Molecular analysis of the responses of *Caenorhabditis elegans* (Bristol N2), *Panagrolaimus rigidus* (AF36) and *Panagrolaimus sp.* (PS 1579) (Nematoda) to water stress

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(ABSTRACT)

This work provides a comparative and genetic analysis of the responses to water stress in desiccation-tolerant and desiccation-sensitive nematodes. *Caenorhabditis elegans*, a model organism for the study of development, aging, and cell biology was shown to be a desiccation-sensitive organism that survives relative humidities above 40% for periods of up to seven days. Transcripts from the desiccation-tolerant species *Panagrolaimus rigidus* AF36 and *sp.*PS1579, which were expressed uniquely during separate desiccation and osmotic stresses, as well as during recovery from exposure to the dual stresses, were cloned. These sequences were used to search for similarities in the genome sequence data of *C. elegans*. Putative anhydrobiotic-related transcripts were identified that potentially encode heat shock protein 70, late embryogenic abundant protein, and trehalose-phosphate synthase. Other putative genes that were identified within eight separate libraries encode proteins involved in transcription (histones), protein biosynthesis (ribosomal proteins, elongation factors), protein degradation (ubiquitin, proteases), and transport and cell structure (actin, collagen). Gene ontology analysis of the cloned transcripts revealed that developmental processes are activated during exposure to the stresses as well as during recovery, which may suggest a rejuvenation process as a key to survival in *Panagrolaimus* nematodes. Genes that were up-regulated during desiccation stress in *C. elegans* were classified as belonging either to an early response (until 12 hours of stress), or to a late response (after 12 hours of stress). The early response was characterized by the up-regulation of a large number of genes encoding mono-oxygenases, which may suggest onset of oxidation stress during desiccation of *C. elegans*. The late response was characterized by the appearance of transcripts encoding proteins of the immune system, heat shock proteins (protein denaturation), and superoxide dismutases (oxidation damage). Genes in *C. elegans* that were down-regulated in response to desiccation stress include those encoding proteases and lysozymes (metabolic shutdown). Genes that encode channel proteins (water homeostasis) were found among the transcripts up-

regulated during recovery of *C. elegans*. The up-regulation of *gpdh-1* and *hmit-1.1*, two transcripts linked to hyperosmotic stress, suggest that osmotic stress is experienced by *C. elegans*. Comparison of these data with those obtained from exposure of *C. elegans* to a range of other stresses showing that the nematode *C. elegans* uses specific transcripts for the desiccation response; transcripts that are not induced in other stresses such as heat, anoxia or starvation. In addition, transcripts regulated during desiccation stress of *C. elegans* were also regulated during dauer formation, which may indicate common stress tolerant mechanisms. Recent studies in mammalian cells and *C. elegans* have shown that microRNAs are able to degrade and to sequester mRNA especially during stress in so called stress bodies. In this study, *C. elegans* microRNA knock-outs showed a significant decrease in desiccation stress survival compared to wild type *C. elegans* which may suggest the importance of microRNAs for stress survival in *C. elegans* and other organisms.

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Literature review

1.1 Significance of work

Anhydrobiosis is the ability of an organism to survive long term exposure to dryness. Under conditions of desiccation the organism dehydrates until all metabolism stops. The addition of water results in rehydration and resumption of growth and normal life activities. Investigating this peculiar phenomena may lead to a better understanding of why water is so important for all biological processes [30]. Previous research on desiccation tolerance focused on microbes (e.g. yeast, cyanobacteria), plants (algae, resurrection plant), and simple invertebrate animals (tardigrade, brine shrimp) [15, 36, 67, 75, 144, 170, 213]. This work constitutes the first comparative analysis of genetic control of desiccation responses in two desiccation-tolerant nematodes (*Panagrolaimus*) and the desiccation-sensitive *Caenorhabditis elegans*. The anhydrobiotic *Panagrolaimus* nematodes are suitable model organisms for this research because they are easy to culture and have multiple differentiated tissues that all undergo desiccation, which implies the existence of an organism-wide mechanism for anhydrobiosis (life without water).

To fully appreciate anhydrobiosis and its ramifications for an organism, it is important to remember that organisms without cellular water cannot carry out fundamental biochemical tasks such as the assembly of proteins or their interactions [30]. Desiccation-tolerant organisms have evolved a number of mechanisms to survive desiccation, including morphological changes (e.g. cyst formation, changes in membrane permeability) and the biosynthesis of stress specific molecules (e.g. trehalose, late abundant embryogenesis (lea) protein). It should be noted, that desiccation-tolerant organisms, when they are in anhydrobiosis, can also withstand major stresses like radiation [204], high pressure [163], heat [35], and vacuum [208]. Investigating the molecular mechanisms of desiccation tolerance is important to understand the involvement of water in cells, and how to preserve biological material in the absence of water [42, 211].

Filenchus polyhypnus was recorded to have survived for 39 years in a desiccated moss sample [127]. Unlike that nematode, most eukaryotic organisms cannot survive such conditions. Being able to preserve organic material for many years at room temperature, like the nematode would have great benefits [20, 211]. The medical field would be able to extend the ability to preserve and to transport organs for transplantation, store blood at room temperature, transport medicine in regions of need without expensive preserving equipment. Furthermore, cells stored in facilities such as the ATCC (American Type Culture Collection) would benefit from being able to store all their cell lines at room temperature, as opposed to expensive cryopreservation.

This potential is recognized by the corporate sector, which have attempted to engineer tissues that can be dried. This field is called anhydrobiotic engineering. First, cell lines

expressing trehalose have been shown to be able to survive moderate desiccation treatments [91, 188]. However, despite this initial success, the mechanism of anhydrobiosis remains unknown.

This work attempts to contribute to our understanding of anhydrobiosis by comparing the genetic responses of desiccation tolerant and desiccation-sensitive nematodes during and after the desiccation stress.

1.2 Anhydrobiosis - background

1.2.1 Definition

In 1702, the Dutch microscopist Anton Van Leeuwenhoek observed the revival of seemingly dead belloid rotifers from a desiccated sample [213]. Nearly 250 years later David Keilin was the first person to describe anhydrobiosis: ‘a particular state of an organism when it shows no visible sign of life and when its metabolic activity becomes hardly measurable, or comes reversible to a standstill’ [99], which he called ‘cryptobiosis’. The state David Keilin describes can be entered through three different ways: anhydrobiosis, cryptobiosis, and anoxybiosis [35]. Anoxybiosis describes a state of an organism when it suffers an acute lack of oxygen. As a result it goes into a latent state and, in contrast to anhydrobiosis and cryptobiosis, does not desiccate. Cryptobiosis is reached when an organism experiences extreme low temperatures. The organism desiccates to avoid formation of water crystals. Anhydrobiosis (life without water) is simply triggered by environmental desiccation. Anhydrobiosis confers an extreme stress tolerance, as an organism not

only survives the lack of water from the environment, but experiences loss of all free water.

Environmental Desiccation is a challenge faced by many animal and plant on land. However desiccation tolerance is used in the literature to describe a wide range of conditions, from partial dryness experienced by intertidial algae to complete dryness of truly anhydrobiotic organism like tardigrades [4]. The best definition is that organisms can only be called anhydrobiotic when they are able to withstand periods of total dryness, and desiccation-sensitive when they require at least 10% water content in their body to survive. Specifically, truly desiccation tolerant organisms should be able to tolerate cellular water content of less than 0.1 grams of water per gram dry mass [35].

1.2.2 Common mechanisms involved in desiccation tolerance

Examples of desiccation tolerance can be found in nearly every phyla [4]. Thus, it is possible that anhydrobiosis evolved more than once, and that there may not be a conserved mechanism of desiccation tolerance. In support of this hypothesis, it has been observed that some organisms synthesize specific sugars upon desiccation stress, while other anhydrobiotic organisms do not [112]. However, despite the synthesis of unique molecules during the desiccation response, there are common mechanisms that can be found among diverse organisms, and the following section describes some of these.

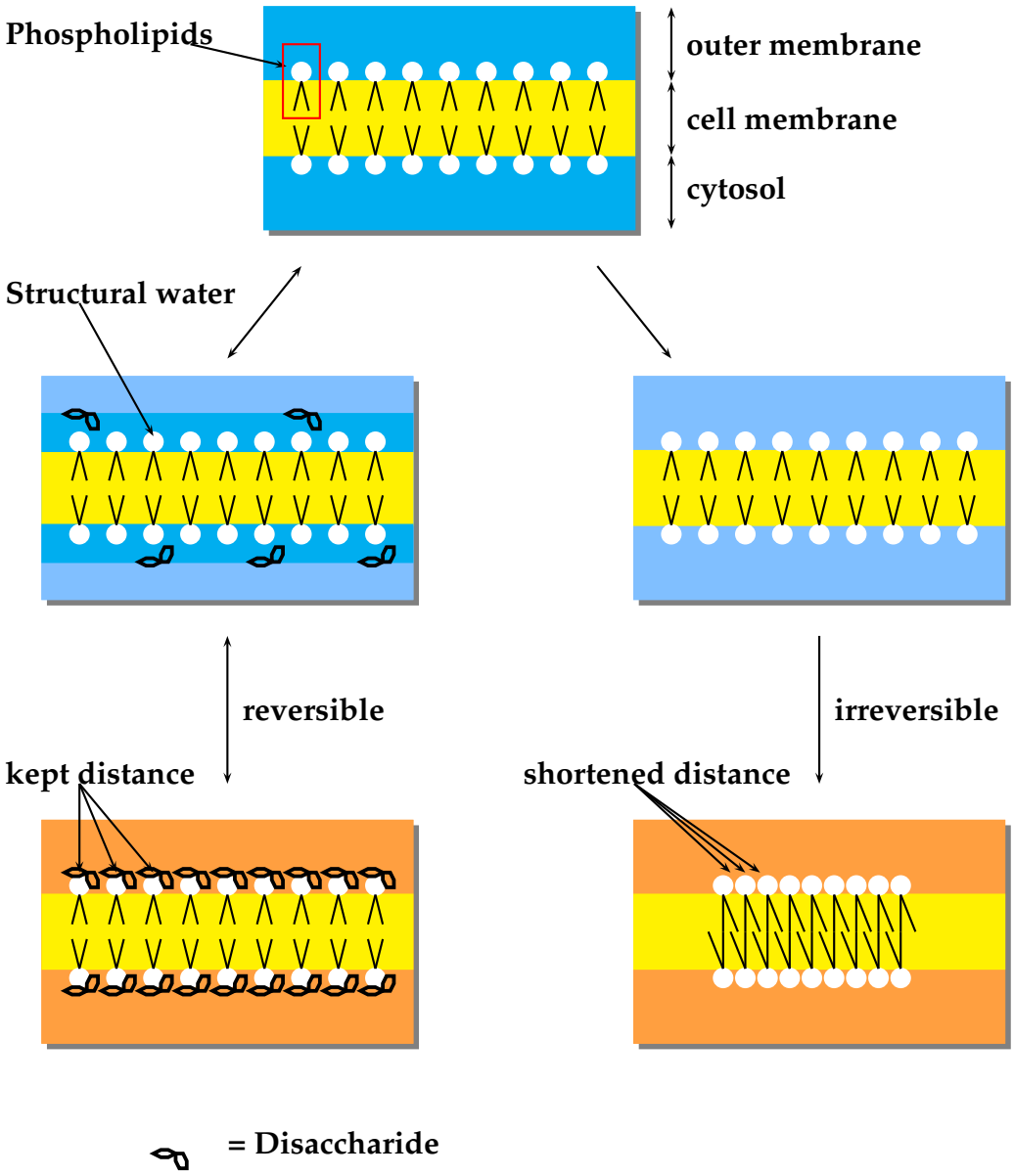


Figure 1.1: Water replacement hypothesis schema - In fully hydrated cells (top figure), membrane lipids are in an undisturbed liquid-crystalline state. Upon water loss (intermediate water contents), the presence of preferentially excluded solutes (sugars) in tolerant cells (middle left figure) keeps the membrane surface preferentially hydrated (indicated by the blue band) and prevents membrane fusion. The absence of these solutes in the sensitive cells (middle right figure) might result in membrane fusion. On further drying, the sugar molecules (i.e. trehalose) in tolerant cells replace water in the hydration shell of the membranes, thereby maintaining the spacing between phospholipid molecules. The bilayer remains in the liquid-crystalline phase (bottom figure left). In sensitive cells, the removal of water from the hydration shell in the absence of sugars results in packing of the phospholipid molecules, which leads to a phase transition into the gel phase. This might lead to irreversible membrane damage (bottom right figure).

Water replacement theory and glassy state hypothesis

A characteristic of many anhydrobiotic organisms during entry into the desiccated state is the synthesis of non-reducing disaccharides such as trehalose. Trehalose is believed to replace the water molecules on the polar head groups of the membrane-phospholipids upon desiccation. There are at least three different pathways describing the biosynthesis of trehalose. The most widely found pathway involves the transfer of glucose from UDP-glucose to glucose 6-phosphate to form trehalose-6-phosphate and UDP. The enzyme catalyzing this reaction is trehalose-phosphate synthase (TPS) [62]. The water replacement [44], and the vitrification or 'glassy state' hypothesis [39] are the best explanations for the roles of non-reducing disaccharides in desiccation stress.

The water replacement hypothesis simply states that nonreducing carbohydrates replace water at membrane surfaces. This is important as it keeps the membranes in their natural, less ordered liquid crystalline state (see Figure 1.1 on page 5). Specifically, when the water is removed around cellular membranes by desiccation, the spatially separated polar headgroups of the phospholipids are packed together, thus increasing the density of the headgroups in the membrane. This increased packing in turn leads to increased opportunities for Van-der-Waals interactions among the hydrocarbon chains [41, 186]. The acylchain interactions increase in the phase transition temperature (Tm); the temperature at which the phospholipid membrane are converted from a highly ordered phase (gel phase) to a less ordered one (liquid crystalline) [40, 212]. This increase has serious consequences, as during entry into and exit from a desiccated state phospholipids have to pass through this phase transition. The simultaneous existence of both phases can lead to phase separation and disrupted membranes [43, 58]. Thus, this transition has to be avoided. Non-reducing disaccharides depress the Tm of membranes during desiccation

such that the membranes are kept in the liquid crystalline phase at room temperature, even though they are dry [44].

The process of vitrification or formation of the 'glassy state' within the cells occurs when the water content in the cytoplasm drops below 0.1 grams of water per gram of dry weight. This state is characterized by a bulk reduction in chemical reactions and molecular diffusion. As the name suggests, the cytoplasm changes from a liquid to an amorphous glass-like state [104]. A glass is a liquid of high viscosity. In a vitrified state molecules have limited motion but are not permitted to aggregate [83, 173]. This produces stability to the living system, in essence the molecules are locked in time and space and avoid interactions that can lead to precipitation [26]. The viscous glass can then be melted again by addition of water. This restores the normal metabolism of the cell. This glass has a specific glass-to-liquid phase transition temperature (Tg), which is dependent, apart from the temperature, on the molecular weight of the carbohydrate, the chemical composition, and the water content of the cytoplasm in the cells [39, 83].

Late embryogenesis abundant (Lea) protein and amphiphiles

The Lea proteins are the best studied proteins associated with desiccation stress. The first Lea protein was discovered in a cotton plant *Gossypium hirsutum* and was found in high abundance in plant seeds [187]. Lea proteins are subdivided into six different groups based on their amino acid sequence [183] and can be found in microorganisms, plants, and animals. Despite 20 years of research on these proteins, their functions are still unclear [187]. One current theory is that Lea proteins prevent aggregation of macromolecules during desiccation and heat stress [74]. A unique characteristic was shown

during hydration of a Lea protein: it changes conformation. In hydrated environments, Lea proteins have an unfolded confirmation while upon desiccation the proteins acquire an alpha-helical and coiled coil structure [73, 183]. Furthermore, it was shown that a recombinant Lea protein (AavLEA) from the nematode *Aphelenchus avenae* did not behave like a classical molecular chaperone, but exhibited a protective, synergistic effect in the presence of trehalose. AavLEA was shown to be able to protect a protein from aggregation due to desiccation and freezing [74].

Amphiphiles are chemical compounds that have both hydrophilic and hydrophobic properties and are normally present in the cytoplasm of the cell. Typical endogenous, biologically relevant amphiphiles are the phenolic substances present in dry seeds, pollen and resurrection plants [21, 143, 160]. Upon desiccation endogenous amphiphilic substances partition into the membrane, whereas rehydration causes the amphiphiles to return to the cytoplasm [72]. The movement of amphiphiles to the membrane, along with non-reducing sugars like trehalose, potentially leads to an increase in the Tm of the membrane. This would have the consequence that, upon rehydration, the membrane would pass through a phase transition and become leaky [82]. The reason amphiphiles are incorporated into the membrane is probably their beneficial function. Amphiphiles such as arbutin have shown to posses antioxidant properties suggesting a compromise between potentially harm the membrane integrity and saving the organism from free oxygen radicals during anhydrobiosis. [144, 160].

Other common responses

The synthesis of osmolytes is another mechanism to compensate for an increase in osmotic pressure that occurs during desiccation and is present also during desiccation. The osmolytes have mainly two important functions: first, balancing intracellular osmotic pressure and second, compensate for perturbation of macromolecules. Typical osmolytes are sugars and polyols, amino acids, methylated ammonium salts, sulfur compounds and urea [145]. Evidence suggests a pH shift to a more acidic environment helps organisms to cope with water stress [48, 185, 199, 201]. Heat shock proteins (HSP) [53, 56], FoxO genes [184], and superoxide-dismutase [37, 193] have also been linked to desiccation stress. Aquaporins [88, 101, 135, 197] and anion channels [51, 103] are specific membrane channels that control cellular osmotic and water homeostasis during desiccation stress. A group of transcription factors up-regulated during desiccation stress, belonging to the Leucine-zipper group have also been identified [49, 190].

Summary of desiccation responses

In summary, the following mechanisms are believed to occur during desiccation. Cellular membranes are kept in a gel-phase during desiccation by non-reducing disaccharides such as trehalose (Water replacement hypothesis). In order to prevent oxidative damage of the membrane during anhydrobiosis, amphiphilic antioxidants are incorporated into the membrane. The stability of proteins and cellular structures are achieved by forming a glassy state via polysaccharides (glassy state hypothesis) and LEA protein biosynthesis. Additionally cellular channels such as aquaporins control the efflux of water form the cell. These are the main mechanisms believed to assure the organism a safe transition into

and out of an anhydrobiotic state.

1.2.3 Organisms under investigation

Mechanisms involved in desiccation tolerance have been investigated in diverse desiccation tolerant organisms such as resurrection plants, nematodes, yeast, and non-desiccation tolerant organisms, such as *Arabidopsis* and *Drosophila*. Here are presented short reviews about discoveries on desiccation stress tolerance research in microbes, plants, and small animals. As anhydrobiosis (water stress) confers also resistance to radiation, salt, and oxidation stress [4, 102] it is possible, that the same protective mechanism is used in other stress responses. Therefore, results from other applicable stresses are included as well.

Microbes

Studies on the cyanobacterium *Anabaena sp.* PCC 7120 have shown that about 300 genes are differentially expressed during desiccation stress [81], and mainly involves genes responsible for DNA repair and NAD synthesis. A proteomic analysis of *Nostoc commune* under UV stress has identified nearly 500 proteins involved in the UV response [60]. One half of these proteins were involved in an early stress response and the other half belonged to the long lasting stress response group. A characteristic stress response, which is also observed in *Pseudomonas putida* [29], is the formation of extrapolysaccharide, which is thought to protect the cell from radiation and rapid water loss, and to stabilize cell structures during desiccation [177]. Water stress protein [162] and scytone-

min, an ultra-violet (UV) protective pigment [68], are two unique stress response elements often found in terrestrial cyanobacteria.

Several *Deinococcus* species exhibit extreme desiccation tolerance [13, 178], as well as resistance to ionizing radiation (IR) [46, 168] and ultraviolet light (UV) [100, 214]. The *Deinococcus* DNA repair mechanism appears to function more efficiently than that of any other known organism. Despite recent progress, the molecular pathways responsible for enhanced DNA repair in *Deinococcus* remain still widely unknown [46, 123].

Escherichia coli has been investigated for osmotic stress responses. The results showed a pool of 152 genes to be regulated, 107 down- and 45 up-regulated. Most of the up-regulated genes are unclassified, unknown or hypothetical [205].

Cellular arrest is a common stress response of the yeast *Saccharomyces cervisiae* subjected to desiccation [170], salt [3] and UV stress [18]. Apart from the common stress-related transcripts (e.g. heat shock proteins) and synthesis of sugars (trehalose), an investigation during starvation stress showed a new mechanism of stress tolerance: the formation of actin bodies [159]. These bodies are hypothesized to provide storage for building blocks of the cytoskeleton after the stress. It is possible that these bodies are also formed upon other stresses. Furthermore, it was shown that trehalose is not necessary for *Saccharomyces cervisiae* to achieve desiccation tolerance [155].

Plants

The resurrection plants such as *Craterostigma plantagineum* [12] and *Myrothamnus flabellifolia* [133] are the best studied examples of anhydrobiosis in plants. During desiccation, expression of a large number of transcripts that involve the protein synthetic machinery, ion and metabolite transport, and membrane biosythesis/repair are detected [144]. Gene products such as late embryogenesis abundant (Lea) protein were identified in the cytoplasm and in chloroplasts, and other stress induced transcripts include genes involved in abscisic acid biosynthesis [116], sugar synthesis for structure and vitrification [194], regulatory proteins such as protein translation initiation factors, homeodomain-leucine zipper genes [54], superoxidase dismutases (SOD) [193] and a gene probably working as a regulatory RNA (for review see [15]).

Arabidopsis thaliana as model system for plants has also been investigated for its response to water stress. Noteworthy here is a study in which *A. thaliana* was exposed to heat and desiccation stress at the same time. The goal was to see whether the response to multiple stress is a sum of each stress response. *Arabidopsis* was shown to synthesize osmolytes during both stresses. Proline was synthesized as a response to heat stress and sucrose as a response to desiccation stress. The investigators queried whether the plant would produce both osmolytes or only one upon combined desiccation and heat stress. *Arabidopsis* produced only sucrose. This result suggests that one stress, in this case desiccation stress, can also confer tolerance to another stress, in this case heat shock [157].

Small animals

All known anhydrobiotic animals share two common characteristics: they are no larger than 5 mm and have no exoskeleton [4]. One of these animals is the tardigrade (or water bear, Tardigrada). During desiccation the animal shrinks, retracting all its extremities until only a round 'tun' is visible with no sign of life. In this state the tardigrade can not only survive desiccation but can also resist radiation, space vacuum and high pressure [102].

Another well studied anhydrobiotic animal is the brine shrimp (e.g. *Artemia salina*), although only the cyst of the animal is desiccation-tolerant. The cyst is a life stage where the organism survives as a latent life form inside a membrane that is sometimes formed by an old cuticle. Research on the cyst has revealed two novel proteins, ferritin and artemin [32], which are synthesized during desiccation stress, in addition to commonly known proteases [140] and heat shock proteins [118].

The largest known anhydrobiotic animal is the African chironomid *Polypedilum vanderplanki* [101, 202, 204]. The only desiccation-tolerant stage of this animal is the larval stage. Anhydrobiosis was shown to be achieved even in the absence of brain function [202], and trehalose biosynthesis could be induced by simply shifting the internal ion concentration [203]. These results suggest that anhydrobiosis is directly triggered by environmental clues and does not require the involvement of a central signaling mechanism triggered by a nervous system.

The desiccation tolerances of *Drosophila* species have also been investigated. A comparison between a laboratory grown population selected for desiccation tolerance and a

population from a desert showed that adaptation to increased desiccation conditions can be achieved by selection [70]. One particular feature of the laboratory strain that could be adaptive to desiccation stress was a prolonged larval stage and a higher accumulation of carbohydrates and water in the body [69]. Another study comparing gene expression analysis between the response to cold and desiccation stress showed differences between the two responses, which supports the idea of specific responses to specific stresses [169]. Furthermore, studies with the fly *Sarcophaga bullata* indicate temporarily distinct responses to acute stresses like desiccation: an early response and a long term response. This finding stands in agreement with the data from studies with cyanobacteria above [217]. In contrast to the *Polypedilum* studies, the work on *Sarcophaga* indicates a role for the central nervous system in the stress response.

In summary the numerous studies in diverse organisms, the synthesis of specific sugars (e.g. trehalose, sucrose) to form the glassy state and to avoid phase transition of the membranes during desiccation stress seems to be a widely utilized defense against desiccation stress. The synthesis of specific proteins to avoid oxidation (e.g. superoxide dismutase), protein denaturation (e.g. heat shock protein), and protein aggregation (e.g. late embryonic abundant protein) is also seen in a large number of organisms in response to desiccation.

Recent studies investigating differences among stress responses at the organism level show that environmental clues and the presence of a signaling system play crucial roles in determining the resulting stress response. Organisms have been shown to induce one response to several different stresses even the response to the single stress could have been induced (e.g. desiccation and freeze response [209], osmotic response and des-

iccation response [203]). Organisms subject to different contemporary stresses by the environment have been shown to induce one response, which confers also resistance to several other stresses, in contrast to induce resistance to each specific experienced stress (e.g. heat stress and desiccation tolerance induction [157]). Studies have also shown that different stages of stress response exist, i.e. an early and a long term response [60]. This suggests a central signalling system in higher organisms to trigger and regulate the appropriate timed response.

1.3 *Caenorhabditis elegans* and *Panagrolaimus*

1.3.1 The phylum Nematoda

Nematodes are a diverse group of invertebrates that exist as parasites or as free-living forms in soil, freshwater, and marine environments. They are bacteriovores, fungivores, omnivores, predators, and plant-parasites. The most general life cycle of a nematode involves an egg, four larval stages (L1 to L4) and the adult. The life cycle of some nematodes includes specialized stages for resisting environmental stresses, such as a protective cyst or dauer [127].

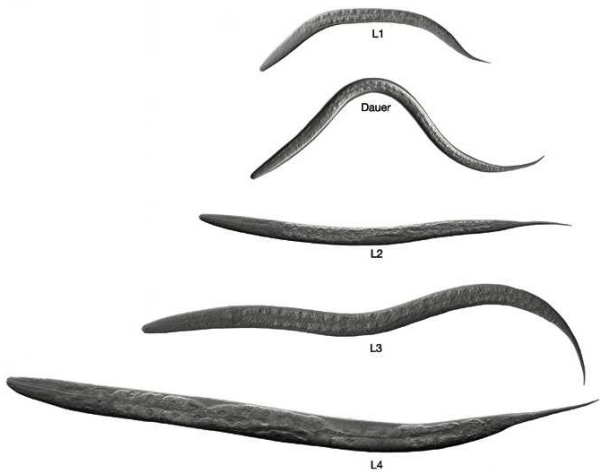
1.3.2 Model systems

Caenorhabditis elegans

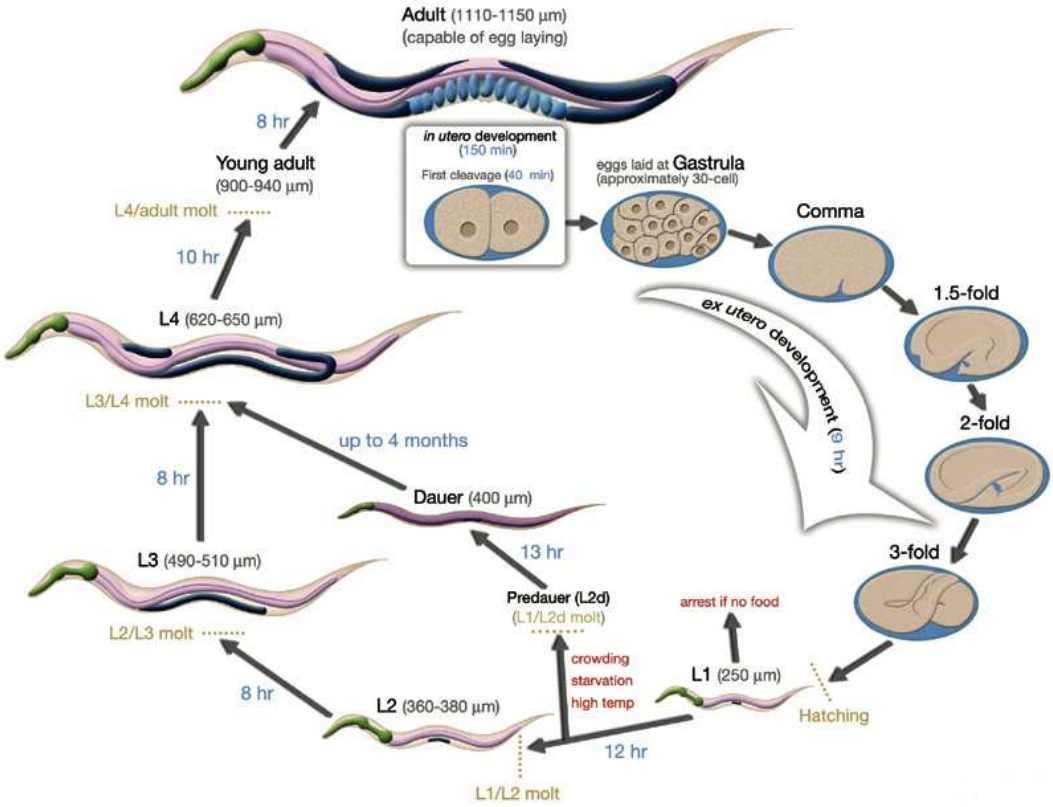
Several attractive features of *C. elegans* biology have contributed to its choice as a model organism for the study of genetics, developmental biology, neurobiology, cell biology and behavior. It is easy to maintain in the laboratory, growing quickly on a bacterial lawn grown on an agar plate. Its small size (about 1.3 mm in length and 80 microns in diameter), invariant anatomy and transparent body have made it possible to describe the complete cell lineage from the single cell embryo to all 956 somatic cells in the adult and also to map all the connections between nerve cells [5].

In 1998 *C. elegans* became the first multicellular organism for which a complete genome sequence was obtained. Its genome has 100 Mb and is about 30 times smaller than the human genome, but it is predicted to contain 21000 protein-coding genes, which is about the number humans have [19, 34]. About 35% of *C. elegans* genes are closely related to human genes. The recent discovery of RNA interference (RNAi) processes added another useful gene manipulation tool (as well as a layer of complexity) for this popular model organism [65].

C. elegans has a short generation time, going from a single-cell egg to an adult in 3.5 days at 20°C (see Figure 1.2 on page 17). The growth period can be easily regulated by temperature. By lowering the growth temperature to 15°C, the development takes about twice as long. After the embryo hatches and goes through four larval stages, it becomes a mature adult. *C. elegans* can also adopt an alternative lifestyle during the mid-first larval stage, called dauer larva. The dauer stage is induced in response to overcrowding or the



(a) Life stages of *C. elegans*



(b) Life cycle of *C. elegans*

Figure 1.2: *Caenorhabditis elegans* life cycle and life stages taken from Wormatlas [5]

absence of adequate food supplies. Dauer larvae can remain viable for as long as three months, while it roams slowly in search of food. Dauer larvae is the most common form of *C. elegans* found in the wild. When adequate food resources are located, the nematode exits the dauer stage and proceeds to larval stage four [87].

The genus *Panagrolaimus*

Members of the genus *Panagrolaimus* are bacteria feeding nematodes that occupy a diversity of niches ranging from Antarctic, temperate and semi-arid soils to terrestrial mosses. Among these exist anhydrobiotic species, which can be divided into fast and slow dehydration strategists, and desiccation-sensitives species [165]. Thus the phylogenetic relationship of the genus *Panagrolaimus* is majorly based on morphometric data and is not fully investigated, so that many *Panagrolaimus* species remained unassigned to species level [165].

Research has concentrated on the freeze-tolerant *Panagrolaimus davidi*. The nematode shows two different cold tolerance mechanisms, one of them being the production of osmolytes to prevent ice crystals, the other being anhydrobiosis. The choice of the mechanism relies exclusively on the environmental stimuli. When the exposure to freeze-stress starts at temperatures close to the freezing point (i.e. -1°C) the nematode dehydrates rather than freezes [209].

Anhydrobiotic *P. superbus* shows a constant synthesis of trehalose during normal life cycle, which is probably one reason why it can tolerate faster desiccation rates than any

other known species from the genus *Panagrolaimus*. The longest documented desiccation period for a *Panagrolaimus* species, which was also able to mate with a fresh culture of the same species after rehydration, is 8.7 years [8].

The two *Panagrolaimus* species chosen for this work are *Panagrolaimus rigidus* (AF36) and *Panagrolaimus sp.* (PS1579). The first stain was isolated in Pennsylvania, USA, by Andreas Fodor and *Panagrolaimus* PS1579 was isolated in California, USA, by Paul Steinberg. Both species were obtained from the *Caenorhabditis* Genetics Center (CGC). The reasons for choosing these two species is their ability to tolerate both osmotic and desiccation stress and the absence of alternative life stages such as cysts or dauer. These two species of *Panagrolaimus* are able to go into anhydrobiosis at every stage of their life cycle. The choice to analyze two species of the same genus was to identify common transcripts used for desiccation tolerance, which are not species specific.

1.3.3 Nematodes and stress

According to Wharton, nematodes have developed five strategies for dealing with environmental stress such as loss of the moisture film or food: (1) high reproductivity or short generation time, (2) survival strategies such as production of eggs, infective larvae, and dauer larvae, (3) mechanisms for synchronizing their life cycle with the availability of food or hosts, (4) physiological triggers which indicate entry into or the presence of a suitable host or food supply, (5) intermediate hosts in the life cycle of parasitic species [207].

C. elegans has been used to study numerous environmental stresses including UV

[141], ionizing radiation [139], osmosis [110], and oxidation [66], outside of its natural stress resistant dauer stage. *C. elegans* can enter the dauer stage upon stresses such as food shortage and overpopulation during the first larval stage. Alternatively dauer can be induced artificially by using a naturally induced pheromone. The dauer stage is characterized by a linear body morphology, as well as entry into a reduced state of metabolism (quiescence). In recent years, mutants impaired for dauer arrest (dauer deficient (*daf*) mutants) have revealed the involvement of guanylyl cyclase, transforming growth factor-β , insulin-like signaling pathways, and hormonal signaling pathways in the regulation of dauer arrest and exit (for review [87]).

A gene family found in *C. elegans* linked to osmotic stress is the *osr*-gene family. These genes are responsible for osmotic sensing by the worm. *C. elegans* mutants in this gene family retain wild type phenotype and gain resistance to or become unable to sense osmotic stress [45, 171]. Adult wild type *C. elegans* was shown to be able to resist osmotic stress up to 550 mM NaCl on agar, if previously adapted. During the stress the worm produced glycerol as an osmolyte. Supplying the worm with trehalose increased its ability to survive osmotic stress. Knock-outs for *age-1*, a gene that confers longevity [182], and *daf-16*, a fork head transcription factor involved in stress resistance [2], also showed an decrease in osmotic stress resistance [110].

Desiccation tolerance has been studied in the insect-parasitic nematode *Steinerma feltiae*. Approximately 400 proteins were found to be differentially regulated in response to desiccation by two dimensional gel electrophoresis. Seven of these were similar to desiccation responsive proteins found in other organisms [129, 148]. Expression analysis revealed that the *Steinerma*, like plants, expressed LEA-like genes [189]. LEA-like gene

expression was subsequently confirmed in the nematode *Aphelencoides avenae* [73] and *C. elegans* [24].

1.4 Non coding RNAs

In 1998 a mechanism was discovered that silenced a gene by using a double stranded RNA (dsRNA) in *C. elegans* [65]. Subsequent studies found that this process was present in organisms ranging from plants to humans [16, 23, 61, 220]. Later small endogenous RNA molecules, known as microRNAs, were discovered within the *lin-4* gene of *C. elegans* [114]. These molecules are gene expression regulators that inhibit translation of target mRNAs. An overview is shown in Figure 1.3 on page 23 and described below.

siRNA

The starting point of gene silencing is the design of double stranded RNA (dsRNA), that is complementary to the mRNA sequence of the target. Once introduced in the organism (plant, fly, cell) as a hairpin, long dsRNA, or as small 22 nt dsRNAs with overhangs, called siRNA, the silencing machinery will process these molecules. The hairpin and the long dsRNA are cut into small 22 nucleotides (nt) RNAs by Dicer, an RNase III enzyme. The resulting small dsRNAs have now two nucleotides overhangs at their 3' ends and a phosphate group at their 5' ends [128]. Those molecules are now identical to the siRNAs. The newly formed siRNAs assemble into an RNA-induced silencing complex, known as the RISC-complex. The complex incorporates only the guide strand. The passenger strand

is believed to function with other Argonaut-like proteins to potentiate the silencing effect in a process called Flamenco [22]. When the loaded RISC complex finds a perfect complementary sequence, the target mRNA is cleaved and degraded by RISC, with cleavage occurring between the 10th and 11th nucleotide of the guide strand. [154]

1.4.1 miRNA

MicroRNAs (miRNAs) are small, untranslated, single-stranded RNA (ssRNA) molecules whose genes are commonly located in the intronic regions of protein coding genes. It is believed that about 100 to 1000 miRNAs exist per genome and regulate about 10,000 to 100,000 genes [117]. These small RNAs (21 nucleotide (nt)) control translation of mRNA by binding to their target mRNA through complementary base pairing. Specifically, a stem loop structure called pri-mRNA is transcribed by Polymerase II and processed by RNase III enzymes like Drosha or Pasha, which excise the stem loop to form the pre-miRNA. Following the transport out of the nucleus by Exportin-5, the RNaseIII-like enzyme Dicer generates the mature miRNA. The mature miRNA is then incorporated into miRNA-induced silencing complex (miRNP) [64].

How the miRNA-complex represses mRNA is unknown. A model suggests that the translation initiation complex protein eIF4G at the m7-capped mRNA initiates a run-off and mRNAs with this protein aggregate in so called P-bodies [63]. From this point the fate of the mRNA is either degradation or storage in P-bodies. Storage of the mRNA can be induced during stress [17]. Degradation starts with the deadenylation by Ccr4:Not1 deadenylase complex. Additionally the GW182 protein binds to the miRNP complex and

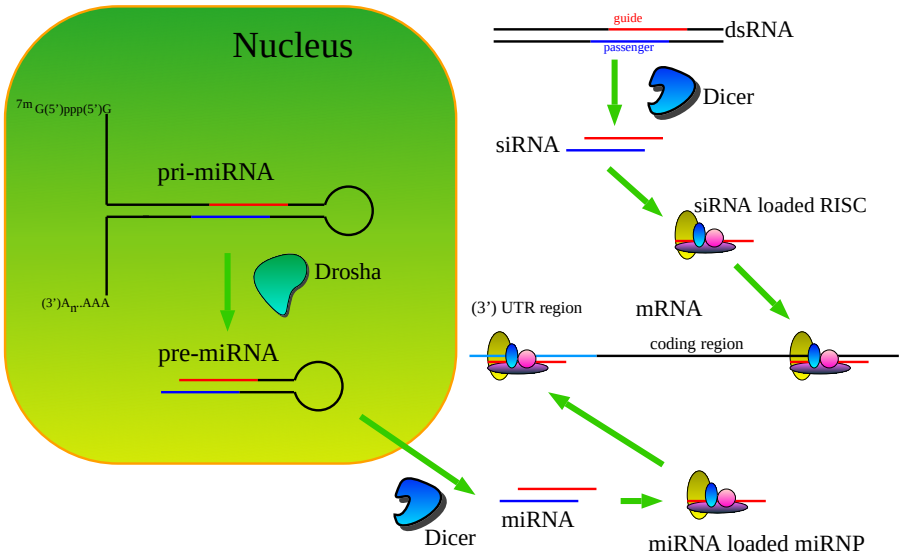


Figure 1.3: Schema of the endogenous and exogenous gene silencing.

promotes the recruitment of degradation promoting molecules [14].

MiRNAs function in a broad range of biological processes. Severe morphological changes have been observed on deregulating miRNA expression levels in plant [198**?**], *C. elegans* [1, 119], zebrafish [71, 216] and mouse [206, 219] models. These data underline the importance of these small RNAs for development and gene regulation. While a large number of conserved and nonconserved miRNAs in many phyla are known, their targets remain unknown. Computational approaches to identify possible targets predict that 30% of genes in a genome are regulated by miRNA [117]. However, only a few targets have been experimentally confirmed. An important scientific challenge remains the identification of *in vivo* targets for specific miRNAs [153].

miRNA and stress

The known functions of microRNA are mainly in development, cancer and diseases [6, 33, 59, 77, 172, 210]. However, microRNAs have been recognized to play a role in stress responses as well. The first link was suggested from a computational target prediction study in *Arabidopsis thaliana*. MicroRNAs were predicted to regulate stress related genes such as superoxide dismutases, laccases and ATP sulphorylases [97]. Specifically, an up-regulation of miR-395 during sulfate starvation in *A. thaliana* underlined microRNA involvement during the stress. Furthermore, in rice, miRNAs are up-regulated during diverse stresses [174, 221] (for review see [150]).

Mir-14 was the first microRNA to be linked with stress responses in an animal system. Deletion of miR-14 causes the mouse cells to be more sensitive towards osmotic stress, and to dampen protein synthesis when exposed to hypothermia [57]. Osmotically stressed mice showed an increase in miR-7b expression, which was demonstrated *in vitro* to inhibit the translation of Fos, a major regulatory protein in the mouse brain [113]. Hypoxia stressed mouse cells revealed regulation of diverse microRNAs, some suggesting a link to tumor-formation [106]. Stress responsive microRNAs were also identified during cellular nutrient deprivation [17, 192], cardiac pressure overload [191], DNA damage, and oncogenic stress [28, 80, 156, 179].

During stress, Argonaute and miRNA localize in processing bodies (PBs or GW bodies). Upon exposure to particular stress stimuli such as oxidative stress, Argonaute and miRNAs become localized to newly assembled structures known as stress granules (SGs) [25, 98, 125, 164, 180]. MicroRNAs appear to also up-regulate genes during stress using

an unknown mechanism [115].

In conclusion, miRNAs are regulated by, involved in, and required for several stress responses. However their mechanism of action during these stresses are largely unknown. As desiccation tolerance is an important response for many organisms, evaluation of the role of mRNAs during desiccation stress is important.

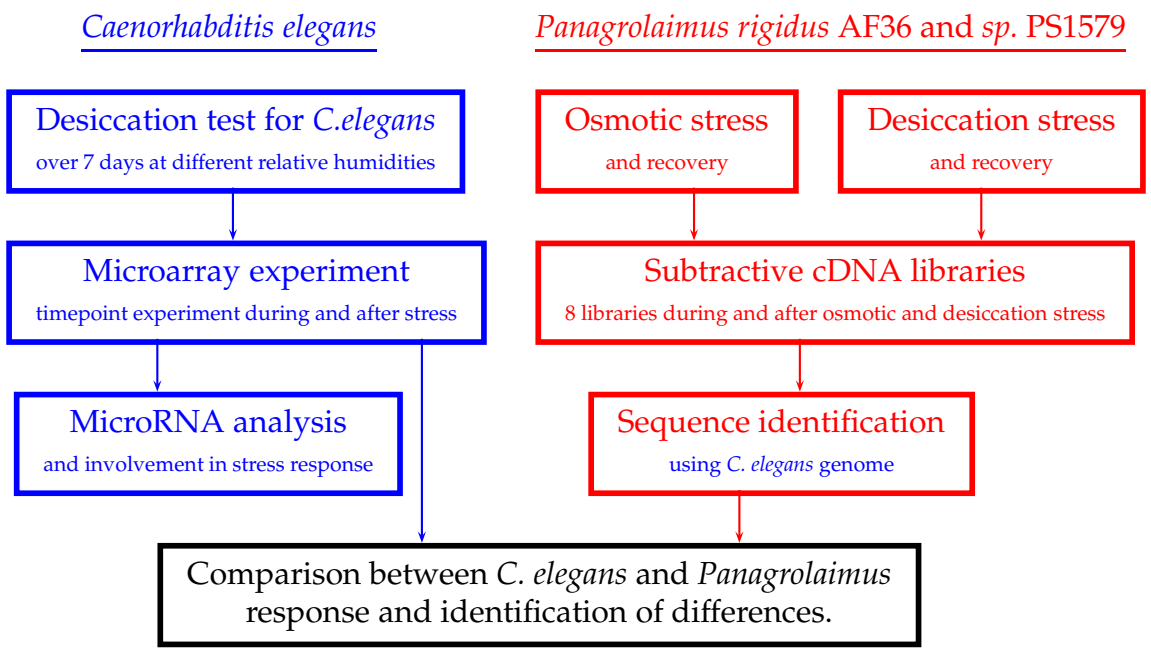


Figure 1.4: Flowchart of the project

1.5 Project overview

This study uses the anhydrobiotic nematodes *Panagrolaimus sp.* PS1579 and *rigidus* AF36 desiccation sensitive *Caenorhabditis elegans* to study water stress. The specific aims of this study are shown in the experimental workflow (Figure 1.4 on page 26) and summarized below:

1.5.1 Aim 1 - Characterize desiccation stress tolerance of *C. elegans*, and desiccation and osmotic stress tolerance of *Panagrolaimus rigidus* AF36 and *sp.* PS1579.

A mixed-stage population of *C. elegans* was desiccated for a period of seven days under different relative humidity conditions. Survival was recorded at different timepoints during the stress. The outcome describes the first analysis of desiccation stress in *C. elegans* and provides the necessary background information for subsequent gene expression experiments.

The nematodes *Panagrolaimus rigidus* AF36 and *sp.* PS1579 were desiccated on cellulose membranes for 48 hours and subsequently rehydrated. Images and survival data were analyzed. A parallel experiment using osmotic stress was performed. The two *Panagrolaimus* species were submitted to sequential desiccation and osmotic stresses in order to determine whether one stress is part of the other stress. For example, a higher survival rate would be expected if the first stress can be considered a pre-adaptation to the second.

1.5.2 Aim 2 - Identify genes from the two *Panagrolaimus* species that are regulated by desiccation and osmotic stress and characterize genetically the stress response.

The first part of this aim was to clone genes that are only expressed during and after the desiccation stress. This was achieved by generating stress and post-stress subtractive

libraries. Specifically, a subtractive cloning method allows cloning of transcripts that were present only during desiccation stress, but not during recovery. Then, using the same method, but switching the sample, a desiccation recovery library was created. This was done with both species, resulting in two “desiccation entry” and two “desiccation recovery” libraries.

In order to identify conserved stress responsive transcripts from the desiccation response libraries, the same procedure described above was used for an osmotic stress response. Altogether, eight libraries were created, all containing sequences of genes uniquely expressed during, and recovery from, osmotic and desiccation stresses. All these libraries were then sequenced and compared with each other in order to find common and unique genes. As the genome of both *Panagrolaimus* species have not been sequenced, the genome data of *C. elegans* and *C. briggsae* were used for the identification of the cloned sequences.

1.5.3 Aim 3 - Analyze the gene expression of *C. elegans* submitted to desiccation stress during a period of five days. Identify highly regulated genes involved in desiccation tolerance response.

C. elegans was submitted to desiccation stress using 60% relative humidity and 16°C for five days. Total RNA from five timepoints during the stress and one during recovery (36 hours after rehydration) were collected for microarray analysis. The expression data were then compared to the subtractive libraries of both *Panagrolaimus* species to find differences and similarities in the desiccation stress response. Furthermore, the dataset was

compared to dauer stage-regulated genes and stress annotated genes in *C. elegans* to identify shared and unique transcripts.

1.5.4 Aim 4 - Asses whether microRNAs are regulated and participate actively in the stress response of *C. elegans*.

Total RNA extracted from the 0 hour, 12 hour, 24 hour and 36 hour timepoints of the previous *C. elegans* desiccation experiment were used to profile 128 *C. elegans* microRNAs. Mir-1, let-7, mir-34, mir-244, mir-265 and mir-273 expression were chosen to prove their involvement during the desiccation stress. The expression data of each microRNA were correlated to the expression of their computational predicted targets. Furthermore, *C. elegans* knock-out mutants of these microRNAs were submitted to desiccation stress at 60% relative humidity and 16°C. Their survival after 36 hours was analyzed and compared to wild type to assess the microRNA involvement during desiccation stress.

Desiccation has been analyzed in different organisms and common features of the tolerance response have been identified. This research adds significant data to this knowledge as it is the first gene expression analysis on a multicellular desiccation tolerant organism and a non-desiccation tolerant organism from the same phylum. The physiology (Aim 1) and gene expression (Aim 2) data describe the response of a desiccation-tolerant organism toward desiccation stress. The analysis of *C. elegans* gene expression over time during desiccation stress (Aim 3) elucidates the timely response of *C. elegans*. Comparing both responses on a genetic level (Aim 3) clarifies the difference between desiccation-tolerant and desiccation-sensitive organisms. Expression analysis of microR-

NAs (Aim 4), known for their capability to regulate a large number of genes, identifies their possible role in desiccation tolerance. Altogether this study results in new insights in desiccation tolerance which potentially can lead to new methods in anhydrobiotic engineering.

Chapter 2

Physiology of desiccation

2.1 Introduction

Chapter 2 describes the response of *C. elegans* and two nematodes from the genus *Panagrolaimus* to desiccation stress (Figure 2.1 on page 32). Additionally, the effects of osmotic stress are investigated in the *Panagrolaimus* species. This study provides the physiological information necessary for studies described in subsequent chapters, which seek to identify genes regulated during and after desiccation stress.

This study is the first reported investigation of *C. elegans* response to desiccation stress. The organism was submitted to several different regimes of relative humidity in order to characterize the degree of tolerance. The results show that *C. elegans* is partially tolerant to desiccation. This is not an unexpected finding as free living nematodes experience periods of dryness in their natural environment. However, they are not able to revive from relative humidity of 0% and thus are not capable of true anhydrobiosis.

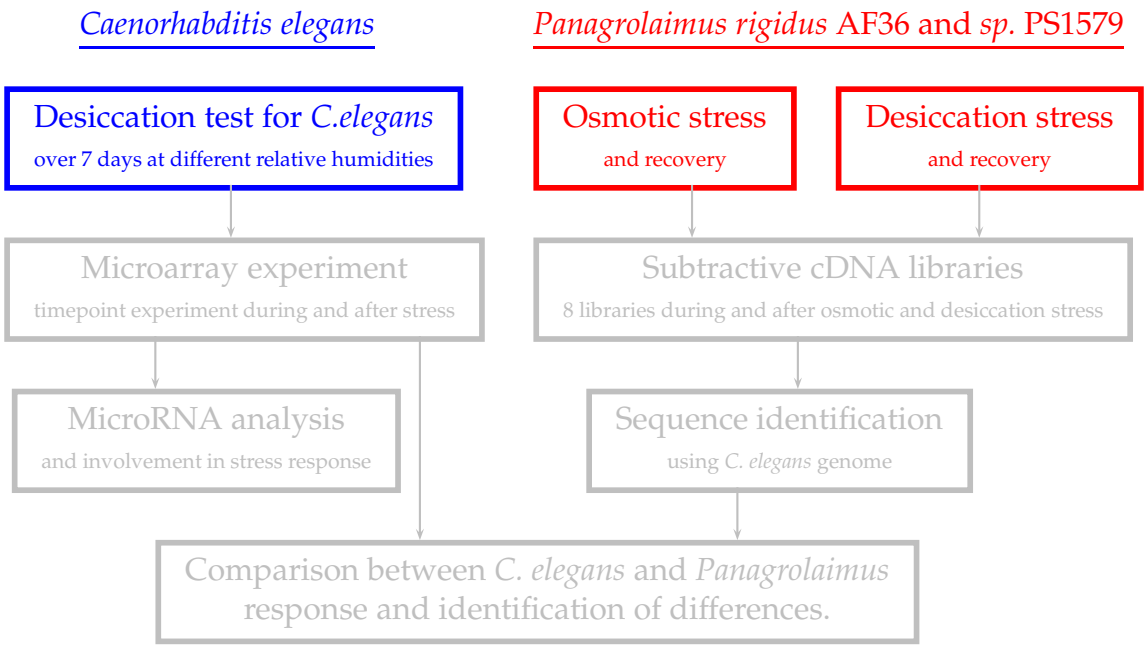


Figure 2.1: Flowchart highlighting Chapter 2 studies.

As a comparison, two anhydrobiotic *Panagrolaimus* nematode species, *rigidus* AF 36 and *sp.* PS1579, were chosen to investigate differential gene expression during desiccation. The use of two closely related nematodes facilitates finding the genes required for desiccation tolerance. The working hypothesis for this work is that a gene found to be regulated in both nematodes during desiccation stress may play a vital role during the stress response and absent in *C. elegans*.

In addition to desiccation stress, osmotic stress was also investigated in both *Panagrolaimus* species. The rationale behind analyzing both osmotic and desiccation stress responses was to further narrow the number of genes to be analyzed. There are a number of commonalities between desiccation and osmotic stress responses: first, both osmotic- and desiccation-stressed organisms go through a shrinkage phenomenon due to water efflux from the body [205, 213]; second, organisms produce osmolytes in hyperosmotic solution and during desiccation [90, 110]; third, desiccation-tolerant organisms show similar morphological changes on both stresses [102, 188]; fourth, metabolic and transcriptional processes are slowed down significantly under both stresses [205].

The similarities in the responses to osmotic stress and desiccation tolerance suggest that the same mechanism(s) may be used in both responses. This hypothesis was investigated using African chironomid *Polypedilum vanderplanki* [161] and *Steinerma* nematode larvae [151]. Specifically, both experiments were performed by submitting one group of animals first to desiccation, and then to osmotic stress, and the second group to the two stresses reversed. In both cases the survival after the second stress was lower than the survival after the first. The outcome suggests two possibilities: either osmotic stress re-

sponse is an integral part of the desiccation stress response, in which case the organisms induce osmotic protective mechanism during desiccation stress, or desiccation tolerance uses mechanisms related to osmotic tolerance, but both responses use their own specific transcriptions and metabolites. This question was addressed in this study by submitting both *Panagrolaimus* species to a sequential desiccation-osmotic stress experiment, and osmotic-desiccation stress experiment and comparing osmotic and desiccation induced transcripts for similarities.

2.2 Materials and Methods

Culture maintenance *Caenorhabditis elegans* (Bristol N2), and the *Panagrolaimus sp.* PS1579 and *rigidus* AF36 were obtained from the *Caenorhabditis* Genetics Center (CGC). Both species were maintained at 25$^{\circ}\mathrm{C}$ on Nematode Growth (NG) agar plates with live bacteria (*Escherichia coli* OP50, uracil auxotrophic strain).

Culture preparation Worm cultures were washed from the agar plate by rinsing twice with S-buffer (0.1 M NaCl, 0.05 M potassium phosphate buffer, pH6.0) and collecting the worm suspension in a centrifuge tube. Following centrifugation (1 min, 200 x g) the supernatant was discarded. The worm pellet was re-suspended in S-buffer. This step was repeated three times. After the last certifugation, the pellet was re-suspended in 5 ml S-buffer and left on ice for at least 5 min. The worm suspension was mixed with 5 ml ice cold 70% sucrose solution and centrifuged for 5 min at 1500 x g. Afterwards the floating worms were put in sterile S-basal buffer. The sample was centrifuged for 3 min at

1000 x g, washed with by S-buffer wash and centrifuged (2 min, 200 x g). The worms were used immediately after cleaning.

Osmotic stress At least 50,000 worms per sample were re-suspended in 5 ml 200 mM NaCl S-buffer solution. The samples were kept agitated at 25$^{\circ}\mathrm{C}$. After two hours preconditioning the NaCl concentration in the tube was increased to 500 mM NaCl. The worms were left in the incubator for an additional 48 hours at which time they were centrifuged (2 min at 200 x g) and washed with S-basal buffer. The worms were left for an additional two hours in S-basal buffer at 25$^{\circ}\mathrm{C}$ and subsequently stained with Sytox green (Molecular Probes, USA) for survival determination. This experiment was done in duplicate.

Desiccation stress *Panagrolaimus* AF 36 and PS1579 were sucrose cleaned. At least 50,000 worms per sample were placed on a 5-cm Nitrocellulose membrane (Millipore). The membrane with the worms was immediately placed in a chamber equilibrated to a relative humidity (RH) of 0% with silica gel. The relative humidity was monitored with an iButton (Maxim Integrated Products, USA). After another 48 hour incubation, a piece of the membrane was cut and visualized under an optical microscope to image the desiccated nematodes (Figure 2.2 on page 38). The remaining membrane was placed in S-buffer for recovery for two hours and stained afterwards for survival determination. For long term desiccation experiments portions of the membrane were removed at predetermined times (typically 24% of the membrane every seven days). These experiments were performed in duplicate.

***C. elegans* desiccation stress tolerance** *C. elegans* was sucrose cleaned and about 80,000 worms were placed on a 5-cm Nitrocellulose membrane (Millipore). Then the membranes were placed in 7 chambers pre-equilibrated to 40%, 50%, 60%, 70%, 80%, 90% and 100% relative humidity respectively. The humidity was regulated in each chamber during the experiment with glycerol solutions. As control a culture of *C. elegans* was kept in S-buffer. The desiccation experiment was performed at 16$^{\circ}\mathrm{C}$. After 12 hours, 24 hours, 36 hours, 48 hours, 60 hours, 72 hours, 5 days and 7 days a piece of the sample was used for survival rate determination. The experiment was done in triplicate.

Stress survival Survival was measured using SYTOX green (Molecular Probes, USA). The nematodes were placed in S-buffer containing 1μM SYTOX green dye. After a 15 min incubation 100-$\mu\mathrm{l}$ aliquots from each sample were aliquoted in 96 well plate (Novagen, USA). For each sample 5 wells were used, paying attention to have at least 100 worms per well. The samples were visualized using a confocal microscope (MS510, Zeiss). A digital image was taken from each well and the images were analyzed manually. The percent survival was determined by counting both the total number of worms as well as the number of dead worms (SYTOX stained). % survival= $\frac{Total\ worms - Dead\ worms}{Total\ worms}$

Sequential stress experiments For the desiccation-osmotic-sequential stress experiment, after the 48 hours of desiccation stress the membrane with the desiccated worms was soaked in 5 ml S-basal buffer supplemented with 200 mM NaCl and left for two hours at 25$^{\circ}\mathrm{C}$. After two hours the NaCl concentration was increased to 500 mM by adding 300 $\mu\mathrm{l}$ 5M NaCl solution. Then the sample were osmotically stress for 48 hours and

treated as described above. For the osmotic-desiccation-sequential stress, worms were stressed in 500 mM NaCL S-buffer as described above for osmotic stress. After 48 hours of osmotic stressed the worms were placed on a nitrocellulose (Millipore, USA) membrane. The membrane was then placed in a chamber equilibrated to 0% relative humidity. The procedure was continued as described for desiccation stress.

2.3 Results

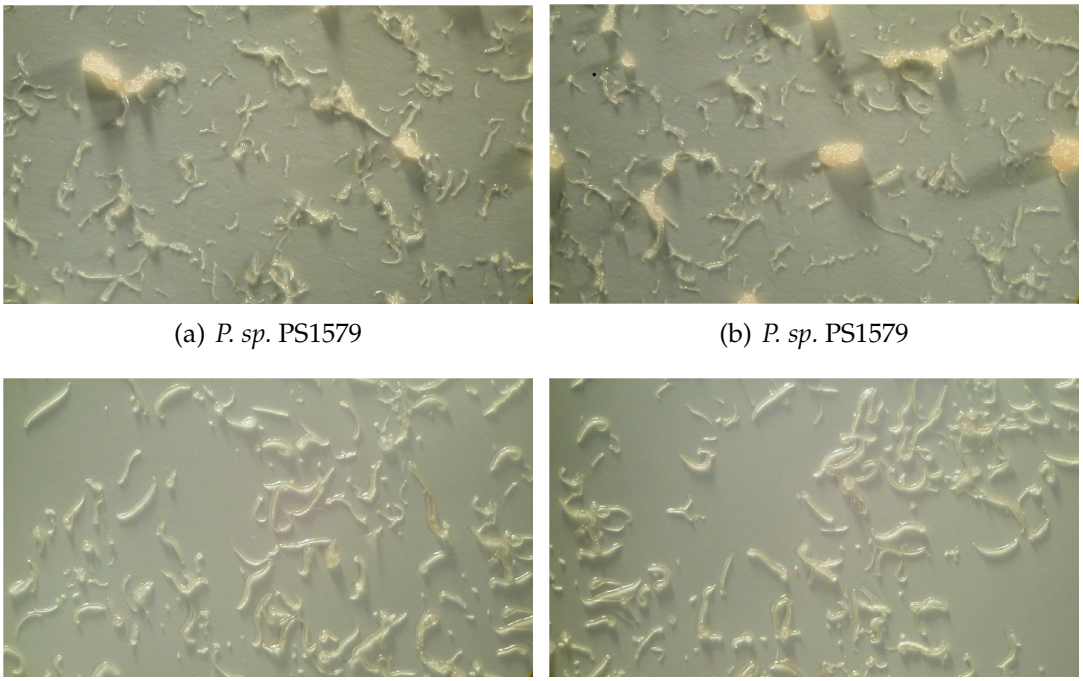
2.3.1 *Panagrolaimus sp.* PS1579 and *rigidus* AF36

Osmotic stress tolerance of *Panagrolaimus* species

The aim was to design an osmotic stress protocol for both *Panagrolaimus* species to be used for a subtractive library study (Chapter 3.4.3). The two *Panagrolaimus* species were pre-adapted for two hours in 200 mM S-buffer and then the salt concentration was increased to 500 mM NaCl. The nematodes were stressed for 48 hours and recovered for two hours in S-buffer. The survival was 87% for *Panagrolaimus rigidus* AF36 and 95% for *Panagrolaimus sp.* PS1579. The osmotic stressed nematodes resumed complete movement in less then two hours.

Desiccation stress tolerance of *Panagrolaimus* species

The aim was to design a desiccation protocol for both *Panagrolaimus* species to be used for the subtractive hybridization library described in Chapter 3.4.3. Both *Panagro-*



(a) *P. sp.* PS1579 (b) *P. sp.* PS1579

(c) *P. rigidus* AF36 (d) *P. rigidus* AF36

Figure 2.2: *Panagrolaimus sp.* PS1579 (a, b) and *rigidus* AF36 (c,d) desiccated on a membrane- *Panagrolaimus rigidus* AF36 and *sp.* PS1579 were desiccated for 48 hours on a Nitrocellulose membrane at 0% relative humidity. To be noted is the aggregation of *Panagrolaimus sp.* PS1579 in contrast to *Panagrolaimus rigidus* AF36.

laimus species were placed on a membrane and desiccated in a chamber calibrated to 0% relative humidity for 48 hours. The nematodes were rehydrated in S-basal for two hours prior to measurement of the survival. The survival was 88% for species *rigidus* AF36 and 93% for species PS1579.

Furthermore, it was noted that *Panagrolaimus sp.* PS1579 preferred to aggregate in comparison to *Panagrolaimus rigidus* AF36 (Figure 2.2 on page 38). Desiccated nematodes needed more than two hours to regain complete movement.

Long term desiccation survival of two *Panagrolaimus* species

In order to assess the resistance of *Panagrolaimus rigidus* AF36 and *sp.* PS1579 to long term 0% relative humidity conditions. *Panagrolaimus sp.* PS1579 and *rigidus* AF36 were treated as described (0% relative humidity) for three weeks, with the survival recorded every seven days (Figure 2.3 on page 40). Species PS1579 showed a higher tolerance for long term desiccation (88% survival after three weeks) than species *rigidus* AF36 (64% survival after three weeks). These results are consistent with published data that state *Panagrolaimus rigidus* AF36 has a lower desiccation tolerance than *sp.* PS1579 [165].

Sequential stress tolerance of *Panagrolaimus* species

Osmotic stress is believed to take place during desiccation. However, the response overlaps between osmotic stress and desiccation are presently not clear. To evaluate the relation between these two stresses both *Panagrolaimus* species were submitted to sequential desiccation stress and osmotic stress. Specifically, desiccated nematodes were immediately placed in a 200 mM NaCl S-buffer solution for two hours followed by an increase to 500mM NaCl and a further incubation of 48 hours. Osmotic stressed nematodes were immediately placed on a membrane and incubated in a 0% relative humidity chamber. Survival was scored after each stress. The results are shown in Figure 2.4 on page 42. Both *Panagrolaimus* species show a high survival ($>$ 85%) after osmotic and desiccation stress. After the second stress the survival of *Panagrolaimus rigidus* AF36 dropped to 40% for the osmotic followed by desiccation stress experiment and 30% for the desiccation followed by osmotic stress. *Panagrolaimus sp.* PS 1579 survial dropped to 45% and 50% respectively.

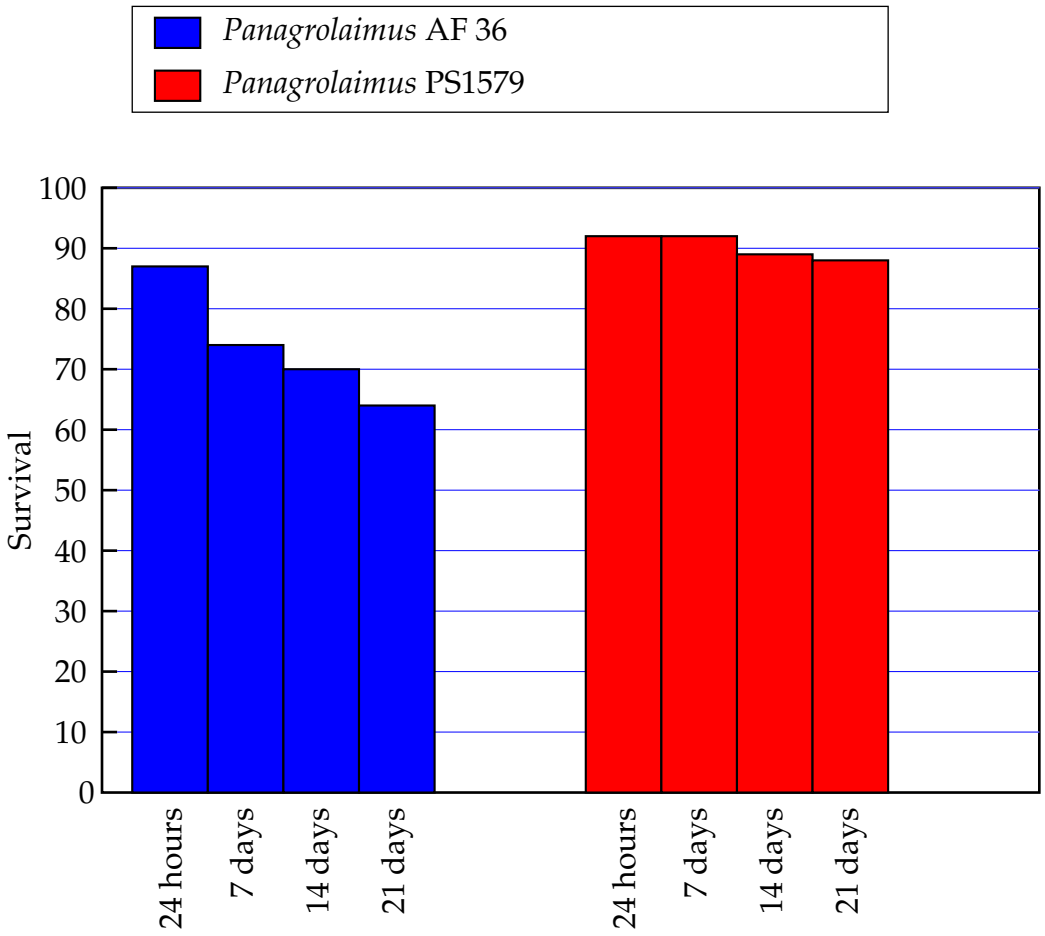


Figure 2.3: Long term survival of *Panagrolaimus sp.* PS1579 and *rigidus* AF36 - Both *Panagrolaimus* species were desiccated on a nitrocellulose membrane in a chamber calibrated to 0% relative humidity for 21 days. Every week a quarter of the meembrane with the nematodes were rehydrated and the survival measured.

2.3.2 *Caenorhabditis elegans*

In order to test desiccation tolerance in *C. elegans*, nematodes of a mixed stage culture were desiccated on a nitrocellulose membrane at 16°C under different relative humidity ranging from 40% to 100% over a period of one week. The result is shown in Figure 2.5 on page 44. The mixed population of *C. elegans* was unable to survive long periods (more than a week) without water. However, *C. elegans* can tolerate high humidities for up to four days.

As control, a culture of *C. elegans* was kept in S-buffer at 16°C, which is in the range of the standard culture condition (11-25°C). The survival of the control (95-100% survival during the experiment) suggests that starvation did not play a role during the desiccation stress. If starvation would have influenced the survival of the stressed nematodes, the survival of the control culture would be expected to drop, too. Development was limited by lowering the temperature to 16°C, so that the developmental time for a full life cycle increased to 4.8 days. For the first 24 hours all survival remained at ∼90%. After this timepoint only nematodes kept at relative humidities of 100% and 90% maintained the survival above 80% until 60 hours. Afterwards the survival dropped to 60% over the next two days. For the samples incubated at relative humidities ranging from 40% to 70%, a drop in the survival can be noted after 24 hours. From 48 hours to 60 hours the survival rate leveled to around 60%. Then it dropped again reaching a 30% survival at 120 hours for the samples incubated at 40% and 50% relative humidity. For the samples incubated at 60% and 70% relative humidity the survival dropped between 40% and 50%. The sample

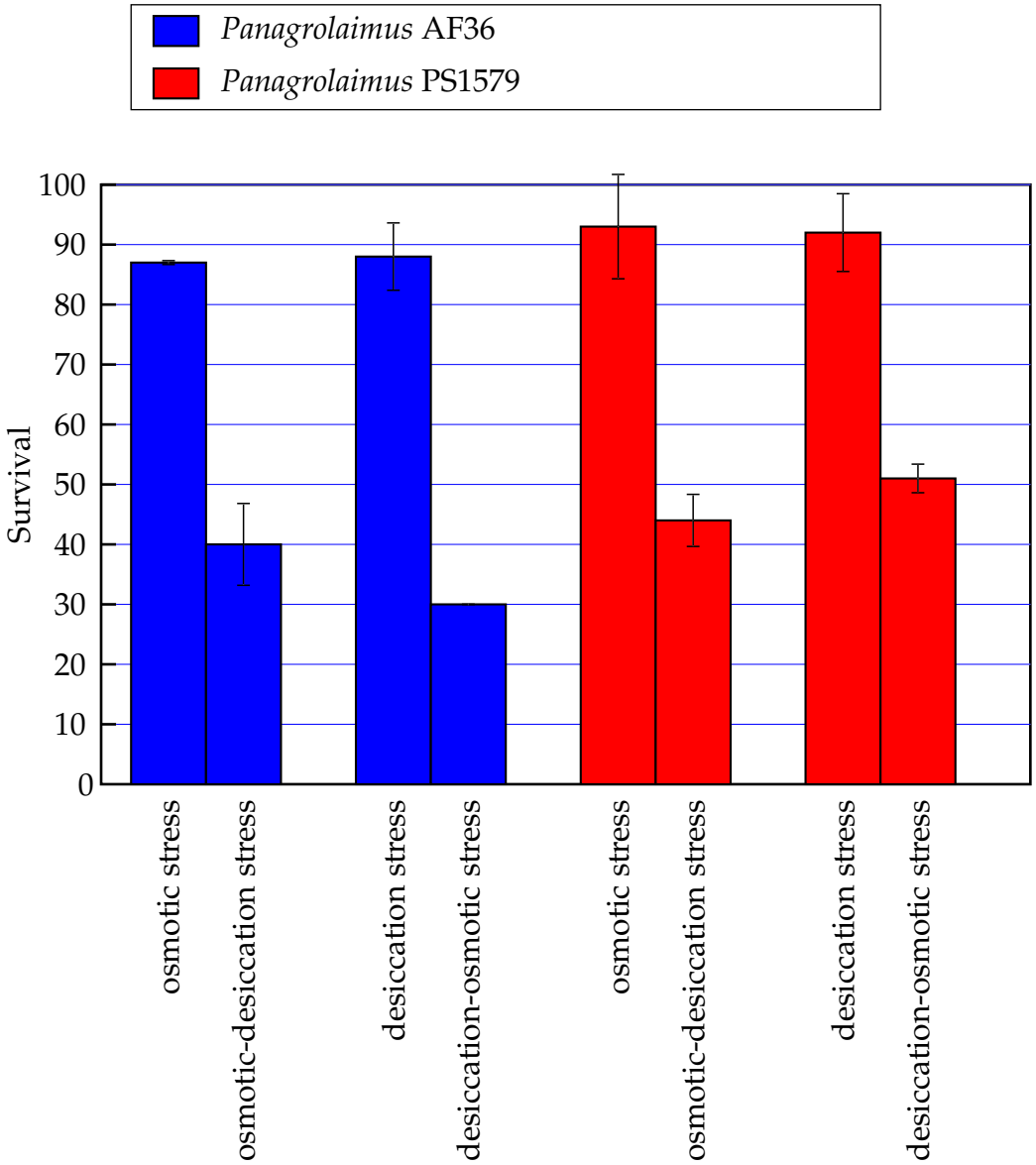


Figure 2.4: Sequential stress survival of *Panagrolaimus rigidus* AF36 and *sp.* PS1579 - One half of a population of *Panagrolaimus rigidus* AF36 and of *Panagrolaimus sp.* PS1579 were submitted to desiccation stress followed by osmotic stress and the another half of the population of both species were submitted to osmotic stress followed by desiccation stress. The survival after each stress is shown in the bar diagram. Error bars show the standard error of two replicates.

incubated at 80% seemed to follow an intermediate path between the samples incubated at 90% and 100% and the ones incubated between 40% and 70%. The survival rate at 80% started dropping at timepoint 36 hours of desiccation and started leveling at timepoint 60 hours. At the end of the experiment the survival rate reached the same level as the samples incubated at 90% and 100%.

2.4 Discussion

Chapter 2 describes the characterization of the response of *Panagrolaimus rigidus* AF36 and *sp.* PS1579 to osmotic and desiccation stress and the desiccation tolerance of *C. elegans*. The two species of *Panagrolaimus* showed remarkable stress tolerance towards harsh desiccation conditions (0% relative humidity). The data collected also suggest *Panagrolaimus sp.* PS1579 has a better stress tolerance then *Panagrolaimus rigidus* AF36 according to the survival.

The hypothesis stated in the introduction that osmotic stress might be an integral part of the desiccation response is contradicted by the survival after the second stress of the nematodes independent from the sequence of stresses. The fact that the nematodes did survive the second stress (30% to 50%), suggests two hypothesis: Either some common mechanisms induced during both stresses are present conferring partial resistance to the second stress, or a certain percentage of the *Panagrolaimus* population induced both stress tolerances.

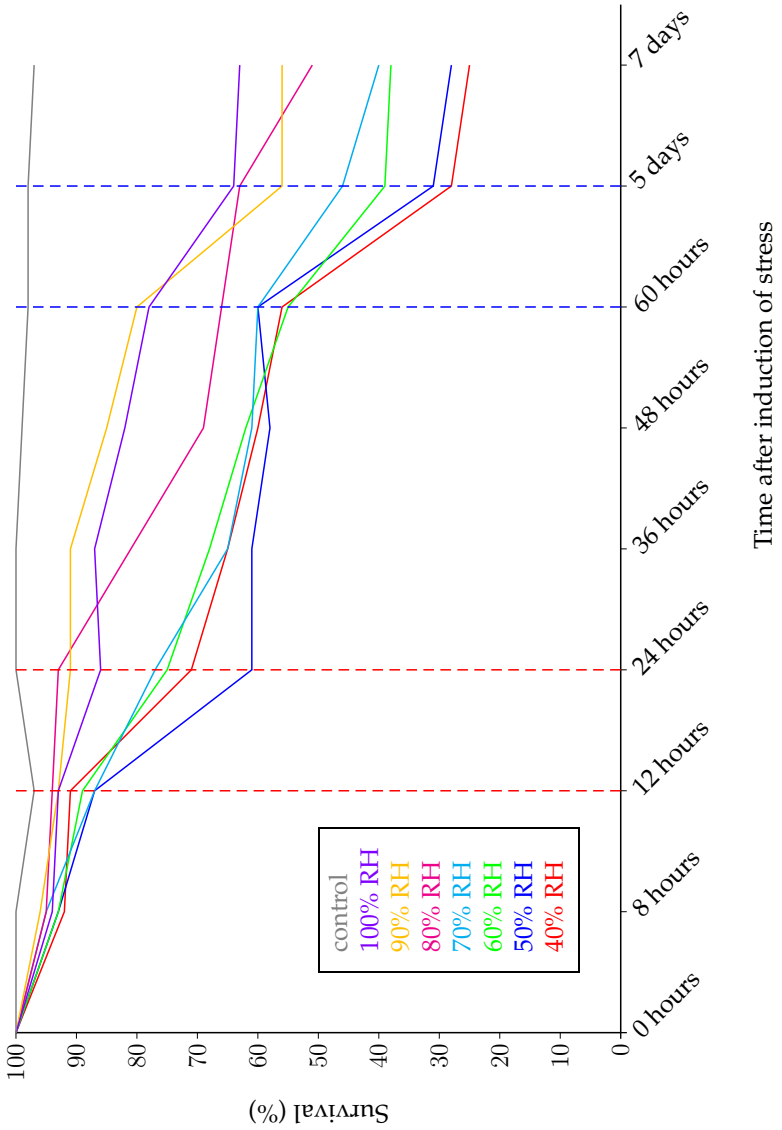
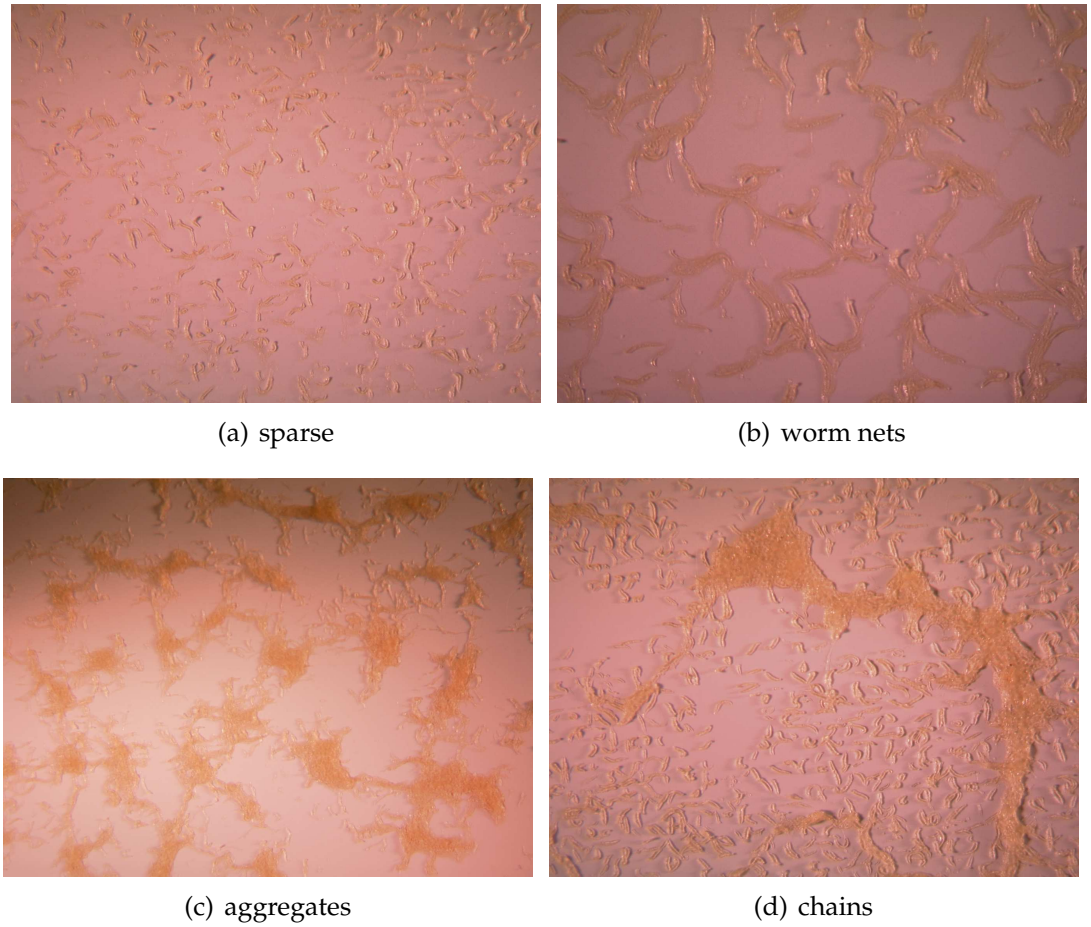


Figure 2.5: *C. elegans* survival under different relative humidities. - A mixed stage population of *C. elegans* was placed on nitrocellulose membrane and incubated in chambers equilibrated at different relative humidities ranging from 40% to 100% at 16°C. The control population was kept in S-buffer. The survival was calculated using the average from three replicates. A noticeable drop is seen between 12 and 24 hours after initiation of the stress for the cultures kept at lower relative humidities.



(a) sparse (b) worm nets

(c) aggregates (d) chains

Figure 2.6: Different examples of *C. elegans* pattern seen on nitrocellulose membrane.

The response of *C. elegans* to desiccation stress was characterized for the first time. The result suggests a weak desiccation tolerance of *C. elegans* compared to *Panagrolaimus* species as *C. elegans* is able to survive only mild desiccation stress, with a survival of less than 30% after seven days at 40% RH.

2.4.1 *Panagrolaimus sp.* PS1579 and *rigidus* AF36

The results obtained in this chapter seem to contradict the characterization of *Panagrolaimus rigidus* AF36 and *sp.* PS1579 as slow desiccation tolerant strategists [165] as long preconditioning time was not necessary to achieve high survival rate at 0% relative humidity. However, the relative humidity in the chamber does not drop immediately to 0% as the membrane with the nematodes is still wet. Consequently the relative humidity for the nematodes does not drop immediately to 0%. This gives the nematodes enough time to induce desiccation tolerance and can be considered a pre-conditioning/adaptation time.

The long-term desiccation experiment shows that both species can survive environments of 0% relative humidity for a long period. However, the result suggests also that the survival drops with time. The differences in the rate might be caused by the way the two worms dried on the membrane. Figure 2.2 on page 38, *Panagrolaimus* PS1579 aggregates, which might help in the retention of water or protection from environmental damage like oxidation stress occurring during anhydrobiosis.

Submitting both *Panagrolaimus* species to desiccation followed immediately by osmotic stress and then from osmotic stress to desiccation stress resulted in a lower survival than

after the first stress. These data suggest that osmotic and desiccation stress responses are independent indicating that the stress cDNA libraries will probably contain only a few overlapping sequences between the stresses.

The fact that the level of *Panagrolaimus* survival after the two stresses did not drop to zero, but were in the range between 40% to 60% suggests common mechanisms between the two stress responses. This might be due to the fact that during desiccation a small osmotic stress due to evaporation of water and increased osmotic pressure due to water efflux from the organism most likely occurs. Another possible explanation is that a certain percentage of worms might have also induced anhydrobiosis or osmotic tolerance mechanisms during the first stress and so survived the second stress. This would imply that osmotic and desiccation response can be induced together or alone depending on the environmental clues. This phenomenon has been shown in other organisms [157, 209]

2.4.2 *Caenorhabditis elegans*

This study was the first large scale analysis of desiccation stress in *C. elegans*. The relative humidity assay showed that *C. elegans*, despite the stress resistant dauer stage, is capable of surviving moderate desiccation. An evident feature for the worm was the tendency to aggregate as placed on the membrane (Figure 2.6 on page 45). This behavior is seen in other nematodes. For example *Ditylenchus dipsaci* forms a ‘nema wool’ on the surface of stored flower bulbs [127].

Analyzing the survival screening, two aspects need to be pointed out: first, the wa-

terfilm, an essential condition for normal life activity of the worm [5], was gone after just one hour at 60% relative humidity, a condition where the majority of the worms were still viable (95% and higher, visual observation). Second, after 24 hours *C. elegans* survival decreased significantly for about 12 hours (approximately 30%), and then stabilized until 60 hours. An explanation might be that *C. elegans* responds to the continuing desiccation stress by inducing a response to loss of water around it aggregating immediately upon placing the worms on the membrane, despite the fact that the membrane is soaking wet. This first action might be followed by a modification of the cuticle to limit water efflux after the waterfilm disappeared. Furthermore, oxidation damage originating from free radicals may be occuring. This triggers the expression of superoxide dismutases (SODs) and DNA repair mechanisms. Due to the loss of water the worm might experience protein aggregation and degradation. This chain of events might trigger at different timepoints additional stress response mechanisms. The survival of the organism depends consequently on how fast it can respond to these inter-related events. A higher atmospheric relative humidity may provide the needed time required for higher survival.

A desiccation-tolerant organism may differ in this perspective as it already responds ahead of time to what kind of challenges are to be expected during the stress, expressing antioxidants or osmolytes. A desiccation-sensitive organism will respond only to the momentary challenges it faces. Once cellular damages reach a critical level, the organism dies. Results from the microarray and the subtractive library might be able to confirm this hypothesis.

2.4.3 Comparison of the physiological data

As expected, the *Panagrolaimus* species are much more desiccation-tolerant than *C. elegans*. Whereas *C. elegans* exhibit 30% survival after seven days at 40% relative humidity, the *Panagrolaimus* species showed a survival of higher with 75% after seven days at 0% relative humidity. Consequently, *C. elegans* can be considered a desiccation sensitive organism. *Panagrolaimus rigidus* AF36 and *sp.* PS1579 can be considered truly anhydrobiotic, as they show survival after a period of 0% relative humidity for 21 days. Among the desiccation tolerant organisms these two species are regarded as slow desiccation strategists as they need a certain period of time to induce their tolerance [165].

Under osmotic stress conditions *C. elegans* and *Panagrolaimus rigidus* AF36 and *sp.* PS1579 seem to show similar behaviors. In an osmotic stress study [110] *C. elegans* was able to survive on an agar plate containing 550 mM salt concentration. *Panagrolaimus rigidus* AF36 and *sp.* PS1579 were shown also in this study to be able to withstand this stress. However, attention must be paid, that *C. elegans* was stressed on solid medium and the two *Panagrolaimus* species were stressed in a liquid environment.

Upon placing the nematodes on the membrane, *C. elegans* shows a tendency to aggregate as seen in Figure 2.6 on page 45. The same behavior is seen as part of the desiccation stress survival of *Ditylenchus dipsaci* [127]. The most likely explanation for this behavior is to slow down the rate of water loss. Furthermore, aggregation is a potential defense mechanism against adverse environmental conditions such as UV or oxidation [158]. The outer nematodes of the aggregation experience those stresses protecting the less exposed nematodes in the aggregate. Thus such an aggregation of *C. elegans* can be induced also by increasing atmospheric oxygen. This behavior was shown to be trig-

gered by neuronal receptors [158]. This supports the hypothesis that aggregation is a complex behavior of nematodes activated by environmental senses.

In summary, the knowledge gained from the *Panagrolaimus* experiments was used in designing the procedure of the subtractive library for osmotic and desiccation stress. Both *Panagrolaimus* species will be desiccated at 0% relative humidity for 48 hours prior to revival in S-buffer for two hours. The osmotic stress was induced by incubating the worms in a 500 mM NaCl S-basal solution for 48 hours with a two hour adaptation at 200 mM NaCl S-basal solution. Recovery from the osmotic stress was for two hours in S-buffer.

The desiccation stress done on *C. elegans* at different relative humidities showed the ability of *C. elegans* to survive moderate desiccation. The relative humidity at about 60% was chosen as the ideal condition for designing the microarray timepoint experiment. This parameter represents the best compromise between severeness of stress (survival) and timeframe before and after the first major decrease in survival takes place. This will make it possible to identify possible changes in the response on genetic level that lead to the decrease in survival and to prove the above stated hypothesis.

Chapter 3

Osmotic and desiccation tolerance of *Panagrolaimus rigidus* AF36 and *sp.* PS1579

3.1 Introduction

Chapter 3 describes the construction of cDNA libraries containing sequences that are involved in the response to, and the recovery from, desiccation and osmotic stress in the anhydrobiotic nematodes *Panagrolaimus rigidus* AF36 and *Panagrolaimus* PS1579 (Figure 3.1 on page 52). Genes that were uniquely expressed during desiccation and osmotic stress were identified by constructing subtractive cDNA-libraries for each stress and recovery condition. Subtractive libraries are made by hybridizing two denatured cDNA populations (probe and control) and then removing the formed hybrids. The remaining unhybridized cDNAs strands represent transcripts uniquely expressed in one population

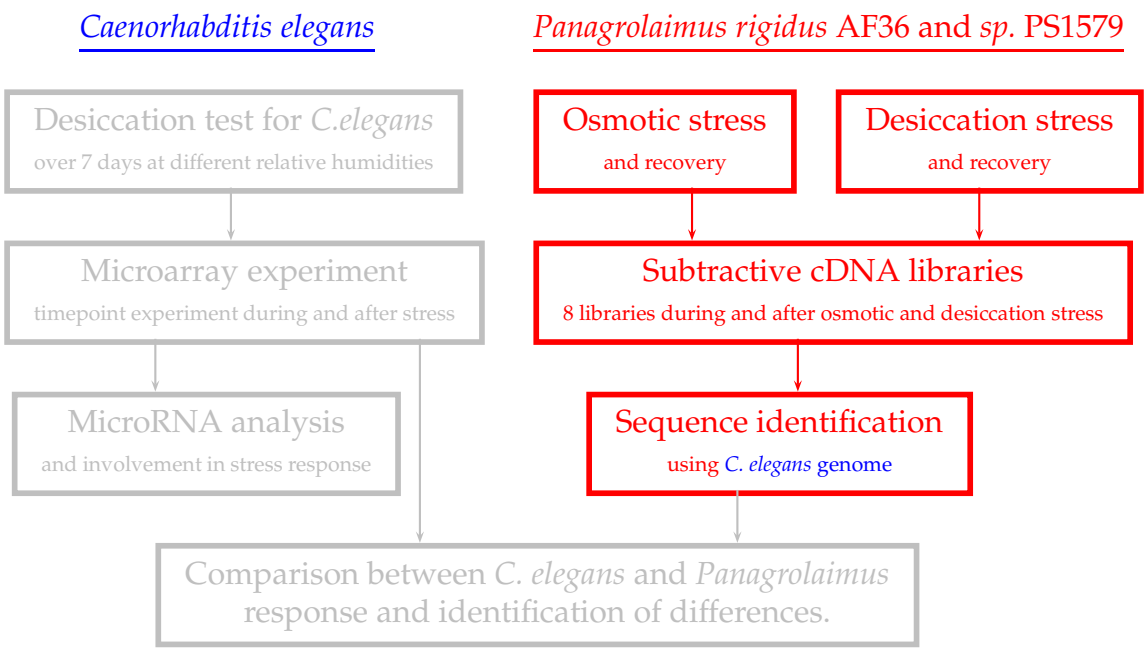


Figure 3.1: Flowchart highlighting Chapter 3 studies.

(the probe), which are then cloned [52, 76]. Eight libraries were generated in this way, each containing transcripts that were specifically expressed in response to desiccation and osmotic stress in the two anhydrobiotic nematode species. As the genomes of both *Panagrolaimus* species used in this study have not been sequenced, the identification of the cloned sequences was done through similarity analysis with the *C. elegans* and *C. briggsae* genomes.

The results of the subtractive library analysis indicated that desiccation and osmotic stress induce non-overlapping sets of genes. Additionally, transcripts induced by the same stress, but from different *Panagrolaimus* species did not show large numbers of overlapping genes.

Table 3.1: Table of the eight generated subtractive cDNA libraries

Species	total RNA from sample	subtracted cDNA	sublibrary
AF36	desiccation stress	post desiccation stress	desiccation stress
	post desiccation stress	desiccation stress	post desiccation stress
	osmotic stress	post osmotic stress	osmotic stress
	post osmotic stress	osmotic stress	post osmotic stress
PS1579	desiccation stress	post desiccation stress	desiccation
	post desiccation stress	desiccation stress	post desiccation
	osmotic stress	post osmotic stress	osmotic stress
	post osmotic stress	osmotic stress	post osmotic stress

3.2 Materials and Methods

Culture maintenance *Caenorhabditis elegans* (Bristol N2), and the *Panagrolaimus* species PS1579 and *rigidus* AF36 were obtained from the *Caenorhabditis* Genetics Center (CGC). Both species were maintained at 25$^{\circ}\mathrm{C}$ on Nematode Growth (NG) agar plates with live bacteria (*Escherichia coli* OP50, uracil auxotrophic strain).

Culture preparation Worm cultures were washed from the agar plate by rinsing twice with S-buffer (0.1 M NaCl, 0.05 M potassium phosphate buffer, pH6.0) and collecting the worm suspension in a centrifuge tube. Following centrifugation (1 min, 200 x g), the supernatant was discarded. The worm pellet was re-suspended in S-buffer. This step was repeated three times. After the last certification, the pellet was re-suspended in 5 ml S-buffer and left on ice for at least 5 min. Then, the worm suspension was mixed with 5 ml ice cold 70% sucrose solution and centrifuged for 5 min at 1500 x g. Afterwards, the

floating worms were put in sterile S-buffer. Then the sample was centrifuged for 3 min at 1000 x g, followed by S-buffer wash. The sample was centrifuged (2 min, 200 x g) and the worms were used immediately.

Osmotic stress The two *Pangrolaimus* species were sucrose cleaned as described above and at least 50,000 worms per sample were re-suspended in 5 ml 200 mM NaCl S-buffer solution. The samples were kept at 25$^{\circ}\mathrm{C}$. After two hours preconditioning the NaCl concentration was increased to 500 mM NaCl by adding 200 $\mu\mathrm{l}$ of a 5M NaCl solution. The worms were left in the incubator for an additional 48 hours. Then half of the sample was used for total RNA extraction, the other half was centrifuged for 2 min at 200 x g and washed with 5 ml S-buffer. The worms were left for other two hours in S-basal buffer at 25$^{\circ}\mathrm{C}$. The worms were centrifuge at 200 x g for 2 min and re-suspended in Trizol (Invitrogen, USA) for total RNA extraction.

Desiccation stress Two cultures of *Panagrolaimus rigidus* AF36 and *sp.* PS1579 were grown over several generations on NGM plates. The nematodes were sucrose cleaned, united to on sample and at least 50,000 worms per sample were placed on a 5-cm nitrocellulose membrane (Millipore, USA). The membranes with the worms were immediately placed in a chamber equilibrated to a relative humidity (RH) of 0% with silica gel. After another 48 hour incubation, half of the membrane was put in Trizol for total RNA extraction. The other half of the membrane was placed in S-buffer for recovery for two hours. Then the sample was centrifuged at 200 x g for 2 min and re-suspended in Trizol (Invitrogen, USA) for total RNA extraction.

Total RNA extraction Total RNA was extracted using the Trizol (Invitrogen, USA) protocol according to the direction of the manufacturer with an additional step at the beginning. Specifically, the Trizol solution with the worms was heated in a waterbath to 65$^{\circ}\mathrm{C}$ for five minutes. Then the sample was placed immediately in a dry ice/ethanol mixture to freeze. After five minutes the sample was placed again in a 65$^{\circ}\mathrm{C}$ waterbath [108, adapted from]. This procedure was repeated three times. Then the sample was centrifuged at 12000 x g for 10 min at 4$^{\circ}\mathrm{C}$. The resulting supernatant was transferred to a new tube and 500 $\mu\mathrm{l}$ isopropyl alcohol was added. The tubes were mixed by repeated inversion. Then the samples were incubate for 10 minutes at -70$^{\circ}\mathrm{C}$ and subsequently centrifuged for 10 min at 12000 x g at 4$^{\circ}\mathrm{C}$. The supernatant was decanted and 75% ethanol (1 ml) was added to wash the RNA pellet. The tubes were centrifuged at 7500 x g for 5 min. The supernatant was decanted and the pellet air dried for 5-10 min. The pellet was then redissolved in 50 $\mu\mathrm{l}$ nuclease-free water (USB, USA). The concentration and purity of the isolated total RNA was assessed by reading the absorption at 260 nm and 280 nm. The integrity was assessed by staining 2 $\mu\mathrm{g}$ total RNA on a 1.2% agarose gel.

Subtractive library In order to clone genes only expressed during and after a stress, the subtractive kit from Clonetech was used (BD PCR-Select cDNA Subtraction Kit, Figure 3.2 on page 58). Specifically, tester cDNA and a driver cDNA were prepared from the extracted total RNA using MMLV reverse transcriptase (Genehunter, USA). Tester cDNA contains the desired transcripts to be cloned. Driver cDNA contains the transcripts the tester cDNA will be subtracted from. In this study, for example, to clone transcripts only

expressed during desiccation and not during recovery, the transcripts extracted during the stress would represent the tester and the transcripts extracted two hours after rehydration would represent the driver. The two cDNA populations were digested with *Rsa*I in order to obtain short, blunt-ended molecules. The tester population was divided in two samples each ligated to different adapters. The driver cDNA was not ligated to adapters. Then the two tester cDNA and the driver cDNA were denatured and each tester cDNA population was mixed with excess driver cDNA. The samples were allowed to hybridize at 68°C for eight hours. Afterwards the two probes were combined and were allowed to continue to hybridize (68°C). The resulting solution was then amplified by two rounds of PCR selective towards double stranded transcripts which had different adapters. This procedure was performed to generate subtractive cDNA populations of transcripts containing uniquely expressed genes during osmotic and desiccation stress and transcripts uniquely expressed after two hours of recovery from both stresses. This procedure was done with both *Panagrolaimus* species generating eight batches of transcripts (Table 3.1 on page 53). A validation of the subtractive library by Southern-blot was not performed.

The large transcripts were purified from smaller transcripts or transcript fragments (less then 200 nucleotides) using Chromaspin columns (Clonetech, USA). The transcripts were cloned in *E . coli* using 'Blunt zero cloning Kit' from Invitrogen (Invitrogen, USA). The resulting clones were sequenced by Agencourt Bioscience (Agencourt Bioscience Corporation, USA). All six frames of the transcripts were translated into amino acid sequences and compared to the virtual translated genome library of *C. elegans* and *C. briggsae* using Ensemble (http://www.ensembl.org/Caenorhabditis-elegans/index.html) [92]. The threshold for positive identification of the transcripts was an E-value of less than 1. Furthermore, the sequences were compared with each other to identify contiguous sequences (con-

tigs) among the libraries, which was done using the program CAP3 Sequence Assembly Program (http://pbil.univ-lyon1.fr/cap3.php) [89].

Stress survival Survival was measured using SYTOX green (Molecular Probes, USA). The nematodes were placed in S-buffer containing 1μM SYTOX green dye. After a 15 min incubation, 100-$\mu\mathrm{l}$ aliquots from each sample were aliquoted onto a 96 well plate (Novagen). For each sample, 5 wells were used, paying attention to have at least 100 worms per well. The samples were visualized using a confocal microscope (MS510, Zeiss). Digital images were taken of each well and the images were analyzed manually. The percent survival was determined by counting both the total number of worms as well as the number of dead worms (SYTOX stained) % survival= $\frac{Total\ worms - Dead\ worms}{Total\ worms}$

Knock-down procedure The genes chosen for selective knockdown are shown in Table 3.2 on page 59. Knock-down was performed using RiboMAX (Invitrogen,USA). Specifically, the desired transcripts from the subtractive libraries were amplified by PCR from the cloned *E. coli* plasmids using the adapter sequences with a T7 promoter region at the 5' end. 100 $\mu\mathrm{l}$ of the PCR product was incubated with RiboMAX solution for 30 min to generate double-stranded RNA (dsRNA). The dsRNA was purified with an ethanol precipitation and re-suspended in 200 $\mu\mathrm{l}$ nuclease-free water. The dsRNA was quantitated by ultraviolet light absorbance and samples were kept at -70$^{\circ}\mathrm{C}$ until used.

The worms were sucrose cleaned and divided in six equal aliquots. The dsRNA was added to the worm suspension to a final concentration of 0.5 $\mu\mathrm{g}/\mu\mathrm{l}$ dsRNA. To the con-

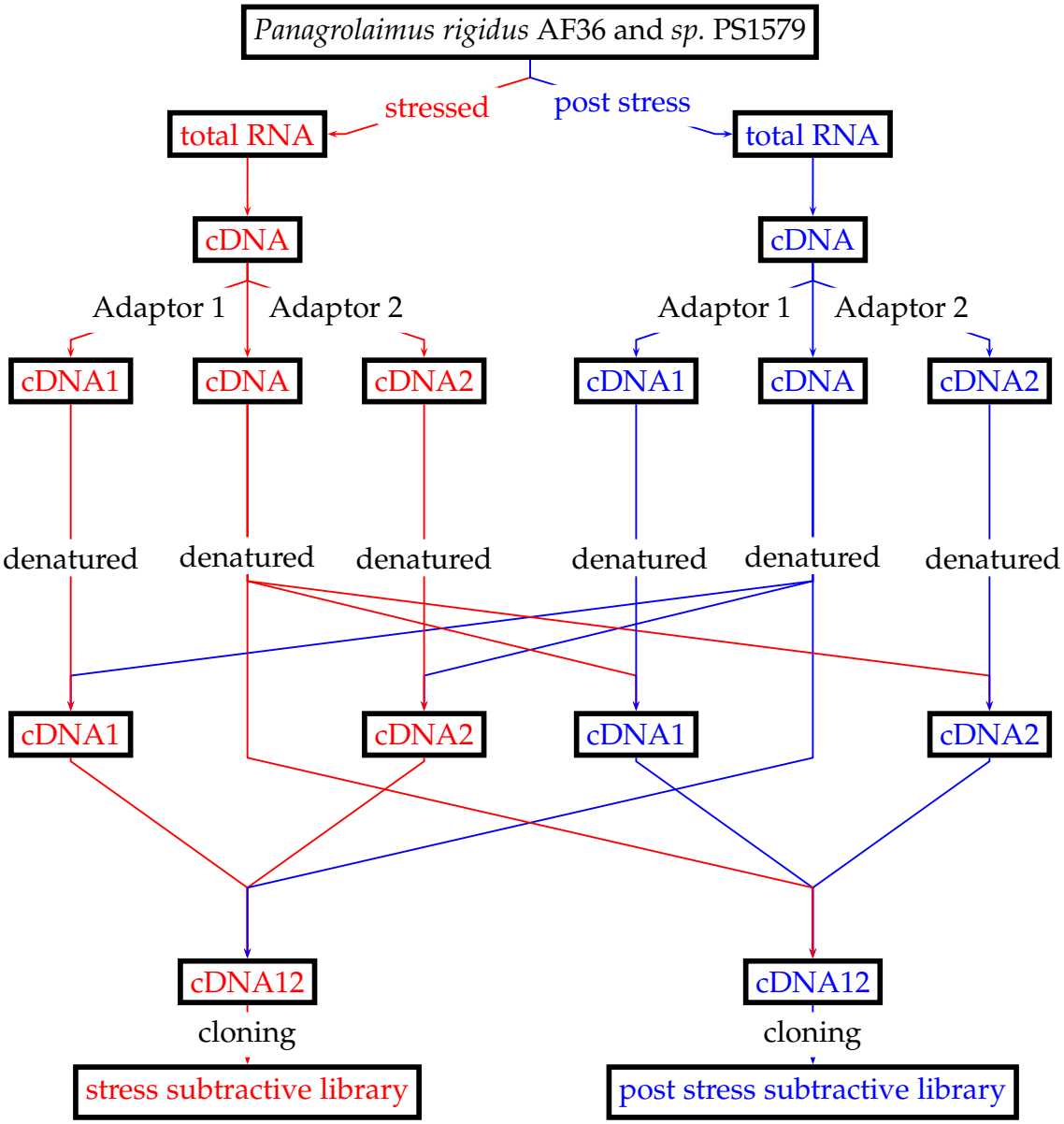


Figure 3.2: Schema of the subtractive libraries as employed in this study

Table 3.2: Transcripts chosen for the knock-down in *Panagrolaimus rigidus* AF36 and *sp.* PS1579. Function information is taken from Wormbase [78].

***Panagrolaimus rigidus* AF36**			
transcript	library	*C. elegans* gene	function
snb-1	des	synaptobrevin	plays a role in vesicle docking and/or fusion
unc-11	des	clathrin-adaptor protein	essential for embryogenesis, locomotion, and pharyngeal pumping; regulates neurotransmitter release by controlling vesicle trafficking and fusion
tps-2	des	trehalose-6-P synthase	Trehalose synthesis
lea-1	post des	LEA related	participates in anhydrobiosis
R05G6.7	osmo	anion-selective channel protein	unknown
***Panagrolaimus* PS1579**			
gene	library	*C. elegans* gene	function
taf-1	post osmo	TBP-associated transcription factor	required for proper embryonic and larval development
lea-1	post des	LEA related	participates in anhydrobiosis
xrn-2	post osmo	5′-3′ exonuclease	unknown
R05G6.7	osmo	anion-selective channel protein	unknown
unc-11	des	clathrin-adaptor protein	essential for embryogenesis, locomotion, and pharyngeal pumping; regulates neurotransmitter release by controlling vesicle trafficking and fusion

trol nuclease-free water was added. The samples were incubated for 12 hours at 16°C. The samples were then submitted to desiccation and osmotic stresses with survival determined as described above. These experiments were done in duplicate. However, a validation of the knock-down by Southern blot was performed, but the results were not conclusive.

Cloned transcript analysis The resulting clones from the subtractive libraries were sequenced by Agencourt Bioscience (Agencourt Bioscience Corporation, USA). In order to identify shared transcripts between the subractive libraries each transcript was compared with each transcript in the library. For this purpose the program CAP3 Sequence Assembly Program (http://pbil.univ-lyon1.fr/cap3.php) [89] was used. The program was designed to assemble transcripts to identify contiguous sequences. In this study the program was used to identify overlapping sequences in the subtractive libraries.
In order to identify cloned transcripts, the translated amino acid sequences of all transcript present in the libraries were compared to the virtual translated genome library of *C. elegans* and *C. briggsae* using Ensembl (http://www.ensembl.org/Caenorhabditis-elegans/index.html) [92]. The threshold for positive identification of the transcripts was an E-value of less than 0.

Gene ontology analysis The identified trascripts of each library were converted to Affymetrix chip nomenclature using the webtool NetAffyx (Affymetrix, USA) in order to import them into DAVID (Database for Annotation, Visualization, and Integrated Discovery, NIAID/NIH, USA) [50]. The program annotated each gene with the gene ontology term from the Gene Ontology Consortium (GO) [9]. The program EASE (Expression Analysis Systematic Ex-

Table 3.3: Count of the number of resulting clones from each subtractive library of *Panagrolaimus rigidus* AF36 and *sp.* PS1579

Library	Number of clones	Number of PS1579	Numbers of AF36
Osmotic stress	140	56	84
Post osmotic stress	103	61	42
Desiccation	240	98	142
Post desiccation	371	192	179
Total	854	407	447

plorer, NAID/NIH, USA) then sorted the list of genes to biological annotation clusters. An Annotation cluster is defined as the identification of terms or phrases that describe a statistically significant number of genes in a list with respect to the number of genes described by the term or phrase in the population of genes from which it is derived. An EASE score is attributed to each gene and Annotation cluster by the program. This score is an adjustment to the Fisher exact probability and weights significance in favor of themes by more genes. For all analysis a high stringency for the Annotation clustering was used. The high stringency setting generates less Annotation clusters with more tightly associated genes in each cluster [85].

3.3 Results

3.3.1 Sequence analysis of the libraries

The data from the subtractive libraries are summarized in Table 3.3 and Table 3.4 on page 61 and page 62. Altogether, 854 transcripts were identified and sequenced from eight subtractive libraries. All alignments were done by comparing the predicted translat-

Table 3.4: Count of the number of identified and unknown transcripts found in the subtractive library of *Panagrolaimus rigidus* AF36 and *sp.* PS1579.

Library	Ensembl	unknown
Osmotic stress	68	72
Post osmotic stress	76	27
Desiccation	93	147
Post desiccation	170	201
Total	407	447

ed sequence of the libraries with the *C. elegans* and *C. briggsae* translated cDNA library. An analysis for overlapping sequences in the eight libraries gave 136 overlapping sequences (see Appendix C on page 205 for a complete list of overlapping sequences), only a few of these had overlapping transcripts between different stresses and between species. Table 3.5 on page 64 lists all overlapping sequences containing sequences found at least in a library from a different *Panagrolaimus* species or a stress library. The identification of the transcripts was performed using the translated amino acid sequence of the transcript and compared to the virtual translated cDNA library from *C. elegans* and *C. briggsae* (E-value lower than 0).

The list contains 18 overlapping sequences and only five sequences were identified. For example, the first overlapping sequences in the table were identified as belonging to an aspartyl protease *asp-1*. This protease is found in three different libraries (post desiccation, osmotic stress, and post osmotic stress).

During the design phase of this work overlapping sequences were anticipated in order to identify conserved stress related sequences. A complete list of all overlapping sequences can be found in Appendix C on page 205. Overlapping sequences number

8 and 114 were the only sequences cloned that are derived from the same stress library (desiccation and post desiccation respectively), but from different species libraries. However, the identification of these sequences gave no positive identification. Overlapping sequences number 15, 37, 64, and 93 contain cloned sequences from the same species and sequences from either both stress libraries or both post stress libraries. Here only overlapping sequences 15 and 37 were able to be identified as *dnj-14* and *act-4* respectively.

These results suggest that either the response to osmotic and desiccation stress is different or the two *Panagrolaimus* species are so phylogenetically divergent that nucleic acid sequences similarities cannot be performed. Therefore, the next approach was to sort the libraries according to their gene identification and sort the overlaps. The result is shown in Figure 3.3 on page 65. A total of 20 overlaps (71 sequences) from 407 (18%) identified sequences were found. A list of the sequences with the corresponding libraries and species can be found in Table 3.6 on page 66.

Table 3.5: Analysis of the overlapping sequences (Overlap) computed by CAP3 software from *Panagrolaimus rigidus* AF36 and *sp.* PS1579. d - desiccation library, p-d - post desiccation library, o - osmotic stress libraries, p-0 - post desiccation library, overlap no - overlapping sequences number. Sequence identification was performed using the translated sequences of the transcripts found in the contig and compared to the translated cDNAs of *C. elegans* and *C. briggsae*. A complete list of overlapping sequence can be found in the Appendix C on page 205. Green are overlapping sequences present in the libary of one species and in multiple stress sublibraries, blue are overlapping sequences from different species but same sublibrary, magenta are overlapping sequences from different species and sublibraries.

AF36	PS1579	d	p-d	o	p-o	Overlap no
	x		x	x	x	C100 (*asp-1*)
	x		x		x	C123 (*ubq-1*)
	x		x	x		C128
	x	x		x		C64, C93
	x	x	x			C90
x			x		x	C15 (*dnj-14*)
x		x	x			C31
x		x		x		C37 (*act-4*)
x		x	x			C43, C60
x	x		x			C114
x	x	x				C8
x	x	x		x		C23
x	x	x	x			C6
x	x	x	x	x		C65 (*rrn-3.1*)
x	x		x		x	C86
x	x	x		x	x	C9

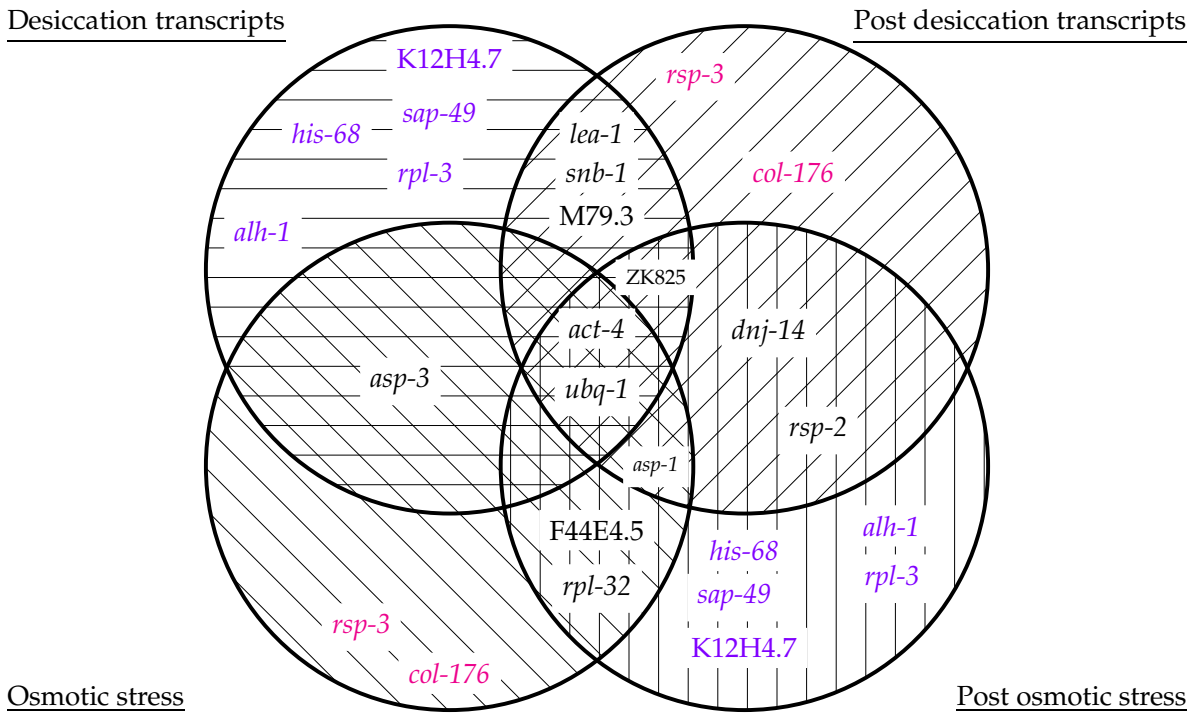


Figure 3.3: Venn diagram of the resulting common identified transcripts found in the *Panagrolaimus* libraries. Abbreviations: act - actin, alh - aldehydedehydrogen, asp - asparyl protease, col - collagen, dnj - Prokaryotic heat shock protein, his - histone, rpl - ribosomal protein large subunit, rps - ribosomal protein small subunit, sap - splicosomal associated protein, snb - synaptobrevin related, ubq - ubiquitin. A list of the all the sequences can be found in Table 3.6 on page 66.

Table 3.6: Common identified sequences in the libraries. Abbreviations: act - actin, alh - aldehydedehydrogen, asp - asparyl protease, col - collagen, dnj - Prokaryotic heat shock protein, his - histone, rpl - ribosomal protein large subunit, rps - ribosomal protein small subunit, sap - splicosomal associated protein, snb - synaptobrevin related, ubq - ubiquitin.

gene symbol	species	Ensembl	E-value	library	no
act-4	AF36	M03F4.2	1.20E-35	salt	c37
act-4	AF36	M03F4.2	2.80E-40	post salt	
act-4	AF36	M03F4.2a.1	2.90E-36	des	c37
act-4	AF36	M03F4.2a.1	2.80E-36	des	c37
act-4	AF36	M03F4.2a.1	2.90E-36	des	c37
act-4	AF36	M03F4.2a.1	1.50E-71	post des	
act-4	PS1579	M03F4.2a.1	1.00E-35	des	
act-4	PS1579	M03F4.2a.1	9.40E-79	post des	
alh-1	AF36	F54D8.3	2.70E-61	des	
alh-1	PS1579	F54D8.3a.1	3.10E-42	post salt	
asp-1	PS1579	Y39B6A.20.1	4.80E-25	post des	C 100
asp-1	PS1579	Y39B6A.20.1	1.90E-25	post salt	C 100
asp-1	PS1579	Y39B6A.20.1	1.80E-25	salt	C 100
asp-1	AF36	Y39B6A.20.1	2.40E-25	post des	C 87
asp-1	AF36	Y39B6A.20.1	2.40E-25	post des	C 87
asp-1	AF36	Y39B6A.20.1	2.30E-25	post des	C 87
asp-1	AF36	Y39B6A.20	1.60E-31	post salt	C19
asp-1	AF36	Y39B6A.20	1.00E-31	post salt	C19
asp-1	PS1579	Y39B6A.20.1	1.40E-70	post des	
asp-1	PS1579	Y39B6A.20.1	1.90E-50	salt	
asp-1	PS1579	Y39B6A.20	1.90E-25	post salt	
asp-1	PS1579	Y39B6A.20.1	1.70E-26	salt	
asp-3	PS1579	H22K11.1	2.60E-26	des	C 70
asp-3	PS1579	H22K11.1	2.80E-27	des	C 70
asp-3	AF36	H22K11.1	1.40E-36	salt	C28
asp-3	AF36	H22K11.1	1.40E-36	salt	C28
col-176	AF36	ZC373.7	0	salt	
col-176	PS1579	ZC373.7	8.20E-21	post des	
dnj-14	AF36	K02G10.8	1.50E-14	post des	C15
dnj-14	AF36	K02G10.8	1.50E-14	post salt	C15
ZK829	AF36	ZK829.4	3.60E-10	post salt	
ZK829	AF36	ZK829.4.1	1.60E-49	post des	
ZK829	PS1579	ZK829.4.1	6.00E-50	des	
his-68	PS1579	T23D8.6	1.40E-36	des	C 67
his-68	PS1579	T23D8.6	1.40E-36	des	C 67

continues on the next page...

Table 3.6: continued

gene symbol	species	Ensembl	E-value	library	no
his-68	AF36	T23D8.6	2.80E-22	post salt	
K12H4.7	PS1579	K12H4.7	0	post salt	
K12H4.7	PS1579	K12H4.7	4.30E-52	des	
lea-1	PS1579	K08H10.1.1	1.60E-10	post des	C 136
lea-1	PS1579	K08H10.1.1	9.20E-11	post des	C 136
lea-1	AF36	K08H10.1.1	9.50E-09	post des	
lea-1	PS1579	K08H10.1.1	5.00E-09	post des	
lea-1	PS1579	K08H10.2a	0.13		
hsp-70	AF36	F44E5.4	2.10E-74	post salt	
hsp-70	AF36	F44E5.4	7.50E-88	salt	
M79.3	PS1579	M79.3	4.5E-19	post des	
M79.3	PS1579	M79.3	9.20E-19	des	C 76
rpl-3	AF36	F13B10.2a.1	1.80E-89	des	
rpl-3	PS1579	F13B10.2d	3.10E-64	post salt	
rpl-32	AF36	T24B8.1.3	5.40E-51	post salt	
rpl-32	AF36	T24B8.1.3	5.00E-25	salt	
rps-2	AF36	C49H3.11.1	3.70E-29	post salt	
rps-2	PS1579	C49H3.11.1	4.00E-30	post salt	
rps-2	PS1579	C49H3.11.1	3.50E-20	post des	
rps-3	AF36	C23G10.3.1	5.10E-78	post des	c 50
rps-3	AF36	C23G10.3.1	5.20E-78	post des	c 50
rps-3	PS1579	C23G10.3.1	1.00E-68	salt	
sap-49	AF36	C08B11.5.2	0.03	des	
sap-49	PS1579	C08B11.5.2	0.21	post salt	
snb-1	AF36	T10H9.4	2.30E-32	post des	
snb-1	AF36	T10H9.4	4.20E-33	des	C7
snb-1	AF36	T10H9.4	4.20E-33	des	C7
ubq-1	PS1579	F25B5.4a.2	2.40E-67	post des	C 123
ubq-1	PS1579	F25B5.4a.2	5.00E-67	post salt	C 123
ubq-1	PS1579	F25B5.4a.2	2.50E-67	post des	C 123
ubq-1	AF36	F25B5.4a.2	5.90E-75	salt	
ubq-1	PS1579	F25B5.4a	5.00E-67	post salt	
ubq-1	PS1579	F25B5.4a.2	4.80E-75	post des	
ubq-1	PS1579	F25B5.4a.2	2.40E-67	post des	
ubq-1	PS1579	F25B5.4c.2	3.00E-55	des	
ubq-1	PS1579	F25B5.4c.2	1.70E-38	post salt	

3.3.2 Gene ontology analysis of the libraries

Each identified transcript in the libraries was assigned a biological process term from the gene ontology database if available [9]. The transcripts with the biological process term were then classified in groups and clustered according to their degree of similarity using the software DAVID and EASE [50, 85]. For example, water homeostasis, osmoregulation, and regulation of body fluids are terms annotated for the transcripts *toc-1*, *sqv-2* and *eft-2*. These transcripts are clustered according to a similarity algorithm forming an Annotation cluster. Furthermore, a transcript can be annotated with different terms, i.e. *toc-1* is annotated with developmental processes, body morphogenesis, signal transduction and osmoregulation, which leads to transcripts being present in multiple Annotation clusters. In order to identify terms which are enriched in the dataset, the program EASE calculates an enrichment score (in -log scale) for each cluster. The score states the overall enrichment score for the group based on the EASE score of each term member. The higher the score, the more enriched is the group. In order to understand what biological processes are involved during and after the desiccation and osmotic stress, each library was clustered using this method.

Desiccation library

Table 3.7 on page 69 lists the first 20 gene ontology terms according to the number of transcripts annotated with the term. The Table 3.8 on page 70 shows the annotation clusters as calculated by EASE according to their enrichment score. Annotation cluster 4 contains the gene ontology term with the most annotated transcripts in the desiccation library (50%). The second most represented Annotation cluster (45%) is cluster 5 containing transcripts annotated to be involved in multicellular development. This cluster is followed by larval development (33%, annotation cluster 1) and cellular protein metabolic processes (33%, Annotation cluster 2). The third most enriched Annotation cluster represent transcripts involved in cytokinesis, embryonic cleavage and cell division.

Table 3.7: First 20 most annotated gene ontology names for the desiccation library.

Term	Count	%	EASE-score
GO:0009987 cellular process	51	62.96%	1.551E-02
GO:0008152 metabolic process	43	53.09%	5.026E-02
GO:0044238 primary metabolic process	40	49.38%	8.901E-03
GO:0044237 cellular metabolic process	40	49.38%	6.611E-03
GO:0032501 multicellular organismal process	38	46.91%	2.286E-02
GO:0032502 developmental process	37	45.68%	2.041E-02
GO:0007275 multicellular organismal development	36	44.44%	1.970E-02
GO:0043170 macromolecule metabolic process	34	41.98%	2.551E-02
GO:0000003 reproduction	28	34.57%	7.622E-05
GO:0009792 embryonic development ending in birth or egg hatching	27	33.33%	1.756E-02
GO:0044260 cellular macromolecule metabolic process	27	33.33%	2.881E-04
GO:0009791 post-embryonic development	27	33.33%	2.548E-05
GO:0009790 embryonic development	27	33.33%	2.324E-02
GO:0040007 growth	27	33.33%	5.408E-04
GO:0019538 protein metabolic process	27	33.33%	3.386E-04
GO:0044267 cellular protein metabolic process	27	33.33%	1.989E-04
GO:0002164 larval development	24	29.63%	1.939E-04
GO:0002119 larval development (sensu Nematoda)	24	29.63%	1.897E-04
GO:0050896 response to stimulus	18	22.22%	2.714E-02
GO:0048856 anatomical structure development	16	19.75%	1.091E-02

Table 3.8: First five most enriched gene ontology clusters for the desiccation library. For Annotation cluster EASE score see Table 3.15 on page 82.

Annotation cluster 1	gene	EASE-score
GO:0009791 post-embryonic development	27	2.55E-05
GO:0002119 larval development (sensu Nematoda)	24	1.90E-04
GO:0002164 larval development	24	1.94E-04
Annotation cluster 2	gene	EASE-score
GO:0044267 cellular protein metabolic process	27	1.99E-04
GO:0044260 cellular macromolecule metabolic process	27	2.88E-04
GO:0019538 protein metabolic process	27	3.39E-04
Annotation cluster 3	gene	EASE-score
GO:0000910 cytokinesis	6	0
GO:0040016 embryonic cleavage	7	0
GO:0051301 cell division	7	0.02
Annotation cluster 4	gene	EASE-score
GO:0044237 cellular metabolic process	40	0.01
GO:0044238 primary metabolic process	40	0.01
GO:0008152 metabolic process	43	0.05
Annotation cluster 5	gene	EASE-score
GO:0007275 multicellular organismal development	36	0.02
GO:0032502 developmental process	37	0.02
GO:0032501 multicellular organismal process	38	0.02

Table 3.9: First 20 most annotated gene ontology names for the post desiccation library

Term	Count	%	EASE-score
GO:0009987 cellular process	52	58.43%	0.02
GO:0032501 multicellular organismal process	47	52.81%	4.06E-05
GO:0008152 metabolic process	46	51.69%	0.02
GO:0044238 primary metabolic process	44	49.44%	9.82E-04
GO:0032502 developmental process	44	49.44%	2.25E-04
GO:0007275 multicellular organismal development	43	48.31%	2.07E-04
GO:0044237 cellular metabolic process	41	46.07%	0.01
GO:0009790 embryonic development	40	44.94%	2.20E-07
GO:0009792 embryonic development ending in birth or egg hatching	39	43.82%	4.14E-07
GO:0043170 macromolecule metabolic process	37	41.57%	0.01
GO:0040007 growth	36	40.45%	3.16E-08
GO:0009791 post-embryonic development	32	35.96%	9.16E-08
GO:0000003 reproduction	32	35.96%	1.51E-06
GO:0065007 biological regulation	30	33.71%	0.03
GO:0050789 regulation of biological process	30	33.71%	0.02
GO:0002164 larval development	29	32.58%	9.62E-07
GO:0002119 larval development (sensu Nematoda)	29	32.58%	9.34E-07
GO:0044260 cellular macromolecule metabolic process	28	31.46%	1.87E-04
GO:0019538 protein metabolic process	26	29.21%	0
GO:0044267 cellular protein metabolic process	26	29.21%	8.65E-04

The percentage of transcripts annotated in this cluster is 7% of the total number of transcripts in the list (7 out of 81).

Post desiccation library

Table 3.9 on page 71 lists gene ontology terms according to the number of transcripts annotated each term. Table 3.10 on page 72 provides the annotation clusters as calculated by EASE.

The gene ontology term most frequent annotated with the transcripts involved primary metabol-

Table 3.10: First five most enriched gene ontology clusters for the post desiccation library. For Annotation cluster EASE score see Table 3.15 on page 82.

Annotation cluster 1	gene	EASE-score
GO:0009791 post-embryonic development	32	9.16E-08
GO:0002119 larval development (sensu Nematoda)	29	9.34E-07
GO:0002164 larval development	29	9.62E-07
Annotation cluster 2	gene	EASE-score
GO:0006412 translation	16	1.28E-09
GO:0044249 cellular biosynthetic process	18	5.54E-07
GO:0010467 gene expression	18	0.03
Annotation cluster 3	gene	EASE-score
GO:0009790 embryonic development	40	2.20E-07
GO:0009792 embryonic development ending in birth or egg hatching	39	4.14E-07
GO:0032501 multicellular organismal process	47	4.06E-05
GO:0007275 multicellular organismal development	43	2.07E-04
GO:0032502 developmental process	44	2.25E-04
Annotation cluster 4	gene	EASE-score
GO:0044260 cellular macromolecule metabolic process	28	1.87E-04
GO:0044267 cellular protein metabolic process	26	8.65E-04
GO:0019538 protein metabolic process	26	0
Annotation cluster 5	gene	EASE-score
GO:0040010 positive regulation of growth rate	21	4.91E-04
GO:0040009 regulation of growth rate	21	5.08E-04
GO:0045927 positive regulation of growth	22	0
GO:0040008 regulation of growth	22	0
GO:0048518 positive regulation of biological process	22	0

Table 3.11: First 20 most annotated gene ontology names for the osmotic stress library

Term	Count	%	EASE-score
GO:0009987 cellular process	27	41.54%	0.06
GO:0032502 developmental process	25	38.46%	5.77E-04
GO:0032501 multicellular organismal process	25	38.46%	0
GO:0007275 multicellular organismal development	24	36.92%	9.94E-04
GO:0044237 cellular metabolic process	21	32.31%	0.05
GO:0009790 embryonic development	19	29.23%	0
GO:0009792 embryonic development ending in birth or egg hatching	18	27.69%	0
GO:0043170 macromolecule metabolic process	18	27.69%	0.09
GO:0000003 reproduction	15	23.08%	0
GO:0044260 cellular macromolecule metabolic process	14	21.54%	0.01
GO:0044267 cellular protein metabolic process	14	21.54%	0.01
GO:0019538 protein metabolic process	14	21.54%	0.01
GO:0040007 growth	13	20.00%	0.04
GO:0010467 gene expression	11	16.92%	0.03
GO:0044249 cellular biosynthetic process	8	12.31%	0
GO:0009058 biosynthetic process	8	12.31%	0.02
GO:0006412 translation	7	10.77%	4.95E-04
GO:0007276 gamete generation	7	10.77%	0.04
GO:0009059 macromolecule biosynthetic process	7	10.77%	0
GO:0019953 sexual reproduction	7	10.77%	0.05

ic processes (50%, annotation cluster 6) and embryonic development (50%, annotation cluster 3). Gene ontology terms clustered in the Annotation cluster 4 (cellular macromolecule metabolic processes), cover 31% of the total number of transcripts found in the post desiccation library. The next most annotated gene ontology terms involve translation (20%, annotation cluster 2).

Osmotic stress library

Table 3.11 on page 73 lists genes ontology terms according to the number of transcripts annotated with the term. The Table 3.12 on page 74 shows the annotation clusters as calculated by

Table 3.12: First five most enriched gene ontology clusters for the osmotic stress library. For Annotation cluster EASE score see Table 3.16 on page 83.

Annotation cluster 1	gene	EASE-score
GO:0032502 developmental process	25	5.77E-04
GO:0007275 multicellular organismal development	24	9.94E-04
GO:0032501 multicellular organismal process	25	0
Annotation cluster 2	gene	EASE-score
GO:0006412 translation	7	4.95E-04
GO:0009059 macromolecule biosynthetic process	7	0
GO:0044249 cellular biosynthetic process	8	0
GO:0009058 biosynthetic process	8	0.02
Annotation cluster 3	gene	EASE-score
GO:0044267 cellular protein metabolic process	14	0.01
GO:0044260 cellular macromolecule metabolic process	14	0.01
GO:0019538 protein metabolic process	14	0.01
Annotation cluster 4	gene	EASE-score
GO:0030154 cell differentiation	5	0.05
GO:0048869 cellular developmental process	5	0.05
GO:0048468 cell development	4	0.06
Annotation cluster 5	gene	EASE-score
GO:0044237 cellular metabolic process	21	0.05
GO:0043170 macromolecule metabolic process	18	0.09
GO:0044238 primary metabolic process	19	0.18
GO:0008152 metabolic process	22	0.19

DAVID according to their enrichment score.

The gene ontology terms mostly annotated from the osmotic stress library involved the development (38%, Annotation cluster 1). The second most annotated terms concerned cellular metabolic processes (32%, annotation cluster 5) and embryonic development, with the latter not included in any enriched annotation cluster, due to the high p-values. Annotation cluster 3 representing transcripts annotated with cellular protein metabolic process represented the next most annotated gene ontology term (27%). The next clusters containing the most annotated transcripts involved regulation of growth (annotation cluster 7 and 8) and translation (10%, annotation cluster 2).

Post osmotic library

Table 3.13 on page 76 list gene ontology term according to the number of transcripts associated with the term. The Table 3.14 on page 77 shows the annotation clusters as calculated by EASE according to their enrichment score.

The biological function gene ontology term most frequent annotated with the transcript found in the post osmotic stress library are cellular and primary metabolic processes (61%). However these terms were not present in the most enriched gene ontology annotation clusters. The next terms were annotated to (embryonic) development (44-50%, annotation cluster 3) and larval development (33%, annotation cluster 1). The next gene ontology terms most frequent annotated with the transcripts are also not present in any enriched annotation cluster and deal with metabolic processes (33%) and biosynthetic processes (23%).

Table 3.13: First 20 most annotated gene ontology names for the post osmotic stress library

Term	Count	%	EASE-score
GO:0008152 metabolic process	43	72.88%	3.76E-07
GO:0009987 cellular process	37	62.71%	0.04
GO:0044238 primary metabolic process	36	61.02%	3.54E-05
GO:0044237 cellular metabolic process	33	55.93%	7.22E-04
GO:0032502 developmental process	30	50.85%	0.01
GO:0043170 macromolecule metabolic process	30	50.85%	9.07E-04
GO:0007275 multicellular organismal development	29	49.15%	0.01
GO:0032501 multicellular organismal process	29	49.15%	0.02
GO:0009792 embryonic development ending in birth or egg hatching	26	44.07%	1.51E-04
GO:0009790 embryonic development	26	44.07%	2.23E-04
GO:0000003 reproduction	22	37.29%	1.31E-04
GO:0009791 post-embryonic development	21	35.59%	7.37E-05
GO:0002164 larval development	20	33.90%	8.80E-05
GO:0002119 larval development (sensu Nematoda)	20	33.90%	8.63E-05
GO:0019538 protein metabolic process	20	33.90%	0
GO:0040007 growth	20	33.90%	0
GO:0043283 biopolymer metabolic process	19	32.20%	0.06
GO:0044260 cellular macromolecule metabolic process	17	28.81%	0.02
GO:0044267 cellular protein metabolic process	16	27.12%	0.04
GO:0006139 nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	15	25.42%	0.05

Table 3.14: First five most enriched gene ontology clusters for the post osmotic stress library. For Annotation cluster EASE score see Table 3.16 on page 83.

Annotation cluster 1	gene	EASE-score
GO:0009791 post-embryonic development	21	7.37E-05
GO:0002119 larval development (sensu Nematoda)	20	8.63E-05
GO:0002164 larval development	20	8.80E-05
GO:0040007 growth	20	0
Annotation cluster 2	gene	EASE-score
GO:0065004 protein-DNA complex assembly	5	2.19E-04
GO:0006333 chromatin assembly or disassembly	5	3.29E-04
GO:0051276 chromosome organization and biogenesis	6	5.95E-04
GO:0006334 nucleosome assembly	4	9.37E-04
GO:0006323 DNA packaging	5	9.83E-04
Annotation cluster 3	gene	EASE-score
GO:0009792 embryonic development ending in birth or egg hatching	26	1.51E-04
GO:0009790 embryonic development	26	2.23E-04
GO:0032502 developmental process	30	0.01
GO:0007275 multicellular organismal development	29	0.01
GO:0032501 multicellular organismal process	29	0.02
Annotation cluster 4	gene	EASE-score
GO:0006812 cation transport	10	7.33E-05
GO:0006811 ion transport	10	0.01
GO:0006810 transport	11	0.32
Annotation cluster 5	gene	EASE-score
GO:0015986 ATP synthesis coupled proton transport	4	0
GO:0006754 ATP biosynthetic process	4	0
GO:0006753 nucleoside phosphate metabolic process	4	0
GO:0046034 ATP metabolic process	4	0
GO:0009206 purine ribonucleoside triphosphate biosynthetic process	4	0
GO:0009201 ribonucleoside triphosphate biosynthetic process	4	0
GO:0009145 purine nucleoside triphosphate biosynthetic process	4	0

3.3.3 Gene knock-down analysis

Several genes were selected for RNAi-based knock-down (Table 3.2 on page 59). One *lea* gene from *Panagrolaimus rigidus* AF 36 and one from *Panagrolaimus sp.* PS1579 and the *tps* gene from *Panagrolaimus rigidus* AF36 were chosen as standard genes known to be involved in desiccation tolerance. For *Panagrolaimus rigidus* AF36, the genes *snb-1*, *unc-11* and R05G6.7 were chosen. For the knock-down experiment on *Panagrolaimus sp.* PS1579, *taf-1* and *xrn-2* genes were chosen, as well as *unc-11* and R05G6.7 from species *rigidus* AF36 in order to determine if transcripts from *Panagrolaimus rigidus* AF36 affected the survival of *Panagrolaimus sp.* PS1579. The RNAi knock-downs were submitted to desiccation and osmotic stress, in order to investigate if these transcripts did indeed affect the survival of both stresses or only the survival of one stress. The choice of a RNAi delivery by soaking was taken because injection would have damaged the cuticle influencing negatively the survival of the worm during stress and feeding would have been compromised by the presence of an additional bacteria which seem to be a symbiont of the two *Panagrolaimus* species.

The result of the knock-downs is shown in Figure 3.4 on page 79 and Figure 3.5 on page 80. The gene knock-down experiment for *Panagrolaimus rigidus* AF36 showed a significant decrease in osmotic stress survival for the *lea-1* and R05G6.8 genes, whereas desiccation stress resulted in significantly reduced survival for the *unc-11* and *tps-2* knock-downs. The gene knock-down experiments for *Panagrolaimus sp.* PS1579 showed significant increases in viability for *lea-1* and *taf-1* knock-downs during osmotic stress. None of the knock-downs showed a significant change in survival due to desiccation stress.

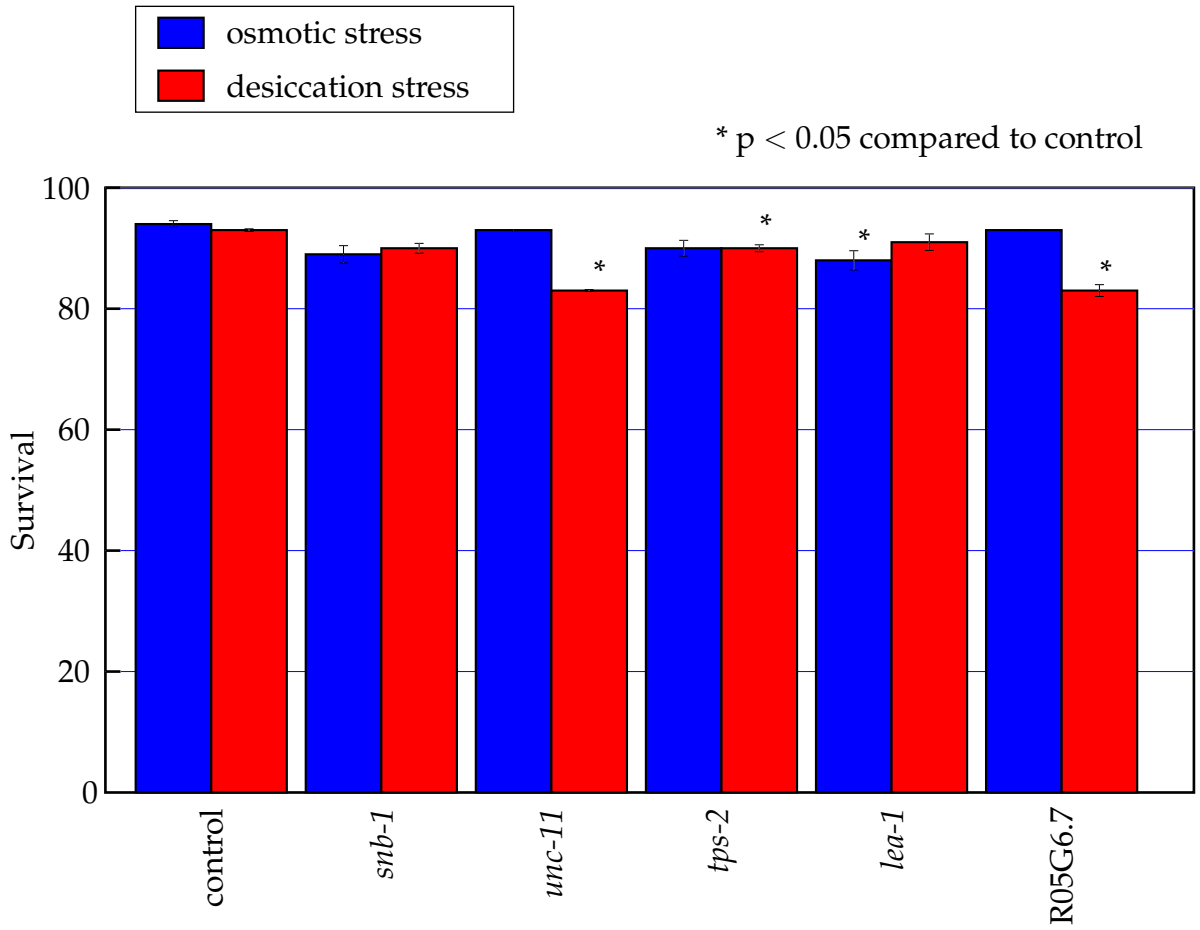


Figure 3.4: Knock-down experiment with *Panagrolaimus rigidus* AF36 - Five *Panagrolaimus rigidus* AF36 cultures were incubated with dsRNA from *lea tps snb-1*, *unc-11* and R05G6.7 respectively for 12 hours. Then the samples were desiccated for 48 hours at 0% relative humidity or osmotic stressed in 500 mM NaCl S-buffer. After two hours of recovery, the survival was measured. Error bars show the standard error of two replicates.

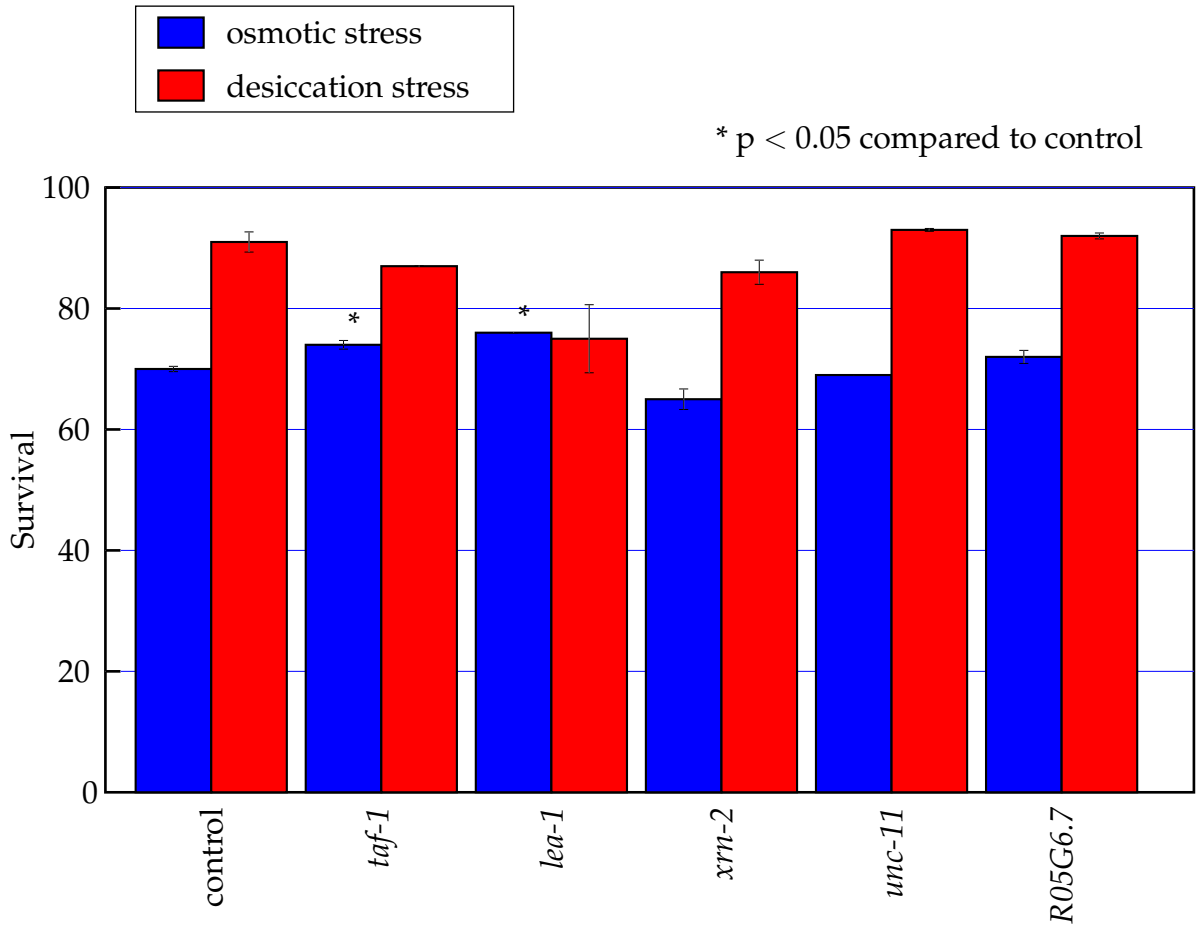


Figure 3.5: Knock-down experiment with *Panagrolaimus sp.* PS1579 - Five *Panagrolaimus sp.* PS1579 cultures were incubated with dsRNA from *lea taf-1 xrn-1*, *unc-11* (from *Panagrolaimus rigidus* AF36 library) and R05G6.7 (from *Panagrolaimus rigidus* AF36 library) respectively for 12 hours. Then the samples were desiccated for 48 hours at 0% relative humidity or osmotic stressed in 500 mM NaCl S-buffer. After two hours of recovery, the survival was measured. Error bars show the standard error of two replicates.

3.4 Discussion

3.4.1 Subtractive library

The large number of clones derived from the subtractive hybridization gives an indication of the complexity of the response to the stresses. The cloned sequences are only from genes uniquely expressed during the stress or the recovery. Differentially expressed genes are not present in the libraries.

Table 3.4 on page 62 lists the number of identified sequences versus the unknown sequences. From the differences between identified and unknown sequences, it can be noted that the post osmotic stress library has the largest number of identified sequences (75%), versus the desiccation stress library, which has the largest number of unidentified sequences (40%). The overall identified transcripts are 407 (47%) from which 294 unique transcripts, which have an assigned gene ontology term. These transcripts were used to analyze the response to osmotic and desiccation stress.

3.4.2 Analysis of the stress and post stress libraries

Desiccation and osmotic stress library

Table 3.15 on page 82 shows the comparison of the five most enriched Annotation cluster as computed by EASE. In both libraries the transcripts annotated with cellular protein metabolic processes and cellular metabolic processes are enriched. Transcripts encoding for proteases, ribosomal proteins, heat shock proteins, ubiquitin, tRNA synthetase and kinases characterize these four clusters suggesting a degradation (proteases, ubiquitin) of proteins as well as synthesis (ribo-

Table 3.15: The gene ontology term with the lowest p-value in the cluster was chosen as the representative of the Annotation cluster. Score is the enrichment score given by the software EASE.

	Desiccation library	score	Osmotic stress library	score
1	Post embryonic development	4.01	Developmental process	3.06
2.	Cellular protein metabolic process	3.57	Translation	2.53
3	Cytokinesis	2.42	Cellular protein metabolic process	1.93
4.	Cellular metabolic process	1.84	Cell differentiation	1.28
5.	Multicellular organismal development	1.68	Cellular metabolic process	0.96

somal proteins, tRNA synthatase).

In the desiccation library, two Annotation clusters annotated with post-embryonic development and multicellular organism development are present among the first five most enriched Annotation cluster. Transcripts found in these two clusters encode mainly for actins, ribosomal proteins, ubiquitin, histone, and kinases. These transcripts combined with the transcripts annotated with the term cytokinesis, suggest either that the worm is reinforcing or adapting its cellular cytoskeleton for anhydrobiosis or it is preparing the cells to reactivate cell cycle after desiccation stress. Ribosomal proteins, and histones might be involved in stabilizing nucleic acids in the cell supporting the hypothesis that the cell is preparing the necessary steps for the exit from anhydrobiosis already during entering into anhydrobiosis. Kinases suggest a signalling process.

Developmental process is the most enriched Annotation cluster term in the osmotic stress library. Transcripts in this cluster encode for proteins such as ribosomal proteins, heat shock proteins, ubiquitin and proteases. A transcript identified as *pod-1* is also present in the cluster. *C. elegans* mutants of this gene show a defect in a-p-polarity and embryos are sensitive to changes of salt concentration. It was noted in destruction of the innermost (lipid rich) layer of the eggshell

Table 3.16: . The gene ontology term with the lowest p-value in the cluster was chosen as the representative of the Annotation cluster. Score is the enrichment score given by the software EASE.

	Post desiccation library	score	Post osmotic stress library	score
1	Post embryonic development	6.36	post embryonic development	3.72
2.	Translation	5.55	Protein-DNA complex assembly	2.96
3	Embryonic development	4.95	Embryonic development	2.73
4.	Cellular macromolecular metabolic process	3.22	Cation transport	2.21
5.	Positive regulation of growth rate	2.94	ATP synthesis coupled transport	1.94

together with ultrastructural abnormalities underneath their lipid rich eggshell layer [175]. This might cause the osmotic sensitivity. Actin is suggested to regulate POD-1 activity. The expression of *Pod-1*, *atn-1*, which encodes for an actin binding protein [11], and *ani-2*, which encodes for a protein important for stabilizing and remodeling actin cytoskeleton [122], suggest a remodelling or reinforcement of the cytoskeleton to cope with osmotic stress. Elongation factors, ribosomal proteins and ubiquitin genes represent the Annotation cluster termed translation. These transcripts suggest protein translation (elongation factor, ribosomal proteins) and localization (ubiquitin) in the cell. The Annotation cluster cell differentiation contains transcripts for cell fate specification *pal-1* [93, 200] and cell migration *mig-2* [121] suggesting cell differentiation to more specialized cells.

Post desiccation and post osmotic stress library

Table 3.16 on page 83 shows the comparison of the five most enriched Annotation clusters as computed by EASE for the post stress libraries. Both libraries contain transcripts annotated with "development". The two annotation clusters post-embryonic development and embryonic development contain transcripts encoding for proteins such as proteases, histones, ribosomal proteins, and ubiquitins. The fact that these transcripts are associated with embryonic and larval (post em-

bryonic) development suggests a global repair mechanism after the stress. This is strengthten by the fact that the post desiccation library contains the enriched Annotation cluster positive regulation of growth rate, which contains nearly the same transcripts as the post-embryonic development term. Transcriptions annotated for translation and cellular macromolecule metabolic processes are enriched also in the post desiccation library. The post-osmotic stress library contains transcripts involved in protein-DNA complex assembly. The two transcripts *taf-1* and *rad-51* needs to be mentioned here. *C. elegans* expresses *taf-1* in all embryonic nuclei, in oocytes and in the adult germ line. Absence of this transcription factor leads to an arrest at the 100 cell stage, without differentiation of the cells. This phenotype is believed to be caused by impaired embryonic mRNA transcripts and reduction of Polymerase II transcripts, which counts also for miRNA transcription. Furthermore *Taf-1* regulates about 14% of genes in yeast and ca. 18% of genes in hamster [196]. *Rad-51* is required for normal chromosomal morphology, univalent formation, dissociation of chiasmata, hyperresistance of meiotic pachytene nuclei to X-irradiation, and the progress of oocytes through diakinesis [176]. These two transcripts together with histones might reassemble the chromatin after the stress. Annotation cluster named Cation transport and ATP synthesis coupled proton transport suggest the use of proton gradient for ATP-synthesis. Two transcripts need to be mentioned here: *atp-2* and *vha-2*. *Atp-2* encodes the active site of the beta-subunit of complex V. Depletion of this protein results in a worm arrested in larval stage 3. Abnormal behavior, impaired locomotion, pharyngeal pumping and dauer formation defective phenotypes were observed [86]. The vacuolar-H-type ATPase encoded by *vha-2* is believed to function in toxin and metabolic waste metabolism and osmoregulation [142].

In summary, desiccation and osmotic stress require a reinforcement or remodeling in the cytoskeleton. Transcripts presently annotated with embryonic development during recovery suggest an overall repair mechanism that is comparable to the larval development/growth of the worm. Specifically, the organism replaces damaged tissues or cells in a process that is normally seen during larval development. As larval development is a fast growing pahse, new cells of tissues

need to be generated, which might be exactely happen during recovery. Furthermore the Annotation cluster “positive regulation of growth” indicate that this process might be limited to repair of tissues and not for additional growth of the worm.

3.4.3 Knock-down experiments

The knock-down experiments showed only a small change in survival of the knock-down worms compared to the control. Considering the number of genes that might be involved in the stress response, a gene can easily be redundant or play only a small role in the overall response. However it is not excluded that the knock-down efficiency was low. The validation of the knockdown experiments by Southern blot failed.

Unc-11 and R05G6.7 from *Panagrolaimus rigidus* AF36 did not influence significantly the survival rate of *Panagrolaimus* PS1579, but did influence significantly the desiccation survival of *Panagrolaimus rigidus* AF36. The first result seen with species PS1579 was expected as these two transcripts were not taken from the spesies library. The result seen with the species *rigidus* AF36 for the transcript *unc-11* is in accordance with the origin of the transcript, the desiccation library. R05G6.7 was taken from the osmotic stress library, thus it did not influence significantly the osmotic response. R05G6.7 is an outer membrane mitochondrial voltage dependent anion selective channel protein [215]. A higher stress upon mitochondrial function during desiccation stress might have influenced negatively the desiccation stress survival.

The survival of the *lea* gene knock-downs was significant for the osmotic stress response and both genes were present during the recovery. The *lea* knock-down transcript from species PS1579 increases the osmotic stress survival. As the transcripts has a very high E-value (0.13) compared

to *lea-1* genes found in species *rigidus* AF36 (5.0E-9, 9.2E-11, 1.6E-10), the possibility exists, that this transcript is not a *lea* gene. However, both *lea* transcripts did not influence significantly the desiccation stress, suggesting that some *lea* transcripts have stress specific functions. *Tps-2*, the trehalose 6-phosphate synthase, found in the desiccation library, exhibited a significant decrease in survival during desiccation stress but not in the osmotic stress, underlining the importance of trehalose during desiccation. Also *taf-1*, a transcription factor, showed a positive response during osmotic stress when knocked-down.

Summarizing, the knowledge gained from these experiments are the following: It was shown, that a large number of genes are involved in the response and recovery of the animal from both stresses. More than half of the cloned transcripts of these genes do not match any known *C. elegans* sequence. The identified sequences from the stress and the post stress libraries showed some overlaps. However, it is not possible to deduce that these are the same transcripts used in different libraries. The gene ontology analyses revealed developmental, structural and protein metabolic processes occuring during osmotic and desiccation stresses. During recovery, desiccation stressed worms express genes for translation, post embryonic development and macromolecular metabolic processes, suggesting repair processes are involved . Gene ontology analysis for the osmotic stress recovery suggests a link between osmotic imbalance and ATPase activation. Chromatin rearrangement and development occurs during osmotic stress recovery as well. The knock-down experiments showed, that the chosen transcripts influence the survival of the *Panagrolaimus* worm, supporting the claim that *unc-11*, *tps-2*, *lea-1*, and R05G6.7 are important for stress survival.

Chapter 4

Desiccation tolerance of *Caenorhabditis elegans*

4.1 Introduction

Chapter 4 describes the gene expression pattern of a mixed population of *C. elegans* undergoing desiccation stress on a nitrocellulose membrane under conditions optimized previously (Chapter 2.4.3 on page 50). The survival was measured and total RNA was extracted for the microarray experiment (Figure 4.1 on page 88).

Briefly, a mixed population of *C. elegans* was submitted to desiccation stress using chambers equilibrated with relative humidities ranging from 40% to 100%. The survival of the culture was monitored every 12 hours for the first 72 hours, then after 5 and 7 days after initiation of the stress. Worms desiccated at lower than 80% relative humidity showed a decrease in survival after 12 hours. Furthermore, the *C. elegans* worms showed a high

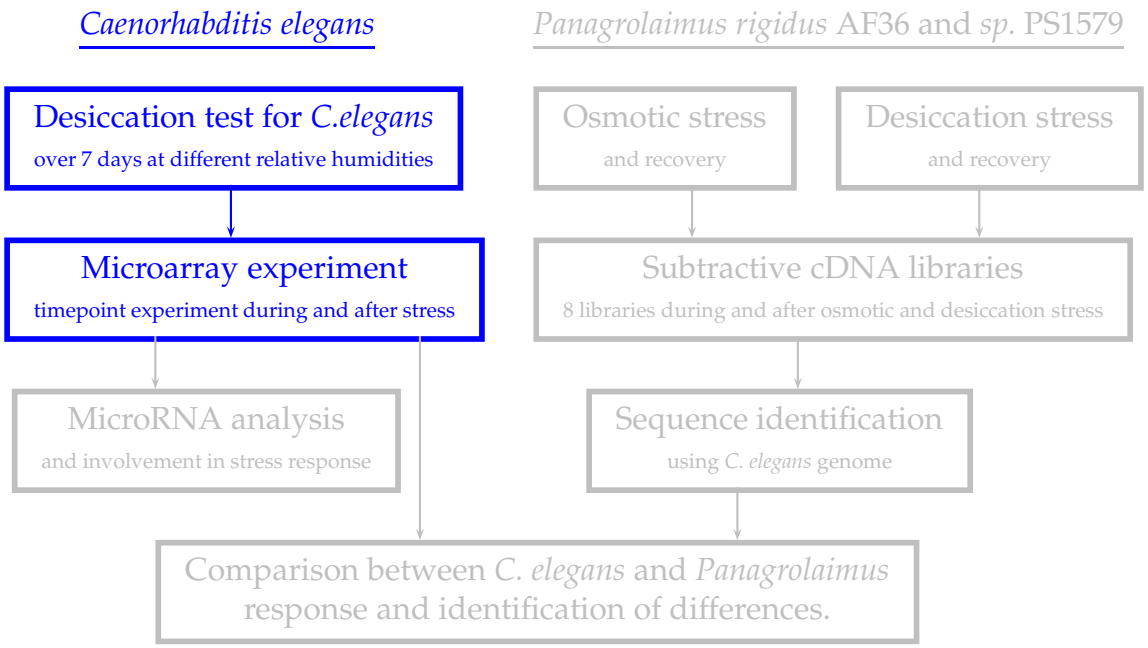


Figure 4.1: Flowchart highlighting Chapter 4 studies.

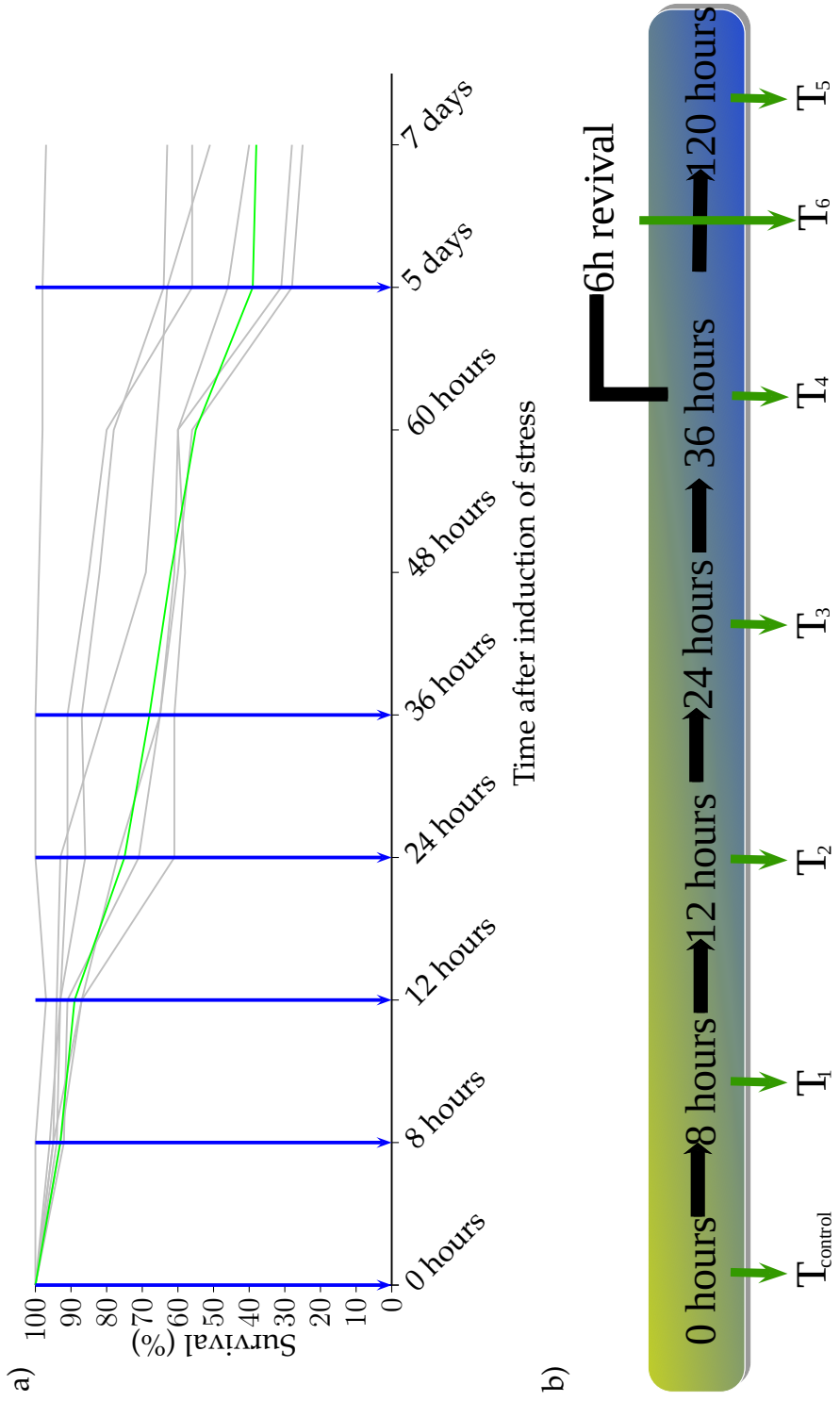


Figure 4.2: Micro-array design - a) Time course survival data. The green line represents the desiccation stress survival of *C. elegans* at 60% relative humidity. The blue arrows indicate the time-points samples were taken for micro-array analysis. b) Schematic design of the microarray timepoints

survival rate ($\sim$ 85%) for the first 12 hours of the stress despite the fact that the water film around the nematode had already disappeared around the first hour of desiccation.

One hypothesis explaining such decrease in survival from the 12 hour timepointis is that organisms might experience different unfavorable conditions simultaneously or sequentially. For example, during desiccation stress, a worm first loses the surrounding water film. Then, water evaporation leaves residual salt in the ground thus increasing the salinity of the surrounding environment. The protective water layer around the worm evaporates, exposing the animal to oxygen damage. The oxygen oxidizes lipids on the cuticle of the worm. Thus, the worm has to defend itself at a certain timepoint not only from desiccation, but also from osmotic and oxidation stress. These stresses might be the reason for a sudden decrease in survival as seen after the 12 hour timepoint.

Additionally, in the previous chapter, the desiccation stress response from anhydrobiotic organisms has shown that numerous biological processes are involved in the response. The data suggest that osmotic stress processes are close to the ones employed by desiccation stress. However, transcripts encoding antioxidant genes were only present in form of a Cytochrome P450 gene. Transcripts annotated to be involved in primary metabolism were found in high abundance (50% of the identified transcripts in the desiccation library) together with transcripts involved in the developmental processes (45%). During recovery, development (50%) and macromolecular metabolic processes (50%) were the most frequently annotated transcripts.

The aim of the transcriptional profiling experiment was to analyze the desiccation response of a desiccation-sensitive nematode, in this case *C. elegans*. The data were com-

pared to the *Panagrolaimus* subtractive hybridization libraries in order to assess differences and similarities.

4.2 Materials and Methods

Culture maintenance *Caenorhabditis elegans* (Bristol N2) was obtained from the *Caenorhabditis* Genetics Center (CGC). The strains was maintained at 25$^{\circ}\mathrm{C}$ on Nematode Growth (NG) agar plates with live bacteria (*Escherichia coli* OP50, uracil auxotrophic strain).

Culture preparation Worm cultures were washed from the agar plate by rinsing twice with S-buffer (0.1 M NaCl, 0.05 M potassium phosphate buffer, pH6.0) and collecting the worm suspension in a centrifuge tube. Following centrifugation (1 min, 200 x g) the supernatant fraction was discarded. The worm pellet was re-suspended in S-buffer. This step was repeated for three times. After the last certifugation, the pellet was re-suspended in 5 ml S-buffer and left on ice for at least 5 min. The worm suspension was mixed with 5 ml ice cold 70% sucrose solution and centrifuged for 5 min at 1500 x g. Afterwards the worms were put in sterile S-basal buffer. The sample was centrifuged for 3 min at 1000 x g, washed with by S-buffer wash and centrifuged (2 min, 200 x g). The worms were used immediately after cleaning.

Microarray desiccation experiment *C. elegans* was sucrose cleaned and at least 80,000 worms were placed on 5-cm nitrocellulose membranes (Millipore, USA). One membrane

for each timepoint was generated and the experiment was repeated three times (biological triplicates). The control membrane was immediately soaked in the Trizol (Invitrogen, USA) solution. The other membranes were placed in a chamber equilibrated to a relative humidity (RH) of 60% with glycerol solution at 16$^{\circ}\mathrm{C}$. After 8, 12, 24, and 36 hours a membrane from the chamber was placed in Trizol solution for total RNA extraction. At timepoint 36 hours after initiation of the stress an additional sample was taken from the chamber and re-hydrated for 30 minutes in S-buffer and transferred onto an NG plate grown with an *E. coli* OP50 lawn. After six hours the nematodes were washed off with S-buffer and sucrose cleaned. The worms were then placed in Trizol for total RNA extraction. The last timepoint was collected after five days since the beginning of the stress.

Total RNA extraction Total RNA was extracted using the Trizol (Invitrogen, USA) protocol according to the direction of the manufacturer with an additional step at the beginning. Specifically, the Trizol solution with the worms was heated in a waterbath to 65$^{\circ}\mathrm{C}$ for five minutes. Then the sample was placed immediately in a dry ice/ethanol mixture to freeze. After five minutes the sample was placed again in a 65$^{\circ}\mathrm{C}$ waterbath. This procedure was repeated three times. Then the sample was centrifuged at 12000 x g for 10 min at 4$^{\circ}\mathrm{C}$. The resulting supernatant was transferred to a new tube and 500 $\mu\mathrm{l}$ isopropyl alcohol was added. The tubes were mixed by repeated inversion. Then the samples were incubate for 10 minutes at -70$^{\circ}\mathrm{C}$ and subsequently centrifuged for 10 min at 12000 x g at 4$^{\circ}\mathrm{C}$. The supernatant was decanted and 75% ethanol (1 ml) was added to wash the RNA pellet. The tubes were centrifuged at 7500 x g for 5 min. The supernatant was decanted and the pellet air dried for 5-10 min. The pellet was then redissolved in 50 $\mu\mathrm{l}$ nuclease-free water (USB, USA). The concentration and purity of the isolated total RNA was assessed by reading the absorption at 260 nm and 280 nm. The integrity was assessed by staining

2 µg total RNA on a 1.2% agarose gel.

Microarray Total RNA from the three biological replicates of each timepoint were mixed together and submitted to the microarray core facility (VBI, Virgina Tech Bioinformatic Institute, USA) for analyses on the whole genome Affymetrix *C. elegans* microarray chips (no. 900383, Affymetrix , USA). Each timepoint was hybridized to three microarray chips to provide technical triplicates. The data were analyzed using the Arrayassist software package (version 5.1.0, Stratagene, USA). The microarray data were imported as CEL and CHP files. A level analysis (RMA), a quality control analysis, and a hybridization control were performed to check the quality of the data. Then the data was converted into a logarithmic scale and a baseline transformation was performed. This step produced a log-ratio dataset from which a significant analysis was performed using the Benjamini-Hochberg FDR method. The replicates were then averaged. The expression data were then clustered in 10 clusters using the K-means.

Knock-down experiment Selected genes *fmo-2*, *sod-5*, *tag-199*, *cyp-13A4*, and *cyp-13A6* were amplified from *C.elegans* cDNA by PCR using the RNAi primers suggested in Wormbase (www.wormbase.org) with an additional *Kpn*I restriction site at the 5'-end. The resulting PCR product was subcloned into the multiple cloning site of the PL4440 feeding vector [181], such that it was flanked by two identical T7 promoters. *E. coli* HT115 (gift of Diya Banerjee, Biological Sciences, Virginia Tech) was transformed with the recombinant plasmid or with control insert-free vector. Sucrose cleaned *C. elegans* were fed IPTG-induced bacteria for 12 hours at 25°C. The worms were then washed off the desiccation

stress as described above was applied. At timepoints 0, 12, 24 and 36 hours after initiation of the stress, worms were collected and stained for survival. Additionally total RNA was extracted from *sod-5* knock-down and the control for knock-down validation. The significance analysis was calculated using the student t-test.

Knock-out The *C. elegans* knock-out strains of the genes *sod-5* (FX1146) and *cyp-13A6* (VC40) were obtained from the Caenorhabditis Genetics Center (CGC). Both cultures showed superficially wild-type phenotype and were maintained at 25$^{\circ}\mathrm{C}$ on Nematode Growth (NG) agar plates with live bacteria (*Escherichia coli* OP50). After being sucrose washed, the mutant worms were submitted to desiccation stress as described for the knock-down experiment. The significance analysis was calculated using the student t-test.

Survival Survival was measured using SYTOX green (Molecular Probes, USA). The nematodes were placed in S-buffer containing 1μM SYTOX green dye. After a 15 min incubation a 100-$\mu\mathrm{l}$ aliquots from each sample were aliquoted on a 96 well plate (Novagen, USA). For each sample 5 wells were used, paying attention to have at least 100 worms per well. The samples were visualized using a confocal microscope (MS510, Zeiss). A digital image was taken from each well and the images were analyzed manually. The percent survival was determined by counting both the total number of worms as well as the number of dead worms (SYTOX stained) % survival= $\frac{Total\ worms - Dead\ worms}{Total\ worms}$

Validation of knock-down To validate the knock-down experiment total RNA from control and *sod-5* knock-down worms were reverse transcribed by MMLV reverse transcriptase (Genehunter). The transcript was then amplified using the forward primer 5'-TGGATATTCTCTCTGATATTGCCAATG-3' and the reverse primer 5'-ATGCAGGAGCGGCAAGAGCAATGA-3'. The gene *gpd-1* was used as a standard, which was amplified using the following forward primer GAATCAACGGTTTCGGAAGA and the reverse primer TCGACAACACGGTTCGAGTA. The semiquantitative PCR was performed as follows: 20 μl PCR solution were amplified for the first 10 cycles (95°C for 30 s, 65°C for 1.5 min, 75°C for 1.5 min) then at every second cycle two μl from the PCR sample was removed for the next 16 cycles. The samples were separated and visualized on a 1.2% agarose gel. The band density of the eighth PCR cycle was measured by 'Quantity ONE' software (Bio-Rad, USA). The ratio of the concentrations from the *sod-5* transcript of the knock-down and the control were compared to the transcript concentration of *gpd-1* in the knock-down and control sample, respectively.The ratio between the *sod-5* knock-down expression and the standard *gpl-1* transcript was calculated. Both ratios were compared to assess the level of knock-down.

4.3 Results

4.3.1 Overview of the transcriptional response to desiccation stress

The goal was to generate an accurate fingerprint of the response of *C. elegans* over time to desiccation stress at 60% relative humidity using a timepoint microarray approach. The first timepoint was chosen immediately after placing *C. elegans* on the membrane and

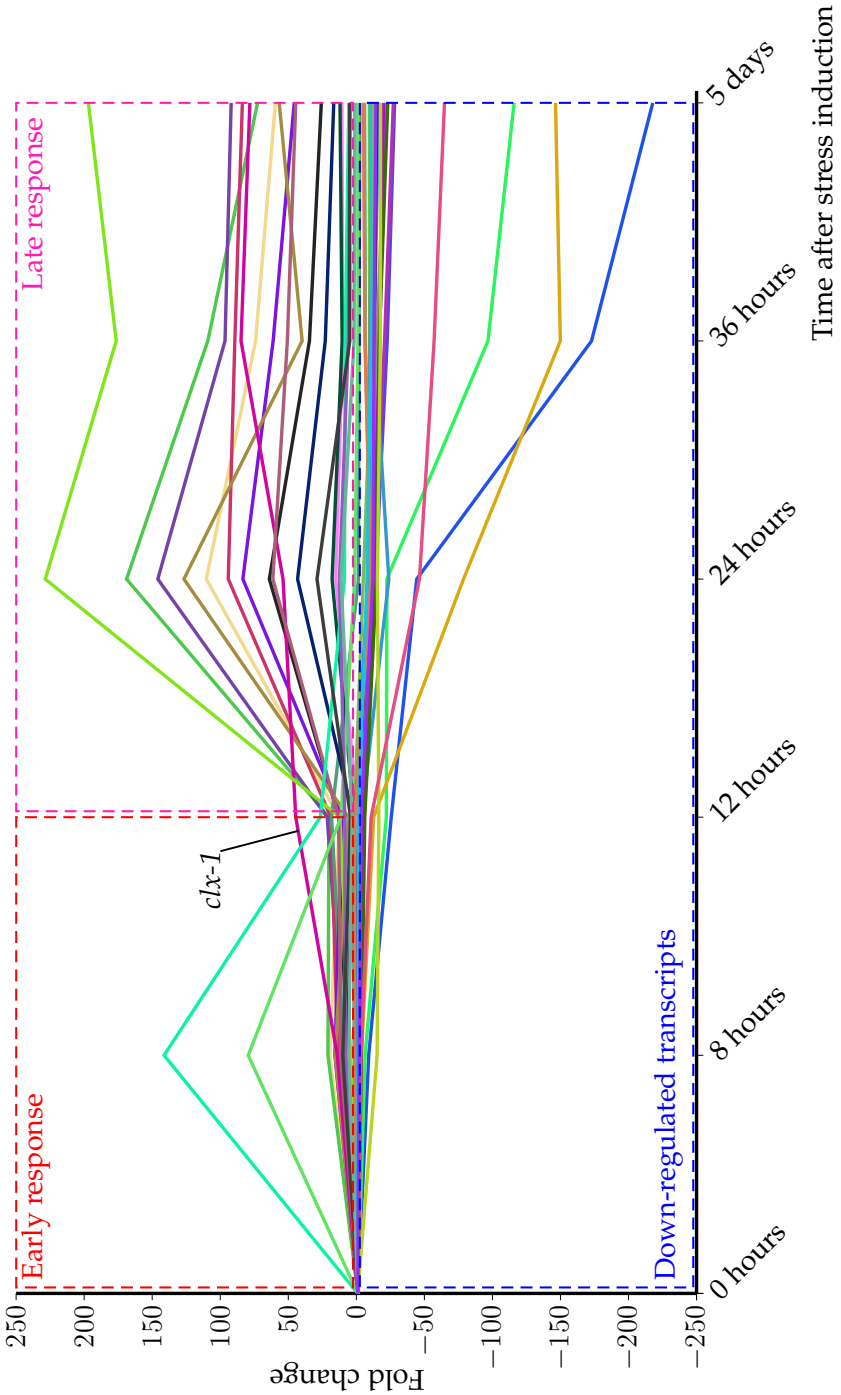


Figure 4.3: Expression profile of the 40 most differentially expressed genes from the *C. elegans* microarray experiment. The transcripts were subdivided in four clusters: early response, late response, down-regulated and revival (not shown here). The expression of the transcript *clx-1* is annotated, as it is the only highly up-regulated transcript, which is up-regulated during the early response and remains up-regulated during the stress.

was used as control. The second timepoint was chosen when the movement of the nematodes stopped (8 hours after initiation of the stress). The third timepoint was chosen at 12 hours, the fourth at 24 hours, the fifth after 36 hours after the initiation of the stress. The sixth timepoint was chosen after the nematodes were desiccated for 36 hours and washed off the membrane on an agar plate filled with *E. coli* OP50. The last timepoint was chosen after the worms had been stressed for 5 days. The chosen timepoints are visualized in Figure 4.2 on page 89. At each timepoint the collected nematodes were placed in Trizol for total RNA extraction.

Figure 4.3 on page 96 shows the expression over time of the 40 most differentially expressed transcripts during desiccation (revival timepoint not included). Two groups of up-regulated transcripts can be identified: genes up-regulated during the first 12 hours, and genes up-regulated after the first 12 hours of desiccation stress. Apart from *clx-1* no other highly up-regulated (more than 10-fold) gene with a positive slope crosses this timepoint. These pattern of expression can be divided in two two response-timeframes, which are referred here as ‘early response’ and ‘late response’.

The down-regulated transcripts did not appear to fall into these two categories. However, the down-regulation of genes started also after 12 hours of desiccation stress. The revival timepoint showed that regulated genes returned to their normal expression levels after recovery from the stress as can be seen in Figure 4.10 on page 115). Furthermore *C. elegans* resumed locomotion and feeding on an agar plate seeded with *E. coli* confirming that *C. elegans* survived the stress and was able to resume normal life activity.

To better analyze the data, the next sections consider the array data subdivided into

different clusters each containing 20 genes. The first cluster contains the 20 most highly up-regulated genes characterizing the 'early response', the second cluster contains the most highly up-regulated transcripts from the 'late response', a third cluster contains the 20 most highly down-regulated genes, and the fourth cluster contains the most up-regulated genes during the revival timepoint compared to the timepoint 36 hours.

Early response genes

Early response genes are up-regulated during the first 12 hours and return almost to their pre-stress levels afterwards as seen in Figure 4.4 on page 100. The majority of these genes (30%) belong to the family of Cytochrome P450 family (*cyp*-genes). A flavin-containing mono-oxygenase (*fmo-2*) and a predicted glutaredoxin protein (F10D7.3) were present. All these genes are mono-oxygenases, which are enzymes that reduce molecular oxygen by incorporating one oxygen atom into its substrate and the other one in water. The *C. elegans* genome has 81 cytochrome P450 genes annotated, but their functions are still unknown [47]. *Fmo-2* is normally expressed in intestinal cells with additional expression in the region of the excretory gland of the head area [149].

Two predicted small molecule kinases (T16G1.4 and T16G1.6) of unknown function are also found among the highly up-regulated genes. Another highly up-regulated gene is R08F11.4, which encodes an uncharacterized SAM-dependent methyltransferase-like protein. *Dct-7* is a predicted protein of unknown function with a length of 64 amino acids of unknown function. Its sequence contains 22 Glycines (30%) and 11 Leucines (16%). It is believed to be controlled by Daf-16 and affects germline tumor [152].

The last gene in the list is *hmit-1.1*, a proton (H+)-dependent myo-inositol transporter, believed to function in the regulation of cell signaling and intracellular osmolarity [79].

Late response genes

Genes belonging to the ‘late response’ cluster showed increased expression levels after 24 hours and then remained up-regulated during the stress (Figure 4.5 on page 101). One member of the heat shock protein family can be found in this list: F08H9.4 *(hsp-16*-family). This heat shock protein (HSP) is thought to act as passive ligand for unfolded proteins to keep them safe from aggregation until the proteins can be refolded by a large (ATP-consuming) HSP [27]. F08H9.4 is mainly expressed in the excretory canal and ventral nerve-cord neurons. The knock-down phenotype of this gene shows a higher pathogen susceptibility than wild-type [167].

Transcript *sod-5* encodes a Cu/Zn superoxide dismutase, which is regulated by Daf-16 and PHA-4 suggesting and involvement of the insulin-like signaling network and the pharyngeal pumping behavior during desiccation stress [84]. *Cdr-3*, which is a putative glutathione S-transferase-like protein and homologue to *cdr-1* [55], and F37B1.4, which encodes for a glutathione S-transferase, are two genes encoding for proteins involved in detoxification of the worm. Three CaeNaCin (a *Caenorhabditis* bacteriocin,*cnc*) encoding genes and three Fungus-Induced Protein (*fipr*) encoding genes represent members of the innate immune response [38]. The remaining genes are uncharacterized.

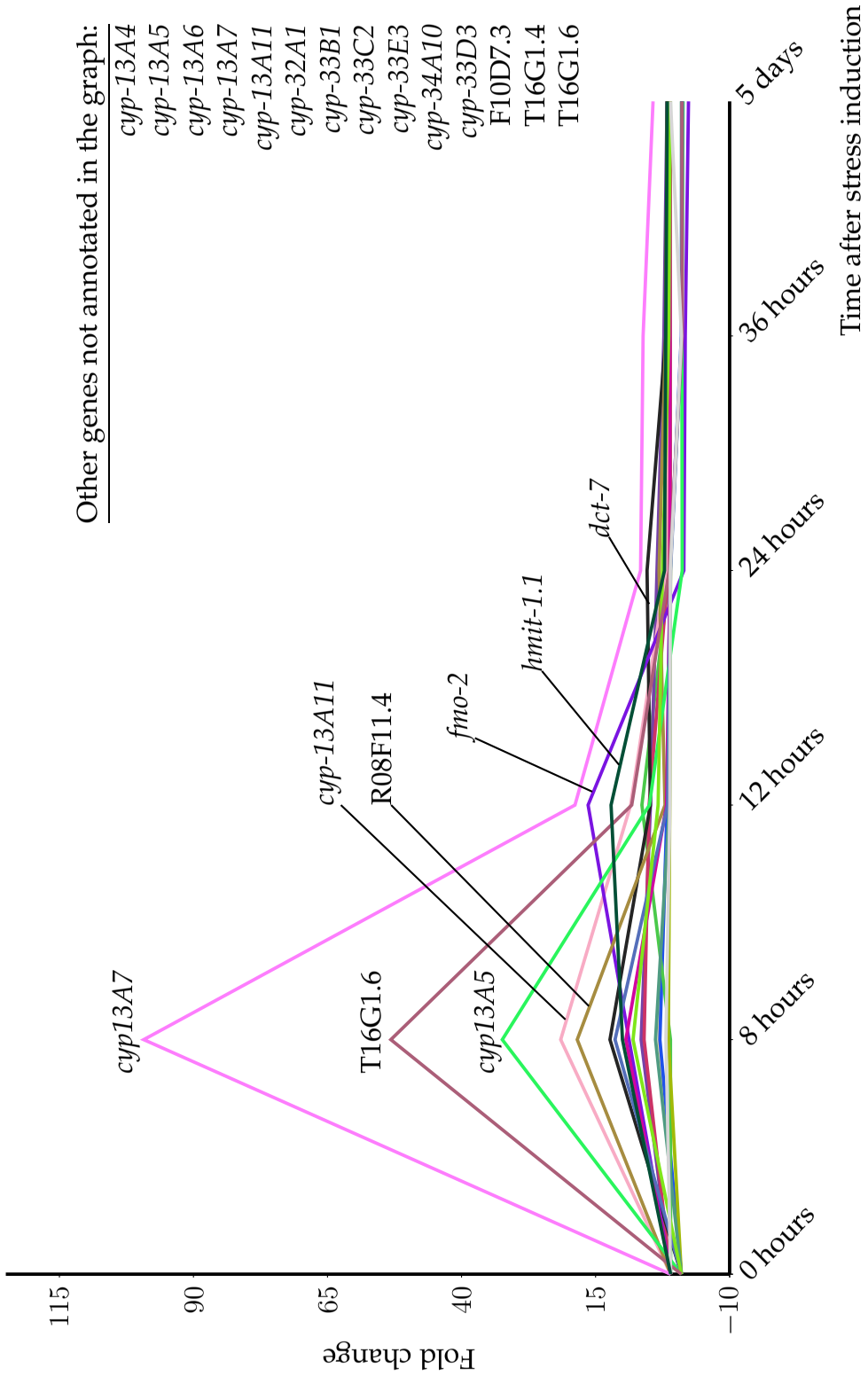


Figure 4.4: The 20 most highly up-regulated genes belonging to the 'early response' during desiccation stress of *C. elegans*.

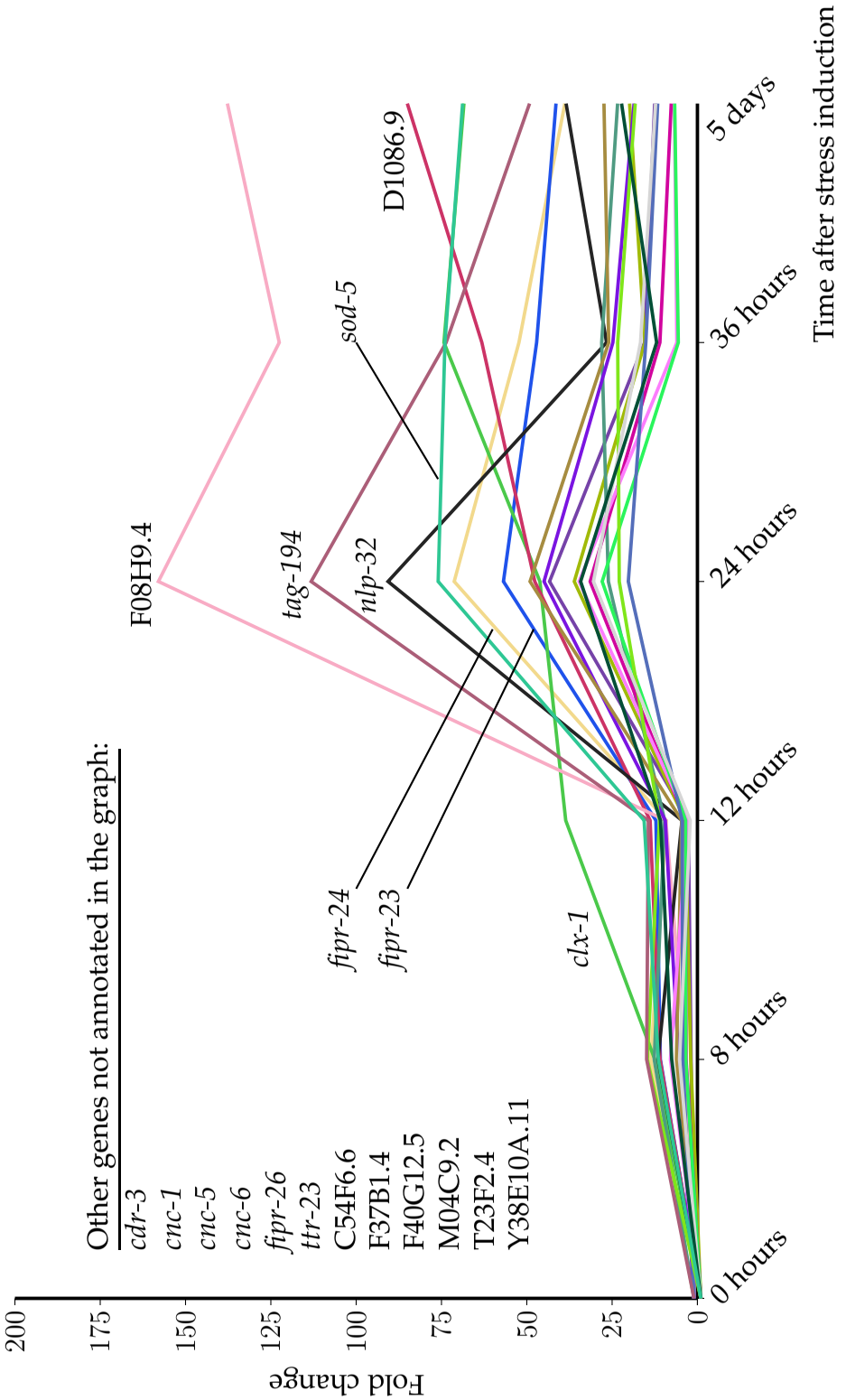


Figure 4.5: The 20 most highly up-regulated genes belonging to the 'late response' during desiccation stress of *C. elegans*.

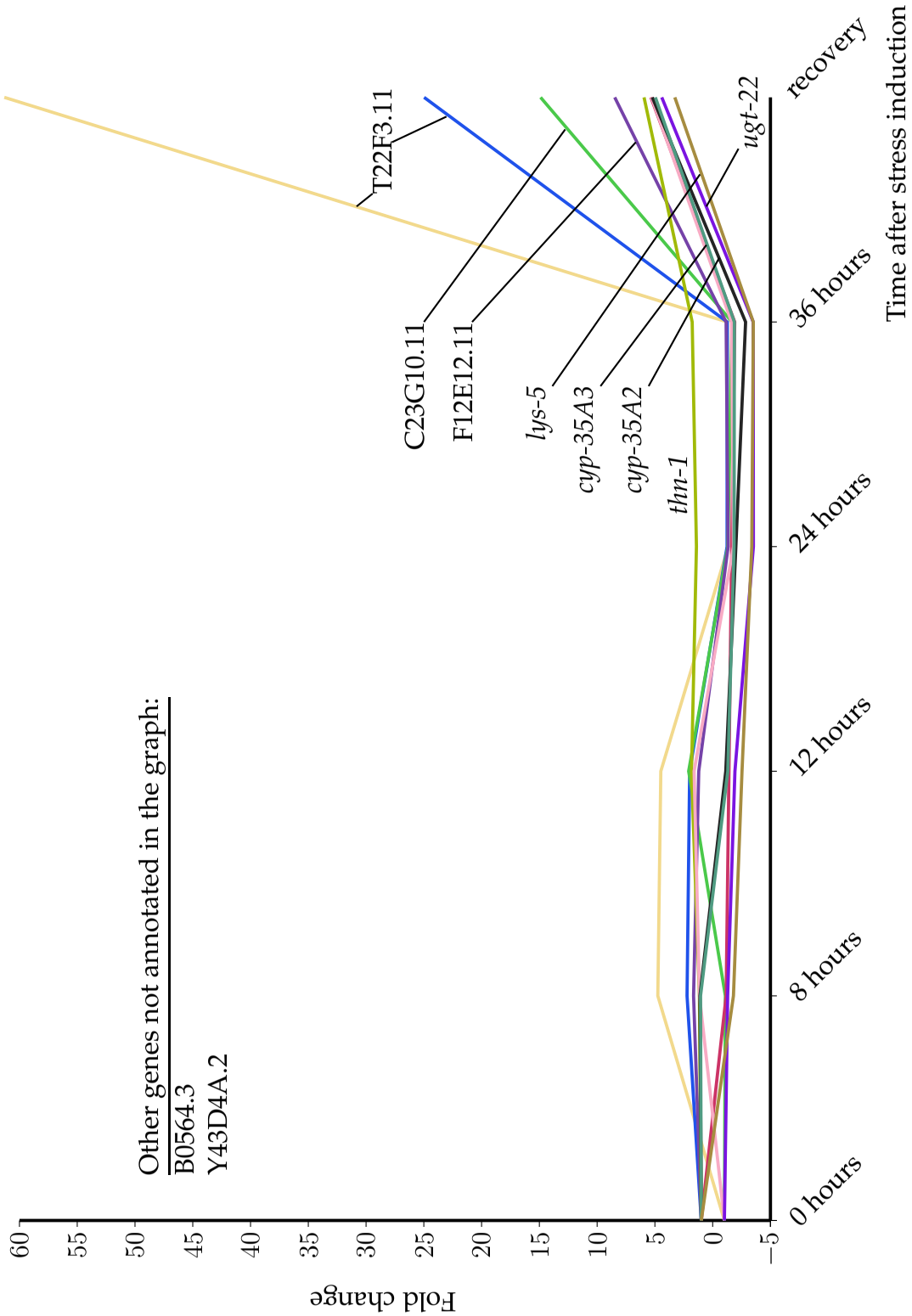


Figure 4.6: The 20 most highly up-regulated genes during desiccation stress recovery of *C. elegans*.

Genes regulated upon stress exit

Genes, which show an increase in expression level compared to timepoint 36 hours and control, are included in this cluster. The gene T22F3.11, which is believed to encode a permease of the major facilitator superfamily, is involved in metabolism, especially in carbohydrate transport [79]. Two genes encoding pathogen related proteins (*thn-1* and *thn-2*) are up-regulated as well. These genes, when knocked-down, increase the pathogen susceptibility of the worm and influences nematode lifespan [137, 166]. Cytochrome P450 genes (*cyp-35A2*, *cyp-35A3*, *cyp-35A4*, and *cyp-35C1*) from another subfamily then the early response cytochrome P450 genes, are found among the up-regulated genes [130, 131]. A putative lysozyme *lys-5* is also up-regulated after the stress. The remaining highly up-regulated genes have not been characterized.

Downregulated genes

The expression profile of the 20 most highly down-regulated genes during the stress is shown in Figure 4.7 on page 104. Three lysozymes (*lys-1*, *lys-4*, *ilys-5*) and three proteases (*cpr-1*, *cpr-4*, F57F5.1) are among the most down-regulated genes. The transcript *ins-7* encodes an insulin-related protein, which is believed to function as an agonist to the daf-2 receptor. Knock-down experiments with this gene showed a longer lifespan and increased dauer formation [137].

Y39b6A.1, a human hornerin homolog, was found to be involved in osmotic stress [111]. F45D11.15 expression was regulated during stress and aging [79]. Other genes encoding for a putative esterase (F46B6.8), lipase (F28H7.3), and phosphatase (Y73B6BL.24)

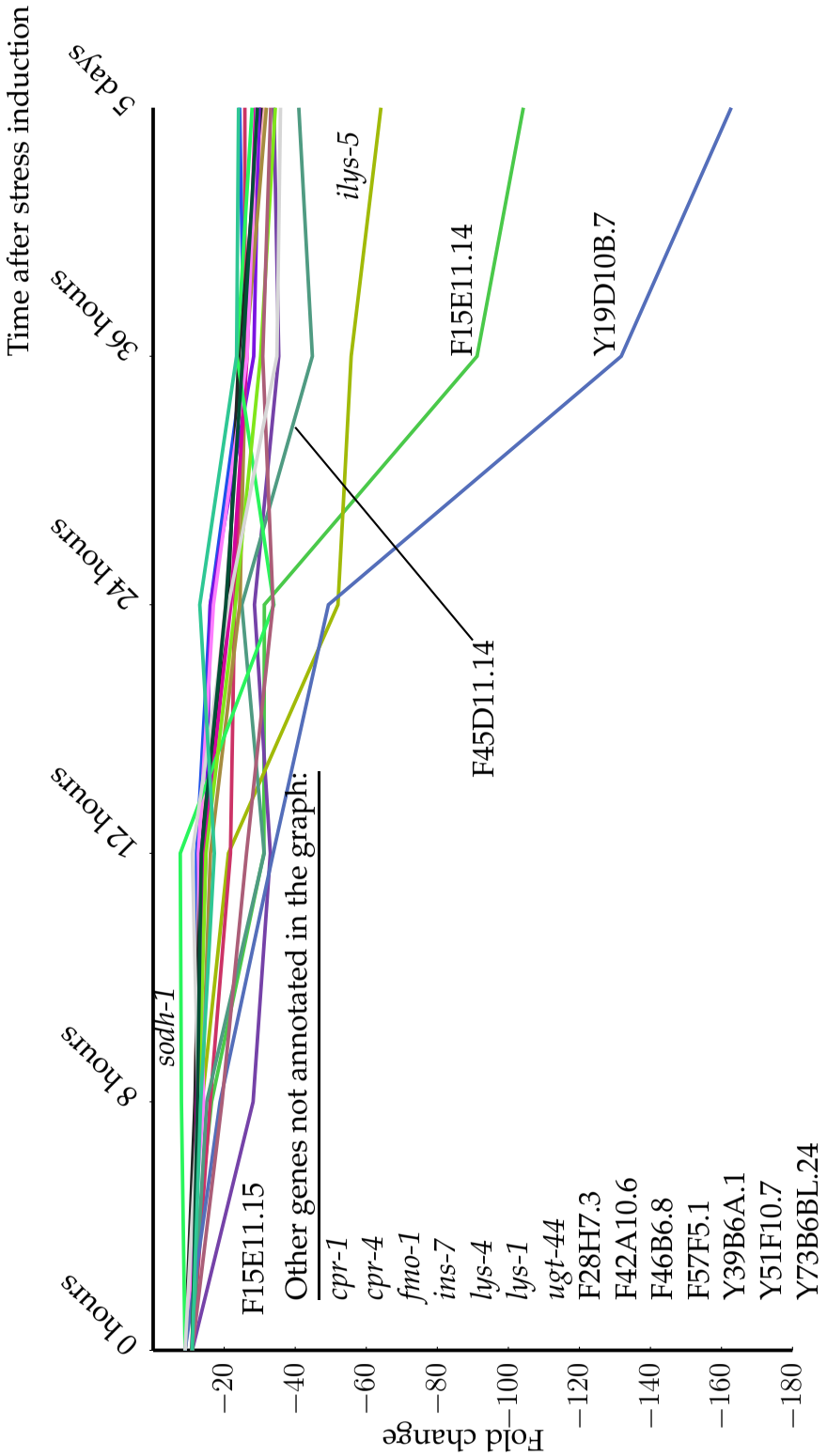


Figure 4.7: The 20 most highly down-regulated genes during desiccation stress of *C. elegans*.

were also present, however nothing is presently known about their function.

4.3.2 Knock-down and knock-out

In order to test the involvement of genes identified in the microarray, RNAi knock-down of selected genes was performed. Specifically, six mixed populations of *C. elegans* were fed with *E. coli* containing the vector PL4440 expressing dsRNA sequences of *sod-5*, *fmo-2*, *cyp-13A4*, *cyp-13A6*, *cyp-13A5* and *tag-199* respectively and a control population with an empty vector. The worms were left on the knock-down plates for 12 hours at 25°C to induce the silencing of the target gene. The worms were then transferred to membranes and submitted to the desiccation stress for 36 hours. After 36 hours of desiccation stress, the survival was measured.

The result is shown in Figure 4.8 on page 106. The lowest survival was recorded by the knock-down experiment of the gene *tag-199* (p=0.06), which encodes an uncharacterized protein. The highest survival was seen by the knock-down experiment of the superoxide dismutase 5 (*sod-5*) (p=0.001).

In order to investigate if the knock-out mutants would show a different phenotype as the knock-downs, knock-outs of the genes *sod-5* and *cyp-13A6* were submitted to the same desiccation procedure. As the result shows, both knock-out mutants exhibited the same survival as their knock-down counterparts. The validation of the knock-down was done by semi-quantitative PCR (polymerase-chain-reaction) using the *sod-5* knock-down and the control. The result is shown in Figure 4.9 on page 107 confirming the successful

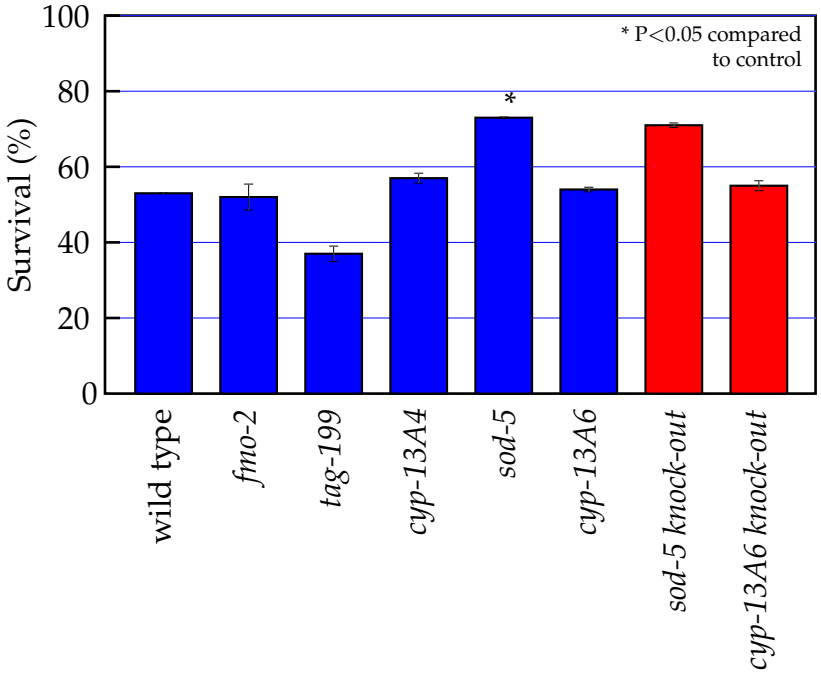


Figure 4.8: Desiccation survival of the *C. elegans* knock-down and the knock-outs. - Five *C. elegans* cultures were knocked-down by feeding. The target genes were *fmo-2*, *tag-199*, *cyp-13A4*, *sod-5*, and *cyp-13A6*. All nematodes were desiccated for 36 hours at 60% relative humidity at 16°C. The knock-down phenotype of *sod-5* showed a significant (*) increase in survival compared to wildtype. The knock-out mutants of the gene *sod-5* and *cyp-13A6* displayed the same phenotype as their knock-down counterparts when submitted to the same desiccation stress. Error bars show the standard error of two replicates.

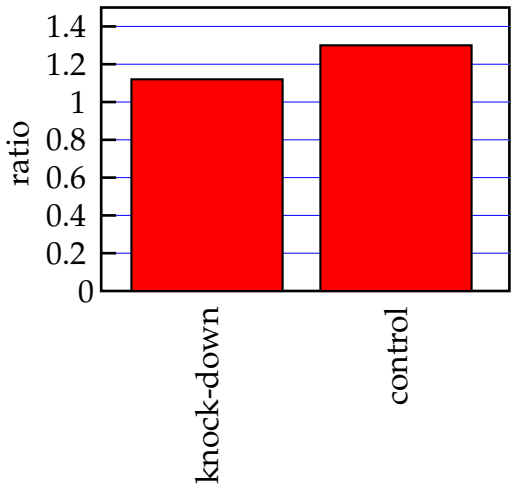


Figure 4.9: Semi-quantitative PCR validation of the *C. elegans* knock-down - Total RNA was extracted from wild type and *sod-5* knock-down and was reverse transcribed. The transcripts of *sod-5* and the standard *gpl-1* were amplified by PCR from wild-type and knock-down. After 8 cycles both PCR products were run on a 1.2% agarose gel. The density of the resulting bands was measured and the ratio between the *sod-5* sample and the *glp-1* sample from wildtype (left bar, control) and knocked-down (right bar, knocked-down) were calculated.

knock-down.

4.4 Discussion

C. elegans is capable of surviving moderate dessication stress for a limited time. Evidence from the gene expression profile suggests that loss of water leads to oxidative stress. This hypothesis is supported by the observation that twelve cytochrome P450 genes and the *fmo-2* gene, all belonging to the mono-oxygenase family were among the 20 most up-regulated transcripts in the early response. *Fmo-2* is expressed in the intestinal cells and head area of the worm. Additionally, another *Fmo-3*, expressed in hypodermis, is three-fold up-regulated during the first 24 hours.

The overexpression of two predicted small molecule kinases (T16G1.4 and T16G1.6) suggests a signalling process, potentially related to the oxidative and/or desiccation stresses. The up-regulation of an H(+)MyoInositol co-Transporter (*hmit-1.1*) involved in osmotic homeostasis [79] is an evidence that the worm is experiencing body water loss.

Around 12 hours after induction of the stress, the highly up-regulated genes (Figure 4.4 on page 100) return to an expression level below 10-fold up-regulation and do not show any increase in expression level during the duration of the stress. The first transcript showing an increase (after eight hours of exposure to stress) in the expression level is *clx-1*, which is a homolog to the human isoform two of collagen alpha-1(II) chain precursor. This gene encodes a protein of unknown function that contains 23 copies of a 15 amino acid

repeat (GAPPSGGPPGPF(D/N)PS). This transcript might have a specific stress related function as the knock-down of this gene in *C. elegans* shows wild type phenotype.

After 12 hours of desiccation stress, another set of genes shows up-regulation (more then 20-fold). The highest up-regulated transcript (160-fold) of this set encodes for a heat shock protein (HSP) family 16 member: F08H9.4. This HSP is expressed in the excretory canal and ventral nerve-cord neurons and is only weakly induced by heat shock [167]. The HSP-16 family are known to be up-regulated long after a stress has begun [95]. HSP-16 proteins are thought to act as passive ligands for unfolded proteins to prevent the latter from aggregating until the proteins can be refolded by a large (ATP-consuming) HSP [27]. Refolding of proteins is supported by the up-regulation of HSP 70, which is a known chaperone [134], after 12 hours of stress (10-fold).

Four *fipr*- and three *cnc*-genes are among the 20 most highly up-regulated genes in the late response. Both gene families are believed to be activated upon infection by parasitic or infectious fungi or bacteria. The expression of these proteins and the heat shock protein suggests that the concentration of denatured macromolecules increases after 12 hours of stress leading to an inflammatory response. The localization of the HSP in the excretory canal might suggest that the worm is excreting the denatured proteins from the body cells.

The up-regulation of a superoxide-dismutase (SOD-5, 75-fold), two glutathione S-transferases (*cdr-3* and *gst-15*, both 30-fold) and SOD-3, a mitochondrial superoxide dismutase [94], (10-fold) reflects the presence of oxidative stress. SOD-5 is regulated by Daf-16 and PHA-4. The latter is a forkhead transcription factor regulating response to dietary restriction and dauer stage. The high expression of SOD-5 may be an indication that the worm is

experiencing dietary restriction and decrease metabolism [7, 31, 147]. This hypothesis is supported by the up-regulation of *ttr-23*, a trans-thyretin-related nematode specific protein. The knock-down nematode phenotype of *ttr-23* exhibits a hyperactive locomotion [124].

The higher survial of the SOD-5 knock-out and knock-down worms during desiccation stress compared to wildtype suggest a non-activation of a pathway or behaivor. *C. elegans* responds to desiccation by slowing down the pharyngeal pumping and primary metabolism regulated by SOD-5 [147]. The knock-out and knock-down may not be able to regulate this behavior due to the missing SOD-5. The worm consequently does not slow down its metabolism, which seems to be beneficial for the stress response. The fact that both knock-down and knock-out show the same phenotype suggests an important role of SOD-5 during desiccation stress and attributes SOD-5 a regulatory function during desiccation stress.

Looking at the highly down-regulated transcripts, three proteases (*cpr-1 cpr-4*, and F57F5.1) and three lysozymes (*ilys-5*, *lys-1*, and *lys-4*) are among the 20 most highly down-regulated genes. A predicted lipase (F28H7.8), a triglyceride lipase-cholesterol esterase (F46B6.8) and a lysosolmal prostatic acid phosphatase (Y73B6BL.24) are also down-regulated. These enzymes are believed to be part of the catabolic metabolism of the organism. Thus their exact function is still unknown. The genes *ugt-44* encoding for a UDP-glucuronosyl-transferase, and *sodh-1* (sorbitol dehydrogenase) is also highly down-regulated. A down-regulation of these transcripts might be also an indication to a change or slowing down of the overall metabolic rate.

Consistent with this hypothesis is also the down-regulation of a daf-2 agonist: *ins-7*. Th transcript encodes an insulin/IGF-1-like peptide. Knock-down phenotype of this gene in *C. elegans* showed a significant increase in life span and an increase frequency of dauer formation [136]. An increase in life span has been shown to be correlated with a low metabolic rate [195, and dauer] [126, 132]

Analyzing the genes up-regulated after 36 hours of desiccation stress and six hours of recovery, two transcripts encoding for two channel proteins are found. T22F3.11 [166], which encodes for a permease, and Bestrophin, a chloride channel [79]. The presence of these two channel proteins suggests a mechanism to reestablish an osmotic homeostasis in the cells of the organism.

Thn-1, which encodes a homolog of the thaumatin family of plant antifungal proteins, is part of the immune system of the worm [166]. The role of this protein is not known in the worm. Thus this transcript represents the seventh highly differentially expressed transcript from the immune system from three different protein families (*cnc*, *fipr* and *thn* family) during the stress response and recovery. A possible explanation for the expression of immune system transcripts is that desiccation stress presents symptoms similar to pathogenic infection.

Two UDP-glucuronosyl-transferases were up-regulated during recovery, suggesting metabolic processes like fat storage are taking place. Fat storage is supported also by the presence of two Cytochrome P450 transcripts. Both Cytochrome P450 (*cyp-35A2* and *cyp-35A3*) are involved in fat storage as their knock-down phenotype shows a reduction in fat storage [10]. Members of the *cyp*-family were already found up-regulated during the ‘early

response'.

Summarizing, the microarray expression data show five main mechanisms employed by *C. elegans* to survive desiccation stress. The first mechanism is a response towards oxidation stress by up-regulating initially *cyp13A* and *fmo* genes, followed by *sod* and *gst* genes. The second mechanism is triggered by the denaturation and aggregation of denatured proteins or macromolecules. Consequently heat shock proteins are expressed to refold and prevent aggregation (HSP70 and F08H9.4). The third mechanism involves the activation of an immune response. The cause of involving the immune response is still unclear: either it is triggered by the denatured proteins or the symptoms of pathogenic infection and desiccation stress are similar. The fourth response is the slowing down of the metabolic rate suggested by the downregulation of *ins-7* and enzymes involved in catabolism and up-regulation of *ttr-23* and *ugt*. The last mechanism is employed to keep water and osmotic homeostasis in the cells or organism by up-regulating *hmit-1.1* during stress and up-regulating a permease and a chloride channel during recovery. Unfortunately, most of the highly up-regulated genes identified during recovery are not characterized suggesting further work is necessary to understand the desiccation stress response in particular the recovery from the stress.

The gene expression data could partially confirms the hypothesis, that the nematode adjusts to desiccation stress by sequentially responding to different unfavorable conditions. Specifically, it was observed a shift in gene expression (early versus late response) around 12 hours after stress, which suggest that the nematode is responding to a change in the stress it is experiencing. This timepoint correlates with the decrease in survival of the worm after 12 hours after initiation of the stress. Further evidence is the presence of

oxidative damage (i.e. *cyp* transcripts) and water loss (*hmit-1.1*), followed by a slowing down of the metabolism (i.e. SOD-5) and protein metabolism (i.e. HSP). However, the sudden (after 12 hours of stress) increase in the expression of transcripts, does not permit the conclusion, that the failure to activate one or more responses (in time before the damage is lethal) leads to the particular decrease in survival after 12 hours of stress. A gene expression analysis from *C. elegans* desiccated at higher and lower relative humidity might help answer this question, as the 12 hour decrease in survival seems to decrease with higher relative humidities.

4.4.1 Desiccation induced trascripts compared to stress annotated transcripts and heat shock proteins (HSPs) from *C. elegans*

The genes annotated with stress in Wormbase [79] as shown in Table 4.1 on page 114. Figure 4.10 on page 115 shows the expression of these genes in the desiccation stress microarray dataset. The majority (90%) of the genes shown in the figure did not change expression level beyond 2-fold, suggesting that *C. elegans* uses transcripts not annotated before to be part of a stress response, a story possibility since the desiccation tolerance of *C. elegans* has not been studied in great detail.

An aquaporin (*aqp-4*), a water channel protein involved in osmotic homeostasis [88, 107], and a peptide transporter (*opt-2* [138]) are down-regulated during the desiccation stress, suggesting avoidance of water loss and cellular permeability for macromolecules. Supporting the presence of osmotic stress in the first 12 hours of the stress is the expression of *gdph-1*, which encodes a glycerol-3-phosphate dehydrogenase up-regulated

Table 4.1: Genes annotated with stress taken from the Wormbase [79]. On the left side of the column is the gene symbol of the gene (if available) an on the right side the sequence name of the gene.

Gene symbol	Ensembl	Gene symbol	Ensembl
abl-1	M79.1	*ifb-1*	F10C1.2
abu-3	F31A3.1	*ire-1*	C41C4.4
abu-4	Y5H2A.3	*kin-29*	F58H12.1
aco-1	ZK455.1	*mek-1*	K08A8.1
age-1	B0334.8	*mtl-1*	K11G9.6
aqp-2	C01G6.1	*mtl-2*	T08G5.10
aqp-3	Y69E1A.7	*opt-2*	K04E7.2
aqp-4	F40F9.9	*osr-1*	C32E12.3
aqp-8	K02G10.7	*oxi-1*	Y39A1C.2
atf-5	T04C10.4	*pas-4*	C36B1.4
atf-6	F45E6.2	*pas-5*	F25H2.9
bag-1	F57B10.11	*pek-1*	F46C3.1
clpp-1	ZK970.2	*pmk-2*	F42G8.3
cnx-1	ZK632.6	*pmk-3*	F42G8.4
crt-1	Y38A10A.5	*pqm-1*	F40F8.7
daf-2	Y55D5A.5	*scl-1*	F49E11.9
elt-2	C33D3.1	*sgk-1*	W10G6.2
ero-1	Y105E8B.8	*sinh-1*	Y57A10A.20
fat-2	W02A2.1	*sip-1*	F43D9.4
frh-1	F59G1.7	*skn-1*	T19E7.2
ftn-1	C54F6.14	*sod-2*	F10D11.1
ftn-2	D1037.3	*sod-3*	C08A9.1
gcs-1	F37B12.2	*uev-2*	F56D2.4
gpdh-1	F47G4.3	*uev-3*	F26H9.7
gsto-1	C29E4.7	*unc-68*	K11C4.5
hsf-1	Y53C10A.12	*unc-78*	C04F6.4
hsp-12.6	F38E11.2	*unc-87*	F08B6.4
hsp-16.2	Y46H3A.3	*vhp-1*	F08B1.1
hsp-16.41	Y46H3A.2	*xbp-1*	R74.3
hsp-4	F43E2.8		

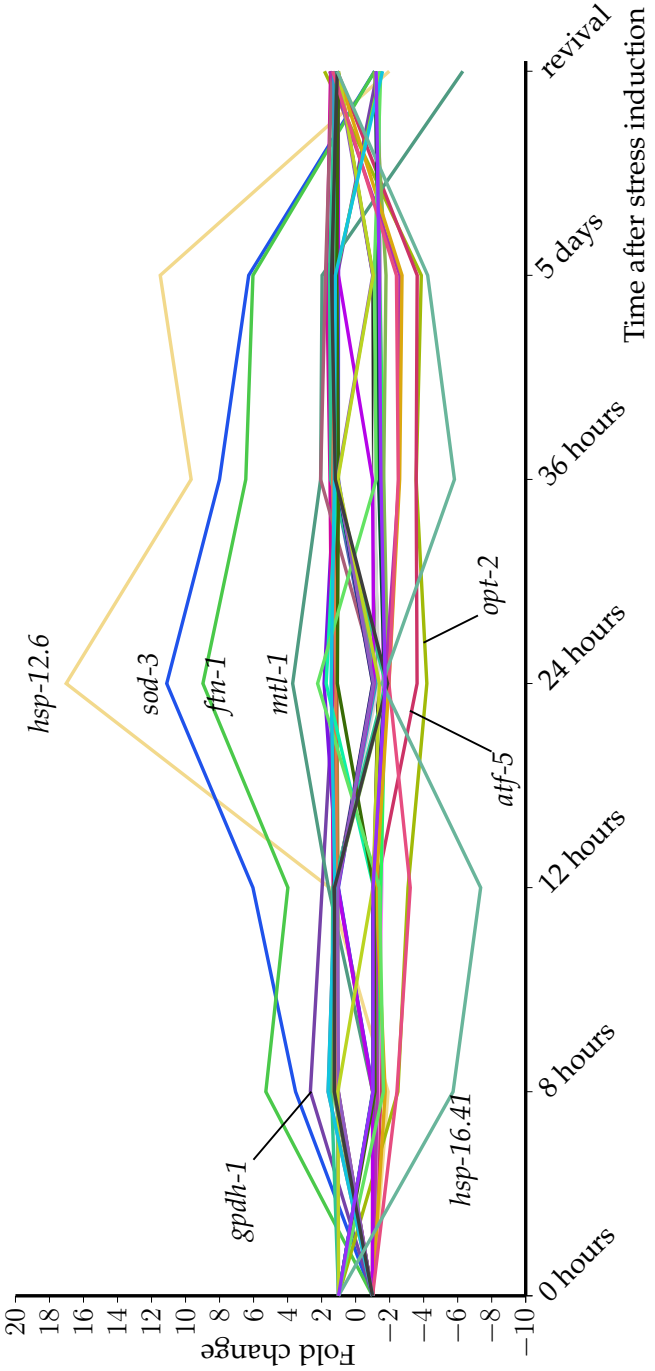


Figure 4.10: The most highly differentially expressed transcripts of *C. elegans* annotated with stress in Wormbase (www.wormbase.org) [79]. For a complete list of the genes see Table 4.1 on page 114.

during hyperosmotic stress [110]. An ubiquitin-encoding gene is found (*uev-3* [96]) also to be up-regulated. Ubiquitin genes were present in all the stress libraries of *Panagrolaimus*, suggesting that ubiquitination is a conserved protein tagging mechanism during (desiccation) stress.

Among the down-regulated genes, two HSP16 members are found. HSP16 family is believed to function by preventing unfolded protein to aggregate. These two HSPs are usually expressed upon heat and environmental stresses [95]. As previously stated, another HSP-16 member (F08H9.4) was highly up-regulated in the desiccation dataset. The difference between the three HSP16 members is the location of expression [167]: F08H9.4 is expressed in the excretory organs of *C. elegans* suggesting the excretion of unfolded proteins or the accumulation of them in particular locations of the nematode body.

Some heat shock proteins have chaperone activity and are induced during desiccation stress. In the *Panagrolaimus* libraries HSP70 along with two co-chaperones (*dnj*) were identified. HSP70 was also found among the most up-regulated genes in the *C. elegans* microarray dataset. The HSP70 RNAi knock-down phenotype in *C. elegans* shows a reduction in life span [134]. HSP60, the second highest up-regulated HSP, is present in the mitochondria and is also involved in the protein unfolding response [218]. HSP12.6 is the third most highly expressed HSP and is a molecular HSP believed to be regulated by DAF-16 and HSF-1 [27]. The presence of these genes suppert the hypothesis, that protein unfolding occurs in desiccation-sensitive and as well in desiccation tolerant nematodes.

This comparative analysis supports the idea that desiccation stress response needs

additional specific transcripts not expressed during other stresses such as heat or osmotic stress. The possibility exists that there are processes ocurring that are similar to dauer formation as well as the response of desiccation tolerant nematodes. These possibilities are examined next.

4.4.2 Comparison with dauer regulated genes

Dauer is a highly stress tolerant stage in the *C. elegans* life cycle [87]. In order to assess whether the desiccation stress response shown by *C. elegans* during desiccation stress resembles the dauer stage stress tolerance, genes highly regulated during the dauer stage [120] were compared to the 275 most highly regulated transcripts during desiccation stress. 612 genes are shared in both data-sets. The representation factor for the two transcript groups is 3.4 ($p < 1.258 \cdot 10E\text{-}17$), indicating that the transcript overlap is higher than expected from the two independent groups. This supports the hypothesis that desiccation stress and dauer stage processes have similarities.

To provide additional insights into the relationship between general stress responses, the dauer transcripts and the desiccation stress transcripts, the similarities between each set was evaluated. Figure 4.11 on page 119 shows that the microarray dataset has more genes in common with dauer stage than with the stress related genes, suggesting that the desiccation response is more similar to dauer stage survival. The dauer stage is the most stress tolerant in *C. elegans*. The worm enters this stage when the nematode experiences unfavorable environments as a defense mechanism. Identifying similarities between dauer expressed genes and genes expressed during desiccation might lead to

common stress specific responses. The list of common transcripts is shown in Table 4.2 on page 120.

One transcript encoding for SOD-3 was common to all three libraries. Among the common transcripts between stress annotated genes and dauer regulated genes, *aqp-4* (Aquaporin) and *opt-2* (oligo peptide transporter) were also among the down-regulated genes during the desiccation response, with *gpdh-1* (glycerol-3-phosphate dehydrogenase) and *mtl-1* (metallothionein) found to be up-regulated during desiccation stress. Two Heat shock proteins (*hsp-12.6* and *hsp-16.41*) and a ferritin (*ftn-1*) were found not to be regulated during dauer.

The transcripts common only to dauer and desiccation stress show the presence of five Cytochrome P450 genes and the *fmo-2* gene, all found in the early response cluster. Also, *fipr-24*, *nlp-32*, *ttr-23*, F08H9.4 from the late response cluster are present in the list. Thus *sod-5* and *clx-1* are missing, suggesting a specific function during desiccation stress.

4.4.3 Comparison with the trascripts of the *Panagrolaimus* subtractive library

The relationship between the stress response in the *Panagrolaimus* species, the dauer regulated genes and the desiccation microarray dataset are shown in Figure 4.12 on page 122 and Table 4.3 on page 123. Only five genes are common between the *Panagrolaimus* subtractive libraries and the desiccation stress transcripts from *C. elegans*. *Nlt-1* and *lys-1*, T25C12.3 were all identified in the *Panagrolaimus* desiccation library and are

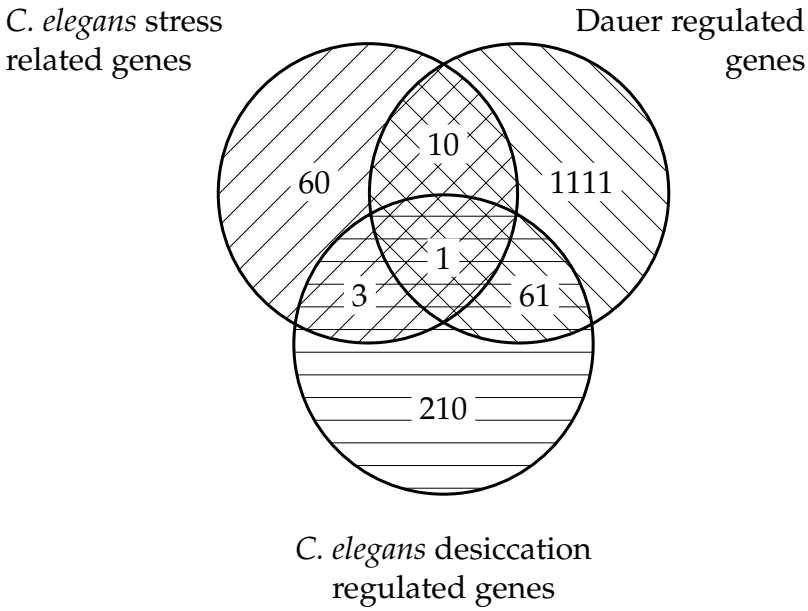


Figure 4.11: Diagram showing the common genes between the dauer regulated genes [120], genes linked to stress in the wormbase, and the 274 most highly differentially expressed genes from the *C. elegans* desiccation microarray experiment.

Table 4.2: Common genes among the following datasets: des - set of the 274 most highly differentially expressed during desiccation stress in *C. elegans*, dauer - set of highly regulated genes during dauer stage [120], stress - set of genes annotated with stress in Wormbase. On the left side of the column is the main name of the gene (if available) an on the right side the sequence name of the gene.

des-dauer			stress-des	
cex-1	F56D1.6	C09H5.2	*ftn-1*	C54F6.14
clec-61	ZK666.7	C18A11.3	*hsp-12.6*	F38E11.2
col-90	C29E4.1	C23G10.11	*hsp-16.41*	Y46H3A.2
cyp-13A4	T10B9.1	C25H3.10		
cyp-13A5	T10B9.2	C45B2.1		
cyp-13A6	T10B9.3	C49F5.7	des-dauer-stress	
cyp-13A7	T10B9.10	C55A1.6	*sod-3*	C08A9.1
cyp-33B1	C25E10.2	F08H9.3		
dct-18	F58G1.4	F08H9.4		
djr-1.2	C49G7.11	F09B9.1	stress-dauer	
dpy-5	F27C1.8	F32G8.3	*aco-1*	ZK455.1
fipr-24	C37A5.8	F40G12.5	*aqp-4*	F40F9.9
fmo-2	K08C7.5	F41F3.3	*daf-2*	Y55D5A.5
gbh-2	M05D6.7	F44G3.10	*gpdh-1*	F47G4.3
gst-3	K08F4.11	F46B6.8	*mtl-1*	K11G9.6
hacd-1	R09B5.6	F48D6.4	*mtl-2*	T08G5.10
hsp-12.3	F38E11.1	F49C12.7	*opt-2*	K04E7.2
inx-15	R12E2.9	F57F4.4	*osr-1*	C32E12.3
lbp-7	T22G5.2	F59A7.2	*scl-1*	F49E11.9
lys-10	F17E9.11	K03H6.2	*sgk-1*	W10G6.2
lys-6	F58B3.3	R12E2.15		
nhx-2	B0495.4	T07G12.5		
nlp-25	Y43F8C.1	T08B1.1		
nlp-32	F30H5.2	T09A12.2		
pho-1	EGAP2.3	T09E11.11		
sqt-1	B0491.2	T25C12.3		
ttr-17	Y5F2A.2	Y38E10A.11		
	C05C10.4	Y38E10A.13		
	C06B3.7	Y51A2B.1		
	C08E8.4	Y6E2A.4		

down-regulated (10-, 24-, and 8-fold respectively) during desiccation stress in *C. elegans*. HSP70, found in the osmotic and post osmotic stress library of *Panagrolaimus* is up-regulated during desiccation stress in *C. elegans*. R12E2.15 found in the osmotic stress library is 8-fold down-regulated during the desiccation stress of *C. elegans*. T25C12.3 and R12E2.15 are both also present in the dauer regulated transcript.

The ribosomal proteins (small and large) shared between the transcripts of the subtractive libraries of *Panagrolaimus* and the transcripts regulated during dauer represent about 50% of the total number of common transcripts between the two sets of genes. This suggest that the stress tolerance of the dauer stage is also due to the developmental processes, as most of these genes are annotated with the gene ontology term post embryonic and larval development. Specifically, the ability to regenerate damaged tissue or cells (differentiation) might be a reason for the heightened tolerance towards stresses in dauer and anhydrobiotic organisms.

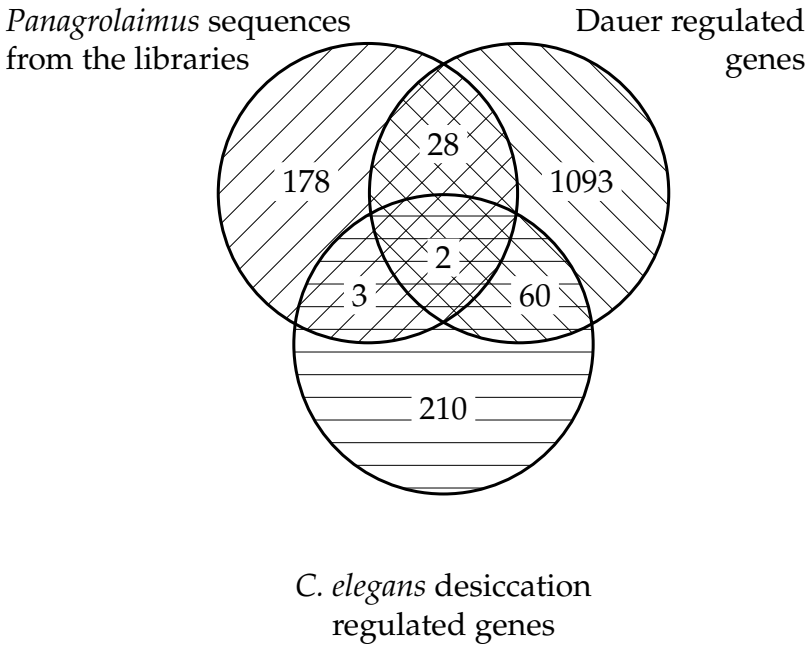


Figure 4.12: Diagram showing the common genes between the dauer regulated genes [120], identified in the subtractive libraries of *Panagrolaimus rigidus* AF36 and *sp.* PS1579, and the 274 most highly differentially expressed genes from the *C. elegans* desiccation microarray experiment.

Table 4.3: Common genes among the following datasets: desiccation - set of the 274 most highly differentially expressed during des stress in *C. elegans*, dauer - set of highly regulated genes during dauer stage [120], sublib - set of transcripts identified in the subtractive libraries of *Panagrolaimus rigidus* AF36 and *sp.* PS1579. On the left side of the column is the main name of the gene (if available) an on the right side the sequence name of the gene.

des-dauer		des- dauer		sublib-des	
cex-1	F56D1.6		C05C10.4	*hsp-70*	C12C8.1
clec-61	ZK666.7		C06B3.7	*lys-1*	Y22F5A.4
col-90	C29E4.1		C08E8.4	*nlt-1*	ZK892.2
cyp-13A4	T10B9.1		C09H5.2		
cyp-13A5	T10B9.2		C18A11.3		
cyp-13A6	T10B9.3		C23G10.11	des-dauer-sublib	
cyp-13A7	T10B9.10		C25H3.10		R12E2.15
cyp-33B1	C25E10.2		C45B2.1		T25C12.3
dct-18	F58G1.4		C49F5.7		
djr-1.2	C49G7.11		C55A1.6		
dpy-5	F27C1.8		F08H9.4	sublib-dauer	
fipr-24	C37A5.8		F09B9.1	*byn-1*	F57B9.5
fmo-2	K08C7.5		F32G8.3	*krs-1*	T02G5.9
ftn-1	C54F6.14		F40G12.5	*lpd-6*	K09H9.6
gbh-2	M05D6.7		F41F3.3	*rpl-11.2*	F07D10.1
gst-3	K08F4.11		F44G3.10	*rpl-12*	JC8.3
hacd-1	R09B5.6		F46B6.8	*rpl-13*	C32E8.2
hsp-12.3	F38E11.1		F48D6.4	*rpl-23*	B0336.10
hsp-12.6	F38E11.2		F49C12.7	*rpl-25.1*	F55D10.2
hsp-16.41	Y46H3A.2		F57F4.4	*rpl-3*	F13B10.2
inx-15	R12E2.9		F59A7.2	*rpl-6*	R151.3
lbp-7	T22G5.2		K03H6.2	*rps-18*	Y57G11C.16
lys-10	F17E9.11		T07G12.5	*rps-2*	C49H3.11
lys-6	F58B3.3		T08B1.1	*rps-20*	Y105E8A.16
nhx-2	B0495.4		T09A12.2	*rps-24*	T07A9.11
nlp-25	Y43F8C.1		T09E11.11	*rps-25*	K02B2.5
nlp-32	F30H5.2		Y38E10A.11	*rps-8*	F42C5.8
pho-1	EGAP2.3		Y38E10A.13	*uvt-5*	F56B6.4
sod-3	C08A9.1		Y51A2B.1		C02E7.6
sqt-1	B0491.2		Y6E2A.4		C02E7.7
ttr-17	Y5F2A.2				C37A2.7

Chapter 5

MicroRNA during desiccation tolerance

5.1 Introduction

Chapter 5 evaluates the potential involvement of microRNAs in desiccation stress (Figure 5.1 on page 125). MicroRNAs (miRNAs) form an evolutionarily conserved class of non-coding regulatory RNAs. The function of a microRNA starts after the hybridization with their target messenger RNAs (mRNAs), inhibiting or enhancing their translation into proteins [64, 115]. MiRNA are involved in numerous aspects of developmental cell biology and have been indicated being involved in cancer and other diseases [6, 33, 59, 77, 172, 210]. However, the connection between microRNAs expression and stress tolerance has recieved little attention [150].

The goal of this work is the identification of miRNAs that are differentially expressed and play a vital role in *C. elegans* during desiccation stress. Specifically, total RNA extracted from the microarray experiment (Chapter 4) at timepoints 0, 12, 24, 36 hours

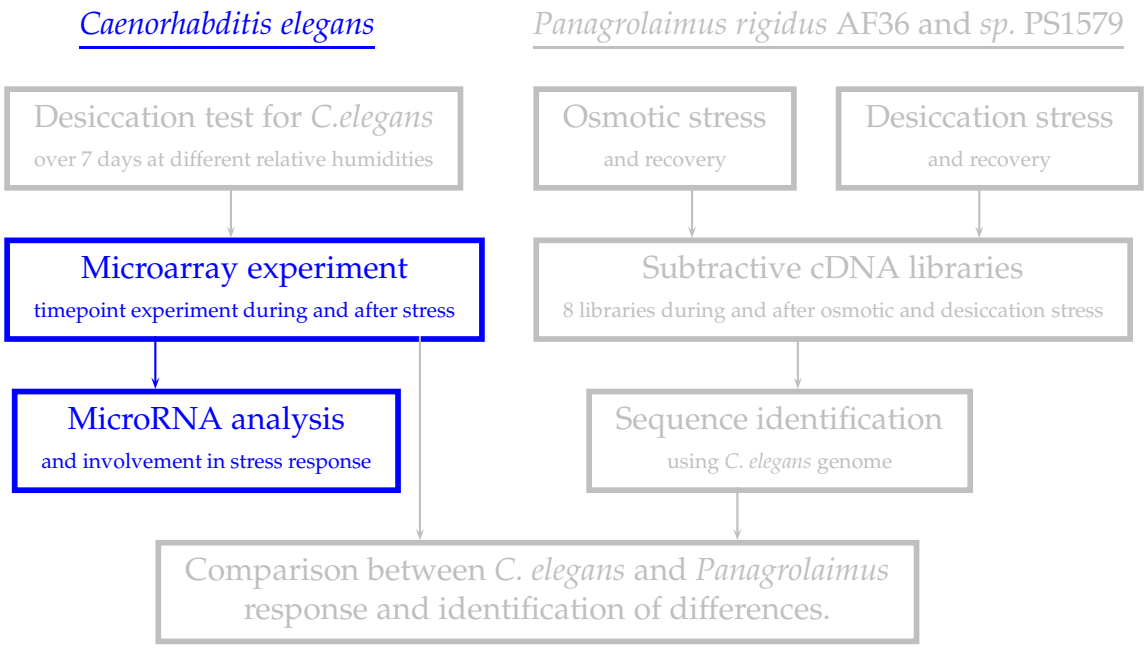


Figure 5.1: Flowchart highlighting Chapter 5 studies.

after initiation of the stress was used to analyze miRNA concentrations. Up-regulated and down-regulated microRNAs over the course of 36 hours were considered potential candidates for being actively involved in the stress response. After identifying potential microRNA candidates, the predicted miRNA targets were computationally identified using the software Pictar [105, 109]. The expression data of the predicted genes were then analyzed using the transcriptional profiling data described in Chapter 4. Furthermore, microRNA knock-outs of these microRNAs were submitted to desiccation stress for 36 hours to validate their involvement in desiccation stress tolerance.

5.2 Materials and Methods

MicroRNA analysis Total RNA collected for the transcriptional profiling experiments described in Chapter 4 were used to measure microRNA expression during the first 36 hours of the desiccation stress. Specifically, total RNA extracted for the microarray hybridizations at timepoints 0, 12, 24 and 36 hours after initiation of the stress were hybridized on a MicroRNA-array chip at Genesensor (Genosensor, USA). The expression was analyzed with ArrayAssist 5.0.1 (Stratagene, USA). The microarray data was imported and a quality control analysis and a hybridization control analysis was perfomed to check the quality of the data. The data was converted into a logarithmic scale and a baseline transformation was perfomed. This step produces a log-ratio dataset from which a significance analysis was perfomed using the Benjamini-Hochberg FDR method. The expression of the three technical replicates on the chip were averaged.

MicroRNA knock-outs The microRNA knock-outs strains of mir-1 (VC576), mir-34 (VC1051), mir-244 (MT16696), mir-265 (MT14661), and mir-273 (MT14347) were obtained from the *Caenorhabditis* Genetics Center (CGC). MicroRNA let-7 knock-out was a gift from Diya Banerjee (Biological Science, Virginia Tech). All strains, were maintained at 25$^{\circ}\mathrm{C}$ on Nematode Growth (NG) agar plates with live bacteria (*Escherichia coli* OP50, an uracil auxotrophic strain) and displayed a superficially wild-type phenotype. The let-7 mutant strain was maintained at 15$^{\circ}\mathrm{C}$. All strains were sucrose washed and submitted to the desiccation stress as described below. The significance analysis was calculated using the student t-test.

Culture preparation Worm cultures were washed from the agar plate by rinsing twice with S-buffer (0.1 M NaCl, 0.05 M potassium phosphate buffer, pH6.0) and collecting the worm suspension in a centrifuge tube. Following centrifugation (1min, 200 x g) the supernatant was discarded. The worm pellet was re-suspended in S-buffer. This step was repeated for three times. After the last certifugation, the pellet was re-suspended in 5 ml S-buffer and left on ice for at least 5 min. The worm suspension was mixed with 5 ml ice cold 70% sucrose solution and centrifuged for 5 min at 1500 x g. Afterwards the floating worms were put in sterile S-basal buffer. The sample was centrifuged for 3 min at 1000 x g, washed with by S-buffer wash and centrifuged (2 min, 200 x g). The worms were used immediately after cleaning.

Desiccation stress To induce desiccation stress the knock-out *C. elegans* strains were sucrose cleaned and at least 80,000 worms per sample were placed on a 5 cm nitrocellulose membrane (Millipore, USA). The membrane with the worms was then immediately placed

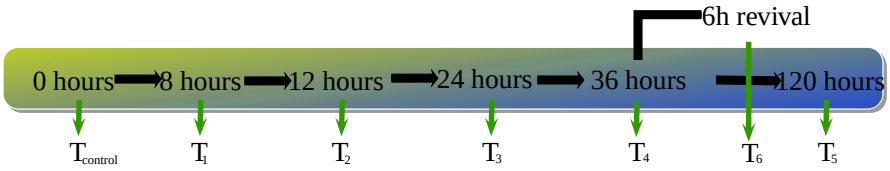
in a chamber equilibrated to a relative humidity (RH) of 60% with a glycerol solution. After 12, 24 and 36 hours a piece of the membrane was cut and prepared for the survival count. This experiment was done in duplicate.

Survival Survival was measured using SYTOX green (Molecular probes, USA). The nematodes were placed in S-buffer solution containing 1μM SYTOX green dye.The samples were left for 15 min and 100 $\mu\mathrm{l}$ aliquotes were placed on a 96 well plate (Novagen, USA). For each sample 5 wells were used, paying attention to have at least 100 worms per well. The samples were visualized using a confocal microscope (MS510, Zeiss). A digital image was taken from each well. The images were analyzed with a computer and survival rate was determined by counting the total number of worms and the dead worms (colored in green).

5.3 Results

The expression levels of 118 out of the 128 screened microRNAs, showed differential expression during the first 36 hours of desiccation stress (Figure 5.2 on page 129). Two microRNAs, mir-34 and mir-265, showed an increase in concentration of 2-fold during the timeperiod of 36 hours. In contrast, mir-244 and mir-273 showed a down-regulation during the same time-frame. Furthermore, regulation of most miRNAs is taking place at 12 hours after the initiation of the stress.

In order to detect possible involvement of these micrRNA during stress, the microR-



time frame	0-12	12-24	24-36
2-fold up	0	28	0
2-fold down	22	0	2
2-fold up	2		
2-fold up		4	
2-fold up	2		
2 fold down	2		

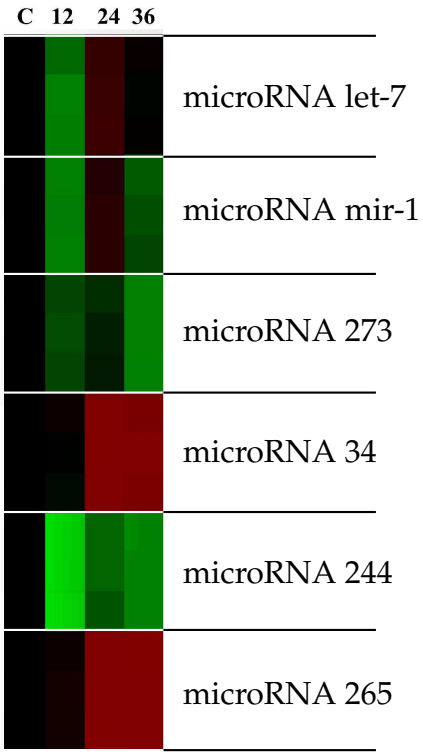


Figure 5.2: Figure on top shows the timepoints of the desiccation tolerance experiment. The table on the bottom left shows the summary of the expression levels of the 128 *C. elegans* microRNAs screened. The figure on the bottom right shows the heat-map of the microRNAs let-7, mit-1, mir-34, mir-244, mir-265, and mir-273 in triplicate.

NA targets of mir-1,mir-34, mir-244, mir-265, and mir-273 were computationally predicted using the PicTar algorithm [105, 109]. As mentioned previously, miRNAs are post-transcriptional regulators that down-regulate translation of their target genes. For a miRNA-mediated process, a decrease in target mRNA levels should be associated with an increase in the specific miRNA. Alternatively, an increase in mRNA could be the result of a decrease in miRNA levels. The expression profile of the predicted microRNA targets are shown in Figures 5.4, 5.5, 5.7, 5.6, and 5.8 on pages 134, 135, 137, 136, and 138 respectively.

The theoretical expression profile for a mir-1 target gene should show an up-regulation during the first 12 hours, followed by a slight decrease for the next 12 hours and an up-regulation again until 36 hours after induction of the stress. A significant increase or decrease should be visible during the first 24 hours of the stress. Only the mir-1 predicted target F35D11.3, encoding for an uncharacterized conserved protein, shown in Figure 5.4 page 134 partially resembles the theoretical expression profile. The optimal expression profile for the mir-34 target gene should show a decrese in the mRNA expression level after 24 hours of stress. The predicted targets *col-98* (collagen protein), C48E7.1 (unknown, but membrane bound), F11E6.3 (unknown protein positively regulating growth), and *twk-18* (potassium channel protein) show a down-regulation after 24 hours of stress suggesting a direct regulation of these transcripts (see Figure 5.5 page 135). The same optimal expression profile should be seen for the targets of mir-265. However, all the predicted targets remain at the basal levels over the entire period of the stress (Figure 5.6 on page 136). The optimal expression profile for a mir-273 target gene should show a moderate up-regualtion begining after 12 hours of stress and again an increase in expression level after 36 hours. Also here none of the predicted targets can be correlated

with the optimal expression pattern (see Figure 5.7 page 137). The optimal expression profile for a mir-244 should follow the same pattern as for mir-273 just starting with an higher up-regulation in the first 24 hours. The only gene following approximately the ideal expression profile is K07F5.15, which encodes a small protein of ancient origin [146] (see Figure 5.8 page 138).

The target prediction approach showed that the prediction algorithm can be used to identify potential targets. However, overall the target gene profiles were poorly matched to the miRNA levels. To further assess the importance of miRNAs during desiccation stress, knock-outs of let-7, mir-1, mir-34, mir-273, mir244, and mir-265 were used to test their tolerance towards desiccation stress. Additionally the microRNA let-7 was also chosen as it is one of the best characterized microRNAs. Additionally a member of the let-7 family was found to be involved in stress tolerance [113].

The results of the knock-out experiments are shown in Figure 5.3 on page 132. The let-7 knock-out displayed a lower survival (about 20%) in each timepoint compared to wild-type. This is due to the phenotype associated with let-7 mutants; when grown above 15°C, a supernumerary moult to a fifth larval stage, L5, occurs. The result is a cuticle bursting and death of the worm. Temperatures below 15°C supresses this phenotype.
Mir-1, showing a decrease and an up-regulation around 24 hours of the stress showed a significant decrese in survival after 12 hours. This result would suggest, that mir-1 is essential for the survival after 12 hours of stress, when the concentration of mir-1 increased two-fold (see microRNA-array heat-map on Figure 5.2 on page 129).

Mir-34 expression is upregulated after 24 hours of desiccation stress. The mir-34 knock-

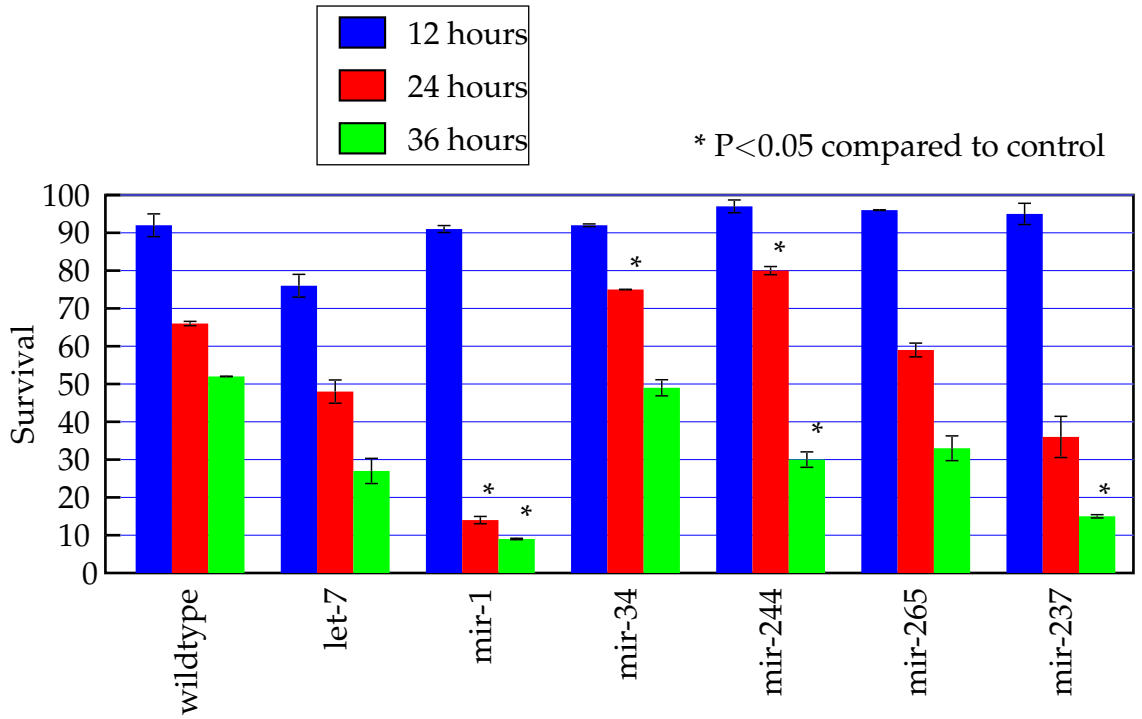


Figure 5.3: Desiccation stress survival rate of microRNA knock-outs let-7, mir-1, mir-34, mir-244, mir-265, and mir-273. All knock-out and wildtype *C. elegans* were desiccated for 36 hours at 60% relative humididty and 16°C. The survival was measured after 12, 24 and 36 hours of desiccation stress. The error bars represent the standard error of duplicates.

out displayed a significantly higher survival than wild-type after 12 hours of stress. The survival then falls to the same level as wild type. Comparing the survival of the mir-34 knock-out with the mir-34 expression profile in wild-type, it can be noted that at the time-point the expression profile of mir-34 shows an up-regulation, the mir-34 knock-out mutant shows a significant increase in survival. Therefore, the absence of the mir-34 after 12 hours of stress significantly changed the stress survival, suggesting an involvement in the stress response. The mir-244 concentration during desiccation stress decreased after 12 hours of stress. The survival of the mir-244 knock-out showed a significant increase in survival after 12 hours of stress followed by a signifcant decrease in survival after 36 hours. Also here a significant change in survival is noted after the concentration of mir-244 in wildtype occured. Mir-265 concentration showed an increase after 24 hours desiccation stress. The survival was lower at each timepoint relative to wild-type, but no significantly, suggesting that mir-265 does not play a major role. Mir-273 levels decreased after 24 hours of stress. The knock-out of mir-273 also displayed a decrease in survival after 24 hours of stress, which was significant after 36 hours. Mir-273-knock-out survival also changed significantly, coinciding with a change in transcriptional level.

5.4 Discussion

The aim of this experiment was to investigate the potential involvement of microRNAs during the first 36 hours of desiccation stress. The expression levels of 128 *C. elegans* microRNAs were analyzed at timepoints 0, 12, 24, and 36 hours after initiation of the stress. Two microRNAs showed an increase and two showed a decrease of at least 2-fold in levels during the 36 hours stress. The microRNAs concentrations change most at the

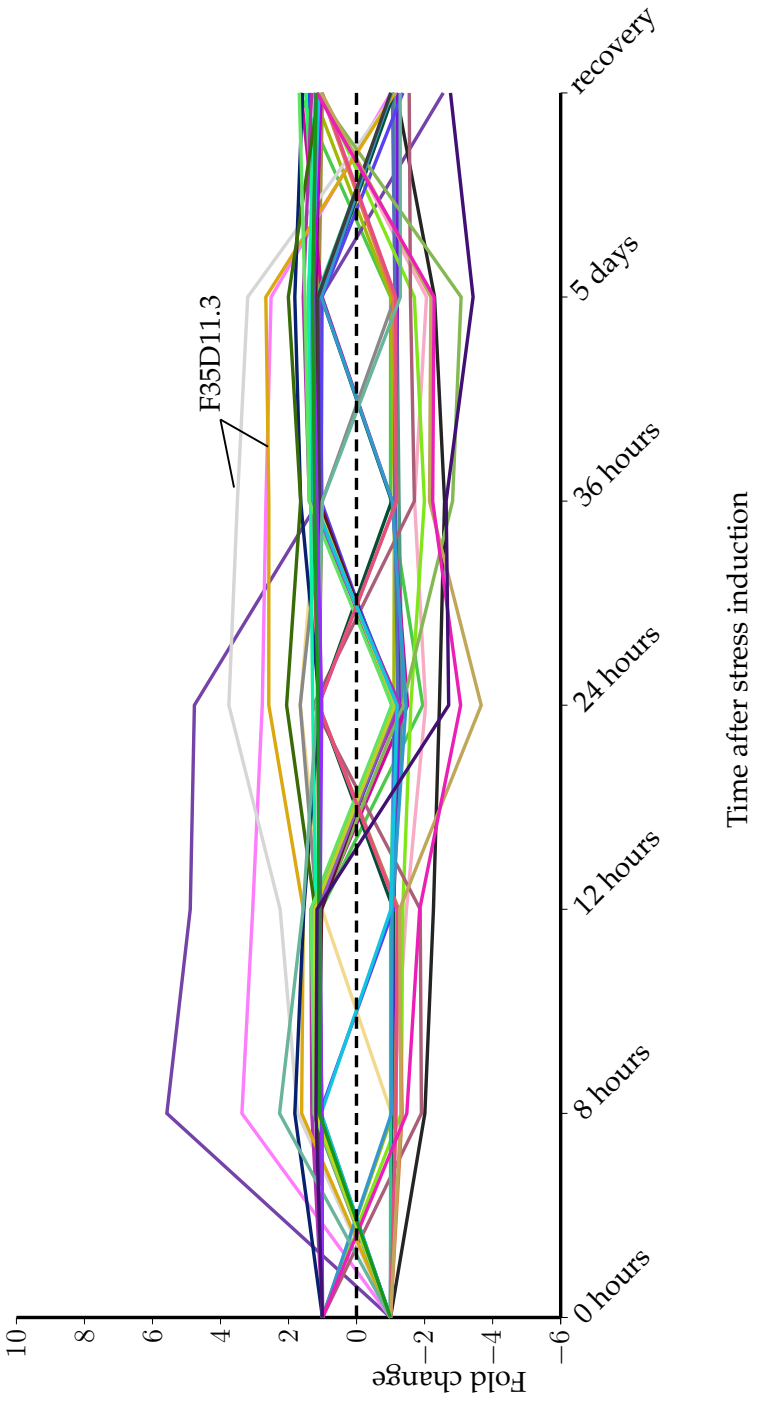


Figure 5.4: Expression profile of computationally predicted mir-1 targets during desiccation. Potential regulated mRNA-targets, which follow the ideal target expression profile according to the expression profile of mir-1 during desiccation stress, are annotated with the gene symbol. The theoretical expression profile for a mir-1 target gene should show an up-regulation during the first 12 hours, followed by a slight decrease for the next 12 hours and an up-regulation again until 36 hours after induction of the stress.

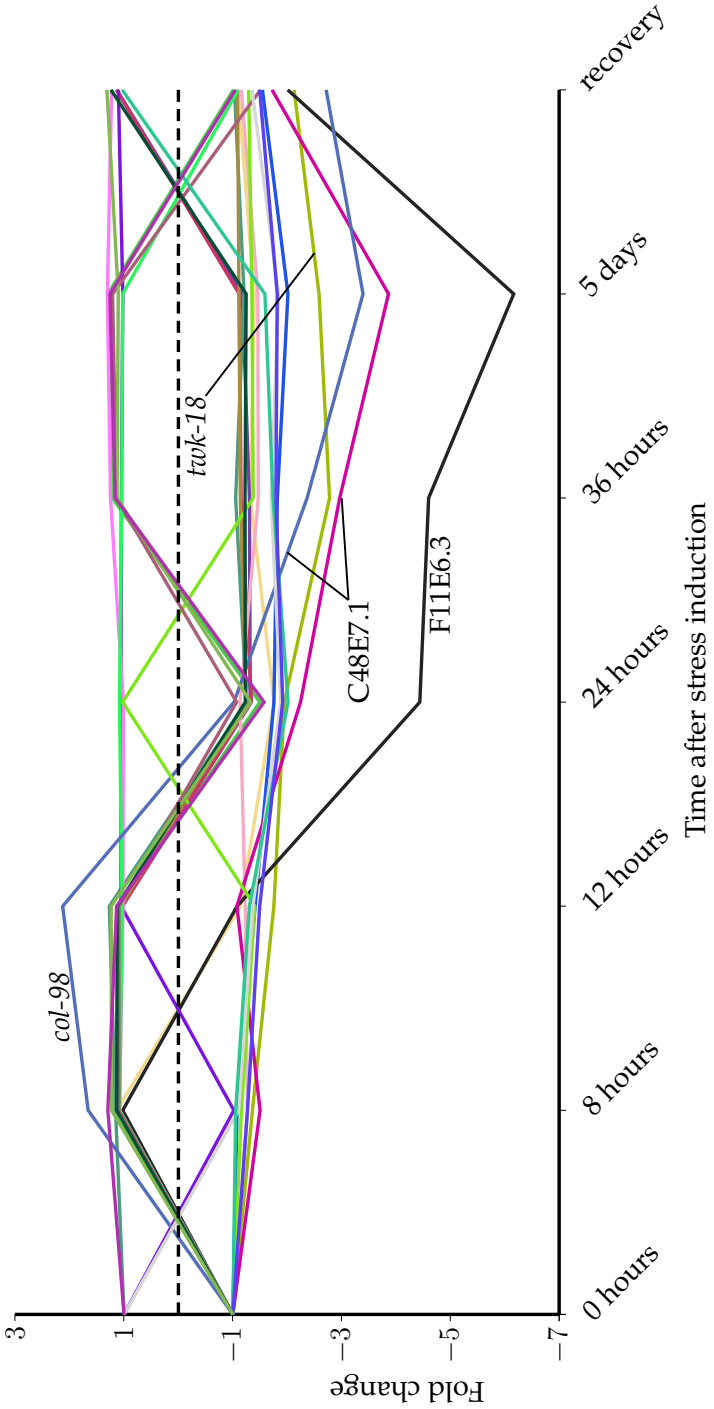


Figure 5.5: Expression profile of computationally predicted mir-34 targets during desiccation. Potential regulated mRNA-targets, which follow the ideal target expression profile according to the expression profile of mir-1 during desiccation stress, are annotated with the gene symbol. The theoretical expression profile for the mir-34 target gene should show a decrease in the mRNA expression level after 24 hours of stress.

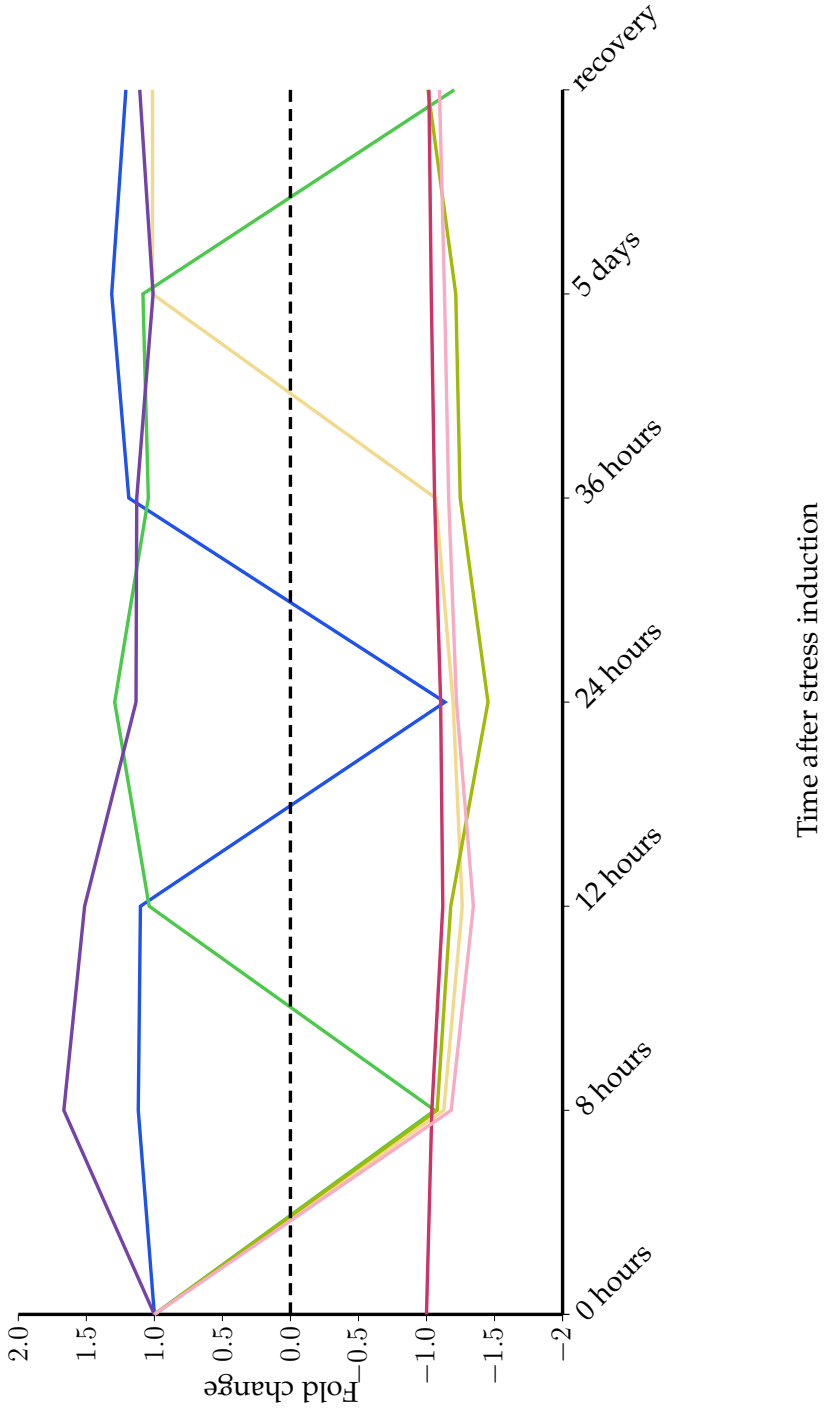


Figure 5.6: Expression profile of computationally predicted mir-265 targets during desiccation. Potential regulated mRNA-targets, which follow the ideal target expression profile according to the expression profile of mir-1 during desiccation stress, are annotated with the gene symbol. The theoretical expression profile for the mir-265 target gene should show a decrease in the mRNA expression level after 24 hours of stress.

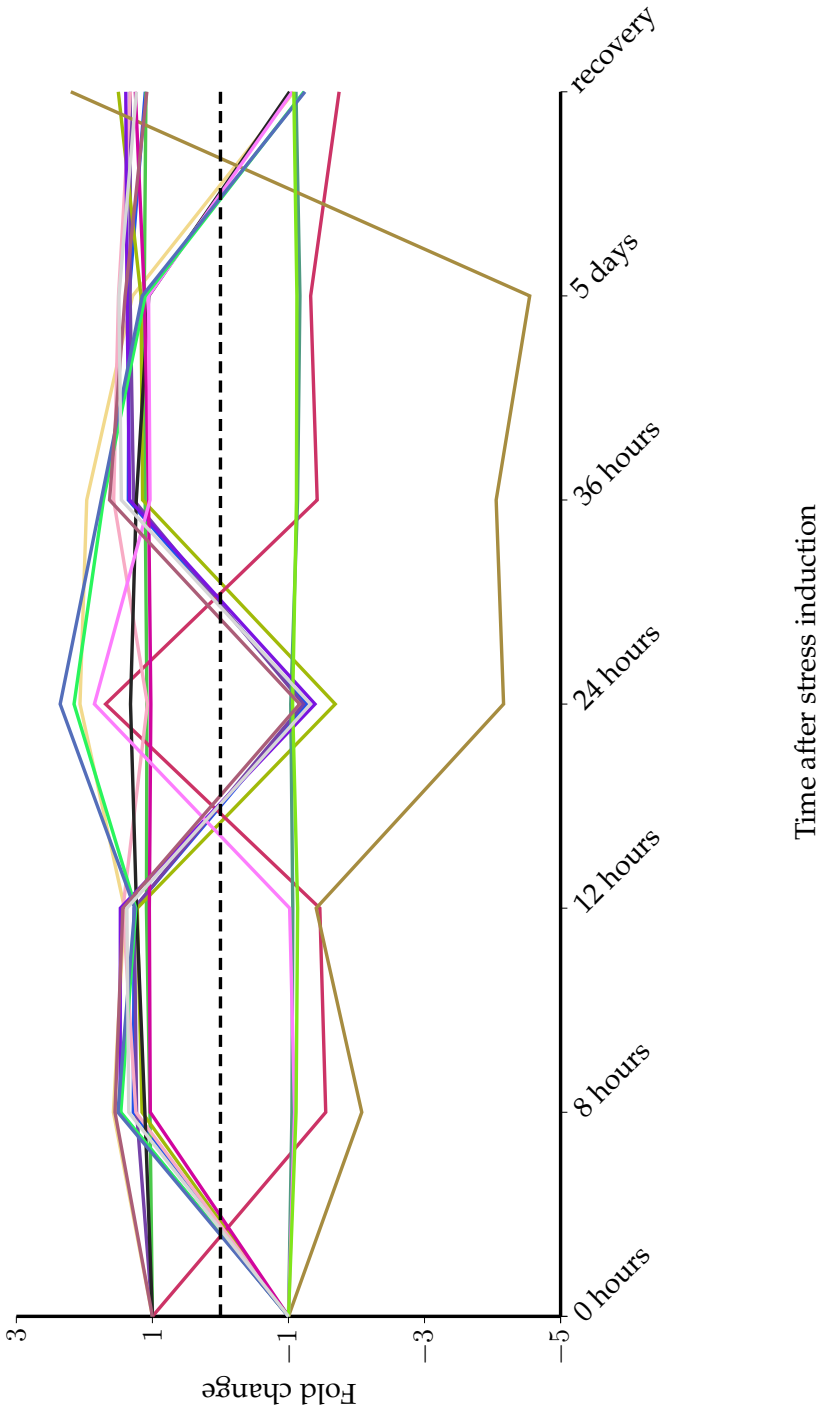


Figure 5.7: Expression profile of computationally predicted mir-273 targets during desiccation. Potential regulated mRNA-targets, which follow the ideal target expression profile according to the expression profile of mir-1 during desiccation stress, are annotated with the gene symbol. The theoretical expression profile for a mir-273 target gene should show an up-regulation beginning after 12 hours of stress and again an increase in expression level after 36 hours.

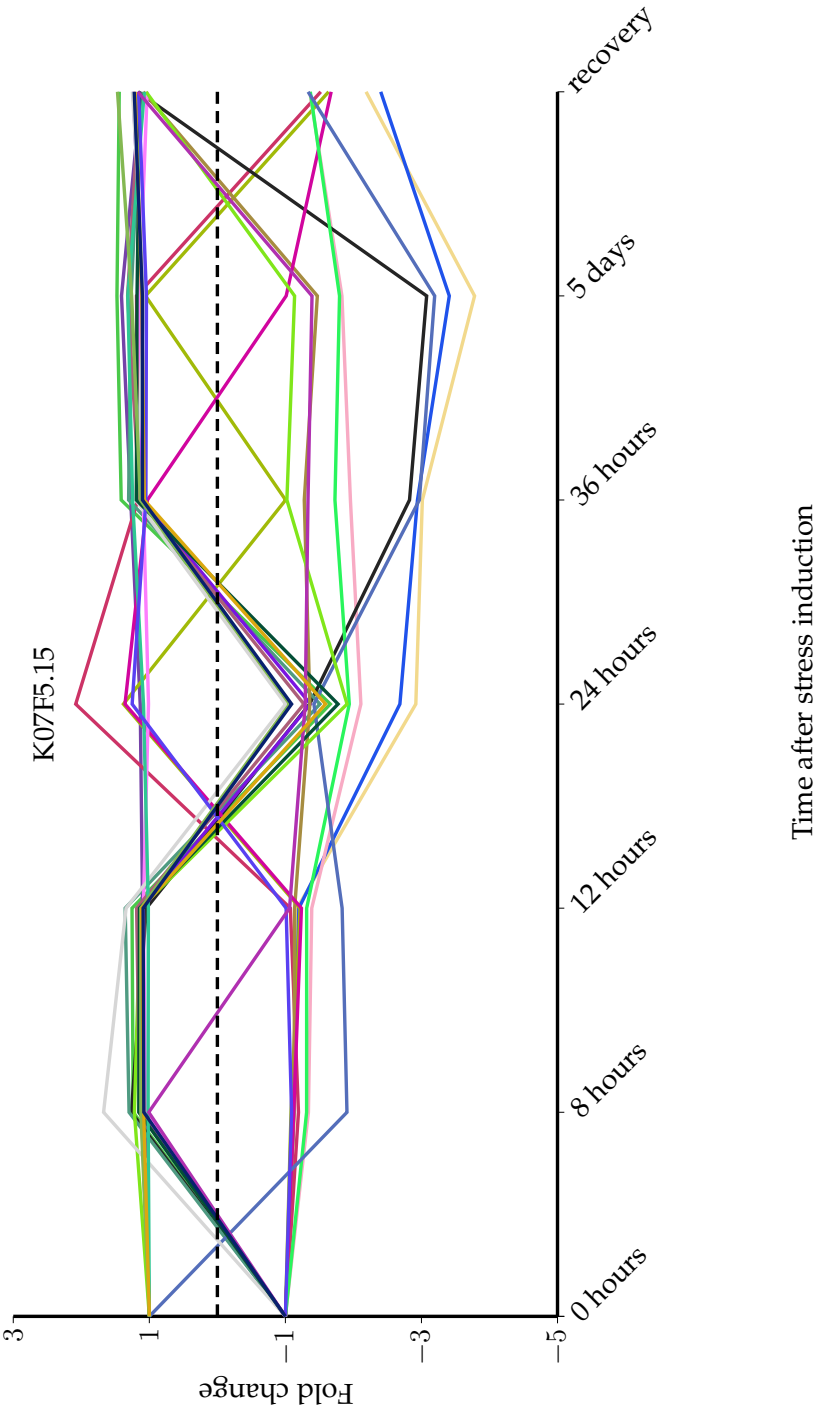


Figure 5.8: Expression profile of computationally predicted mir-244 targets during desiccation. Potential regulated mRNA-targets, which follow the ideal target expression profile according to the expression profile of mir-1 during desiccation stress, are annotated with the gene symbol.The theoretical expression profile for a mir-244 target gene should show a up-regulation beginning after 12 hours of stress and again an increase in expression level after 36 hours.

12 hour timepoint emphasizing again the early and late response phase.

The target identification of the microRNAs were first computationally predicted using the desiccation microarray data generated previously (Chapter 4) resulting in a few mRNA-miRNA correlations. A regulatory trend from the expression profile of the predicted targets could only be detected for mir-1, mir-34, and mir-244 targets.

The knock-out experiment confirmed the involvement of microRNAs during stress tolerance. Mir-1 showed the most severe phenotype (lowest tolerance), followed by mir-273. Mir-35 and mir-244 knock-outs showed initial higher survival than wild-type and the mir-265 knock-out did not show any significant phenotype. While it is not clear how microRNAs are influencing the desiccation response, a correlation between changes in concentration of the microRNA and survival can be detected. Mir-1, mir-34 and mir-244 appear to have potential targets identified in the micro-array dataset what Table in Figure 5.2 on page 129, that are only up-regulated 5-fold, thus being in the range of a potential suppression by their miRNAs. MicroRNA during stress are thought to be able to sequester mRNA in stress bodies and/or degrade mRNAs. A microRNA knock-out is probably not capable to sequester or degrade a set of mRNAs, which might be the cause for a lower survival. Considering the results obtained from the single gene knock-downs in *C. elegans* and the *Panagrolaimus* species, which showed a maximal 10% difference in survival compared to wild-type (see Figure 3.4 on page 79 and 4.8 on page 106), suggesting the involvement of more transcripts getting degraded by a microRNA. A recent study [98] proposed that stress bodies are formed during stress in mammalian cells. These bodies segregate mRNA hybridized with micrRNA and storing them in stabilizing form until the stress is over. Therefore, a target identification looking at mRNA expression level from a micro-array

might not detect the segregation by microRNA. Basically, mRNA is getting sequestred in stress bodies and thus are kept presumably intact and do not change in concentration due to missing degradation. An accumulation of specific mRNAs in stress bodies puts them ‘out of the system’ for the translation mashinery of the cell. However, once the cell gets disrupted and total RNA is extracted, these sequestred mRNA are counted as being availabe for the cell.

Summarizing, it was shown that micrRNA concentration change during the desiccation stress in the first 36 hours of stress with a transition at 12 hours coincidin with a change in survival. Selected micro-RNAs were shown to modulate survival negatively and positively, at least for short period of time. More attention needs to be given to how micrRNAs are involved in nematode stress tolerance.

Chapter 6

Conclusion

6.1 Introduction

6.1.1 Aim 1

The first aim of this study was to characterize desiccation tolerance in *C. elegans* and the *Panagrolaimus rigidus* AF36 and *sp.* PS1579. *C. elegans* was found to survive moderate desiccation for four days. During this timeframe a noticeable drop in survival at 24 hours was recorded. This finding suggests that at this timepoint a major change (i.e. additional stress) is experienced by *C. elegans*. The two *Panagrolaimus* species showed remarkable survival in a 0% relative humidity condition. *Panagrolaimus sp.* PS1579 showed a better stress tolerance than *rigidus* AF36. Osmotic stress tolerance was tested using salt concentrations comparable to sea water. The survival was close to 90% for both species, a remarkable osmotic tolerance for a terrestrial nematode. Sequential stress analysis showed that exposure to one stress confers only partial tolerance to the other stress. The conclusion drawn from this observation is that osmotic and desiccation

stresses have some related mechanisms in common, but are predominantly independent responses.

6.1.2 Aim 2

The second aim was to identify genes from *Panagrolaimus rigidus* AF36 and *sp.* PS1579 that are uniquely expressed during and after osmotic and desiccation stresses. Eight cDNA subtractive libraries were constructed and sequenced. Gene identification was done using the cDNA libraries from *C. elegans* and *C. briggsae*. The identification were successful for 48% of the 854 sequences found in all libraries. The investigation of complementary sequences in the library resulted in only two sequences being identified in both speciess within the same stress libraries. Comparison of the identified sequences between the libraries resulted in few matches, reinforcing the differences between osmotic and desiccation stress responses. This led to the conclusion that the mechanism of desiccation and osmotic tolerance have some common features, though on the transcriptional level the two responses are very different. Gene ontology analysis identified an enrichment of transcripts annotated with development in all libraries suggesting a replacement of damaged material by the organism after osmotic or desiccation stress. Three *lea* transcripts in post desiccation library and a *tps* sequence in the desiccation library were found, as well genes shown to be important for osmotic tolerance in *C. elegans*.

6.1.3 Aim 3

The third aim of this study was to identify the response of *C. elegans* to desiccation stress. The result showed a large number of genes being differentially expressed. Plotting the expression data against time, differentially expressed genes could be separated into four groups: (1) ‘early response’ genes up-regulated mainly in the first 24 hours, (2) ‘late response’ genes up-regulated after 24 hours, (3) down-regulated genes, and (4) genes up-regulated during the recovery. The first group is enriched with mono-oxygenses, the second was characterized by two heat shock proteins, *sod-5* and genes belonging to the innate immune system. The third group was characterized by lysozymes and proteins involved in macromolecular metabolism. The fourth group was characterized by mono-oxygeneses, transport and channel proteins.

Transcripts regulated during desiccation stress by *C. elegans* were found to be regulated in the dauer stage. Thus transcripts from *C. elegans* known to be involved in other stress responses are not highly expressed during desiccation stress. Furthermore, *C. elegans* was found to not up-regulate any of the transcripts identified in the desiccation subtractive library. These findings lead to the conclusion, that *C. elegans* is using a unique set of transcripts to respond to desiccation stress. The transcripts regulated during desiccation stress in *C. elegans* suggest a response to different experienced stresses such as osmosis, protein degradation, and oxidation, which are known to occur during the stress indicating more likely a sum of different responses rather than an organized overall response as seen by the anhydrobiotic nematodes.

6.1.4 Aim 4

The fourth aim of this work was to assess whether microRNAs are actively involved in desiccation stress tolerance. Specifically, the expression of 118 microRNAs from *C. elegans* were measured in the first 36 hours of desiccation stress. The result showed four up- and down-regulated microRNAs over the entire timeframe of 36 hours. Other microRNAs showed an up- or down-regulation around timepoint '12 hours'. Computational target validation and correlation with the microarray data was not conclusive. Therefore, *C. elegans* microRNA-knockouts from six microRNAs (let-7, miR-1, miR-34, miR-244, miR-265, miR-273) were submitted to desiccation stress and their survival were scored. The result showed a linear decrease for let-7 knock-out, which was attributed to its phenotype. The lowest survival or stress tolerance was shown by mir-1 knock-out. A correlation between the micro-RNA concentration change time-point and the time-point of decreased survival in the knock-outs was detected. MicroRNAs therefore play a vital role during stress.

6.2 Comparison of the data

Immediately after placing either *Panagrolaimus* or *C. elegans* on a nitrocellulose membrane, they aggregate. In this behavior, the two nematodes do not differ. It was recently shown [158] that this is a defense mechanism natural to the nematode due to environmental stimuli.

After this initial aggregation, *Panagrolaimus* and *C. elegans* differ. *Panagrolaimus rigidus* AF36 and *sp.* PS1579 survive relative humidities as low as 0% for more than a week. *C.*

elegans without entering the dauer stage only survives a few days at high relative humidities. The transcription profile of *C. elegans* reveals that it experiences osmotic and oxidative stress in the first 12 hours. Then the nematode slows down its metabolism and movement. Specific HSPs are expressed against denatured and aggregated protein. Protein denaturation or other stimuli cause proteins of the immune system to be released/up-regulated. The *Panagrolaimus* species go into anhydrobiosis and consequently shut down their metabolism. However, the transcripts expressed during the stress suggest that the organism is also preparing for rehydration. Specifically, transcripts involved in cell cycle and development are enriched. This suggests, that after revival, the cells of the organism are ready to restart their cell cycle activities and repair mechanisms. In addition, protein metabolic processes are taking place during desiccation. The presence of aspartyl-protease transcripts and ribosomal transcripts suggest the synthesis of new proteins or the organism prepares to degrade the denatured proteins after the re-hydration. Ribosomal proteins may also be used to protect nucleic acids, especially mRNAs from getting damaged. The presence of some of these ribosomal RNAs during dauer stage in *C. elegans* might confirm this theory of ribosomal units having stress protective function, maybe in stress granules. Trafficking and structural changes are also taking place during desiccation in the *Panagrolaimus* species. Only a protein concentration comparison between nematodes in anhydrobiosis and after anhydroiosis could confirm if certain mRNAs are protected or translated during the stress.

Upon rehydration, *C. elegans* re-establishes the cell homeostasis by expressing permases and anion channel proteins. Also the primary and fat metabolism is activated. *Panagrolaimus* species, upon re-hydration, are activating translational processes, translating transcripts bound previously by the ribosomal proteins. The synthesis of macro-

molecules suggests the presence of repair mechanisms for damaged tissues. This is further confirmed by transcripts involved in development and growth.

6.3 Future perspective

Future investigations based on this research should look at the role of the insulin-mediated pathways, especially, the role of *sod-5* during desiccation stress. This transcript is up-regulated during desiccation stress, but it is not regulated during dauer. DAF-16 and PHA-4 are regulating SOD-5 and are involved in life span extension of the animal.

The role of the ribosomal proteins during stress should be addressed. The key to anhydrobiosis or severe stress survival may rest in the possibility to regenerate or repair damaged tissue on a large scale, which is probably achieved by re-initiating a developmental-like process in anhydrobiotic organisms. This mechanism might start from the ribsomal units.

Together with the ribosomal proteins, the involvement of microRNA needs to be addressed as well. The results obtained in this study support the involvement of microRNA in stress survival. However, the mechanism is not fully understood. That microRNAs are able to sequester mRNA and potentially with the help of ribosomal proteins protect the transcripts during stress, gives microRNAs a unique potential role in stress response pathways.

Members of the immune system of *C. elegans* were found to be up-regulated during stress. This fact suggests that it might be a response to denatured proteins or perturbed cell homeostasis. To identify mechanisms employed by anhydrobiotic organisms to prevent activation of an immune response during non-pathogenic stress may contribute to finding new methods in the fight against diseases.

The most work intensive part of this research was the identification of all transcripts found in the subtractive library and in the desiccation response of *C. elegans*. The full knowledge of the stress response transcriptome will potentially lead to the successful anhydrobiotic engineering of biological tissues.

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Appendix A

Gene ontology table

A.1 Desiccation library

Table A.1: Complete list of all biological process gene ontology (GO) terms from the desiccation subtractive library of *Panagrolaimus* AF36 and PS1579. 81 genes were annotated with at least one GO term. Term - GO number GO term, Count - number of transcripts annotated with this GO term in the library, % - % of the total number of annotated transcripts in the library.

Term	Count	%	EASE-score
GO:0009987 cellular process	51	62.96%	1.551E-02
GO:0008152 metabolic process	43	53.09%	5.026E-02
GO:0044238 primary metabolic process	40	49.38%	8.901E-03
GO:0044237 cellular metabolic process	40	49.38%	6.611E-03
GO:0032501 multicellular organismal process	38	46.91%	2.286E-02
GO:0032502 developmental process	37	45.68%	2.041E-02
GO:0007275 multicellular organismal development	36	44.44%	1.970E-02

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Table A.1: continued

Term	Count	%	EASE-score
GO:0043170 macromolecule metabolic process	34	41.98%	2.551E-02
GO:0000003 reproduction	28	34.57%	7.622E-05
GO:0009792 embryonic development ending in birth or egg hatching	27	33.33%	1.756E-02
GO:0044260 cellular macromolecule metabolic process	27	33.33%	2.881E-04
GO:0009791 post-embryonic development	27	33.33%	2.548E-05
GO:0009790 embryonic development	27	33.33%	2.324E-02
GO:0040007 growth	27	33.33%	5.408E-04
GO:0019538 protein metabolic process	27	33.33%	3.386E-04
GO:0044267 cellular protein metabolic process	27	33.33%	1.989E-04
GO:0002164 larval development	24	29.63%	1.939E-04
GO:0002119 larval development (sensu Nematoda)	24	29.63%	1.897E-04
GO:0050896 response to stimulus	18	22.22%	2.714E-02
GO:0048856 anatomical structure development	16	19.75%	1.091E-02
GO:0006508 proteolysis	16	19.75%	8.724E-07
GO:0007610 behavior	14	17.28%	6.566E-02
GO:0022414 reproductive process	14	17.28%	5.921E-03
GO:0009058 biosynthetic process	12	14.81%	1.576E-02
GO:0044249 cellular biosynthetic process	10	12.35%	2.006E-02
GO:0048513 organ development	10	12.35%	4.962E-02
GO:0048731 system development	10	12.35%	7.247E-02
GO:0003006 reproductive developmental process	10	12.35%	2.235E-02
GO:0040035 hermaphrodite genitalia development	9	11.11%	2.499E-02
GO:0007548 sex differentiation	9	11.11%	4.698E-02
GO:0048806 genitalia development	9	11.11%	2.675E-02
GO:0009059 macromolecule biosynthetic process	8	9.88%	1.693E-02
GO:0040016 embryonic cleavage	7	8.64%	2.370E-03
GO:0006412 translation	7	8.64%	1.488E-02

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Table A.1: continued

Term	Count	%	EASE-score
GO:0051301 cell division	7	8.64%	1.540E-02
GO:0006996 organelle organization and biogenesis	7	8.64%	8.535E-02
GO:0000910 cytokinesis	6	7.41%	1.552E-03
GO:0040032 post-embryonic body morphogenesis	6	7.41%	9.470E-02
GO:0009886 post-embryonic morphogenesis	6	7.41%	9.666E-02
GO:0006259 DNA metabolic process	6	7.41%	8.342E-02
GO:0006082 organic acid metabolic process	5	6.17%	9.222E-02
GO:0019752 carboxylic acid metabolic process	5	6.17%	9.222E-02
GO:0006520 amino acid metabolic process	4	4.94%	9.071E-02
GO:0006334 nucleosome assembly	3	3.70%	2.971E-02
GO:0006333 chromatin assembly or disassembly	3	3.70%	7.821E-02
GO:0000281 cytokinesis after mitosis	3	3.70%	1.205E-03
GO:0031497 chromatin assembly	3	3.70%	3.628E-02
GO:0065004 protein-DNA complex assembly	3	3.70%	6.510E-02
GO:0033205 cytokinesis during cell cycle	3	3.70%	1.598E-03
GO:0032506 cytokinetic process	2	2.47%	3.805E-02
GO:0000920 cell separation during cytokinesis	2	2.47%	3.805E-02

Table A.2: Complete list of all Annotation clusters as computed by DAVID from the desiccation stress subtractive library of *Panagrolaimus* AF36 and PS1579. GO number GO term, count - number of transcripts annotated with this GO term in the library.

Annotation cluster 1	count	EASE-score
GO:0009791 post-embryonic development	27	2.55E-05
GO:0002119 larval development (sensu Nematoda)	24	1.90E-04
GO:0002164 larval development	24	1.94E-04

Annotation cluster 2	count	EASE-score
GO:0044267 cellular protein metabolic process	27	1.99E-04
GO:0044260 cellular macromolecule metabolic process	27	2.88E-04
GO:0019538 protein metabolic process	27	3.39E-04

Annotation cluster 3	count	EASE-score
GO:0000910 cytokinesis	6	0
GO:0040016 embryonic cleavage	7	0
GO:0051301 cell division	7	0.02

Annotation cluster 4	count	EASE-score
GO:0044237 cellular metabolic process	40	0.01
GO:0044238 primary metabolic process	40	0.01
GO:0008152 metabolic process	43	0.05

Annotation cluster 5	count	EASE-score
GO:0007275 multicellular organismal development	36	0.02
GO:0032502 developmental process	37	0.02
GO:0032501 multicellular organismal process	38	0.02

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Table A.2: continued

	count	EASE-score
Annotation cluster 6	count	EASE-score
GO:0000281 cytokinesis after mitosis	3	0
GO:0033205 cytokinesis during cell cycle	3	0
GO:0007049 cell cycle	4	0.34
GO:0022402 cell cycle process	3	0.56
Annotation cluster 7	count	EASE-score
GO:0003006 reproductive developmental process	10	0.02
GO:0040035 hermaphrodite genitalia development	9	0.02
GO:0048806 genitalia development	9	0.03
GO:0007548 sex differentiation	9	0.05
GO:0048513 organ development	10	0.05
GO:0048731 system development	10	0.07
Annotation cluster 8	count	EASE-score
GO:0040032 post-embryonic body morphocountsis	6	0.09
GO:0009886 post-embryonic morphocountsis	6	0.1
GO:0010171 body morphocountsis	6	0.1
Annotation cluster 9	count	EASE-score
GO:0006334 nucleosome assembly	3	0.03
GO:0031497 chromatin assembly	3	0.04
GO:0065004 protein-DNA complex assembly	3	0.07
GO:0006333 chromatin assembly or disassembly	3	0.08
GO:0006325 establishment and/or maintenance of chromatin architecture	3	0.13
GO:0006323 DNA packaging	3	0.13

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Table A.2: continued

	count	EASE-score
GO:0051276 chromosome organization and biocountsis	3	0.25
GO:0065003 macromolecular complex assembly	3	0.25
GO:0022607 cellular component assembly	3	0.32

Annotation cluster 10	count	EASE-score
GO:0006520 amino acid metabolic process	4	0.09
GO:0006082 organic acid metabolic process	5	0.09
GO:0019752 carboxylic acid metabolic process	5	0.09
GO:0006519 amino acid and derivative metabolic process	4	0.13
GO:0009308 amine metabolic process	4	0.18
GO:0006807 nitrogen compound metabolic process	4	0.19

Annotation cluster 11	count	EASE-score
GO:0040010 positive regulation of growth rate	14	0.1
GO:0040009 regulation of growth rate	14	0.11
GO:0048518 positive regulation of biological process	16	0.14
GO:0045927 positive regulation of growth	15	0.14
GO:0040008 regulation of growth	15	0.17
GO:0050789 regulation of biological process	18	0.8

Annotation cluster 12	count	EASE-score
GO:0018987 osmoregulation	4	0.15
GO:0042592 homeostatic process	5	0.15
GO:0048878 chemical homeostasis	4	0.2
GO:0065008 regulation of biological quality	5	0.21
GO:0030104 water homeostasis	3	0.37
GO:0050878 regulation of body fluid levels	3	0.38

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Table A.2: continued

	count	EASE-score
Annotation cluster 13	count	EASE-score
GO:0006281 DNA repair	3	0.24
GO:0006974 response to DNA damage stimulus	3	0.25
GO:0006950 response to stress	4	0.26
GO:0009719 response to endogenous stimulus	3	0.26
Annotation cluster 14	count	EASE-score
GO:0018991 oviposition	4	0.32
GO:0033057 reproductive behavior in a multicellular organism	4	0.32
GO:0019098 reproductive behavior	4	0.33
GO:0032504 multicellular organism reproduction	4	0.34
GO:0048609 reproductive process in a multicellular organism	4	0.34
Annotation cluster 15	count	EASE-score
GO:0007242 intracellular signaling cascade	3	0.81
GO:0007154 cell communication	4	1
GO:0007165 signal transduction	3	1
Annotation cluster 16	count	EASE-score
GO:0043687 post-translational protein modification	4	0.95
GO:0006796 phosphate metabolic process	3	0.98
GO:0006793 phosphorus metabolic process	3	0.98
Annotation cluster 17	count	EASE-score
GO:0006810 transport	7	0.98
GO:0051234 establishment of localization	7	0.99

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Table A.2: continued

	count	EASE-score
GO:0051179 localization	7	0.99

A.2 Post desiccation library

Table A.3: Complete list of all biological process gene ontology (GO) terms from the post desiccation subtractive library of *Panagrolaimus* AF36 and PS1579. 89 genes were annotated with at least one GO term. Term - GO number GO term, Count - number of transcripts annotated with this GO term in the library, % - % of the total number of annotated transcripts in the library.

Term	Count	%	P-value
GO:0009987 cellular process	52	58.43%	0.02
GO:0032501 multicellular organismal process	47	52.81%	4.06E-05
GO:0008152 metabolic process	46	51.69%	0.02
GO:0044238 primary metabolic process	44	49.44%	9.82E-04
GO:0032502 developmental process	44	49.44%	2.25E-04
GO:0007275 multicellular organismal development	43	48.31%	2.07E-04
GO:0044237 cellular metabolic process	41	46.07%	0.01
GO:0009790 embryonic development	40	44.94%	2.20E-07
GO:0009792 embryonic development ending in birth or egg hatching	39	43.82%	4.14E-07
GO:0043170 macromolecule metabolic process	37	41.57%	0.01
GO:0040007 growth	36	40.45%	3.16E-08
GO:0009791 post-embryonic development	32	35.96%	9.16E-08
GO:0000003 reproduction	32	35.96%	1.51E-06
GO:0065007 biological regulation	30	33.71%	0.03
GO:0050789 regulation of biological process	30	33.71%	0.02
GO:0002164 larval development	29	32.58%	9.62E-07
GO:0002119 larval development (sensu Nematoda)	29	32.58%	9.34E-07
GO:0044260 cellular macromolecule metabolic process	28	31.46%	1.87E-04
GO:0019538 protein metabolic process	26	29.21%	0
GO:0044267 cellular protein metabolic process	26	29.21%	8.65E-04

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Table A.3: continued

Term	Count	%	EASE-score
GO:0045927 positive regulation of growth	22	24.72%	0
GO:0009058 biosynthetic process	22	24.72%	4.25E-08
GO:0048518 positive regulation of biological process	22	24.72%	0
GO:0040008 regulation of growth	22	24.72%	0
GO:0040010 positive regulation of growth rate	21	23.60%	4.91E-04
GO:0040009 regulation of growth rate	21	23.60%	5.08E-04
GO:0009059 macromolecule biosynthetic process	19	21.35%	1.16E-10
GO:0044249 cellular biosynthetic process	18	20.22%	5.54E-07
GO:0010467 gene expression	18	20.22%	0.03
GO:0006412 translation	16	17.98%	1.28E-09
GO:0005975 carbohydrate metabolic process	7	7.87%	0.01
GO:0051301 cell division	6	6.74%	0.06
GO:0009308 amine metabolic process	5	5.62%	0.06
GO:0044262 cellular carbohydrate metabolic process	5	5.62%	1.60E-02
GO:0000910 cytokinesis	5	5.62%	0.01
GO:0006807 nitrogen compound metabolic process	5	5.62%	0.07
GO:0043284 biopolymer biosynthetic process	4	4.49%	0
GO:0000074 regulation of progression through cell cycle	3	3.37%	0.09
GO:0006414 translational elongation	3	3.37%	0.01
GO:0007271 synaptic transmission, cholinergic	2	2.25%	0.07

Table A.4: Complete list of all Annotation clusters as computed by DAVID from the post desiccation stress subtractive library of *Panagrolaimus* AF36 and PS1579. GO number GO term, count - number of transcripts annotated with this GO term in the library.

Annotation cluster 1	count	EASE-score
GO:0009791 post-embryonic development	32	9.16E-08
GO:0002119 larval development (sensu Nematoda)	29	9.34E-07
GO:0002164 larval development	29	9.62E-07
Annotation cluster 2	**count**	**EASE-score**
GO:0006412 translation	16	1.28E-09
GO:0044249 cellular biosynthetic process	18	5.54E-07
GO:0010467 count expression	18	0.03
Annotation cluster 3	**count**	**EASE-score**
GO:0009790 embryonic development	40	2.20E-07
GO:0009792 embryonic development ending in birth or egg hatching	39	4.14E-07
GO:0032501 multicellular organismal process	47	4.06E-05
GO:0007275 multicellular organismal development	43	2.07E-04
GO:0032502 developmental process	44	2.25E-04
Annotation cluster 4	**count**	**EASE-score**
GO:0044260 cellular macromolecule metabolic process	28	1.87E-04
GO:0044267 cellular protein metabolic process	26	8.65E-04
GO:0019538 protein metabolic process	26	0
Annotation cluster 5	**count**	**EASE-score**
GO:0040010 positive regulation of growth rate	21	4.91E-04

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Table A.4: continued

	count	EASE-score
GO:0040009 regulation of growth rate	21	5.08E-04
GO:0045927 positive regulation of growth	22	0
GO:0040008 regulation of growth	22	0
GO:0048518 positive regulation of biological process	22	0
Annotation cluster 6	count	EASE-score
GO:0044238 primary metabolic process	44	9.82E-04
GO:0044237 cellular metabolic process	41	0.01
GO:0008152 metabolic process	46	0.02
Annotation cluster 7	count	EASE-score
GO:0009308 amine metabolic process	5	0.06
GO:0006807 nitrogen compound metabolic process	5	0.07
GO:0006519 amino acid and derivative metabolic process	4	0.13
Annotation cluster 8	count	EASE-score
GO:0000074 regulation of progression through cell cycle	3	0.09
GO:0051726 regulation of cell cycle	3	0.12
GO:0022402 cell cycle process	4	0.3
GO:0007049 cell cycle	4	0.36
Annotation cluster 9	count	EASE-score
GO:0030104 water homeostasis	4	0.15
GO:0050878 regulation of body fluid levels	4	0.15
GO:0018987 osmoregulation	4	0.16
GO:0048878 chemical homeostasis	4	0.21
GO:0065008 regulation of biological quality	5	0.22

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Table A.4: continued

	count	EASE-score
GO:0042592 homeostatic process	4	0.36
Annotation cluster 10	count	EASE-score
GO:0040032 post-embryonic body morphocountsis	5	0.24
GO:0009886 post-embryonic morphocountsis	5	0.24
GO:0010171 body morphocountsis	5	0.26
Annotation cluster 11	count	EASE-score
GO:0006082 organic acid metabolic process	4	0.26
GO:0019752 carboxylic acid metabolic process	4	0.26
GO:0006520 amino acid metabolic process	3	0.3
Annotation cluster 12	count	EASE-score
GO:0022414 reproductive process	9	0.28
GO:0040035 hermaphrodite genitalia development	6	0.3
GO:0048806 genitalia development	6	0.31
GO:0048513 organ development	7	0.38
GO:0007548 sex differentiation	6	0.4
GO:0003006 reproductive developmental process	6	0.43
GO:0048731 system development	7	0.45
Annotation cluster 13	count	EASE-score
GO:0007017 microtubule-based process	3	0.31
GO:0007010 cytoskeleton organization and biocountsis	3	0.45
GO:0051649 establishment of cellular localization	4	0.48
GO:0051641 cellular localization	4	0.49
GO:0046907 intracellular transport	3	0.51

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Table A.4: continued

	count	EASE-score
Annotation cluster 14	count	EASE-score
GO:0040018 positive regulation of multicellular organism growth	4	0.53
GO:0035264 multicellular organism growth	4	0.64
GO:0040014 regulation of multicellular organism growth	4	0.64
Annotation cluster 15	count	EASE-score
GO:0006810 transport	13	0.54
GO:0051234 establishment of localization	13	0.62
GO:0051179 localization	13	0.71
Annotation cluster 16	count	EASE-score
GO:0018991 oviposition	3	0.61
GO:0033057 reproductive behavior in a multicellular organism	3	0.61
GO:0019098 reproductive behavior	3	0.62
GO:0032504 multicellular organism reproduction	3	0.63
GO:0048609 reproductive process in a multicellular organism	3	0.63
Annotation cluster 17	count	EASE-score
GO:0019219 regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	3	0.99
GO:0031323 regulation of cellular metabolic process	3	0.99
GO:0019222 regulation of metabolic process	3	1

A.3 Osmotic stress library

Table A.5: Complete list of all biological process gene ontology (GO) terms from the osmotic stress subtractive library of *Panagrolaimus* AF36 and PS1579. 65 genes were annotated with at least one GO term. Term - GO number GO term, Count - number of transcripts annotated with this GO term in the library, % - % of the total number of annotated transcripts in the library.

Term	Count	%	EASE-score
Term	Count	%	PValue
GO:0009987 cellular process	27	41.54%	0.06
GO:0032502 developmental process	25	38.46%	5.77E-04
GO:0032501 multicellular organismal process	25	38.46%	0
GO:0007275 multicellular organismal development	24	36.92%	9.94E-04
GO:0044237 cellular metabolic process	21	32.31%	0.05
GO:0009790 embryonic development	19	29.23%	0
GO:0009792 embryonic development ending in birth or egg hatching	18	27.69%	0
GO:0043170 macromolecule metabolic process	18	27.69%	0.09
GO:0000003 reproduction	15	23.08%	0
GO:0044260 cellular macromolecule metabolic process	14	21.54%	0.01
GO:0044267 cellular protein metabolic process	14	21.54%	0.01
GO:0019538 protein metabolic process	14	21.54%	0.01
GO:0040007 growth	13	20.00%	0.04
GO:0010467 gene expression	11	16.92%	0.03
GO:0044249 cellular biosynthetic process	8	12.31%	0
GO:0009058 biosynthetic process	8	12.31%	0.02
GO:0006412 translation	7	10.77%	4.95E-04
GO:0007276 gamete generation	7	10.77%	0.04
GO:0009059 macromolecule biosynthetic process	7	10.77%	0

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Table A.5: continued

Term	Count	%	EASE-score
GO:0019953 sexual reproduction	7	10.77%	0.05
GO:0048869 cellular developmental process	5	7.69%	0.05
GO:0030154 cell differentiation	5	7.69%	0.05
GO:0048468 cell development	4	6.15%	0.06

Table A.6: Complete list of all Annotation clusters as computed by DAVID from the osmotic stress subtractive library of *Panagrolaimus* AF36 and PS1579. GO number GO term, count - number of transcripts annotated with this GO term in the library.

Annotation cluster 1	count	EASE-score
GO:0032502 developmental process	25	5.77E-04
GO:0007275 multicellular organismal development	24	9.94E-04
GO:0032501 multicellular organismal process	25	0

Annotation cluster 2	count	EASE-score
GO:0006412 translation	7	4.95E-04
GO:0009059 macromolecule biosynthetic process	7	0
GO:0044249 cellular biosynthetic process	8	0
GO:0009058 biosynthetic process	8	0.02

Annotation cluster3	count	EASE-score
GO:0044267 cellular protein metabolic process	14	0.01
GO:0044260 cellular macromolecule metabolic process	14	0.01
GO:0019538 protein metabolic process	14	0.01

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Table A.6: continued

	count	EASE-score
Annotation cluster 4	count	EASE-score
GO:0030154 cell differentiation	5	0.05
GO:0048869 cellular developmental process	5	0.05
GO:0048468 cell development	4	0.06
Annotation cluster 5	count	EASE-score
GO:0044237 cellular metabolic process	21	0.05
GO:0043170 macromolecule metabolic process	18	0.09
GO:0044238 primary metabolic process	19	0.18
GO:0008152 metabolic process	22	0.19
Annotation cluster 6	count	EASE-score
GO:0009791 post-embryonic development	10	0.13
GO:0002119 larval development (sensu Nematoda)	8	0.31
GO:0002164 larval development	8	0.31
Annotation cluster 7	count	EASE-score
GO:0040008 regulation of growth	9	0.17
GO:0040010 positive regulation of growth rate	7	0.31
GO:0040009 regulation of growth rate	7	0.31
GO:0048518 positive regulation of biological process	8	0.35
GO:0045927 positive regulation of growth	7	0.45
Annotation cluster 8	count	EASE-score
GO:0040008 regulation of growth	9	0.17
GO:0050789 regulation of biological process	11	0.58
GO:0065007 biological regulation	11	0.66

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Table A.6: continued

	count	EASE-score
Annotation cluster 9	count	EASE-score
GO:0009653 anatomical structure morphocountsis	4	0.48
GO:0040014 regulation of multicellular organism growth	3	0.49
GO:0035264 multicellular organism growth	3	0.49
Annotation cluster 10	count	EASE-score
GO:0006810 transport	6	0.73
GO:0051234 establishment of localization	6	0.78
GO:0051179 localization	6	0.83

A.4 Post osmotic stress library

Table A.7: Complete list of all biological process gene ontology (GO) terms from the post osmotic stress subtractive library of *Panagrolaimus* AF36 and PS1579. 59 genes were annotated with at least one GO term. Term - GO number GO term, Count - number of transcripts annotated with this GO term in the library, % - % of the total number of annotated transcripts in the library.

Term	Count	%	EASE-score
GO:0008152 metabolic process	43	72.88%	3.76E-07
GO:0009987 cellular process	37	62.71%	0.04
GO:0044238 primary metabolic process	36	61.02%	3.54E-05
GO:0044237 cellular metabolic process	33	55.93%	7.22E-04
GO:0032502 developmental process	30	50.85%	0.01
GO:0043170 macromolecule metabolic process	30	50.85%	9.07E-04
GO:0007275 multicellular organismal development	29	49.15%	0.01
GO:0032501 multicellular organismal process	29	49.15%	0.02
GO:0009792 embryonic development ending in birth or egg hatching	26	44.07%	1.51E-04
GO:0009790 embryonic development	26	44.07%	2.23E-04
GO:0000003 reproduction	22	37.29%	1.31E-04
GO:0009791 post-embryonic development	21	35.59%	7.37E-05
GO:0002164 larval development	20	33.90%	8.80E-05
GO:0002119 larval development (sensu Nematoda)	20	33.90%	8.63E-05
GO:0019538 protein metabolic process	20	33.90%	0
GO:0040007 growth	20	33.90%	0
GO:0043283 biopolymer metabolic process	19	32.20%	0.06
GO:0044260 cellular macromolecule metabolic process	17	28.81%	0.02
GO:0044267 cellular protein metabolic process	16	27.12%	0.04

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Table A.7: continued

Term	Count	%	EASE-score
GO:0006139 nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	15	25.42%	0.05
GO:0009058 biosynthetic process	14	23.73%	6.92E-05
GO:0048518 positive regulation of biological process	13	22.03%	0.1
GO:0044249 cellular biosynthetic process	13	22.03%	2.38E-05
GO:0016043 cellular component organization and biogenesis	11	18.64%	0.01
GO:0022414 reproductive process	10	16.95%	0.03
GO:0006812 cation transport	10	16.95%	7.33E-05
GO:0006811 ion transport	10	16.95%	0.01
GO:0006259 DNA metabolic process	9	15.25%	2.32E-04
GO:0009059 macromolecule biosynthetic process	9	15.25%	5.78E-04
GO:0006996 organelle organization and biogenesis	8	13.56%	0.01
GO:0006412 translation	8	13.56%	5.38E-04
GO:0006091 generation of precursor metabolites and energy	6	10.17%	0.06
GO:0051301 cell division	6	10.17%	0.01
GO:0051276 chromosome organization and biogenesis	6	10.17%	5.95E-04
GO:0065004 protein-DNA complex assembly	5	8.47%	2.19E-04
GO:0065003 macromolecular complex assembly	5	8.47%	0.01
GO:0040016 embryonic cleavage	5	8.47%	0.02
GO:0006333 chromatin assembly or disassembly	5	8.47%	3.29E-04
GO:0006325 establishment and/or maintenance of chromatin architecture	5	8.47%	9.83E-04
GO:0006323 DNA packaging	5	8.47%	9.83E-04
GO:0009056 catabolic process	5	8.47%	0.05
GO:0022607 cellular component assembly	5	8.47%	0.01

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Table A.7: continued

Term	Count	%	EASE-score
GO:0006754 ATP biosynthetic process	4	6.78%	0
GO:0006753 nucleoside phosphate metabolic process	4	6.78%	0
GO:0006732 coenzyme metabolic process	4	6.78%	0.03
GO:0051188 cofactor biosynthetic process	4	6.78%	0.02
GO:0051186 cofactor metabolic process	4	6.78%	0.06
GO:0031497 chromatin assembly	4	6.78%	0
GO:0009206 purine ribonucleoside triphosphate biosynthetic process	4	6.78%	0
GO:0009205 purine ribonucleoside triphosphate metabolic process	4	6.78%	0
GO:0009201 ribonucleoside triphosphate biosynthetic process	4	6.78%	0
GO:0009199 ribonucleoside triphosphate metabolic process	4	6.78%	0
GO:0015992 proton transport	4	6.78%	0
GO:0015986 ATP synthesis coupled proton transport	4	6.78%	0
GO:0006164 purine nucleotide biosynthetic process	4	6.78%	0.01
GO:0006163 purine nucleotide metabolic process	4	6.78%	0.01
GO:0009165 nucleotide biosynthetic process	4	6.78%	0.03
GO:0043285 biopolymer catabolic process	4	6.78%	0.02
GO:0009152 purine ribonucleotide biosynthetic process	4	6.78%	0.01
GO:0006119 oxidative phosphorylation	4	6.78%	0.01
GO:0009150 purine ribonucleotide metabolic process	4	6.78%	0.01
GO:0009145 purine nucleoside triphosphate biosynthetic process	4	6.78%	0
GO:0009144 purine nucleoside triphosphate metabolic process	4	6.78%	0

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Table A.7: continued

Term	Count	%	EASE-score
GO:0009142 nucleoside triphosphate biosynthetic process	4	6.78%	0
GO:0009141 nucleoside triphosphate metabolic process	4	6.78%	0
GO:0046034 ATP metabolic process	4	6.78%	0
GO:0009117 nucleotide metabolic process	4	6.78%	0.05
GO:0009108 coenzyme biosynthetic process	4	6.78%	0.01
GO:0030163 protein catabolic process	4	6.78%	0.01
GO:0055086 nucleobase, nucleoside and nucleotide metabolic process	4	6.78%	0.06
GO:0006334 nucleosome assembly	4	6.78%	9.37E-04
GO:0006818 hydrogen transport	4	6.78%	0
GO:0009057 macromolecule catabolic process	4	6.78%	0.04
GO:0009260 ribonucleotide biosynthetic process	4	6.78%	0.01
GO:0009259 ribonucleotide metabolic process	4	6.78%	0.01
GO:0006260 DNA replication	3	5.08%	0.07
GO:0048522 positive regulation of cellular process	3	5.08%	0.07
GO:0051656 establishment of organelle localization	3	5.08%	0.09
GO:0051640 organelle localization	3	5.08%	0.09

Table A.8: Complete list of all Annotation clusters as computed by DAVID from the post osmotic stress subtractive library of *Panagrolaimus* AF36 and PS1579. GO number GO term, count - number of transcripts annotated with this GO term in the library.

Annotation cluster 1	count	EASE-score
GO:0009791 post-embryonic development	21	7.37E-05

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Table A.8: continued

	count	EASE-score
GO:0002119 larval development (sensu Nematoda)	20	8.63E-05
GO:0002164 larval development	20	8.80E-05
GO:0040007 growth	20	0
Annotation cluster 2	count	EASE-score
GO:0065004 protein-DNA complex assembly	5	2.19E-04
GO:0006333 chromatin assembly or disassembly	5	3.29E-04
GO:0051276 chromosome organization and bioAnnotation clustersis	6	5.95E-04
GO:0006334 nucleosome assembly	4	9.37E-04
GO:0006323 DNA packaging	5	9.83E-04
GO:0006325 establishment and/or maintenance of chromatin architecture	5	9.83E-04
GO:0031497 chromatin assembly	4	0
GO:0065003 macromolecular complex assembly	5	0.01
GO:0022607 cellular component assembly	5	0.01
Annotation cluster 3	count	EASE-score
GO:0009792 embryonic development ending in birth or egg hatching	26	1.51E-04
GO:0009790 embryonic development	26	2.23E-04
GO:0032502 developmental process	30	0.01
GO:0007275 multicellular organismal development	29	0.01
GO:0032501 multicellular organismal process	29	0.02
Annotation cluster 4	count	EASE-score
GO:0006812 cation transport	10	7.33E-05
GO:0006811 ion transport	10	0.01
GO:0006810 transport	11	0.32

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Table A.8: continued

	count	EASE-score
Annotation cluster 5	count	EASE-score
GO:0015986 ATP synthesis coupled proton transport	4	0
GO:0006754 ATP biosynthetic process	4	0
GO:0006753 nucleoside phosphate metabolic process	4	0
GO:0046034 ATP metabolic process	4	0
GO:0009206 purine ribonucleoside triphosphate biosynthetic process	4	0
GO:0009201 ribonucleoside triphosphate biosynthetic process	4	0
GO:0009145 purine nucleoside triphosphate biosynthetic process	4	0
GO:0009205 purine ribonucleoside triphosphate metabolic process	4	0
GO:0009199 ribonucleoside triphosphate metabolic process	4	0
GO:0009142 nucleoside triphosphate biosynthetic process	4	0
GO:0009144 purine nucleoside triphosphate metabolic process	4	0
GO:0009141 nucleoside triphosphate metabolic process	4	0
GO:0006818 hydrogen transport	4	0
GO:0015992 proton transport	4	0
GO:0009152 purine ribonucleotide biosynthetic process	4	0.01
GO:0009150 purine ribonucleotide metabolic process	4	0.01
GO:0009260 ribonucleotide biosynthetic process	4	0.01
GO:0006164 purine nucleotide biosynthetic process	4	0.01
GO:0009259 ribonucleotide metabolic process	4	0.01
GO:0006163 purine nucleotide metabolic process	4	0.01
GO:0006119 oxidative phosphorylation	4	0.01
GO:0009108 coenzyme biosynthetic process	4	0.01
GO:0051188 cofactor biosynthetic process	4	0.02
GO:0009165 nucleotide biosynthetic process	4	0.03
GO:0006732 coenzyme metabolic process	4	0.03
GO:0009117 nucleotide metabolic process	4	0.05

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Table A.8: continued

	count	EASE-score
GO:0051186 cofactor metabolic process	4	0.06
GO:0055086 nucleobase, nucleoside and nucleotide metabolic process	4	0.06
GO:0015672 monovalent inorganic cation transport	4	0.15
GO:0016310 phosphorylation	4	0.61
GO:0006796 phosphate metabolic process	4	0.77
GO:0006793 phosphorus metabolic process	4	0.77

Annotation cluster 6	count	EASE-score
GO:0030163 protein catabolic process	4	0.01
GO:0043285 biopolymer catabolic process	4	0.02
GO:0009057 macromolecule catabolic process	4	0.04
GO:0009056 catabolic process	5	0.05

Annotation cluster 7	count	EASE-score
GO:0040035 hermaphrodite genitalia development	6	0.11
GO:0048806 genitalia development	6	0.12
GO:0007548 sex differentiation	6	0.16
GO:0003006 reproductive developmental process	6	0.18
GO:0048513 organ development	6	0.27
GO:0048731 system development	6	0.32

Annotation cluster 8	count	EASE-score
GO:0048518 positive regulation of biological process	13	0.1
GO:0045927 positive regulation of growth	12	0.12
GO:0040008 regulation of growth	12	0.14
GO:0040010 positive regulation of growth rate	10	0.19
GO:0040009 regulation of growth rate	10	0.19

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Table A.8: continued

	count	EASE-score
GO:0065007 biological regulation	16	0.55
GO:0050789 regulation of biological process	15	0.58
Annotation cluster 9	**count**	**EASE-score**
GO:0051234 establishment of localization	13	0.16
GO:0051179 localization	13	0.21
GO:0006810 transport	11	0.32
Annotation cluster 10	**count**	**EASE-score**
GO:0008219 cell death	3	0.12
GO:0016265 death	3	0.12
GO:0048468 cell development	3	0.37
GO:0030154 cell differentiation	3	0.58
GO:0048869 cellular developmental process	3	0.58
Annotation cluster 11	**count**	**EASE-score**
GO:0051656 establishment of organelle localization	3	0.09
GO:0051640 organelle localization	3	0.09
GO:0051649 establishment of cellular localization	3	0.55
GO:0051641 cellular localization	3	0.56
GO:0019953 sexual reproduction	3	0.9
Annotation cluster 12	**count**	**EASE-score**
GO:0040014 regulation of multicellular organism growth	4	0.4
GO:0035264 multicellular organism growth	4	0.4
GO:0040018 positive regulation of multicellular organism growth	3	0.58

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Table A.8: continued

	count	EASE-score
Annotation cluster 13	count	EASE-score
GO:0040032 post-embryonic body morphoAnnotation clustersis	3	0.53
GO:0009886 post-embryonic morphoAnnotation clustersis	3	0.53
GO:0010171 body morphoAnnotation clustersis	3	0.54
GO:0009653 anatomical structure morphoAnnotation clustersis	3	0.89
Annotation cluster 14	count	EASE-score
GO:0010468 regulation of Annotation cluster expression	4	0.89
GO:0006355 regulation of transcription, DNA-dependent	3	0.94
GO:0006351 transcription, DNA-dependent	3	0.94
GO:0032774 RNA biosynthetic process	3	0.95
GO:0045449 regulation of transcription	3	0.96
GO:0019219 regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	3	0.96
GO:0031323 regulation of cellular metabolic process	3	0.96
GO:0006350 transcription	3	0.97
GO:0019222 regulation of metabolic process	3	0.97
GO:0016070 RNA metabolic process	3	0.99

Appendix B

Similarity results

```
Query=  SL1_15307_A01_seqqqq_q (Untrimmed) Agencourt Bioscience Corporation ABI
        (860 letters)

  Translating both strands of query sequence in all 6 reading frames

Database:  Caenorhabditis_elegans.CEL160.42.pep.all.fa
           26,439 sequences; 11,641,465 total letters.
Searching....10....20....30....40....50....60....70....80....90....100% done

                                                                      Smallest
                                                                        Sum
                                                                High  Probability
Sequences producing High-scoring Segment Pairs:                 Score  P(N)      N


      *** NONE ***
```

Table B.1: Similarity analysis of the sequences in the subtractive libearies - The cloned trascripts were translated into amino acid sequences using all 6 reading frames and compared to the virtual translated cDNA library of *C. elegans* using Ensembl (http://www.ensembl.org/Caenorhabditis-elegans/index.html) [92]. SL1-15307-A01-seqqqq-q represents the clone in the library, Score respresents the conservation score and P(N) respresents the E-value, HSP stands for the High scoring sequence.

```
Query=  SL1_15307_A02_seqqqq_q (Untrimmed) Agencourt Bioscience Corporation ABI
        (833 letters)

  Translating both strands of query sequence in all 6 reading frames

Database:  Caenorhabditis_elegans.CEL160.42.pep.all.fa
           26,439 sequences; 11,641,465 total letters.
Searching....10....20....30....40....50....60....70....80....90....100% done

                                                                      Smallest
                                                                        Sum
                                                                High  Probability
Sequences producing High-scoring Segment Pairs:                 Score  P(N)      N

R10E11.2.1 pep:known chromosome:CEL160:III:9782053:978311...      143  2.2e-10   1
R10E11.2.2 pep:known chromosome:CEL160:III:9782060:978311...      143  2.2e-10   1
Y38F2AL.4 pep:known chromosome:CEL160:IV:2314588:2315987:...      143  2.2e-10   1
R10E11.8.3 pep:known chromosome:CEL160:III:9781139:978176...      132  4.5e-09   1
R10E11.8.1 pep:known chromosome:CEL160:III:9781148:978195...      132  4.5e-09   1
R10E11.8.2 pep:known chromosome:CEL160:III:9781150:978176...      132  4.5e-09   1



>R10E11.2.1 pep:known chromosome:CEL160:III:9782053:9783119:1 gene:R10E11.2
            transcript:R10E11.2.1
        Length = 161

  Minus Strand HSPs:

 Score = 143 (55.4 bits), Expect = 2.2e-10, P = 2.2e-10
 Identities = 30/35 (85%), Positives = 31/35 (88%), Frame = -3

Query:   336 GTTQQPRLFVGMILSLIFCEVLGLYGMIVALILSS 232
            GT QQPRLFVGMIL LIF EVLGLYGMIVALIL +
Sbjct:   126 GTAQQPRLFVGMILILIFSEVLGLYGMIVALILGT 160
```

Appendix C

List of overlapping sequences

```
  Number of segment pairs = 718256; number of pairwise comparisons = 280543
'+' means given segment; '-' means reverse complement

Overlaps            Containments  No. of Constraints Supporting Overlap

******************** Contig 1 ********************
SL1_15307_A06.seq+
SL1_15307_A05.seq+
******************** Contig 2 ********************
SL1_15307_B02.seq+
SL1_15307_A08.seq+
******************** Contig 3 ********************
SL1_15307_B04.seq+
                    SL1_15307_A04.seq+ is in SL1_15307_B04.seq+
******************** Contig 4 ********************
SL1_15307_B11.seq+
                    SL3_15307_G12.seq+ is in SL1_15307_B11.seq+
******************** Contig 5 ********************
SL1_15307_C03.seq+
SL1_15307_C08.seq+
******************** Contig 6 ********************
SL1_15307_C06.seq+
SL5_M13F_E06.seq+
```

Table C.1: List of overlapping sequences. Contig - number of overlapping sequence (Contig 1 - Overlapping sequence 1), SL1-15307-A06.seq represents location of the clone containing the sequence in the library, + - - reading direction,

```
                    SL5_M13F_H07.seq+ is in SL5_M13F_E06.seq+
                    SL8_M13F_D07.seq+ is in SL5_M13F_H07.seq+
                    SL8_M13F_H12.seq+ is in SL8_M13F_D07.seq+
SL4_15307_D06.seq-
SL4_15307_F03.seq-
****************** Contig 7 ******************
SL1_15307_C12.seq+
SL1_15307_B12.seq+
****************** Contig 8 ******************
SL1_15307_E09.seq+
SL5_M13F_E11.seq+
****************** Contig 9 ******************
SL1_15307_F02.seq+
                    SL7_M13F_G07.seq+ is in SL1_15307_F02.seq+
SL3_15307_A11.seq+
****************** Contig 10 ******************
SL1_15307_F09.seq+
SL3_15307_D08.seq+
****************** Contig 11 ******************
SL1_15307_G02.seq+
                    SL3_15307_H11.seq+ is in SL1_15307_G02.seq+
SL1_15307_G07.seq+
SL1_15307_G05.seq+
****************** Contig 12 ******************
SL1_15307_G06.seq+
                    SL1_15307_G04.seq+ is in SL1_15307_G06.seq+
****************** Contig 13 ******************
SL1_15307_H01.seq+
                    SL1_15307_H02.seq+ is in SL1_15307_H01.seq+
****************** Contig 14 ******************
SL1_15307_H03.seq+
SL1_15307_H04.seq+
SL3_15307_G05.seq+
****************** Contig 15 ******************
SL1_15307_H09.seq+
                    SL2_15307_B10.seq+ is in SL1_15307_H09.seq+
****************** Contig 16 ******************
SL2_15307_A01.seq+
                    SL2_15307_B05.seq+ is in SL2_15307_A01.seq+
                    SL2_15307_C12.seq+ is in SL2_15307_A01.seq+
****************** Contig 17 ******************
SL2_15307_A05.seq+
SL2_15307_C06.seq+
****************** Contig 18 ******************
SL2_15307_B06.seq+
                    SL2_15307_D02.seq+ is in SL2_15307_B06.seq+
****************** Contig 19 ******************
SL2_15307_D06.seq+
                    SL2_15307_C03.seq+ is in SL2_15307_D06.seq+
****************** Contig 20 ******************
SL2_15307_E05.seq+
```

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                    SL2_15307_F05.seq+ is in SL2_15307_E05.seq+
****************** Contig 21 ********************
SL2_15307_E07.seq+
                    SL2_15307_E08.seq+ is in SL2_15307_E07.seq+
****************** Contig 22 ********************
SL2_15307_F04.seq+
SL2_15307_F10.seq+
****************** Contig 23 ********************
SL2_15307_F11.seq+
SL5_M13F_F09.seq+
****************** Contig 24 ********************
SL2_15307_H05.seq+
SL3_15307_A07.seq+
****************** Contig 25 ********************
SL2_15307_H07.seq+
                    SL2_15307_E09.seq+ is in SL2_15307_H07.seq+
****************** Contig 26 ********************
SL2_15307_H10.seq+
SL3_15307_C03.seq+
****************** Contig 27 ********************
SL3_15307_A03.seq+
                    SL3_15307_A04.seq+ is in SL3_15307_A03.seq+
                    SL3_15307_A12.seq+ is in SL3_15307_A04.seq+
                    SL3_15307_B10.seq+ is in SL3_15307_A04.seq+
****************** Contig 28 ********************
SL3_15307_B05.seq+
SL2_15307_G07.seq+
****************** Contig 29 ********************
SL3_15307_B08.seq+
                    SL3_15307_B03.seq+ is in SL3_15307_B08.seq+
****************** Contig 30 ********************
SL3_15307_C08.seq+
                    SL3_15307_C09.seq+ is in SL3_15307_C08.seq+
****************** Contig 31 ********************
SL3_15307_C11.seq+
                    SL4_15307_G03.seq+ is in SL3_15307_C11.seq+
                    SL4_15307_H12.seq+ is in SL3_15307_C11.seq+
                    SL1_15307_D02.seq+ is in SL3_15307_C11.seq+
                    SL3_15307_E12.seq+ is in SL3_15307_C11.seq+
SL1_15307_D09.seq+
****************** Contig 32 ********************
SL3_15307_D01.seq+
SL1_15307_G09.seq+
****************** Contig 33 ********************
SL3_15307_D02.seq+
SL3_15307_F05.seq+
                    SL3_15307_H09.seq+ is in SL3_15307_F05.seq+
****************** Contig 34 ********************
SL3_15307_D06.seq+
SL3_15307_F01.seq+
****************** Contig 35 ********************
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SL3_15307_D10.seq+
                    SL1_15307_E04.seq+ is in SL3_15307_D10.seq+
******************** Contig 36 ********************
SL3_15307_E03.seq+
SL1_15307_D01.seq+
******************** Contig 37 ********************
SL3_15307_E06.seq+
                    SL3_15307_E07.seq+ is in SL3_15307_E06.seq+
                    SL2_15307_D12.seq+ is in SL3_15307_E07.seq+
                    SL3_15307_E04.seq+ is in SL3_15307_E06.seq+
******************** Contig 38 ********************
SL3_15307_F09.seq+
                    SL3_15307_D11.seq+ is in SL3_15307_F09.seq+
******************** Contig 39 ********************
SL3_15307_G01.seq+
SL3_15307_F07.seq+
******************** Contig 40 ********************
SL3_15307_H02.seq+
SL1_15307_D12.seq+
******************** Contig 41 ********************
SL3_15307_H06.seq+
                    SL3_15307_H12.seq+ is in SL3_15307_H06.seq+
******************** Contig 42 ********************
SL3_15307_H10.seq+
                    SL3_15307_E01.seq+ is in SL3_15307_H10.seq+
******************** Contig 43 ********************
SL4_15307_A03.seq+
SL4_15307_F02.seq+
                    SL4_15307_A11.seq+ is in SL4_15307_F02.seq+
SL1_15307_F05.seq+
                    SL4_15307_F05.seq+ is in SL1_15307_F05.seq+
                    SL6_M13F_C09.seq+ is in SL1_15307_F05.seq+
                    SL6_M13F_C11.seq+ is in SL1_15307_F05.seq+
******************** Contig 44 ********************
SL4_15307_A04.seq+
                    SL1_15307_D05.seq+ is in SL4_15307_A04.seq+
SL1_15307_C04.seq+
                    SL1_15307_E11.seq+ is in SL1_15307_C04.seq+
******************** Contig 45 ********************
SL4_15307_A08.seq+
SL6_M13F_A08.seq+
******************** Contig 46 ********************
SL4_15307_B01.seq+
SL4_15307_D08.seq+
                    SL4_15307_B05.seq+ is in SL4_15307_D08.seq+
SL6_M13F_E08.seq+
SL6_M13F_A07.seq-
                    SL4_15307_A12.seq- is in SL6_M13F_A07.seq-
******************** Contig 47 ********************
SL4_15307_B06.seq+
SL6_M13F_E04.seq+
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****************** Contig 48 ******************
SL4_15307_B07.seq+
SL6_M13F_D07.seq+
****************** Contig 49 ******************
SL4_15307_B10.seq+
SL4_15307_B09.seq+
                   SL6_M13F_D12.seq+ is in SL4_15307_B09.seq+
****************** Contig 50 ******************
SL4_15307_C04.seq+
SL6_M13F_F01.seq+
****************** Contig 51 ******************
SL4_15307_C05.seq+
                   SL4_15307_H11.seq+ is in SL4_15307_C05.seq+
****************** Contig 52 ******************
SL4_15307_C06.seq+
SL6_M13F_G07.seq+
****************** Contig 53 ******************
SL4_15307_C10.seq+
                   SL4_15307_G05.seq+ is in SL4_15307_C10.seq+
****************** Contig 54 ******************
SL4_15307_C11.seq+
                   SL4_15307_E03.seq+ is in SL4_15307_C11.seq+
****************** Contig 55 ******************
SL4_15307_D09.seq+
                   SL4_15307_D10.seq+ is in SL4_15307_D09.seq+
****************** Contig 56 ******************
SL4_15307_E02.seq+
                   SL4_15307_F01.seq+ is in SL4_15307_E02.seq+
****************** Contig 57 ******************
SL4_15307_E04.seq+
                   SL4_15307_F10.seq+ is in SL4_15307_E04.seq+
****************** Contig 58 ******************
SL4_15307_E10.seq+
SL4_15307_C02.seq+
****************** Contig 59 ******************
SL4_15307_E12.seq+
SL4_15307_E11.seq+
****************** Contig 60 ******************
SL4_15307_G01.seq+
                   SL6_M13F_A11.seq+ is in SL4_15307_G01.seq+
                   SL6_M13F_A12.seq+ is in SL6_M13F_A11.seq+
                   SL3_15307_F12.seq+ is in SL4_15307_G01.seq+
                   SL4_15307_A10.seq+ is in SL4_15307_G01.seq+
SL6_M13F_B08.seq+
                   SL6_M13F_A04.seq+ is in SL6_M13F_B08.seq+
****************** Contig 61 ******************
SL4_15307_G09.seq+
                   SL4_15307_E07.seq+ is in SL4_15307_G09.seq+
SL6_M13F_G06.seq+
SL6_M13F_D04.seq+
                   SL4_15307_H09.seq+ is in SL6_M13F_D04.seq+
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****************** Contig 62 ******************
SL4_15307_H02.seq+
SL6_M13F_B05.seq+
****************** Contig 63 ******************
SL4_15307_H07.seq+
                    SL6_M13F_F08.seq+ is in SL4_15307_H07.seq+
SL4_15307_B04.seq+
****************** Contig 64 ******************
SL5_M13F_A07.seq+
                    SL7_M13F_B12.seq+ is in SL5_M13F_A07.seq+
****************** Contig 65 ******************
SL5_M13F_A09.seq+
SL5_M13F_D05.seq+
SL7_M13F_B06.seq+
SL7_M13F_C02.seq+
                    SL8_M13F_F03.seq+ is in SL7_M13F_C02.seq+
                    SL9_M13F_A05.seq+ is in SL8_M13F_F03.seq+
                    SL8_M13F_E08.seq+ is in SL8_M13F_F03.seq+
                    SL9_M13F_A03.seq+ is in SL8_M13F_F03.seq+
                    SL9_M13F_D02.seq+ is in SL7_M13F_C02.seq+
                    SL5_M13F_G07.seq+ is in SL7_M13F_C02.seq+
                    SL7_M13F_C11.seq+ is in SL7_M13F_C02.seq+
                    SL5_M13F_A01.seq+ is in SL7_M13F_C02.seq+
                    SL9_M13F_D12.seq+ is in SL7_M13F_C02.seq+
                    SL5_M13F_B11.seq+ is in SL7_M13F_C02.seq+
SL5_M13F_B08.seq-
SL5_M13F_G01.seq-
SL4_15307_D11.seq-
SL1_15307_G01.seq-
                    SL4_15307_F04.seq- is in SL1_15307_G01.seq-
                    SL1_15307_F08.seq- is in SL1_15307_G01.seq-
****************** Contig 66 ******************
SL5_M13F_A12.seq+
SL5_M13F_F01.seq+
****************** Contig 67 ******************
SL5_M13F_B01.seq+
SL5_M13F_F03.seq+
****************** Contig 68 ******************
SL5_M13F_B12.seq+
                    SL5_M13F_C12.seq+ is in SL5_M13F_B12.seq+
****************** Contig 69 ******************
SL5_M13F_C06.seq+
SL5_M13F_F07.seq+
****************** Contig 70 ******************
SL5_M13F_D01.seq+
SL5_M13F_D08.seq+
****************** Contig 71 ******************
SL5_M13F_D04.seq+
                    SL5_M13F_H08.seq+ is in SL5_M13F_D04.seq+
****************** Contig 72 ******************
SL5_M13F_D09.seq+
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                    SL5_M13F_E09.seq+ is in SL5_M13F_D09.seq+
****************** Contig 73 ********************
SL5_M13F_D10.seq+
SL5_M13F_B07.seq+
SL5_M13F_D11.seq+
                    SL5_M13F_E12.seq+ is in SL5_M13F_D11.seq+
                    SL5_M13F_D12.seq+ is in SL5_M13F_E12.seq+
****************** Contig 74 ********************
SL5_M13F_E04.seq+
                    SL5_M13F_F12.seq+ is in SL5_M13F_E04.seq+
****************** Contig 75 ********************
SL5_M13F_F11.seq+
                    SL5_M13F_F10.seq+ is in SL5_M13F_F11.seq+
****************** Contig 76 ********************
SL5_M13F_G03.seq+
SL8_M13F_C03.seq+
****************** Contig 77 ********************
SL5_M13F_G05.seq+
                    SL5_M13F_A10.seq+ is in SL5_M13F_G05.seq+
****************** Contig 78 ********************
SL5_M13F_H06.seq+
                    SL5_M13F_G11.seq+ is in SL5_M13F_H06.seq+
****************** Contig 79 ********************
SL6_M13F_A10.seq+
SL6_M13F_D10.seq+
****************** Contig 80 ********************
SL6_M13F_B06.seq+
                    SL6_M13F_F02.seq+ is in SL6_M13F_B06.seq+
****************** Contig 81 ********************
SL6_M13F_B10.seq+
                    SL6_M13F_C05.seq+ is in SL6_M13F_B10.seq+
SL6_M13F_B01.seq-
SL4_15307_F07.seq-
****************** Contig 82 ********************
SL6_M13F_D02.seq+
SL6_M13F_B11.seq+
****************** Contig 83 ********************
SL6_M13F_D03.seq+
SL6_M13F_F04.seq+
****************** Contig 84 ********************
SL6_M13F_E09.seq+
                    SL6_M13F_E02.seq+ is in SL6_M13F_E09.seq+
SL6_M13F_E06.seq+
****************** Contig 85 ********************
SL6_M13F_E12.seq+
SL6_M13F_E05.seq+
****************** Contig 86 ********************
SL6_M13F_F12.seq+
SL4_15307_E05.seq+
SL4_15307_B02.seq+
SL6_M13F_A05.seq+
```

```
                    SL2_15307_D05.seq+ is in SL6_M13F_A05.seq+
                    SL9_M13F_G02.seq+ is in SL6_M13F_A05.seq+
                    SL7_M13F_F07.seq+ is in SL6_M13F_A05.seq+
****************** Contig 87 ******************
SL6_M13F_G03.seq+
                    SL6_M13F_F03.seq+ is in SL6_M13F_G03.seq+
SL6_M13F_G04.seq+
****************** Contig 88 ******************
SL6_M13F_G09.seq+
SL8_M13F_H10.seq+
SL8_M13F_D09.seq+
****************** Contig 89 ******************
SL6_M13F_H03.seq+
                    SL8_M13F_D12.seq+ is in SL6_M13F_H03.seq+
SL8_M13F_G07.seq-
****************** Contig 90 ******************
SL6_M13F_H07.seq+
SL8_M13F_B02.seq+
                    SL5_M13F_F02.seq+ is in SL8_M13F_B02.seq+
                    SL7_M13F_A02.seq+ is in SL5_M13F_F02.seq+
****************** Contig 91 ******************
SL6_M13F_H08.seq+
                    SL8_M13F_A07.seq+ is in SL6_M13F_H08.seq+
****************** Contig 92 ******************
SL7_M13F_A01.seq+
SL5_M13F_E08.seq+
****************** Contig 93 ******************
SL7_M13F_A09.seq+
SL5_M13F_F04.seq+
****************** Contig 94 ******************
SL7_M13F_B03.seq+
SL7_M13F_B02.seq+
****************** Contig 95 ******************
SL7_M13F_B08.seq+
                    SL7_M13F_E05.seq+ is in SL7_M13F_B08.seq+
****************** Contig 96 ******************
SL7_M13F_B09.seq+
                    SL7_M13F_E02.seq+ is in SL7_M13F_B09.seq+
****************** Contig 97 ******************
SL7_M13F_C10.seq+
SL7_M13F_C04.seq+
****************** Contig 98 ******************
SL7_M13F_E10.seq+
                    SL7_M13F_A07.seq+ is in SL7_M13F_E10.seq+
****************** Contig 99 ******************
SL7_M13F_E12.seq+
SL7_M13F_G03.seq+
****************** Contig 100 ******************
SL7_M13F_F12.seq+
SL7_M13F_D05.seq+
SL9_M13F_E10.seq+
```

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****************** Contig 101 ******************
SL7_M13F_G10.seq+
                    SL7_M13F_H11.seq+ is in SL7_M13F_G10.seq+
SL7_M13F_H09.seq+
****************** Contig 102 ******************
SL7_M13F_H04.seq+
SL7_M13F_F04.seq+
****************** Contig 103 ******************
SL7_M13F_H08.seq+
                    SL7_M13F_E11.seq+ is in SL7_M13F_H08.seq+
****************** Contig 104 ******************
SL8_M13F_A01.seq+
SL9_M13F_G05.seq+
****************** Contig 105 ******************
SL8_M13F_A02.seq+
                    SL8_M13F_C11.seq+ is in SL8_M13F_A02.seq+
SL9_M13F_B12.seq+
****************** Contig 106 ******************
SL8_M13F_A11.seq+
SL8_M13F_A09.seq+
****************** Contig 107 ******************
SL8_M13F_A12.seq+
SL8_M13F_G11.seq+
SL8_M13F_F05.seq+
                    SL9_M13F_A11.seq+ is in SL8_M13F_F05.seq+
SL9_M13F_F01.seq+
                    SL9_M13F_A12.seq+ is in SL9_M13F_F01.seq+
****************** Contig 108 ******************
SL8_M13F_B09.seq+
SL9_M13F_B08.seq+
****************** Contig 109 ******************
SL8_M13F_C04.seq+
SL8_M13F_H02.seq+
                    SL8_M13F_G10.seq+ is in SL8_M13F_H02.seq+
                    SL9_M13F_F09.seq+ is in SL8_M13F_H02.seq+
                    SL8_M13F_C02.seq+ is in SL8_M13F_H02.seq+
SL9_M13F_B05.seq+
SL9_M13F_B06.seq+
SL8_M13F_G01.seq-
                    SL9_M13F_B09.seq- is in SL8_M13F_G01.seq-
                    SL9_M13F_A09.seq- is in SL8_M13F_G01.seq-
****************** Contig 110 ******************
SL8_M13F_C08.seq+
SL8_M13F_A03.seq+
                    SL8_M13F_A04.seq+ is in SL8_M13F_A03.seq+
****************** Contig 111 ******************
SL8_M13F_C09.seq+
SL8_M13F_B01.seq+
                    SL8_M13F_C01.seq+ is in SL8_M13F_B01.seq+
SL8_M13F_E05.seq-
****************** Contig 112 ******************
```

```
SL8_M13F_D03.seq+
SL9_M13F_D04.seq+
****************** Contig 113 ******************
SL8_M13F_D05.seq+
                    SL6_M13F_H12.seq- is in SL8_M13F_D05.seq+
                    SL8_M13F_C12.seq+ is in SL8_M13F_D05.seq+
****************** Contig 114 ******************
SL8_M13F_D06.seq+
                    SL6_M13F_E03.seq+ is in SL8_M13F_D06.seq+
****************** Contig 115 ******************
SL8_M13F_D11.seq+
SL8_M13F_D10.seq+
                    SL8_M13F_E10.seq+ is in SL8_M13F_D10.seq+
                    SL9_M13F_B10.seq+ is in SL8_M13F_D10.seq+
SL9_M13F_B03.seq+
SL8_M13F_F01.seq-
****************** Contig 116 ******************
SL8_M13F_E03.seq+
                    SL9_M13F_F05.seq+ is in SL8_M13F_E03.seq+
SL8_M13F_H03.seq+
SL8_M13F_H06.seq+
SL9_M13F_B04.seq+
                    SL9_M13F_D09.seq+ is in SL9_M13F_B04.seq+
SL9_M13F_E08.seq+
SL9_M13F_C03.seq-
SL8_M13F_E11.seq-
****************** Contig 117 ******************
SL8_M13F_E04.seq+
                    SL9_M13F_D03.seq+ is in SL8_M13F_E04.seq+
****************** Contig 118 ******************
SL8_M13F_E06.seq+
SL9_M13F_F03.seq+
****************** Contig 119 ******************
SL8_M13F_E07.seq+
SL9_M13F_C12.seq+
****************** Contig 120 ******************
SL8_M13F_E09.seq+
SL9_M13F_C11.seq+
****************** Contig 121 ******************
SL8_M13F_E12.seq+
SL8_M13F_D08.seq+
SL9_M13F_C09.seq+
****************** Contig 122 ******************
SL8_M13F_F04.seq+
                    SL8_M13F_D02.seq+ is in SL8_M13F_F04.seq+
SL8_M13F_H09.seq+
                    SL9_M13F_D11.seq+ is in SL8_M13F_H09.seq+
SL8_M13F_B06.seq-
                    SL8_M13F_G04.seq- is in SL8_M13F_B06.seq-
****************** Contig 123 ******************
SL8_M13F_F10.seq+
```

```
SL8_M13F_B10.seq+
                    SL7_M13F_H02.seq+ is in SL8_M13F_B10.seq+
****************** Contig 124 ******************
SL8_M13F_F11.seq+
SL9_M13F_E04.seq+
SL9_M13F_F10.seq+
****************** Contig 125 ******************
SL8_M13F_H11.seq+
                    SL6_M13F_G11.seq+ is in SL8_M13F_H11.seq+
SL8_M13F_C10.seq+
SL9_M13F_F02.seq-
****************** Contig 126 ******************
SL9_M13F_A04.seq+
SL9_M13F_E05.seq+
****************** Contig 127 ******************
SL9_M13F_A07.seq+
                    SL9_M13F_D08.seq+ is in SL9_M13F_A07.seq+
****************** Contig 128 ******************
SL9_M13F_A08.seq+
                    SL7_M13F_C05.seq+ is in SL9_M13F_A08.seq+
****************** Contig 129 ******************
SL9_M13F_B01.seq+
                    SL9_M13F_C06.seq+ is in SL9_M13F_B01.seq+
****************** Contig 130 ******************
SL9_M13F_B07.seq+
SL9_M13F_E02.seq+
****************** Contig 131 ******************
SL9_M13F_B11.seq+
SL9_M13F_C02.seq+
****************** Contig 132 ******************
SL9_M13F_D10.seq+
                    SL9_M13F_G07.seq+ is in SL9_M13F_D10.seq+
                    SL9_M13F_B02.seq+ is in SL9_M13F_D10.seq+
****************** Contig 133 ******************
SL9_M13F_E01.seq+
                    SL9_M13F_C08.seq+ is in SL9_M13F_E01.seq+
****************** Contig 134 ******************
SL9_M13F_E03.seq+
                    SL9_M13F_E09.seq+ is in SL9_M13F_E03.seq+
****************** Contig 135 ******************
SL9_M13F_E12.seq+
SL9_M13F_E11.seq+
****************** Contig 136 ******************
SL9_M13F_G03.seq+
SL9_M13F_G06.seq+
****************** Contig 137 ******************
SL9_M13F_G04.seq+
SL9_M13F_C01.seq+
```

Appendix D

Sequences in the sublibrary

Table D.1: List of clones from the subtractive libraries sorted according to the overlapping sequences listed in Appendix C on page 205. no - Number of clone in the library, Condition - sublibrary, Ensembl - most similar gene in *C. elegans*, Trace - location of the clone containing the transcripts, Overlap no - Number of the overlapping sequence from Table C on page 205. The complete list is available as Microsoft Excel file on request.

no	Condition	Strain	Ensembl	E-value	Description	Trace	Overlap no
672	post osmotic	PS1579	T09A5.11	1.70E-26	Oligosaccharyl-transferase, beta subunit	SL1-15307-A06	C 01
673	post osmotic	PS1579	T09A5.11.2	7.00E-28	Oligosaccharyl-transferase, beta subunit	SL1-15307-A05	C 01
26	des	AF36	F28C6.6	8.30E-10	suf-1 - (SUppressor-of-Forked (Drosophila) homolog)	SL1-15307-C03	C 05
27	des	AF36	F28C6.6	8.00E-10	suf-1 - (SUppressor-of-Forked (Drosophila) homolog)	SL1-15307-C08	C 05
92	des	AF36	none			SL1-15307-F09	C 10
111	des	AF36	none			SL3-15307-D08	C 10
846	osmotic	PS1579	Y39B6A.20.1	1.80E-25	asp-1 - (ASpartyl Protease)	SL7-M13F-D05	C 100
695	post osmotic	PS1579	Y39B6A.20.1	1.90E-25	asp-1 - (ASpartyl Protease)	SL7-M13F-F12	C 100
500	post des	PS1579	Y39B6A.20.1	4.80E-25	asp-1 - (ASpartyl Protease)	SL9-M13F-E10	C 100

Table D.2: List of clones from the subtractive libraries sorted according to the identified gene in *C. elegans*. no - Number of clone in the library, Condition - sublibrary, Ensembl - most similar gene in *C. elegans*, Trace - location of the clone containing the transcripts, Overlap no - Number of overlapping sequence from Table C on page 205. The complete list is available as Microsoft Excel file on request

no	Condition	Strain	Ensembl	E-value	Description	Trace	Overlap
2	des	PS1579	B0272.3	3.50E-45	3-hydroxyacyl-CoA dehydrogenase [KOG2304]	SL5-M13F-D09	C 72
3	des	PS1579	B0272.3	5.10E-42	3-hydroxyacyl-CoA dehydrogenase [KOG2304]	SL5-M13F-E09	C 72
58	des	PS1579	M28.5.2	4.90E-41	60S ribosomal protein 15.5kD/SNU13, NHP2/L7A family (includes ribonuclease P subunit p38), involved in splicing [KOG3387]	SL5-M13F-B06	
212	des	AF36	T04C12.4.2	1.10E-58	act-3 - (ACTin)	SL3-15307-G06	
563	post salt	AF36	M03F4.2	2.80E-40	act-4 - (ACTin)	SL2-15307-C11	
749	salt	AF36	M03F4.2	1.20E-35	act-4 - (ACTin)	SL2-15307-D12	c37 in
54	des	AF36	M03F4.2a.1	2.80E-36	act-4 - (ACTin)	SL3-15307-E04	c37 in
55	des	AF36	M03F4.2a.1	2.90E-36	act-4 - (ACTin)	SL3-15307-E06	c37
56	des	AF36	M03F4.2a.1	2.90E-36	act-4 - (ACTin)	SL3-15307-E07	c37 in
57	des	PS1579	M03F4.2a.1	1.00E-35	act-4 - (ACTin)	SL5-M13F-C09	
564	post des	AF36	M03F4.2a.1	1.50E-71	act-4 - (ACTin)	SL4-15307-H08	
320	post des	PS1579	M03F4.2a.1	9.40E-79	act-4 - (ACTin)	SL9-M13F-C05	
530	post salt	PS1579	C36A4.9a.3	2.50E-63	Acyl-CoA synthetase [KOG1175]	SL7-M13F-H05	

Appendix E

Microarray data from *Caenorhabditis elegans*

The CEL and CHP files of the microarray data are currently stored on a DVD.

Appendix F

MicroRNA expression table

Table F.1: MicroRNA expression during the first 36 hours of desiccation - Total RNA from the microarray experiment at timepoint 0, 12, 24, and 36 hours after the initiation of the desiccation stress was used to measure the expression of microRNAs. The expression data was then analyzed for 2-fold increase or decrease during the different timeframes. The average of three technical replicates and the max and minimum value are shown here.

changes	0	12	24	36
0.5 and 2 fold 0 to 36				
miR-265	0.549 (0.542 to 0.56)	0.981 (0.979 to 0.984)	1.019 (0.975 to 1.063)	1.518 (1.474 to 1.544)
miR-34	0.565 (0.553 to 0.585)	0.923 (0.901 to 0.958)	1.077 (1.043 to 1.145)	1.198 (1.143 to 1.232)
miR-273	1.169 (1.16 to 1.182)	1.28 (1.238 to 1.315)	0.831 (0.776 to 0.876)	0.493 (0.476 to 0.508)
miR-244	2.21 (2.067 to 2.354)	0.885 (0.845 to 0.929)	1.115 (1.078 to 1.157)	0.687 (0.677 to 0.701)
0.5 fold 0 to 12				
miR-234	1.36 (1.288 to 1.451)	0.95 (0.942 to 0.956)	0.972 (0.966 to 0.98)	1.028 (0.977 to 1.098)
miR-76	1.05 (0.999 to 1.114)	0.829 (0.811 to 0.842)	1.11 (1.049 to 1.16)	0.95 (0.906 to 1.015)
miR-231	1.34 (1.277 to 1.413)	0.951 (0.91 to 1.03)	1.049 (1.015 to 1.116)	0.915 (0.9 to 0.926)
miR-233	1.052 (0.984 to 1.137)	0.849 (0.82 to 0.904)	0.948 (0.923 to 0.996)	1.12 (1.092 to 1.163)
miR-250	1.441 (1.374 to 1.507)	0.967 (0.928 to 1.016)	1.033 (1.005 to 1.053)	0.923 (0.866 to 0.982)
miR-232	1.589 (1.525 to 1.669)	0.973 (0.908 to 1.035)	1.027 (0.994 to 1.058)	0.97 (0.955 to 0.984)
miR-43	1.327 (1.315 to 1.336)	0.985 (0.971 to 0.993)	1.001 (0.953 to 1.031)	0.999 (0.945 to 1.065)
miR-70	1.424 (1.368 to 1.495)	1.113 (1.088 to 1.132)	0.887 (0.853 to 0.948)	0.874 (0.836 to 0.894)

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Table F.1: continued

changes	0	12	24	36
miR-50	1.227 (1.174 to 1.303)	0.971	0.999 (0.986 to 1.025)	1.001 (0.976 to 1.039)
miR-124	1.412 (1.393 to 1.43)	1.059 (1.031 to 1.093)	0.94 (0.874 to 1.016)	0.941 (0.886 to 0.973)
miR-244	2.21 (2.067 to 2.354)	0.885 (0.845 to 0.929)	1.115 (1.078 to 1.157)	0.687 (0.677 to 0.701)
miR-42	1.221 (1.218 to 1.223)	0.914 (0.878 to 0.938)	1.086 (1.058 to 1.124)	0.862 (0.82 to 0.921)
miR-83	1.491 (1.402 to 1.573)	1.005 (0.98 to 1.037)	0.995 (0.975 to 1.031)	0.93 (0.918 to 0.946)
miR-47	1.316 (1.283 to 1.352)	1.045 (1.027 to 1.07)	0.708 (0.689 to 0.729)	0.955 (0.942 to 0.979)
cel-lin-4	1.167 (1.111 to 1.23)	0.903 (0.869 to 0.922)	1.001 (0.966 to 1.068)	0.999 (0.954 to 1.062)
miR-79	1.533 (1.458 to 1.639)	0.922 (0.882 to 0.973)	0.896 (0.882 to 0.906)	1.078 (1.044 to 1.113)
miR-2	1.373 (1.357 to 1.386)	0.719 (0.676 to 0.747)	1 (0.955 to 1.046)	1 (0.938 to 1.057)
miR-228	1.081 (1.065 to 1.097)	0.801 (0.787 to 0.814)	1.089 (1.078 to 1.097)	0.919 (0.877 to 0.95)
miR-86	1.043 (0.987 to 1.124)	0.756 (0.724 to 0.775)	1.07 (1.008 to 1.19)	0.957 (0.893 to 1.035)
miR-1	1.119 (1.071 to 1.15)	0.881 (0.871 to 0.888)	1.189 (1.161 to 1.245)	0.752 (0.719 to 0.813)
miR-77	1.602 (1.504 to 1.662)	1.006 (0.966 to 1.05)	0.994 (0.96 to 1.052)	0.99 (0.958 to 1.019)
miR-75	1.385 (1.353 to 1.434)	0.946 (0.883 to 0.984)	1.051 (1.008 to 1.112)	0.949 (0.924 to 0.981)
0.5 fold 24 to 36				
miR-273	1.169 (1.16 to 1.182)	1.28 (1.238 to 1.315)	0.831 (0.776 to 0.876)	0.493 (0.476 to 0.508)
miR-267	1.034 (1.005 to 1.051)	1.272 (1.237 to 1.304)	0.966 (0.953 to 0.985)	0.581 (0.575 to 0.593)
2 fold 0 to 24				
miR-265	0.549 (0.542 to 0.56)	0.981 (0.979 to 0.984)	1.019 (0.975 to 1.063)	1.518 (1.474 to 1.544)
miR-34	0.565 (0.553 to 0.585)	0.923 (0.901 to 0.958)	1.077 (1.043 to 1.145)	1.198 (1.143 to 1.232)
2 fold 12 to 24				
miR-76	1.05 (0.999 to 1.114)	0.829 (0.811 to 0.842)	1.11 (1.049 to 1.16)	0.95 (0.906 to 1.015)
miR-231	1.34 (1.277 to 1.413)	0.951 (0.91 to 1.03)	1.049 (1.015 to 1.116)	0.915 (0.9 to 0.926)
miR-233	1.052 (0.984 to 1.137)	0.849 (0.82 to 0.904)	0.948 (0.923 to 0.996)	1.12 (1.092 to 1.163)
miR-250	1.441 (1.374 to 1.507)	0.967 (0.928 to 1.016)	1.033 (1.005 to 1.053)	0.923 (0.866 to 0.982)
miR-84	0.968 (0.947 to 1.006)	0.888 (0.87 to 0.916)	1.63 (1.544 to 1.726)	1.032 (1.001 to 1.088)
miR-232	1.589 (1.525 to 1.669)	0.973 (0.908 to 1.035)	1.027 (0.994 to 1.058)	0.97 (0.955 to 0.984)
miR-74	0.989 (0.921 to 1.051)	0.928 (0.887 to 0.974)	1.179 (1.119 to 1.266)	1.011 (0.996 to 1.029)
miR-244	2.21 (2.067 to 2.354)	0.885 (0.845 to 0.929)	1.115 (1.078 to 1.157)	0.687 (0.677 to 0.701)
miR-42	1.221 (1.218 to 1.223)	0.914 (0.878 to 0.938)	1.086 (1.058 to 1.124)	0.862 (0.82 to 0.921)
miR-85	1 (0.95 to 1.053)	0.919 (0.91 to 0.931)	1.165 (1.11 to 1.207)	1 (0.965 to 1.049)
cel-let-7	0.975 (0.902 to 1.038)	0.817 (0.788 to 0.841)	1.139 (1.035 to 1.238)	1.025 (0.973 to 1.101)

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Table F.1: continued

changes	0	12	24	36
cel-lin-4	1.167 (1.111 to 1.23)	0.903 (0.869 to 0.922)	1.001 (0.966 to 1.068)	0.999 (0.954 to 1.062)
miR-44	1.103 (1.076 to 1.135)	0.95 (0.922 to 0.989)	1.05 (1.005 to 1.102)	0.872 (0.825 to 0.915)
miR-2	1.373 (1.357 to 1.386)	0.719 (0.676 to 0.747)	1 (0.955 to 1.046)	1 (0.938 to 1.057)
miR-228	1.081 (1.065 to 1.097)	0.801 (0.787 to 0.814)	1.089 (1.078 to 1.097)	0.919 (0.877 to 0.95)
miR-38	1.075 (1.029 to 1.133)	0.91 (0.894 to 0.918)	1.1 (1.078 to 1.142)	0.925 (0.886 to 0.98)
miR-81	1.034 (1.017 to 1.064)	0.966 (0.946 to 0.996)	1.05 (1.025 to 1.084)	0.966 (0.939 to 0.994)
miR-86	1.043 (0.987 to 1.124)	0.756 (0.724 to 0.775)	1.07 (1.008 to 1.19)	0.957 (0.893 to 1.035)
miR-34	0.565 (0.553 to 0.585)	0.923 (0.901 to 0.958)	1.077 (1.043 to 1.145)	1.198 (1.143 to 1.232)
miR-1	1.119 (1.071 to 1.15)	0.881 (0.871 to 0.888)	1.189 (1.161 to 1.245)	0.752 (0.719 to 0.813)
miR-82	0.974 (0.939 to 1.003)	0.949 (0.931 to 0.981)	1.123 (1.091 to 1.155)	1.026 (0.991 to 1.07)
miR-40	1.051 (1.02 to 1.096)	0.952 (0.941 to 0.971)	1.003 (0.971 to 1.046)	0.997 (0.954 to 1.061)
miR-75	1.385 (1.353 to 1.434)	0.946 (0.883 to 0.984)	1.051 (1.008 to 1.112)	0.949 (0.924 to 0.981)
miR-54	1.056 (1.028 to 1.088)	0.917 (0.911 to 0.924)	1.024 (0.977 to 1.083)	0.976 (0.94 to 1.047)
miR-55	0.975 (0.909 to 1.038)	0.84 (0.81 to 0.876)	1.136 (1.098 to 1.211)	1.025 (0.961 to 1.106)
miR-72	0.994 (0.944 to 1.037)	0.81 (0.807 to 0.813)	1.109 (1.004 to 1.228)	1.006 (0.965 to 1.062)
miR-80	0.966 (0.926 to 1.023)	0.82 (0.801 to 0.852)	1.161 (1.127 to 1.202)	1.034 (1.019 to 1.063)
miR-71	1.093 (1.06 to 1.139)	0.935 (0.903 to 0.977)	1.065 (1.019 to 1.123)	0.894 (0.858 to 0.935)
2 fold 12 to 36				
miR-265	0.549 (0.542 to 0.56)	0.981 (0.979 to 0.984)	1.019 (0.975 to 1.063)	1.518 (1.474 to 1.544)
miR-233	1.052 (0.984 to 1.137)	0.849 (0.82 to 0.904)	0.948 (0.923 to 0.996)	1.12 (1.092 to 1.163)
miR-2	1.373 (1.357 to 1.386)	0.719 (0.676 to 0.747)	1 (0.955 to 1.046)	1 (0.938 to 1.057)
miR-34	0.565 (0.553 to 0.585)	0.923 (0.901 to 0.958)	1.077 (1.043 to 1.145)	1.198 (1.143 to 1.232)