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Carsten Horn · Ernst A. Wimmer

A versatile vector set for animal transgenesis

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Abstract Genetic manipulation of a series of diverged arthropods is a highly desirable goal for a better understanding of developmental and evolutionary processes. A major obstacle so far has been the difficulty in obtaining marker genes that allow easy and reliable identification of transgenic animals. Here, we present a versatile vector set for germline transformation based on the promiscuous transposons *mariner*, *Hermes* and *piggyBac*. Into these vectors, we introduced a potentially universal marker system that is comprised of an artificial promoter containing three Pax-6 homodimer binding sites. This promoter drives strong expression of spectral variants of the enhanced green fluorescent protein (EGFP) in larval, pupal, and adult photoreceptors. Using special filter sets, the yellow (EYFP) and cyan (ECFP) variant are fully distinguishable and therefore represent a separable pair of markers. Furthermore, we adapted a simple plasmid-based transposition assay system to enable quick functional tests of our vectors in different arthropod species before employing them in more laborious germline transformation experiments. Using this system we demonstrate that our vectors transpose in both *Drosophila melanogaster* and *Drosophila virilis*.

Keywords Green fluorescent protein · Pax-6 · Transformation marker · Transposable elements · Transposon mutagenesis

Introduction

Transgenic animals can be generated by the use of transposable elements. P-element transformation is by far the best utilized system in flies (Engels 1996). However, specific host factor requirements restrict P-element mobility to species closely related to *D. melanogaster* (Rio and Rubin 1988). In contrast, a series of other DNA-mediated transposable elements that have recently been discovered are widespread in the animal kingdom, and independence of host factors could be shown (Lampe et al. 1996). The *Tc1*/*mariner* element *Mos1* derived from *D. mauritiana* (Medhora et al. 1991) is able to transpose in the mosquito *Aedes aegypti* (Coates et al. 1998), in zebrafish (Fadool et al. 1998), in chicken (Sherman et al. 1998), and can mediate trans-kingdom transposition into the parasitic protozoan *Leishmania* (Gueiros-Filho and Beverley 1997). Two further completely unrelated transposons, the *hAT* element *Hermes* derived from the housefly *Musca domestica* (Warren et al. 1994) and the TTAA element *piggyBac* derived from the lepidopteran cabbage looper *Trichoplusia ni* (Cary et al. 1989), functionally transpose in series of different insect embryos (Sarkar et al. 1997a; Lobo et al. 1999), and have shown their ability to cross genera and orders of insects in germline transformation experiments (Handler et al. 1998; Jasinskiene et al. 1998; Berghammer et al. 1999; Hediger et al. 2000; Pinkerton et al. 2000; Toshiki et al. 2000). Helper plasmids providing transposase sources for these three transposons have been developed and shown to mediate transposition of modified, non-autonomous elements (O'Brochta et al. 1996; Coates et al. 1998; Handler and Harrell 1999). Thus, the means for germline transformation of different arthropod species are provided.

However, a major obstacle in the use of these transposons has been the difficulty in obtaining marker genes that allow easy and reliable identification of transgenic animals. Species-specific transformation markers can be generated by first isolating visible mutations in the species of interest, then cloning the corresponding gene, and finally rescuing the mutant phenotype by incorporation of a wild type copy of this gene through transformation. Although straightforward, this procedure is laborious and risky, and the same considerable investment must be put into every species. Therefore, a universal marker that

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C. Horn · E.A. Wimmer (✉)
Lehrstuhl für Genetik, Universität Bayreuth,
Universitätsstrasse 30 NWI, 95447 Bayreuth, Germany
e-mail: ernst.wimmer@uni-bayreuth.de
Tel.: +49-921-555813, Fax: +49-921-552710

could serve to follow gene transfer in any species would provide a major advantage. In Berghammer et al. (1999), a novel eye-specific and potentially universal transformation marker was presented that is based on the enhanced green fluorescent protein (EGFP; Tsien 1998) and an artificial promoter that is responsive to the evolutionary conserved transcription factor Pax-6 (3xP3; Sheng et al. 1997). In the companion paper (Horn et al. 2000), we show the high sensitivity of the marker and indicate the advantages that markers with gene expression in pre-adult stages might have for non-model organisms. This will be of particular interest to the field of evolutionary developmental biology and for modern pest management programs.

Here, we present further developments on our potentially universal germline transformation systems based on the 3xP3-EGFP marker. First we demonstrate the suitability of the yellow (EYFP) and cyan (ECFP) spectral variants of EGFP as additional independent and completely distinguishable transformation markers. Second we introduce vectors for a convenient and versatile two-step cloning procedure consisting of a complete set of transformation vectors based on the three promiscuous transposons *mariner*, *Hermes*, and *piggyBac*, and the three spectral variants EGFP, EYFP, and ECFP. Third we present an adaptation of our transformation systems to quickly examine its usability in series of different arthropod species by simple plasmid-based embryonic transposition assays.

Materials and methods

Plasmid construction

All plasmids were constructed using standard molecular biology techniques as described by Sambrook et al. (1989).

pSLfa1180fa – a versatile cloning shuttle vector

pSLfa1180 was constructed by introducing the annealed oligos FSASHNDF (5′-AGCTGGCCGGCCTAGGCGCGCCA-3′) and FSASHNDR (5′-AGCTTGGCGCGCCTAGGCCGGCC-3′) containing the rare octa-cutter restriction sites *Fse*I and *Asc*I into the 5′ most site (*Hin*dIII) of the multiple cloning site (MCS) of pSL1180 (Pharmacia). Similarly pSL1180fa was constructed by introducing the annealed oligos FSASECOF (5′-AATTCGGCCGGCCTAGGCGCGCC-3′) and FSASECOR (5′-AATTGGCGCGCCTAGGCCGGCCG-3′) into the 3′ most site (*Eco*RI) of the MCS of pSL1180. Plasmids containing the desired sequence of restriction sites (*Fse*I-*Asc*I-*Hin*dIII-MCS for pSLfa1180 and MCS-*Eco*RI-*Fse*I-*Asc*I for pSL1180fa) were determined by sequence analysis. The versatile cloning vector pSLfa1180fa containing *Fse*I and *Asc*I restriction sites flanking the MCS of pSL1180 (Pharmacia) was constructed by ligating the 1.4 kb *Sca*I fragment from pSLfa1180 to the 2.0 kb *Sca*I fragment from pSL1180fa.

Set of universal transformation vectors

To introduce the rare octa-cutter restriction sites *Asc*I and *Fse*I into pSL-3xP3-EGFP (Horn et al. 2000) the oligos FSASNRBF (5′-CGAGATCGGCCGGCCTAGGCGCGCC-3′) and FSASNRBR (5′-GTACGGCGCGCCTAGGCCGGCCGATCTCG-3′) were annealed and cloned between the *Bsi*WI and *Nru*I sites, thereby restoring the sites and generating pSL-3xP3-EGFPaf. pSL-3xP3-EYFPaf was constructed by replacing *egfp* with a 0.7 kb *Sal*I/*Not*I fragment from pEYFP-1 (Clontech). The sequence between the *Kpn*I and *Bam*HI sites of pEYFP-1 had previously been deleted by restriction with *Asp*718 and *Bam*HI, Klenow fill-in, and religation. pSL-3xP3-ECFPaf was then constructed by replacing *eyfp* with a 0.7 kb *Pin*AI/*Not*I fragment from pECFP (Clontech). Universal transformation vectors were prepared by introducing transformation marker genes into the *mariner*, *piggyBac* and *Hermes* transposon plasmids pBS*Mos*1 (Medhora et al. 1991), p3E1.2 (Cary et al. 1989) and pHermes[EGFP] (Pinkerton et al. 2000) as follows: pMos{3xP3-EGFPaf}, pMos{3xP3-EYFPaf} and pMos{3xP3-ECFPaf} were constructed by cloning the 1.3 kb *Eco*RI/*Nru*I fragment (blunted with Klenow) from pSL-3xP3-EGFPaf, pSL-3xP3-EYFPaf and pSL-3xP3-ECFPaf, respectively, into pBS*Mos*1 cut with *Sal*I and blunted with Klenow. The modified *mariner* transformation vectors containing a 0.6 kb deletion in the transposase open reading frame, pMos{3xP3-EGFPafm}, pMos{3xP3-EYFPafm} and pMos{3xP3-ECFPafm}, were constructed similarly except for cutting pBS*Mos*1 (Medhora et al. 1991) with *Sal*I and *Nru*I. pBac{3xP3-EGFPaf}, pBac{3xP3-EYFPaf} and pBac{3xP3-ECFPaf} were constructed by cloning the respective 1.3 kb *Eco*RI/*Nru*I fragments (blunted with Klenow) into p3E1.2 (Cary et al. 1989) cut with *Hpa*I. Modified *piggyBac* transformation vectors containing a 0.8 kb deletion in the transposase open reading frame, pBac{3xP3-EGFPafm}, pBac{3xP3-EYFPafm} and pBac{3xP3-ECFPafm}, were constructed by cloning a 100 bp *Fse*I-*Bgl*II fragment from pSLfa1180fa into *Fse*I and *Bgl*II cut pBac{3xP3-EGFPaf}, pBac{3xP3-EYFPaf} and pBac{3xP3-ECFPaf}, respectively. For all *mariner* and *piggyBac* constructs, the transcriptional direction of the inserted marker gene was chosen to be in opposite orientation to the transcriptional direction of the endogenous transposase. pHer{3xP3-EGFPaf}, pHer{3xP3-EYFPaf} and pHer{3xP3-ECFPaf} were constructed by cloning the respective 1.3 kb *Eco*RI/*Nru*I fragments into pHermes[EGFP] (Pinkerton et al. 2000) cut with *Hin*dIII, blunted with Klenow, and recut with *Eco*RI. P-Element derived transformation vectors, pP{w^{+mC}, 3xP3-EYFPaf} and pP{w^{+mC}, 3xP3-ECFPaf} were constructed by cloning the respective 1.3 kb *Eco*RI/*Nru*I fragments (blunted with Klenow) into pCaSpeR4 (Thummel and Pirotta 1992) cut with *Stu*I. The insertion variants orientated with maximal distance between the 3xP3 promoter and the promoter of the "mini"-*white* gene were chosen. Germline transformation experiments were performed as described in the companion paper (Horn et al. 2000).

Donor plasmids for the transposition assay

mariner, *piggyBac* and *Hermes* derived donor plasmids containing an *E. coli* origin of replication, and a kanamycin resistance gene were prepared as follows: pSLfa1180fa-OriKan was constructed by cloning a 2.7 kb *Eco*RI/*Hin*dIII fragment from pKhsp82 (Coates et al. 1996) into pSLfa1180fa cut with *Eco*RI and *Hin*dIII. pMos{3xP3-EGFPaf-OriKan}, pBac{3xP3-EGFPaf-OriKan} and pHer{3xP3-EGFPaf-OriKan} were constructed by cloning a 2.8 kb *Fse*I fragment from pSLfa1180fa-OriKan into *Fse*I cut pMos{3xP3-EGFPaf}, pBac{3xP3-EGFPaf} and pHer{3xP3-EGFPaf}, respectively.

Epifluorescence microscopy

EGFP, EYFP and ECFP marker fluorescence in *Drosophila* compound eyes was observed employing the Leica MZ FLIII fluorescence stereomicroscope with the planachromatic 0.5× objective. Documentation was carried out using the Leica WILD MPS52 camera and the Ektachrome P1600 color reversal film (Kodak). Depending on the nature of the fluorescing protein different filter systems were applied. Each filter system consists of excitation and emission filters. Bandpass emission filters restrict the detectable wavelength to a defined spectral width, whereas longpass emis-

Table 1 Filter sets for different spectral variants of GFP

Filter set	Excitation filter (λ_{max}/spectral width)	Emission filter (λ_{min} or λ_{max}/spectral width)
GFP2	480 nm/40 nm	510 nm
V	425 nm/40 nm	475 nm
GFP3	470 nm/40 nm	525 nm/50 nm
YellowGFP	500 nm/20 nm	535 nm/30 nm
CyanGFP	436 nm/20 nm	480 nm/40 nm

sion filters allow light above a defined wavelength to pass through. We used the longpass filter sets GFP2 (GFP Plus) and V (Leica, Bensheim), and the bandpass filter sets GFP3 (GFP Plant; Leica, Bensheim), YellowGFP (Chroma 41028; AHF analysentechnik AG, Tübingen), and CyanGFP (Chroma 31044v2; AHF analysentechnik AG, Tübingen). Spectral properties are summarized in Table 1. For simultaneously working with different spectral variants, stereomicroscopes with easily changeable filter sets, like the Leica MZ FLIII with its rotatable filter holder carrying four filter sets at a time, are advantageous.

Transposition assay

Transposition assays were carried out as described by Coates et al. (1997) and Sarkar et al. (1997a, b). The assay has been modified to directly employ derivatives of our universal transformation vectors as donor plasmids. In addition to the target vector pGDV1 (Coates et al. 1997; Sarkar et al. 1997a, b), the donors pMos{3xP3-EGFPaf-OriKan}, pBac{3xP3-EGFPaf-OriKan}, and pHer{3xP3-EGFPaf-OriKan} were employed with the helper plasmids pKhsp82-MOS (Coates et al. 1998), phsp-pBac (Handler et al. 1999) and pKhsp82Hermes (Sarkar et al. 1997a), respectively. Transposition events from the donor to the target plasmid provide the target plasmid with the ability to replicate in *E. coli* and kanamycin resistance. This allows a simple selection for transposition events, when donor, helper, and target plasmid are injected into early insect embryos. The assay comprises the experimental steps of plasmid injection, plasmid isolation, amplification and analysis. Microinjections into pre-blastoderm embryos were performed as described (Rubin and Spradling 1982) using a final concentration of 250 ng/µl donor plasmid, 500 ng/µl helper plasmid and 1.0 µg/µl target plasmid. *D. virilis* embryos derived from a *white* mutant stock (15010–1051.53; The National *Drosophila* Species Resource Center, Bowling Green, Ohio) were treated the same way as *D. melanogaster* embryos except for a prolonged collection time (40 min at 25°C versus 30 min at 18°C). Injected embryos were incubated in a humid environment for 16–18 h at 18°C. Then, a heat shock of 1.5 h at 37°C was applied to induce transcription of the transposase gene at the stage of germ band retraction. After an incubation period of 2 h at room temperature the isolation of plasmid DNA from an average of 100 injected embryos was carried out by rinsing off the hydrocarbon oil with heptane, collecting the embryos with a needle, and homogenizing them in 10 µl HM buffer (0.5% SDS, 0.08 M NaCl, 0.16 M Sucrose, 0.06 M EDTA, 0.12 M TrisHCl pH 9.0). After addition of 90 µl HM buffer and 30 min incubation at 65°C, 14 µl 8 M potassium-acetate solution was added and everything was incubated for 30 min on ice. The suspension was then centrifuged (10 min 14,000 rpm at room temperature) and the supernatant carefully transferred into a new tube. After ethanol precipitation, the DNA was resuspended in 10 µl TE buffer (Sambrook et al. 1989). DNA solution containing the equivalent of 10 embryos (1 µl) was used to transform 25 µl of competent *E. coli* DH10B cells by electroporation. Then, 1 ml SOC medium (Sambrook et al. 1989) was added to transformed cells. After an incubation time of 1 h at 37°C, 0.5% (v/v) of the culture were plated onto LB plates (Sambrook et al. 1989) containing ampicillin (100 µg/ml) to determine the donor plasmid titer. The remaining culture volume was pelleted, resuspended in 100µl of LB medium (Sambrook et al. 1989) and plated onto LB plates containing chloramphenicol (10 µg/ml) and kanamycin (25 µg/ml). Double resistant clones were analyzed for the presence of transposition products by restriction enzyme analysis and DNA sequencing with the following internal transposon primers: MLR (5′-TTCGACAGTCAAGGTTGACACTTCACAAGG-3′) and MRF (5′-AAGACGATGAGTTCTACTGGCGTGGAATC-C-3′) for *mariner*, PLR (5′-CAGTGACACTTACCGCATTGACA-AGCACGC-3′) and PRF (5′-CCTCGATATACAGACCGATAAA-ACACATGC-3′) for *piggyBac*, HLR (5′-AATGAATTTTTTGTT-CAAGTGGCAAAGCAC-3′) and HRF (5′-AAAATACTTGCAC-TCAAAAGGCTTGACACC-3′) for *Hermes*. The sequences revealed that true transposition events, as marked by specific target site duplications at the insertion sites, could be recovered for all three transposons in both *D. melanogaster* and *D. virilis*.

Results and discussion

Berghammer et al. (1999) and Horn et al. (2000) present a novel, potentially universal transformation marker for use in arthropod transgenesis. However, for the development of sophisticated tools for the genetic analysis of non-model organisms, more than one transformation marker is desirable. Due to the absence of marked balancer chromosomes for these organisms, two component systems such as GAL4-based ectopic expression (Brand and Perrimon 1993), transposon mutagenesis (Cooley et al. 1988), or “enhancer trap” screens (O’Kane and Gehring 1987) need individual labeling of the different components.

3xP3-EYFP and 3xP3-ECFP serve as independent and separable transformation markers

Therefore, we asked if the different available spectral variants of EGFP, the yellowish variant EYFP, and the cyan variant ECFP (Clontech), could also serve as transformation markers when driven by the 3xP3 promoter construct. Figure 1 shows that both EYFP and ECFP can serve as transformation markers. Like EGFP, both spectral variants are more sensitive transformation markers than “mini”-*white* (data not shown; Horn et al. 2000), are receptive to slight position effects (Fig. 1B, C, G, H; Horn et al. 2000), mark pre-adult stages (data not shown; Horn et al. 2000), and can be detected in the presence of eye pigments, when outcrossed to a wild type *Drosophila* strain (Fig. 1E, J; Berghammer et al. 1999). Comparing many different lines, EYFP mediated fluorescence seems on average at least as strong, if not stronger than EGFP, whereas ECFP mediated fluorescence is weaker, which makes it harder to detect in the presence of eye pigments (Fig. 1J).

To see if 3xP3-EYFP and 3xP3-ECFP can serve as independent and separable transformation markers, we viewed fly heads with 3xP3 driven expression of the different spectral variants EGFP, EYFP and ECFP with a series of various filter sets (Table 1). As shown in Fig. 2, EGFP is detected with all filter sets tested and can therefore not be distinguished clearly from EYFP or ECFP. The longpass filters GFP2 or V (Leica) show higher sen-

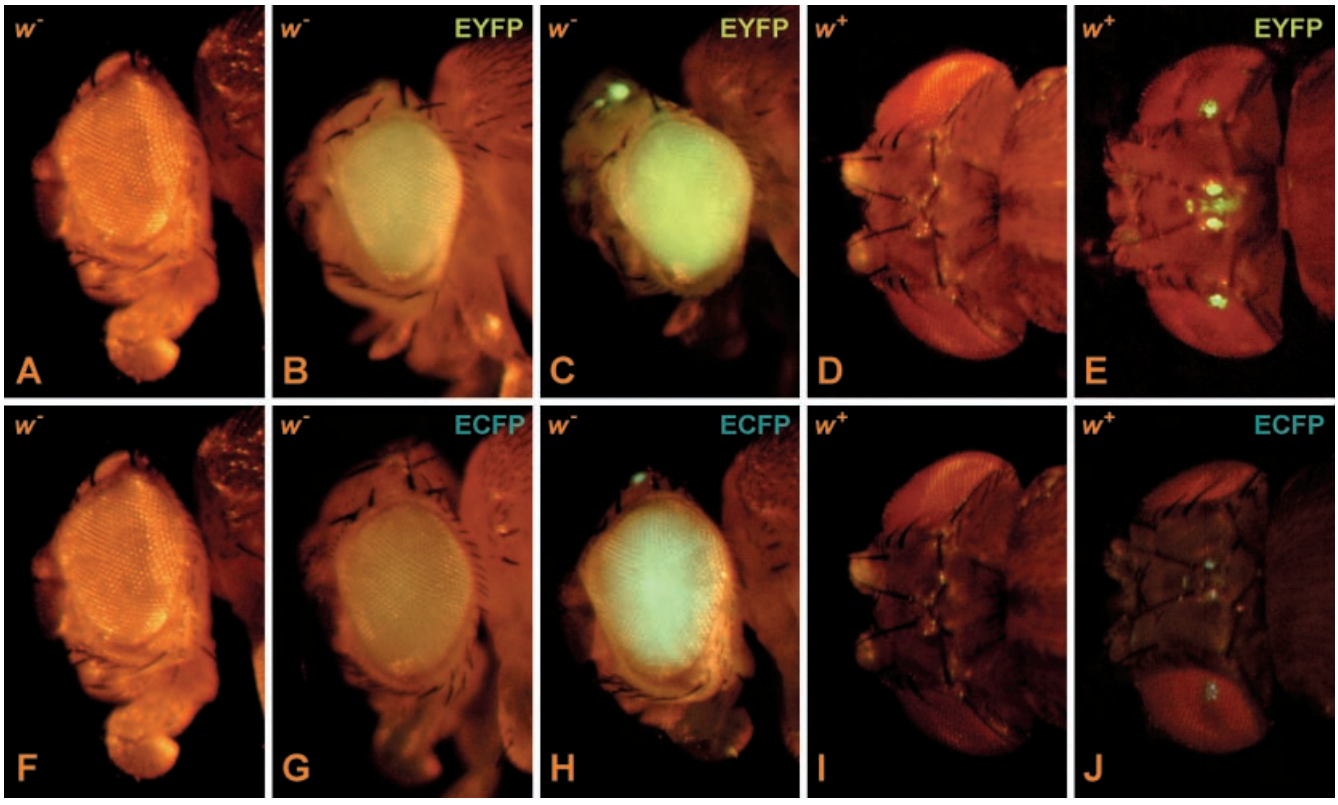

Fig. 1A–J 3xP3-EYFP and 3xP3-ECFP marker gene expression in a *Drosophila white* (*w*) and w^+ background. Flies were observed with the GFP2 (*upper panels*) and V (*lower panels*) filter sets. In comparison to non-transgenic *w* mutant flies (**A**, **F**), the range of 3xP3-EYFP (**B**, **C**) and 3xP3-ECFP (**G**, **H**) marker gene expression of different Dm[Bac{3xP3-EYFP}] and Dm[Her{3xP3-ECFP}] lines suggests position effect suppression. When outcrossed to the wild type Oregon R strain, EYFP (**E**) and ECFP (**J**) fluorescence can only be detected in the pseudopupil of the compound eye and the ocelli. **D**, **I** Non-transgenic Oregon R control flies observed with the corresponding filter set

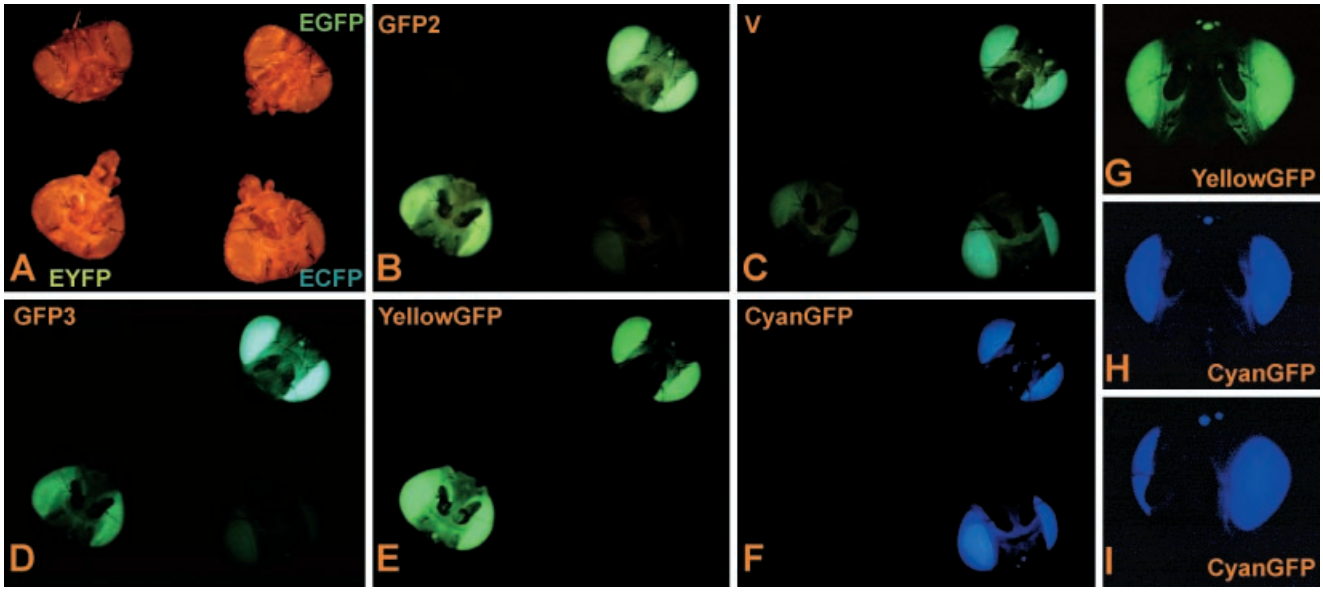

Fig. 2A–I Comparison of EGFP, EYFP and ECFP fluorescence detection properties using different filter sets. In panels **A**–**F**, the same *w* control (*upper left*), Dm[Mos{3xP3-EGFP}] (*upper right*), Dm[Bac{3xP3-EYFP}] (*lower left*) and Dm[Her{3xP3-ECFP}] (*lower right*) transgenic fly heads are grouped together. Observations by illumination with cold light source (**A**) or with the filter sets GFP2 (**B**), V (**C**), GFP3 (**D**), YellowGFP (**E**) and CyanGFP (**F**). In a fly carrying both the 3xP3-EYFP and the 3xP3-ECFP marker, both markers can be detected (**G**, **H**). No quenching of ECFP expression due to EYFP presence can be detected, as a sister fly carrying only the 3xP3-ECFP marker (**I**) shows similar levels of ECFP fluorescence

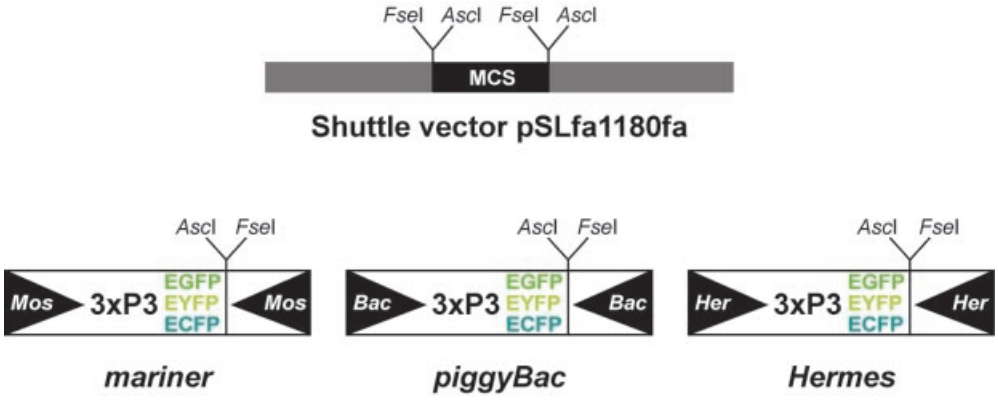

Fig. 3 Two-step cloning procedure using the cloning shuttle vector pSLfa1180fa. Complicated constructs will first be assembled in the multiple cloning site (MCS) of the cloning shuttle vector which contains all possible hexa-cutter sites and *Not*I. Then the final construct can be introduced into one or several ones of the nine different transformations vectors which are best suited for the purpose chosen

sitivity for EYFP or ECFP, respectively (Fig. 2B, C), without completely blocking detection of the other spectral variant. These longpass filters have the advantage of being highly luminous, which allows for easier sorting of insects under the dissecting scope. However, they cannot completely distinguish between the spectral variants (Fig. 2B, C). Of the bandpass filters, the YellowGFP and CyanGFP filters (Chroma) allow complete separation of 3xP3-EYFP and 3xP3-ECFP transformants (Fig. 2E, F), which will be important for the individual identification of two component genetic systems. Admittedly, the low luminosity of the bandpass filters makes insect handling more difficult and might only allow detection of medium to strong expression lines. Thus, for general use, the longpass filters GFP2 and V (Leica) are more convenient. However, occasional resorting to the bandpass filters YellowGFP and CyanGFP (Chroma) will be required when definite distinction between EYFP and ECFP is necessary. Therefore, stereomicroscopes with easily changeable filter sets are advantageous for simultaneously working with different spectral variants.

To test whether both EYFP and ECFP can be detected when concurrently expressed in the same fly eyes, we crossed 3xP3-EYFP and 3xP3-ECFP fly lines. In the resulting flies carrying both constructs, we could detect EYFP (Fig. 2G) as well as ECFP (Fig. 2H). ECFP-fluorescence was as strong as in non-EYFP expressing sibling flies (Fig. 2I), which indicates that the presence of EYFP does not quench ECFP-mediated fluorescence. Thus, 3xP3-EYFP and 3xP3-ECFP present an independent and distinguishable marker pair that can be employed for two component genetic systems.

Versatile vector set based on *mariner*, *Hermes*, and *piggyBac* transposons

The use of unrelated transposable elements should confront the problem that certain transposition events might be repressed in certain species. Therefore, we chose the *Tc1*/*mariner* element *Mos1* (Medhora et al. 1991), the *hAT* element *Hermes* (Warren et al. 1994), and the TTAA element *piggyBac* (Cary et al. 1989) for the development of a diverse set of transformation vectors. Into the backbone of all three transposons we introduced all three spectral variants to generate the nine basic transposition vectors: pMos{3xP3–EGFPaf}, pMos{3xP3–EYFPaf}, pMos{3xP3–ECFPaf}, pHer{3xP3–EGFPaf}, pHer{3xP3–EYFPaf}, pHer{3xP3–ECFPaf}, pBac{3xP3–EGFPaf}, pBac{3xP3–EYFPaf}, pBac{3xP3–ECFPaf}. The "af" in these vectors indicates restriction sites for the rare octa-cutter enzymes *Asc*I and *Fse*I, which allow simple cloning into the complete set of vectors. To avoid having to reclone a complicated effector construct, we developed a versatile two-step cloning procedure, which is based on the cloning shuttle vector pSLfa1180fa (Fig. 3). This vector contains all possible hexa-cutter sites, and the site for *Not*I in its polylinker, which is flanked on either end with the rare octa-cutter restriction sites *Fse*I and *Asc*I. Thus, any complicated construct of interest can first be pasted together in the shuttle vector pSLfa1180fa, and then easily transferred into the transformation vector of choice as an *Fse*I, an *Asc*I, or *Fse*I-*Asc*I fragment. This two-step cloning procedure allows for maximum versatility, since the choice of transformation vector might change depending on the species one would like to transform (see below), and the marker choice might depend on what other components need to be introduced.

All presented data were generated with this first set of transformation vectors. When producing the first set of *mariner* and *piggyBac* transformation vectors, we introduced the marker into a single restriction site of the transposon, thereby disrupting the open reading frame of the transposase. Despite the fact that this disruption rendered the transposon constructs non-autonomous, all coding information for the respective transposon is still included. In order to avoid that cryptic splicing or genetic rearrangements might cause the reconstitution of a functional transposase, we generated a new series of *mariner* and *piggyBac* constructs, in which 600 bp or 800 bp , respectively, of the transposase open reading frame have been removed: pMos{3xP3–EGFPafm}, pMos{3xP3–EYFPafm}, pMos{3xP3–ECFPafm}, pBac{3xP3–EGFPafm}, pBac{3xP3–EYFPafm}, pBac{3xP3 –ECFPafm}. Since these vectors have the same features, and work as well as the original set, we will only provide this safer version of the vectors in the future. Actually, for *mariner* constructs the transformation rate increased from 4% (Horn et al. 2000) to 10% (transformation rate as per-

centage of fertile injection survivors producing fluorescent offspring). This might be due to the reduced size, or the use of symmetrically sized transposon ends (350 bp) on either side of the marker. For the *Hermes* constructs no modification was introduced, as they were originally generated in such a way that only the ends of the transposons were used (Pinkerton et al. 2000).

Transposition assay for quick evaluation of transposon usage

We cannot expect that all transposons will be active and stable in all species. Transposase activity might be blocked, or the presence of related transposons might destabilize insertions. However, by starting with a series of three unrelated transposable elements, we expect to overcome particular problems some transposons might have in certain species. Since we do not know which transposons can be employed in which species, it might be useful, especially in arthropods with long generation times, to initially evaluate which transposon will allow transposition before starting time-consuming germline transformation experiments. Therefore, we adapted our vectors to be used in a simple plasmid-based transposition assay for evaluation of the mobility of transposable elements in the embryonic soma of a variety of arthropods (Coates et al. 1997; Sarkar et al. 1997a, b). To generate the donor plasmids pMos{3xP3–EGFPaf-OriKan}, pHer{3xP3–EGFPaf-OriKan}, and pBac{3xP3–EGFPaf-OriKan} we introduced into our pMos{3xP3–EGFPaf}, pHer{3xP3–EGFPaf}, and pBac{3xP3–EGFPaf} constructs a kanamycin resistance gene (Kan^R) and an *Escherichia coli* origin (Ori) of replication. In the presence of a helper plasmid providing transposase activity, the transposon construct, including the Ori/Kan^R component, can jump onto another plasmid. The target plasmid used, pGDV1 (Coates et al. 1997; Sarkar et al. 1997a, b), is derived from *Bacillus subtilis*, cannot grow in *E. coli*, but carries a chloramphenicol resistance gene (Cam^R). When all three types of plasmids, donor, helper, and target, are injected together into syncytial blastoderm embryos of the arthropod species of interest, successful transposition can be detected, when after re-isolation of the plasmid DNA, Kan^R and Cam^R plasmids are amplified in *E. coli*. To exclude recombination events, restriction analysis and sequencing are used to finally confirm true transposition (Fig. 4A; detailed description in Materials and methods). Furthermore, the transposition assay system can be modified to test for insertion stability. When performing the assay without helper construct, and therefore without transposase source, transposition can only take place if the embryo contains active transposase endogenously which will destabilize the insertion on the donor plasmid, and allow integration into the target plasmid.

To examine the functionality of our transposition assay system, we injected *Drosophila melanogaster* and *Drosophila virilis* embryos with the respective donor, helper and target combination for *mariner*, *Hermes*, and

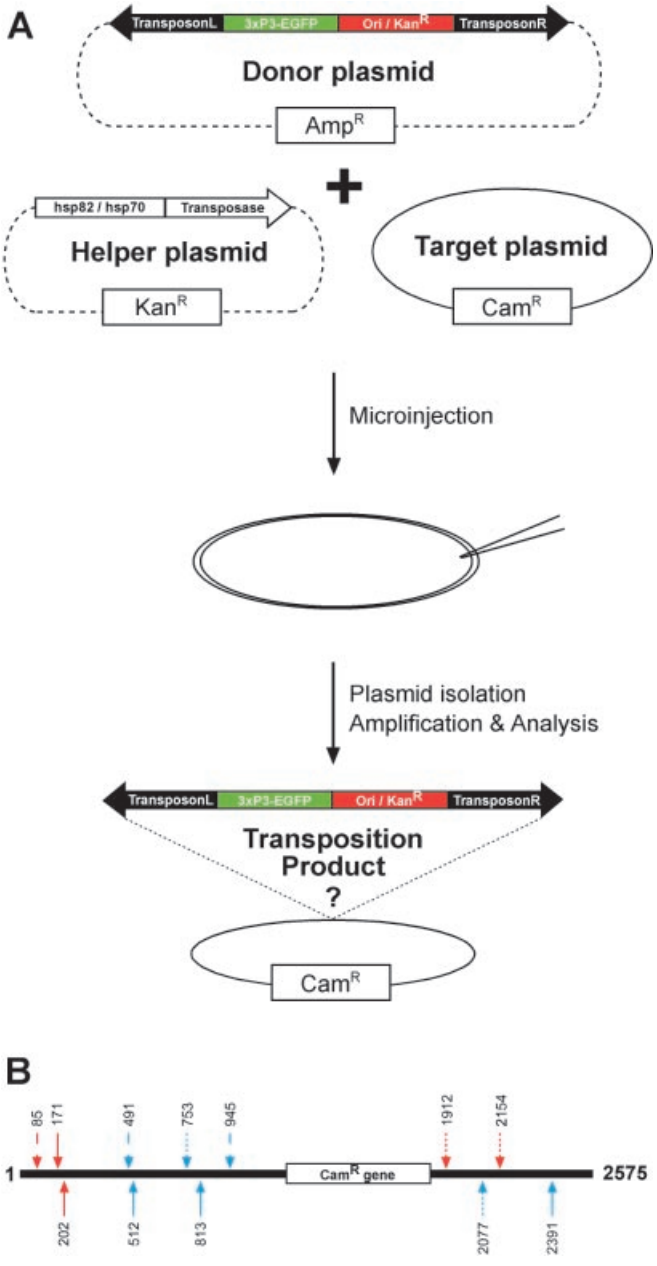

Fig. 4A, B Plasmid-based simple transposition assay for a quick functional test of transformation vectors. **A** Principle of the transposition assay (Coates et al. 1997; Sarkar et al. 1997a, b) which is based on three components: (1) the donor plasmid carrying the transposable element interrupted by the 3xP3-EGFP marker, a kanamycin resistance gene and an *E. coli* origin of replication; (2) the target plasmid carrying a chloramphenicol resistance gene but lacking an *E. coli* origin of replication; (3) the helper plasmid providing transposase activity. All three plasmids are co-injected into preblastoderm embryos of the species of interest. Transposition events from the donor plasmid to the target plasmid result in a transposition product capable of replication in *E. coli* and able to grow in the presence of both kanamycin and chloramphenicol. **B** *mariner*, *piggyBac* and *Hermes* universal transformation vector derivatives were used as donors and transposition products were recovered both in *D. melanogaster* (*blue arrows*) and *D. virilis* embryos (*red arrows*). Sequence analysis revealed the exact integration position within the pGDV1 target plasmid. Arrows indicate *mariner*, dashed arrows *piggyBac* and dotted arrows *Hermes* insertions; *arrows above the bar* designate that the 5′ end of the transposon is to the left, *below the bar* that the 5′ end is to the right)

piggyBac transposons. For all three transposons we could detect true transposition events in both species tested (Fig. 4B), which indicates that our transposition assay system is suitable for quick evaluation of transposon activity. Transposition in *D. melanogaster* was expected as all three transposable elements had already been shown to transpose (Coates et al. 1997; Sarkar et al. 1997a; Lobo et al. 1999), and had been successful in germline transformation (Garza et al. 1991; O'Brochta et al. 1996; Berghammer et al. 1999; Handler and Harrell 1999). In *D. virilis*, transposition of *mariner* was expected, as germline transformation with this element had already been successful (Lohe and Hartl 1996); transposition of *Hermes* was anticipated as germline transformation with the related *hobo* element had previously been shown (Lozovskaya et al. 1996); *piggyBac* had not previously been tested, but based on our positive transposition results this transposable element seems also applicable to *D. virilis*.

The potentially universal application of our marker system (Berghammer et al. 1999), the sensitivity of our marker (Horn et al. 2000), the promiscuity of the employed transposable elements (O'Brochta and Atkinson 1998), the versatility of our transformation vector set, and the possible quick evaluation of our transformation vectors present a valuable basis for transgenesis experiments in many different animal species. This will be of great interest to basic as well as applied science. The field of integrated pest management will be provided with a technique that allows a more detailed study of the biology of pest insects and at the same time provides a means to actually modify these insects in a useful manner. Furthermore, comparative developmental biology, evolutionary biology, animal ecology, and behavioral biology are provided with a molecular methodology that will enhance their analytical possibilities.

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