Mol. Cells *26*, 1-4, July 31, 2008

Minireview

Molecules and Cells

Training Molecularly Enabled Field Biologists to Understand Organism-Level Gene Function

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A gene's influence on an organism's Darwinian fitness ultimately determines whether it will be lost, maintained or modified by natural selection, yet biologists have few gene expression systems in which to measure whole-organism gene function. In the Department of Molecular Ecology at the Max Planck Institute for Chemical Ecology we are training "molecularly enabled field biologists" to use transformed plants silenced in the expression of environmentally regulated genes and the plant's native habitats as "laboratories." Research done in these natural laboratories will, we hope, increase our understanding of the function of genes at the level of the organism. Examples of the role of threonine deaminase and RNA-directed RNA polymerases illustrate the process.

INTRODUCTION

Genes can function at any of the many levels in which all biological phenomena are embedded: cell, tissue, organ, organism, population, species, community, ecosystem. Whether a gene is lost, maintained or modified by natural selection depends on how its expression influences the Darwinian fitness of the organism. Despite the recognized importance of the whole-organismic function of genes, biologists are better equipped to understand the biochemical function of gene products which may or may not relate to their function at the organismic level. The first hypothesis of a gene's function usually comes from sequence similarities with other biochemically characterized proteins. An example illustrates how such annotations can be distracting. The abundant water-soluble proteins found in vertebrate eyes, dubbed "crystallins", are highly taxon-specific proteins; their sequences are identical to common metabolic enzymes, such as aldehyde dehydrogenases or transketolases (Sax et al., 1996). Their enzymatic activity is likely secondary, if not irrelevant to their function at the level of the organism, which is to create a transparent, structurally supported environment for the eye.

New structural functions for metabolic enzymes in a specialized tissue such as the eye are likely unusual in that the new function is readily apparent to the researcher collecting the sample. In most cases, figuring out a whole-organismic function will require an intimate understanding of the organism. The paucity of whole-organism expression systems and our incomplete understanding of the natural history of organisms used in our biochemical expression systems have hampered our ability to understand whole-organismic gene function. For example, the best developed eukaryotic expression system, namely yeast, is poorly understood at an organismic level because we know very little about its life outside the laboratory cultures in which it is kept, and as a consequence, a large fraction of the yeast genome remains functionally uncharacterized, now more than a decade after the availability of its complete genomic sequence. These observations underscore the fact that in the "-omics" era, we have lost our ability to train biologists with a "feel for the organism". The study of natural history lost its clout when the "-omic" tool box (genomic, transcriptomic, proteomic, metabolomic, etc.), used to pry open a handful of genome-enabled organisms, invalidated "biological intuition".

But change is underway. Technology-savvy biologists have so refined the "omic" tools that they can be used with almost any organism. The rapid pace of sequencing is blurring the boundaries between "model" and "non-model"; transcript arrays and their analysis are commercial commodities; and proteome and metabolomic analyses are common phrases used to market new analytical instrumentation. Only transformation still requires substantial effort. As a consequence, knowledge of natural history is regaining its cachet and the appreciation of our biological legacy is again on the rise. With sequencing costs plummeting, the once-elusive quantification of Darwinian fitness in a currency of sequence similarity becomes affordable. Think of Darwinian evolution as a stream of nucleotides flowing forward in time. Organisms move the genetic stream forward; their Darwinian fitness measures the success of this transmission. Organisms are not passive conduits but, rather, the Fed Ex employees of the genetic stream, finding new ways to make their genetic deliveries to the next generation more successful-more timely, more reliable, more economical.

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Received May 14, 2008; accepted May 16, 2008; published online May 20, 2008

Keywords: Darwinian fitness, direct defenses, indirect defenses, jasmonic acid signaling, natural selection, threonine deaminase

Integrating molecular biology into ecology: training molecularly enabled field biologists

Training a generation of "molecularly enabled" field biologists, who are adept at using the "-omic" tool box and able to discern subtle changes in an organism, will likely be a greater challenge than navigating regulatory roadblocks or solving other empirical challenges. Many graduate training programs still reflect the unhappy divorce that split biology departments along cellular-molecular and ecological-evolutionary lines.

The overarching scientific objective of the Department of Molecular Ecology at the MPI for Chemical Ecology is to integrate advances in molecular biology into the study of ecological interactions; in turn, ecologists' whole-organismic expertise can be integrated into the study of gene function. To accomplish this, we are training the next generation of whole-organismic biologists in an environment that offers them access to and support in the use of molecular tools. Such tools enable ecologically important traits to be dissected and manipulated under real-world conditions. As such, we are training molecularly enabled field biologists and to implement this change, we developed molecular tool boxes for two native plant species (*Nicotiana attenuata* and *Solanum nigrum)* and set about trying to understand the genes that are important for the Darwinian fitness of these plants in their native habitats in the Great Basin Desert of North America. Our research interests focus on the traits that allow plants to survive in nature and as a consequence the Department's field station in Utah in the southwestern USA plays a central role in the research: all research projects start with hypotheses originating from field work and end with tests of function, carried out after the release of transformed plants at this station.

Our research has shown that these two native plants use many dynamic defense strategies to defend themselves against herbivores and pathogens, without poisoning their natural enemies, pollinators, or other mutualists, and to manage competition with other plants (Kessler and Baldwin, 2001; 2007; Kessler et al., 2006; Rayapuram and Baldwin, 2007; Schmidt et al., 2004; Steppuhn et al., 2004). Plants defend themselves by increasing the production of secondary metabolites that decrease the performance of herbivores. Some of these chemical defenses directly influence insect behavior, reducing feeding, growth, and reproduction by inhibiting digestive enzymes or producing toxins that interfere with some aspect of an herbivore's physiology (Paschold et al., 2007; Steppuhn and Baldwin, 2007; Zavala et al., 2004). Others function as indirect defenses by attracting the natural enemies of the herbivores to the attacked plants (Baldwin et al., 2006; Kessler and Baldwin, 2001; Paschold et al., 2006). Volatile organic compounds released by herbivore-attacked plants are known to be attractive to predators and parasitoids (Dicke, 2000) and function defensively under natural conditions (Kessler and Baldwin, 2001). In addition to attracting natural enemies of the herbivores, the release of volatile organic compounds can function as a direct defense by repelling the ovipositing herbivores (De Moraes, 2001; Kessler and Baldwin, 2001).

Using the tools of molecular biology, the defensive functions of secondary metabolites were uncovered: simple RNA interference (RNAi) constructs driven by constitutive promotors made it possible to silence secondary metabolites without simultaneously affecting plant growth (Steppuhn et al., 2004). However, because heterotrophy evolved before photosynthesis, plants have always had to cope with the ravages of consumers and are expected to use all aspects of their metabolism, not just secondary metabolism, to defend themselves. Tests of the defensive function of primary metabolites will require more sophisticated gene-silencing tools to discern their role in defense against or tolerance of herbivore attack. Subtle silencing tools will allow silencing to be both tissue-specific and controlled at very precise times: the goal is to minimize the influence of gene silencing on growth and development while determining herbivore performance and resistance under natural conditions (Kang et al., 2006).

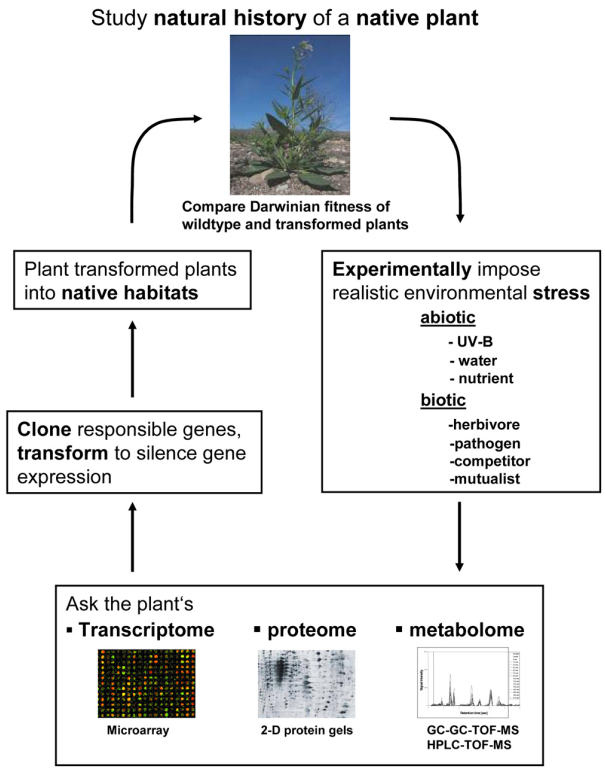

Fig. 1. Work flow for the unbiased analysis of traits that are important for a plant's survival and performance in the "real world". *Nicotiana attenuata*, native tobacco species, is exposed to several abiotic or biotic stresses in its native habitats. Then the plants are harvested and used to mine candidate genes related to stress response using different molecular tools. The candidate genes are cloned and transformed to silence their expression. The transgenic plants are transferred to 'native habitats' and characterized to find the function of silenced genes.

Student training will be as important as meeting the technical requirements of silencing genes. When unbiased transcriptional responses are used to "ask the plant" which genes are regulated in response to herbivore attack, the plant provides testable hypotheses about which genes are important in tolerance or defense. Gene annotations classify genes and specify their putative biochemical function based on sequence similarity. These annotations are extremely valuable but they should be viewed with caution as they do not exclude biochemical functions or functions at other levels. Students will need to learn to ignore these annotations and feel comfortable using an experimental approach that inverts the normal sequence of events in the biological discovery process (Fig. 1). Instead of proceeding step-by-step, from gene, to transcript, protein, metabolite, glasshouse phenotype and only when the plants are fully characterized, to a field test, field tests will need to be carried out earlier in the analysis. Two examples of this approach highlight the advantage of conducting field trials early in the discovery process and learning to ignore the annotations of genes available in the databases.

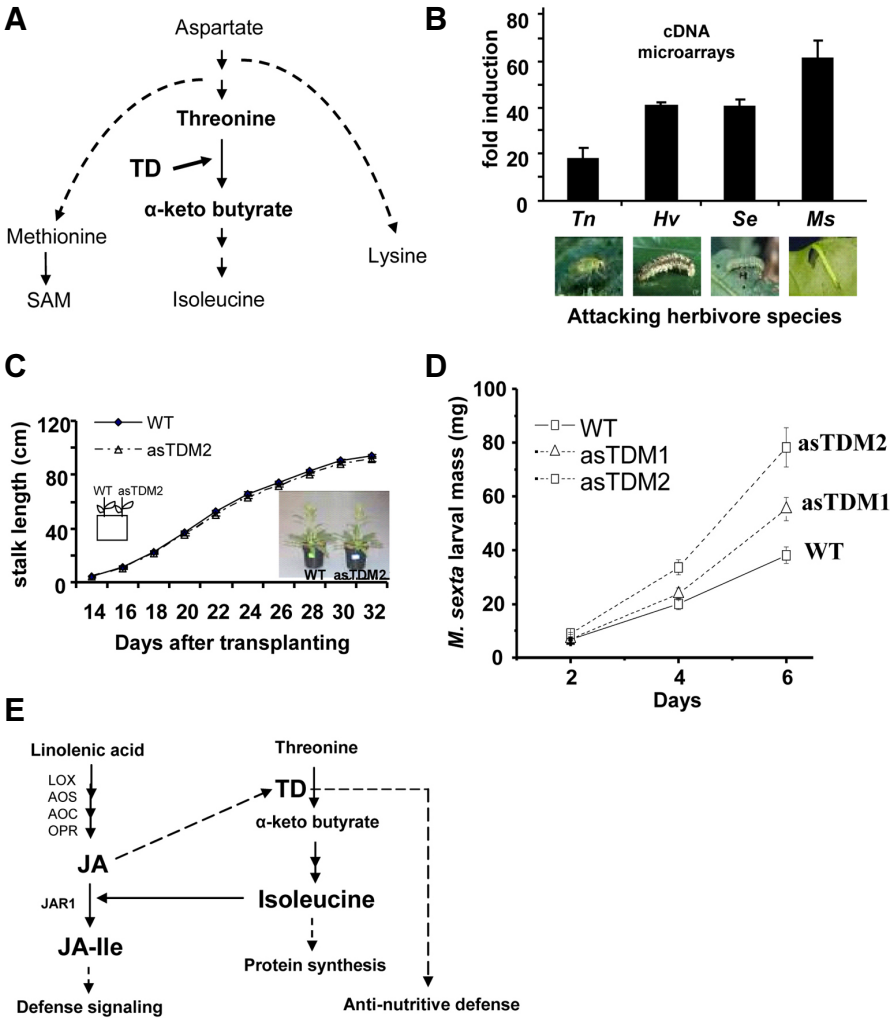

Fig. 2. Silencing threonine deaminase (TD) identified a new herbivore-defense signal. (A) The aspartate metabolic pathway of higher plants. Threonine deaminase functions biochemically to synthesize the essential amino acid isoleucine (Ile) by catalyzing the deamination of threonine (Thr). (B) TD is dramatically up-regulated after herbivore attack. *Tn, Tupiocoris notatus* (scckfly); *Hv, Heliothis virescens* (tobacco budworm); *Se, Spodoptera exigua* (beet armyworm); *Ms, Manduca sexta* (tobacco hornworm). (C) Comparison of growth rates of wild-type (WT) and TD-silenced transgenic plants grown in individual pots or competing with each other in the same pot. Plants transformed to silence this herbivory-induced increase in TD transcript accumulation are unaffected in their growth. (D) TD-silenced transgenic plants are highly susceptible to attack from *Manduca sexta* larvae. (E) The role of TD in plant defense. TD either plays a role as a post-ingestive direct defense, reducing Thr availability in the guts of larvae, or is involved in defense signaling, both of which turn out to be the case. Dashed arrows represent signal transduction pathways. *LOX*, lipoxygenase; *AOS*, allene oxide synthase; *AOC*, allene oxide cyclase; *OPR*, 12-oxo-phytodienoic acid reductase; *JAR1*, JA-amino synthetase; *TD*, threonine deaminase; JA, jasmonic acid; JA-Ile, jasmonic acid-isoleucine conjugate.

Examples: the role of threonine deaminase and small RNAs in plant defense signaling

Threonine deaminase (TD) plays an essential role in isoleucine (Ile) biosynthesis (Fig. 2A) but surprisingly, when unbiased transcriptional approaches (differential display RT-PCR) were used to compare the transcriptomes of insect-attacked leaves with those of unattacked leaves, TD transcript levels were found to be dramatically increased (Schittko et al., 2001). This observation was confirmed by extensive microarray analyses (Fig. 2B). Wounding increased transcript levels modestly, but when wounds are treated with herbivore-specific elicitors, such as the fatty acid amino acid conjugates found in herbivores' oral secretions (Halitschke et al., 2001), transcript levels were dramatically increased. When plants were transformed with a fragment of the 3′ untranslated region of the *N. attenuata* TD in an antisense orientation, some plants had normal growth rates (Fig. 2C) (demonstrating that normal amino acid metabolism was unaffected), but were silenced in their herbivory-induced TD transcript accumulation. When larvae of the specialist lepidopteran herbivore, *Manduca sexta*, were placed on these plants, their growth rates were significantly higher than those feeding on wild-type plants (Fig. 2D), which provided the clue that they were impaired in defense signaling (Kang et al., 2006).

Detailed analysis of the phytohormones activated during herbivore attack revealed that TD supplied the Ile required to conjugate jasmonic acid (JA) at the site of herbivore attack; the Ile in turn is used to synthesize a jasmonic-acid conjugate (JA-Ile). JA-Ile was subsequently shown to play a central role in activating herbivore defense signaling (Fig. 2E; Kang et al., 2006; Wang et al., 2007). Additional proteomic analyses demonstrated that TD and arginase play a post-ingestive defensive role in tomato by catalytically degrading the essential amino acids, threonine and arginine (Chen et al., 2005).

Similar procedures were used to discover a central role for small RNAs in mediating plant responses (Pandey and Baldwin, 2007). In these experiments, plants were silenced in three different RNA-directed RNA polymerases (RdR1-3) in the *N. attenuata* genome, and planted into the plant's native habitats. Different RdRs were found to play roles in herbivore resistance, UV-B protection and growth (Pandey and Baldwin, 2007; 2008; Pandey et al., 2008a; 2008b). As long as gene silencing doesn't dramatically alter plant growth, a single field trial can allow a trained researcher - one with an intimate understanding of the plant's natural history - knowing the stresses a plant faces in its native habitats, to rapidly identify environmental phenotypes. For these experiments to succeed, biologists need both whole-organism expression systems as well as an organ-

ism-level understanding of those unicellular organisms currently used for gene expression. These expression systems should include all the real-world challenges that an organism typically faces: ideally the organism's native habitat should be used as a laboratory.

CONCLUDING REMARKS

Clearly there will be much to be learned both by "asking the plant" and by using "omic" tools to decipher the plant's answer in the genes, proteins, and metabolites that it regulates differently when attacked by herbivores. If we are sufficiently forward thinking to ignore the gene annotations, to silence these regulated responses in ways that don't dramatically influence growth and then to ask the community of herbivores that naturally attack plants whether the plant is more resistant to or tolerant of herbivore attack, we will undoubtedly learn much that is new about how plants survive in the real world (Fig. 1).

ACKNOWLEDGMENTS

We thank Emily Wheeler for editorial comments. This work was supported by the Max Planck Society.

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