Insect Cold-Hardiness: New Advances Using Gene Screening Technology

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Abstract. The goldenrod gall moth *Epiblema scudderiana* uses the freeze avoidance strategy of winter cold hardiness. In recent studies we have begun to explore the changes in gene expression that support subzero survival, using both cDNA library screening and cDNA array screening technologies. Screening of a library prepared from cold-exposed larvae showed the cold-responsive up-regulation of EsMlp, a LIM protein with a probable role in myogenesis. Heterologous screening using *Drosophila* cDNA arrays proved highly effective in identifying multiple genes that responded to –20° C exposure, in particular highlighting the up-regulation of six plasma membrane transporters. Heterologous screening is an excellent search tool for seeking new genes/proteins that support stress tolerance in comparative animal systems, provided that appropriate validation and follow-up techniques are used.

Introduction

Many insects use the freeze avoidance strategy of winter survival and achieve deep supercooling by the production of antifreeze proteins and the accumulation of high concentrations of carbohydrate protectants such as glycerol. Research in my lab uses the goldenrod gall moth, *Epiblema scudderiana* (Lepidoptera, Olethreutidae), as a model for studies of the metabolic adaptations that underlie insect freeze avoidance, often comparing and contrasting these with the responses of the freeze tolerant goldenrod gall fly, *Eurosta solidaginis* (Diptera, Tephritidae) (Storey and Storey, 1992). Both overwinter as final stage larvae in galls on the woody stems of goldenrod and, as such, they are often exposed above the snow-

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line to the full force of ambient winter temperatures, which can fall to at least –30° C in the Ottawa area. Recent studies in my lab have been exploring the role of gene expression in phenomena, including freeze tolerance, anaerobiosis and hibernation (for review see Storey, 2003, 2004, this volume; Larade and Storey, 2002). Our recent work with both insect species has focused on the role of gene expression in adaptation to cold and/or freezing, and the present article presents highlights from some new studies of cold-induced gene expression in *E. scudderiana* derived from cDNA library and cDNA array screening.

Cold-induced Gene Expression: cDNA Library Screening

Changes in gene expression that support winter cold hardiness in insects could be triggered and regulated in one of two ways: (1) as seasonal responses that are initiated by photoperiod and/or thermoperiod cues and mediated by hormone signals, oftentimes as part of an obligatory winter diapause (particularly in univoltine species), or (2) as direct responses to low temperature exposure. The production of antifreeze proteins is a well-known example of the first mechanism as is the accumulation of the enzymatic machinery needed for cryoprotectant biosynthesis so that glycerol can be quickly synthesized when stimulated by cold exposures below 5° C. Indeed, a high proportion of gene expression responses that support insect cold hardiness are likely in place well before the first exposures to subzero temperatures occur. Our initial analysis of gene responses to acute cold exposure in both *E. scudderiana* and *E. solidaginis* actually supports this because we found relatively few genes that were putatively up-regulated from either cDNA library screening for cold-responsive genes in *E. scudderiana* or differential display PCR analysis of freeze-responsive genes in *E. solidaginis* (Bilgen, 1998; Bilgen et al., 2001).

Our first studies of cold-induced gene expression in *E. scudderiana* involved construction and screening of a cDNA library made from autumn-collected, cold-acclimated *E. scudderiana* using radiolabeled cDNA probes made from 15° C-acclimated (control) larvae versus larvae acclimated to 4° C for two weeks (cold-exposed) (Bilgen et al., 2001). Only three candidate clones were isolated as putatively up-regulated and only one of these encoded a full length protein that could be identified. This clone was shown to be the *E. scudderiana* homologue of the *Drosophila* gene *Mlp60A* and was named *EsMlp* (Genbank accession number AF206698). The translated amino acid sequence of EsMlp protein contained 94 amino acids and was 79% identical with the *Drosophila* protein. Like Mlp60A, EsMlp contained a single copy of the characteristic cysteine-rich

consensus sequence of LIM proteins that forms a pair of zinc fingers, as well as a glycine-rich region that further assigned it to the CRP subgroup of LIM proteins. Northern blotting revealed that *EsMlp* mRNA transcripts were elevated within eight hours when larvae were transferred from 4° C to –20° C, reaching 2-fold higher within 24 hours. Transcript levels also rose by approximately 3-fold over the autumn/winter to peak in February before falling to about 70% of autumn values in April. Western blotting revealed that EsMlp protein levels also increased by 3-to-4-fold in midwinter as compared with autumn levels but surprisingly, EsMlp protein in the larvae was 8.5-fold higher in April than in November. LIM proteins are involved in myogenesis during both embryogenesis and metamorphosis and play roles in the development and maintenance of cytoarchitecture. *E. scudderiana* larvae are in diapause over the early winter months, so it is unlikely that metamorphic changes in muscle structure are occurring, but *EsMlp* up-regulation might have one of two functions over the winter: (1) a role in temperature-dependent restructuring of muscle protein composition, as typically occurs in poikilotherm acclimation to changing temperatures; or (2) a role in diapause development in the preparatory phases preceding the extensive myogenesis that will occur in the spring. Peak levels of EsMlp protein in April and the fact that the protein has a nuclear targeting signal and a zinc finger motif (often found in transcription factors) suggest that it may be involved in regulating the transcriptional program leading up to spring metamorphosis.

cDNA Array Screening for Cold-responsive Genes

The use of cDNA arrays for gene screening is currently one of the hottest techniques in biology and offers multiple benefits, including (1) simultaneous assessment of the responses by hundreds of genes, most of them identified, (2) detection of transcripts that are present in low copy number (library screening favors abundant transcripts), (3) relative ease of sample preparation and data quantification, and (4) the ability to assess both individual gene responses and cumulative responses by groups of genes (e.g., families, pathways, or cascades) (Eddy and Storey, 2002). This latter capacity is particularly important in studies of biochemical adaptation where we are seeking insights into the types of metabolic responses that underlie organismal responses to diverse environmental stresses. Recently, we have applied heterologous probing (array produced from one species—human, rat, *Drosophila*—but screened with cDNA from another) to analyze stress-induced gene expression in several systems. The technique has been criticized for several reasons: (1) cross-hybridization with spots on the array

is always less than 100%, (2) due to differential hybridization capacities comparisons of responses between genes are compromised, and (3) a small possibility of false positive matches exists (i.e., binding to cDNA that is not its homologue). However, the reality is that species-specific arrays are never going to be available for all organisms and that heterologous probing, if correctly validated, is a superb search tool. A substantial percentage of genes are highly conserved across phylogeny and even at considerable phylogenetic distances, strong cross-hybridization can occur. For example, after optimizing hybridization and washing conditions, we achieved cross-hybridization with human 19K cDNA arrays (Ontario Cancer Institute) that was 85–90% for cDNA from hibernating mammals, 60–80% for freeze-tolerant frogs, and 18% for the marine snail *Littorina littorea* (a freeze- and anoxia-tolerant species) (Storey, 2003; Eddy and Storey, 2002; Larade and Storey, 2002). For snails this percentage seems low, and in addition, only 10.6% of the genes that hybridized were designated as putatively up-regulated. However, with 19,000 genes on the array, the result still provided over 300 "hits" of putatively anoxia-responsive genes for future study.

In new studies we used Drosophila microarrays produced by the Canadian Drosophila Microarray Centre at the University of Toronto (containing cDNAs for 7,222 genes, www.flyarrays.com) to screen for gene expression responses to cold exposure in *E. scudderiana.* Clearly, this involved heterologous probing and is subject to the above concerns. However, after optimization of hybridization and washing conditions, we achieved 89% cross-hybridization between *E. scudderiana* cDNA and the Drosophila chip, which allowed us to evaluate the expression of over 6,400 genes on the array. The data discussed below assesses the effects of acute subzero temperature exposure on gene expression; controls were September-collected E. scudderiana larvae that were acclimated to 15° C for two weeks and experimental animals were larvae that were then acutely moved from 15 to –20° C and held for 24 hours. Total RNA was isolated from each group and first strand cDNA synthesis was performed in the presence of cyanine 3 or cyanine 5 fluorescent dyes. Labeled cDNA from control and experimental conditions was hybridized with the cDNA array for 16 hours at 30° C. After washing, fluorescence readings at two wavelengths were taken using a Vertek scanner, and Arraypro software was used to quantify fluorescence intensity and calculate the ratio of cy3:cy5 binding by each spot. Analysis of the data showed that 4.7% of cross-reacting genes were putatively up-regulated in the experimental condition by 1.5-fold or greater, whereas a similar number were down-regulated; hence, about 90% of transcripts are unaltered by cold exposure.

All data derived from heterologous probing require verification by other methods to confirm up-regulation, and ongoing studies in our lab are focused on selected genes or gene families that were highlighted by the array screening. A general methodology for verification is proving highly effective. For any new gene, this begins with a virtual trip to Genbank to retrieve sequences from several other species. From these, a conserved consensus sequence is derived and used to design a cDNA probe (commercially synthesized) that is used with the PCR reaction to retrieve and amplify the species-specific PCR product. After sequencing to confirm its identity, the species-specific product is used as the probe to measure relative mRNA levels under multiple conditions via quantitative PCR (or Northern blotting). With the further use of peptide antibodies designed and produced from the translated species-specific amino acid sequence, we can also analyze accompanying stress-induced changes in protein levels.

Genes that were highlighted from array screening as being strongly up-regulated by –20° C exposure of *E. scudderiana* larvae included a rather large number of plasma membrane transporters, among them:

1. ***Concentrative nucleoside Na^+ transporter 2 (CNT2):*** CNTs cotransport sodium and nucleosides unidirectionally against the nucleoside concentration gradient (Cabrita et al., 2002) and function in both salvage pathways for nucleic acid synthesis and in the transport of nucleosides that have important roles in signaling (e.g., adenosine plays a key role in metabolic rate depression; Storey, 2004, this volume).
2. ***Na (K) Cl cotransporter:*** These electroneutral cotransporters are nearly ubiquitous and have an important role in sodium chloride absorption/secretion and in cell volume regulation during hypertonic and other volume challenges (Kaplan et al., 1996).
3. ***Na, Pi cotransporter:*** Inorganic phosphate has critical roles, both in intermediary metabolism and in the structure of macromolecules. However, due to the negative electrochemical potential across the cell membrane, inorganic phosphate cannot enter cells by diffusion and hence is imported by Na^+-dependent Pi cotransporters that have important roles in regulating phosphate availability (Werner and Kinne, 2001).
4. ***Na-Ca exchanger (NCX):*** These exchangers are found in the plasma membrane of most cells and are integral to Ca^{2+} homeostasis (Omelchenko et al., 2003). In heart, for example, NCX provides the principal mechanism for Ca^{2+} extrusion after contraction. NCX may also function in Ca^{2+} influx, particularly under pathophysiological conditions. Isoform 1 is found in a

macromolecular complex with protein kinase A, its anchoring protein AKAP, protein kinase C, and protein phosphatases 1 and 2A. Beta-adrenergic mediated phosphorylation of NCX increases exchanger current.

5. ***Dicarboyxylate transporter (cation-independent):*** This sodium-independent transporter in *Drosophila* was named INDY (I'm not dead yet) because mutation of the gene to create a disfunctional protein resulted in a two-fold extension of the average adult lifespan (Inoue et al., 2002). INDY transports a variety of tricarboxylic acid cycle intermediates, including citrate and succinate, and it is hypothesized that dysfunction of INDY reduces substrate availability for energy metabolism, resulting in life extension effects similar to those achieved by caloric restriction.
6. ***Monocarboxylate transporter (MCT):*** Monocarboxylic acids such as lactate and pyruvate play central roles in metabolism and are often transported between tissues that produce versus catabolize these compounds. Their transport across the plasma membrane is via proton-linked MCTs that occur in especially high levels in white muscles and are also up-regulated by increased work load in heart (Halestrap and Price, 1999).

Coordinated up-regulation of a variety of plasma membrane transporters in response to acute subzero exposure is an intriguing response that has not previously been reported as a part of cold acclimation. Up-regulation may be needed to enhance the overall levels of transporters in the cold or perhaps to alter the relative levels of different isoforms of each transporter. Further studies will verify and explore changes in both mRNA and protein levels of each of these transporters and aim to discover how they contribute to reestablishing homeostasis at low temperature.

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