Transgenic approaches to study wing color pattern development in Lepidoptera

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The extremely diverse lepidopteran wing patterns make useful models to study the evolution of development and the molecular changes that enable it. Until now, the implication of candidate genes in the differentiation of color patterns has relied primarily on correlational evidence, *i.e.*, gene expression patterns in a developing wing mapping closely to the adult color pattern. The use of transgenic techniques in the Lepidoptera, including the manipulation of gene expression, will finally allow researchers to test hypotheses of gene function at various levels of the patterning hierarchy, from signaling ligands and transcription factors to pigment enzymes. Here we present an overview of transgenic techniques employed in lepidopteran systems and highlight areas where current and future research will provide exciting opportunities to deepen our understanding of the mechanisms of morphological evolution.

Introduction

The striking variety of colors and patterns found on the wings of butterflies and moths presents researchers with unique opportunities to investigate mechanisms of evolution. Selective pressures on wing patterns such as sexual selection,[1] species recognition[2] and mimicry[3–5] have been documented and detailed. Our understanding of these ultimate pressures provides a unique lens through which to examine the evolution of genes and developmental pathways that control these color patterns. Conversely, immunohistolocalization studies of gene expression have provided the majority of the data about the development of wing color patterns. These studies have proposed the co-option of networks of developmental genes[6–8] as well as of enzymes that may be involved in pigment synthesis pathways.[9] In addition, the somatic transformation of epidermal cells of the forewings of *Junonia coenia* showed the direct involvement of the homeotic gene, *Ultrabithorax*, in the modification of hindwing from forewing patterns in this butterfly.[10] By approaching wing color pattern studies from both ecological and molecular vantage points, we hope to bridge the gap between genotype and phenotype and in particular shed light on the interplay between ultimate and proximate factors that lead to the evolution of novelty.

Lepidopteran wing patterns combine several characteristics that make them attractive for this kind of integrative research. The enormous phenotypic diversity present in the 180 000 or so species of described Lepidoptera provides endless opportunities to explore the underlying developmental basis of

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Fig. 1 Examples of various wing color patterns found among the butterflies. (a) *Bicyclus anynana*, (b) *Heliconius hecale*, (c) *Pieris rapae*, (d) an Hairstreak, (e) *Idea sp.*, (f) *Junonia coenia*, (g) *Caligo atreus*, and (h) *Caligo sp.*.(Photo credits to William Piel—a, b, c, d, e, g, h; and Andrew Stoehr—f).

patterns that range from spots and eyespots to bands, to intervenular or vein dependent patterns (Fig. 1a–h). Much of this diversity has already been organized and catalogued in the multiple phylogenies of lepidopteran genera and families[11–15] making it possible to investigate hypotheses of ancestry and novelty, as well as of parallelism and convergence in the patterns. In addition, these patterns develop on a single epithelial cell layer, the wing epidermis, providing a simple morphogenetic system in which to study developmental mechanisms, *e.g.* diffusion and cell signaling. Finally, important genetic resources are now available including detailed gene maps in *Bombyx mori*[16] and several *Heliconius* species,[17,18] EST libraries in *Bombyx*,[19,20] *Heliconius*,[21] and *Bicyclus*,[22] BAC libraries for each of these species and several other butterflies and moths, and the *Bombyx* genome projects.[23,24]

With new genetic resources also come opportunities to study mechanisms such as gene network co-option events (Fig. 2a), to investigate changes in the *cis*-regulatory DNA of candidate genes, as well as changes in the expression of upstream regulators. These are the up-and-coming, exciting areas of research in the evolution of development of wing color pattern.[25] In addition to color patterns, however, lepidopteran wings present the opportunity to study more fundamental developmental patterning processes such as the establishment of the anterior posterior axis of the wing (Fig. 2b) and the differentiation of the venation system. Mechanisms of vein differentiation are fairly well understood in *Drosophila*[26–28] but little is known about them in other insects, most with more complex venation patterns. Because many wing patterns in Lepidoptera are associated with veins, additional knowledge of the molecular underpinning of vein development in this group of insects will inevitably further our understanding of the development of the color patterns.

In order to fully exploit all the molecular resources currently available and to implicate candidate genes in novel functions associated with patterning or coloring a wing, new molecular tools are necessary, many of which hinge on our ability to manipulate the genome of the lepidopteran species in question. Here, we will detail the available methodologies to transform tissues in the Lepidoptera, as well as methods to ectopically express or repress genes. Later, we will discuss some of the future directions for continuing the study of wing pattern development in butterflies.

Germ line transformation

The ability to stably transform the germ line is a powerful tool to elucidate and test gene function. Within the Lepidoptera, only a handful of species have been successfully transformed, including the moths *Bombyx mori* [29] and the Pink Bollworm[30] and the butterflies, *Bicyclus anynana*[31] (Fig. 1a) and *Junonia coenia* (J.M. Marcus, personal communication; Fig. 1f). Additional experiments in the moths *Helicoverpa zea*,[32,33] the tobacco budworm, *Heliothis virescens*,[34] and the potato tuber moth, *Phthorimaea operculella*,[35] have successfully produced transgenic embryos but were not tested for stable germ line transformation. A variety of vectors have been used for germ line transformations including the transposable elements; *piggyBac*,[29–31,34] *Hermes*,[31] *Hobo*[32] and *Minos*,[36] as well as a baculovirus[37] and a combined technique using *piggyBac* and a virus in *Bombyx mori*.[38] These vectors have all provided transformation rates less than or equal to 10% although survivorship of the injected embryos varied widely. The versatility of vectors such as *piggyBac*[39,40] and of universal promoters that when coupled to fluorescent reporter proteins can be used as markers for transgenesis[41] (Fig. 2c) should encourage researchers working in other lepidopteran species to experiment with transgenesis.

Somatic transformation

Ectopic expression or knockdown of genes by RNA interference (RNAi) has successfully been accomplished, in a non-heritable fashion, by somatic cell transformations. The techniques used to produce ectopic expression can be divided between *in vitro* approaches, where tissues are dissected and treated in culture, and *in vivo* approaches. Since *in vitro* techniques do not allow the normal development of tissues, they are perhaps most useful for testing transgene constructs before use in the more time consuming technique of germ line

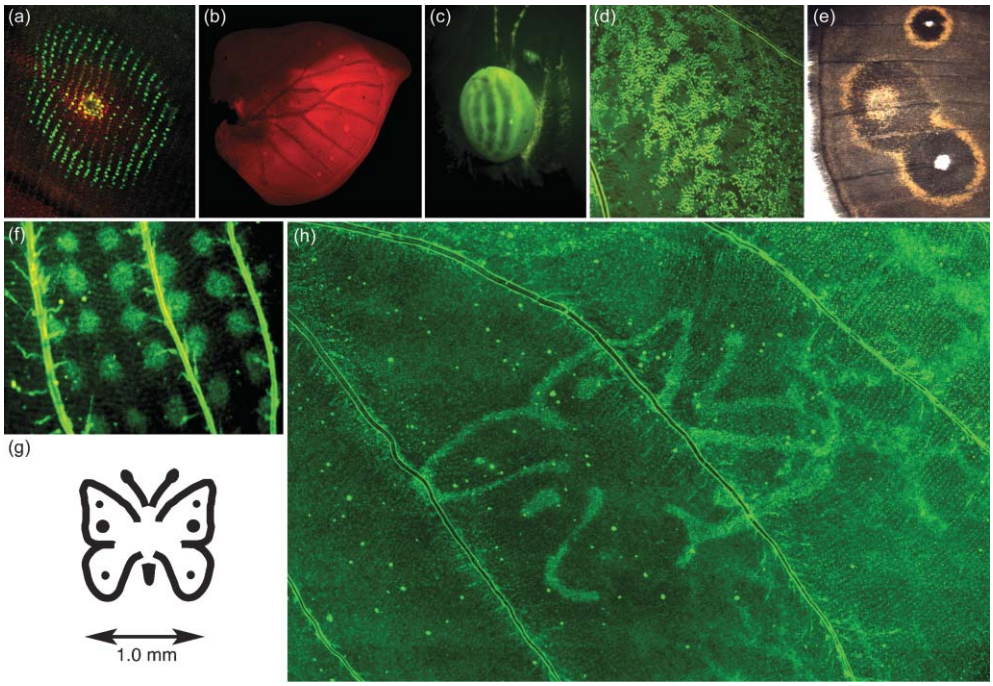

Fig. 2 Gene expression in the wings of butterflies and functional genetic tools. (a) Antibody stainings for Engrailed (green) and Distal-less (red) in an eyespot of *Bicyclus anynana* illustrate genetic co-option in the development of a morphological novelty. (b) Antibody staining for Engrailed in the larval wing of *Bicyclus anynana* suggests a conserved role for *engrailed* in establishing the anterior-posterior axis of the wing. (c) Expression of EGFP in the adult eye of *Bicyclus anynana* using the 3xP3 synthetic promoter. (d) Expression of EGFP in the pupal wing of *Bicyclus anynana* following electroporation of a DNA plasmid, containing the *EGFP* sequence under the control of the *Drosophila Hsp70* promoter, and after a pupal heat-shock. (e) An ectopic eyespot induced by damage to the pupal wing in *Bicyclus anynana*. This phenomenon could be due to the molecular mechanism of wound healing employing the same morphogens involved in signaling from eyespot foci.[8] (f) Fine spatial control of transgene induction in the developing pupal wing is produced when *EGFP*, under the control of the same heat-shock promoter mentioned above, is activated by means of a laser heat shock through a grid stencil. (g) Pattern of micro-stencil used to create expression pattern in the next panel. (h) EGFP expression in a pupal wing of a transgenic *B. anynana* illustrating the complexity of patterns that can be produced with the laser induction method.

transformation. An *in vitro* approach for the biolistic introduction of transgenes (using a gene-gun) has been used successfully in several tissues of *Bombyx mori* including ovarioles,[42,43] embryos, and larval wing discs.[43] *In vitro* electroporation has also been successfully used to transform embryos and larval tissues in a variety of lepidopteran species.[32,33,44,45] In contrast, far fewer experiments have produced *in vivo* transgene expression. Work in our lab has shown that electroporation of a reporter construct can result in EGFP expression in pupal wing epithelial cells of *Bicyclus anynana* (Fig. 2d) but that adult wing patterns can be affected with the process of gene delivery.[46] Also, a combination of sonoporation and lipofection produced transformed tissues in fifth instar larvae of *Bombyx mori*.[47] Several viral vectors have been used to introduce transgenes into larval tissues of *J. coenia*[10,48] leading to a mosaic of transformed adult wing cells and little damage due to the viral injection process. Recently, a somatic transformation vector derived from the *J. coenia* densovirus has been used to transform embryos in multiple lepidopteran species.[49] *In vivo* techniques for studying wing pattern development must be approached carefully, however, since damage to the wing disc epithelium especially during the pupal stage is known to induce ectopic patterns in some lepidopteran species[4,8,50] (Fig. 2e). Nonetheless, *in vivo* somatic gene expression techniques represent an important tool for investigating gene function in the lepidopteran wing.

RNAi has become a widespread tool for investigating gene function, and is especially useful in non-model insect systems[51–53] where other techniques have not been developed. In the Lepidoptera, techniques used to introduce RNAi into somatic tissues include infection of larval tissues with viruses expressing anti-sense RNA,[54] injection of double stranded RNA into all developmental stages[55–60] and in one species, feeding double-stranded RNA (dsRNA) to *Epiphyas postvittana*.[61] A variety of gene targets have been knocked down including a transcription factor[54] and a pigment pathway enzyme.[57] Additionally, RNAi has been successful in lepidopteran species that have not yet been successfully manipulated using other techniques.[55,57,60] These studies suggest that RNAi could be used to explore gene function in wing pattern development in a broader collection of species than is currently being investigated. On the other hand, it is likely that many other experiments have been attempted in the Lepidoptera and not reported due to failure in observing an altered phenotype. The general consensus in the community (as per discussions at the 7th International Workshop on the Molecular Biology and Genetics of the Lepidoptera in 2006) seems to be that results differ from gene to gene, that usually very large quantities of double stranded RNA need to be injected in order for a phenotype to be seen, and that gene knockdown in the early embryo

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may be easier to achieve than in differentiated tissues due to the ease of penetration of double stranded RNA into cells at the syncytial stage.

Control of gene expression

The expression of transgenes has been accomplished in several ways and represents a special challenge for studying wing pattern development. Constitutive viral promoters,[10,35,43,45,48] synthetic promoters, such as *3xP3* that lead to gene expression in the eyes[31,62](Fig. 2c) and promoters such as the *Bombyx A3* actin promoter[29,30,36] have been used to induce transgene expression in a variety of tissues in Lepidoptera. Given, however, that many of the candidate genes identified in wing pattern development have pleiotropic effects that may result in lethality if constitutively expressed,[63] there is a need to control the spatial and temporal expression of the transgenes. Promoters and enhancers that provide this control of gene expression include tissue specific promoters and/or enhancers and inducible promoters. Currently, the number of tissue specific regulatory sequences identified in the Lepidoptera is very limited. Some of these sequences have lead to tissue specific expression of transgenes in the chorion,[42] silkglands[37] and brain[44,64] of *Bombyx mori*, but other tissue specific enhancers, such as wing enhancers, are unknown.

The yeast GAL4/UAS system is widely used in *Drosophila* and vertebrate systems to provide temporal and spatial control of transgenes. A recent study using the GAL4/UAS expression system in *Bombyx* showed that a particular GAL4 line can drive reporter gene expression within the silk glands,[65] yet the current unavailability of wing specific enhancers limits the usefulness of this system for research into wing pattern development. The GAL4/UAS system also requires the creation of large stocks of transformed animals, which is not feasible for many labs. Inducible promoters that give the researcher control of gene expression are, therefore, most promising for wing pattern research, at least in these early days. Within the Lepidoptera, the *Drosophila hsp70* promoter has been most widely used, both in moths[64,66] and in butterflies.[67] The *hsp82* promoter has also displayed inducible gene expression in *P. operculella* embryos while *hsp70* did not show strong expression.[35] In fact, several common promoters used successfully in certain lepidopteran species do not function at similar levels in others including *hsp70*,[35] *3xP3*,[49] and the *Drosophila polyubiquitin* promoter.[49] This underscores some of the difficulties facing researchers as they try to bring transgenic techniques into a new system and suggests that promoter activity should be evaluated in each species. Once a responsive heat-shock inducible promoter is found, however, it is now possible to activate genes in controlled temporal and, importantly, fine spatial scale in the cells of the butterfly pupal wing, using laser mediated heat shocks[67] (Fig. 2f–h). This technique provides the control of transgene expression necessary for investigating wing pattern development while circumventing the need for tissue specific promoters.

Given that many butterflies display phenotypic plasticity, *i.e.*, they modify their patterns of gene expression in response to environmental parameters such as temperature,[68] the activation of transgenes *via* a global or local heat-shock should always be compared to similarly treated wild type animals and non treated controls (both wildtype and transgenic animals) to tease apart the effect of a general plastic response from the ectopic expression of a candidate gene.

Future directions

The future for transgenic approaches to the study of wing pattern development is promising. As mentioned, versatile vectors provide the tools to enable additional lepidopteran species to be transformed. The broadening pool of lepidopterans where functional genetic studies can be performed will allow us to study the evolution of gene function as opposed to solely looking at the evolution of gene expression patterns. Likewise, new genomic resources will provide ever more candidate genes whose roles in wing pattern development can be investigated. Techniques that allow candidate genes to be ectopically expressed on the wing, such as the laser mediated heat shock induction of genes attached to *hsp70* promoters, provide localized ectopic gene expression that will inform our understanding of gene function. These transgenic phenotypes will, therefore, test the sufficiency of candidates in pattern development or pigment pathway control. Additionally, RNAi techniques[69] will allow the testing of candidate gene necessity in wing pattern development. For this application, transgenic RNAi has several advantages over conventional RNAi techniques. Several of the candidate genes implicated in wing color patterning (*e.g. Distal-less* and *engrailed*) have other known roles in wing development. The use of transgenic RNAi combined with ectopic expression systems, such as the laser induction method, may provide the spatial control necessary to tease apart confounding pleiotropic effects of these genes, which conventional RNAi approaches cannot do. Transgenic RNAi will also allow the creation of stable experimental lines that can be used across generations, without the need to manipulate every test individual.

Beyond these tools for testing known candidate genes, transgenic techniques can be used in the future to identify novel candidate genes through gene disruption studies,[70] and novel enhancer regions through the creation of enhancer-trap lines.[71] Additionally, using genomic sequence information from BAC libraries or genome projects, candidate regulatory regions can be identified *via* phylogenetic shadowing[72] or other bioinformatics methods[73] and further tested for function using a reporter construct in a transgenic line. These functional studies will allow investigators to break through the present boundary of what is known in *Drosophila* and identify unique Lepidopteran patterning genes or *cis*-regulatory sequences that underlie the evolution of novel wing patterns. Finally, transgenic techniques may eventually allow researchers to manipulate wing patterns in order to test the ways in which changes in gene expression might interact with more ultimate phenomena such as species recognition, sexual selection and predator/prey interactions.

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