Minos-mediated transgenesis in the pantry moth *Plodia interpunctella*

Short title: Minos transgenesis in *Plodia* pantry moths

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Abstract

Transposon-mediated transgenesis has been widely used to study gene function in Lepidoptera, with *piggyBac* being the most commonly employed system. However, because the *piggyBac* transposase originates from a lepidopteran genome, concerns about potential endogenous activity and remobilization raise questions about its suitability for long-term genomic modifications across a wide range of species. As an alternative, we tested the effectiveness of the *Minos* transposase, a *Tc1/mariner* family element derived from a dipteran insect, for stable germline transformation in the pantry moth *Plodia interpunctella*. By injecting a *Minos*-based construct along with *in vitro* synthesized *Minos* transposase mRNA into syncytial embryos, we observed somatic activity of the *3xP3::EGFP* eye/glia reporter gene in only 1 out of 14 (7.1%) injected G_0 individuals that reached the pupal stage. Nonetheless, pooled in-crossing of G_0 individuals resulted in high rates of germline transmission of the transgene, with 23.6% of G_1 pupae showing *3xP3*-driven eye fluorescence. Together with a previous study in *Bombyx* silkworms, these data suggest that *Minos*-mediated transgenesis yields practical rates of germline integration in Lepidoptera, providing an alternative tool for functional genomics in species where *piggyBac* may not be optimal.

Introduction

There is growing interest in developing new laboratory model systems and methods for the study of functional genomics in Lepidoptera, an enormous insect lineage where the silkworm *Bombyx mori* has been a focal species for the study of gene function. The pantry moth *Plodia interpunctella* is a pest of stored food with life history traits that make it suitable for economical and long-term maintenance of genetic lines. We and others have previously established methods for non-homologous repair mediated CRISPR mutagenesis, CRISPR short edits based on homologous recombination, and *piggyBac*-mediated transgenesis in this organism (Heryanto, Mazo-Vargas, et al. 2022; Heryanto, Hanly, et al. 2022; Shirk et al. 2023). Thus far, we have failed to insert a transgenic cassette of several kilobases using CRISPR-related techniques based on homologous repair and non-homologous plasmid insertion. Thus, transposon-based transgene random insertion remains a valuable method in *Plodia*, and we expect that the further optimization of such methods will potentiate fundamental studies of lepidopteran biology, notably because the reagents used in these

experiments – the source of transposase mRNA and the transgene donor plasmids – are presumably transferable to new species without modification.

One potential limitation of transgenesis in Lepidoptera as currently implemented, however, is that the favored *piggyBac* transposase originated from a lepidopteran species, the cabbage looper *Trichoplusia ni* (Fraser et al. 1985). Because lepidopteran laboratory species are expected to have some level of endogenous *piggyBac*-family transposon activity, this could elicit remobilization of newly inserted cassettes and may cause unwanted effects such as movements of transgenes in the genome, genomic instability that would compromise germline function and reduce fertility, or selection of transgene insertions at sites that are transcriptionally repressed in tissues of interest. Consistent with these potential issues, two studies documented that *piggyBac* transgenes can be remobilized in *Bombyx* and hinder the interpretability of reporter assays (Wang et al. 2015; Jia et al. 2021).

Here we investigated the suitability of an alternative transposon-based method, using the *Minos* transposase of the *Tc1/mariner* derived from the dipteran *Drosophila hydei*, a system that has been widely used for transgenesis in insects (Franz and Savakis 1991; Loukeris et al. 1995; Loukeris et al. 1995; Kapetanaki et al. 2002; Pavlopoulos et al. 2004; Metaxakis et al. 2005) and other invertebrates (Zagoraiou et al. 2001; Hozumi et al. 2010; Kontarakis and Pavlopoulos 2014; Jackson et al. 2024; Kuo et al. 2024). Importantly, a previous study found that the *Minos* transposase can mediate efficient germline integration in *Bombyx* embryos when delivered as an *in vitro* transcribed mRNA (Uchino et al. 2007). We transferred these methods to *Plodia* which resulted in efficient germline transmission of a fluorescent marker, and thus confirmed that *Minos* can mediate stable transgenesis in another lepidopteran laboratory system. We discuss strategies for the rapid generation of transgenic lines in *Plodia* and provide detailed methods to facilitate the use of this technique in other species.

Material & Methods

Microinjection reagents for *Minos* transgenesis

The donor plasmid carrying the transgenesis marker, *pMi(3xP3::EGFP-SV40)*, as well as the *Minos* transposase transcription template plasmid *pBlueSKMimRNA* (Pavlopoulos et al. 2004) were sourced from Addgene (*RRID:Addgene_102540; RRID:Addgene_102535*). Plasmids were amplified in 50 mL liquid cultures of Luria-Bertani broth with 100 µg/mL Ampicillin, and plasmid DNA was purified with the ZymoPURE II Plasmid Midiprep Kit (Cat# D4200). Plasmids were eluted in 120 µL of elution buffer, yielding concentrations >4,800 ng/µL of plasmid DNA.

To linearize the *pBlueSKMimRNA* plasmid for mRNA synthesis, the plasmid was digested using the *Not*I-HF restriction enzyme (New England Biolabs). The linearized DNA was first purified by gel extraction using the Zymoclean Gel DNA Recovery Kit (Zymo Research) and further purified using the Zymo DNA Clean & Concentrator-25 kit with an elution volume of 13 µL of DNA elution butter. Subsequently, the HiScribe T7 ARCA mRNA with tailing kit (New Englands Biolabs) was used for transcription and capping of *Minos* transposase mRNA, followed by purification using the MEGAClear Transcription Clean-Up Kit (Thermo Fisher Scientific). The purified mRNA was eluted in 50 µL of the provided elution

buffer, which was pre-heated to 65°C and incubated on the filter column for 10 minutes in a 65°C oven. After quantification with Nanodrop (Thermo Fisher Scientific), the transposase mRNA was divided into > 520 ng/µL one-time use aliquots and stored at -70°C.

Injection mixes were freshly prepared before injection and consisted of 400 ng/µL *Minos* transposase mRNA, 200 ng/µL donor plasmid, and 0.05% Phenol Red (1:10 dilution of a 0.5% cell-culture grade Phenol Red solution, Sigma-Aldrich), brought to a 5 µL final volume with 1x *Bombyx* injection buffer (pH = 7.2, 0.5 mM NaH_2PO_4, 0.5 mM Na_2HPO_4, 5 mM KCl).

Plodia **stock**

The white-eyed *wFog* laboratory strain of *Plodia interpunctella* was used to facilitate the screening of *3xP3*-driven fluorescence in pupal and adult eyes (Heryanto, Hanly, et al. 2022; Heryanto, Mazo-Vargas, et al. 2022). This strain consists in the genome background of the *Dundee* strain (NCBI genome accession GCA_900182495.1), into which a *white* gene *c.737delC* mutation originating from the *Piw3* strain (NCBI genome accession: GCA_022985095.1) was introgressed during two generations of backcrossing and several rounds of sib-sib inbreeding (Heryanto, Hanly, et al. 2022).

Plodia **husbandry**

The *wFog* strain and the transgenic broods were reared in the laboratory from egg to adulthood in an incubator set at 25°C with 40-70% relative humidity and a 14:10 h light:dark cycle, following methods made available via the Open Science Framework repository (Heryanto et al. 2025). Briefly, egg laying was induced by CO_2 narcosis of adult stock in an oviposition jar, and a weight boat containing 10-12 mg eggs was placed in a rearing container containing 45-50 g of wheat bran diet with 30% glycerol (**Table S1**). This life cycle spans 29 days from fertilization to a reproductively mature adult stock at 25°C.

Microinjection of *Plodia* syncytial embryos

The general procedure for microinjection as well as a list of suggested equipment is available online (Heryanto, Mazo-Vargas, et al. 2022) and summarized below. *Minos* capped mRNA and the donor plasmid were mixed into a 5 µL aliquot (400 : 200 ng/µL mRNA:DNA) and kept on ice shortly before the procedure. Eggs were collected in an oviposition jar following CO_2 narcosis of a stock of *wFog* strain adults, aligned on parafilm, and microinjected between 20-50 min after egg laying (AEL) using borosolicate pulled glass needles and an air pressure microinjector. Eggs were then sealed with cyanoacrylate glue and left to develop at 25°C in a closed tupperware with a wet paper towel.

Fluorescent screening and imaging

All life stages were screened under an Olympus SZX16 stereomicroscope equipped with a Lumencor SOLA Light Engine SM 5-LCR-VA lightsource, mounted to a trinocular tube connected to an Olympus DP73 digital color camera. Chroma Technology filter sets for the detection of EGFP (ET470/40×, ET510/20m) were used to screen *3xP3::EGFP* fluorescence. An initial screening of G_0 fluorescence in injected eggs is optimal at 96-112 h AEL, a time window where *3xP3*-driven fluorescence in presumptive glia and ocelli is discernible in the larval head and body but precedes hatching time at 120h AEL, assuming incubation at 25°C.

Earlier times of screening show weaker fluorescence and confounding signals, likely due to the episomal expression of residual plasmids in large vitellophages (Heryanto, Mazo-Vargas, et al. 2022). Pupae of white-eyed mutant moths are most useful to select transgenic founders, but can be difficult to isolate from the larval diet. To alleviate this, strips of cardboard are added to larval containers that contain fifth instar larvae, as the corrugations within this material are the preferred pupation site that facilitates the collection of pupae (Shim and Lee 2015). Pupae were isolated from these lodges and placed on double-sided tape dabbed with task wipe fibers for screening. Fluorescent and brightfield images were acquired using the same fluorescent microscopy set-up using the Olympus DP74 camera, and overlaid using the Screen blending mode in Adobe Illustrator. Brightfield images of transgenic G_2 adult eyes (**Fig. 1F**) were taken using a Keyence VHX- 5000 digital microscope.

Crossing

Pupae selected for crossing were each moved to a 1.25 oz Plastic Souffle Cup containing about 1 cm^2 of paper towel for adult emergence. All G_0 individuals were intercrossed together, which consisted of 1 fluorescent positive individual and 13 fluorescent negative individuals. G_1 individuals were sexed as pupae to ensure controlled mating and crosses. For backcrosses, each G_1 individual was crossed with 3 or 4 unmated individuals of the opposite sex from the control genetic background (*wFog*) (**Table 1**). Crosses were set up either as adults or late stage pupae, where the plastic cup was transferred to a bed of Plodia diet in a rearing container (Heryanto, Hanly, et al. 2022). Once wandering L5 were visible, cardboard strips were added to the diet container for pupation. Pupae were then isolated from the corrugated cardboard grooves, as well as from the diet that were identified by frass laden silk cocoons. The resulting G_2 were backcrossed to non-transgenic white-eyed moths (*wFog*).

Results

To test for the efficacy of *Minos*-mediated transgenesis in *Plodia* embryos, we microinjected 341 eggs between 20-50 mins after egg laying (AEL). This injection time window, as well as the microinjection technique and rearing conditions were comparable to our previous experiments using the *Hyperactive piggyBac* transposase (*HyPBase*) in *Plodia* (Heryanto, Mazo-Vargas, et al. 2022). The injection mix consisted of capped poly-A tailed *Minos* mRNA, mixed with a donor plasmid for the integration of the *3xP3::EGFP* transgenesis marker cassette. About 10% of G_0 embryos showed *3XP3*-driven fluorescence upon initial screening (**Table 1**), and this injection batch generated only 14 pupae, one of which showed mosaic EGFP expression in its eyes. The low number of pupae obtained may be due to poor hatching conditions in this batch rather than genotoxicity, as survival of late embryos and hatchling is sensitive to humidity conditions. Next, we in-crossed all the 14 G_0 adults by pooling them in a rearing container for random mating, and recovered 250 G_1 pupae, 23.6% of which showed transgene activity (**Fig. 1C**). Out of the 59 pupae with eye fluorescence, 10 showed an additional, unexpected fluorescence in pupal oenocytes (Stendell 1912; Shirk and Zimowska 1997; Makki et al. 2014), visible as ventral metameric belts in the second to seventh abdominal segments (**Supplementary Figure 1**). This activity is likely due to an identical

insertion shared between these 11 siblings, dubbed the oenocyte-fluorescent allele below. To further confirm the transmissibility of the G_1 insertion alleles, we generated four backcross and in-crosses, which generated 32% transgenic G_2 offspring individuals in total (**Table 1**). In the first backcross, a positive male was mated with 4 non-transgenic white-eyed females (*wFog*), yielding 70.8% transgenic offspring. In-crossing a transgenic female to negative males from the G_1 pool recovered 67.3% transgenic G_2 offspring. Finally, two separate crosses each involved the mating of four negative G_1 females to one transgenic male that showed both *3xP3* and oenocyte fluorescent activities. These two crosses generated about 53.5% transgenic G_2 pupae.

A further examination of the *3xP3::EGFP* transgene activity in the G_2 generations revealed fluorescence patterns identical to the ones previously reported (Heryanto, Mazo-Vargas, et al. 2022) with *piggyBac*-mediated integration, consistent with glial expression in embryos, and ocelli expression in larvae (**Fig. 1D-E, Supplementary Video 1**). In adults of the wFog (white-eyed background), the retinal expression of EGFP was strong enough to reveal a green tint of the eyes, visible without fluorescent microscopy equipment (**Fig. 1F**).

Discussion

The feasibility of transgenesis experiments depends not only on the germline integration rate of the transposase system, but also on other logistic criteria such as the injection, rearing and crossing efforts. Below, we elaborate on these aspects and provide experimental considerations with the overarching goal to facilitate implementation in *Plodia* and other lepidopteran organisms.

We detected lower rates of mosaic fluorescence with *Minos* compared to previous *HyPBase* experiments, with 10% of the G_0 injected eggs and 7% of surviving pupae showing a *3xP3* signal compared to 22% of injected eggs and 32% of surviving pupae with *HyPBase*. While prone to future optimization, these preliminary results indicate that *Minos*-mediated transposition rates may not be as efficient in the soma as the ones reported with the *HyPBase*. This is likely because *HyPBase* was genetically engineered to boost activity of the original *T. ni* piggyBac transposase (Yusa et al. 2011).

We decided to ignore whether G_0 founders showed transgenesis marker activity, and treated all surviving G_0 adults as potential germline carriers of the transgene. In order to establish a G_1 stock, all 14 surviving adults from the injected batch were transferred to a larval diet container and allowed to mate randomly as a pool of founders. This approach generated G_1 individuals with *3XP3*-driven fluorescence, with 23.6% of 250 screened pupae showing EGFP expression in their eyes. This pooled G_0 crossing scheme presents some caveats. First, not being able to trace how many individuals reproduced, we could not accurately measure germline integration rates as previously done in other insects (Gregory et al. 2016; Heryanto, Mazo-Vargas, et al. 2022). Second, this approach risks generating heterogenous lines with multiple insertion sites.

To mitigate these issues, individual backcrossing of each G_0 founder to uninjected adults of the opposite sex would be preferable, but this represents a significant effort.

Nevertheless, we suggest that a G_0 pooling approach would be appropriate as an initial assessment of feasibility, or for transgenesis experiments where positional effects or multiple copies are not interfering with interpretation. This is reasonable, for example, if a transgene is designed to fluorescently label structures for qualitative imaging. For more quantitative assays, or for expression-reporter assays that may be influenced by insertion sites, it would nonetheless be possible to backcross G_1 founders over several generations, and then assess whether transgene segregation occur in the mendelian ratios expected from single parental copies (Heryanto, Mazo-Vargas, et al. 2022). In other words, pooled in-crossing of *Minos*-injected G_0 individuals can be a practical way to establish transgenic lines in *Plodia* and other insects, and we recommend future experimenters to not limit G_0 crossing efforts only to individuals in which somatic activity of the transgenesis marker was detected.

Finally, we must caution that the successful screening of *3xP3*-driven fluorescence requires relatively translucent tissues at most stages. With that being said, in species where tissue opacity may block fluorescence, or where a white-eyed mutant stock is not available, it should still be possible to screen for *3xP3* activity in the larval lateral ocelli (stemmata), or in early pupal eyes before melanization occurs (Thomas et al. 2002; Özsu et al. 2017).

Conclusions

Our data presented here, together with a previous report in *Bombyx* (Uchino et al. 2007), show that *Minos* transgenesis is applicable to lepidopteran systems provided it is possible to inject early syncytial embryos and maintain relatively inbred lines. Here, the ease of rearing and the ability to induce egg laying with CO_2 narcosis in *Plodia* allowed two experimenters to microinject 341 embryos during a 3 hour session, using a single stock of reproductively mature moths, and the pooled crossing scheme fostered the recovery of fluorescent G_1 individuals without time-intensive crossing efforts. *Minos*-mediated transgenesis could be considered as a key transgenesis in Lepidoptera, given the risks of transgene remobilization and instability associated with more widely used *piggyBac* transposases of lepidopteran origin.

Acknowledgements

We thank Jasmine Alqassar and Christa Heryanto for maintaining *Plodia* cultures, Patricia Hernandez for providing access to microscopy equipment, and the Institutional Biosafety Committee at the George Washington University for administering the approval of Plodia rearing and transgenesis protocols in the Martin lab (protocol #IBC-24-261).

Figures

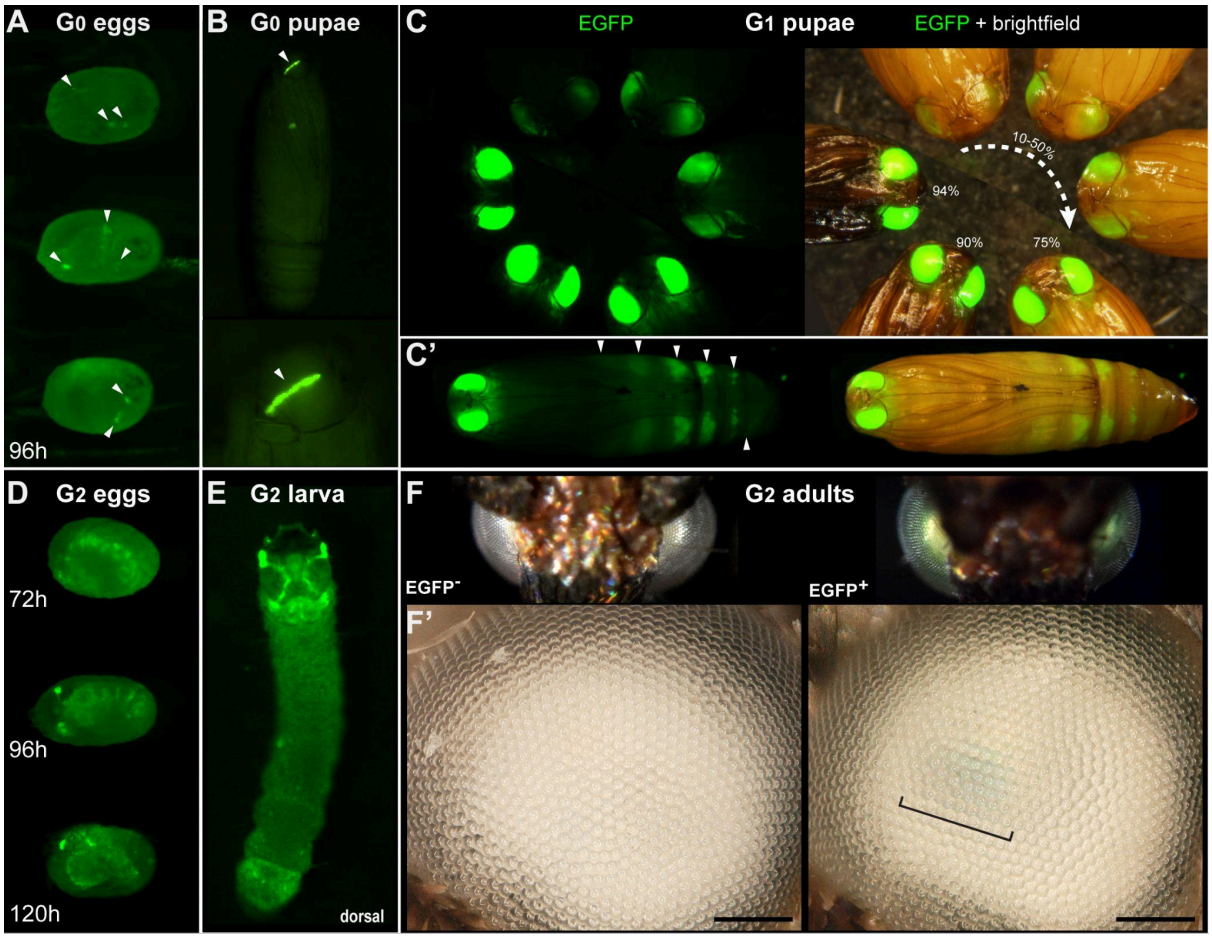
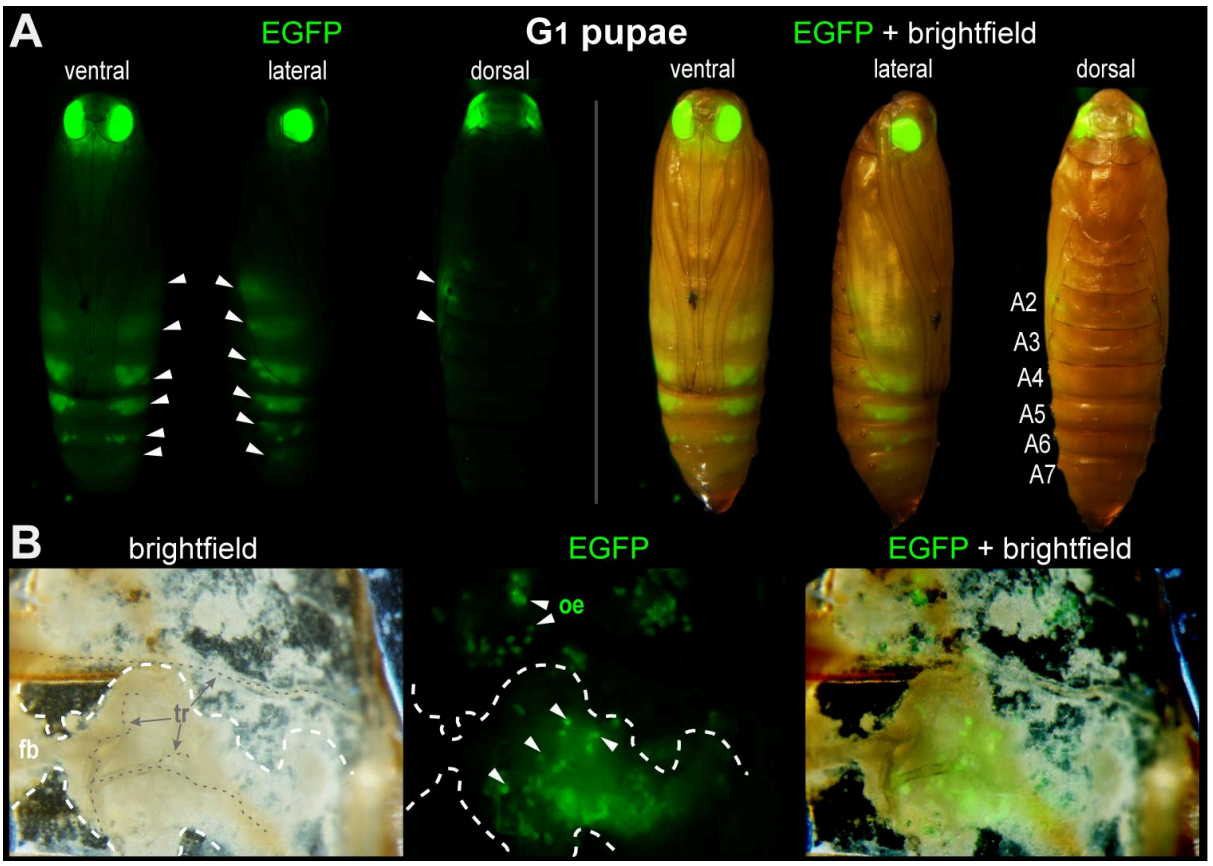


Figure 1. Transgenic *Plodia* carrying the *3xP3::EGFP* cassette via *Minos*-mediated transgenesis. (A) G_0 eggs from injections of the donor plasmid *pMi[3xP3::EGFP]* and *Minos* transposase mRNA. Three examples of *3xP3* activity are shown at 96 hrs after egg laying (AEL). **(B)** Mosaic *3xP3::EGFP* expression in the G_0 pupal eye: 1 out of 14 pupae (7%) exhibited this positive EGFP phenotype. **(C)** Non-mosaic expression of EGFP in G_1 pupal eyes. EGFP intensity increases with the developmental stage of the pupae. Right panel depicts an overlay of EGFP and brightfield images taken separately, and pupal stages (indicated by percentages) were estimated based on eye, wing, tarsal, and cuticle pigmentation (Zimowska et al. 1991). **(C')** 10 out of 59 G_1 pupae positive for *3xP3::EGFP* showed ectopic expression in abdominal segments (white arrowheads) in addition to the eyes. **(D)** G_2 eggs express EGFP in the expected *3xP3*-driven activity in the developing ocelli and presumptive glia detectable as early as 72 hrs AEL until 120 hrs before hatching (at 25°C). **(E)** *pMi[3xP3::EGFP]* expression in a first instar G_2 larva shows strong activity in head glia and lateral ocelli. **(F-F')** Adult G_2 individuals positive for *3xP3::EGFP* have green eyes under normal white light, due to strong EGFP expression. Comparison of an G_2 adult negative (left) and positive (right) for *3xP3::EGFP* under white light illumination and without fluorescence filters (F': magnified, lateral views of corresponding eyes). Black bracket points to a visible green tint in the inner retinal tissue. Scale bars = 100 µm.



Supplementary Figure 1. Ectopic oenocyte expression of *pMi[3xP3::EGFP]* in a portion of G_1 progeny. (A) 10 out of 59 (17%) G_1 pupae positive for *3xP3::EGFP* also showed fluorescent activity in metameric belts of cells restricted to the abdominal segments A2 to A7 (white arrowheads). **(B)** Dissection of the pupal cuticle with internal tissues and fat body partially removed. Fluorescence is detected in large cells embedded under the cuticle and in proximity to tracheal projections. These cells have been described as oenocytes in the closely related phycitine moth *Ephestia kuehniella* (Stendell 1912). Abbreviations: tr, trachea ; fb, fat body; oe, oenocytes.

Supplementary Video 1. Transgenic fluorescent detection in hatchlings.
pMi[3xP3::EGFP] fluorescence activity in G_2 first-instar larvae shortly before (left) and during hatching (right).

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