

Ecological genomics: making the leap from model systems in the lab to native populations in the field

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Recent reviews have emphasized the need to incorporate genomics into ecological field studies to further understand how species respond to changing environmental conditions. Genomic tools, such as cDNA (complementary DNA) microarrays, allow for the simultaneous analysis of gene expression of thousands of genes from all or part of an organism's genome (the transcription profile), thereby revealing the genetic mechanisms that underlie species' responses to environmental change. However, despite their potential, two major limitations have hindered the incorporation of microarrays and other genomic tools into field studies: (1) the limited availability of microarrays for ecologically relevant, non-model species and limited financial resources for developing new microarrays; and (2) concern that high sensitivity of gene expression to even subtle alterations in environmental conditions will hinder detection of relevant changes in field measures of transcription profiles. Here, we show that with cross-species hybridizations of microarrays developed for a closely related model organism, an appropriate experimental design, and sufficient replication, transcriptional profiling can successfully be incorporated into field studies. In this way, relevant changes in gene expression with changing environmental conditions can be detected.

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Recent technological advances in molecular biology have provided biologists with an unprecedented glimpse of the genomes of living organisms by allowing global assessment of variation in gene sequences and gene expression. A number of recent reviews have outlined the potential for understanding organisms across scales from genes to ecosystems by applying genomic methodologies to ecological studies (Jackson *et al.* 2002; Feder and Mitchell-Olds 2003; Purugganan and Gibson 2003; Hofmann *et al.* 2005; Garrett *et al.* 2006). One such methodology holds great promise for making substantial contributions to the field of ecology. Transcriptional profiling assesses expression levels (mRNA concentrations) for all or part of an organism simultaneously, allowing for gene-by-gene comparisons of expression. Variations in gene expression can be linked to phenotypic variation, thereby advancing our understanding of the adaptive importance of traits and gene functions (Vasemagi and Primmer 2005; Oleksiak 2002) and their consequences for higher-level ecological processes.

Gene expression microarrays – collections of hundreds to thousands of known and unknown cDNA (complementary DNA) gene sequences printed on a small slide – are often used to compare transcription profiles in tissues collected from individuals of a group of organisms subjected to different experimental treatments or at various stages of development. The variations in expression levels represented on the microarrays are used to assess the impacts of environmental conditions or development on gene function. Gene expression microarrays have been used to measure and compare transcription profiles of many different taxa and, in doing so, genes and gene networks have been identified that underlie ecologically important traits, such as those linked to cold adaptation (Van Buskirk and Thomashow 2006), salinity tolerance (Gong *et al.* 2005), insecticide resistance (David *et al.* 2005), and defense against pathogens (Zeidler *et al.* 2004).

Most of these studies have focused on model organisms for which gene expression microarrays and other molecular tools are readily available. Yet, measuring the genome-level expression of ecologically important non-model species in their natural environment is likely to yield the most relevant insights into the molecular mechanisms underlying species responses to changing environmental conditions. Despite the potential for genomics to provide a better understanding of linkages between gene expression, phenotype, and the environment, few field studies have taken the next step – namely, including genomic tools, such as transcriptional profiling based on gene expression technology. This is due in part to the lack of

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financial resources required for such studies (although the cost is decreasing), but is also the result of two major perceived limitations: (1) the lack of existing tools, such as gene expression microarrays, and gene-function data for most non-model organisms; and (2) the difficulty of detecting ecologically relevant changes in gene expression under variable field conditions.

Although initiatives such as the National Science Foundation's Tree of Life are leading to increased funding for the sequencing of new organisms, most genomic information, including gene sequences, gene libraries, and studies of gene function, has been developed for a small and select subset of model species (eg *Arabidopsis*, rice, *Drosophila*). The lack of genomic information and tools for ecologically relevant, non-model species can be overcome in three ways: (1) by studying model organisms in natural environments (Jackson *et al.* 2002); (2) by applying genomic tools developed for model organisms to closely related, non-model organisms (Hofmann *et al.* 2005); or (3) by developing gene expression microarrays for non-model organisms. The first option is the least desirable, because it is unclear how relevant the responses of model organisms to environmental conditions will be for non-model organisms of interest. On the other hand, cross-species DNA hybridizations of probe and microarray cDNA (Panel 1) developed for model species have been made among a variety of organisms, including several species in the family Solanaceae (Moore *et al.* 2005),

and between *Arabidopsis thaliana* (mouse-ear cress), *Thellungiella halophila* (Gong *et al.* 2005), and *Thlaspi caerulescens* (Plessl *et al.* 2005). This option requires that the non-model organism of interest is closely related to a model organism for which gene expression microarrays and related genomic tools have been developed. The third option, developing a microarray that is specific to a non-model organism, is gradually becoming possible with the commercial production of custom-made expression arrays (eg NimbleGen, Affymetrix) and the introduction of techniques such as SuperSage arrays (Matsumura *et al.* 2006) that do not rely on cross-species hybridization of DNA samples. However, the time and financial cost involved in developing these approaches for measuring transcriptional profiles in non-model organisms are still major barriers for many research programs. The application of genomic tools developed for model organisms therefore has the potential to be a powerful, less expensive, and more immediate option for many ecologists.

The second limitation is the challenge of moving beyond highly controlled environments in the lab or greenhouse to natural field settings. Expression levels of genes can be highly sensitive to environmental conditions, and thus field measures of transcription profiles may incorporate a large amount of background variation that could make it difficult to detect significant changes in expression patterns in response to experimental treatments. Indeed, transcriptional profiling studies to date have been limited to either lab or greenhouse settings in order to control for environmental variation. Here, we demonstrate that with an appropriate microarray experimental design and sufficient replication it is possible to detect experimental treatment signals in gene expression in field studies as well.

By overcoming the limitations described above, ecologists can begin to incorporate transcriptional profiling and other genomic tools into their field studies. Transcriptional profiling will be especially relevant for those examining the impacts of environmental change, where changes in gene expression patterns can be compared between individuals subjected to different environmental conditions. For the first test case of the feasibility of applying gene expression microarrays to a non-model organism in an ecological field study, we measured transcriptional profiles of the grass species *Andropogon gerardii* as part of a long-term field experiment in which precipitation variability was altered – a key prediction of global climate change models (IPCC 2001). Previous results from this experiment indicated that higher variability in precipitation resulted in a host of ecological responses, including lower annual net primary productivity and soil CO₂ flux and higher plant species diversity (Knapp *et al.* 2002). These ecosystem and community responses were driven by responses of the dominant C₄ grasses, primarily *A. gerardii* (Knapp *et al.* 2002). Because the responses of *A. gerardii* can be linked directly to changes in tallgrass prairie structure and function (Smith and Knapp 2003),

Panel 1: Cross-species DNA hybridization

Typically, cDNA microarray experiments are designed to measure the abundance of gene transcripts of individuals under different experimental conditions. A cDNA microarray is generated by spotting single strands of PCR-amplified cDNA library clones onto a glass slide. Signal data are obtained by hybridizing labeled cDNA from experimental samples of the same species (eg maize on maize) with the fabricated microarray slide. RNA is extracted from the experimental plants and used to synthesize cDNA labeled with one of two colored dyes in a reverse transcription process. These cDNA strands will then hybridize to single-stranded cDNA molecules attached to the microarray slide if the nucleotide sequences of the two molecules are similar. A molecule that does not match the sequence of any cDNA on the array will be washed off, because its double-stranded nature depends on reasonably accurate pairing of complementary nucleotides. The remaining hybridized molecules will fluoresce when excited with a laser, allowing quantification of transcript abundance for that particular sequence.

The process is identical in a cross-species DNA hybridization. However, because the hybridization of labeled targets and array molecules is dependent on sequence similarity, fewer sequences (ie spots) will fluoresce due to lower overall sequence similarity between the two species. Thus, there should be a negative relationship between the level of genetic divergence and hybridization rate. For species that are only distantly related to model organisms, the cross-species array approach may be less profitable than alternatives such as SAGE arrays (Matsumura *et al.* 2006) that can be produced for any taxa in the absence of genomic or cDNA sequence data. However, for relatively closely related taxa there is an abundance of information on transcription available through this approach.

this dominant grass is an ideal focal species for assessing how altered gene expression patterns and consequent potential changes in phenotype in a single species can underpin responses at the ecosystem level. We used cDNA microarrays developed for a close relative of *A. gerardii* – maize (*Zea mays*) – to compare transcription profiles between individuals subjected to ambient and altered patterns of precipitation. Because of the potential for higher environmental variability in a field setting, we included more replicates than is typical for plant microarray experiments in the laboratory. Our goal was to determine if the cumulative effects of altered precipitation over a growing season would result in statistically significant changes in gene expression patterns in individuals of *A. gerardii*.

■ Materials and methods

Field experiment

We examined the effect of variation in rainfall on gene expression in *A. gerardii* by sampling plants growing in rainfall manipulation plots (RaMPs; Figure 1) established at Konza Prairie Biological Station in the Flint Hills region of northeastern Kansas (39°05' N, 96°35' W). Precipitation inputs to native tallgrass prairie in the RaMPs were controlled by transparent roofs that collected ambient rainfall and a system of storage tanks and sprinklers that permitted rainfall to be applied to plots at experimentally controlled amounts and intervals (Fay *et al.* 2002). Two experimental treatments were imposed: ambient plots were watered at the same frequency and with the same amount of precipitation as fell outside the plots, and altered plots were watered at intervals 50% longer than ambient patterns. The latter treatment results in fewer but larger individual rainfall events, because the same total amount of precipitation was applied as in the ambient treatment. Altered and ambient plots were arranged in a randomized complete block design, with two replicates in each of three blocks.

During the growing season in which we conducted this microarray experiment, the altered precipitation plots experienced lower average growing season volumetric soil water content (19%) relative to ambient plots (27%), even though both received similar total amounts of rainfall. Prior to leaf tissue sampling for microarray analysis on September 15, 2003, ambient plots received five pre-



Figure 1. A rainfall manipulation plot (RaMP) consists of native tallgrass prairie covered by a rainout shelter that intercepts and stores rainfall for either the application of ambient precipitation or for altered precipitation, in which the interval between ambient rainfall events is increased by 50%. Inset: *A. gerardii* leaf samples were flash-frozen in liquid nitrogen in the field for later RNA extraction and microarray analysis of transcription profiles.

cipitation events (four out of five events of >20 mm) between the end of August and the genomic sampling date in mid-September. During the same time period, the altered treatment plots only received one precipitation application, 25 days prior to genomic sampling. Thus, at the time of sampling, plants were exposed to a season-long history of different precipitation regimes, with a threefold difference in soil moisture between ambient and altered treatments on the tissue collection day.

Microarray experimental design

We collected a total of 48 leaf tissue samples for transcription profiling. A 10 cm section of green tissue was removed from the youngest fully expanded leaf from four plants per plot for each treatment and immediately frozen in liquid nitrogen (Figure 1, inset), then stored at -80°C . To minimize the differences between plants assayed on the same microarray, we collected samples from morphologically and developmentally similar plants (ie similar height, not flowering) in adjacent ambient and altered precipitation plots. The two plants in a given pair were sampled within 5 minutes of one another. We paired samples from each of the six ambient plots and the six adjacent altered plots for a total of 24 pairs of plants. Twelve pairs of plants were collected in the morning and 12 in the afternoon.

Total RNA was extracted from the plant samples, purified and converted to cDNA with the Array900 3DNA detection kit (Genisphere, Hatfield, PA). The maize microarrays used in the study presented here contained 10 536 expressed sequence tags (ESTs), which represent fragments of the active genes from maize libraries constructed in two labs (Nakazono *et al.* 2003): Stanford

Unigene Set 1 developed by the Maize Gene Discovery Project (www.maizegdb.org) and the Schnable laboratory at Iowa State University (www.plantgenomics.iastate.edu/maizechip/). We paired plant samples from adjacent plots of different treatments on each microarray and assigned each of the two dye types to half of the replicates within each treatment category to prevent confounding of dye and treatment. We estimated gene expression levels at each of the 10 536 ESTs on the maize microarray by scanning fluorescence levels with a GenePix 4000B (Molecular Devices Corp, Sunnyvale, CA) microarray scanner. Because eight of the pairs of plants yielded less than the minimum of 2 mg of RNA required by the

Array900 kit, we only analyzed data from 16 of the 24 pairs of plants sampled.

Gene expression analyses

Prior to analysis, we transformed the data so that the range of raw fluorescence values was the same for each dye label (Cy3 and Cy5; Wolfinger *et al.* 2001) and removed genes with combined intensities of less than 100 fluorescence units from further analysis. Genes were also excluded from the analysis if they showed insufficient fluorescence levels to be scored on less than three arrays. This resulted in 1743 gene predictions being considered not hybridized. The remaining data were analyzed for differences in gene expression between the two precipitation treatments on a gene-by-gene basis, using mixed model analyses of variance (Wolfinger *et al.* 2001). The data were normalized with the following mixed model ANOVA (SAS Institute Inc, Cary, NC):

$$y_{ij} = \mu + A_i + D_j + (AxD)_{ij} + e_{ij}$$

where y_{ij} is the fluorescence intensity from the plant cDNA on the i th array and labeled with the j th dye, μ is the sample mean, A_i is the effect of the i th array ($i = 1-16$), D_j is the effect of the j th dye, $(AxD)_{ij}$ is the array-dye interaction and e_{ij} is the stochastic error. We analyzed the residuals (r) with a second mixed model ANOVA on a by-gene basis, using the following model statement:

$$r_{ijlmm} = \mu + A_i + W_l + T_m + P_n + D_j + (WxP)_{ln} + (TxP)_{mm} + A_i(TP)_{mm} + e_{ijlmm}$$

where W_l is the effect of the l th precipitation treatment, T_m is the effect of the m th time period, and P_n is the effect of the n th block (random effect). Because statistical tests for thousands of genes are performed in microarray analyses, experiment-wise error rates must be addressed. We controlled the experiment-wise false discovery rate using the QVALUE software to estimate Q values (Storey and Tibshirani 2003).

Results

Over 83% (8793) of the cDNAs on the maize microarrays cross-hybridized with cDNAs from *A. gerardii* (Figure 2). For a majority of these putative genes on the maize chip (8091), no differential expression due to the altered precipitation treatment was detected (Figure 2a). However, 702 genes did exhibit statistically significant ($Q \leq 0.05$) increases or decreases in transcription in response to altered precipitation treatments (Figure 2b; WebTable 1). The changes in transcription ranged from 0.1 to 24-fold for differentially downregulated genes and from 0.2 to 35-fold for upregulated genes (values are scaled in Figure 2b; Table 1; and WebTable 1). Variations in expression among individuals was relatively low (Table 1). If the altered precipitation treatment did result in significant

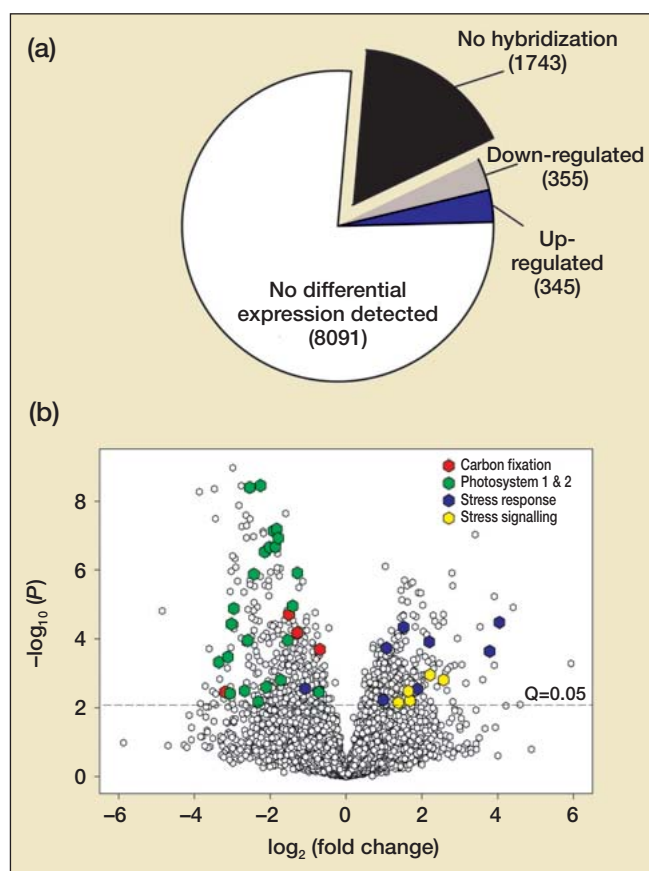


Figure 2. Transcriptional profile of *A. gerardii* cDNA extracted from plants subjected to ambient and altered precipitation patterns. (a) Number of maize microarray genes out of 10 536 that did not hybridize with *A. gerardii* cDNA, that hybridized but for which differential expression was not detected, or that were differentially up- or downregulated. (b) Volcano plot of gene expression in altered versus ambient precipitation regimes (x-axis: least squares mean of log-transformed fold change in expression levels in ambient vs altered leaf tissue) and the associated statistical significance level by gene (y-axis). Each dot represents a different gene. Putative genes related to photosynthesis, carbon fixation, and stress response are highlighted in color. Values to the left of zero on the x-axis indicate higher gene expression for ambient precipitation; values to the right of zero indicate higher gene expression for altered precipitation. Values above the $Q = 0.05$ line on the y-axis indicate statistically significant up- or down-regulation of gene expression.

Table 1. Partial list of genes in *A gerardii* that were differentially expressed in the altered precipitation treatment

GenBank accession number	GenBank sequence matching Putative protein	E value	\log_2 (fold change)					Number of plants changing transcription	
			mean	SE	CV	range	Q value	up	down
BM080630	emb X54075.1 ZMHSP18K1 maize mRNA for an 18kDa heat shock protein	0	3.7916	0.5493	38.33	1.46–4.63	0.0129	5	0
AI491543	gb AF236373.1 <i>Zea mays</i> hypersensitive-induced response protein (HIR1) mRNA, complete cds	0	-1.574	0.3251	50.30	-2.0–1.2	0.0467	3	11
AI782966	gb AY722708.1 <i>Zea mays</i> clone SPK1 putative salt-inducible protein kinase	0	1.5868	0.3352	97.26	-1.8–6.1	0.0096	13	1
BM267937	HORVU photosystem I reaction center subunit II, chloroplast precursor	1E-50	-1.9737	0.3576	49.05	-4.0–0.72	0.0220	1	14
BM381413	_MAIZE photosystem I reaction centre subunit N, chloroplast precursor (PSI-N)	5E-46	-2.0276	0.3208	34.88	-2.9–1.0	0.0332	1	14
AW352495	ribulose 1,5-bisphosphate carboxylase/oxygenase [<i>Zea mays</i>]	2e-49	-1.7691	0.4021	85.0	-4.29–1.52	0.0201	2	12

Putative gene functions and colors correspond to those in Figure 2b. Putative gene functions and descriptions are based on Blast search results from sequence matches. E-values indicate likelihood by chance of the sequence match between the maize cDNA and DNA of the putative gene in the global genetic database GenBank (www.ncbi.nlm.nih.gov/Genbank/index.html). Positive and negative \log_2 (fold change) values indicate significant up- and down-regulation, respectively. A Q-value ≤ 0.05 indicates significant fold change in gene expression with the altered treatment.

up- or down-regulation of genes, then the result was generally consistent across all replicates (Table 1).

The most striking pattern of gene down-regulation in response to increased time between precipitation events was a decrease in transcription of genes coding for proteins involved in photosynthesis and carbon fixation (Figure 2b; Table 1). Twenty-four different genes involved in either photosystem I or II were significantly downregulated in the altered versus ambient precipitation regimes, including those for chlorophyll *a-b* binding protein (forming a light harvesting complex which captures and transforms light energy), oxygen-evolving enhancer protein 3-1 (part of the photosystem II protein complex), and photosystem I reaction center subunits V, XI, II, and N. The expression of genes coding for rubisco and fructose-bisphosphate aldolase, both of which are important proteins for carbon fixation in C_4 plants – decreased over twofold under the altered rainfall regime. Moreover, there was a substantial decrease in expression of a putative HIR1 gene involved in the hypersensitive response, a programmed cell death response to infection by pathogens.

Genes that were upregulated in altered relative to ambient treatment individuals included many that are linked to proteins involved in plant stress response and signaling, including six heat-shock proteins (Figure 2b; Table 1). A gene putatively involved in osmoregulation and ion homeostasis (WebTable 1, GenBank accession number AI622346) increased in expression by a factor of ten. We also detected upregulation of genes for two proteins – alcohol dehydrogenase and ascorbate peroxidase – known to be similarly upregulated in response to high salinity in maize (Wang *et al.* 2003) and rice (Kawasaki *et al.* 2001). There

were no statistically significant shifts in gene transcription for genes putatively associated with senescence (WebTable 1, GenBank accession numbers AW017610, AI586828).

Discussion

In this study, we show for the first time that it is possible to overcome two important limitations to incorporating genomic tools, such as microarrays, into ecological field studies. These are the lack of genomic tools and information for ecologically relevant non-model organisms and environmental sources of variation inherent to the field. Genomics can therefore be moved from the realm of model organisms in the lab to that of native organisms in the field.

Using this approach, we have shown that transcriptional profiling using cDNA microarrays, in which individual plants subjected to different environmental treatments are compared directly, can be employed to detect important changes in gene expression patterns in native plants in a natural field setting. Indeed, despite expectations of significant variation in gene expression due to cross-hybridizing *A gerardii* cDNA with maize cDNA, as well as that associated with more variable environmental conditions in the field, we were able to detect consistent and significant up- and down-regulation of a number of genes, particularly those associated with photosynthesis, carbon fixation, and stress response. Importantly, the responses we observed at the gene level are consistent with observed physiological responses to the altered precipitation treatments (Fay *et al.* 2002; Knapp *et al.* 2002) and, more generally, to water stress (Heckathorn *et al.* 1997).

The selection of an appropriate model species microar-

ray for this study was important. Had we used a less closely related organism (eg *Arabidopsis*), we would probably have observed lower hybridization rates and garnered less information. Indeed, a major limitation to applying gene expression microarrays for a model organism to a closely related non-model organism is the limited number of species that can be studied. However, such an approach is of relatively low cost and does enable the measurement of transcriptional profiles in ecologically relevant species now, pending the development of additional, species-specific microarrays or other genomic tools. These species-specific microarrays will provide greater flexibility, since they can be developed for virtually any species. Unfortunately, they require a substantial investment of time and financial resources, which currently limits their development and application in ecological studies.

It is well known that phenotypic expression can be profoundly influenced by subtle changes in gene expression (Darvasi 2003). Our study shows that the application of genomic tools to ecological field studies can potentially provide information on which genes may be ecologically relevant under different environmental conditions and could be the basis for future studies examining linkages between changes in gene expression, phenotype (physiology), and ecosystem-level processes. Given the proper experimental approach, ecologists can begin to exploit the abundant information available through genomics and bioinformatics, link ecosystem-level changes with underlying shifts in the genome of its living components, and better understand how organisms are likely to respond to a rapidly changing world.

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