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What five insects told us about how a native plant copes with real-world problems

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Five native insects have taught us about traits that are essential for the survival of a native tobacco plant, *Nicotiana attenuata*, which lives in the Great Basin Desert of the USA. The five insects come from different feeding guilds, attack different tissues at different developmental stages and have revealed different traits that are essential for plant survival. For example, this plant recognizes attack from a specialized Lepidopteran herbivore (*Manduca sexta*) by the particular chemistry of the herbivore's saliva, and uses this recognition to tailor a complicated 6-layered defense response that involves a remodeling of the plant's transcriptome, metabolome and proteome, as well as some of its life history traits. The plant traits revealed by the remaining four insects will be described below, and how these insects will be used as high-through-put (HTP) screeners of large plantations of transformed and recombinant inbred lines of this native plant, to reveal the function of genes required for survival in nature. Insects are some of the best plant biologists on our planet and it behooves plant biologists to rediscover the lost art of natural history discovery to take advantage of the unique skill-sets of insects.

The plant and its insect herbivore community *Nicotiana attenuata* (coyote tobacco: Solanales: Solanaceae) is a diploid tobacco native to the Great Basin Desert of North America. It enjoys a unique position scientifically, because hundreds of transgenic lines have been studied over the past two decades to understand the fitness consequences of precisely defined changes in gene expression for plants growing in their native habitats. For more than 30 years, the plant has been an ecological model system to study the complex plant-mediated interactions that occur in nature and to elucidate the genetic basis of traits important for the Darwinian fitness of plants. The choice of this diploid, largely selfing plant as a model system is grounded in its natural history and unusual germination behavior. Dormant seeds, survivors of decades or even centuries of microbial challenges in soil, germinate synchronously into the nitrogen-rich, competition-free environments that are commonly found after fires. Dense *N. attenuata* populations then develop, in which plants are exposed not only to intense conspecific competition, but also to attack from as many as 34 different herbivore and pathogen taxa that colonize these post-fire populations from adjacent unburned areas. These are the ecological conditions in which agriculture started, as slash-and-burn agriculture, and are the conditions that all our crops face. However, most crop taxa have not originated from post-fire adapted native species and are unlikely to be pre-adapted to the conditions of the agricultural niche. By understanding the specific traits that this native tobacco plant has evolved to optimize its fitness in the post-fire environment in which it times its germination and growth from long-lived seedbanks, we hope to learn what will be required to make our crop plants more self-sufficient, less dependent on agricultural inputs. *Nicotiana attenuata* hosts a diverse insect herbivore community that includes several specialists on the Solanaceae: *Corimelaena extensa* (Hemiptera: Thyreocoridae), *Epitrix hirtipennis*, and *E. subcrintia* (Coleoptera: Chrysomelidae), two sympatric sibling species of *Trichobaris*, *T. compacta*, and *T. mucorea* (Coleoptera: Curculionidae), *Tupiocoris notatus*, (Hemiptera: Miridae), *Manduca quinquemaculata*, and *M. sexta* (Lepidoptera: Sphingidae) as well as the generalist herbivores *Spodoptera spp.* (Lepidoptera: Noctuidae), *Trimerotropis spp.* (Orthoptera: Acrididae), and *Oecanthus spp.* tree crickets (Orthoptera: Gryllidae) and the opportunistic generalist herbivores *Empoasca spp.* (Hemiptera: Cicadellidae) and *Heliothis spp.* (Lepidoptera: Noctuidae). *Geocoris pallens* is a common predator of herbivores on *N. attenuata*. Five of these insects (four herbivores and one predator) have proven to be very useful in eliciting and responding to plant traits, characteristics that make them very useful as High-Through-Put (HTP) screeners and sentinel insects for large forward-genetic Recombinant Inbred Line (RIL) populations that will be described after these five insects are introduced.

The herbivores: Manduca, Empoasca, Tupiochorus, and Trichobarus spp *Manduca spp.* The tobacco and tomato hornworms (*M. sexta* and *M. quinquemaculata*) have evolved an unprecedented capacity to detoxify the signature defense metabolite of the genus, *Nicotiana*, namely: nicotine. These nicotine-adapted larvae excrete the vast majority of the nicotine that they ingest with their food, but also co-opt a small fraction of the ingested nicotine for their own defense [1]. *M. sexta* larvae are found on *N. attenuata* plants because the adult moths oviposit while nectaring on the flowers. Hence, this insect plays dual roles in the lives of *N. attenuata* plants, as a devastating herbivore during the larval stages and as an important pollinator as an adult, vectoring gametes among the spatially separated plant populations that occur after fires, likely adding important genetic diversity to the seeds that must survive the decades of dormancy in the seed banks. Attack from these nicotine-adapted larvae is specifically recognized by the plant, when fatty acid amino acid conjugates (FACs) in the larval oral secretions are introduced into wounds when the larvae feed. This FAC-mediated recognition by the plant results in a six-layered suite of defense, avoidance, and tolerance responses (summarized in an iBiology talk available at https://www.ibiology.org/plant-biology/studying-plants-ecological-interactions-genomics-era-story-nicotiana-attenuata/#part-2), which engages all aspects of the plant's physiology. As such, *Manduca* larvae are ideal elicitors of ecologically important plant phenotypes and these phenotypes can be elicited by introducing larval oral secretions into plant wounds, a treatment that can be readily accomplished in a HTP manner for the large numbers of plants in a MAGIC population. *Empoasca spp.* These piercing-sucking leafhoppers "eavesdrop" on the jasmonate (JA)-mediated signaling capacities of their host plants, preferentially selecting those hosts that are deficient in jasmonate accumulations [2]. Given that JA signaling mediates the vast majority of plant defenses, it is particularly intriguing that this natural phenotyping "bloodhound" specifically targets JA signaling, apparently independently of the downstream defense metabolites that are regulated by JA signaling. Hence this insect can be used as a phenotyping tool to rapidly identify JA-deficient accessions in natural populations and the large number of plants in MAGIC populations. All that is required is an accurate quantification of *Empoasca* feeding damage after an experimental release of these herbivores into a population. *Tupiocoris notatus*. These free-living mirid herbivores manipulate the plant's cytokinin signaling, and in the process have lifted a page from the playbook of endophytic insects. Endophytic insects, because they are embedded within plant tissues, cannot easily move to other tissues, but instead have evolved the ability to modify their hosts' physiology to create metabolic "sinks" in the very tissues they infest. They do this in part by manipulating cytokinin signaling, which can transform a "source", which is a plant organ that normally produces sugars–like

a mature leaf–into a "sink", where sugars are stored or consumed, as in fruits and young leaves. These mirids produce their own cytokinins, transferring these phytohormones to plants during feeding to increase food quality and minimize the activation of senescence processes that are part of a plant's generalized defense response [3]. In the field, *T. notatus* preferentially attacks leaves with transgenically increased CK levels, and plants with transgenetically abrogated CK-perception are less tolerant of *T. notatus* feeding damage. Hence, by monitoring mirid feeding damage on the many plants of MAGIC populations, we would be able to rapidly identify RILs altered in cytokinin production, detoxification and perception. Again, all that is required is an accurate quantification of *Tupiocoris* feeding damage after an experimental release of these herbivores into a population. *Trichobaris mucorea*. The adult females of this weevil species oviposit their eggs into basal parts of elongating stems, early in the growing seasons as plants begin their transitions from vegetative to reproductive growth. The hatched neonate weevils burrow into stems to feed on the pith of elongating stems, all without damaging the plant's vasculature. These weevils manage to complete their life cycles with few negative consequences for their hostplant's fitness, and adults emerge after overwintering in completely senescent stems, to mate and oviposit on a next generation of plants. Larvae move up and down the stem, eliciting the production of phenolic defenses in the pith, eating fresh pith as the stems grow, and consuming their own frass in the process [4–6]. While unstudied, the larvae's coprophagic behavior is likely important for the larvae's ability to complete all four instars and pupate into an adult while only feeding on the limited amount of pith available in plant stems. Recent unpublished data suggests that the larvae use its bacterial microbiome to combat fungal infections that can be acquired while feeding in stems, in a manner reminiscent of that of its host plant's use of bacterial microbiomes to combat fungal phytopathogens (as described in [7] and in Lee *et al.*, unpublished). The larvae's bacterial microbiome appears to be transferred to the pith via the egg, and eggs are readily sterilized by a superficial bleach treatment. Hence, by comparing the fate of experimental ovipositions of both sterile and nonsterile eggs into the stems of the plants of a MAGIC population, one could potentially identify genetic loci involved in the recruitment of entomopathogenic fungi as plant defenses, as well as loci that facilitate the coprophagic behaviors of the larvae, which in turn likely allow plants to tolerate *Trichobaris* infestations without significant fitness consequences. The predators: *Geocorus* spp. *G. pallens* and *G. punctipes* can be found on *N. attenuata* or neighboring plant species throughout *N. attenuata*'s habitat in the Great Basin Desert. The adults and larvae are acutely responsive to the volatiles emitted from *N. attenuata* after herbivory, learning to associate the release of particular mono- and sesquiterpenes and green leaf volatiles with the presence of feeding herbivores (their prey), and use this information to optimize their hunting behavior and thereby mediating an important indirect defense for the plant [8,9]. A simple, HTP egg predation bioassay has been developed that provides quantitative information about the feeding behavior of these otherwise difficult-to-observe "big-eyed" hemipteran predators in the field, and this assay was recently used to successfully input the genomic location for a linalool-synthase from a RIL population (Li *et al.*, in review). We plan to use this egg-predation assay and the feeding behavior of native *Geocorus* predators with MAGIC populations planted into natural habitats to identify genes that regulate this important means of indirect defense for plants. In summary, these four herbivorous and one carnivorous insect species respond to and elicit remarkably complex traits in plants, traits that would be onerous, if not impossible, to identify in a HTP manner. Forward-genetic tools that capture the natural genetic variation of a species are one of the most successful means of identifying genetic controls over phenotypes that are useful for phenotype manipulations. This is because natural selection selects for traits that increase an organisms' Darwinian fitness, and the genes responsible for natural variation have already been tested

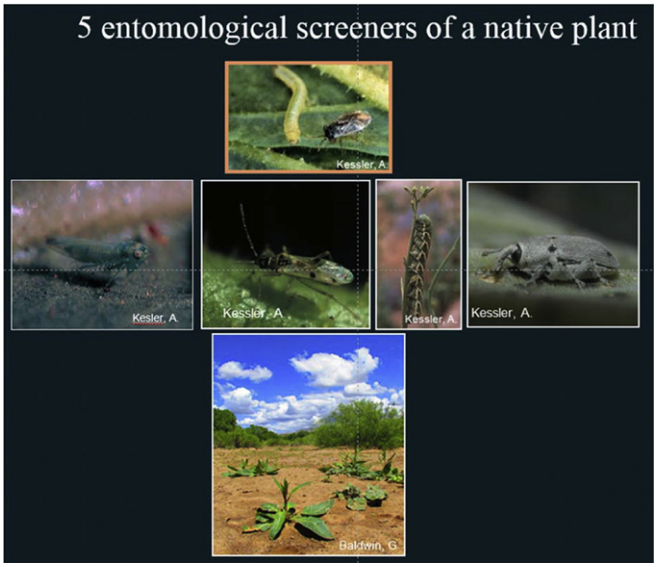

Fig. 1 Five entomological screeners of a native plant. *Nicotiana attenuata* plants growing in a native population in the Great Basin Desert (bottom photo: G. Baldwin) are regularly attacked by *Empoasca spp.*, *Tupiochorus notatus*, *Manduca spp.*, and *Trichobarus spp.* insect herbivores (middle photos: A. Kessler), which in turn are attacked by *Geocorus spp.* (top photo: A. Kessler) hemipteran predators.

under real-world conditions. By providing HTP means of screening, the large number of plants required by these forward-genetics tools, sentinel insects can provide plant biologists with a means of understanding the genetics behind the regulation of complex traits responsible for the ecological sophistication of plants.

The forward-genetics tool: The magic population Linkage or QTL mapping, and association mapping or GWAS are the two most commonly used forward-genetics statistical frameworks to query the genetic architecture of underlying traits. While GWAS can identify associative SNPs with one base pair precision, the procedure has limitations that make it difficult to use with the highly structured natural populations of *N. attenuata*. One of the primary assumptions of GWAS is that there should be no structure in the population, that is, the population must interbreed freely. A decade ago, a nine-year effort was initiated to create a 26-parent Multiparent Advanced Generation Inter Cross (MAGIC) population that captures the natural variation of the *N. attenuata* species. More than 230 accessions from seed collections representing 60 sites across the species' range were phenotyped in a glasshouse common-garden experiment, using comprehensive metabolomics, growth and morphology screens to identify 26 parental accessions that captured the species ecological phenotypic diversity. These diverse founder plants were used to create the MAGIC population, which involved all possible unidirectional binary matings, four generations of intercrossing and six generations of inbreeding of 1950 RILs to ensure ~99% homozygosity across all loci. Pending successful funding, we plan to plant this MAGIC population into two field sites in the Great Basin Desert, and recruit the services of the above-mentioned five entomological screeners (Fig. 1) to identify genetic loci that regulate the complicated ecological traits that native plants use to optimize their fitness in natural environments.

Disclosure of interest The author declares that he has no competing interest.

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Évolution et spécificité des interactions insectes hôtes–insectes parasitoïdes

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Charles Darwin parlait ainsi de certains insectes hyménoptères parasitoïdes: « Je ne peux pas me persuader qu'un Dieu bénévolent et omnipotent aurait créé volontairement les Ichneumonidae avec l'intention expresse qu'ils se nourrissent du corps vivant des chenilles. » Mais qu'est-ce qu'un parasitoïde? Il s'agit d'un organisme au mode de vie à moitié parasite–il se développe au contact et aux dépens de son hôte–et à moitié prédateur, car son développement conduit généralement à la mort de l'hôte, dont il consomme les tissus. Les parasitoïdes sont pour la majorité des insectes. On les rencontre dans six ordres d'insectes différents, mais plus particulièrement dans l'ordre des Hyménoptères, au sein duquel 80 % des espèces seraient des parasitoïdes. Ils parasitent des hôtes arthropodes et sont donc des régulateurs naturels de leurs populations ainsi que des auxiliaires importants en lutte biologique. Ils ont en effet l'avantage d'être plus spécifiques que les prédateurs. Les parasitoïdes peuvent se développer à la surface de l'hôte (« ectoparasitoïdes ») ou à l'intérieur du corps de leur hôte (« endoparasitoïdes »). Ils peuvent être solitaires (un seul parasitoïde adulte émergera de l'hôte) ou grégaires (plusieurs parasitoïdes se développeront dans un même hôte). Ce résumé présente trois aspects des recherches de l'équipe sur les interactions immunitaires entre des guêpes endoparasitoïdes et leurs hôtes drosophiles. La réponse immunitaire au parasitisme est décrite dans un premier temps pour apporter le contexte nécessaire. Face à un corps étranger de grande taille comme un œuf d'endoparasitoïde, la plupart des insectes mettent en place une réponse d'encapsulement. Elle met en jeu des cellules circulantes ou hémocytes, parmi lesquelles les plasmatocytes qui formeront une première couche de cellules autour de l'œuf du parasitoïde. Sur cette couche cellulaire viendront s'agréger des lamellocytes qui formeront les couches cellulaires suivantes. Les lamellocytes sont typiques du sous-groupe *melanogaster* de drosophiles et ils sont produits essentiellement en réponse au parasitisme. L'encapsulement implique aussi la mélanisation de la capsule qui fait intervenir des enzymes appelées phénol oxydases (PO; proenzymes PPO). PPO3 est produite par les lamellocytes, PPO1 et PPO2 étant stockées sous forme de cristaux dans les cellules à cristaux qui font partie des hémocytes. PPO1 et PPO2 sont activées par une cascade de protéines à sérines régulée par des serpines. Les radicaux cytotoxiques produits par la cascade PO (conduisant à la mélanisation) sont supposés tuer l'œuf ou la larve du parasitoïde. Les parasitoïdes du genre *Leptopilina*, qui se développent dans des drosophiles, peuvent cibler ces différents composants immunitaires. Ils injectent par exemple des molécules (Rho GAPs) capables d'inhiber les GTPases Rac1 et Rac2 de *D. melanogaster*, qui sont nécessaires à la réaction d'encapsulement, ou des serpines qui inhibent l'activation des phénol oxydases. Dans la larve de drosophile, stade parasité par les *Leptopilina*, les hémocytes sont présents dans deux compartiments: (i) des îlots sessiles sous-cuticulaires au niveau de chaque segment larvaire, dans lesquels on trouve des plasmatocytes et des cellules à cristaux–les lamellocytes peuvent se transdifférencier à partir des plasmatocytes sessiles mis en circulation après parasitisme–; (ii) la glande lymphatique, composée d'une série de lobes postérieurs contenant des prohémocytes et de deux lobes antérieurs contenant des prohémocytes dans la zone médullaire et des hémocytes différenciés dans la zone corticale. La différentiation est contrôlée par des signaux produits par un groupe de cellules formant le PSC (*Posterior Signaling Center*). Notre équipe s'intéresse aux bases génétiques de la résistance de *D. melanogaster* à un type de souche du parasitoïde *Leptopilina boulardi*. Nous avons pu identifier un candidat pour le gène majeur de résistance caractérisé (un gène à deux allèles, l'allèle résistant étant dominant sur l'allèle sensible), *edl/mae*, qui code pour une protéine contenant un domaine SAM d'interaction protéine–protéine. La protéine Edl/mae est un interactant et un régulateur de Pointed P2 et Aop/Yan, deux protéines jouant un rôle dans le développement et la différentiation/prolifération cellulaire. L'analyse du phénotype immunitaire entre souche résistante et sensible met en évidence deux événements importants dans la réponse immunitaire (proportion des types hémocytaires en circulation, phénotype lié à la glande lymphatique) plus précoces dans la souche résistante que dans celle sensible. Ceci suggère un rôle important du déroulement temporel de la libération des hémocytes en circulation dans la résistance de l'hôte. On retrouve cette question du déroulement temporel de la réponse d'encapsulement en comparant des espèces de drosophiles. Nous avons en effet étudié la réponse de l'espèce invasive ravageuse *D. suzukii* aux parasitoïdes locaux comme *Leptopilina heterotoma*. L'encapsulement par *D. melanogaster* commence dès le dépôt de l'œuf du parasitoïde et est achevé 48 h après parasitisme, la capsule formée étant ensuite visible dans la larve et l'adulte de drosophile. Chez *D. suzukii*, la larve de parasitoïde n'est ni mélanisée ni entourée de cellules 48 h après parasitisme. Les premiers signes d'encapsulement n'apparaissent