Fluorescent transformation markers for insect transgenesis

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Received 7 July 2001; received in revised form 12 November 2001; accepted 21 December 2001

Abstract

The first effectively achieved germ-line transformations of non-drosophilid insects were based on mutant rescue of eye color phenotypes. However, for most insect species neither visible mutants nor corresponding cloned genes are available. Therefore, the development of broadly applicable and reliable transformation markers will be of great importance to fully exploit the enormous potential transgenic insect technology has to offer. Here we review transposon-mediated germ-line transformation approaches that employ green fluorescent protein (GFP) variants to identify successful gene transfer. Furthermore, we provide novel data on the use of DsRed as an additional red fluorescent transformation marker for insect transgenesis. In conclusion, fluorescent proteins controlled by suitable strong promoters possess ideal characteristics to serve as transformation markers for a wide range of insect species. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Green fluorescent protein; EGFP; ECFP; EYFP; DsRed; Germ-line transformation; Transposable elements; Transposon mutagenesis; Enhancer trapping

1. Introduction

Insect transgenesis will allow great advances in our comprehension of the molecular and biochemical basis of insect biology and therefore constitutes an important tool for basic research in genetics, zoology, and ecology. In addition, it will present new and efficient strategies to control pest insect populations or might enable us to change the pest status of certain insects (O’Brochta and Atkinson, 1998). Hopes of creating transgenic insects were raised when almost 20 years ago germ-line transformation was first demonstrated in the vinegar fly, Drosophila melanogaster, by the use of the transposable element P (Spradling and Rubin, 1982). Unfortunately, several years of fruitless efforts in many different species finally proved that the P-element is non-functional outside of the Drosophilidae (Handler et al., 1993). However, the search for alternative and more promiscuous transposable elements, most notably Minos, mariner, Hermes, and piggyBac, has been very rewarding and in the past seven years medically and agriculturally important species of three different insect orders have been successfully transformed (recently reviewed in: Handler and James, 2000; Handler, 2001; Atkinson et al., 2001. In order to apply these germ-line transformation systems to a broad range of insect species, the development of new marker systems that allow easy and reliable identification of transgenic individuals will now be of key importance.

2. Eye color genes as transformation markers

The first efficient germ-line transformations in non-drosophilid insect species utilized marker genes that allowed the identification of transformants by mutant-rescue selection similar to typical transformation experiments in D. melanogaster (Rubin and Spradling, 1982). In these cases the marker gene represents the wild-type allele of a gene that, when mutated, causes a recessive, visible but viable phenotype. After introduction of the wild-type gene into the corresponding mutant insect strain by transformation, the wild-type allele complements the recessive mutation and the visible phenotype is rescued. Commonly used marker genes are eye color genes responsible for the production of screening pigments in the compound eye. Mutations in these genes cause obvious phenotypes that are readily distinguished
from wild-type. Insect strains carrying these eye color mutations usually grow well in laboratory cultures and many of the corresponding genes are reasonably small (2 to 3 kb). Therefore, eye color genes present suitable markers for insect transgenesis which allow easy detection of transformed individuals (recently reviewed in: Sarkar and Collins, 2000).

Transformation of the Mediterranean fruit fly, Ceratitis capitata (Loukers et al., 1995; Handler et al., 1998; Michel et al., 2001), and the yellow fever mosquito, Aedes aegypti (Jasinskiene et al., 1998; Coates et al., 1998), greatly benefited from the existence of eye color mutations and the availability of cloned wild-type genes for mutant-rescue selection. In C. capitata a null mutation in the white eye locus could be complemented by a cloned wild-type copy of this gene (Zwiebel et al., 1995). The same gene has subsequently been used to transform the closely related oriental fruit fly, Bactrocera dorsalis, for which white eyed strains also exist (Handler and McCombs, 2000), similar to the use of the D. melanogaster white gene in transformations of D. virilis (Lozovskaya et al., 1996; Lohe and Hartl, 1996; Gomez and Handler, 1997). To transform Ae. aegypti, the D. melanogaster cinnabar gene was employed to rescue the white eye phenotype of an Ae. aegypti strain mutant in the kynurenine hydroxylase gene (Cornel et al., 1997). Also for the red flour beetle Tribolium castaneum, a transformation system was recently established which is based on the rescue of a strain carrying the eye color mutation vermilionwhite by the cloned Tc vermilion gene (Lorenzen et al., 2002).

Eye color genes have a number of desirable characteristics to serve as easily detectable transformation markers. However, the lack of suitable recipient mutant strains for most insects of medical and agricultural importance limits their application. Although such mutant-rescue transformation markers can in principle be generated for any species, the procedure of first isolating visible mutations in the species of interest, then cloning the corresponding gene, and finally rescuing the mutant phenotype, is laborious and risky with the same considerable investment required for every new species. Moreover, mutant strains are inbred and thus tend to be weaker than robust wild-type strains. This can cause lower survival rates in transformation experiments (Pinkerton et al., 2000) or become problematic when establishing homozygous transgenic lines (Atkinson et al., 2001). Furthermore, the development of transgene-based pest management programs will rely on competitive wild-type-like organisms which emphasizes the need for marker systems applicable in wild-type backgrounds.

3. Drug resistance genes as markers for insect transgenesis

Initially the search for dominant-acting selection markers that could act independently of pre-existing mutant strains focused on genes which confer drug resistance (recently reviewed in: french-Constant and Benedict, 2000). Actually the first transformed line of the malaria mosquito Anopheles gambiae was established using the bacterial neo gene encoding neomycin phosphotransferase II as selectable marker (Miller et al., 1987). The selection was based on resistance to Geneticin® (G418), but further success was limited to two additional reports in Aedes triseriatus (McGrane et al., 1988) and Ae. aegypti (Morris et al., 1989), which were not effectively repeated. So far, resistance markers like neo (Steller and Pirrotta, 1985), opd encoding an organophosphate hydrolase (Benedict et al., 1995), or the Resistance to dieldrin (Rdl) gene (Stilwell et al., 1995) have efficiently been used only in D. melanogaster where independent transformation markers allowed the determination of optimal conditions for the selection. Resistance to a given drug or antibiotic varies considerably within a wild-type insect population. In addition, transposon-mediated insect transgenesis does not allow us to specifically target transgenes to certain genomic positions. As a result, transformation experiments produce individuals that have various numbers of insertions at different insertion sites. Due to chromosomal position effects, the expression level of the transformation marker will differ significantly between independently transformed individuals. Therefore, the selection for drug resistance is problematic and prone to false positives or false-negatives (i.e., death of weakly expressing transformants).

Moreover, the toxicity of many drugs is undesirable for use in routine techniques such as stock maintenance due to the constant problem of accidental or cumulative exposure of research personnel to the drugs. In addition, there should be concern about the accidental release of a transgenic insect carrying a drug resistance marker. This is an even greater problem for applications in pest management programs that actually aim at the release of transgenic insects. Insecticide resistance (Hemingway and Ranson, 2000) and antibiotic-resistance (Monroe and Polk, 2000) already present major problems for human welfare that should not be escalated by the use of drug resistance transformation markers.

4. Transgenic insects identified by GFP-based transformation markers

Due to the random integration characteristics of transposon-based transgenesis approaches, an ideal transformation marker must allow for reliable detection at greatly varying expression levels. Moreover, the marker should be dominant and visible in wild-type backgrounds. To develop such a marker the gene encoding the green fluorescent protein (GFP) from the jellyfish Aequorea victoria (Prasher et al., 1992) is well suited,
since it is easily detectable in vivo and has proven functional in different tissues of many heterologous systems (reviewed in: Tsien, 1998). However, until recently GFP was mostly used as a spatial reporter for gene expression or as a fusion tag for in vivo protein localization (Chalfie et al., 1994; Plautz et al., 1996; Brand, 1999), but not as a primary marker for the selection of transformed insects. Wild-type GFP actually has two disadvantages for the routine use as a transformation marker: it is relatively insoluble and its excitation maximum lies within the UV-spectrum. Long-term exposure to UV-light, however, can damage the examined live organisms.

With the introduction of more soluble red shifted mutant GFP-variants like enhanced GFP (EGFP; Cormack et al., 1996; Yang et al., 1996; Clontech Laboratories, Inc. Palo Alto, CA), both these problems have been eliminated. EGFP has an excitation peak of 488 nm and can therefore be excited with more harmless blue light and actually fluoresces 35 times more intensely than wild-type GFP. A detailed review about the advantages of GFP variants as markers for transgenic insects has recently been published (Higgs and Lewis, 2000), which also includes the use of GFP for viral approaches to insect manipulation. In our review, we will focus on the most recent uses of fluorescent markers for transposon-based transformation of different insect species (Table 1).

Due to the ideal properties of EGFP, it was the first fluorescent variant to be employed in different laboratories as a primary transformation marker for insect transgenesis. In order to reliably detect single-copy insertions of transgenes, EGFP needs to be driven by a strong promoter. For this purpose both constitutive and tissue-specific promoters have been used. EGFP-based markers were first tried in combination with an eye color marker in D. melanogaster to examine its applicability (Handler and Harrell, 1999; Horn et al., 2000). Actually, both studies came to the conclusion that EGFP-based transformation markers are more sensitive and reliable than the eye color gene, “mini”-white, which is routinely used as a transformation marker in this species. This is probably due to a higher susceptibility of the “mini”-white gene to position effect suppression. Depending on the promoter, different genes seem to be affected quite differently by position effects, even if they are linked and inserted at the same chromosomal position (Bhadra et al., 1998). As a general rule, EGFP-based marker genes seem to be less susceptible to complete suppression than eye color genes (Handler and Harrell, 1999; Horn et al., 2000).

EGFP-based gene constructs were then successfully tested for their use as sole markers to identify gene transfer in species that had previously been transformed with eye color markers: C. capitata, B. dorsalis (A. Handler, personal communication) and Ae. aegypti (Pinkerton et al., 2000). EGFP-based markers have also now been effectively applied in many insect species that had no previously established transformation protocols (Table 1). The successful transformation of species from three different orders (Diptera, Lepidoptera, and Coleoptera) indicates how widely applicable EGFP-based transformation markers are for insect transgenesis.

One of the great advantages of fluorescent transformation markers is their suitability for wild-type organisms. This is crucial for insect species having none or only weak visible mutant strains and especially important for potential applications in pest management programs. Not only will it be essential that released insects are competitive and healthy, but the fluorescence transformation marker also has the potential to serve as a genetic marker for field detection after release (Hagler and Jackson, 2001). Spectrofluorometric assays can provide sensitive and fast analyses for applied purposes (Handler and Harrell, 2001a).

However, there are also some general problems with the use of fluorescent markers for insect transgenesis. Prolonged exposure to intense illumination during screening for transgenic insects might actually prove fatal for some insects. Therefore, the use of strong promoters for EGFP expression is recommended to allow fast and reliable detection. Another problem is autofluorescence of some biological materials like ingested food, the Malpighian tubules, the chitinous exoskeleton, or necrotic tissue. In this respect, the use of longpass emission filters can help to distinguish yellowish autofluorescence from the green EGFP-fluorescence (Brand, 1995). Another potential problem is strong melanization of adult cuticle which can obstruct detection of EGFP expressed in internal tissues. Fortunately, many embryonic, larval, or pupal stages are more translucent and, depending on the promoter used to drive EGFP expression, might be applicable for transformant identification. The use of very young stages, however, is not recommended, since fluorescent proteins need some time to mature by internal cyclization and oxidation (Davis et al., 1995). Moreover, maternal expression of EGFP from heterozygote mothers may be detected in embryos not carrying the marker gene. Depending on the duration of embryonic development and the particular transformation marker, the stages just before larval hatching might actually be best to screen for transgenics. This will not only speed up the detection of transgenics, which might be important for insects with longer generation times, but will also eliminate the rearing of almost all G1 progeny, which can be a significant advantage when rearing species on expensive foods.

5. EGFP under the control of constitutive promoters

Constitutive promoters active in all cells provide the advantage of allowing selection of transformants at all
Table 1
Insect transgenesis mediated by fluorescent-marked transposon vectors

<table>
<thead>
<tr>
<th>Marker</th>
<th>Transposon</th>
<th>Species transformed</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PUbnlsEGFP</td>
<td>piggyBac</td>
<td>Drosophila melanogaster</td>
<td>Handler and Harrell, 1999</td>
</tr>
<tr>
<td></td>
<td>piggyBac</td>
<td>Anastrepha suspensa</td>
<td>Handler and Harrell, 2001a</td>
</tr>
<tr>
<td></td>
<td>piggyBac</td>
<td>Ceratitis capitata</td>
<td>A. Handler, pers. comm.</td>
</tr>
<tr>
<td></td>
<td>piggyBac</td>
<td>Bactrocera dorsalis</td>
<td>A. Handler, pers. comm.</td>
</tr>
<tr>
<td></td>
<td>piggyBac</td>
<td>Lucilia cuprina</td>
<td>Heinrich et al., 2002</td>
</tr>
<tr>
<td></td>
<td>piggyBac</td>
<td>Anopheles albimanus</td>
<td>A. Handler, pers. comm.</td>
</tr>
<tr>
<td>PUbDsRed1</td>
<td>piggyBac</td>
<td>Drosophila melanogaster</td>
<td>Handler and Harrell, 2001b</td>
</tr>
<tr>
<td></td>
<td>piggyBac</td>
<td>Anastrepha suspensa</td>
<td>A. Handler, pers. comm.</td>
</tr>
<tr>
<td>actin5C:EGFP</td>
<td>Hermes</td>
<td>Drosophila melanogaster</td>
<td>Pinkerton et al., 2000</td>
</tr>
<tr>
<td></td>
<td>Hermes</td>
<td>Siromyox calcitrans</td>
<td>O’Brochta et al., 2000</td>
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<tr>
<td></td>
<td>Minos</td>
<td>Aedes aegypti</td>
<td>Pinkerton et al., 2000</td>
</tr>
<tr>
<td></td>
<td>Hermes</td>
<td>Culex quinquemansius</td>
<td>Catteruccia et al., 2000</td>
</tr>
<tr>
<td>hr5-ie1:EGFP</td>
<td>piggyBac</td>
<td>Anopheles gambiae</td>
<td>Grossman et al., 2001</td>
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<tr>
<td>act88:GFP</td>
<td>Hermes</td>
<td>Culex quinquemansius</td>
<td>P. Atkinson and M. Allen, pers. comm.</td>
</tr>
<tr>
<td>BmA3EGFP</td>
<td>piggyBac</td>
<td>Bombyx mori</td>
<td>Tamura et al., 2000</td>
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<td></td>
<td>piggyBac</td>
<td>Pectinophora gossypiella</td>
<td>Peloquin et al., 2000</td>
</tr>
<tr>
<td>3xP3–EGFP</td>
<td>piggyBac, Hermes, mariner</td>
<td>Drosophila melanogaster</td>
<td>Horn et al., 2000</td>
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<tr>
<td></td>
<td>piggyBac</td>
<td>Drosophila simulans</td>
<td>E. Rosato, pers. comm.</td>
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<tr>
<td></td>
<td>piggyBac</td>
<td>Drosophila pseudoobscura</td>
<td>E. Rosato, pers. comm.</td>
</tr>
<tr>
<td></td>
<td>piggyBac, Hermes</td>
<td>Drosophila virilis</td>
<td>D. Bopp, P. Wittkopp, pers. comm.</td>
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<td></td>
<td>Minos</td>
<td>Musca domestica</td>
<td>Hediger et al., 2001</td>
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<td></td>
<td>piggyBac</td>
<td>Ceraatitis capitata</td>
<td>I. Livadaras, C. Savakis, pers. comm.</td>
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<td></td>
<td>piggyBac</td>
<td>Aedes aegypti</td>
<td>Kokozza et al., 2001</td>
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<td></td>
<td>piggyBac</td>
<td>Anopheles stephensi</td>
<td>Ito et al., 2002</td>
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<td></td>
<td>piggyBac</td>
<td>Bombyx mori</td>
<td>Thomas et al., 2002</td>
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<td></td>
<td>piggyBac</td>
<td>Tribolium castaneum</td>
<td>Berghammer et al., 1999</td>
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<tr>
<td></td>
<td>piggyBac</td>
<td>Harmonia axyridis</td>
<td>H. Kuwayama and T. Niimi, pers. comm.</td>
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<tr>
<td>3xP3–EYFP</td>
<td>piggyBac, Hermes, mariner</td>
<td>Drosophila melanogaster</td>
<td>Horn and Wimmer, 2000</td>
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<td></td>
<td>piggyBac</td>
<td>Tribolium castaneum</td>
<td>M. Klingler, pers. comm.</td>
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<td>3xP3–ECFP</td>
<td>piggyBac, Hermes, mariner</td>
<td>Drosophila melanogaster</td>
<td>Horn and Wimmer, 2000</td>
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<tr>
<td></td>
<td>piggyBac</td>
<td>Tribolium castaneum</td>
<td>M. Klingler, pers. comm.</td>
</tr>
<tr>
<td>3xP3–DsRed</td>
<td>piggyBac</td>
<td>Drosophila melanogaster</td>
<td>(this publication)</td>
</tr>
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</table>

Stages: embryonic, larval, and adult. The D. melanogaster polyubiquitin promoter was successfully utilized to generate the transformation marker PUbnlsEGFP and to identify transformants of D. melanogaster (Handler and Harrell, 1999), the Caribbean fruit fly, Anastrepha suspensa (Handler and Harrell, 2001a), and the Australian sheep blowfly, Lucilia cuprina (Heinrich et al., 2002). In transgenic D. melanogaster and A. suspensa, fluorescence was detectable throughout development, whereas in transgenic L. cuprina, fluorescence seems restricted to embryos, larvae, and ovaries, since EGFP expression was undetectable in most other adult tissues. In PUbnlsEGFP, EGFP is fused to a nuclear localization signal, which facilitates the nuclear import of EGFP (Handler and Harrell, 1999). The mediated subcellular localization of the fluorescent protein allows for a clearer identification of transformants against a common background of autofluorescence which is usually not nuclear
established. Yet, for comparative studies in the
field of evolutionary developmental biology or comparative zoology, a universal transformation system is desirable, since it should be applicable to all eye-bearing animals. Consistent with the general function of Pax-6 in the differentiation of photoreceptor cells (Sheng et al., 1997), the artificial 3xP3 promoter construct mediates EGFP expression in all larval, pupal, and adult eyes of insects so far analyzed. This tissue-specific promoter can, therefore, like the constitutive promoters mentioned above, be used to identify transgenic insects at all stages (Horn et al., 2000). Fluorescence in the larval eyes can usually be detected at the end of embryogenesis before the larvae hatch. This is especially important for species with great larval appetite like the silkworm B. mori, where the 3xP3–EGFP marker allows identification of transgenic G1 progeny as late embryos based on green fluorescence in the developing larval stemmata (Thomas et al., 2002).

Expression in optical systems like the eyes allows fluorescent markers to be also detected in animals with thick and melanized cuticles. In this respect, simple eyes like dorsal ocelli or larval stemmata are ideal since all photoreceptors are observed through one lens and fluorescence can be detected simultaneously. In compound eyes of wild-type insects, the ommatidia are usually optically insulated from each other by eye pigments and fluorescence can only be detected in those ommatidia that point straight towards the observer. This allows for identification of adult transgenics in wild-type backgrounds of different vinegar flies (E. Rosato, personal communication), the malaria mosquito, An. stephensi (Ito et al., 2002), the silkworm, B. mori (Thomas et al., 2002; Uhlířová et al., 2002), and the red flour beetle, T. castaneum (Berghammer et al., 1999). However, in some species, like the house fly, Musca domestica, or the yel-

6. 3xP3–EGFP as universal transformation marker for insect transgenesis

Eye development of all metazoan animals is governed by an evolutionary conserved genetic circuitry which is under the control of the transcriptional activator Pax-6/Eyelass (Callaerts et al., 1997). Multimerization of an in vitro-selected, optimized Pax-6 homodimer binding site (P3) had been shown to mediate photoreceptor specific gene expression in D. melanogaster (Sheng et al., 1997). These discoveries allowed Berghammer et al. (1999) to develop a transformation marker based on a single transcription factor-activated, artificial promoter. Three tandem repeats of the P3 site in front of a TATA box (3xP3) have been employed to drive strong, eyespecific expression of EGFP (Berghammer et al., 1999; Horn et al., 2000). This marker construction (3xP3–EGFP) has been embedded into several broad range transposons providing a versatile vector set for animal transgenesis (Horn and Wimmer, 2000). The small size (1.3 kb) of the 3xP3–EGFP marker provides an additional advantage which allows for small transposon constructs resulting in high transformation rates.

3xP3–EGFP-based transformation systems have by now been applied to generate and identify transgenic individuals of three different insects orders (Table 1). The evolutionary conserved “master regulator” function of Pax-6 in eye development of metazoa (Callaerts et al., 1997) actually suggests that the 3xP3–EGFP marker should be applicable to all eye-bearing animals. Consistent with the general function of Pax-6 in the differentiation of photoreceptor cells (Sheng et al., 1997), the artificial 3xP3 promoter construct mediates EGFP expression in all larval, pupal, and adult eyes of insects so far analyzed. This tissue-specific promoter can, therefore, like the constitutive promoters mentioned above, be used to identify transgenic insects at all stages (Horn et al., 2000). Fluorescence in the larval eyes can usually be detected at the end of embryogenesis before the larvae hatch. This is especially important for species with great larval appetite like the silkworm B. mori, where the 3xP3–EGFP marker allows identification of transgenic G1 progeny as late embryos based on green fluorescence in the developing larval stemmata (Thomas et al., 2002).

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localized. This can be especially important to identify transformants that show low levels of EGFP expression due to position effects.

Another commonly used constitutive promoter to drive EGFP is derived from the D. melanogaster actin5C gene. The resulting transformation marker actin5C:EGFP works well at all developmental stages of D. melanogaster as well as in the mosquitoes Ae. aegypti, Anopheles stephensi, and Culex quinquefasciatus (Pinkerton et al., 2000; Catteruccia et al., 2000; Allen et al., 2001). However, it mediates only low and non-uniform EGFP expression levels in the stable fly, Stomoxys calcitrans (O’Brochta et al., 2000), which indicates that the D. melanogaster actin5C promoter might not be the best choice for broad range applications in different insect species.

For the first stable germline transformation of lepidopteran species of the silkworm, Bombyx mori (Tamura et al., 2000), and the pink bollworm, Pectinophora gossypiella (Peloquin et al., 2000), the B. mori A3 cytoplasmic actin gene promoter (BmA3) was chosen to drive EGFP. For both species transformants could be identified successfully, but unexpectedly the marker BmA3:EGFP was not detectable in embryos. Furthermore, the BmA3 promoter is predominantly active in the midgut (Mange et al., 1997), which makes it difficult to reliably identify transformants since autofluorescence of many insect foods can mask low-level fluorescence and only allows the detection of strongly expressing individuals with potentially multiple insertions. This might, however, be less of a problem with other fluorescent markers like DsRed (see below) whose excitation causes less autofluorescence of biological tissues (Handler and Harrell, 2001b).

The above-mentioned fluorescent markers have allowed the transformation of diverse insect species for which no genetic markers had previously been established. Yet, for comparative studies in the fields of evolutionary developmental biology or comparative zoology, a universal transformation system is desirable, since it would allow the examination of the very same transgene construct in a series of diverged insect species. However, transformation markers based on constitutive promoters have so far only been applied to closely related species, and it is questionable if any such promoter can be functional across a wide range of insect orders. Natural promoters are usually complex, comprising many binding sites for several regulatory transcription factors. In order to be fully active in a series of diverged species, all trans-acting factors of such a natural promoter need to be evolutionarily conserved. This is less likely, the more complex the promoter is structured.
low fever mosquito, *Ae. aegypti*, adult eye pigmentation quenches the fluorescence completely (Hediger et al., 2001; Kokoza et al., 2001). Therefore, if adults are to be screened to identify transgenic individuals marked with the 3xP3–EGFP marker, the use of eye color mutants is recommended. Then fluorescence in all ommatidia of large compound eyes can be observed simultaneously (Figs. 1 and 2). In wild-type *M. domestica* and *Ae. aegypti*, 3xP3–EGFP-mediated eye fluorescence can be detected during larval and pupal stages (Hediger et al., 2001; Kokoza et al., 2001). This indicates that 3xP3–EGFP-based transformation systems can be applied to competitive wild-type strains and not only to potentially fitness-impaired mutant lines.

The choice of fluorescent marker and developmental stage for identifying transgenics might very well depend on the timing and extent of cuticle formation and melanization as well as eye development and pigmentation. For most insect species, late embryonic and larval stages will probably be best, both with respect to marker detection as well as for handling procedures. At these stages, the 3xP3–EGFP marker mediates additional fluorescence in the central nervous system (CNS), part of the peripheral nervous system, the anal pads and the hindgut of *D. melanogaster* (Horn et al., 2000). Most of these expression domains can also be detected in other insect species (Hediger et al., 2001; Kokoza et al., 2001; Ito et al., 2002). The transgene-mediated CNS fluorescence seems evolutionary conserved and can be observed in lepidoptera as well as coleoptera (Thomas et al., 2002; A. J. Berghammer, M. Klingler and T. Niimi, personal communication), which might also allow the application of 3xP3–EGFP in insect species with eyeless larval stages. Thus, 3xP3–EGFP might be able to serve as a truly universal marker for insect transgenesis, which in combination with promiscuous transposons provides the ideal means for comparative studies in the fields of developmental biology, evolutionary biology, behavioral biology, and animal ecology.

7. Potential toxicity of GFP

Results from mammalian cell culture experiments indicate that the *A. victoria* GFP and its variants can cause cytotoxicity when expressed at high levels (Hanazono et al., 1997). So far this doesn’t seem to be a particular problem for the use of GFP as a transformation marker in insects. Nonetheless, a potential toxicity problem has been experienced when transforming the RED strain of *Ae. aegypti* with a polyubiquitin- or actin5C-driven EGFP marker, since all strongly expressing transgenic G1 individuals died as pupae and only one low-expressing line could be established (A.A. James, personal communication). However, at the moment it is not clear whether this effect is indeed due to high levels of EGFP expression. In contrast, neither in *D. melanogaster* nor in wild-type *Ae. aegypti*, did expression of actin5C:EGFP cause any obvious effects on viability (Pinkerton et al., 2000). Also, no demonstrable effect on survival has been detected so far in insect transformations with the marker 3xP3–EGFP, even when high expression causes intense fluorescence in the eye and central nervous system (A. Berghammer, M. Klingler, C.H., E.A.W., unpublished results). Recently, the gene for a green fluorescent protein from the anthozoan sea feather *Renilla reniformis* (Ward and Cormier, 1979) has been cloned, humanized (hrGFP; Stratagene, La Jolla, CA) and shown to be less toxic in mammalian cell culture experiments than *A. victoria* GFP variants (Felts et

![Fig. 1](http://www.zeiss.de/C12S67BE0045ACF1?Open)
ECFP, EYFP, and DsRed present independent transformation markers for the development of multicomponent genetic systems. Comparison of DsRed, EYFP, and ECFP fluorescence detection using different filter sets. All six panels show the same white-eyed Dm[Bac{3xP3–DsRed}] (lower middle), Dm[Bac{3xP3–EYFP}] (upper left), and Dm[Her{3xP3–ECFP}] (upper right) transgenic fly heads. Observations by illumination with a cold light source (a) or with the filter sets Cy3.5/DsRed (b), YellowGFP (c), CyanGFP (d), GFP2 (e), V (f). For information on Dm[Bac{3xP3–EYFP}] and Dm[Her{3xP3–ECFP}] flies see Horn and Wimmer (2000).
8. ECFP and EYFP as distinguishable transformation markers

GFP and EGFP have commonly been used in model organisms as reporter genes for enhancer/promoter analyses, to label certain tissues or cells, and as a fusion tag for subcellular protein localization in vivo (Tsien, 1998). Many of these constructs might also be useful to study the biology of non-model insects. However, the use of a GFP labeling construct in combination with a GFP-based transformation marker might be problematic. Spatially restricted markers active in defined tissues might be better in this respect than constitutive markers, but ideally different, discernable fluorescent molecules should be used for reporter genes and transformation markers.

A blue mutant variant of GFP, EBFP, has fluorescence excitation and emission maxima of 383 and 445 nm, respectively (Patterson et al., 1997). These are different enough from EGFP to use specific filter sets to clearly discern EBFP from EGFP. However, the low quantum yield and the rapid photobleaching of EBFP are unfavorable characteristics for its use as a transformation marker when many individuals have to be scored and exposure times cannot be reduced to a minimum. Moreover, the excitation with UV light is neither ideal for live biological samples nor for the skin and eyes of research personnel.

Another more stable bluish mutant of GFP, the cyan fluorescing variant ECFP, was developed with excitation and emission maxima of 434 and 477 nm, respectively (Patterson et al., 2001). This variant can therefore be excited with more innocuous blue light and its stability allows its use in transformation markers (Horn and Wimmer, 2000). Yet, the spectral variant ECFP is not unambiguously discernable from EGFP and is therefore only of limited use in combination with GFP or EGFP-containing constructs. However, using specific filter sets (Table 2) ECFP can be discriminated from a yellowish mutant variant of GFP, EYFP, which has excitation and emission maxima of 514 and 527 nm, respectively (Cubitt et al., 1999). Both ECFP and EYFP show reasonably high quantum yields and photobleaching times (Patterson et al., 2001) that allow their application as independent and separable markers for insect transgenesis (Horn and Wimmer, 2000).

9. Red fluorescing DsRed as transformation marker

ECFP and EYFP can be employed as a marker pair to use one fluorescent protein for transgenic selection and the other for gene expression studies or tissue labeling. Nevertheless, ECFP is a less sensitive marker than EGFP and excitation with short wavelength blue light causes increased autofluorescence. Moreover, their use would require the exchange of GFP or EGFP for EYFP in every previously established construct of interest. In order to use the already existing GFP and EGFP constructs, an additional separable fluorescent marker is desirable. Such a marker became available with the isolation of the red fluorescent protein DsRed (drFP583) from a coral-like sea anemone of the genus Discosoma (Matz et al., 1999). DsRed has about 23% amino acid sequence identity to A. victoria GFP with conserved amino acids in the immediate vicinity of the fluorescence chromophore (Wall et al., 2000; Yarbrough et al., 2001). DsRed has excitation and emission maxima of 558 and 583 nm, respectively (Matz et al., 1999). Its high resistance to photobleaching and high quantum yield (Baird et al., 2000), as well as its longevity (Matz et al., 1999) provide ideal characteristics for a transformation marker.

For insect transgenesis approaches, we and others (Handler and Harrell, 2001b) have started to apply the humanized variant DsRed1 (Clontech Laboratories, Inc., Palo Alto, CA) which shows the same fluorescence properties as DsRed (drFP583; Matz et al., 1999). Handler and Harrell (2001b) drove DsRed1 expression with the D. melanogaster polyubiquitin promoter to identify transgenic D. melanogaster larvae and adults. They could show that PUBsRed1-mediated red fluorescence is consistently brighter and more easily detectable at lower magnifications than EGFP expression. A high signal-to-noise ratio helps the identification of transformants, since DsRed fluoresces outside the range of the autofluorescence of most biological tissues (Clontech, 1999).

We placed DsRed1 under the control of the artificial 3xP3 eye promoter and tested its usability as a transformation marker in D. melanogaster. In adult white mutant vinegar flies, strong red fluorescence can be easily detected in the compound eyes and ocelli (Figs. 1b and 2b). In compound eyes of wild-type vinegar flies, the DsRed marker could not only be detected in those ommatidia that point straight towards the observer as described for EGFP (Berghammer et al., 1999), but also in neighboring ommatidia (data not shown). The partial

<table>
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<tr>
<th>Filter set</th>
<th>Excitation filter (λ_{max}/spectral width)</th>
<th>Emission filter (λ_{max} or λ_{max}/spectral width)</th>
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<tr>
<td>Longpass filters:</td>
<td></td>
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<tr>
<td>GFP2</td>
<td>480 nm/40 nm</td>
<td>510 nm</td>
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<td>V</td>
<td>425 nm/40 nm</td>
<td>475 nm</td>
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<td>Bandpass filters:</td>
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<tr>
<td>Cy3.5/DsRed</td>
<td>565 nm/30 nm</td>
<td>620 nm/60 nm</td>
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<td>YellowGFP</td>
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<td>CyanGFP</td>
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loss of optical isolation is probably due to the fact that tissues absorb less energy at longer wavelength, which allows some DsRed detection despite the presence of eye pigments. For the same reason DsRed expressed in the adult brain can be detected through the slightly melanized head cuticle, whereas EGFP fluorescence is quenched (compare Fig. 1b with 1c).

To see how early in development DsRed could serve as a transformation marker, we checked for 3xP3-mediated red fluorescence in embryonic and larval stages. Consistent with the slow maturation time of DsRed (Baird et al., 2000), we have not been able to detect 3xP3–DsRed as early as EGFP, which could be recognized in the developing larval eyes of stage 16 embryos (Horn et al., 2000). Nevertheless, even in the fast-developing D. melanogaster embryo (egg activation to hatching in about 22 h at 25°C), 3xP3–DsRed could be clearly identified in the Bolwig organs (larval eyes) at the end of embryogenesis just before hatching. The fluorescence was not fully mature at this stage, but identification was reliable due to the absence of autofluorescence. Thus, we expect that 3xP3–DsRed will also be detectable in late embryos of slower-developing insect species. In D. melanogaster, red fluorescence can be observed during larval stages in the CNS, part of the peripheral nervous system, the anal pads and the hindgut (data not shown). Compared to 3xP3–EGFP-mediated green fluorescence, the patterns appear delayed, but are otherwise as described (Horn et al., 2000). At room temperature, DsRed takes days to reach maximal fluorescence and maturation proceeds through a green fluorescent intermediate (Baird et al., 2000). However, we could not see this intermediate green fluorescence with any of our filter sets (Table 2). It is probably below our in vivo detection level, as maximal green fluorescence is supposedly less than one percent of the mature red fluorescence (Baird et al., 2000).

Due to the fluorescence properties described above, DsRed will be highly efficient as a marker for insect transgenesis. Moreover, DsRed fluorescence has been shown to be stable in biological tissues for several weeks (Matz et al., 1999). The longevity and resistance to photobleaching should allow the stable genetic marking of insects, which will be ideal for field applications like sterile insect technique (SIT) programs (Peloquin et al., 2000). Actually, the combination of DsRed with universally applicable promoters would be ideal for initial release studies, since such a harmless transgene could allow monitoring of its spread in wild-type populations, and its widespread applicability would allow the detection of potential horizontal transfers to other species.

10. Combining DsRed as a transformation marker with EGFP as a reporter for gene expression studies

The longevity of DsRed provides a great quality for a transformation marker, but it is disadvantageous to its use as a reporter to study temporally regulated genes. Together with the slow maturation time, which is in the order of days (Baird et al., 2000; Handler and Harrell, 2001b), this precludes the use of DsRed as a reporter of short-term gene expression. Moreover, the tendency to form aggregates presents a serious impediment to using DsRed as a fusion tag for in vivo protein localization (Baird et al., 2000). Recently, a mutant variant, DsRed2, has been described that is more soluble, matures faster, has a reduced tendency to form aggregates and seems to be less toxic (Clontech, 2001a). Otherwise, DsRed2 has the same fluorescence properties as DsRed1. However, the maturation time of about 24 h is still somewhat too long for a reporter gene.

Another DsRed mutant called E5 or “Fluorescent Timer” (Terskikh et al., 2000; Clontech, 2001b) displays initial bright green fluorescence before it matures to red and can be detected after a few hours. This variant can function as a reporter with an internal fluorescent clock, since it provides both spatial and temporal information on gene expression. The fluorescent state of green, yellow (overlap of green and red) or red indicate both gene activation and down-regulation (Terskikh et al., 2000). The use of the green–red fluorescent timer as a reporter gene can probably be combined with ECFP as a distinguishable marker for transgenesis.

Considering the vast amount of diverse GFP/EGFP reporter gene and fusion tag constructs already available, the best recommendation to prevent interference is, at present, the use of GFP-based in vivo reporters in combination with either DsRed1- or DsRed2-based transformation markers. Using specific filter sets (Table 2), EGFP and DsRed can be unambiguously discerned (Fig. 1), which will allow their independent detection even if they were expressed in the same tissue at the same time.

11. ECFP, EYFP, and DsRed as independent and separable transformation markers for multi-component genetic systems

In addition to their use as a marker pair for transgenic gene expression studies, EGFP and DsRed will also serve as separable transformation markers for the development of genetic multi-component systems. Due to the absence of marked “balancer” chromosomes in non-model insects, the different components will require individual labeling. Thus, methods originally developed in D. melanogaster such as first approaches to a transgenic sterile insect technology (Thomas et al., 2000; Heinrich and Scott, 2000) or the GALA/UAS- and tTA/TRE-based ectopic expression systems (Brand and Perrimon, 1993; Bello et al., 1998) can be transferred to different insect species. Since ectopic expression of genes can cause dominant lethality or sterility, these systems are made inducible by splitting activator and effec-
tor activities into two separate, benign constructs. These can then independently be used to generate and establish transgenic insect lines. Only after crossing the two transgenes together, the tissue-specific activator will drive ectopic expression of the effector and the consequences can be analyzed.

In addition to the introduction of transgenes into the genome of different insect species, dominantly marked transposons can also be employed as mutagenic agents to identify gene functions. Through genetic analysis, components of many different biological processes can be identified and interrelated. A basic problem has long been how to obtain molecular information for a gene identified by the phenotype of its mutation. Transposon-mediated mutagenesis presents an ideal solution. The integration of a transposon into a gene will mutate and tag it at the same time. The gene corresponding to the mutation can then easily be cloned by inverse PCR (Ochman et al., 1988). The efficient method of mutagenesis by transposon tagging relies on the development of two elements: one element, called “jumpstarter”, encodes a transposase that mobilizes a second element, called “mutator”, which integrates randomly into the genome and thereby causes mutations (Cooley et al., 1988). Fundamental to this approach is the use of two different types of transposons that cannot cross mobilize each other, as well as a clearly distinguishable marking of the two elements.

Moreover, transposon mobility also provides a way to sample the genome for enhancer sequences (O’Kane and Gehring, 1987). Transposon-mediated mutagenesis and enhancer trapping can actually be performed simultaneously in the same screen, as long as the mutator element contains, in addition to the dominant transformation marker, a reporter gene driven by a basal promoter. When the mutator element integrates near a specific enhancer element, the basal promoter will respond and reporter gene expression will be driven in an enhancer-dependent pattern.

Transposon mutagenesis and enhancer trapping screens can be performed to identify genes for many different biological traits. Furthermore, when the enhancer trap screen is combined with a binary ectopic expression system (see above) such a screen will not only identify interesting enhancers, but at the same time provide tools to drive gene expression in many different embryonic, larval, or adult tissues (Brand and Perrimon, 1993). For this purpose, the reporter gene in the mutator element is replaced by a gene for a heterologous transcriptional activator like yeast GAL4 (Brand and Perrimon, 1993) or the bacterial–viral fusion tTA (Bello et al., 1998). For the identification of the enhancer trap, these activators then need to drive the expression of a secondary reporter, e.g., EGFP, for in vivo analysis or the bacterial lacZ gene, whose activity can easily be detected by histochemical stainings of fixed tissues. The major advantage of this more elaborate mutagenesis system is that once an enhancer of interest has been identified, it can be used to express other genes. The transgenic line carrying the GAL4 or tTA enhancer trap can drive tissue-specific expression of any cloned gene which has been linked to UAS or TRE promoter sequences, respectively (Brand and Perrimon, 1993; Bello et al., 1998).

To develop such an enhancer trap expression system, actually three separable transformation markers are needed. One to mark the jumpstarter element, one for the mutator element, and one for the reporter or effector constructs. Ideally, three distinguishable markers will allow us to clearly identify, separate, and stably establish novel mutator insertions. Fig. 2 illustrates that the spectral variants ECFP, EYFP, and DsRed can be unambiguously discerned from each other by the use of specific filter sets (Table 2). They therefore represent independent and separable transformation markers that can be employed for the development of genetic multi-component approaches.

For the transposon mutagenesis, it will not matter which element is used with which transformation marker, as long as the mutator element carries a highly sensitive marker that is least susceptible to position effect suppression. This is important to obtain an unbiased insertional coverage of the entire genome. Therefore, EYFP- or DsRed-based markers will probably be preferable, since their fluorescence is somewhat more easily detectable than ECFP. Nevertheless, if the transposon mutagenesis also serves as an in vivo enhancer trap screen to identify genes active in certain larval or adult tissues, then novel insertions and enhancer trap effects need to be detected simultaneously and non-invasively. As mentioned above, EGFP or EYFP will probably be the best reporters to identify enhancer traps in live organisms. If one wants to avoid frequent switching between different filter sets while screening, the marker chosen to identify novel insertions of the mutator element needs also to be based on EYFP. This, however, will exclude markers based on constitutively active promoters, since the potentially ubiquitous fluorescence of the transformation marker could mask the reporter fluorescence. The use of tissue-specific promoters would at least allow the identification of enhancer traps for tissues the transformation marker is not expressed in. Thus, the more expression of the transformation marker is restricted, the more tissues will be screenable. Widely applicable enhancer trap expression systems based on the three fluorescent markers 3xP3–ECFP, 3xP3–EYFP, and 3xP3–DsRed are currently under construction in our laboratory (C.H. and E.A.W., unpublished).

12. Epifluorescence stereomicroscopy

Figs. 1 and 2 depict 3xP3–EGFP, 3xP3–ECFP, 3xP3–EYFP, and 3xP3–DsRed marker fluorescence in the
compound eyes of *D. melanogaster* white mutants. The fluorescence was observed with the Leica fluorescence stereomicroscope MZ FLIII, whose rotatable filter changer can hold up to four different filter sets. This is advantageous when different spectral variants are analyzed at the same time and rapid filter changes are required. The MZ FLIII uses a separate excitation beam path for the fluorescence illumination. This triple beam technology makes dichroic filters dispensable, which are needed in other fluorescence microscopes to introduce the excitation light into the observation light path. Thus, for the MZ FLIII stereomicroscope, each filter set consists of one excitation and two emission filters (Table 2) for the single illumination and two parallel observation beams, respectively.

Longpass emission filters allow light above a defined wavelength to pass through, whereas bandpass emission filters restrict the detectable wavelength to a defined spectral width. In our studies, we use the longpass filter sets V (Leica, Bensheim, Germany) and GFP2 [(GFP Plus) Leica, Bensheim, Germany], as well as the bandpass filter sets Cy3.5/DsRed [Chroma #41021; AHF analysentechnik AG (#F41-035), Tübingen, Germany], YellowGFP [Chroma #41028; AHF analysentechnik AG, Tübingen, Germany], and CyanGFP [Chroma #31044v2; AHF analysentechnik AG, Tübingen, Germany]. If required for other microscope setups, the corresponding dichroic filters for the Chroma filter sets are described at: [http://www.chroma.com/data/fsc-index.cfm](http://www.chroma.com/data/fsc-index.cfm).

The bandpass filters Cy3.5/DsRed, YellowGFP, and CyanGFP allow complete separation of 3xP3–DsRed, 3xP3–EYFP, and 3xP3–ECFP transformants (Fig. 2a–d). This will be important for the individual identification of the different components of complex genetic systems. However, the low luminosity of the YellowGFP and CyanGFP bandpass filters makes insect handling difficult and might only allow detection of medium-to-strong expression lines. The longpass filters GFP2 or V show significantly higher sensitivity for EYFP or ECFP, respectively, but they do not completely block detection of the other spectral variants (Fig. 2e,f). Yet these longpass filters have the advantage of being highly luminous, which allows for easier sorting of insects under the dissecting scope. Thus, for general use, the longpass filters GFP2 and V are more convenient.

For the detection of DsRed fluorescence the bandpass filter Cy3.5/DsRed is highly luminous, which allows its use for both routine insect handling and reliable separation from ECFP and EYFP fluorescence. Handler and Harrell (2001b) used a Texas Red™ filter set and Chroma Technology Corp., Brattleboro, VT, recommends also some TRITC and Phycoerythrin filters ([http://www.chroma.com/rfp.html](http://www.chroma.com/rfp.html)). All these filters allow reliable detection of DsRed and some of the sets with a wider bandpass emission filter might even be more sensitive. However, when definite distinction between DsRed, EYFP, and ECFP is required, the Cy3.5/DsRed filter is recommended in combination with YellowGFP and CyanGFP (AHF analysentechnik AG, Tübingen, Germany; Figs. 1b and 2b).

The triple-beam technology of the Leica MZ FLIII should be perfect for detecting fluorescence markers in most tissues. However, the angle between illumination and observation beams can be problematic for detection of fluorescence in complex optical systems like certain compound eyes. Since the fluorescent protein is expressed in the photoreceptor cells, it is maximally excited if the illuminating light enters the ommatidium parallel to its optical axis, and again the fluorescent light can be detected best if the optical axes of observation beam and ommatidium are aligned (“cat’s eye” effect). This cat’s eye effect is not observed in the case of *D. melanogaster* (see Figs. 1 and 2). But in *T. castaneum*, for example, the triple-beam setup of the MZ FLIII does not allow sensitive detection of 3xP3–EGFP-mediated green fluorescence in the compound eyes of wild-type or white-eyed *pearl* mutant beetles (M. Klingler, personal communication). For a sensitive detection of fluorescence in the compound eyes of this species, a stereomicroscope like the Leica MZ12 or the Nikon SMZ1500 are more favorable (Table 3). In these stereomicroscopes, fluorescence illumination is guided through one of the observation beams, thus allowing excitation light and emission light to enter and leave the ommatidia on the same optical path. Consistent with this, fluorescence in the eyes of adult *T. castaneum* can only be detected in the observation beam that is used for excitation, but not in the other (M. Klingler, personal communication). Kramer Scientific Corp., Valley Cottage, NY, has developed a fluorescence module for the Leica MZ12 that guides the fluorescence illumination through both observation beams, thereby achieving a more homogeneous illumination and detection of fluorescent specimen. Although this setup is well suited for ommatidial displaying the cat’s eye effect, it is, however, somewhat less luminous than the original Leica MZ12 fluorescence module. On the other hand, the Kramer Scientific fluorescence module includes a sliding carriage for a rapid change of filter sets (which is not possible with the Leica MZ12 fluorescence module). This allows convenient detection of different fluorescent markers in the adult eyes of *T. castaneum*. For the SMZ1500, Nikon supplies the fluorescence module P-FLA that allows a quick and easy change of four filter blocks by using a slide lever. The Nikon setup provides excellent optics, is highly sensitive for fluorescence detection and suitable for detection of fluorescent variants in ommatidia with and without cat’s eye effect.
Transposon mutagenesis will provide an ideal means to link function with the explosion of DNA sequence information that the field of insect genomics will soon generate. Functional genomics will heavily rely on possibilities to identify gene expression patterns and to characterize mutations of the proposed genes to understand their molecular and physiological roles. Ectopic expression systems will allow us to examine the effects gene activities have for particular tissues. Independent of transposon mutagenesis approaches, the newly discovered phenomenon of gene silencing by double-stranded RNA, also called RNA interference (RNAi), will provide an additional tool to identify gene functions (Barstead, 2001). RNAi techniques have already been successfully applied in D. melanogaster (Carthew, 2001), T. castaneum (Lewis et al., 2000) and the milkweed bug Oncopeltus fasciatus (Hughes and Kaufman, 2000).

Moreover, a method for targeted gene knockouts using intermediate transgene constructs has recently been described for D. melanogaster (Rong and Golic, 2000, 2001). The transfer and generalization of this technology to other insect species appears feasible given the availability of broadly applicable transformation systems. The use of fluorescence-based transformation markers in this approach might actually allow automated sorting to identify rare recombinant individuals. Complex object parametric analyzers and sorters are currently developed to identify and sort D. melanogaster embryos and larvae based on size and the presence or absence of fluorescent labels (Furlong et al., 2001; Union Biometrica, Somerville, MA). Applications in the nematode worm Caenorhabditis elegans have shown that correct sorting requires only few fluorescing cells per individual. Plans exist to even develop a sorter for eggs and embryos of the zebrafish Danio rerio (Union Biometrica, Somerville, MA). Some of these sorters might be adjustable to also sort translucent embryos or larvae of other insect species and might at some point replace the labor-intensive screening for transformants by automated identification of transgenic individuals.

The required marker genes and broad-range transposons (Atkinson et al., 2001; Handler, 2001) to develop such methodologies for a wide range of insect species are available. However, all efficient insect transgenesis approaches to date have been based on microinjection of embryos. This often encompasses dechorionation and slight desiccation, which is not tolerated by all insect embryos. Therefore, microinjection techniques need to be further improved. Mosquito embryos, for example, do not need to be dechorionated or desiccated (Morris, 1997). Moreover, other DNA delivery methods like biolistics (Kravariti et al., 2001) or electroporation (Swartz et al., 2001) need to be further explored. So far, these techniques have not been very effective in transforming D. melanogaster, which is attributed to the low amount of DNA delivered (Baldarelli and Lengyel, 1990; Kamdar et al., 1995). However, transient expression indicates that plasmid DNA can successfully be introduced into insect embryos by biolistics and electroporation (Leopold et al., 1996; Mialhe and Miller, 1994).

Improvements in DNA delivery and targeting might eventually allow the establishment of novel transformation techniques that could extend transgenesis to more insect species.

Presently, insect transgenesis seems very promising. Detailed molecular studies will be possible on many biological processes of a diverse set of species. In addition, this technology will provide a means to modify and influence wild populations of medically and economically important insect species. However, projects that bear the risk or actually require the release of transgenic insects into the environment should be planned with utmost care given the general lack of experience and the intense public scrutiny to which such projects will be subjected. Therefore, stringent safety assessment is required for the transport and particularly the release of genetically modified insects. Already at the initial stages of this methodology, molecular and population geneticists, entomologists, ecologists, as well as pest management specialists need to coordinate their efforts along with regulatory agencies to establish a safe use of the great potential transgenic insects have to offer.
Acknowledgements

We are very grateful to Brigitte Jaunich for technical assistance and express our thanks to Al Handler and Martin Klingler for valuable comments on the manuscript. We extend our thankful appreciation to those who shared unpublished results. We thank Christian F. Lehner and the members of the Lehrstuhl Genetik for support, encouragement, and discussions during the course of our work, which is supported within the priority program ‘International Research into the Development of Sustainable Agriculture and Forestry’ by the Robert Bosch Foundation providing a junior professorship and independent research group to E.A.W. The DFG-Graduiertenkolleg ‘Ecological Significance of Natural Compounds and other Signals in Insects — from Structure to Function’ supports F.S.P. by a Ph.D. stipend. C.H. is a fellow of the Fonds der Chemischen Industrie.

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