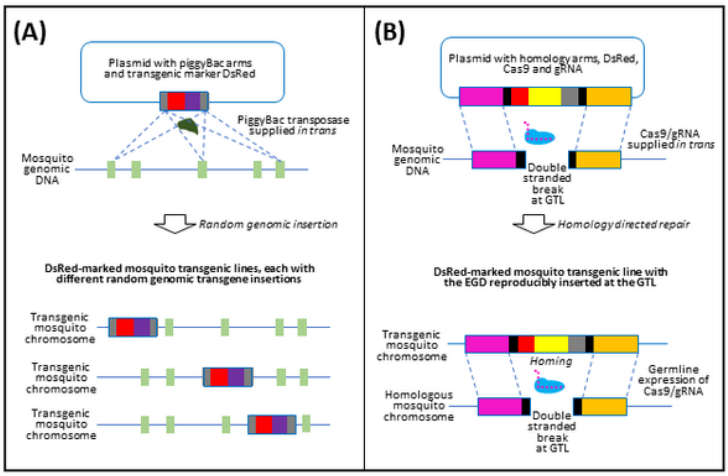

Figure 1

Differences in transforming mosquitoes using random or precise genomic insertion. (A) In this example of random integration, the PiggyBac transposon system is used to create transgenics containing a transgene consisting of the DsRed fluorescent marker under the control of an ocular promoter (red) and effector gene and its regulatory elements (purple). In germline injections, PiggyBac transposase (dark green) is supplied *in trans* either as protein or from a helper plasmid. This acts on the inverted terminal repeats of the PiggyBac arms (grey) of the transformation plasmid to integrate the transgene at any genomic location consisting of the DNA sequence TTAA (green). This means that each transgenic generated from such injections will potentially have random insertions of the transgene at TTAA sites anywhere in the genome. (B) In an approach involving precise genomic insertion, the CRISPR-Cas9 genome editing system is used to create transgenics containing an EGD that consists of 5' (pink) and 3' (orange) homology sequences flanking the GTL (black), the DsRed fluorescent marker under the control of an ocular promoter (red), Cas9 under the control of a germline promoter (yellow), and the gRNA under the control of the ubiquitous and constitutive U6 promoter (dark grey). In germline injections, the Cas9/gRNA (light blue shape/dotted pink line) ribonucleoprotein is supplied *in trans* either as protein or from a helper plasmid to generate a double stranded break in the GTL. The germline cell detects the cut and instigates homology directed repair, which uses the 5' and 3' homologous sequences flanking of the GTL of the transgenic mosquito chromosome to repair the double stranded break and, in doing so, transfers a copy of the EGD into the GTL of the homologous chromosome. Thus, any transgenic line using this method will always contain the EGD at the GTL so that in subsequent generations the EGD is capable of homing to non-transgenic homologous chromosome to bias its own inheritance.

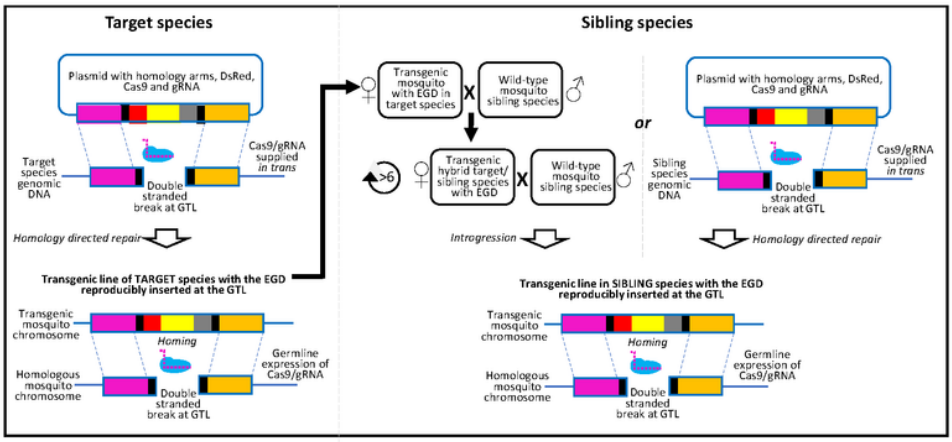

Figure 2

Generation of transgenic mosquitoes containing the same EGD at a conserved GTL in target and sibling species, either using *de novo* transformation or introgression via backcrossing. The target species is transformed using a homology directed repair-based approach to insert the EGD at the GTL, which is conserved in both the target species and sibling species. To produce a sibling species with the EGD at the conserved GTL, two approaches are possible. In the first, the EGD that was originally generated in the genetic background of the target species is crossed to the sibling species to produce interspecific fertile female hybrids and repeatedly backcrossing to the parental sibling species. The second possibility is *de novo* transformation of the sibling species, where the same EGD integrated in the target species is inserted into the conserved GTL of the sibling species.