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Differential display analysis of gene expression in invertebrates

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Abstract. Screening for differentially expressed genes is a straightforward approach to study the molecular basis for changes in gene expression. Differential display analysis has been used by investigators in diverse fields of research since it was developed. Differential display has also been the approach of choice to investigate changes in gene expression in response to various biological challenges in invertebrates. We review the application of differential display analysis of gene expression in invertebrates, and provide a specific example using this technique for novel gene discovery in the nematode *Caenorhabditis elegans*.

Key words. Invertebrate; cadmium; insect; differential display; genomics.

Introduction

Elucidation of the changes in gene expression associated with biological processes is a central problem in a variety of biological systems. Screening for differentially expressed genes is a straightforward approach to study the molecular basis for alterations in levels of transcription. In recent years, a number of approaches have been developed for the analysis of differential gene expression, including differential display, expressed sequence tags sequence analysis, the generation of subtractive libraries, microarray and gene chip analyses, and serial analysis of gene expression. This article will review recent progress in applying differential display analysis in invertebrate systems, and provide some guidance to scientists who may wish to apply differential display technology in their research. A number of examples where differential display has been used in a variety of invertebrates and several areas of application are described.

Survey of differential display used in invertebrates

Differential display has been shown to be a powerful tool for the analysis of complex systems since it was developed [1]. For invertebrates, many researchers have successfully used differential display to identify genes that are critical for development, mediate immune responses, and cellular responses to a variety of chemical and environmental stresses. Table 1 summarizes recent studies where differential display has been used to identify differentially expressed genes in invertebrates. As with higher organisms (e.g. mammals), differential display of invertebrate gene expression has been used in the fields of genetics, immunology, developmental biology and toxicology.

Insects represent a large class of arthropods, comprising three quarters of all known animal species. Thus, the majority of invertebrate differential display studies have been applied to insects. In *Drosophila melanogaster*, the technique of differential display has been used to identify an antibacterial protein and genes expressed in the follicle cells with potential roles in axis formation [2, 3]. Studies examining juvenile hormone-inducible genes showed that the developmental expression of these genes corresponds to the abundance profile of juvenile hormone in vivo [4]. In studies of the regulation of sleep and walking, differential display identified transcripts that are expressed differentially depending on behavioral state and circadian time [5]. In addition, differential display of *Drosophila* identified a novel anoxia-regulated gene that has an important role in the regulation of tissue responsiveness to oxygen deprivation [6].

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Table 1. Survey of differential display used in invertebrates.

Organism (Species)	Major Concept/Research Area	References
Termite (*Hodotermopsis japonica*)	population genetics	21
Honeybee (*Apis mellifera*)	molecular genetics	10
	nervous system	9
Mosquito (*Anopheles gambiae*)	immune system	11, 13
Mosquito (*Aedes aegypti*)	immune system	12
	molecular genetics	14
Fruit fly (*Drosophila melanogaster*)	development	4
	molecular genetics	3, 5, 6
	immune system	2
Nematode (*Brugia malayi*)	parasitology	33
Nematode (*Caenorhabditis elegans*)	toxicology	34, 36
Nematode (*Heterodera glycines*)	parasitology	32
Silkworm (*Bombyx mori*)	molecular genetics	16
	immune system	17
	development	15
Moth (*Trichoplusia ni*)	immune system	18–20
Sphinx moth (*Manduca Sexta*)	sensory reception	22
Spruce budworm (*Choristoneura fumiferana*)	endocrine system	7
Flagellate (*Leishmania mexicana mexicana*)	parasitology	27
Flagellate (*Trypanosoma brucei rhodesiense*)	parasitology	26
Sporozoan (*Plasmodium falciparum*)	parasitology	28
Snail (*Biomphalaria glabrata*)	immune system	25
	parasitology	23, 24
Soft-shell clam (*Mya arenaria*)	toxicology	30, 31
Coral (*Acropora cervicornis*)	toxicology	29
Bean bug (*Riptortus clavatus*)	molecular genetics	8

In addition to *Drosophila melanogaster*, several invertebrate species have been used to study genes induced in response to juvenile hormone. In spruce budworm, *Choristoneura fumiferana*, six genes were identified using differential display whose level of expression increased in response to juvenile hormone I exposure [7]. In bean bug, *Ritortus clavatus*, juvenile hormone induces termination of diapause, and reproductive maturation in adult female. Hirai et al. used differential display to isolate four new juvenile hormone-responsive complementary DNAs (cDNAs) in the fat body of the bean bug [8].

In studies of several species of honeybee, Kamikouchi et al. were able to isolate a gene expressed preferentially in the mushroom bodies of the brain of the worker honeybee *Apis mellifera L*. [9]. In addition, one study used differential display to identify differential gene expression between queens and workers during the caste determination process in *A. mellifea* [10].

To investigate the molecular mechanism of malaria parasite infection, several groups have used differential display to isolate immune response genes in mosquito. In this regard, a number of differentially expressed messenger RNAs (mRNAs) of the vector mosquito, *Anopheles gambiae*, were identified [11]. In addition, immunity peptides from mosquito cell lines including three defensin A isoforms from *Aedes aegypti* and a defensin D from *Aedes albopictus* were isolated by Gao et al. [12]. Moreover, Vizioli et al. isolated an immune-responsive, antimicrobial peptide, gambicin, from the malaria vector *Anopheles gambiae* [13]. Morlais and Severson used an integrated, targeted approach based on bulked segregant and differential display analysis to identify genes within genome regions containing quantitative trait loci that determine the susceptibility of *Aedes aeggp* to the malarial parasite *Plasmodium gallinaceum* [14].

In silkworm, differential display has been used to study diapause determination and immune response. To understand the molecular mechanism of diapause determination in early embryogenesis of the silkworm, mRNAs from diapause and non-diapause eggs were compared [15]. In addition, several genes whose expression increased in response to diapause hormone were identified in the developing ovaries of the silkworm *Bombyx mori* [16]. To study immune response in silkworm, Yang et al. cloned an antibacterial protein, cecropin D, from the fat bodies of *B. mori* larvae immunized with bacteria [17].

Differential display was applied to find new components of the immune systems in the moth *Trichoplusia ni*. Genes from *Trichoplusia ni* that are induced by a bacterial challenge were identified [18–20]. Kang et al. compared mRNA populations from last-instar larvae injected with bacteria to those of untreated larvae [18]. The group later identified a peptidoglycan recognition protein, which has both human and mouse homologues, whose expression was restricted to organs of the immune system. This suggests a common origin of the invertebrate and vertebrate innate immune systems [19].

Almost all termite species possess a soldier caste, and it is thought that there are ubiquitous mechanisms of soldier differentiation throughout isopteran species. Miura et al. used *Hodotermopsis japonica* termites as a social insect model to study the proximate mechanisms of caste determination [21]. The group performed differential display to identify genes specifically expressed in the soldiers caste of the damp wood termite, comparing mRNA from the heads of mature soldiers and to those of pseudergates (worker caste). The gene, SOL1, increased expression during soldier termite differentiation [21]. SOL1 expression is limited to the termite mandibular glands. Miura et. al were able to show that the technique of differential display is applicable to reveal the proximate mechanisms of caste determination in termites and other social insects.

In sphinx moth *Manduca sexta*, differential display was used to isolate antennal-specific mRNAs from the male sphinx moth in an attempt to investigate the role of insect antennae in the olfactory system [22]. The group identified an olfactory-specific glutathione S-transferase. They suggested that it might play an important role in protecting the olfactory system from harmful xenobiotics, and by inactivating aldehyde odorants, which are specific components of the *M. Sexta* sex pheromone [22].

In addition to applications in insects, differential display is used to study the interaction between host snails and parasites. Several studies have identified changes in gene expression in the snail *Bomphalaria glabrata* following infection with *Schistosoma mansoni* [23, 24]. Recently, Schneider and Zelck used differential display to identify differentially expressed mRNAs between schistosome-resistant and schistosome-susceptible snail hemocytes [25].

In contrast to identifying differentially expressed genes in the infected host, several studies have used differential display to isolate differentially expressed genes in infecting protozoan parasites. For example, Milner et al. studied *Trypanosoma brucei rhodesiense*, the causative agent of the acute form of human sleeping sickness. They identified a serum-resistance-associated protein that is preferentially expressed in cell lines resistant to the trypanosome lytic factor [26]. In addition, a new developmentally regulated gene from amastigotes of *Leishmania mexicana mexicana* has been isolated [27]. Moreover, differential display has been used to identify differences between mRNAs representing different asexual erythrocytic stages of *Plasmodium falciparum* [28].

In marine invertebrates, differential display has been used to identify genes that are responsive to environmental toxicants. For example, differentially expressed mRNAs have been identified in the soft-shell clam, which was exposed to dioxin, and coral that was exposed to permethrin and copper [29–31].

In nematodes, several studies have applied differential display to study gender-specific, oxidative stress-inducible and metal stress-responsive genes. Llado et al. investigated gender-specific gene expression in the plant parasitic nematode *Heterodera glycines* [32]. In addition, Michalski et al. used differential display and the electronic subtraction approach for the analysis of gender-specific gene expression in the mosquito-borne filarial nematode *Brugia malayi* [33]. To investigate the effects of oxidative stress on gene expression in nematodes, Tawe et al. applied differential display and identified four oxidative stress-responsive genes. They identified the detoxification enzyme glutathione S-transferase in the non-parasitic nematode *Caenorhabditis elegans* [34]. In the filarial nematode *Onchocerca volvulus*, Liebau et al. applied differential display to identify the glutathione S-transferase (*Ov*-GST-3) that is differentially expressed following exposure to oxidative stress [35]. Liao and Freedman used differential display to identify cadmium-responsive genes in the nematode *C. elegans* [36]. Fifty-three differentially expressed mRNAs, which correspond to the products of 32 independent genes, were identified.

The nematode *C. elegans* as a model system for toxicological research

C. elegans was used to investigate molecular mechanisms associated with heavy metal exposure. *C. elegans* provides an excellent model system for obtaining an integrated picture of cellular, developmental and molecular aspects of transition metal toxicity. The adult hermaphrodite is composed of 959 somatic cells and contains highly differentiated muscle, nervous, digestive and reproductive systems [37]. High levels of evolutionary conservation have been observed between *C. elegans* and higher organisms in genes related to regulatory and developmental pathways [38–40]. In addition, homologues of many of the proteins induced as part of the stress response in vertebrates have been identified in *C. elegans*. These include metallothionein, superoxide dismutase, ubiquitin, low and high molecular weight heat shock proteins, glutathione S transferase and catalase [41–46]. One of the major advantages to using *C. elegans* as a model system to identify metal-responsive genes is the magnitude of mRNA and genomic DNA sequence data available [47]. The entire sequence of the *C. elegans* genome has been determined and annotated [48].

To investigate the mechanism by which cadmium affects gene expression in multicellular organisms, differential display was used for the identification of cadmium-responsive gene from *C. elegans*. The transition metal cadmium is considered a serious occupational and environmental health threat. It is continuously introduced into the atmosphere through the smelting of ores and the burning of fossil fuels [49]. Toxicological responses of cadmium

exposure include kidney damage, respiratory diseases, neurological disorders, and lung, kidney, prostate and testicular cancer [50]. Cadmium induces intracellular damage via (i) nonspecific inactivation and denaturation of proteins, by binding to free sulfhydryl residues; (ii) displacement of zinc cofactors from a variety of proteins and (iii) generation of reactive oxygen species, which ultimately oxidize DNA, proteins and lipids. To attenuate the toxic effects of cadmium, cells respond by increasing the steady-state levels of a variety of proteins. The functions of these proteins include the repair of intracellular damage and the removal of the toxicant. To remove the toxicants (i.e. reactive oxygen species, denatured proteins, cadmium) cells activate the transcription of genes that encode proteins that scavenge reactive oxygen species [51–55]. Damaged proteins are repaired or degraded through interactions with low and high molecular weight heat shock proteins and ubiquitin [56, 57]. Cadmium is removed from the cell through chelation by metallothionein, or is exported by means of metal ion pumps [58–60].

Discovery and identification of novel cadmium-responsive genes

The overall strategy used for discovery of novel cadmium-responsive genes in *C. elegans* is presented in figure 1. RNA samples from control *C. elegans* and those exposed to cadmium for 8 and 24 h were first reverse-transcribed with one of four sets of degenerate anchored 3′ oligo(dT) primers. After reverse transcription, each of 20 decamers was paired with one of four $T_{12}MN$ anchored primers, and then used to amplify cDNA prepared from untreated and cadmium-treated *C. elegans* (80 reactions for each cDNA pool; 240 total reactions). Included in the reaction was an ^{35}S-radiolabeled deoxynucleotide used to visualize the amplified products by autoradiography. Amplified products generated from different populations of mRNAs (i.e. cadmium-treated and untreated) were then compared in a side-by-side fashion after gel electrophoresis. The products of this study are the 3′ ends of cDNAs whose levels of expression are changed following cadmium exposure.

In separate experiments, individual primer pairs produced >95% equivalent banding patterns (i.e. reproducibly amplified cDNA fragments). In contrast, patterns were markedly different when alternative primer pairs were used. All amplification experiments were performed in duplicate using RNA prepared from duplicate populations of cadmium-treated and untreated *C. elegans*. Only cDNAs whose levels of expression were affected by cadmium in duplicate experiments, or at multiple cadmium exposure times were selected for further analysis, of which 53 cDNAs were successfully isolated.

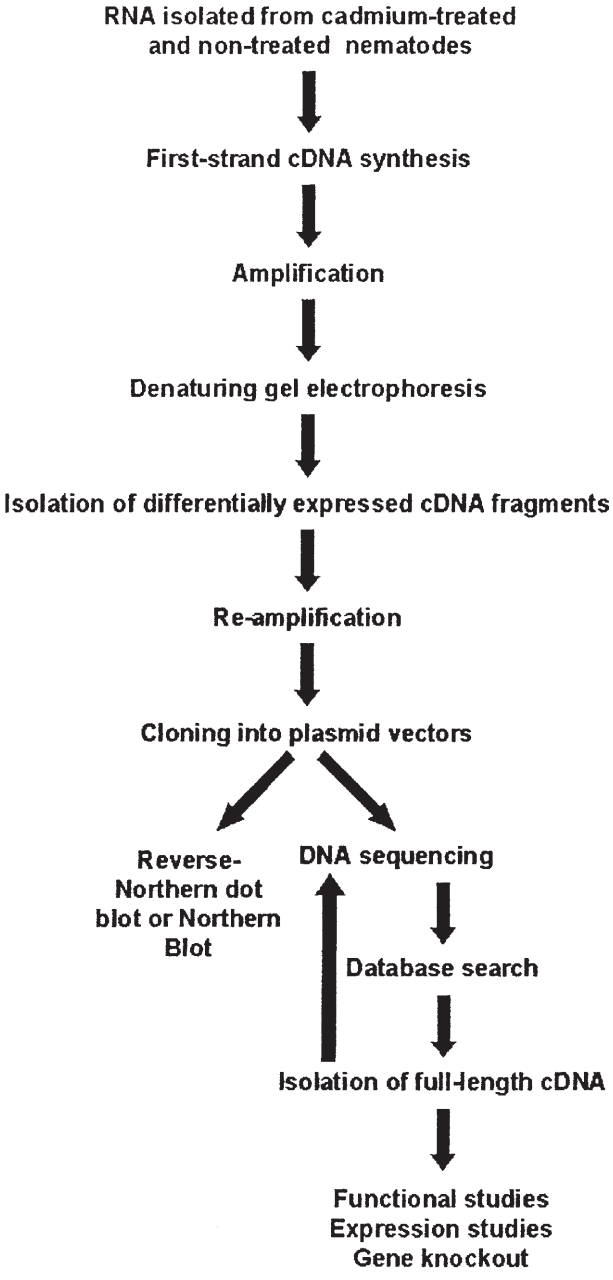

Figure 1. Strategy for discovery and characterization of cadmium-responsive genes in *C. elegans*.

Subsequently, these cDNAs were sequenced, and the sequence data were analyzed.

Cautions and modifications taken to execute differential display analysis

The advantages of differential display are numerous: (i) only a small amount of starting material is required; (ii) simplicity is a feature of all key techniques; (iii) it is very

sensitive in detecting induced and repressed gene expression; it allows (vi) 'side-by-side' comparisons of multiple samples and (v) rapid identification of a cDNA and confirmation of the result; and (vi) the differentially displayed cDNAs can be rapidly isolated and sequenced. Thus, a unique mRNA sequence can quickly be obtained and compared with those in databases. These characteristics make this technique increasingly popular for detecting and isolating differentially expressed genes. Differential display has several additional advantages for studies of gene expression in invertebrates. Primarily, prior DNA or mRNA sequence information is not required for the amplification of differentially expressed mRNAs. For the majority of invertebrate species little or no sequence data is currently available. Thus, differentially display may be the only technique available for the identification of large numbers of differentially expressed mRNAs.

Although differential display offers significant advantages, many investigators note drawbacks and limitations. One of the major difficulties with differential display is the potentially high rate of false positives, amplified cDNAs that do not represent mRNA whose level of expression is altered [61]. The high incidence of false positives may occur for several reasons. These include (i) chromosomal DNA contamination in RNA samples; (ii) the use of short primers and low annealing temperatures in amplification reactions and (iii) cloning errors arising from contaminating DNA present in cDNAs excised from gels. To eliminate false positives, it is recommended that minimally duplicate experiments be performed; triplicate or quadruplicate replicates will further reduce the number of false positive results. Ideally, RNA should be prepared from replicate samples then used in the reverse-transcription reaction [62]. In addition, the use of longer anchor primer (≥ 22 nucleotides) or a 'hot start' in amplification reactions have been shown to improve the reproducibility in differential display analyses [63–65]. To determine whether the RNA samples have chromosomal DNA contamination, control amplification reactions should be performed that do not contain a reverse transcriptase step [62].

Several methods have been developed to confirm altered gene expression in products derived from eluted differential display cDNAs. These approaches include Northern blot analyses, using the eluted differential displayed cDNA as probe; reverse-Northern with Southern blot, slot- or dot-blotted clones, in combination with amplified RNA as probe [66]. For large-scale screening of candidate cDNA fragments, reverse-Northern analysis is preferred [67]. To confirm changes in expression of the cadmium-responsive gene identified in *C. elegans,* both conventional Northern blot and reverse-Northern dot blot analyses were used. The reverse-Northern analysis, confirmed that 49 of the cognate RNAs identified by differential display showed two- to six-fold increases in levels of expression following cadmium exposure (fig. 2). In our experience, the reverse-Northern analysis appears to be a more sensitive assay than conventional Northern blotting. The lack of sensitivity in the conventional Northern analysis for the confirmation of differentially expressed mRNAs may be due to the short lengths of cDNA used as probe. The differentially displayed cDNA fragments (<360 bp. in the *C. elegans* study) usually fail to generate ^{32}P-labeled probes with sufficient specific activities to detect low abundance mRNAs [68].

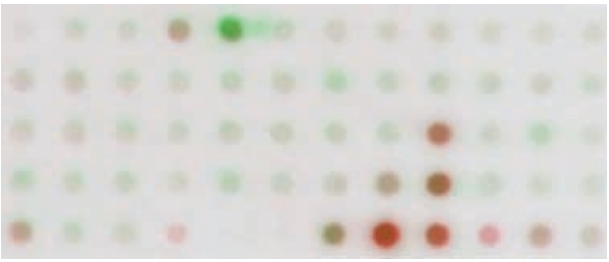

Figure 2. False color image of reverse-Northern dot blot of cadmium-responsive genes from *C. elegans*. Red, cadmium-inducible gene; green, cadmium-repressive gene.

The majority of cDNA fragments isolated using differential display typically contain 3′-untranslated regions of the mRNAs, and not a large amount of coding region sequence. For invertebrates, large amounts of genomic or expressed sequence tag (EST) sequence data are not available in public databases. Thus, labor-intensive full-length cDNA screening or RACE may be needed to obtain sufficient qualities of sequence data that can be used for sequence homology searches, gene classification and protein prediction [66]. In order to identify proteins that are encoded by the cadmium-responsive mRNAs identified by differential display, the data that were available as part of the *C. elegans* Genome Project data were used. The nucleotide sequences of many of the cadmium-responsive mRNAs are identical to EST clones and predicted cDNAs identified by the project. By assembling contigs consisting of the differentially expressed cDNA sequence and the related *C. elegans* EST sequences, longer open-reading frames were generated and could then be analyzed. Similar strategies may be used in the future as genomic and EST sequence data become available for other invertebrates (e.g. schistosoma, mosquito, *Drosophila*, other *Caenorhabditi*).

Conclusion

Despite its limitations and the development of a variety of methods for high throughput analysis of differential gene expression (i.e. microarrays), differential display is still the most widely used approach for analysis of gene ex-

pression. Since it is based on simple, well-established techniques, it can be employed in virtually any standard molecular biology laboratory. This makes differential display easily applicable for most researchers in diverse fields. Consequently, it is possible for researchers who study invertebrates to compare the molecular changes associated with differentiation; exposure to environmental agents (hormones, toxicants, infectious agents, environmental changes) and animal activity.

The application of differential display for the isolation of novel genes associated with various biological conditions in invertebrates will contribute to our understanding of the molecular mechanisms of homologous biological processes in higher organisms. However, differential display is the first of many steps required to elucidate molecular mechanisms. Differential display detects changes in mRNAs associated with a biological challenge. However, it does not indicate the mechanism of altered gene expression. Therefore, further functional characterization of a particular gene of interest should be performed. These include expression of the gene products for functional studies, mapping the modulated genes, determination of cellular patterns of expression, gene knockout studies and the identification of functional homologues in other species.

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