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Molecular Genetics of Mosquito Resistance to Malaria Parasites

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Abstract Malaria parasites are transmitted by the bite of an infected mosquito, but even efficient vector species possess multiple mechanisms that together destroy most of the parasites present in an infection. Variation between individual mosquitoes has allowed genetic analysis and mapping of loci controlling several resistance traits, and the underlying mechanisms of mosquito response to infection are being described using genomic tools such as transcriptional and proteomic analysis. Malaria infection imposes fitness costs on the vector, but various forms of resistance inflict their own costs, likely leading to an evolutionary tradeoff between infection and resistance. *Plasmodium* development can be successfully completed only in compatible mosquito–parasite species combinations, and resistance also appears to have parasite specificity.

Studies of *Drosophila*, where genetic variation in immunocompetence is pervasive in wild populations, offer a comparative context for understanding coevolution of the mosquito–malaria relationship. More broadly, plants also possess systems of pathogen resistance with features that are structurally conserved in animal innate immunity, including insects, and genomic datasets now permit useful comparisons of resistance models even between such diverse organisms.

Abbreviations

EST	Expressed sequence tag
NBS-LRR	Nucleotide binding site and leucine-rich repeat-containing protein
TLR	Toll-like receptor
ROS	Reactive oxygen species
SA	Salicylic acid

1 Introduction

The genus *Plasmodium* has more than 100 species which infect birds, reptiles and diverse mammals including humans. Different *Plasmodium* species utilize different mosquito genera for transmission, and a reptile *Plasmodium* is even transmitted by a sandfly. The vectors of human malaria parasites, however, comprise relatively few species of the *Anopheles* genus. Despite sometimes daily contact with malaria parasites in human bloodmeals, other mosquito species in malaria endemic areas never sustain infection to serve as vectors of human disease. Within the few permissive species in nature, only a proportion of females are actually involved in transmission, and even in permissive individuals, only a small proportion of parasites survive to complete successful development. Thus, malaria transmission in nature passes through a specific and narrow conduit that limits parasite numbers in many ways. Certainly the efficiency of the system should not be underestimated, because the transmission rate is sufficient to maintain malaria as one of the major public health problems of the world. However, it is reasonable to ask just how robust and stable the malaria transmission system really is, and whether there are unexploited weaknesses that could be manipulated to reduce malaria transmission below the level of population maintenance (Vernick and Waters 2004). With this goal in mind, a body of knowledge has been generated describing the molecular basis of the vector–parasite interaction and genetic variation for suppression of parasite development within permissive mosquito species.

2
Sporogonic Development

Female mosquitoes first take a bloodmeal several days after adult emergence, and continue to feed every few days thereafter. If a bloodmeal is taken from an animal carrying a compatible species of *Plasmodium*, the sporogonic stage of the malaria life cycle is initiated. But what does 'compatible' mean in this context? As with any host–pathogen interaction, compatibility requires that the parasite both finds the specific resources, such as cellular receptors, nutrients, and developmental signals, required to reproduce itself, and develops even in the presence of host immune defenses.

The human host, the vector host, and the parasite have competing evolutionary agendas, with the host seeking to eliminate infection and the parasite striving for efficient transmission. Infectious disease is generally characterized by partial host immunity, with some, but not all, individuals in a population infected at any given time. This model appears to hold for the system at hand, because mosquitoes possess mechanisms that limit, but generally do not completely prevent, parasite development (Luckhart et al. 1998; Han et al. 2000). Thus, only a small minority of malaria parasites that enter the mosquito develop completely (Gouagna et al. 1998; Vaughan et al. 1994). These baseline mechanisms already defeat most malaria parasites, so increasing their efficiencies or introducing other additive or synergistic mechanisms can result in complete resistance (Collins et al. 1986; Osta et al. 2004; Vernick et al. 1995).

This review concentrates on the second point above: vector host defenses that can suppress successful parasite development, genetic variation in those defenses, and potential molecular mechanisms underlying those defenses. Successful development requires that the virulence machinery of the parasite be a good molecular fit for the vector, allowing the parasite to acquire resources and ultimately reproduce. However, a compatible interaction does not mean a harmless one, and there is evidence that malaria parasites decrease vector reproductive fitness (discussed below). This fitness cost to the mosquito probably in part drives the mosquito to mount an active immune response. Efficient resistance to a pathogen can also be passive, and it is likely that genetic variation of critical mosquito molecules can yield parasite-resistant vector phenotypes by making the vector functionally invisible to the parasite at key developmental junctures, analogous to human genotypes with mutant CCR5 receptor for HIV invasion (Dean et al. 1996; Huang et al. 1996), or lacking the Duffy receptor for malaria merozoite invasion of erythrocytes (Miller et al. 1976; Zimmerman et al. 1999).

The sexual phase of the malaria life cycle is initiated when a proportion of parasites replicating mitotically in vertebrate host erythrocytes make a developmental switch to a terminally differentiated nonreplicating sexual form

called the gametocyte (Fig. 1). Gametocytes, the only parasite stage infective for mosquitoes, are ingested by the mosquito with the bloodmeal. Within minutes in the mosquito midgut, gametocytes generate gametes that undergo fertilization to produce zygotes. Over the next 24 h, each zygote transforms into a nondividing motile form called the ookinete. This form exits the gut lumen and invades cells of the midgut epithelium by an uncharacterized mechanism. The ookinete traverses the epithelial cell, exits through the basolateral membrane and lodges between the plasma membrane and basal lamina where within hours it transforms to a rounded oocyst. Over the next 10–20 days, depending on the parasite species, about 5,000 sporozoites form within the oocyst. At the end of this period of latency, the mature oocyst releases the sporozoites into the mosquito hemocoel, and a small proportion will survive to invade the salivary glands. In subsequent bloodmeals, the mosquito injects sporozoites along with saliva into a new vertebrate host to establish infection and complete the transmission cycle.

The merits of the parasite and mosquito species and strain combinations commonly used as experimental systems have been reviewed (Sinden 1997; Vernick 1998). It is worth noting that these are mostly laboratory models that do not represent natural vector–parasite combinations, and thus much of what we see in the laboratory has not been confirmed in the natural transmis-

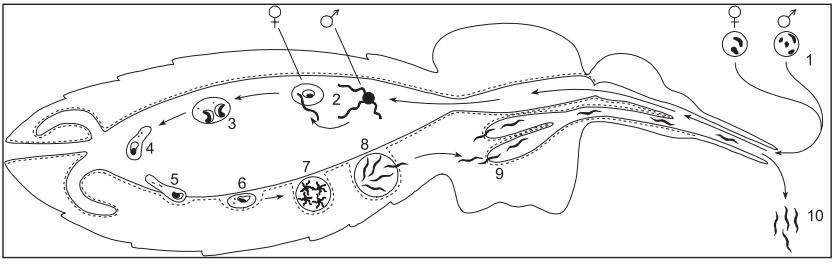

Fig. 1 Malaria parasite development in the mosquito vector. *1,* Mature gametocytes, the infective stage for the vector, are ingested in the bloodmeal. *2,* Within minutes, gametocytes produce gametes that undergo fertilization. *3,* Zygotes. *4,* Over approximately 24 h, zygotes transform into the motile ookinete stage. *5,* Ookinetes enter midgut epithelial cells from the midgut lumen and migrate to the basolateral membrane, where they exit the midgut cell. *6,* Ookinetes lodge in a position outside the plasma membrane of the epithelial cell but underneath the basal lamina (*dotted line*), where they transform into oocysts. *7,* Oocysts grow in the same location over the next 10–20 days and begin a process of differentiation to produce sporozoites. *8,* Mature oocysts rupture to release sporozoites into the hemocoel. *9,* Sporozoites invade the salivary glands, where they can remain infective for the life of the mosquito. *10,* Sporozoites are introduced along with saliva during a bloodmeal upon a vertebrate host, where they can establish a new infection

sion system. In fact, immune responses measured in non-natural laboratory models are known to differ from the natural species combinations (Tahar et al. 2002).

3 Genetically Selected Systems of Malaria Resistance

Melanotic Encapsulation in the Hemocoel A genetic line of *An. gambiae* has been artificially selected against the simian parasite, *P. cynomolgi* (Collins et al. 1986), with resistance manifested as melanotic encapsulation of otherwise ultrastructurally normal parasites after they exit from the midgut cell into the space between the basolateral plasma membrane and the midgut basal lamina, beginning 16 h post bloodmeal (Collins et al. 1986; Paskewitz et al. 1988). Hemocytes did not appear to be directly involved in the encapsulation process (Paskewitz et al. 1988). However, insect midgut basal lamina is probably nonselectively permeable to molecules below a threshold size (Reddy and Locke 1990), so there is likely passive diffusion of melanization substrates and enzymes into the space occupied by extracellular ookinetes. This view is supported by the observation that capsules were thickest on the side of the parasite facing the hemocoel (Paskewitz et al. 1988). Only after the capsule completely surrounded the ookinete did the parasite degenerate ultrastructurally.

A model for in vivo encapsulation was developed using charged Sephadex beads. Negatively charged beads were encapsulated much more efficiently by the resistant *An. gambiae* line than by susceptible mosquitoes (Paskewitz and Riehle 1994), whereas positively charged or neutral beads were encapsulated with equally high efficiency in both genetic lines. The efficiency of bead melanization decreased with mosquito age in both genetic lines, but under the appropriate conditions the bead melanization assay allowed 80%–90% of female mosquitoes tested to be phenotypically assigned to the correct genetic strain (Chun et al. 1995). The efficiency of bead melanization was enhanced by bloodmeal in resistant, but not in susceptible, mosquitoes.

The bead assay established that the components producing the divergent encapsulation responses of resistant and susceptible mosquitoes were present in the hemocoel rather than being a feature of midgut epithelial cells. The inverse correlation between efficiency of bead melanization and mosquito age suggested that the encapsulation phenotype requires a factor that can be present in limiting amounts. This is consistent with the observation that melanization in mosquitoes of the resistant line was more efficient against low parasite numbers, also suggesting the involvement of a finite component that could be titrated by parasite number (Vernick et al. 1989).

New World and Asian strains of *P. falciparum* were efficiently encapsulated by the resistant mosquito line, but *P. falciparum* strains of African origin were not (Collins et al. 1986). Similarly, the African species *P. ovale* and *P. malariae* (along with close simian relative *P. brasilianum*) also failed to be efficiently encapsulated. The geographic range of *An. gambiae* is limited to sub-Saharan Africa. Thus, one interpretation is that parasites sympatric with *An. gambiae* have evolved local adaptations to evade recognition or effector functions of the encapsulation response. However, because these observations were made using cultured parasite strains and laboratory mosquito colonies, the genetic fidelity of either to their original natural populations could be questioned. Encapsulation of *P. falciparum* has been observed in wild *An. gambiae* in Africa (K. D. Vernick, unpublished results; Schwartz and Koella 2002), and thus it is a natural phenotype even in the sympatric combination, although at low frequency. Finally, a line of *An. dirus* was selected in which resistance to the rodent malaria parasite, *P. yoelii*, was manifested as melanotic encapsulation controlled by a polygenic mechanism (Somboon et al. 1999).

Intracellular Ookinete Lysis in the Midgut Epithelial Cell Genetic lines of *An. gambiae* were selected to be resistant and susceptible to the avian parasite, *P. gallinaceum* (Vernick et al. 1995). Ultrastructurally, the resistant phenotype was manifested as the degeneration of ookinete cellular organelles and lysis of the parasite quickly following ookinete invasion of midgut epithelial cells. The initial number of ookinetes invading the midgut epithelium was similar in both genetic lines, suggesting that parasite killing resulted from an intracellular mechanism rather than an interaction within the bloodmeal in the midgut lumen. A genetic crossing experiment suggested that the resistance mechanism was controlled by a single main locus with dominant effect.

Whether the resistance mechanism acted against other species of *Plasmodium* was not determined, although there is no reason to believe that the response would be generalizable to human malarias as the lytic response to *P. gallinaceum* essentially represents the wild-type response of *An. gambiae* to this parasite. However, it would be interesting to know whether the lytic killing mechanism can be addressed against ookinetes of other *Plasmodium* species if they are introduced in the same bloodmeal with *P. gallinaceum*. Such an experiment would distinguish whether compatible parasite species avoid being killed by intracellular lysis because they evade recognition and induction of lysis or because they are resistant to the lytic response.

Several features appear to distinguish the lytic mechanism of parasite killing from the melanotic encapsulation response described above. Ookinetes killed by lysis did not become melanized but rather suffered rapid degeneration of cellular organelles and apparent necrosis. Encapsulated parasites appeared ultrastructurally normal until the melanotic capsule was complete,

after which time they began to degenerate. Lytic killing was intracellular, with ookinetes rarely reaching the basolateral cell membrane, while encapsulation occurred after apparently healthy parasites had exited the basolateral boundary of the epithelial cell into the extracellular lymph compartment. To determine conclusively whether a relationship exists, however, it will be necessary to understand the underlying biochemistry of both systems.

Lines of *An. atroparvus* resistant and susceptible to *P. berghei* were selected in which oocyst number in resistant midguts was close to zero, and in which the infection phenotype was under polygenic control (van der Kaay and Boorsma 1977). Initial ookinete invasion of the midgut epithelium was similar between the selected lines (Sluiters et al. 1986). The subsequent failure of ookinetes to develop in the resistant line was ascribed to ookinete 'degeneration', and was not described further. Another genetic system selected in *An. gambiae* for resistance to *P. berghei* similarly was said to result from 'degenerated' sporogonic stage parasites (Al-Mashhadani et al. 1980; Al-Mashhadani and Davisdson 1976). It is not known if these two resistant systems are related in any way to the lytic response of *An. gambiae* against *P. gallinaceum*. Lines of *An. stephensi* with decreased *P. falciparum* oocyst numbers were produced by genetic selection (Feldmann et al. 1990; Feldmann and Ponnudurai 1989). The mechanism was unknown, but was based on polygenic control with influence from a possible cytoplasmic factor (Feldmann et al. 1998).

Other Resistance Mechanisms Mosquito resistance to strains or species of malaria parasite, whether active or passive, serves as part of the ecological isolating mechanisms that define parasite niche boundaries. One example is the natural resistance of *An. gambiae* to *P. gallinaceum* described above. In that case, segregating genetic variation allowed the selection of pure susceptible and resistant lines that facilitated study of the mechanism. Another example is the natural resistance of *Culex pipiens* to infection with *P. gallinaceum*, in which parasites (in the form of gametocytes) inoculated into the hemocoel of *C. pipiens* developed ectopically until the 3-day-old oocyst stage but then degenerated and died (Weathersby and McCall 1968). Parasites inoculated into the susceptible species, *Aedes aegypti*, underwent normal (albeit ectopic) development, including sporozoite invasion of salivary glands. When *Ae. aegypti* were given an infective bloodmeal and then parabiotically joined to *C. pipiens* by a capillary to create a common hemolymph, only about 2% of the joined *Ae. aegypti* supported parasite development as compared to about 95% of unjoined *Ae. aegypti* controls, suggesting that the resistance of *C. pipiens* was caused by an diffusible toxic humoral factor (Weathersby and McCroddan 1982). Vector–parasite compatibility or its absence may be enforced at multiple steps during parasite development (Alavi et al. 2003).

An important example of resistance is the apparent failure of culicine mosquitoes to serve as vectors of mammalian malaria parasites despite frequent exposure to infective bloodmeals in natural transmission zones. Among the few publications on the topic, it was reported that a proportion of laboratory reared *C. bitaeniorhynchus* mosquitoes that fed upon infected human volunteers were susceptible to *P. falciparum*, *P. vivax* and *P. malariae* to the sporozoite stage (Williamson and Zain 1937a, 1937b). Another report described infection of the culicine mosquito *Mansonia uniformis* with *P. falciparum* by experimental feeding on an infected human volunteer (Cheong et al. 1963). In no reported case, however, did *P. falciparum* infected culcicines become infective to vertebrates, although the barriers to transmission are currently unknown.

The genetic and molecular basis of barriers to malaria infection and transmission in different mosquito species remains largely unexplored. This subject could now be profitably examined with new genomic tools, and in this regard the *Ae. aegypti* genome sequence will be a useful complement to the *An. gambiae* sequence. One does not have to look far (at least from humans) to find a recent example of pathogen host range restriction. Chimpanzees can be infected with HIV, but unlike humans rarely progress to AIDS-like disease (Balla-Jhagjhoorsingh et al. 2003; Davis et al. 1998; Novembre et al. 1997). It was proposed that greatly reduced variation observed in the MHC class I gene of chimpanzees is the product of a selective sweep caused by a widespread ancient infection by an HIV relative, and that modern populations of chimpanzees are descended from survivors of that pandemic (de Groot et al. 2002). Other primates avoid disease by blocking HIV replication early after cell invasion, by action of the cellular factor, *TRIM5*α (Stremlau et al. 2004).

In mosquitoes, the species barriers to malaria could fall into a number of categories. They could be physiological, such as a thicker peritrophic matrix; cellular, if host molecules that the parasite needs to bind such as cell surface invasion receptors, developmental signaling ligands or nutritive factors are too diverged; immune, such as recognition or effector molecules that the parasite has not adapted to resist or evade. There may be important differences in gene regulatory pathways that affect any of the above. Remarkably, considering that some nonvector mosquito taxa are closely related to vector taxa, none of these possibilities seems insurmountable for the parasite, so in addition to asking how the barriers work, it is also worth asking how they have persisted as functional barriers.

4
Genetic Mapping of Resistance Loci

To date, genetic analysis of malaria resistance phenotypes has used two experimental approaches. The first approach mapped resistance in inbred laboratory lines of *An. gambiae* infected with simian or rodent malaria or charged beads. The second strategy mapped infection intensity in pedigrees of wild-caught *An. gambiae* infected with its natural parasite *P. falciparum*.

Resistance in Laboratory Strains Resistant and susceptible strains of *An. gambiae* selected in the laboratory to encapsulate or permit development of simian malaria parasites were intercrossed for genetic mapping by linkage analysis using microsatellite markers. Mapping in five backcross families challenged with *P. cynomolgi* B malaria parasites identified one major and two minor quantitative trait loci (QTLs) associated with melanotic encapsulation of the parasite. Examination of these backcross progeny suggested a dominant effect of resistance alleles with a single locus as the major genetic determinant of encapsulation. The major QTL, *Pen1*, is located on chromosome arm 2R and accounted for approximately 54% of the variability in encapsulation response (Zheng et al. 1997). A similar study used the same inbred mosquito lines to map encapsulation of negatively charged Sephadex beads, identifying a QTL that mapped to the same region as the *Pen1* locus. In addition, encapsulation of beads and rodent malaria parasites (*P. berghei*) were shown to have similar modes of inheritance: dominant, autosomal, and controlled by a single major gene (Gorman et al. 1997). Finer scale mapping of the *Pen1* locus has identified 48 putative genes (Thomasova et al. 2002). Genetic analysis of *Pen1* remains incomplete at present.

Of the two minor QTLs, *Pen2*, was located on chromosome arm 3L and explained approximately 13% of the variation in encapsulation. The third QTL, *Pen3*, was linked to *Pen1* on chromosome 2 and affected encapsulation of *P. cynomolgi* B only slightly. Attempts to map another immune related trait, infection intensity (that is, sum total of viable plus encapsulated oocysts) in this laboratory system suggested there was no simple genetic component of inheritance, and no overlap with encapsulation QTLs (Zheng et al. 1997).

Recently, efforts to map the encapsulation trait in the same resistant and susceptible strains of *An. gambiae* challenged with a different simian parasite, *P. cynomolgi* Ceylon (it is unclear whether *P. cynomolgi* B and Ceylon differ as strains or species), suggested that different QTLs are involved in encapsulation of different parasites and that resistance to this parasite is incompletely recessive (Zheng et al. 2003). Three QTLs, *Pcen2R*, *Pcen3R* and *Pcen3L*, were identified for resistance to *P. cynomolgi* Ceylon and explained 13%, 16% and 26% of the variation in encapsulation, respectively. Two of the QTLs (*Pcen2R*

and *Pcen3L*) were indistinguishable from *Pen3* and *Pen2*, the minor QTLs identified in *P. cynomolgi* B encapsulation. However, *Pcen3R* was a novel QTL not involved in resistance to *P. cynomolgi* B and *Pen1*, the major *P. cynomolgi* B locus did not contribute to the encapsulation of *P. cynomolgi* Ceylon. These results were generally consistent with previous genetic studies that also found distinct genetic mechanisms underlying encapsulation of these two parasites (Vernick and Collins 1989; Vernick et al. 1989).

Resistance in Natural Populations Inheritance of resistance to *P. falciparum* was examined in natural populations of *An. gambiae* in Mali, west Africa (Niare et al. 2002). In an initial study, eggs were collected from single wild-caught female mosquitoes, and the resulting families were raised under environmentally controlled conditions. Three such random families were fed the same infected human blood and the difference in oocyst number among families was measured. There were significantly different infection distributions among families fed on the same blood, which demonstrated that there were frequent alleles segregating in nature with an effect on susceptibility to parasite infection. Consequently, a mapping experiment was performed.

Genetic mapping of infection intensity (total oocyst number) in two wild pedigrees of *An. gambiae* from Mali infected with natural *P. falciparum* identified a major resistance locus in one family and a minor locus in another family. The major effect QTL (*Pfin1*), located on chromosome arm 2L, explained almost 90% of the parasite-free mosquitoes in the segregating pedigree, and was semidominant. Mosquitoes that were resistant homozygotes (by microsatellite marker) at *Pfin1* had mean 0.17 oocysts per mosquito, while susceptible homozygotes had 50.6 oocysts, indicating that natural resistance mechanisms can be quite efficient. The second QTL, *Pfin2*, was located on chromosome arm 2R near *Pen1* and showed recessive inheritance of the resistance phenotype. Despite their proximity on chromosome 2R, *Pen1* and *Pfin2* are likely to be distinct genes given the differences in their associated phenotypes (encapsulation vs. intensity, respectively) and the fact that encapsulation associated with *Pen1* was strongest against allopatric non-African malaria parasites, while *Pfin2* was discovered due to its effect on sympatric African parasites. This work on wild pedigrees represented the first concrete evidence of resistance alleles segregating in nature, and was one of the first studies to use any wild populations in identifying QTLs (Niare et al. 2002). The genetic analysis protocol established in this work is now being used to carry out a multiplexed genome-wide screen of *An. gambiae* infected with natural *P. falciparum* in Mali to determine the number and frequency of such natural refractory genotypes, and to capture alleles in extant lines for laboratory studies. A combination of single nucleotide polymorphism genotyping, finer scale microsatellite genotyping and carefully selected candidate

gene analyses are being used to identify the loci underlying *Pfin1* and *Pfin2*. *Pfin1* is now restricted to an approximately 6-Mb chromosomal interval. Efforts to identify candidate genes are aided by a current expressed sequence tag (EST) project focused on immune responsive transcripts and subsequent microarray analysis of gene expression during malaria infection.

Despite identifying major QTLs for both encapsulation and infection intensities using the laboratory and field experimental approaches, no single causative locus has yet been isolated. Complete genome sequence, increasing functional characterization of genes and their protein products, finer scale genetic mapping, and continual dissection of mosquito innate immunity should allow more informed choice of candidate genes in the effort to identify malaria resistance genes.

5 Mosquito Transcriptome and Proteome

Transcriptome The initial route taken to describe the molecular immune system of mosquitoes was the identification of individual transcriptionally responsive genes (Dimopoulos et al. 1998; Richman et al. 1997). The first transcriptome-based projects described immune ESTs from a mosquito hemocyte-like cell line (Dimopoulos et al. 2000) and from whole-mosquito subtractive hybridization (Oduol et al. 2000). These and other projects have recently yielded the resources for larger scale transcriptional profiling studies of mosquito immunity.

Microarrays fabricated from normalized cDNA libraries of an *An. gambiae* hemocyte-like cell line were hybridized with labeled cDNA produced from in vitro treated cultured cells, or from in vivo treated mosquitoes (Dimopoulos et al. 2002). Infection of mosquitoes by *P. berghei* induced the expression of 24 genes and repressed the transcription of 10. Most of the malaria-induced genes in mosquitoes were also induced in the cell line by microbial elicitors. The malaria-repressed genes in mosquitoes were mostly repressed or unchanged in response to microbial elicitors in the cell line. Among the approximately eight genes induced in mosquitoes by malaria, but not by sterile or septic injury, were two genes, *isocitrate dehydrogenase* and a dsRNA-binding RNase, that were also not induced by any treatment in the cell line.

The cell-line derived microarray was also used to analyze gene expression profiles of the above described artificially selected mosquito lines that are susceptible or resistant to malaria parasites through the melanotic encapsulation response. Some redox-related genes displayed elevated expression in the resistant line as compared to either susceptible or wild-type G3 colony mosquitoes, suggesting that the resistant line could be under constitutive

oxidative stress (Kumar et al. 2003). The difference between genetic lines was manifested after an uninfected bloodmeal, with little additional effect of malaria parasite infection. This is reminiscent of the increased encapsulation of negatively charged beads after a normal bloodmeal (Chun et al. 1995). The induced gene set was enriched for genes of the mitochondrial genome, particularly those involved in mitochondrial respiration, and several nuclear genes including *thioredoxin reductase*, mitochondrial *thioredoxin*, and *xanthine dehydrogenase*. Basal expression of *catalase*, a gene involved in the clearance of oxidative free radicals, is higher in the susceptible than the resistant line. The *catalase* gene is near the *Pen3* QTL involved in parasite encapsulation, which was a minor locus for *P. cynomolgi* B but a major effect locus for *P. cynomolgi* Ceylon.

EST libraries highly enriched for repressed and induced sequences of the immune transcriptome of whole *An. gambiae* mosquitoes (Oduol et al. 2000) were spotted on microarrays and expression profiles were analyzed (J. Xu, J. Li, F. Oduol, M. Riehle and K.D. Vernick, unpublished results). Transcriptional profiles in response to the Gram-negative bacterial immune elicitor lipopolysaccharide were almost entirely distinct from the response to malaria or injury. A significant coexpressed cluster of genes was induced by injury but repressed by malaria infection, suggesting that a counter-inflammatory response may be caused by malaria parasites. The repression began soon after the infective bloodmeal, before ookinete invasion of the midgut epithelium, and expanded during midgut invasion, indicating the existence of malaria-related molecular signals that may prime the mosquito host for infection.

Whole-genome expression profiles in *Drosophila* have revealed many immune-responsive genes. In one study, microbial infection by *Escherichia coli*, *Mycobacterium luteus*, and the fungus *Beauvaria bassiana* drove the transcriptional response of 400 genes (230 induced and 170 repressed) on Affymetrix oligonucleotide array (De Gregorio et al, 2001). Of the 400, 368 were not previously characterized as immune-related genes. Among the observations were 28 new inducible small peptides, which could be new antimicrobial effectors or cytokine-like signaling molecules. The list of immune-responsive genes was arbitrarily truncated at 400, and was not claimed to be inclusive.

A related study also used an Affymetrix oligonucleotide array and the same pathogens to reveal a list of 543 genes that were upregulated at least twofold by microbial challenge (Irving et al. 2001). This number comprised 4% of the total 13,600 genes assayed. Downregulated genes were not explicitly reported in this study (although the raw data are available), but based on the ratio of up-to-down regulated genes in the previous study, there might be approximately 400 repressed genes, suggesting a conservative estimate of 7% of the *Drosophila* genome devoted to immune defense. It is striking that transcrip-

tional expression of such a large proportion of the genome can be influenced by just three distinct pathogens. (Even more strikingly, approximately 25% of *Arabidopsis* genes can be differentially regulated during pathogen infection; Tao et al. 2003.) There were some differences but no clear expression signatures specific to the different pathogen responses in the *Drosophila* data. Most of the *Drosophila* genes with the greatest immune induction encoded hemolymph factors, including recognition proteins, antimicrobial peptides, serine protease, and protease inhibitors. Although there are hundreds of protease genes in the *Drosophila* genome, only 26 of them were induced by immune challenge. Overall, 47% of the immune-induced genes had no known function. Undoubtedly, some of these are generalized stress-responsive genes.

Proteome Relatively few studies have been done directly on proteins involved in mosquito–parasite interactions. The hemolymph is one major site of interest, because all sporogonic malaria stages contact the hemolymph. Hemolymph is the physiological location of functions involving pathogen recognition, signaling cascades, and effector activity, and is probably the major compartment of the mosquito immune response.

Molecular cloning and two-dimensional gel electrophoresis studies of hemolymph identified hemolymph proteins, including some that were altered by inoculation of saline or Sephadex beads (Chun et al. 2000; Gorman et al. 2000; Han et al. 1999). One of the altered proteins was the serine protease AgSp14D1, which is a relative of *Drosophila* Easter and *Manduca* prophenoloxidase activating enzyme, and may be involved in immune recognition and signaling (Paskewitz et al. 1999). The *AgSp14D1* gene is located near the mapped location of the *Pen3* locus involved in melanotic encapsulation.

There is an effort underway to catalog the hemolymph proteome of *An. gambiae* by mass spectrometry and two-dimensional electrophoresis (F. Oduol, J. Li and K.D. Vernick, unpublished results). Hundreds of proteins have been identified to date, including the expected prophenoloxidases, thioester motif-containing proteins (aTEPs), and serine proteases, and also many proteins with no known function. Comparisons between resting-state and malaria-infected hemolymph have identified a number of proteomic differences, including differences in absolute and relative protein abundance, mass shifts that probably signify cleavage events, and small charge shifts that probably signify phosphorylation or other post-translational modifications involved in immune signaling.

6
Fitness Costs and Evolution of Malaria Resistance

Relatively little work has been done to examine the nature of the evolutionary relationship between malaria parasites and mosquito vectors, although an obvious expectation is that each organism places selective pressures on the other. In order for an antagonistic coevolutionary relationship between host and pathogen to arise, at least two conditions must be satisfied: (1) host genetic variants should differ in susceptibility to infection; and (2) infection should be detrimental to host fitness. Both criteria appear to be met in the mosquito–malaria system. Moreover, the intensity of selective pressure reciprocally imposed between *Anopheles* and *Plasmodium* is probably enhanced by the regularity with which mosquito hosts are exposed to the parasite and the taxonomic specificity of the host–parasite relationship. Most mosquito species are associated with only one or two closely related *Plasmodium* species in nature, and any given mosquito has a high probability of encountering the parasite over her lifetime.

Evidence for coevolutionary adaptation is also provided by the observation that the *Plasmodium* life cycle can be successfully completed only in compatible mosquito–parasite species combinations, while *Plasmodium* infections of non-natural mosquito hosts can fail at any of a number of stages (Alavi et al. 2003; Billingsley and Sinden 1997). Resistance, at least by encapsulation, also appears to include a component of parasite specificity, based on large genetic differences underlying encapsulation of *P. cynomolgi* B, *P. berghei*, and Sephadex beads, as compared to *P. cynomolgi* Ceylon. Transcriptional profiles of a panel of parasite response genes were different for *P. falciparum* and the rodent parasite *P. berghei* (Tahar et al. 2002). One could speculate that, in the case at least of encapsulation, a shared effector cassette may be controlled by distinct upstream recognition and signaling modules responsive to different malaria parasite species or strains. Caution is warranted in drawing generalizations from these studies because neither *P. cynomolgi* nor *P. berghei* are transmitted by *An. gambiae* in nature. Physiological competence for encapsulation (based on the *Pen1* bead phenotype) is further uncoupled from actual parasite encapsulation by the observation that 90% of wild-caught *An. gambiae* in Tanzania encapsulated Sephadex beads, while less than 1% of naturally infected mosquitoes carried encapsulated parasites (Schwartz and Koella 2002), again leaving room for the action of specific coevolved recognition functions.

There is a body of evidence indicating that malaria infection imposes significant fitness costs on the vector. With regard to direct mortality, laboratory studies have been equivocal, sometimes suggesting no infection-dependent mortality (Chege and Beier 1990; Robert et al. 1990) and in other cases reach-

ing the opposite conclusion (Klein et al. 1982, 1986). However, under laboratory conditions with protection from nutritional and environmental stress, pathogens may not yield the best measure of mortality, particularly if the effects are subtle. Indeed, natural infections typically display lower oocyst numbers than experimental infections in the laboratory (Medley et al. 1993), indicating the probable existence of tradeoffs or infection-limiting factors in nature that are eliminated in the laboratory. In a study of natural populations, increased mortality was attributed to higher oocyst burdens, possibly due to the metabolic cost of infection (Lyimo and Koella 1992).

Sporozoite-infected mosquitoes probe a bloodmeal host more often than uninfected ones, and also spend more time probing (Rossignol et al. 1984, 1986; Wekesa et al. 1992). A proposed explanation was that sporozoite-infected salivary glands produce less apyrase (a platelet aggregation inhibitor) than do uninfected glands (Rossignol et al. 1984). Moreover, mosquitoes in nature positive for *P. falciparum* circumsporozoite protein (i.e., probably bearing sporozoites) bite more people per night than uninfected mosquitoes (Koella et al. 1998). Mosquitoes with parasitized salivary glands had a significantly higher feeding-associated mortality in nature (Anderson et al. 2000), which may result from greater exposure to human host defensive measures (e.g., swatting) due to the altered mosquito feeding behavior. The long latency of the oocyst before sporozoite release may defer some of the fitness costs of infection, and associated selection pressure, until late in the vector's life, after most eggs have already been laid (Koella 1999).

The presence of *Plasmodium* oocysts in the midgut has been correlated with reduced mosquito fecundity in laboratory and natural systems (Hogg and Hurd 1995a, 1995b, 1997). Decreased fecundity was also associated with alterations in utilization of the yolk protein vitellogenin by ovaries in infected versus uninfected mosquitoes (Ahmed et al. 2001; Hogg et al. 1997). It was observed that soon after development of the ovarian terminal follicles in *An. stephensi*, a significantly greater proportion of the follicles underwent resorption in infected as compared to uninfected mosquitoes, thus reducing the size of the resulting egg batch (Carwardine and Hurd 1997). The mechanism underlying the resorption appears to be apoptosis of the follicles (Hopwood et al. 2001). Thus, destruction of the follicles probably explains the reduced vitellogenin uptake by ovaries of infected mosquitoes, and the consequent reduced fecundity. These observations add malaria to the list of pathogens that subvert host reproduction to conserve critical host resources (Koella 1999).

There are probably functional tradeoffs between the costs of infection and resistance, and one category of fitness cost is the cost of resistance itself. There is likely a metabolic cost to mounting a defense response, and a parasitized host may be energetically compromised and have fewer resources available for defense against other pathogens (Ahmed et al. 2002; Brey 1994). There

could also be a fitness cost from collateral damage to self caused by immune effectors, which has precedent in host defense systems such as vertebrate inflammation in sepsis (Cohen 2002; Ohta and Sitkovsky 2001) and probably the insect melanotic encapsulation response, in which reactive oxygen species in resistant mosquitoes may be harmful to both parasite and host (Armitage et al. 2003; Kumar et al. 2003; Moret and Schmid-Hempel 2000; Nappi et al. 1995). The melanotic encapsulation response might also compete with other critical functions such as eggshell and cuticle tanning (Ferdig et al. 2000; Johnson et al. 2001).

A related consideration is that the lack of robust natural resistance may at least in part result from indirect or direct parasite modulation of host defenses. Indirect effects could result from bloodmeal-related factors. For example, some malaria-responsive *An. gambiae* genes are transcriptionally regulated by an infected bloodmeal beginning before actual ookinete invasion of the midgut (J. Xu, J. Li, F. Oduol, M. Riehle, and K.D. Vernick, unpublished results; Bonnet et al. 2001; Tahar et al. 2002). This early expression could represent a response to soluble parasite-produced immune elicitors, to the quality of the bloodmeal derived from an infected vertebrate host, or to immune signaling molecules from the infected vertebrate host that might influence mosquito immune signaling pathways (Luckhart et al. 2003). Finally, the parasite may actively and directly manipulate components of host defenses. This mechanism has numerous precedents, for example insect polydnavirus proteins that inhibit host immune hemocyte function (Li and Webb 1994), the Yop virulence factors of *Yersinia pestis* that block host cell phagocytosis (Andersson et al. 1996; Rosqvist et al. 1991), plant fungus subversion of an antimicrobial compound to disrupt immune signaling (Bouarab et al. 2002), bacterial suppression of the production of antibiotic peptides in *Drosophila* (Fauvarque et al. 2002; Lindmark et al. 2001), malaria inhibition of dendritic cell functions to leave the host vulnerable to repeated reinfection (Ocana-Morgner et al. 2003), and the downregulation by herpesviruses, cytomegalovirus and HIV-1 of surface class I MHC protein to block cytotoxic T lymphocyte activity (Cohen et al. 1999; Ploegh 1998).

7
Evolution of Immune Genes in *Drosophila* Populations

Evolutionary forces acting on insect immune systems have been most thoroughly studied in *Drosophila*, where molecular and phenotypic analyses suggest that genetic variation in immunocompetence is pervasive in wild populations. Comparable data have not yet been obtained from the *Anopheles–Plasmodium* system. It has been recognized for some time that natural

populations of *D. melanogaster* harbor genetic variation in the ability to resist parasitization by endoparasitic wasps (Carton and Bouletreau 1985; Hughes and Sokolowski 1996; Kraaijeveld and Godfray 1997). Although resistance to parasitization in wild flies is very low, artificial selection for true-breeding fly lines exhibiting high rates of wasp egg encapsulation has been successful (Carton et al. 1992; Kraaijeveld and Godfray 1997) and attributable to a small number of genes with large effects (Benassi et al. 1992; Orr and Irving 1997). In at least one case, the resistance phenotype seemed to result from increased hemocyte production (Kraaijeveld et al. 2001). More recently, genetic variability in antibacterial immunity has been documented among distinct genetic lines derived from a wild *D. melanogaster* population (Lazzaro et al. 2004). Extreme lines in this study differed by 10 phenotypic standard errors in bacterial load sustained following infection, although no single candidate immune-response gene explained more than 16% of the observed variance. The apparent difference in the genetic architecture of resistance to parasitoids and bacteria is suggestive, but it may be exaggerated by differences in the experimental approach. The parasitoid work relied on lines artificially selected for resistance, such that mutations conferring large phenotypic effects could rapidly dominate the selected population and be readily detected by recombination mapping. In contrast, the candidate gene-based approach taken in the antibacterial work tends to have more power to detect small allelic effects, although the genetic basis for much of the observed phenotypic variance remains undetermined. A rigorous comparison of the structure of variation in defense against parasitoids and microbes will have to wait until comparable experiments have been executed with both pathogens.

Given the importance of immunocompetence to organismal fitness, it is not apparent why variation in quality of the immune response is allowed to persist in natural populations. One possibility is that functional variation in *Drosophila* immunity genes exists as a result of functional tradeoffs. *D. melanogaster* larvae selected for enhanced encapsulation of parasitoid wasp eggs have been shown to be poor competitors under resource-limited conditions (Fellowes et al. 1998; Kraaijeveld and Godfray 1997), perhaps due to a twofold greater investment in generating encapsulation-competent hemocytes in resistant relative to susceptible lines (Kraaijeveld et al. 2001). Increased male courtship activity has been suggested to decrease the rate with which avirulent *E. coli* are cleared from the hemocoel of *D. melanogaster* adults (McKean and Nunney 2001). In the latter case, no mechanistic connection between the resistance phenotype and the fitness cost has been defined, although hormonal differences induced by mating have been suggested to compromise immune capacity in *Tenebrio* (Rolff and Siva-Jothy 2002).

Another class of tradeoff models posits that a given host allele may confer increased resistance to one pathogen but decreased resistance to another. In-

nate immune systems may be especially prone to this type of tradeoff because relatively few proteins may be responsible for recognizing and combating a large diversity of potential pathogens. This model has not been well tested in *Drosophila*, but limited empirical supporting evidence has been generated with the crustacean *Daphnia magna* and its bacterial pathogen *Pasteuria ramosa*. Genetically distinct *Daphnia* clones derived from wild-collected females were most susceptible to infection by *Pasteuria ramosa* isolated along with the particular mother that founded the *Daphnia* line, suggesting that varying *Pasteuria* strains were differentially able to infect specific host genotypes (Carius et al. 2001). A similar experiment examining pathogenesis of pea aphids, however, did not find any evidence for tradeoffs due to pathogen specificity (Ferrari et al. 2001). Provided there is no universally 'best' defense allele at a locus, allelic differences in efficacy against various pathogens could, in principle, allow the maintenance of host variation as a function of pathogen diversity. If relevant pathogen diversity decreases, however, this model collapses into a more traditional host–pathogen coevolutionary 'arms race' in which evolved host resistance to a pathogen drives the pathogen to evolve means of overcoming host defenses, in turn selecting for enhanced resistance in the host, perpetuating the cycle ad infinitum (Dawkins and Krebs 1979).

While allelic differences in response to varying pathogens have not been rigorously tested in *Drosophila*, molecular sequence data from a subset of *Drosophila* immune response genes reflect rapid evolution characteristic of host–pathogen coevolutionary interactions. As a functional class, immune-related genes diverge between species more quickly than nonimmune genes in *Drosophila* (Schlenke and Begun 2003). A similar observation has been made with respect to vertebrate immune response genes (Murphy 1993) and plant *R* genes (Lehmann 2002), suggesting that frequent fixation of adaptive amino acid variants is a common property of innate defense molecules. One striking incidence of apparent *Drosophila*–pathogen coevolution occurs with Relish, a transcription factor fundamentally important to the induction of antibacterial responses (Hedengren et al. 1999). Relish shows a highly significantly accelerated rate of amino acid substitution, with most substitutions concentrated in the autoinhibitory domain of the protein (Begun and Whitley 2000). Dredd, the caspase that physically interacts with Relish to cleave the autoinhibitory domain (Stöven et al. 2003) also shows a highly accelerated rate of amino acid substitution (Schlenke and Begun 2003). Thus, one hypothesis is that bacterial molecules injected into the host cell via Type III secretion systems (Cornelis and Van Gijsegem 2000) might interfere with Relish activation, forcing the host proteins into a coevolutionary arms race to retain functionality.

Rapid amino acid divergence is not characteristic of all innate immune proteins, however. Pioneering studies of sequence variation in *D. melanogaster*

Cecropin antibacterial peptide genes revealed little evidence of adaptive evolution (Clark and Wang 1997; Date et al. 1998; Ramos-Onsins and Aguadè 1998). A later study suggested that directional selection may operate on variation generated by gene conversion between the tandemly repeated *Attacin A* and *B* antibacterial peptide genes in *D. melanogaster* (Lazzaro and Clark 2001), and more comprehensive analysis of sequence polymorphism and divergence in multiple *D. melanogaster* antibacterial peptides showed consistent indications of positive selection on antibacterial peptide genes as a functional class (Lazzaro and Clark 2003). But the rate of amino acid divergence between *D. melanogaster* and *D. simulans* in antibacterial peptides is actually lower than the substitution rate between the two species in nonimmunity genes. Instead, *D. melanogaster* peptides harbor a slight excess of nonconservative amino acid polymorphism in proteolytically processed, but not in mature antibiotic, peptide domains (Lazzaro and Clark 2003). The data from the peptide genes are not consistent with co-evolutionary arms races or selectively maintained hypervariability, but may be consistent with frequency-dependent or fluctuating selection. Finally, peptidoglycan recognition proteins appear to evolve primarily under purifying selection (Jiggins and Hurst 2003).

Drosophila immune response has been proposed as a model for description of anti-*Plasmodium* reactions in anopheline mosquitoes. Much of the existing data support the comparison, although there may also be important differences between the biological contexts of *Drosophila* immunity to parasitoids and bacteria compared to antimalarial responses in mosquitoes. *Drosophila* are plagued by several species of parasitoid wasps and an undetermined number of distinct bacteria, while *Anopheles–Plasmodium* relationships are highly specific. The likelihood of any individual *Drosophila* encountering a given pathogen species in nature is probably small, whereas anophelines in many parts of the world are virtually assured of ingesting a bloodmeal containing *P. falciparum* or *P. vivax*. These differences imply that *Anopheles–Plasmodium* relationships may specifically coevolve even where *Drosophila* immune systems are forced to maintain a strong component of generality. This hypothesis does not predict that mosquito immune response genes will be invariant, only that the variation will be tailored to variation in *Plasmodium* genotypes.

8
Models and Mechanisms of Plant Disease Resistance

Like insects, plants lack a circulating immune system and rely exclusively on a form of innate immunity to defend themselves from pathogen attack. The best-understood form of plant disease resistance is known as gene-for-gene resistance. It is so named because of early observations that particular loci in

plant hosts (called *R* genes for Resistance) conferred resistance to pathogens carrying particular genes called avirulence genes (Flor 1955). Typically, a single *R* gene confers resistance to only a single pathogen strain that carries the cognate avirulence gene. This seems rather odd, as one would expect that the pathogens would quickly lose the avirulence genes. Avirulence genes actually encode virulence factors that promote pathogenicity on hosts lacking the appropriate *R* genes, providing a selection pressure for pathogen populations to retain them. Gene-for-gene resistance is effective against a wide range of pathogens, including viruses, bacteria, oomycetes, fungi, nematodes, and aphids (Dangl and Jones 2001). Resistance is associated with an enormous number of inducible defense responses, including the hypersensitive response, a form of programmed cell death that occurs in cells in direct contact with the pathogen.

At one time, the ligand–receptor model of gene-for-gene resistance was popular. This model proposes that *R* genes encode receptors for proteins or metabolites produced by pathogen avirulence genes. Binding of these molecules by the *R* proteins then triggers activation of defense responses. Isolation of *R* genes from many plant species over the last 10 years has revealed that they comprise a few classes (Ellis et al. 2000). So far, the largest class is composed of cytoplasmic proteins with nucleotide binding sites and leucine-rich repeats (NBS-LRR proteins). Other classes consist of membrane anchored proteins with LRR domains outside the membrane, or receptor kinases containing LRRs. A few *R* genes that do not fit any of these types have also been isolated. The cytoplasmic location of NBS-LRR proteins makes sense since many plant pathogens transport virulence factors into the plant cytoplasm. For example, plant pathogenic bacteria use Type III secretion systems similar to those of *Yersinia* and *Salmonella* to transport proteins into the host cytoplasm (Casper-Lindley et al. 2002; Collmer et al. 2000).

LRR-containing proteins are also components of several aspects of host defense in animals. Toll-like receptors (TLRs) are animal LRR proteins involved in sensing molecular patterns characteristic of broad groups of pathogens, such as lipopolysaccharide of Gram-negative bacteria. Distinct from most plant NBS-LRR proteins, the ligand-binding LRR region of TLRs is extracellular, and thus TLRs recognize extracellular pathogen molecules or signals derived from them, and transduce the immune signal into the cell. However, there are less well-described plant *R* genes that are also membrane anchored, with extracellular LRRs. TLRs are structurally conserved in invertebrate and vertebrate animals, and include mosquito representatives (Christophides et al. 2002; Imler and Zheng 2004), although it is not yet clear if they play a role in mosquito response to malaria parasites. More recently, it has been found that there are also intracellular NBS-LRR proteins in animals that, similarly to plant NBS-LRR proteins, bind cytosolic pathogen-derived molecules and

transduce an immune signal. Despite some semantic confusion in their names, animal NBS-LRR proteins all possess an LRR region that is probably the site of ligand-binding and an NBS that mediates oligomerization and probably activation, and finally a variable domain that confers protein interaction with distinct downstream effectors (Chamaillard et al. 2003; Inohara and Nunez 2003).

Once the plant *R* genes were in hand, many groups tried to detect binding of *R* proteins to their cognate pathogen avirulence proteins. In virtually all cases, these efforts failed dismally, casting doubt on the validity of the ligand–receptor model. An additional problem with the ligand–receptor model is that it requires plants to carry a large number of *R* genes, one for each pathogen virulence factor that it could encounter in its environment. Analysis of the complete genome sequence of the model plant *Arabidopsis thaliana* revealed only 149 NBS-LRR proteins (Meyers et al. 2003), a number that seems inadequate for the task. These difficulties led to the formulation of a new model, called the Guard Hypothesis (Dangl and Jones 2001; Van der Biezen and Jones 1998). In this model, *R* proteins guard important host proteins that may be targeted by pathogen virulence factors. Interference with the guarded protein by a pathogen factor is detected by the *R* protein, and this triggers activation of defenses. This explains why direct interactions between *R* proteins and avirulence proteins have not been easy to find. In the Guard Hypothesis, the number of *R* genes required is not much larger than the number of plant proteins that are possible targets of pathogen virulence factors.

The Guard Hypothesis predicts that there should be plant proteins that interact with R proteins and with the cognate pathogen avirulence proteins. Recently, two proteins that may be guarded by *R* proteins have been identified in *Arabidopsis*. *PBS1* encodes a serine-threonine protein kinase that is degraded by the bacterial protein AvrPphB, which is a cysteine protease (Shao et al. 2003). PBS1 kinase activity is required for recognition of AvrPphB by the *R* gene *RPS5*, but not for the activity of other *R* genes (Swiderski and Innes 2001; Warren et al. 1999). *RIN4* is degraded in the presence of AvrRpt2, the cognate avirulence protein of the *R* gene *RPS2*. In the other example, RIN4 forms complexes with either of AvrRpt2 or RPS2 in vivo. Removal of RIN4 is sufficient to trigger a defense response, and this depends on RPS2 (Axtell and Staskawicz 2003; Mackey et al. 2002, 2003). Evidence that pathogens benefit from interfering with putative guarded proteins such as RIN4 and PBS1 in host backgrounds lacking appropriate *R* genes would provide additional support to the Guard Hypothesis.

There is evidence that many pathogen avirulence genes contribute to virulence in hosts lacking cognate *R* genes. For example, AvrRpt2 enhances virulence of *Pseudomonas syringae* pv. *tomato* DC3000 in hosts lacking *RPS2*,

and inhibits activation of host defense responses (Chen et al. 2000). However, the molecular mechanisms by which such bacterial virulence factors contribute to virulence are not understood.

Gene-for-gene recognition of pathogen attack sets off a cascade of responses with profound effects on the responding cells. Nitric oxide and reactive oxygen species (ROS) are produced within a few hours. They may be toxic to pathogens and have been implicated in signaling. The source of most of the ROS is an NADPH oxidase similar to the enzyme responsible for ROS production in mammalian phagocytes (Torres et al. 2002). Nitric oxide is required for the hypersensitive response triggered by *R* genes in *Arabidopsis* (Delledonne et al. 1998), and is produced by the P-protein of glycine decarboxylase (Chandok et al. 2003). It is not yet known if loss of this activity compromises resistance.

Gene-for-gene resistance in plants is associated with production of small signal molecules such as salicylic acid (SA), which is required for expression of many defense-related genes. This signaling pathway contributes to resistance to various pathogens, but the extent to which gene-for-gene resistance depends on its function is not yet clear. Some of the molecular machinery involved in responding to the SA signal has been elucidated, and constitutes an interesting paradigm for signal transduction. Most SA-dependent responses require the activity of NPR1, an ankyrin-repeat protein (Cao et al. 1997). In the absence of SA, NPR1 is located in the cytoplasm in an oligomeric form due to disulfide bridges between monomers. Increased SA concentrations cause a change in the redox balance, resulting in de-oligomerization of NPR1, which allows it to move into the nucleus (Mou et al. 2003). Once there, it interacts with specific transcription factors (Despres et al. 2000; Zhang et al. 1999), which are required for activation of expression of certain SA-regulated defense genes (Zhang et al. 2003). This process is reminiscent of the mammalian IκB/NFκB system and a similar mechanism in insects in which binding of IκB to NFκB holds it in the cytoplasm until an appropriate signal occurs, at which time the released NFκB translocates to the nucleus (Baeuerle and Baltimore 1996; Hoffmann 2003).

The fact that there is a fitness cost to *R* gene mediated resistance has long been known to crop breeders who deploy *R* genes in the field. A recent study has documented that the presence of the *R* gene RPM1 in an otherwise isogenic background results in a seed yield reduction of 9% (Tian et al. 2003), although the resistance allele is strongly favored when pathogenesis is prevalent. Thus, the frequencies of resistance/susceptible alleles can cycle with the frequency of pathogenesis (Stahl et al. 1999). Deleterious effects of *R* genes could be a result of low-level *R* protein activity in the absence of pathogens, leading to low-level expression of plant defense responses. Some *R* proteins such as RPS2 have been shown to have activity in the absence of pathogens (Tao et al. 2000).

The fitness costs of *R* genes likely contribute to the enormous polymorphism in *R* gene repertoire in wild populations, as fitness costs are balanced against benefits of resistance.

There are far more NBS-LRR proteins in plants (149 in *Arabidopsis* to hundreds in rice) than in humans (~25), where they have only recently been identified. This class of intracellular defense molecule has not yet been described in invertebrates and may be absent in them, although human and invertebrate genomes each encode approximately 10 membrane anchored TLRs. Thus, although it is too soon to generalize, it appears that plant defense responses may be biased towards detection of intracellular attack as compared to animal defenses, perhaps consistent with differences in body plans of the organisms (Girardin et al. 2002).

9 Conclusions

Animals and plants all possess innate, genetically encoded mechanisms of host defense against pathogens. These mechanisms comprise the first line of host defense in vertebrates, and the only known line of defense in other organisms. Innate host defense systems have largely been described as responses to bacteria, viruses and fungi, pathogens that are radically different from the host. In these cases, it may be relatively straightforward for the host to recognize pathogen-associated molecular patterns that distinguish pathogen from self, and to develop effectors with specific activity against, for instance, bacterial but not eukaryotic cell membranes. Eukaryotic hosts infected with protozoan pathogens, such as mosquitoes infected by malaria parasites, lose some of these advantages. It is probably more difficult to design a defensin-like effector that forms pores in parasite but not host plasma membrane when the biophysical properties of both membranes are much more similar.

How do mosquito vectors of malaria respond to infection with malaria parasites? Because of the specificity of the association, and the likely fitness costs to mosquitoes, this probably represents an antagonistic coevolutionary relationship. Consequently, mosquito responses to malaria parasites are more likely to include pathogen-specific components, compared to the characteristically general antimicrobial responses. We do not know whether other protozoan pathogens that may infect mosquitoes in nature, such as microsporidia, are seen by the mosquito as immunologically similar to malaria parasites. Thus, it is not yet possible to say whether mosquito responses to malaria are also effective against other pathogens, or if the antimalaria response has been shaped by selection imposed by other pathogen infections and may be consequently constrained by a requirement for generality.

In the mosquito–malaria system, we do not yet know the immune elicitors, mechanisms of immune recognition and signaling, or effectors. In the laboratory model system of melanotic encapsulation, genetic loci have been mapped and there is a preliminary picture of some of the physiological features. The genetic evidence suggests that even this model response appears to be tailored to specific parasite species and possibly strains. In any case, encapsulation as described in the laboratory model does not appear to be representative of actual mosquito resistance to malaria in nature. Perhaps this is not surprising since, at least in the genetically selected line under current study, efficient encapsulation appears to be costly to the host. It is possible that individual components of the multigenic encapsulation machinery might be utilized in natural populations in different ways, which may be consistent with finding natural resistance traits (*Pfin2*) that map to the same apparent locus as one of the encapsulation loci (*Pen1*). The recent completion of the *An. gambiae* genome sequence and current work on the *Ae. aegypti* sequence offer new tools to dissect the mosquito–malaria interaction. Genomic tools are transforming the ability to analyze transcriptional and protein responses to infection, and to genetically identify and analyze the evolved mechanisms that determine mosquito resistance to malaria in nature.

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