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United States Department of Agriculture — Agricultural Research Service: advances in the molecular genetic analysis of insects and their application to pest management†‡

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Abstract: USDA-ARS scientists have made important contributions to the molecular genetic analysis of agriculturally important insects, and have been in the forefront of using this information for the development of new pest management strategies. Advances have been made in the identification and analysis of genetic systems involved in insect development, reproduction and behavior which enable the identification of new targets for control, as well as the development of highly specific insecticidal products. Other studies have been on the leading edge of developing gene transfer technology to better elucidate these biological processes though functional genomics and to develop new transgenic strains for biological control. Important contributions have also been made to the development and use of molecular markers and methodologies to identify and track insect populations. The use of molecular genetic technology and strategies will become increasingly important to pest management as genomic sequencing information becomes available from important pest insects, their targets and other associated organisms.
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1 INTRODUCTION

The development of molecular biological tools for the analysis of eucaryotic genomes, and the analysis of *Drosophila melanogaster* Meig in particular, has given great hope to the extension of these studies to insects of economic and medical importance. The molecular analysis of *D melanogaster* has been aided in great part by the extensive genetic knowledge and techniques available for this species after a century of research. For other insect species, molecular studies and techniques have provided a short-cut to understanding the genetic basis of all biological processes which have the potential for use in the control of pest insect populations or enhancement of beneficial species.

These advances in the molecular genetic analysis of insects have occurred in three main areas. First is the identification and isolation of genetic elements involved in development, reproduction and behavior, second is the development of gene transfer technology to analyze gene structure–function relationships and to create new strains for biological control, and third is the use of molecular markers and methodologies to identify and track insect populations. Research performed in US Department of Agriculture, Agricultural Research Service (ARS) laboratories has been on the forefront of many of these studies, and is poised to further extend and utilize this information as part of genomics and functional genomics projects. Especially important will be the application of genomic sequence information to identify and isolate target molecules for novel and efficient conventional strategies for the biological control of insect pests, and

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the development of genetically transformed strains for biocontrol of pests and enhancement of beneficial species.

This review will focus on only a few of the contributions by ARS researchers to insect molecular genetics, emphasizing those that are likely to have an important impact on the future of insect pest management. These relate to the molecular identification, analysis and utilization of genes important to insect biochemistry and development, and genes useful for gene transfer vector methodology. Genes and genetic systems involved in development provide important targets for pest management, and gene transfer technology provides sophisticated tools for their analysis, especially in functional genomics, and the eventual utilization of these targets in biological control programs.

2 USE OF INSECT GENES FOR PLANT DEFENSE MECHANISMS

The long-term goal for many of the studies of gene activity in insects is to identify, isolate and manipulate molecules or pathways that can be used as targets for the control of insect populations. For some, control will be manifested by the expression of insect genes, or interfering molecules, in host plants. Some of these genes were originally identified by the biochemical analysis of enzymatic processes important to normal development pathways. One of the most important of these is the chitinase gene, which plays a critical role in chitin degradation in the insect gut during larval molting, making it an important potential target for insect pest management. Research at the Grain Marketing and Production Research Center, USDA-ARS, Manhattan, KS has been on the forefront of these studies using the tobacco hornworm, *Manduca sexta* Joh, as a model.[1] Molecular analysis of chitinase began with the isolation of a cDNA clone,[2] which was subsequently used as a probe to isolate genomic clones.[3] Both studies indicated that a single gene for chitinase existed in the hornworm, but having a somewhat complex organization consisting of at least 11 exons spanning a region of approximately 11 kb.

A novel use of the insect chitinase gene, which holds promise for several methods of pest control, was having it expressed in the host plant of two species, the tobacco hornworm and the tobacco budworm, *Heliothis virescens* F.[4] The hope was that the inappropriate presence of chitinase in plant-feeding insects would be deleterious to their growth or viability. The budworms were the more adversely affected when fed tobacco transformed with the hornworm chitinase alone, but both insect species were significantly affected when transgenic tobacco was also treated with sub-lethal doses of *Bacillus thuringiensis* Berliner (*Bt*) toxin. Improvements of such strategies can be expected with further studies and modifications of gene activity and expression levels in the host plant, use of more highly effective genes, and use in combination with other control systems. Indeed, a recent modification of this strategy has shown that transgenic maize, expressing the chicken glycoprotein, avidin, that binds tightly to the vitamin biotin, are toxic to stored-product insects.[5] These studies establish the important principle that our knowledge of insect biology and biochemistry can be used to create pest-resistant host organisms, and this should prove to be one of the most promising strategies for future pest management.

3 DEVELOPMENTAL MECHANISMS IN BEETLES

Management of beetle species (both harmful and beneficial) continues to be a vital research need for ARS. The order Coleoptera is the largest in the animal kingdom in terms of number of species, and contains many species of great economic significance. There is a relative paucity of knowledge of the genetics and molecular biology of members of this important group. As a result of research conducted by the ARS and others over the past 15 years, we have accumulated a relatively detailed genetic case history for a representative of this order, the red flour beetle, *Tribolium castaneum* Herbst, which has become the only beetle species, thus far, amenable to sophisticated molecular genetic dissection and manipulation.[6–10] As a result of this research, several genes and gene pathways critical for normal beetle development have been identified and characterized. Some of these genes could represent direct targets for pest management, while in other cases, basic biological information derived from study of developmental pathways could indirectly enhance efforts to design novel biotechnology for pest suppression. In time, continued progress in genomic analysis of representatives of the Coleoptera, Lepidoptera, Hymenoptera and possibly other orders, will augment current knowledge of the *Drosophila* and *Anopheles* (dipteran) genomes and provide a diverse set of insect sequences for pest management-related gene mining.

3.1 Hybrid incompatibility and maternal selfish genes

Medea factors are maternally acting, selfish genes that operate by maternal kill of hatchlings, in combination with zygotic rescue from the maternal lethal effect.[11] It is this self-rescuing property of *Medea* factors that accounts for their 'selfish' behavior, and explains why they are predicted to be invasive in populations.[12] Such a larvicidal mechanism is of great interest for potential application to insect pest control, but is unknown in any invertebrate species outside the genus *Tribolium*. Although apparently confined to this genus, *Medea* factors are widespread in natural populations. The original discovery was made in a strain of *T castaneum* from Singapore, but such factors have subsequently been found in wild populations from North and South America, Europe, Africa, Asia and Australia.[13] One

of the *Medea* genes has been positionally cloned, and appears to encode a protein required for normal synaptic transmission (Beeman RW, unpublished). However, the precise mechanism of larvicidal action remains unclear.

Medea-related incompatibility systems involve more than just the *Medea* genes themselves. The self-rescuing properties of *Medea* genes disappear or are overridden in the presence of the hybrid incompatibility factor *H*. The *H* gene was first reported in *T castaneum* strains from India[14] and was subsequently shown to be incompatible with either of the *Medea* genes *M1* or *M4*.[15] The *H* gene by itself has no observable effects on viability, fecundity, fertility or longevity, but beetles that inherit copies of both the *H* gene and *Medea* gene (either *M1* or *M4*) invariably die prior to adult maturation. Before we can be fully equipped to contemplate the full range of potential strategies for pest insect suppression, we need a more detailed understanding of naturally occurring mechanisms such as these that limit insect survival and population growth.

3.2 Genetic regulation of embryonic differentiation

Basic studies on genetic regulation in insects have dual benefits. They lead directly to new knowledge and deeper understanding of insect biology, and they also open new realms of possibility for inventing biologically based solutions to real-world problems, such as the paucity of precision-targeted, environmentally non-intrusive options for natural control of pest insects.

Twenty years ago the sophistication of genetic dissection in *Drosophila* was so far ahead of that for any other invertebrate that it was difficult to document the broader relevance of *Drosophila* by extending studies to other species. Thus, there was a tendency to assume that developmental mechanisms operative in *Drosophila* would be typical or predictive of those in other taxa. Our studies of gene pathways controlling embryonic differentiation in *Tribolium* were the first to reveal, by detailed genetic and molecular analyses, which aspects of embryonic development were conserved. At the same time, these studies hinted at the diversity of insect developmental genetic mechanisms and the degree to which *Drosophila* might be atypical.

The first major discovery to emerge from study of homeotic genes in beetles was the existence of a single homeotic gene complex or HOM-C, in which determining genes for body segments along the entire anterior-to-posterior axis were grouped,[16] rather than being divided into two widely separated clusters as in *Drosophila*. The subsequent discovery of single clusters in animals as evolutionarily distant as mammals revealed that the beetle arrangement was the general rule,[17] and that the separation of the cluster into two in *Drosophila* was probably peculiar to this dipteran lineage.

More detailed comparisons between beetle and fly with respect to two homeotic genes that direct the differentiation of the posterior abdomen revealed that in beetles one of these genes (*Abdominal*) has a predominant role while the other gene (*extra urogomphi*) has a minor role in only the most posterior region of the abdomen.[18] In contrast, the two genes have a more equal division of control of abdominal development in the more highly differentiated *Drosophila*. It would appear that the control domain of the major abdominal HOM-C gene shrank while that of its lesser partner increased correspondingly in compensation in the more highly evolved insect lineages.[19] This evolutionary progression may have been related to the evolution of a long-germ mode of development (more highly predetermined and synchronized) in the higher Diptera, as opposed to the short-germ development of more primitive insects and other arthropods, in which the elaboration of the body segments is more progressive, the posterior-most segments not appearing until the more anterior ones have fully elaborated.

Analyses of other HOM-C genes revealed other types of divergence between beetles and flies. Mutagenesis screens revealed that the *maxillopedia* gene in *Tribolium* mutated easily and frequently to dominant, gain-of-function forms, in which the gene appeared to be expressed at inappropriate times or in tissues where it was normally silent.[20,21] Such mutations were never found in the corresponding *Drosophila* gene. This discrepancy suggests that the two genes might have evolved very different modes of regulation in different insect taxa. Evidence suggests that the *Drosophila* gene is atypical in that its regulatory signals for tissue-specific expression are located within an intron rather than in the 5′ region.[22] Moreover, the *maxillopedia* gene is essential for normal embryonic development in *Tribolium*, but the corresponding *Drosophila* gene seems to have no function whatsoever in *Drosophila* embryos.

As illustrated by these few examples, studies of the molecular genetic regulation of embryonic differentiation in *T castaneum* have led to unexpected new insights into insect developmental mechanisms and provided a glimpse of the nature and extent of the evolutionary diversity between orthologous genes in different insect taxa. Equally important, they have facilitated the development of tools for genetic manipulation and gene discovery that have an impact on unrelated research efforts. For example, balancer chromosomes that were developed to facilitate the manipulation of particular target genes are equally useful for manipulating many other genes. Similarly, mutational screens targeted to particular homeotic or segmentation genes involved in embryonic differentiation have contributed to the construction of high-density, whole genome linkage maps that, in turn, enable us to positionally clone many other *Tribolium* genes, and eventually will reveal more about the degree of synteny and gene conservation within the order Coleoptera.

4 TRANSPOSABLE ELEMENTS FOR GENE-TRANSFER VECTORS

The development of germ-line transformation methodology in *D melanogaster*, based on the *P*-element transposon vector, was a critical turning point in the use of that species for genetic manipulation and analysis.[23] It provided a means of manipulating components of the *Drosophila* genome, as well as those of other organisms, as recombinant DNA to study their structure–function relationships when introduced by transformation into the *Drosophila* genome. When this methodology was first reported by Rubin and Spradling,[24] there was great optimism that it could easily be extended to other insects of agricultural and medical importance for comparative genetic analysis, and also be a means of manipulating insects for control. Indeed, the current relevance of insect genetic transformation is particularly significant owing to increasing genomic sequence information. Transformation methods allow the most incisive functional analysis of this information by phenotypic analysis of integrated genes and by insertional mutagenesis of host genomes. This information can be used directly to isolate and study new biological targets for insect control, such as chitinase genes described previously, or by creation of transgenic strains for improved biocontrol by strategies such as the sterile insect technique (SIT). SIT is one of the most widely used biocontrol methods that relies on the mass release of sterile male insects, rendering mated females in the field non-reproductive, and it is possibly the most significant method for insect biocontrol designed by an ARS scientist.[25] However, while SIT is highly effective, it is also costly and has inherent inefficiencies in terms of sexing and male sterilization, as well as marking to identify released insects. Transformation technology has great potential for creating new strains that are genetically marked and result in female lethality and male sterility. Transgenic strains will also allow new approaches that result in conditional lethality or sterility of released insects and their offspring.

Given the potential importance of insect transformation, several laboratories within and outside of ARS devoted considerable resources to duplicating this methodology, primarily in tephritid flies, mosquitoes and several moth species (see Handler and O'Brochta[26]). None were successful in repeating *P*-mediated gene transfer outside of *Drosophila*, though the numerous variables associated with gene transfer made it quite difficult to determine the limiting factor(s). Considering transposon vector function to be most critical, a simple quantitative assay was developed that could rapidly assess vector mobility in the host insect embryo. This was modified from an *in vitro* excision assay for the *P*-element[27] that measured precise *P* excision from plasmids injected into embryos based upon restoration of expression of a marker gene (β-galactosidase). Tests with this transient embryonic excision assay in several drosophilid and nondrosophilid insects indicated that the *P* vector had no, or very restricted function outside of the Drosophilidae.[28,29] This provided a turning point for laboratories worldwide, who realized that for efficient transformation of their species of interest, either the *P* system would have to be modified or new vectors would have to be discovered and tested.

The excision assay actually provided a method of assessing vector functionality and test modifications that might ameliorate restrictions on mobility. In this respect, factors involved in *P* repression, incomplete mRNA intron-splicing, and *P* transposon activation by γ-irradiation were tested.[29,30] Transformation tests and excision assays in the Caribbean fruit fly, *Anastrepha suspensa* Loew showed that *P* mobility could be enhanced by using a transposase cDNA (having the three introns deleted) and adding *Drosophila* nuclear extracts that could provide an inverted repeat binding protein. Although these modifications did not sufficiently improve the *P* system for routine use in nondrosophilids, the embryonic excision assays and subsequent transposition assays are now a primary method for testing the function of a variety of vectors in insects.

4.1 *hobo* and *hobo*-related transposable elements

With use of the *P* system for general insect transformation proving unlikely, the next system under consideration was the *hobo* transposon that had also been discovered in *D melanogaster*[31] and was being developed for transformation in that species.[32] While it remained uncertain whether *hobo* would be any more effective than *P* in nondrosophilid insects, it was found to be a member of a wide-ranging family of transposons that included *Activator* from maize and *Tam3* from the snapdragon, which suggested that it might have less restricted mobility properties, and related elements might exist in other insect species of interest.[33,34] To assess *hobo* function in tephritid fruit flies and noctuid moths, ARS scientists took the lead in developing *hobo* excision assays that were tested in several species.[35–37] These assays could detect both precise and imprecise excisions, and *hobo* excision was demonstrated in all species. Notably, imprecise excision occurred in the absence of *hobo* transposase, indicating that a cross-mobilizing source of transposase existed in the host species. Transposition assays, which monitored transposon excision and transposition into a target plasmid, also indicated that *hobo* might have at least low level vector function,[37,38] and *hobo* was subsequently used successfully for the first germ-line transformation in *D virilis*,[39] and for the first time in the corn earworm, *Helicoverpa zea* (Boddie).[37] There was also evidence for the first transformation of the Caribbean fruit fly (Handler AM, unpublished), but none of the *hobo* transformations occurred at high frequencies, and only the *Drosophila* transformations were verified as *hobo*-mediated integrations.

Cross-mobilization of *hobo* in excision assays suggested that functionally related elements exist in nondrosophilid dipterans and lepidopterans, and these might provide more robust transformation vectors in their hosts and related species. To identify and isolate these *hobo*-related elements (HRE), PCR-based gene amplification methods were used, with common amino acid motifs from *hobo* and *Ac* as priming sites. Accordingly, HREs were isolated from the moths *H zea* and *H virescens* F,[40] and the tephritid fruit flies *Anastrepha suspensa* Loew, *Bactrocera dorsalis* (Hendel), *B cucurbitae* (Coquillet) and *Ceratitis capitata* Weid.[36] Full-length genomic elements were eventually isolated for HREs from *B dorsalis* (named *hopper*[41]) and *B cucurbitae* (Moser B, Perera OP and Handler AM, unpublished) and these are currently being tested for functionality that would allow their use as vectors. Interestingly, both these elements exist in both bactrocerid species, and BLAST database searches[42] indicate that *hopper* is most closely related to *Ac*-like elements recently discovered in the human genome.[43]

Similar efforts in testing *hobo* and isolating related elements were carried out by O'Brochta (who was trained as an ARS post-doctoral associate) and colleagues,[44] who discovered the *hobo-Ac*-related element, *Hermes,* in the housefly, *Musca domestica* L.[45] *Hermes* has since been found to be a highly effective transformation vector for several nondrosophilid insect species.[46,47]

4.2 The *piggyBac* transposable element

While testing of *hobo* and HREs were ongoing, collaborations were initiated with ARS scientists to test the mobility properties of another transposable element isolated from the cabbage looper, *Trichoplusia ni* Huebn, cell line TN-368.[48] This was the IFP2 element (later re-named *piggyBac*) that had transposed from the *T ni* genome into that of an infecting AcNPV baculovirus. Preliminary embryonic excision and transposition assays in flies and moths[49] suggested that *piggyBac* might have a wide range of vector function, and germ-line transformation tests were initiated in the Mediterranean fruit fly, *C capitata*,[50] which is the primary quarantine pest in the continental USA. Initial transformations in a Medfly *white eye* mutant strain using a *white*$^+$ marked *piggyBac* vector and a *piggyBac*-regulated helper transposase resulted in six transgenic lines at a relatively low transformation frequency of approximately 3–5%. This was the second transposon-mediated transformation of the Medfly, and the first demonstration of a moth vector system functioning in a dipteran species, which was highly encouraging for the broader use of *piggyBac* in other insects. The *piggyBac*/*white*$^+$ system was shortly thereafter used to transform another major tephritid pest, the Oriental fruit fly *B dorsalis*.[51]

The more widespread use of *piggyBac* depended on continued testing in other species, increasing the frequency of transformation, and developing marker systems that would allow screening for transformants in species lacking visible mutations and their cloned wild-type allele (known as mutant-rescue selection). To begin this endeavor, *piggyBac* transformation was attempted in *D melanogaster* using a heat shock-regulated transposase and a green fluorescent protein (GFP) marker system.[52] Transformation frequencies increased from ~3% to 26% using a *hsp70*-transposase helper (phspBac), and a polyubiquitin-regulated enhanced GFP (EGFP) marker gene engineered into the vector pB[PUbnlsEGFP] proved to be much more effective than the *white* marker. Both the phspBac helper and GFP markers are now routinely used for *piggyBac* transformation in numerous insect species spanning three insect orders by laboratories world-wide.[47,53,54] These insects include six dipterans, including three tephritid fruit flies and two mosquitoes, as well as two moth species, and a beetle. Ongoing insect transformation research by ARS scientists includes transformation of the Hessian fly, *Mayetiola destructor* Say (Shukle R, pers comm) and the red flour beetle *T castaneum* (Lorenzen M and Beeman RW, unpublished).

4.3 Markers for transformant selection and systems for detection of released insects

The pB[PUBnlsEGFP] vector was subsequently used for transformation of *A suspensa*, which was important since no visible mutations exist for this species.[55] Transformants were quickly selected, and a promising observation was that GFP expression was most intense from the thoracic flight muscle, and could be detected nearly four weeks after death. This suggested that GFP could be an effective genetic marker for released insects. Unambiguous marking of insects used in release programs, such as SIT, is a significant problem, since identification is necessary to assess program effectiveness to ensure pest-free zones and for risk assessment of released transgenic insects. Current use of fluorescent powder is less than ideal owing to a loss of powder after grooming, transfer during mating, and health concerns for workers.[56] A major effort is currently underway to test GFP-marked strains in pink bollworm and the Mediterranean fruit fly for SIT release programs. Experimental release of pink bollworms transformed with a *piggyBac*/EGFP vector[57] has been approved by USDA-APHIS-PPQ and initial studies have begun. For Medfly, numerous *piggyBac*/EGFP strains already exist, but release studies await integrating the vector into the VIENNA-7 temperature sensitive lethal (tsl) genetic-sexing strain currently being used for SIT programs (Handler AM, Franz G and Robinson AS, unpublished).

Additional marker systems are also a high priority to ensure insect identification, to distinguish insects from separate releases, and for a variety of basic gene expression studies necessary for functional genomics studies. Several mutant variants of the original GFP are available, although overlapping emission spectra can cause some confusion in distinguishing their

expression. The fluorescent protein most distinct from GFP is a red fluorescent protein isolated from the coral, *Discosoma striata*, known as DsRed.[58] To test DsRed for transformant selection and as a potential marker for released flies, the vector pB[PUb-DsRed1] was created and transformed into *Drosophila* and *A suspensa*.[59] DsRed fluorescence was significantly more intense than GFP, and could be detected in *A suspensa* up to 60 days after death. An additional benefit to both GFP and DsRed markers is that they are completely foreign genes to insects, and any ambiguity in visible detection could be resolved by molecular tests for the transgene.

5 PROSPECTS FOR THE USE OF GENOMICS AND PROTEOMICS FOR PEST MANAGEMENT

As we have described, ARS has been in the forefront of molecular genetic studies in agricultural insects that have provided information important to pest management.[60] Studies on host-plant resistance will provide some of the most promising tools for directly and specifically safeguarding economically important plants, and these strategies will certainly be extended to animals and beneficial insects. New and highly specific targets for host resistance will be identified and isolated by pioneering research with the red flour beetle, *T castaneum*. Beyond *Drosophila*, the genetic basis for primary developmental decisions is best understood in this insect, which now serves as a general model for other insect species. Much of this research, however, has depended upon conventional methods of analyzing and utilizing specific genetic elements or systems through straightforward biochemical and genetic approaches. It is now important to recognize that further advances in understanding insect genetics and its applications will occur at an extremely fast pace as information is provided by genomic sequencing, proteomics and bioinformatics. Advances in pest management will not only rely on genomics projects on insects, but projects on their plant and animal host organisms, associated micro-organisms, predators and parasitoids will be of equal importance. Genes and gene products that were unknown or whose isolation was intractable will now be discovered by database searches and rapid screening of DNA microchip arrays. This will afford an enormous amount of genetic material and information that will reveal both target molecules and pathways, and thus development of new control agents for pest management. Central to the understanding and use of this information and reagents will be genetic transformation technology in insects and plants, and ARS has been a leader in developing these methods.

One of the more promising scenarios for the use of genomics and bioinformatics in pest management is based on the recent isolation of odorant receptors in *Drosophila*, achieved by developing database search algorithms for the predicted receptor protein structure.[61] Use of the *Drosophila* sequences and search tools rapidly resulted in selecting similar receptor sequences from the *Anopheles gambiae* Giles sequencing project,[62] and clearly this can be extended to all species having the relevant sequence information available. A critical component of these studies, however, was the functional identification of these genes, which could only be achieved by studying their expression patterns and activity in genetically transformed insects. These receptors, and those involved in mate-finding, will eventually be used to screen for highly specific antagonistic and agonistic binding molecules that can disrupt or enhance foraging or reproduction in conventional control programs. Alternatively, beneficial insects, including parasitoids, predators and sterile males used in release programs, may be genetically transformed with modified receptor genes that enhance these processes. Similar strategies may be used for a variety of receptors that respond to insect hormones during development and reproduction.

In addition to these prospective uses of genomic information, strategies are already being modeled and tested in *Drosophila* for the use of transgenic insects for biological control.[54] Some of these will improve existing programs such as SIT by creating strains that allow genetic-sexing (due to female lethality) or male sterility in response to temperature change or chemical treatment. Other strategies will result in direct control by creating transgenic strains that die along with their offspring in response to changes in diet or environmental conditions. Risk assessment issues for the ecological safety of such programs must be addressed, and is also a high research priority for ARS.

These are only a few examples of how genomics and associated fields will revolutionize our ability to control insect populations and behavior. Just as human genomics is on the vanguard of understanding human disease and drug discovery, insect genomics will be able to follow the same paradigm for pest management. ARS geneticists working on the most important pest and beneficial insect systems are already making a significant contribution to this effort, and it is expected that their involvement in discovery and application of genomics information will continue at the forefront of this endeavor.

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