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昆虫遗传转化品系的常用标记

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摘要: 遗传转化标记是将遗传修饰昆虫从野生型种群中分辨出来的根据,遗传转化昆虫的鉴定、转化品系的维持及其遗传稳定性的监测都依赖于可靠的标记系统,发展易于应用和监测的转化标记能够极大地促进害虫遗传防治的相关研究。用于遗传修饰昆虫的转化标记主要有昆虫眼睛颜色标记基因、抗药性标记基因和荧光蛋白标记基因等。非果蝇类昆虫首个遗传转化品系的鉴定是通过眼睛颜色突变而实现,但大多数昆虫物种没有可用的突变体或缺少相应基因的信息,从而限制了眼睛颜色标记的应用。抗药性基因标记虽然能够通过对转化昆虫进行集体选择而大幅度提高筛选转化体的效率,但由于其鉴定的准确性不高且存在安全性问题,未得到广泛应用。荧光蛋白标记基因的发展则显著拓宽了能够转化的昆虫种类。从水母分离的绿色荧光蛋白(GFP)经突变方法获得了多种不同荧光性质的突变体,经人为修饰后与适宜的强启动子构成转化标记载体,能够有效鉴定更多昆虫物种的遗传转化个体,其中应用较多的是增强型绿色荧光蛋白(EGFP)。此外,从珊瑚属海葵中分离得到的红色 DsRed 标记基因提供了多样化的红色荧光蛋白选择,在某些生物中 DsRed 与 GFP 联合应用的表现明显优于 GFP 突变体,所以其应用前景也非常广泛。本文着重从眼睛颜色、抗药性和荧光蛋白等 3 个方面阐述了标记基因的发展历史与现状,并对其今后的发展方向进行了展望。

关键词: 遗传修饰昆虫; 转化标记; 眼睛颜色标记; 抗药性标记; 荧光蛋白标记

Commonly used transformation markers in genetically modified insects

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Abstract: Transformation markers offer a tool to distinguish the genetically modified insects from wild types. Both the identification of transformants and the maintenance of transformed lines depend on reliable transformation makers. In addition, the evaluation of the genetic stability of released genetically modified insects needs strong and stable markers. Thus the development of broadly applicable, easily detectable and reliable transformation markers will facilitate the study of genetic pest management. In general, eye color genes, drug resistance genes and fluorescent protein genes can be used as markers in genetically modified insects. The first efficient identification of a non-drosophilid insect transformation line was based on the rescue of eye color mutant phenotypes. However, for most insect species, the application of eye color markers is limited because of the lack of suitable recipient mutant strains and less information on related genes. Markers based on drug resistance genes can improve the screening efficiency of transformants, but the selection for drug resistance is problematic and prone to have false positives or negatives with potential biosecurity problems. Fluorescent protein gene markers significantly facilitate the development of stable insect transformation lines. The green fluorescent protein (GFP, isolated from the jellyfish *Aequorea victoria*) and its variants with various fluorescent characteristics can be combined with suitable, strong promoters to serve as transformation markers for a wide range of insect species and guarantee the reliable screening of the transformants. In this

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category, the enhanced green fluorescent protein (EGFP) was mostly used. Besides, the red fluorescent protein (DsRed), isolated from the mushroom coral, *Discosoma striata*, provides a selection of red fluorescent proteins with better performance than GFP mutants. This paper reviews the history and status of transformation markers including eye color genes, drug resistance genes and the fluorescent protein genes. The potential roles of transformation markers in genetic pest management are also discussed.

Key words: genetically modified insect; transformation marker; eye color gene; drug resistance gene; fluorescent protein gene

昆虫遗传转化技术是将携带外源基因的转座子导入到目标昆虫的基因组,使其获得特定表型的分子生物学操纵手段。昆虫遗传转化研究对于深入了解昆虫生理和行为意义重大,同时已成为一种新型有效的害虫控制策略。自 1982 年首次在黑腹果蝇 *Drosophila melanogaster* 中应用 P-元件实现胚胎转化(Rubin & Spradling, 1982)之后,昆虫遗传转化研究逐渐兴起并得到广泛重视,随后开发了各种转座元件如 *Minos*、*Mariner*、*Hermes* 和 *piggyBac* 等,并被成功应用于重要医学和农业害虫的遗传防治研究中。各种转化标记的发展显著促进了昆虫遗传修饰的研究,大大拓宽了能够被转化的昆虫种类(Atkinson *et al.*, 2001; Handler, 2001a; Handler & James, 2000)。遗传转化标记是将遗传修饰昆虫从野生型种群中分辨出来的根据,遗传转化昆虫的鉴定、转化品系的维持及其遗传稳定性的监测都依赖于可靠的标记系统,发展易于应用和监测的转化标记能够极大地促进害虫遗传防治的相关研究。用于遗传修饰昆虫的转化标记主要有昆虫眼睛颜色标记基因、抗药性标记基因和荧光蛋白标记基因等(Alphey, 2002)。

1　眼睛颜色基因转化标记

各种控制眼睛颜色基因的发掘,丰富了昆虫遗传修饰研究的眼睛颜色标记。早期对果蝇眼睛颜色突变的研究揭示了编码色氨酸加氧酶的 *vermilion* 基因(Searles *et al.*, 1990; White *et al.*, 1996)和编码犬尿氨酸—单加氧酶的 *cinnabar* 基因(Cornel *et al.*, 1997; Warren *et al.*, 1996)均参与色素产生的过程。在黑腹果蝇和地中海实蝇 *Ceratitis capitata* 中,*white* 基因负责编码昆虫复眼中色素引入和组装的 ABC 转运蛋白(Bhalla, 1968; Ewart *et al.*, 1994)。通常,这些在复眼中能产生色素的基因如 *white* (*w*)、*vermilion* (*v*)和 *cinnabar* (*cn*)等均可用作遗传修饰昆虫研究的眼睛颜色标记基因。野生型基因突变的等位基因会影响昆虫复眼的颜色,将这些基因引入适宜的野生昆虫中,即可产生可见的复眼表现型差异(Rubin & Spradling, 1982)。这些基因大多为 2~3 kb,其突变基本不会造成昆虫适合度的降低,过量表达对生物体也无害。同时,对其检测无需特殊的检测系统,所以眼睛颜色标记基因更易于被接受,从而得到广泛应用。

眼睛颜色突变体及其相应基因用作评价性标记体系,促进了果蝇和其他昆虫遗传修饰技术的发展(Lorenzen *et al.*, 2002),黑腹果蝇的首次胚胎转化和黑果蝇 *Drosophila virilis* 转化品系的获得均依赖于可见眼睛颜色标记系统的应用(Gomez & Handler, 1997; Rubin & Spradling, 1982)。地中海实蝇(Handler *et al.*, 1998; Loukeris *et al.*, 1995; Michel *et al.*, 2001)和埃及伊蚊 *Aedes aegypti* (Coates *et al.*, 1998; Jasinskiene *et al.*, 1998)的首次成功转化很大程度上得益于眼睛颜色突变体的存在和用于突变—拯救选择的野生型基因的克隆及可用性。在地中海实蝇中,白色眼睛基因座中的一个无效突变可被克隆的野生型拷贝所补充(Zwiebel *et al.*, 1995),随后相似的基因也用于转化同样存在白色眼睛品系的橘小实蝇 *Bactrocera dorsalis* (Handler & McCombs, 2000)。黑腹果蝇 *cn* 基因可以拯救埃及伊蚊突变品系的白色眼睛表型品系。对于赤拟谷盗 *Tribolium castaneum*,通过克隆其 *v* 和 *c* 基因建立了基于携带眼睛颜色突变 *vermillion*^{white} 拯救的转化体系。应用眼睛颜色标记基因进行遗传修饰的昆虫物种如表 1 所示。

表 1　采用眼睛颜色标记的遗传修饰昆虫

Table 1　Genetically modified insects using eye colour genes as transformation markers

转化的物种 Species transformed	转座元件 Transposons	参考文献 References
地中海实蝇 *Ceratitis capitata*	*Minos*	Loukeris *et al.*, 1995
地中海实蝇 *Ceratitis capitata*	*piggyBac*	Handler *et al.*, 1998
地中海实蝇 *Ceratitis capitata*	*Hermes*	Michel *et al.*, 2001
地中海实蝇 *Ceratitis capitata*	*Minos*	Zwiebel *et al.*, 1995
埃及伊蚊 *Aedes aegypti*	*Hermes*	Jasinskiene *et al.*, 1998
埃及伊蚊 *Aedes aegypti*	*Mariner*	Coates *et al.*, 1998
橘小实蝇 *Bactrocera dorsalis*	*piggyBac*	Handler & McCombs, 2000
黑果蝇 *Drosophila virilis*	*Hobo*, *Mariner*	Lohe & Hartl, 1996; Lozovskaya *et al.*, 1996
黑腹果蝇 *Drosophila melanogaster*	*P element*	Rubin & Spradling, 1982

2 抗药性基因转化标记

最初对单独发挥作用的显性选择标记的研究主要集中于抗药性基因,如对新霉素类似物有抗性的磷酸转移酶基因 *NPT II*(Steller & Pirrotta, 1985)、对对硫磷有抗性的有机磷脱氢酶基因 *opd*(Benedict *et al.*,1995; Phillips *et al.*,1990),以及对有机氯杀虫剂狄氏剂 dieldrin 有抗性的 *Rdl* 基因(Ffrench-Constant *et al.*,1991)等。抗药性标记基因最早在冈比亚按蚊 *Anopheles gambiae* 中得以应用,冈比亚按蚊的第一个转化品系是应用编码新霉素羧酸酯酶的 *neo* 基因作为选择标记而建立起来(Miller *et al.*,1987),拥有 *neo* 基因的转化品系可以获得对氨基糖苷类抗生素 G418 的抗性。然而,由于在黑腹果蝇中能够确定基于 G418 抗性筛选的较佳条件,该基因标记只在黑腹果蝇中成功应用(Steller & Pirrotta,1985)。

对于大部分昆虫来说,筛选到适宜的抗药性基因遗传转化标记,可以对试验昆虫进行集体选择,从而大幅度提高筛选转化体的效率,这种优势使其成为可见眼睛颜色标记之外的另一个重要转化标记。然而,抗药性标记的广泛应用还存在诸多问题。首先,转化体筛选的准确性。野生型昆虫种群对某些药物或抗生素的抗性具有波动性;同时,转座子介导的遗传修饰昆虫并不能将特定的靶基因转化到特定的基因组位置上,所以转化试验将会得到不同数量的插入子插入到不同位点的多种转化体;此外,由于位置抑制效应的差异,不同转化体之间转化标记的表达水平也存在明显差异。因此,在没有其他可用标记的昆虫物种中应用抗药性标记筛选转化体,易筛选出未转化成功的假阳性个体,或误杀大多数转化成功的假阴性个体。其次,安全性。很多药物都具有毒性,且操作过程需要研究人员暴露于药物中,所以该技术不被广泛接受。同时,转化品系的维系传代需要依靠抗药性的选择,转化品系的天然抗性选择机制会随世代的增加而加强,而抗药性标记可能使连锁的转化基因具有选择性优势,因此对以释放遗传修饰昆虫为最终目的的害虫治理项目而言,其将面临更大的抗性问题。目前,杀虫剂抗性(Hemingway & Ranson,2000)和抗生素抗性(Monroe & Polk,2000)已成为威胁人类健康的严重问题,而抗药性标记的使用将会使现有的局势变得更为严峻。

3 荧光蛋白转化标记

转座子介导的昆虫遗传修饰研究方法具有随机插入的特性,所以要想对转化个体进行准确检测,就需要应用在不同表达水平均能被稳定监测的遗传转化标记。该种标记基因应具有显性表达、非破坏性、野生型背景中可见等特性。从水母 *Aequorea victoria*(Prasher *et al.*,1992)中分离得到的编码绿色荧光蛋白(green fluorescent protein,GFP)基因具备转化标记的基本特性,GFP 在多种不同的有机体中均可显示出亮绿色的荧光,且在有机体不同组织中表达的绿色荧光易于被监测(Tsien, 1998)。GFP 自被发现以来,以其良好的荧光特性成为被广泛使用的报告基因或体内蛋白定位的融合标签(Brand,1999; Chalfie *et al.*,1994; Cubitt *et al.*,1995; Plautz *et al.*,1996)。然而,由于野生型 GFP 的相对不可溶性和位于紫外光谱内激发峰的限制,尤其是长时间暴露在紫外光条件下不适宜筛选活体生物等因素,限制了其在遗传修饰昆虫鉴定和筛选中的应用。

随着更可溶性 GFP 突变品系如增强型 GFP(EGFP)(Cormack *et al.*,1996; Yang *et al.*,1996)的发展,上述问题基本得以解决。EGFP 激发峰为 488 nm,能够在更无害的蓝光下被激发,强度比野生型 GFP 提高 35 倍,适合快捷无损伤检测。在黑腹果蝇中,EGFP 标记与眼睛颜色基因标记联合应用,验证了 EGFP 对该物种的适用性(Handler & Harrell,1999; Horn *et al.*,2000),并证实 EGFP 遗传转化标记比其常规转化标记即眼睛颜色基因标记"mini"-*white* 更加灵敏、可靠。以埃及伊蚊为靶标的验证结果与黑腹果蝇相似(Pinkerton *et al.*, 2000),可能与"mini"-*white* 基因受位置抑制效应更强有关。此外,由于启动子的不同,即使基因连锁插入到相同的染色体位置上,不同基因受位置抑制的效应也可能存在明显差异(Bhadra *et al.*,1998)。通常,EGFP 基因标记比眼睛颜色基因标记受到完全性抑制的可能性更小(Handler & Harrell,1999; Horn *et al.*,2000)。EGFP 具有可溶性更佳、受蓝光激发、不易受完全性位置抑制等特性,是首个被广泛应用的荧光变体,也是目前昆虫遗传修饰研究的主要转化标记。Higgs & Lewis (2000)详细综述了 GFP 突变品系作为遗传修饰昆虫标记的优势,Horn *et al.*(2002)也指出其优势之一就是能应用野生型

生物体,这对于缺少可见型突变品系或突变品系很弱的昆虫物种至关重要。双翅目、鳞翅目和鞘翅目等 3 个目不同物种的成功转化,表明 EGFP 可以被用作昆虫遗传修饰的转化标记(表 2~4)。

然而,荧光标记在昆虫遗传修饰研究中仍存在一些问题。首先,筛选遗传修饰昆虫过程中长时间的强光照射可能会导致昆虫死亡;其次,很多组织器官如马氏管、几丁质外骨骼或坏死组织的自发光可能会干扰转化体的检测;再次,成虫表皮高强度的黑化会阻碍对其内部组织表达的 EGFP 的监测。很多昆虫的胚胎、幼虫或蛹期阶段比较透明,根据胚胎的发育历期以及遗传转化标记经过内部环化和氧化达到成熟所需的时间(Davis *et al.*,1995)推测,幼虫孵化之前的阶段可能是筛选遗传修饰昆虫的最佳时期。在该阶段进行荧光筛选不仅能够达到快速检测的目的,而且避免了饲养全部 G1 代遗传修饰昆虫,这对幼虫食材珍贵但食量大或世代周期很长的物种而言非常重要。为了更准确地监测单拷贝插入的转化基因,可以借助强启动子驱动 EGFP 的高效表达。同时,根据研究的具体需求,组成型和组织特异性的启动子都可用来构建 EGFP 的独立标记系统。

表 2　采用荧光蛋白基因作为转化标记的双翅目昆虫

Table 2　A list of Dipteran species genetically modified using fluorescent protein genes as transformation markers

转化标记 Markers	转化物种 Species transformed	转座元件 Transposons	参考文献 References
PUbnlsEGFP	黑腹果蝇 *Drosophila melanogaster*	*piggyBac*	Handler & Harrell,1999
	加勒比按实蝇 *Anastrepha suspensa*	*piggyBac*	Handler & Harrell,2001a
	铜绿蝇 *Lucilia cuprina*	*Minos*	Heinrich *et al.*,2002
	淡色按蚊 *Anopheles albimanus*	*piggyBac*	Perera *et al.*,2002
	铜绿蝇 *Lucilia cuprina*	*piggyBac*	Scott *et al.*,2004
	螺旋蝇 *Cochliomyia hominivorax*	*piggyBac*	Allen *et al.*,2004
	地中海实蝇 *Ceratitis capitata*	*piggyBac*	Schetelig *et al.*,2009
	昆士兰果实蝇 *Bactrocera tryoni*	*piggyBac*	Raphael *et al.*,2010
	加勒比按实蝇 *Anastrepha suspensa*	*piggyBac*	Schetelig & Handler,2013
PUbDsRed1	黑腹果蝇 *Drosophila melanogaster*	*piggyBac*	Handler & Harrell,2001b
	昆士兰果实蝇 *Bactrocera tryoni*	*piggyBac*	Raphael *et al.*,2010
PUbDsRed	地中海实蝇 *Ceratitis capitata*	*piggyBac*	Schetelig *et al.*,2009
	加勒比按实蝇 *Anastrepha suspensa*	*piggyBac*	Schetelig & Handler,2013
actin5C:EGFP	黑腹果蝇 *Drosophila melanogaster*	*Hermes*	Pinkerton *et al.*,2000
	厩螯蝇 *Stomoxys calcitrans*	*Hermes*	O′Brochta *et al.*,2000
	埃及伊蚊 *Aedes aegypti*	*Hermes*	Pinkerton *et al.*,2000
	斯氏按蚊 *Anopheles stephensi*	*Minos*	Catteruccia *et al.*,2000
	致倦库蚊 *Culex quinquefasciatus*	*Hermes*	Allen *et al.*,2001
3*xP*3:EGFP	黑腹果蝇 *Drosophila melanogaster*	*piggyBac*, *Hermes*, *Mariner*	Horn *et al.*,2000
	家蝇 *Musca domestica*	*piggyBac*	Hediger *et al.*,2001
	埃及伊蚊 *Aedes aegypti*	*piggyBac*	Kokoza *et al.*,2001
	斯氏按蚊 *Anopheles stephensi*	*piggyBac*	Ito *et al.*,2002
	白纹伊蚊 *Aedes albopictus*	*piggyBac*	Labbé *et al.*,2010
3*xP*3:EYFP	黑腹果蝇 *Drosophila melanogaster*	*piggyBac*, *Hermes*, *Mariner*	Horn & Wimmer,2000
3*xP*3:ECFP	黑腹果蝇 *Drosophila melanogaster*	*piggyBac*, *Hermes*, *Mariner*	Horn & Wimmer,2000
3*xP*3:DsRed	黑腹果蝇 *Drosophila melanogaster*	*piggyBac*	Horn *et al.*,2002
	埃及伊蚊 *Aedes aegypti*	*Tn5*	Rowan *et al.*,2004
	黑腹果蝇 *Drosophila melanogaster*	*piggyBac*	Sarkar *et al.*,2006
	黑腹果蝇 *Drosophila melanogaster*	*piggyBac*	Horn & Wimmer,2000
*Hsp*83-DsRed	铜绿蝇 *Lucilia cuprina*	*piggyBac*	Concha *et al.*,2011
actin5C:DsRed	斯氏按蚊 *Anopheles stephensi*	*piggyBac*	Nolan *et al.*,2002
*hr*5-*ie*1:EGFP	冈比亚按蚊 *Anopheles gambiae*	*piggyBac*	Grossman *et al.*,2001
*hr*5-*ie*1-DsRed2	地中海实蝇 *Ceratitis capitata*	*piggyBac*	Gong *et al.*,2005
	地中海实蝇 *Ceratitis capitata*	*piggyBac*	Fu *et al.*,2007
*ie*1: GpGreen, PhiYFP and J-Red	墨西哥按实蝇 *Anastrepha ludens*	*piggyBac*	Condon *et al.*,2007
β2-EGFP	斯氏按蚊 *Anopheles stephensi*	*piggyBac*	Catteruccia *et al.*,2005
hsp:EGFP	橄榄实蝇 *Bactrocera oleae*	*Minos*	Koukidou *et al.*,2006

表 3 采用荧光蛋白基因作为转化标记的鳞翅目昆虫

Table 3 A list of Lepidoptera species genetically modified using fluorescent protein genes as transformation markers

转化标记 Markers	转化物种 Species transformed	转座元件 Transposons	参考文献 References
PUbnlsEGFP	苹果蠹蛾 *Cydia pomonella*	*piggyBac*	Ferguson *et al.*,2011
actin5C:EGFP	苹果蠹蛾 *Cydia pomonella*	*Hermes*	Ferguson *et al.*,2011
*BmA*3:EGFP	家蚕 *Bombyx mori*	*piggyBac*	Tamura *et al.*,2000
	棉红铃虫 *Pectinophora gossypiella*	*piggyBac*	Peloquin *et al.*,2000
	苹果蠹蛾 *Cydia pomonella*	*piggyBac*	Marec *et al.*,2005
	家蚕 *Bombyx mori*	*Minos*	Uchino *et al.*,2007
	苹果蠹蛾 *Cydia pomonella*	*Hermes*	Ferguson *et al.*,2011
	苹果蠹蛾 *Cydia pomonella*	*piggyBac*	Ferguson *et al.*,2011
	亚洲玉米螟 *Ostrinia furnacalis*	*piggyBac*	Liu *et al.*,2012
3*xP*3:EGFP	家蚕 *Bombyx mori*	*piggyBac*	Thomas *et al.*,2002
	家蚕 *Bombyx mori*	*piggyBac*	Osanai-Futahashi *et al.*,2012
	丛林斜眼褐蝶 *Bicyclus anynana*	*Hermes*, *piggyBac*	Marcus *et al.*,2004
3*xP*3:DsRed	家蚕 *Bombyx mori*	*piggyBac*	Royer *et al.*,2005
DsRed2	棉红铃虫 *Pectinophora gossypiella*	*piggyBac*	Walters *et al.*,2012
*hr*5-*ie*1-DsRed2	家蚕 *Bombyx mori*	*piggyBac*	Tan *et al.*,2013
hsp:GFP	苹果蠹蛾 *Cydia pomonella*	*piggyBac*	Ferguson *et al.*,2011

表 4 采用荧光蛋白基因作为转化标记的鞘翅目昆虫

Table 4 A list of beetles (Coleoptera) genetically modified using fluorescent protein gene as transformation markers

转化标记 Markers	转化物种 Species transformed	转座元件 Transposons	参考文献 References
3*xP*3:EGFP	赤拟谷盗 *Tribolium castaneum*	*piggyBac*, *Hermes*	Berghammer *et al.*,1999
	赤拟谷盗 *Tribolium castaneum*	*Minos*	Pavlopoulos *et al.*,2004
	异色瓢虫 *Harmonia axyridis*	*piggyBac*	Kuwayama *et al.*,2006
	赤拟谷盗 *Tribolium castaneum*	*piggyBac*	Lorenzen *et al.*,2007
3*xP*3:ECFP	异色瓢虫 *Harmonia axyridis*	*piggyBac*	Kuwayama *et al.*,2014
3*xP*3:DsRed	异色瓢虫 *Harmonia axyridis*	*piggyBac*	Kuwayama *et al.*,2014

3.1 组成型启动子驱动的 EGFP

遗传修饰昆虫转化载体构建程序中应用强启动子驱动 EGFP 的表达,有利于准确检测单拷贝插入子。组成型启动子在所有细胞中都有活性,所以能够在昆虫发育的所有阶段(包括胚胎、幼虫和成虫)筛选转化体。Handler & Harrell (1999、2001a)成功地应用黑腹果蝇 *polyubiquitin* 启动子驱动 EGFP 的表达,构建了 PUbnlsEGFP 转化标记,在黑腹果蝇和加勒比按实蝇 *Anastrepha suspensa* 整个发育阶段中实现了荧光的表达。该标记载体的 EGFP 被融合到一核定位信号上,荧光蛋白的亚细胞定位利于准确地从非核定位的自发荧光背景中鉴定转化体。这对由位置效应而导致 EGFP 低表达水平的转化体的鉴定尤为重要。

另一种常用的驱动 EGFP 的组成型启动子来自黑腹果蝇 *actin5C* 基因。转化标记 *actin5C*:EGFP 在黑腹果蝇、埃及伊蚊和斯氏按蚊 *Anopheles stephensi* 各发育阶段的表现均很好(Catteruccia *et al.*, 2000; Pinkerton *et al.*, 2000),但只能介导厩螯蝇 *Stomoxys calcitrans* 低水平非均质性的 EGFP 表达(O′Brochta *et al.*, 2000),表明 *actin5C* 启动子可能并非应用于各物种的最佳启动子。鳞翅目的家蚕 *Bombyx mori* (Tamura *et al.*, 2000)和棉红铃虫 *Pectinophora gossypiella* (Peloquin *et al.*, 2000)的第一次系统的胚胎转化,是选用家蚕 *actin BmA*3 作为启动子驱动 EGFP 的表达。虽然通过 EGFP 的表达成功鉴定了这 2 个物种的转化体,但在其胚胎期并未检测到 *BmA*3:EGFP 标记的表达。此外,尽管 *BmA*3 启动子在中肠的活性比较明显(Mange *et al.*, 1997),但很多昆虫食物的自发光现象导致只能检测到转化基因多重插入的个体中强烈表达的 EGFP,因此中肠是转化体难以有效鉴定的组织之一。而其他的荧光标记,如 DsRed 造成生物组织自发光的现象则较少(Handler & Harrell, 2001b)。

3.2 驱动眼睛特异性荧光表达的通用转化标记 3*xP*3-EGFP

多细胞动物的眼睛发育都受到进化保守遗传通路的控制,而这个通路受转录激活因子 *Pax*-6/

Eyeless 的调控(Callaerts *et al.*,1997),*Pax*-6 结合位点 P3 调节光受体特异性基因的表达(Sheng *et al.*,1997)。基于此,Berghammer *et al.*(1999)在单转录因子激活的人工启动子的基础上发展了一个通用转化标记,即将 3 个 P3 位点的串联重复序列置于 TATA 同源物(3*xP*3)的前边,驱动眼睛特异性 EGFP 的强表达(Horn *et al.*,2000)。3*xP*3 与 EGFP 联合,最初在赤拟谷盗和果蝇中应用成功(Berghammer *et al.*,1999)。3*xP*3-EGFP 标记载体主要在赤拟谷盗的眼睛和脑中表达,并且在整个生活周期均能表达 EGFP 和 DsRed(图 1;Lorenzen *et al.*,2007)。Sheng *et al.*(1997)应用人工 3*xP*3 启动子构建的载体也能够介导 EGFP 在其受测昆虫的幼虫、蛹和成虫眼睛中表达,这与 *Pax*-6 常规功能相一致,所以该组织特异性启动子与组成型启动子相似,可用于鉴定转化昆虫的所有发育阶段(Horn *et al.*,2000)。3*xP*3-EGFP 只有 1.3 kb,而较小的转座载体通常能产生更高的转化效率。值得一提的是,3*xP*3-EGFP 标记能够在 G1 代转化昆虫的胚胎发育末期产生可检测到的表达(图 1A),从而实现转化个体的鉴定,省却了将所有实验昆虫饲养至成虫的繁琐工序,该标记对幼虫食量较大或人工饲料成本较高的昆虫具有重要价值。

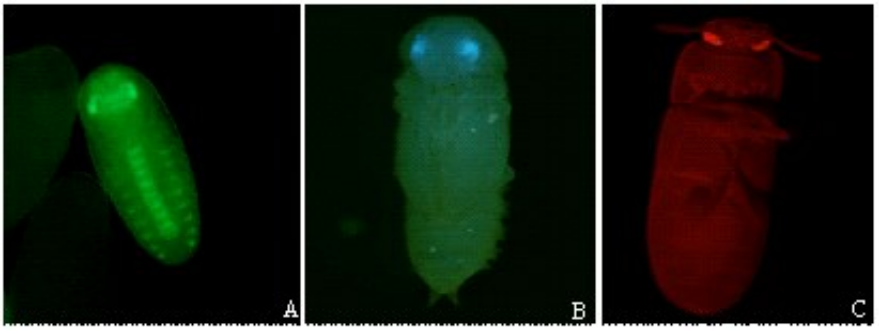

图 1　3*xP*3 驱动 EGFP 和 DsRed 在赤拟谷盗中表达
(由北卡罗莱纳州立大学昆虫学系 Marce Lorenzen 教授提供)

Fig.1　3*xP*3 drive EGFP or DsRed expression in *Tribolium castaneum*
(photo courtesy from Dr. Marce Lorenzen, Entomology Department, North Carolina State University)
A: EGFP 在胚胎中表达; B: EGFP 在蛹中表达; C: DsRed 在成虫中表达。
A: EGFP expression in embryo; B: EGFP expression in pupae; C: DsRed expression in adult.

多细胞动物眼睛发育中 *Pax*-6 的"主调节器"功能,揭示 3*xP*3-EGFP 标记可以应用到所有具有眼睛的动物中。野生型昆虫复眼的小眼通常通过眼睛色素相互隔离,所以只能在朝向观察器的小眼中检测到荧光(图 1C)。这对于鉴定野生型黑腹果蝇、斯氏按蚊(Ito *et al.*,2002)、家蚕(Thomas *et al.*,2002)和赤拟谷盗的转化成虫难度不大(Berghammer *et al.*,1999);但其他物种如家蝇 *Musca domestica* 或埃及伊蚊成虫眼睛的色素会将荧光完全屏蔽或猝灭,从而导致鉴定的失败(Hediger *et al.*,2001;Kokoza *et al.*,2001)。然而,在野生型家蝇和埃及伊蚊的幼虫和蛹阶段,能够检测到 3*xP*3-EGFP 介导的眼睛荧光的表达(Hediger *et al.*,2001;Kokoza *et al.*,2001),表明 3*xP*3-EGFP 的转化标记体系既能用于野生型品系,也能用于突变品系。

荧光标记在视觉系统如眼睛中的表达,使得其在具有很厚或黑化表皮的动物中也能被检测到(图 1B)。荧光标记的选择和转化个体鉴定的最佳发育阶段的确定,很大程度上依赖于昆虫外表皮的形成和黑化以及眼睛发育和色素形成的时间与程度。对于大多数昆虫而言,程序操作和荧光检测的最佳时期可能都是胚胎末期和幼虫期,这限制了 3*xP*3-EGFP 标记在该阶段视觉系统不发达的昆虫中的应用。然而,研究证实 3*xP*3-EGFP 标记能够介导荧光在黑腹果蝇胚胎末期或幼虫期中枢神经系统、部分外周神经系统、肛板和后肠中的表达(Horn *et al.*,2000),在鞘翅目和鳞翅目昆虫中也观察到中枢神经系统中荧光的表达(Thomas *et al.*,2002)。这拓展了 3*xP*3-EGFP 标记在幼虫阶段没有眼睛或视觉系统不发达昆虫中的应用。迄今为止,以 3*xP*3-EGFP 为基础的转化系统已用于 3 个目昆虫转化个体的生产和鉴定,这充分表明人工构建的 3*xP*3-EGFP 标记与转座子联合具有广泛的适用性(Horn & Wimmer,2000;Horn *et al.*,2002)。

3.3 荧光蛋白的毒性

哺乳动物细胞培养试验结果表明,水母 GFP 及其突变体的高水平表达能够造成对细胞的毒性(Hanazono *et al.*,1997),但毒性问题对 GFP 作为昆虫转化标记应用的影响并非特别严重,仅以 *polyubiquitin* 或 *actin5C* 驱动的 EGFP 标记转化埃及伊蚊 RED 品系时表现出了毒性,因此只能建立 EGFP 低表达品系,所有高表达的转化 G1 后代在蛹期全部死亡。该种效应是由高水平表达的 EGFP 造成还是由针对特定品系转化方法中的不同参数造成尚不明确。在黑腹果蝇和野生型埃及伊蚊中,*actin5C*:EGFP 的表达均未对其生育力造成明显不利影响(Pinkerton *et al.*,2000)。同时,3*xP*3-EGFP 标记即使在眼睛和中枢神经系统中高水平表达并产生强烈的荧光,也未发现其对转化昆虫的存活率存在显著性影响(Berghammer *et al.*,1999)。

对于遗传不育释放项目而言,不仅要考虑遗传修饰昆虫的生育能力,而且要考虑释放昆虫与野生型昆虫的竞争力以及转化品系的稳定性。通常,荧光转化标记是否对转化昆虫的寿命、繁殖力、生育力或适合度造成一定的影响,对评估项目的效益具有决定性意义。鉴于 GFP 的潜在毒性,组织特异性启动子驱动的荧光转化标记可能更适于遗传不育释放项目的研究。因为组成型启动子介导的荧光在转化昆虫毒性敏感组织中表达的可能性更大,而组织特异性启动子驱动的荧光在限定空间或组织内表达,可以避免对遗传修饰昆虫关键敏感组织的不利影响。如从海洋珊瑚虫海鳃 *Renilla reniformis* 中克隆的另一绿色荧光蛋白基因(Ward & Cormier,1979)经人为修饰(hrGFP; Stratagene)后,在哺乳动物培养试验中的毒性低于水母 GFP 突变体(Felts *et al.*,2000)。天然珊瑚虫 GFP 作为生物学标记比水母 GFP 具有更大的优势和更广阔的应用前景。在光吸收方面,珊瑚虫 GFP 的消光系数比野生型水母 GFP 高 5 倍,比人源化红移转变的水母蛋白高 2.5 倍。然而,有关 hrGFP 在昆虫转化中的应用还未见报道。

3.4 EBFP、ECFP 和 EYFP 转化标记

在模式生物中,GFP 和 EGFP 通常用作分析增强子或启动子的报告基因,以标记特定的组织或细胞,或作为体内亚细胞蛋白定位的融合标签(Tsien,1998)。非模式昆虫的深入研究也迫切需要 GFP 或 EGFP 的表达载体。然而,这些表达载体与 EGFP 转化标记联合应用可能会产生一些干扰问题,所以报告基因和转化标记的研究仍需发展多样化、可区分的荧光分子。

EBFP 是 GFP 的一个蓝光突变系,其荧光的激发峰和发射峰分别为 383 和 445 nm (Patterson *et al.*,1997)。基于 EBFP 与 EGFP 的光谱差异,足以应用特异性过滤装置清晰地将 EBFP 从 EGFP 中区分出来。然而,EBFP 的量子产率低,光褪色较快,所以当需要鉴定的个体数量很多或照射时间较长时,EBFP 并不适宜用作转化标记。GFP 的另一个更稳定的突变系为青色荧光突变系 ECFP,其激发峰和发射峰分别为 434 和 477 nm (Patterson *et al.*,2001)。该突变品系能够用更无害的蓝光进行激发,且稳定性强,适宜用作转化标记(Horn & Wimmer,2000)。但 ECFP 的光谱不能与 EGFP 完全分开,所以限制了其与带有 GFP 和 EGFP 载体的联合应用。应用特异性的过滤装置能够将 EGFP 从 GFP 的黄色突变品系 EYFP 中完全区分出来,EYFP 的激发峰和发射峰分别为 514 和 527 nm (Cubitt *et al.*,1995)。ECFP 和 EYFP 的量子产率和光褪色时间特性较佳(Patterson *et al.*,2001),可用作独立的遗传修饰昆虫转化标记(Horn & Wimmer,2000)。各种荧光蛋白及突变体的激发峰和发射峰值如表 5 所示,GFP 突变体及 DsRed 表达的荧光如图 2 所示。

表 5 用于遗传修饰昆虫的荧光蛋白特性

Table 5 Properties of the fluorescent proteins used in genetically modified insects

荧光蛋白种类 Species of fluorescent proteins	激发峰 Excitation max. (nm)	发射峰 Emission max. (nm)	参考文献 References
Aequorea victoria GFP	395	509	Cormack *et al.*, 1996; Prasher *et al.*, 1992
EGFP	488	507	Cormack *et al.*, 1996; Yang *et al*, 1996
Renilla reniformis GFP	498	509	Ward & Cormier, 1979
EBFP	383	445	Patterson *et al.*, 1997
ECFP	434	477	Patterson *et al.*, 2001
EYFP	514	527	Cubitt *et al.*, 1995
Discosoma striata DsRed	558	583	Matz *et al.*, 1999
DsRed1	558	583	Handler & Harrell, 2001b
DsRed2	561	587	Handler & Harrell, 2001b

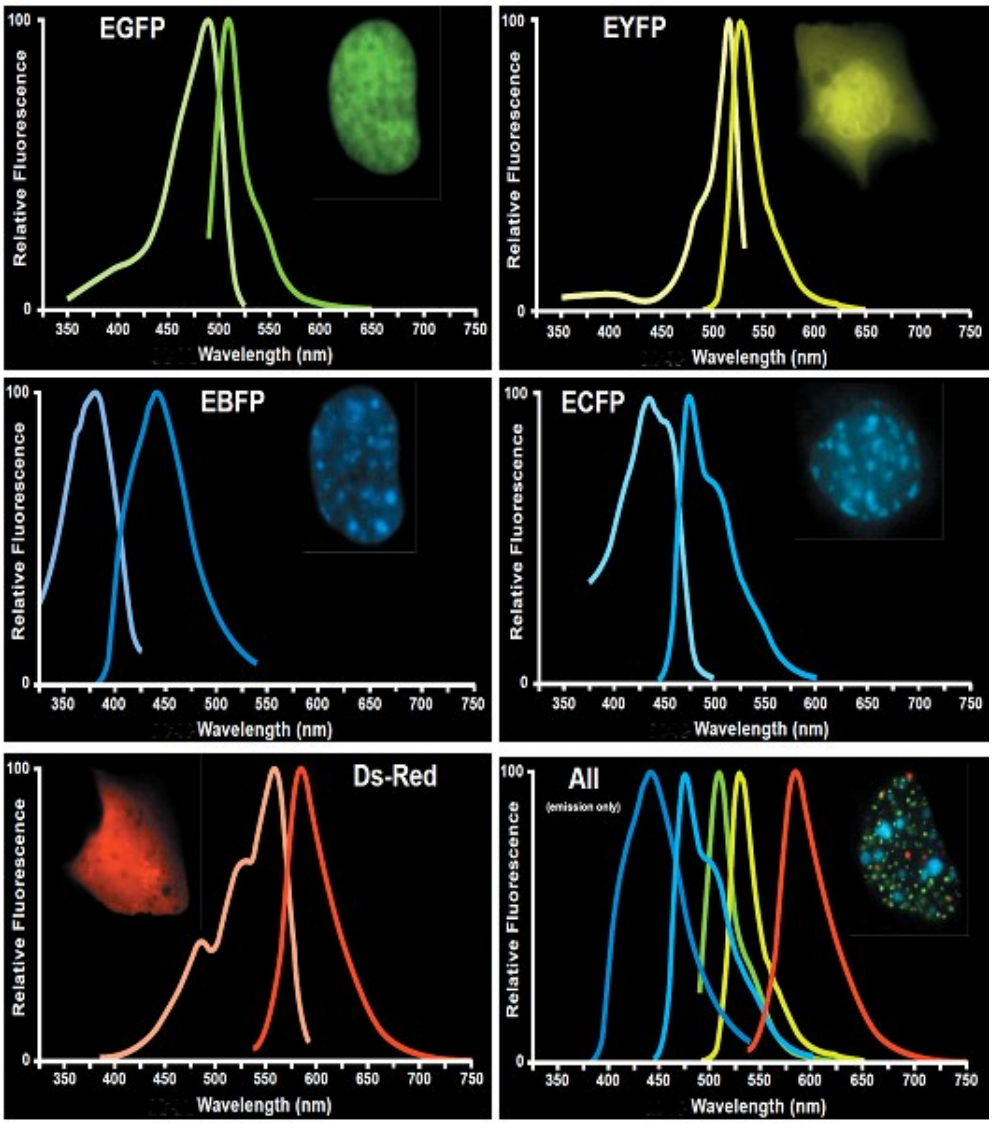

图 2　荧光突变体的荧光颜色及激发峰值(Patterson *et al.*,2001)

Fig.2　Fluorescent color of GFP variants and Ds-Red and their excitation max. (Patterson *et al.*,2001)

3.5　红色荧光 DsRed 转化标记

从珊瑚属海葵 *Discosoma striata* 中分离的红色荧光蛋白 DsRed (drFP583),是另一种可用的荧光标记(Matz *et al.*,1999)。DsRed 与水母 GFP 荧光发色团附近的保守性氨基酸序列具有 23%的相似性(Wall *et al.*, 2000; Yarbrough *et al.*, 2001)。DsRed 的激发峰和发射峰分别为 558 和 583 nm。较高的光褪色抗性、高量子产率以及较长的寿命是其作为转化标记的理想特性。更为重要的是,DsRed 在多数生物组织中表达的荧光都在自发光范围以外,更利于转化体的准确鉴定。但是,DsRed 的成熟时间较长,在遗传修饰转化昆虫的鉴定过程中不能像 EGFP 一样在胚胎发育期就能被检测到(Baird *et al.*,2000; Horn *et al.*,2000)。

人工修饰过的突变系 DsRed1 与 DsRed 具有相似的荧光特性(Matz *et al.*,1999)。Handler & Harrell (2001b) 采用果蝇 *polyubiquitin* 启动子驱动 DsRed1 的表达以鉴定遗传修饰的黑腹果蝇幼虫和成虫,结果显示,PUbDsRed1 介导表达的红色荧光比较明亮,并且与 EGFP 相比,更低数量级的 DsRed1 表达量也能被监测,而较高的信噪比有利于转化体的鉴定。Horn & Wimmer (2000) 利用人工 3*xP*3 眼睛启动子驱动 DsRed1 的表达,检测其在黑腹果蝇中作为转化标记的适用性,结果表明,在成虫白色突变品系和野生型黑腹果蝇的复眼和单眼中均能轻易地检测到强烈表达的红色荧光,且透过轻微黑化的头壳也能在成虫脑中检测到 DsRed1 的表达,而 EGFP 的绿色荧光则被阻断。在澳大利

亚铜绿蝇 *Lucilia cuprina* 双元件系统中,通过杂交双杂合子品系(Double heterozygous line)筛选双纯合子品系(Double homozygous line),由于亲代的雄虫和雌虫分别含有一个拷贝的 ZsGreen 和 DsRed,经过减数分裂后子代可能含有不同荧光蛋白类型和拷贝数(图 3)。ZsGreen 和 DsRed 均由强组成型启动子 *Lchsp*83 驱动,因此双拷贝 *Lchsp*83-DsRed 幼虫即使在白光照下也能被看出 DsRed 的表达(图 3A);在 GFP2 滤镜下 ZsGreen 绿色荧光会受到红色荧光的干扰(图 3B);而 GFP-NB(Narrow broad)滤镜则屏蔽了红色荧光,更容易筛选出双拷贝 *Lchsp*83-ZsGreen 的幼虫(图 3C);再结合 DsRed 滤镜筛选双拷贝 *Lchsp*83-DsRed 的幼虫(图 3D)。

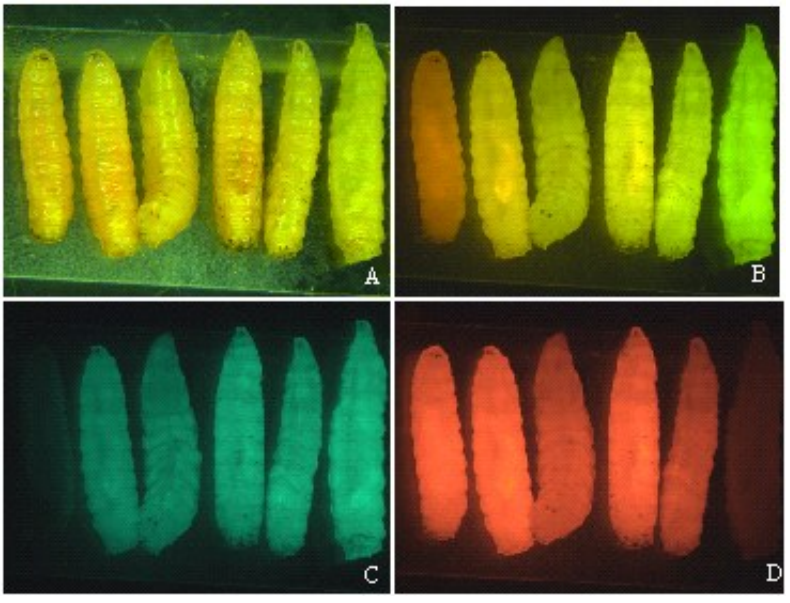

图 3 澳大利亚铜绿蝇 3 龄幼虫的双元件系统荧光图片

Fig.3 Fluorescence images of *Lucilia cuprina* 3rd instar larvae from two components system

驱动载体为 *pBac*-LcHsp83-ZsGreen-TubpA-Lsbnk promoter-tTAopt-SV40;效应载体为 *pBac*-LcHsp83-DsRed-tetO-Lchsp70-Chtra-Lshid [ala2]。幼虫从左到右携带的荧光蛋白类型和拷贝数分别为 2R/0G、2R/1G、1R/1G、2R/2G、1R/2R、0R/2G,其中 R=DsRed,G=ZsGreen,0、1、2 为拷贝数,从左到右第 4 头幼虫为双纯合子品系。照片采用 Leica DFC500 拍摄,采用的滤镜:A.白光;B.GFP2;C.GFP-NB;D.DsRed。

Driver: *pBac*-LcHsp83-ZsGreen-TubpA-Lsbnk promoter-tTAopt-SV40; Effector: *pBac*-LcHsp83-DsRed-tetO-Lchsp70-Chtra-Lshid [ala2]. The fluorescent protein type and copy number carried by 1st to 6th larvae (from left to right) are: 2R/0G, 2R/1G, 1R/1G, 2R/2G, 1R/2R, 0R/2G, here R=DsRed, G=ZsGreen, 0,1,2=copy number, and the 4th larvae is from double homozygous line. The pictures were taken using Leica DFC500 and the filters are: A. White light; B. GFP2; C. GFP-NB; D. DsRed.

另一突变品系 DsRed2 具有与 DsRed1 相似的荧光特性,且可溶性更好,成熟更快,形成多聚物的可能性更低,甚至毒性更低。然而,作为适宜的报告基因,24 h 左右的成熟时间依然较长。DsRed 的另一突变系 E5,也称作“荧光计时器”(Terskikh *et al.*,2000),能够在几小时后检测到荧光信号,成熟之前由最初的绿色荧光变为红光荧光。该标记目前的功能是用作内部荧光时钟的报告基因,可以检测基因表达的时空动态。荧光显示的绿色、黄色(绿色和红色叠加)或红色状态,表明基因的活化和下调表达的情况(Terskikh *et al.*,2000)。绿色—红色荧光计时器,作为报告基因可与 ECFP 联合应用,并作为昆虫遗传修饰研究的可辨认标记,但是目前还没有成功应用的报道。

4 结语

自 1982 年科学家成功转化出首例遗传修饰的果蝇以来,昆虫遗传修饰技术因其潜在的广泛应用前景而成为研究热点。昆虫遗传修饰技术的开发与应用离不开性状优良的标记基因。作为遗传修饰转化载体构建的关键组成部分之一,标记基因对于遗传修饰昆虫转化体的准确鉴定和转化昆虫稳定性的监测具有重要意义,开发可靠性高、稳定性好、应用面广的转化标记基因,对于充分挖掘遗传修饰技术的潜力非常重要。眼睛颜色基因转化标记的多数特征虽然比较理想(Sarkar & Collins,2000),但多数重要的卫生害虫和农业害虫缺少适宜的受体突变品系,从而限制了该标记的应用。尽

管理论上各物种都能产生突变—恢复转化标记,但突变株的获得、相应基因的克隆、突变表型的最终恢复等一系列步骤往往需要耗费大量的时间和人力物力。抗药性基因标记不易获得,且在转化昆虫的鉴定过程中存在诸多准确性和安全性方面的问题。因此,要对更多的昆虫物种进行广泛而深入的遗传修饰研究,就需要开发性能更佳的适宜野生型背景使用的标记系统。

荧光蛋白基因能够在野生型背景转化后代中起作用(Tsien,1998),通过突变方法获得的多种不同荧光性质的突变体,因具有快速、简便、低毒等特点而得以广泛应用,其中应用较多的是 EGFP 和 DsRed 标记基因。组成型和组织特异性的启动子都可用来构建 EGFP 的独立标记系统以驱动 EGFP 的高效表达,但由于天然启动子均来源于特定的物种而具有物种特异性,因此,每个组成型启动子的荧光转化标记只能应用到近缘物种。此外,绿色荧光蛋白的自发光现象也限制了其在某些物种中的应用。红色荧光蛋白 DsRed 造成生物组织自发光的现象则较少(Handler & Harrell,2001b),更利于转化体的准确鉴定;在某些生物中与 GFP 联合应用的表现优于 GFP 突变体,所以应用前景很广泛。DsRed 荧光在生物组织中长达数周的寿命(Matz *et al.*,1999)和光褪色的抗性,也是不育昆虫释放技术在田间应用的理想特性(Peloquin *et al.*,2000),能用于稳定监测野生型种群的扩散和其在野外环境中与其他物种间的水平传播。然而,DsRed 较长的成熟时间限定了转化体鉴定的阶段,阻碍了 DsRed 作为报告基因在短期基因表达研究中的应用(Baird *et al.*,2000; Handler & Harrell,2001b)。鉴于大量不同的 GFP/EGFP 报告基因和融合标签载体都已经可用,针对具体的转化物种,需要根据物种的具体情况选择适合的荧光转化标记,避免假阳性或假阴性现象,或通过更换标记逐一将其解决。目前规避干扰的最好方法就是联合应用以 GFP 为基础的体内报告基因与以 DsRed1 或 DsRed2 为基础的转化标记。即使 EGFP 和 DsRed 在相同的组织中同时表达,应用特异性的过滤装置也能够将其完全区分开,从而进行独立的鉴定和监测。

除了眼睛颜色标记基因、抗药性标记基因和绿色荧光蛋白及上文中提到的突变体外,还有 ZsGreen 等其他的荧光蛋白标记和蛹颜色标记(McCombs & Saul,1995; Wappner *et al.*,1995)。基于水母 GFP 的开发,在其他生物如珊瑚、海葵、水螅、甲壳类动物甚至低等脊索动物中相继发现了 GFP 样蛋白(Wiedenmann *et al.*,2009),荧光光谱覆盖蓝色到远红光,使荧光蛋白的适用范围不断扩大。更多更有效的荧光蛋白和其他标记基因的获得,以及更适宜特定物种的转化系统和检测技术的发展,大大提高了对任何一种昆虫进行遗传修饰改造的可能性。

昆虫遗传修饰技术为基因表达调控、生物大分子相互作用、胚胎发育以及发展生物传感器等研究创造了条件,同时为农林害虫和媒介害虫的防治提供了新的思路。应用遗传修饰手段获得的不育昆虫释放技术是一种可控制甚至根除靶标害虫的环境友好型防控措施。为了保障释放昆虫的最佳防控效果,要求遗传修饰转化昆虫中的转化标记除不影响靶标物种的竞争性和适合度之外,还需要具有良好的遗传稳定性,以便于对其长期监测,达到灵活调控释放不育昆虫与野生昆虫的比例,获取最佳防控效果的目标。然而,遗传修饰昆虫的释放尤其是携带致死基因的昆虫的释放还存在一定的风险,所以在监测释放昆虫环境稳定性的同时,需要监控其在物种间的水平传播,避免对生物多样性、生态环境和人体健康产生潜在的不利影响。

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