Worms under stress: unravelling genetic complex traits through perturbation

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Thesis

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A mi padre

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1

General Introduction

Miriam Rodriguez

Dimidium facti qui coepit habet: sapere aude, incipe
("who has begun is half done: dare to know, dare to begin!")

Epistularum liber primus. Horace 20 BC.

C. elegans as a model organism

Caenorhabditis elegans (*Nematoda; Rhabditidae*) is a free-living, non-parasitic soil related nematode. *C. elegans* has been studied for decades since Sidney Brenner [1] introduced it as a model organism in a time that mainly bacteria were used in molecular biology experiments. This small nematode, with a body size of approximately 1mm, comprises many characteristics that make it one of the most widely studied model organisms for genetic studies among others. Besides *C. elegans* relatively simple body structure, another advantage of using this model is its small genome size. The genome of *C. elegans* contains approximately 100 Mbp distributed over 6 pairs of chromosomes. Furthermore the existence of two genders, hermaphrodite and male, offers the possibility for generating inbred or recombinant offspring. *C. elegans* is easy to maintain in the laboratory and it has a relatively short life cycle. The complete life cycle from egg to adult stage occurs in 2.5 days at 20°C, at the same temperature the average life span is 3 weeks. In the laboratory *C. elegans* feeds on *Escherichia coli* bacteria. Furthermore these worms can be safely stored at -80˚C for several years [2] and are able to survive and be recovered by re-suspension in fresh medium at room temperature.

The manipulation of *C. elegans* in the laboratory is relatively easier than in other model organisms. For instance a genetic modification treatment such as RNAi treatment is integrated simply by feeding nematodes with *E. coli* containing the RNAi of interest [3].

Given the genetic tractability of *C. elegans* as an experimental organism, it is not surprising that *C. elegans* was the first multicellular organism to have its genome completely sequenced in 1998 [4]. This landmark became an essential step in the way towards the sequencing of the human genome.

The tiny and transparent worm has been used extensively in many areas of genetics, developmental and evolutionary biology, and complex disease research [5-7]. Its simplicity does not however compromise the fact that this nematode can be used as a representative for higher organisms, such as humans (chapter 2).

Genetical genomics studies in *C. elegans*

Over the past decade *C. elegans* has increasingly been used as a model organism for genetical genomics studies [8, 9].

Genetical genomics approach combines genetic linkage analysis with genome wide gene expression. It can be used to compare gene expression levels analyses in different genotypes (natural genetic variation) under certain environmental disturbances. In my thesis I compared different genotypes in *C. elegans* by using recombinant inbred lines (RILs). These RILs were derived from a cross between parental wild types Bristol N2 (Bristol) and CB4856 (Hawaii) [8], the two most genetically divergent strains in *C. elegans* [10]. Using this population, Quantitative Trait Loci (QTLs) can be mapped to identify the loci

associated with phenotypic variation of a certain trait. When gene expression phenotypes are considered quantitative traits on a genome-wide scale, they are named expression quantitative trait loci (eQTLs).

eQTL methods take advantage of subtle natural genetic variation to identify the loci that underlie natural variation in gene expression. This approach not only informs about the relationships between genes but also the kind of relation (i.e. regulatory) characterized by direction, strength and statistical probability of each relationship is defined by the QTL. This technique represents a powerful tool to reveal up- or down-regulation of genes associated with a particular phenotype in a certain condition or treatment. The recent development of an open-source database containing the most relevant QTLs described in *C. elegans* has facilitated enormously the access to these results [11].

PANACEA project as the primer of this thesis

The PANACEA project is meant to use *C. elegans* as a model for complex human diseases [http://www.panaceaproject.eu/UK/]. The project consists of a consortium of six European groups sharing different expertise's and following a common goal: the quantitative pathway analysis of natural variation in complex disease signaling in *C. elegans.*

The objective of the project was the development of quantitative genetic approach of natural variation in complex disease signaling in *C. elegans* in order to identify novel alleles and their regulators. In particular, *C. elegans* vulval development was used as a model for cancer signaling [12-15]. PANACEA focused on Ras, Wnt and Notch pathways. Ras, Wnt and Notch signaling are essential pathways in vulval development of which human orthologs were shown to be involved in several complex diseases in humans such as cancer [16-19]. We performed a quantitative pathway analysis in a collection of RILs.

The focus of my thesis

Since the beginning of the 20th century when the word *Genetics* was coined by William Bateson [20], scientist all over the world have shown their curiosity and tried to find the mechanisms that are standing behind a certain phenotypic trait. The work of all these notable geneticists, starting with G. J. Mendel [21-23], has contributed for decades to unravel the genetic architecture of species. Many of these studies have been essential to reach the current level of knowledge, however every single study in genetics is worth to be taken into consideration since all of them have contributed to our understanding and paved the way towards new findings.

Nowadays, we are in the “genetics age” and we seem to have uncovered almost all the information about the genetics of thousands of species. Yet many questions can be formulated since there are still many complex traits that we are not able to decipher yet.

In my opinion, the beauty of genetics resides in the fine combination between a highly specialized and coordinated complex established for millions of years, and the flexibility, which is inherent to the whole system that makes it dynamic and changeable. The fact of finding out a tiny drop in this immense ocean appeared to me a marvelous challenge.

Many are the approaches one can choose to dive into the genetic architecture of an organism. In this thesis I present one of them based on the reaction of genetic systems to a certain perturbation producing a stress response that disturbs the balance of that genetic system. The basis of this approach can be simplified with the model shown in Figure 1.

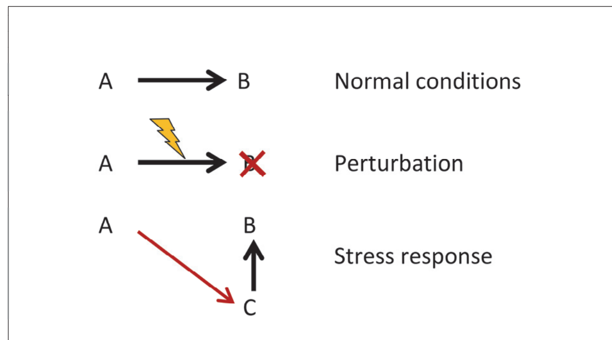

Figure 1: Schematic representation of stress response after a certain perturbation. In normal conditions product A determines activity of product B. When a perturbation alters this system, product B is altered, consequently stress response acts repairing the damage by activating an alternative route via product C to restore the situation in normal conditions.

Genetic perturbation has been studied for decades in different organisms. The ability to compare stable genetic systems to perturbed ones has been developed as a very strong tool to get a better understanding of the plasticity of the genetic system and to get a better knowledge about the complex genetic processes [24]. Perturbation allows uncovering hidden phenotypic variations translated from expression profiles as a result of stress responses.

In these studies both forward as well as reverse genetics experiments have been conducted in order to relate the three sectors of the keystone of genetics: genotype, phenotype and environment.

Outline of this thesis

During the progress of this thesis I have investigated the genetic architecture of complex traits using perturbation at three different levels: i) **genomic perturbation**, as a result of natural genetic variation in RILs, ii) **genetic perturbation**, performed by introgressed fragments of exogenous genome into a different background (Introgression Line; ILs) and gene silencing by induced mutants, and iii) **environmental perturbation** (stress conditions).

In this thesis I showed how the use of induced perturbation provides a promising tool to gain a better insight into complex genetic processes in *C. elegans*. I also illustrated what perturbation in *C. elegans* can teach us about more complex systems, such as complex human diseases [8, 17, 25-27]. I have studied the genetic control of responses to the aforementioned perturbations over a range of different traits; from life cycle traits to gene expression profiles. Moreover I have combined these perturbations together in order to increase the potential of these tools to get the maximal information out of them (Figure 2).

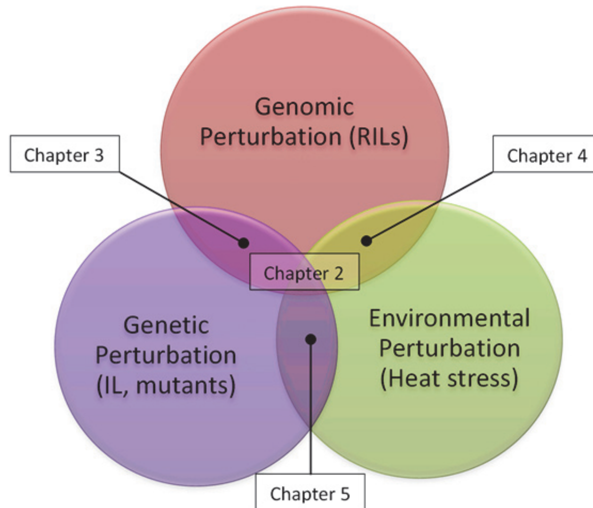

Figure 2: Thesis sketch. Representation of the different types of perturbations described in this thesis and how they have been combined in the thesis chapters.

Many studies of stress response in *C. elegans* have been essential to define the genetic architecture of complex pathways and they could be extrapolated to other organisms. In **chapter 2** of this thesis I present an analysis of recent studies on stress and perturbation in *C. elegans*. The chapter describes how those studies have contributed to a better understanding of stress response and its relation with biological mechanisms occurring in complex human diseases.

The first experimental chapter (**chapter 3**) described in this thesis presents a combination of genetic variation and genetic perturbation. Here we included the loss-of-function mutant *bar-1 (ga80)* in a set of RILs.

Induced mutant analyses in *C. elegans* wild type N2 have been widely studied in order to obtain a better understanding of human disease pathways. However, induced mutations in a single genetic background and single mutations are of limited value to understand related pathways due to the complexity of disease pathways and their natural variation.

We analyzed a collection of RILs derived from a cross between *C. elegans* N2 *bar-1(ga80)* and wild type CB4856. We studied *C. elegans* vulval development and focused on Wnt pathway genes, which human orthologous were shown to be involved in several complex diseases [28].

By crossing N2 *bar-1(ga80)* with CB4856 strain, we revealed hidden genetic modifiers affecting germline/vulval development. The results suggest a number of candidate genes, which appear to be novel polymorphic modifiers of Wnt signalling.

Phenotypically, *bar-1(ga80)* mutant RILs exhibited strong genetic variation of vulval induction. We described an abnormal vulval induction in *bar-1* mutant RILs. QTL analysis showed a correlation between vulval induction and genotype and revealed the effect of novel polymorphic modifiers as well as targets of these modifiers. The trans-regulatory eQTL band in *bar-1* included RILs suggested common modifiers of Wnt pathway. After analyzing, the results point at the polymorphic gene *sqv-2,* which encodes a galactosyltransferase II as a promising candidate for a vulva development modifier. We showed in this chapter how this gene gets uncovered as a vulva development regulator when the β-catenin BAR-1 is dysfunctional, describing a possible new role of *sqv-2* different than already described in the biosynthesis of chondroitin and heparan sulfate [29].

In this approach we designed a new way to describe genetic networks of complex pathways combining induced mutant RILs with gene expression QTLs. By inducing mutants in a combination of the two most divergent *C. elegans* strains, we uncovered cryptic variation in two genetic backgrounds. It is anticipated that this method may become a powerful tool to identify novel regulators in complex human disease signaling.

Regarding the combination of genetic variation and environmental perturbation, we investigated the genetic variation of heat-shock recovery, i.e. hormesis effects on life span and offspring, and associated loci in *C. elegans*. This published data is presented in the **chapter 4** of this book.

Thermo-stress can induce an increase in life span in *C. elegans* [30]. There is a strong genetic component involved in this increase, based on the fact that a better genetic disposition to stress resistance confers a positive selection [31]. To gain insight into the subtle effects of natural variants, the genetics of heat-shock recovery, life span and offspring, we exposed RILs to heat-stress. We used a subset of RILs to study the genetic variation of the heat response. The results show that there is natural variation in hormetic effects on life span for heat-shock and that this variation leads to modulation of life span.

Chapter 5 presents a combination of environmental and genetic perturbation, concretely heat stress in *C. elegans* combined with introgression lines (ILs). These lines were derived from crossings between parental wild types N2 (Bristol) and CB4856 (Hawaii) and they contained a unique known fragment from CB4856 (Hawaii) genome introgressed in a N2 (Bristol) background [32].

Other groups within the worm community have previously pointed at *Chromosome IV* to contain the loci responsible for stress response [33]. In this last experimental chapter we

demonstrated the importance of this chromosome and a locus related with heat stress response phenotype.

In order to proof a causal role of a locus distal to chromosome *IV*, we selected a set of ILs containing a fragment of CB4856 (Hawaii) genome introgressed in a N2 (Bristol) background around this candidate locus in chromosome *IV* and we studied gene expression in normal and heat stress conditions in the ILs as well as in the parental lines. This study confirms the role of the locus distal to chromosome *IV* in heat stress response. To my knowledge this is the first time that an eQTL has been confirmed by a different approach measuring detailed expression of genes, which loci are located on the *trans*-band defined by the eQTL studies.

Despite it is an apparently simple organism, *C. elegans* has been widely studied already for decades. However there is still a long way towards the complete understanding of its biology.

This thesis is an attempt to gain insight not only into *C. elegans* biology but also in the biology of complex signaling in other organisms up to humans using molecular perturbation as an approach.

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2

C. elegans stress response and its relevance to complex human disease and aging

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Abstract

Many organisms have stress response pathways, components of which share homology with players in complex human disease pathways. Research on stress response in the nematode *Caenorhabditis elegans* has provided detailed insights into the genetic and molecular mechanisms underlying complex human diseases. In this review we focus on four different types of environmental stress responses, heat shock, oxidative stress, hypoxia and osmotic stress, and how these can be used to study the genetics of complex human diseases. All four types of responses involve the genetic machineries that underlie a number of complex human diseases like cancer and neurodegenerative diseases, including Alzheimer's and Parkinson's. We highlight the types of stress response experiments required to detect the genes and pathways underlying human disease and suggest that studying stress biology in worms can be translated to understanding human disease and provide potential targets for drug discovery.

C. elegans as a model for complex human disease and aging

Since its introduction in the early 1970's [1], the nematode *C. elegans* (*Nematoda: Rhabditidae*) has been instrumental as a platform for biological research and the implementation of a vast array of technologies [2, 3]. The tiny and transparent worm has been used extensively in many areas of genetics, developmental and evolutionary biology, and complex disease research [4-6]. *C. elegans* serves as an important model for human diseases because it has many biological features in common with humans such as the development of muscles, nerves, and digestive tract and the production of sperm and eggs [7]. Although relatively short-lived (approximately 3 weeks), worms do age, and studying this process has been informative for understanding human aging [8]. Many signalling pathways underlying life span elongation, apoptosis (Glossary), and complex behaviours are conserved between worms and mammals [9-11]. Most notably is the insulin/insulin-like growth factor signalling pathway, which involves the forkhead transcription factor DAF-16, a key regulator of life span changes in response to environmental and gonadal stimuli [8].
C. elegans has received much attention as a model for complex human diseases, including cancer, neurodegenerative, and mitochondrial disease [12-15]. It is also an effective model species for studying complex human neurological diseases [11]. Moreover, *C. elegans* has orthologues of amyloid precursor protein, suggesting worms may be a good model for studying Alzheimer's disease [16]. A specific example of a finding from worms that has been successfully translated into an improvement in human health comes from studies on the kindlin protein family. Mutations in one member of this family, Kindlin-1, lead to Kindler syndrome in humans, which is characterized by skin blistering. The defect in Kindler patients suggested a role of Kindlin-1 in integrin adhesion, but it was in *C. elegans* that the interaction between UNC-112, the ortholog of mammalian kindlins, and integrin was demonstrated [17]. This discovery paved the way to the development of more efficient therapy for this rare disease, and it demonstrates the relevance of using *C. elegans* to understand human disease.

C. elegans stress pathways as a model for complex disease pathways in humans

Many common stress-induced effects on physiology, gene expression, and signalling pathways among animals, including *C. elegans* and mammals, have been found [18]. For instance, heat shock experiments showed that the genes *hsf-1* and *daf-16* are part of the heat shock response and affect life span in *C. elegans* [19]. Homologues of these genes play a key role in the development of age-related diseases in humans [20]. Lack of oxygen induces the transcription factor HIF-1 in *C. elegans,* which protects the germline from apoptosis by antagonizing the function of CEP-1, the homologue of the human tumour suppressor p53 [21]. Studying these effects in *C. elegans* with mutations in genes that have human homologs (e.g., *daf-18* is the homolog of the human tumor suppressor PTEN and *daf-16* is the ortholog of human FOXO (Forkhead transcription factor) provides a tractable genetic system to explore stress response and its relation to disease in humans.

Here we review how *C. elegans* pathways and genes underlying the stress response to heat shock, oxidative stress, hypoxia, and osmotic stress can inform research on complex human disease pathways. Table 1 shows which type of stress experiments are used to identify and characterize genes in *C. elegans*, their human homologs, and the type of disease these genes are associated with.

Hitherto, stress response studies in *C. elegans* have mainly been conducted from the view of understanding the genetics of longevity, neurobiology, and developmental biology. However stress in worms affects genes and pathways that share a high homology with humans and play an important role in various complex diseases. As our knowledge of both the genetics of human disease and stress response in worms has increased, it is now possible to draw comparisons between worms and humans that may suggest new avenues for research or illuminate previously unknown connections in complex human diseases. Therefore we recommend continuing to study stress biology in the worm with an eye toward understanding complex diseases in humans.

Heat shock

Following a heat shock all cells exhibit a heat-shock response, which is a program of stress-inducible gene expression, to prevent cellular degeneration and increase thermal tolerance. The heat-shock response has been well studied in *C. elegans*, revealing the involvement of three neuroendocrine signaling pathways [22]: the nuclear hormone receptor (NR) pathway, the transforming growth factor-β (TGF-β) pathway, and the insulin-like growth factor (IGF)/insulin-like signaling (ILS) pathway, which is the most thoroughly studied. The ILS pathway is involved in elongating life span by regulating the entry of the transcription factors DAF-16 and heat shock factor-1 (HSF-1) into the nucleus. Under normal conditions, DAF-16 and HSF-1 are phosphorylated, which keeps them inactive and restricted to the cytoplasm. Under stress conditions the ILS pathway promotes the entry into the nucleus of these factors and, in consequence, their transcription factor activity [23]. HSF-1 regulates the heat-shock response by controlling the expression of small heat shock proteins (HSP), which are molecular chaperones that function to maintain cellular proteostasis in eukaryotes and prevent protein and cellular damage following stress [24]. For instance HSF-1 and HSP protect *C. elegans* from heat-stroke-associated neurodegeneration [25].

Recent studies have demonstrated that both DAF-16 and HSF-1 are required for life span extension mediated by ILS, but DAF-16 acts during nematode adulthood, whereas HSF-1 is more active during early development in larva stages [26]. It appeared that the activity of HSF-1 is regulated at an early step by ILS via two HSF-1 regulators, DDL-1 and DDL-2 (*daf-16-dependent* longevity genes) [27]. Inhibition of DDL-1/2 increases longevity and thermo tolerance. DDL-1/2 negatively regulate HSF-1 activity by forming a protein complex with HSF-1, which is affected by the phosphorylation status of DDL-1 (homologous to human *coiled-coil domain–containing protein 53*). The formation of the protein complex and the phosphorylation of DDL-1 are controlled by ILS [27]. DAF-16 and HSF-1 play an important role in aging and age-related disease in humans. DAF-16 is orthologous to human FOXO3A, which has been shown to be strongly associated with human longevity [28]. A number of other aging phenotypes, like prevalence of cancer and cardiovascular disease and loss of various physical and cognitive functions, are also associated with the FOXO3A genotype [28]. This is supported by many other studies

showing that polymorphisms in FOXO3A are associated with the ability to reach a very old age in humans [29]. In addition to its effects on aging, there is much evidence that the Insulin/IGF-1 signalling (IIS) pathway (the human equivalent of ILS in worms) is a major regulatory axis underlying cancer in humans [30]. The IIS pathway can induce cellular proliferation in both healthy conditions and cancer [31].
As in worms, HSF1 also plays an essential role in stress responses by maintaining proteostasis in humans through regulation of insulin-like signaling and other age-related pathways [20]. The ability of HSF to bind DNA is inhibited by acetylation at Lys^{80} [32], which is regulated by the de-acetylase SIRT1. SIRT1 regulates cell survival (apoptosis mechanism), inflammation, and metabolism through stress activation by de-acetylation of different factors such as p53, NF-κB, and different FOXO family members. Severe stress-mediated activation of SIRT1 likely leads to negative regulation of p53 [33] and subsequent cancer formation. A recent study demonstrates that HSF1 also regulates specific transcription programs of certain types of human cancer [34].

In addition to aging and cancer, the heat-shock response, in combination with the ILS pathway, is also involved in protein aggregation in both worms and humans. When raised at 25°C, nematodes expressing polyglutamine (polyQ) in muscle accumulate protein aggregates and become paralyzed [35]. Aberrant protein aggregation is a key characteristic of neurodegenerative diseases, such as Alzheimer's disease, which is associated with the misassembly and aggregation of the $A\beta_{1\text{-}42}$ peptide. In *C. elegans* aggregation of $A\beta_{1\text{-}42}$ was reduced when aging was slowed by decreased ILS activity. The downstream transcription factors HSF-1 and DAF-16 regulate opposing disaggregation and aggregation activities [36]. This suggests that therapeutics, which prevent the age-related decline in proteostasis and promote the upregulation of chaperones would slow down disease manifestation and, in turn, accelerate proteostasis collapse.
Taken together, we conclude that conducting heat-shock experiments in *C. elegans* provides fundamental insights into the role of HSPs, HSF-1, and the ILS pathway and may be useful for understanding cancer, aging, and age-related neurodegenerative diseases in humans.

Oxidative Stress

High doses of reactive oxygen species (ROS) cause oxidative stress. In most cells, the primary source of ROS is the mitochondrion due to inefficiencies in oxidative phosphorylation. Oxidative damage is especially known to disrupt proteostasis, but it can also affect lipids, membranes, and DNA. In *C. elegans*, environmental perturbations that induce ROS can lead to reduced levels of dopamine, an important neurotransmitter, which is released by nerve cells. One study looked at the stress response to paraquat, an herbicide that can lead to the formation of ROS [37], and its effect on neurite outgrowth in *C. elegans* [38]. They studied the effect of mutations in the genes *pink-1* and *lrk-1* to paraquat sensitivity. Both genes encode putative kinases highly similar to human PINK1 (phosphatase and tensin (PTEN) homolog-induced putative kinase 1) and LRRK2 (leucine-rich repeat kinase 2), mutations in which have been associated with Parkinson's disease [39]. They demonstrated that *lrk-1* suppressed all phenotypic aspects of the *pink-1* mutation, suggesting PINK-1 may antagonize LRK-1 in humans. Many other chemical perturbations causing ROS have been used in *C. elegans* to model Parkinson's disease [13],

illustrating the utility of studying oxidative stress in worms to understand human neurodegenerative diseases.

Mutations in *mev-1*, encoding the *C. elegans* cytochrome b subunit of the mitochondrial respiratory chain complex II (ubiquinol-cytochrome c reductase), result in increased sensitivity to oxidative stress. *mev-1* is orthologous to the human Isoform 1 of Succinate DeHydrogenase Cytochrome b (SDHC). Recent studies showed that the *mev-1(kn-1)* mutation, which results in an amino acid substitution at the 71st position from glycine to glutamate (G71E), dramatically reduced the mitochondrial complex II activity. The accumulation of ROS is two times greater in *mev-1(kn-1)* worms relative to wildtype, and consequently the mutant animals have shorter life spans. *Mev-1(kn-1)* worms show reduced glutathione concentrations, and this metabolic imbalance might be caused by the role played by succinate dehydrogenase (SDH) in the citric cycle.

In humans, mutations in mitochondrial enzymes from SDH family genes cause a genetic predisposition to develop certain types of tumors [40]. SDH deficiencies might trigger hypoxic conditions, resulting in increased activity of hypoxia inducible factor, a transcription factor involved in regulating cellular oxygen balance that can cause changes in cells and metabolism, and in some cases plays an essential role in angiogenesis, metastasis, cell proliferation, etc. [41]. This factor and other features of hypoxia stress are discussed below.

Hypoxia and CO_2 fluctuation

Low levels of O_2 (hypoxia) can lead to decreased metabolic rate, increased glycolysis, and pausing or slowing of the cell cycle. Oxygen levels are severely affected by disease states like cancer and various heart and lung diseases, where cells and tissues suffer from very low oxygen conditions (pathological hypoxia) [42].

Work in *C. elegans* has greatly increased the knowledge about the underlying mechanisms of hypoxic effects. In contrast to mammals, nematodes do not have specific respiratory organs but instead depend on diffusion for exchange of O_2 and CO_2. The body cavity is filled with fluid, which allows for rapid exchange of gases and chemicals across the cells. Hypoxia activates hypoxia inducible factor 1 (*hif-1)* in *C. elegans* [43, 44]. In normal O_2 conditions, the protein HIF-1 is targeted for degradation by a prolyl-hydroxylase encoded by *egl-9* in *C. elegans,* the mammalian ortholog of this gene is EGLN/PHD [45].

C. elegans shows a wide CO_2 tolerance compared to other animals. However different studies have shown that *C. elegans* avoids high levels of CO_2 [46]. Furthermore it was found that the intensity of CO_2 avoidance was suppressed under starvation conditions. Food-sensing pathways are closely related with O_2 and CO_2 sensitivity in *C. elegans*. One of the most important pathways related with food sensing is the ILS pathway. It is known that high activity of the ILS pathway is directly correlated with a well-fed state.

In *daf-2*, *pdk-1* or *akt-1* mutants, all of which mimic starvation conditions, CO_2 avoidance was suppressed. However in *daf-2::daf-16* double mutants, where the starvation signal was suppressed, the worms did show a high avoidance to CO_2. This could suggest that starvation suppresses CO_2 avoidance by down-regulating the ILS pathway, activating translocation of DAF-16 into the nucleus to act as a transcription factor [46]. This response is common to stress conditions, which suggests that stress conditions such as starvation, heat shock, etc. may be linked to a disruption in O_2/CO_2 levels. Furthermore, hypoxia is

also associated with oxidative stress, highlighting another link between these stress response pathways (Figure 1) [47].

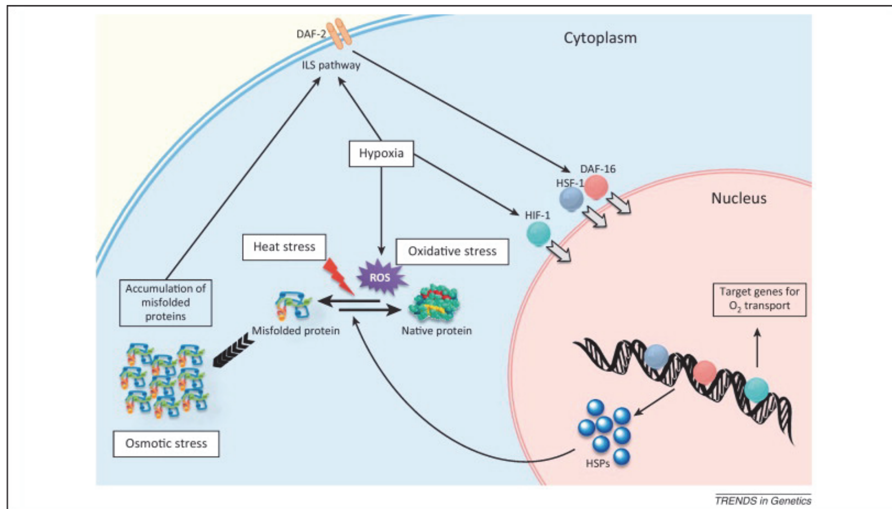

Figure 1. Schematic representation of stress response in *C. elegans* cell. Hypoxia, heat stress and oxidative stress provoke an abnormal conformation of native proteins. The accumulation of misfolded proteins leads to a proteostasis imbalance in the cytoplasm resulting in cellular osmotic stress. In these conditions the stress response mechanism is activated mainly by enhancing insulin-like signaling (ILS) pathway. The trans-membrane receptor DAF-2 initiates the intracellular signaling that directs the transcription factors DAF-16 and HSF-1 into the nucleus where they activate the gene expression of heat shock proteins (HSPs). HSPs are molecular chaperones that help re-establish proteostasis by rescuing misfolded proteins. Hypoxia activates stress response through ILS and expression of hypoxia inducible factor (HIF-1) and its entry into nucleus to act as a transcription factor to activate the expression of target genes for O2 transportation.

Like in worms, hypoxic environments activate HIF in humans (HIF is the ortholog of HIF-1 in *C. elegans*). This activation plays a central role in tissue repair, ischemia, and cancer [48]. The hydroxylation of the HIF proline residue, an evolutionary conserved mechanism, leads to degradation of HIF by the von Hippel-Lindau tumor suppressor protein (VHL). In hypoxic conditions, the proline hydroxylation and degradation of HIF are decreased. HIF then activates target genes to increase oxygen transport. Within tumors, hypoxia can lead to HIF-1α (consisting of subunits HIF1a, HIF2a and HIF3a), overexpression of which has been shown to increase patient mortality in different types of cancer [49].

Osmotic stress

Hypertonic or osmotic stress induces protein damage by aggregation. Under desiccated conditions resulting from hypertonic stress, the loss of water leads to an intracellular ionic imbalance, causing protein aggregation. Studies in *C. elegans* suggested that nematodes may have independent pathways to control proteotoxic effects and survive osmotic shock [50, 51]. In hyper-osmotic stress conditions, *gpdh-1*, which encodes a glycerol-3-phosphate dehydrogenase (GPDH-1), is strongly up-regulated. GPDH-1 induces *de novo* biosynthesis

of glycerol, leading to a rapid accumulation of organic osmotic glycerol in cells. This is a typical effect of hyperosmotic stress in cells [52]. Expression of *gpdh-1* is regulated by two GATA transcription factors, *elt-2* and *elt-3* [53]. Both factors are also required for other developmental as well as non-developmental processes [53, 54]. The enzymatic activity of GPDH-1 is regulated by osmotic regulatory genes, like *osm-7*, *osm-11,* and *osm-8,* which have been described as critical regulators in osmotic disorders [55]. In *C. elegans*, disruptions in osmotic regulatory genes lead to physiological responses similar to the response to hyperosmotic stress conditions [52, 53]. Recent studies showed that osmotic regulatory genes regulate osmotic stress resistance independent from other stress response mechanisms [56, 57]. It has been reported that the response mediated by *gpdh-1* is very specific, and its activation occurs rapidly after the osmotic shock (<15 minutes) and at relatively low levels of salt (200mM NaCl) [55]. This is in contrast to the osmotically induced accumulation of damaged proteins, which occurs only at high salt concentrations (>500mM NaCl) and takes approximately 1 hour. Both mechanisms (glycerol production and accumulation of damaged proteins) could be employed by cells against different levels of osmotic stress, that is, they are not cooperative, but rather work independently in different situations depending on stress conditions. Because the accumulation of damaged proteins is an important feature of diseases such as Alzheimer's and Parkinson's [56], insight into the molecular mechanisms of osmotically induced protein damage in *C. elegans* may help to genetically unravel these complex phenotypes. We suggest that the mechanisms of glycerol production and accumulation of damaged proteins in worms might be useful targets for developing potential Alzheimer's and Parkinson's therapeutics.

Table 1. Types of stress experiments to identify and characterize genes in *C. elegans*, their human homologs, and the types of disease with which these genes are associated.

Stress	*C. elegans* gene	Human homolog/ortholog	Disease	Reference
Heat	*daf-18*	PTEN	Cancer	[67, 68]
Heat	*daf-16*	FOXO3A	Cancer	[28]
Heat	*hsf-1*	Hsf1	Cancer	[24, 69]
Oxidative	*pink-1*	PINK1	Parkinson's	[38]
Oxidative	*lrk-1*	LRRK2	Parkinson's	[38]
Oxidative	*sod-1, -2, -3*	SOD1, 2, 3	Amyothrophic lateral sclerosis (ALS)	[70-72]
Hyperoxia	*gcy-35*	NPR-3	Skeletal overgrowth	[73, 74]
Hypoxia	*hif-1*	HIF	Ischemia, Cancer	[45, 48, 75, 76]
Hypoxia	*vhl-1*	VHL	Cancer	[77]
Hypoxia	*egl-9*	EGLN	Cancer	[45]
Hypoxia/Heat	*cep-1*	P53	Cancer	[21]
Osmotic	*gpdh-1*	GPD1	Transient infantile hypertriglyceridemia	[52, 78]
Osmotic	*elt-2, elt-3*	GATA4-6	Pancreatic agenesis and cardiac defects	[79, 80]
Osmotic	*osm-12*	BBS7	Bardet-Biedl syndrome	[53]
Osmotic/oxidative/Heat	*skn-1*	NFE2 (and others)		[52]

Implications for drug discovery

The mechanisms of stress response we presented demonstrate that different stressors share a common and well-conserved molecular response to changes in proteostasis in cells. There are several oncological, neurodegenerative, and metabolic disorders that are triggered by an accumulation of misfolded proteins as a result of cellular stress. Therefore, controlling misfolded protein imbalance may be a therapeutic tool to control damage that leads to complex human diseases. Many of the diseases related with proteostasis are often associated with aging [57]. During aging, the accumulation of protein aggregates leads to an amplification of protein damage that contributes to cellular toxicity.

Stress response analysis in *C. elegans* offers the opportunity to discover new receptors and targets for drugs to treat various diseases. Heat-shock experiments can be used to investigate the genetics and molecular biology of inhibitors of HSF-1. This type of experiment further opens up new ways for assessing if small HSPs or certain protein aggregates or aggregation mechanisms are druggable. For instance specific activators of HSF1 such as geldanamycin were effective in both polyglutamine (polyQ) disease models as well as other neurodegenerative disease models [58]. Along these same lines, many strategies are emerging to restore proteostasis to alter the clinical course in complex age-associated diseases in humans [59], which can be investigated using heat shock experiments in *C. elegans*.

New trends in therapeutics are pointing at small molecule-pharmacological chaperones as proteostasis regulators. These regulators have been shown to increase the capacity of cells to correctly re-fold damaged proteins [59]. Many proteostasis regulators have been described recently that control HSF1 and in turn up-regulate levels of cytoplasmic HSPs. More recently, the identification of around 300 chemical inducers of the heat-shock response was reported by screening for HSF-1 dependent activators of expression of chaperones in human cells [60]. This suggests that pharmacological manipulation of the heat-shock response could be of benefit in a variety of multiple conformational diseases like Alzheimers' disease and Parkinsons' disease.

Currently, clinical trials are testing chaperone inhibitors such as an inhibitor of HSP in combination with proteasome inhibitors on cancer cell fate to control ER-Golgi homeostasis (see www.clinicaltrials.org) based on the fact that a high proteostasis capability may lead to a proliferation of cancer cells. It was demonstrated that the adaptive biology to proteostasis by developing an effective stress response mechanism, such as overexpression of chaperones like HSP-70, confers protection from proteotoxicity in cases of polyQ aggregates in Huntington's disease [61] or α–synuclein aggregates in Parkinson's disease pathogenesis [62].

Furthermore human HSF1 plays an important role in cancer cells, and its aberrant regulation can lead to disrupted signaling and malignant changes in DNA, protein, and energy metabolism thus promoting tumorigenesis [63]. Very interestingly, recent studies in humans have shown how HSF1 not only regulates disease though classic heat shock response and therefore HSPs, but also regulates transcriptional programs specific to malignant cells [34]. Hypoxia experiments in *C. elegans* focus on HIF-1, which is directly relevant to human disease. HIF1α activity has been used to induce angiogenesis (the growth

of new capillary blood vessels) for use in ischemic disease (disease of reduced blood supply). In models of hind limb ischemia, active HIF1α was shown to be beneficial alone or in combination with bone marrow-derived angiogenic cells [64, 65]. More information on drug development on the basis of HIF and the response to hypoxic stress can be found in [66].

Concluding remarks

Although there are clear limitations to using *C. elegans* stress response as a model for human disease, we suggest nematodes are an ideal first proxy for studies that can be followed-up on in closer models like rodents. One of the *C. elegans* limitations is that in the ILS pathway *C. elegans* only has one receptor for both insulin and IGF-1, whereas mammals have separate insulin and IGF1 receptors. The central role of this signaling pathway in many stress response and complex diseases poses a challenge to translating knowledge from worms to humans. Worms also lack specific respiratory organs, which is important to consider when hypoxia experiments are used to gain insight into human disease. Further, current *C. elegans* experiments are carried out in a single genotype, Bristol N2, which might lead to biased conclusions.

Despite these drawbacks, *C. elegans* provides researchers with a rapid, versatile system for exploring features of complex human diseases. The conservation of the components of the pathways responding to heat shock, hypoxia, osmotic, and oxidative stress makes these experiments valuable endeavors into understanding the genetics underlying various human diseases. As such, stress response experiments in *C. elegans* might provide potential new insights towards the development of new therapies that are translatable to the clinic.

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3

Uncovering genotype specific variation of Wnt signaling in *C. elegans*

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Abstract

The Wnt/β-catenin pathway is highly conserved among metazoans and plays an essential role in important cellular functions such as specialization, migration, adhesion and development. Mutant analyses of this pathway in *C. elegans* strain Bristol N2 have been widely studied. However mutations in a single genetic background do not reveal genome-wide allelic effects in natural populations, which often control complex traits. To gain more insight into the effect of the genetic background mutant screens should be performed in different genotypes.

This study investigates whether mutations in different genotypes reveal hidden polymorphic regulators in the Wnt/β-catenin pathway. Therefore we studied the phenotypic effects of *bar-1(ga80)* in a population of different *C. elegans* genotypes. Each genotype carries the *bar-1* mutation in a genetic mosaic background resulting from the recombination of two of the most divergent *C. elegans* strains genotypes: Bristol N2 and Hawaii CB4856. We quantified genome-wide gene expression and measured the vulval induction index across all genotypes. Quantitative genetics analysis identified loci on chromosome *I* and *II*, which are associated with vulval development phenotypes.

We show that natural genetic variation provides a powerful means to study the cryptic variation harbouring new players in Wnt/ β-catenin signalling.

Introduction

The vast majority of genetic analyses in *Caenorhabditis elegans* has been carried out using mutational knock-outs. The phenotypic effects of such mutations are usually studied in a single genotype, the conventional wild type strain Bristol N2. However, the phenotypes of induced mutations can vary widely depending on the genetic background [1, 2]. Moreover, induced mutations in one genetic background do not reveal the, often subtle, allelic effects that segregate in natural populations and contribute to phenotypic variation of complex traits [3].

For this reason there is a scanty knowledge about the phenotypic effect of a mutation in strains other than Bristol N2. This touches upon fundamental aspects if we want to understand the natural genetic architecture of complex phenotypic traits. Single mutations induced in Bristol N2 should be complemented with experimental manipulations, where the same mutation is introgressed into different genetic backgrounds. In particular this might be interesting in order to understand the function of disease genes and signalling pathways in more complex organisms, like mammals, for which *C. elegans* is an important model species. Over the past decade *C. elegans* has increasingly been used as a model organism for quantitative genetic studies aiming to unravel the genetic architecture of complex traits [4-10].

In the present study we addressed this issue for the Wnt/β-catenin signalling pathway by investigating the phenotypic effects of *bar-1(ga80)* loss-of-function in different *C. elegans* genotypes.

Wnt signalling is widely conserved along metazoans. The Wnt pathway controls vital biological functions such as intercellular communication, adhesion, cell migration, and cell specification. In *C. elegans* Wnt signaling is displayed in two different pathways, the canonical Wnt pathway, through β-catenin/BAR-1 effector, and the non-canonical Wnt pathway, which is β-catenin independent. The canonical Wnt signaling pathway plays an essential role in cell fate determination, cell polarity and migration in *C. elegans* [11]. In the canonical Wnt pathway WNT proteins are recognized by the trans-membrane receptor members of the Frizzled family. This recognition allows the disassembly of the destruction complex formed by APR-1, PRY-1 and GSK-3 homologs of the human proteins APC, Axin and GSK3 respectively. The destruction complex acts by rescuing BAR-1 in absence of WNT protein signal, and by facilitating its ubiquitination and subsequent degradation by the proteasome. When the complex is disassembled by activation of Frizzled, BAR-1 is dephosphorylated and released into the cytoplasm, where it accumulates. Some of the BAR-1 enters the nucleus, where it acts as a transcriptional co-activator of the transcription factor POP-1 (TCF) [12].

In the case of cell fate determination, vulval development in *C. elegans* has become an important model process to study. Here, Wnt signaling acts as an extracellular signal that controls vulval fate in the Vulval Precursor Cells (VPC) [13]. At early larval stages VPCs P3.p to P8.p localize in the ventral mid-body region. They undergo one of the three vulval cell fates 1°, 2°, or 3°. Cell fates 1° and 2° determine the formation of the vulva opening structure, while 3° cell fate is adopted by the companion cells that, after dividing one time, will fuse with the main syncytial epidermis that surrounds the animal, called hyp7. Other Pn.p cells will not divide and fuse directly with hyp7, so-called F cell fate [14]. In order to

determine division and subsequent following to one of the 3 vulval cell fates, P3.p to P8.p express the Hox gene *lin-39*, this expression is regulated by the Wnt pathway through β-catenin BAR-1 [15]. Wnt signaling via *bar-1* is necessary to maintain expression of *lin-39* in the P(3-8).p [16]. *lin-39* covers 2 functions in vulva development, it prevents VPCs from fusion with hyp7 and stimulates their cell division [17, 18]. In *lin-39* loss-of-function mutants P(3-8).p fuse to the epidermal syncytium hyp7 that surrounds the animal [14]. In *bar-1(ga80)* loss-of-function mutants the cell fate determination turns out to be altered with the formation of an aberrant vulva, which is not functional in most individuals. However *bar-1(ga80)* mutants can still reproduce because self-fertilized eggs hatch inside the progenitor resulting in a "bag of worms", when the cuticle of the mother is disrupted the offspring is released [14]. Prior to bagging and during oogenesis, *bar-1(ga80)* mutants show a characteristic phenotype of a bump in the cuticle at the place of the vulva, called protruding vulva (Pvl). Our observations showed that worms having protruding vulva, end up bursting at the vulva in later stages of the adulthood, probably due to the non-functional vulva unable to release eggs.

Concerning cell migration, research has provided ample support for the migration of the QL neuroblast progeny [16, 19, 20]. Forward genetic studies have revealed the mechanisms involved in this cell migration process. In the first larval stages there are two Q neuroblasts located in similar positions at the distal end of the worm. These QR (right) and QL (left) neuroblasts have to divide and migrate in opposite directions in the worm body. During the beginning of L1 development QL migrates a relatively short distance towards the posterior section of the body and divides generating neurons, while QR migrates a longer distance in opposite direction, to the anterior section of the body and also divides generating anterior neurons. Mutations in β-catenin *bar-1* (loss-of-function) showed an aberrant migration of QL, which adopts the same pattern as QR and also migrates towards the anterior section of the nematode body (in gain-of-function *bar-1* is the QR the one that inverts the direction) [12].

Another well-known example of cell migration controlled by Wnt signaling is the migration of the distal tip cells (DTCs) during gonad formation in *C. elegans*. Gonad migration occurs in 3 phases in L2, L3 and L4 stages respectively. The migratory movements of DTCs, in both anterior and posterior gonad arms, determine the morphology of the adult hermaphrodite gonad. The precise reproductive function is closely related to the correct migration of the gonad. There are many genes described as being related to gonad migration [21].

In humans, Wnt signaling pathway is crucial for development and tissue homeostasis [22]. Additionally, the Wnt pathway is strongly involved in cell proliferation [23]. Previous studies showed that the mis-regulation (hyper activation) of the Wnt pathway drives tumor formation [24-27] and evidence is emerging suggesting a relation between the Wnt pathway and metabolic disorders [28] and neurodegenerative diseases [29, 30].

This study attempts to gain insight into the quantitative subtle effects of natural alleles on the Wnt pathway. We have performed an experiment combining genotypic and phenotypic effects of a specific mutation affecting Wnt signalling pathway, *bar-1(ga80)* included in *C. elegans* Recombinant Inbred Lines (RILs) [8] and introgression lines (ILs) sets [4]. Mutation included recombinant inbred lines (miRILs) and Narrow Introgression Lines (miNILs) were generated as a result of crossing CB4856 and *bar-1(ga80)* (N2 genome

background). The miRILs are of great use for studying the effect of the *bar-1(ga80)* mutation in multiple combinations of the two different genetic backgrounds CB4856 and N2. We created a set of 50 miRILs to study the effect of a Wnt pathway mutation in natural variant mosaics (Figure A2). These miRILs were phenotyped and the results were used for QTL mapping in order to identify the loci and potential candidate genes affecting the *bar-1* phenotype.

We conducted a genome-wide analysis of the transcriptome using latest generation microarray in combination with QTL mapping techniques. In order to verify the polymorphic loci we phenotyped introgression lines containing a fragment of CB4856 introgressed in a N2 background in chromosome *I* and *II*. The cross progeny was allowed to self-fertilize for two generations. The obtained miNILs were genotyped to confirm the presence of the introgression and *bar-1* mutation and the ones containing both features in homozygosity were phenotyped for vulval development. To confirm the detected QTL we used the miNILs harbouring selected loci.

Materials and methods

Selection of *bar-1* mutation

In order to obtain the most informative phenotype of the Wnt pathway in mutant induced RILs, different mutants (with a mutation induced into an N2 background) where crossed with CB4856. The mutants used for this purpose were: *pry-1 (mu38)*, *lin-39 (n-1490)*, *bar-1 (ga80)*, and *bar-1 (gf)*.

The crosses consisted of mating males from CB4856 with mutant hermaphrodites. Selection of individuals from F1 was based on the expected phenotypes (e.g. multivulva (Muv), protruding vulva (Pvl), gonad migration defective, etc.). In the next generation after self-fertilization (F2) individuals were again selected based on suppression or enhancement of the expected phenotype. After the two rounds of selection, the individuals were allowed to self-fertilize for up to 12 generations yielding Recombinant Inbred Lines containing the mutation (miRILs). After screening the offspring of all these crosses, the selection of the final mutant was based on the feasibility of the crosses that showed the most informative phenotype.

miRILs generation

Mutation included recombinant inbred lines (miRILs) were generated by mating 4-5 *bar-1(ga80)* hermaphrodites with 12-15 CB4856 males. The F_1 generation was singled out on fresh NGM plates and allowed to self until F_2 offspring. F_2 *bar-1(ga80)* mutants were selected according to their protruding vulva (pvl) phenotype and singled out on fresh NGM plates to self until F_3 generation. From each clonal F_3 population one animal was singled out to produce the F_4 generation. This procedure was repeated until F_{12} generation eventually producing 50 miRILs. The miRILs were kept and maintained at 20°C on 55 mm NGM plates seeded with *E.coli* OP50. Strains were subsequently frozen and kept in liquid nitrogen to prevent any mutation accumulation or laboratory adaptation.

miRILs were initially phenotyped for the protruding vulva and later genotyped to assess homozygosity of the *ga80* allele using primer pairs OTS33/ OTS36 (OTS33: AGATAGACACACACACACAA; OTS36: GAGCATTGTTGCATGTTGGA).

Vulval induction index

The Vulva Induction index (VI) is a phenotypic readout for Wnt pathway activity, the higher the pathway activity, the more cells are induced and *vice versa*. VPCs P3.p to P8.p were checked for vulval development fate 1° and 2°. In wild type N2, VI is 3 while in the vulvaless phenotype VI is 0, in multi-vulva VI>3 and in *bar-1 (ga80)* is 2.3 ± 0.8 significantly different from wild types N2 and CB4856 (two-side t-test $p = 0.003$). In 55% of *bar-1* miRILs we also noticed an ex-vagination at the vulva opening, a phenotype called Protruding vulva (Pvl) [14] and in extreme cases the animals presented a bursting at the vulva that we named Exploding vulva (Exv).

The VI was determined by counting the induced vulval cells in late L3 to mid L4 hermaphrodites as described in [31] divided by the total number of counted animals. Deviations from the wild type pattern of 3 cells induced was regarded as vulval cell induction variant and in the case for *bar-1(ga80)* is referred as vulvaless (VI < 3).

miNILs genotyping

In order to generate mutant included (narrow) introgression lines (miNILs), we selected *bar-1(ga80)* virgin hermaphrodites in the L2/L3 stage and 3 to 5 late L4/adult males of each of the introgression lines (ILs) on two 12-wells plates (Greiner Bio-one, product No. 665180) containing nematode growth medium (NGM) with *E. coli* strain OP50 as a food source [32], and we allowed them for insemination. The crosses were performed at 20˚C. Males were removed when the hermaphrodites reached the adult stage. From six different crosses, 60 young nematodes (F1) were transferred to separate wells of a 12-wells plate. The hermaphrodites of these crosses were allowed to self for one generation in order to produce a new generation (F2). From these F2, hermaphrodites were singled to produce F3 by self-fertilization. The progeny generated after F3 nematodes was genotyped. Nematodes were washed off with 1.0 mL of M9 [32], collected in 1.5 mL Eppendorf tubes, and centrifuged for 20 seconds to spin down. Supernatant was discarded until approximately 25 µL of pellet. From these samples, DNA was isolated as described by Holterman *et al*., 2006 [33]. Fragment Length Polymorphism (FLP) mapping was carried out using the SNPs on the two ends of the introgression (Table 1). SNPs were chosen from a set previously described by Doroszuk *et al*. (2009) [4].

Table 1. Chromosome *II* ILs. ILs containing a fragment of CB4856 (grey) introgressed into Bristol N2 (black) genomic background in Chromosome *II*. The SNPs used for genotyping are indicated in yellow.

			Lines				
SNP marker name	**Genomic position (bp)**	**SNP marker number**	**N2**	**CB**	**ewIR20**	**ewIR21**	**ewIR23**
pkP2101	176721	**21**					
pkP2135	1270097	**22**					
pkP2136	1683953	**23**					
pkP2114	2755074	**24**					
egPE201	3403575	**25**					
egPF202	4147051	**26**					
egPG203	4800868	**27**					
pkP2147	5761743	**28**					
pkP2106	6427387	**29**					
egPJ204	7257517	**30**					
pkP2151	8195651	**31**					
egPL205	8748358	**32**					
pkP2109	9401070	**33**					
pkP2124	10414073	**34**					
pkP2110	11180836	**35**					
egPP206	11752128	**36**					
egPQ207	12630696	**37**					
pkP2116	13235559	**38**					
egPS208	13980541	**39**					
egPT209	14758293	**40**					

miNILs phenotyping

Eggs were obtained using a highly concentrated bleach solution (6 mL NaClO, 0.5 mL NaOH (10 M), 3.5 mL MQ). Gravid hermaphrodites were bleached, followed by the re-suspension of the eggs in M9 liquid medium and dropped in a 6cm plate (procedure called micro-bleaching). Two replicates were included per line.

The eggs were allowed to hatch and reach adult stage for 48-72 hours at 20˚C. After this time the following characteristics were scored: 1) total number of worms, 2) number of worms presenting wild type (normal vulva) phenotype, 3) number of worms presenting protruding vulva phenotype (Pvl), 4) number of worms presenting exploding vulva phenotype (Exv), 5) number of worms presenting bagging phenotype, and 6) occurrence of offspring (eggs or larva).

Transcript profiling

The transcript profiles of the most genetically divergent miRILs (36 mRILs in total) were measured using genome-wide microarrays. The transcript levels of the two parental strains, *bar-1(ga80)* and CB4856, used for the generation of the miRILs were measured twice. We used mixed populations to isolate RNA.

RNA isolation was conducted using the RNeasy Micro Kit from Qiagen (Hilden, Germany), we optimized the protocol provided with the kit. In order to have the highest yield of *C. elegans* culture pellets, the lysis procedure was performed before the protocol. To frozen nematode pellets, 150 µl RLT buffer, 295 µl RNAse-free water, 800 µg/ml proteinase K and 1% ß-mercaptoethanol was added after subsequently incubated in a Thermomixer (Eppendorf, Hamburg, Germany) at 55°C and 1200 rpm for one hour. After centrifugation the protocol ‘Purification of Total RNA from Animal and Human Tissues’ provided with the kit was carried out.

RNA labeling, was done following the Agilent ‘Two-Color Microarray-Based Gene Expression Analysis; Low Input Quick Amp Labeling’ -protocol, version 6.0, starting from step 5. The extracted RNA was processed for microarray performance; the platform used for that purpose was *C. elegans* (V2) Gene Expression Microarray 4x44K (Agilent technologies), following manufacturer instructions.

Agilent High Resolution C Scanner was used to scan the microarrays. The settings used for this purpose were the ones that are recommended in the above mentioned manual. Data was extracted with the Agilent Feature Extraction Software version 10.5, following manufacturers’ guidelines.

Normalization and processing was done with the “Limma” package for the “R” statistical programming environment. No background correction of the RNA-array data was performed as recommended by [34]. For within-array normalization of the RNA-array data we applied the Loess method [35] and for between-array normalization the Quantile method was used [36]. The obtained normalized intensities were used for further analysis.

Expression (e)QTL mapping

QTLs were found by using a single marker model on the average Vulval Induction index (VI) per genotype. The two most significant markers for VI were used as co-factors in a Multiple QTL Model (MQM), using a 6 marker exclusion window around the co-factor. Genome wide thresholds were determined by a 1000 permutations per trait per genome wide scan. We listed the most significant p-value per scan and from this list took the 950th most significant p-value as threshold.

Gene expression markers were added by taking the genes with the strongest and most significant *cis*-eQTL effect and using them as markers. In this way we could place extra markers increasing the total number of markers to 124. These markers were only used for finding gene expression QTLs.

Expression QTLs were detected by single marker mapping on the normalized log2 single channel data using the combined FLP and gene expression marker-set (GEMs). QTL mapping method was also described in previous studies [8, 37]

The threshold was determined by permutation. We used 45,220 permutations (one for each spot) to correct for multiple testing. The threshold was set to the -log10(p) level by which the number of spots with an eQTL from the permutation set divided by the number of spots with an eQTL of the original set was less than 5%. FDR 0.05, -log10(p) > 4.4.

eQTL confirmation using introgression lines

We confirmed 2 *trans*-bands related to vulva induction on chromosome *I* and *II*. In order to confirm these eQTL we used introgression lines containing a locus of CB4856 introgressed in N2 genomic background and we crossed these lines with *bar-1(ga80)*. Using this method we aimed to detect the possible modifier locus of the Wnt pathway that, according to our QTL analysis is polymorphic between N2 and CB4856. In this way we isolated the possible modifier expressing it in a mutant *bar-1* background.

NILs containing introgressions of CB4856 into a N2 genome in the regions of interest (chromosome *I* and *II*) were used. Males from these lines were selected to mate with *bar-1(ga80)* hermaphrodites. Consecutive selection of successful crossings was done in F1 and F2 generation. After F3 a selection on expected (different from wild-type, e.g. clumsy movement, abnormal vulva, etc.) phenotype was done. After few generations of single animal isolation, we obtained inbred lines. Once we obtained the miNILs they were phenotyped and genotyped (Supplementary table S1).

For the phenotyping of the new miNILs, vulva morphogenesis was the only considered phenotype due to its efficient scoring and differentiation. We phenotyped the new miNILs for vulva morphology following *visual* morphological criteria to classify them as i) normal vulva (wild type), ii) protruding vulva (Pvl) or iii) exploded vulva (Exv).

Results

Mutant Induced Recombinant Inbred Lines

We selected a final set of genetically most diverse 50 miRILs (Figure 1). This selection was based on the SNP genotypes and reduced the number of too many repetitive genotypes while maintaining genotypic diversity. These miRILs were phenotyped and the results were used for QTL mapping.

We observed that the genotypic distribution per marker was not equal across the genome (Figures 2 and 3). Chromosomes *II* and *IV* showed equal distribution for each allelic type, whereas in chromosome *III* N2 alleles prevailed over the CB4856 ones. The largest distortions were found on chromosomes *I*, *V* and *X*. On chromosome *X* the N2 genotype predominated as it would be predicted since N2 alleles are linked to the *bar-1* mutation, which is located on this chromosome. On the top of chromosome *I* the N2 genotype is over-represented, possibly because of the genome incompatibility between N2 and CB4856 [38]. The complete fixation of the CB4856 genotype at marker 60 was unexpected and could indicate an interaction between this locus and the *bar-1* mutation.

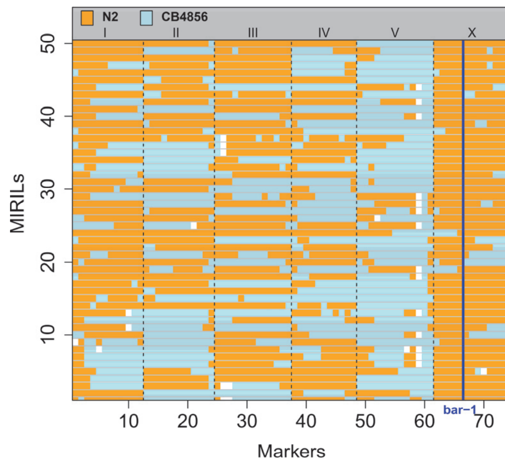

Figure 1. miRILs overview. Genotypes of the 50 miRIL population. Chromosomes are indicated by roman symbols in the top grey bar. N2/bar-1(ga80) genotype is shown in orange and CB4856 genotype in light blue. The physical position of bar-1 on chromosome X is shown by the vertical blue line. Marker numbers are on the x-axis, miRIL numbers are on the y-axis. Chromosome borders are shown as dashed black lines.

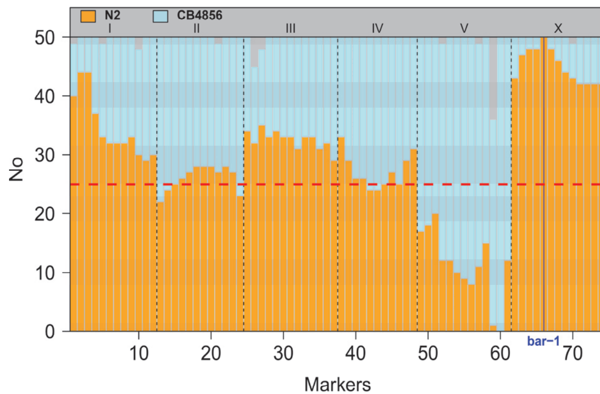

Figure 2. Genotypic distribution per marker. Number of individuals (x-axis) with N2/*bar-1(ga80)* genotype (orange) or CB4856 genotype (light blue) per marker (y-axis). Not available data are shown in grey. Chromosomes are indicated by roman symbols in the top grey bar. Chromosome borders are shown as dashed black lines. Red horizontal dashed lines shows the point where 50% of the lines would have the N2/*bar-1(ga80)* genotype and 50% would have the CB4856 genotype. The physical position of *bar-1* on chromosome *X* is shown by the vertical blue line.

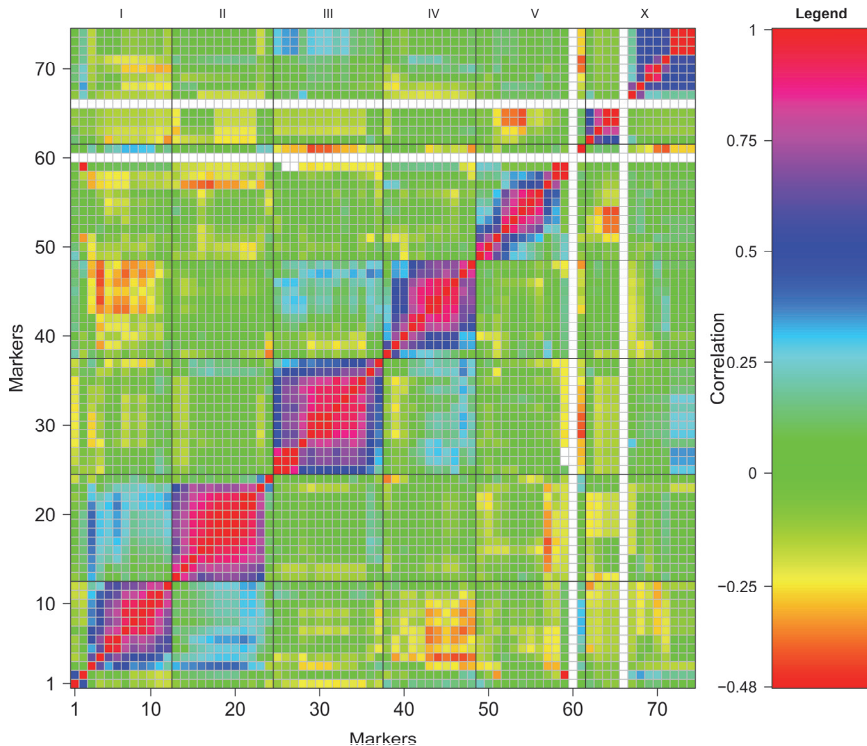

Figure 3. Correlation between markers. FLP marker number is shown on the x- and y-axis. Correlation shown as a colour range shown by the legend. Most markers on the same chromosome are highly correlated, whereas between chromosomes markers are not correlated.

Phenotypic variation

Vulva Index (VI) was screened in the miRILs. We measured variation in VI across the miRILs. A heritability of ~93% (mean sum of square between lines / (mean sum of square between lines + mean sum of square within lines)) shows that the variation between the miRILs is largely caused by the genetic background. About 60% of the miRILs had a VI phenotype between 2.4 and 3, comparable to one of the parents (Figure 4). About 30% of the lines had a significantly higher VI than *bar-1(ga80)* and were phenotypically indistinguishable from CB4856. This means that at least one locus with a CB4856 allele, which counter-acts the *bar-1* loss-of-function effect on VI must be present. Another 30% of the miRILS had a VI phenotype comparable to the *bar-1(ga80)*. Interestingly, the other miRILS showed a lower VI than *bar-1(ga80)*. Two miRILs, BR39 and BR57 showed hardly any induction and about 10 miRILs have only 1 to 2 cells induced on average.

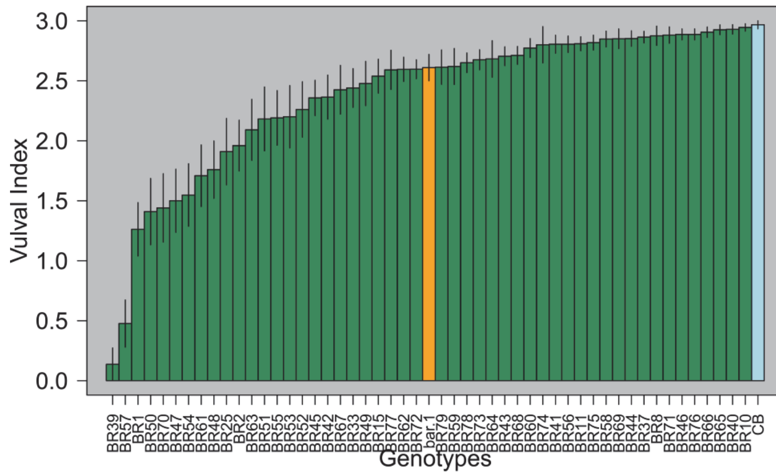

Figure 4. Distribution of the Vulval Index (VI) in the *bar-1* mutation included Recombinant Inbred Lines (miRILs). miRILs are shown on the x-axis, VI on the y-axis. *bar-1(ga80)* mutant highlighted in orange, CB4856 highlighted in light blue. The *bar-1* mutant and CB4856 have a significantly different VI (p<0.005; two-side t-test unequal variance assumed).

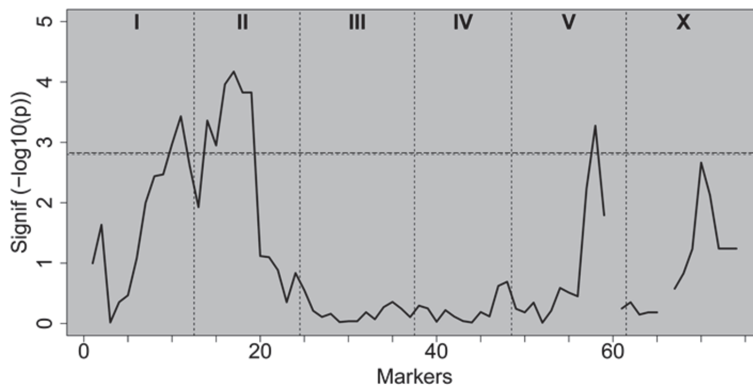

Figure 5. QTL profile of Vulval Index (VI). Markers on the x-axis. Chromosomes are shown on top, chromosome borders are indicated by the dotted vertical lines. Permutation (0.05) threshold is shown by the dashed horizontal line. The significance and the effect of the link per marker are shown on the y-axis. To draw the line per trait, the -log10 (p-value) multiplied by the sign of the QTL effect was used. The effect is the effect of the N2/*bar-1(ga-80)* allele compared to the average phenotype of the population.

QTL results of the modified phenotypes

We found two major QTLs, on chromosome *I* and chromosome *II* (Figure 5). Two other QTLs were found, one on chromosome *V* and another on chromosome *X*. These QTLs are however less reliable as the markers are not equally distributed across these loci.

Gene expression variation by genetical genomics

We measured the genome wide transcription profiles of 36 *bar-1(ga80)* miRILs to investigate the effects of *bar-1(ga80)* mutation on gene expression regulation. With this data we could map eQTLs and investigate which loci are modifying the *bar-1* related gene expression patterns.

With a single marker model we found almost 2350 genes to have an eQTL (Table 2, Figure 6). About 50% of these genes were in *cis* (a local polymorphism close to the causal gene of the eQTL) and the other half were in *trans* (a polymorphism distant from the causal gene of the eQTL). Most of the *trans*-acting eQTLs were grouped in two major *trans*-bands. These co-located with the QTLs for VI phenotype on chromosome *I* and *II*. The presence of these two major *trans*-bands points towards the existence of at least two polymorphic modifiers between CB4856 and N2/bar-1(ga80), which are dependent on the *bar-1* mutation.

Table 2. Number of spots/genes with an eQTL at two different FDR thresholds. Number of genes does not equal the number of spots as some genes are represented by multiple spots. The total number of genes is also split up in those that have a *cis* and those that have a *trans* eQTL.

At –log10(p)	3.9 FDR0.1	4.4 FDR0.05
Spots	3432	2392
Genes	2349	1629
Cis	1212	1025
Trans	1169	622

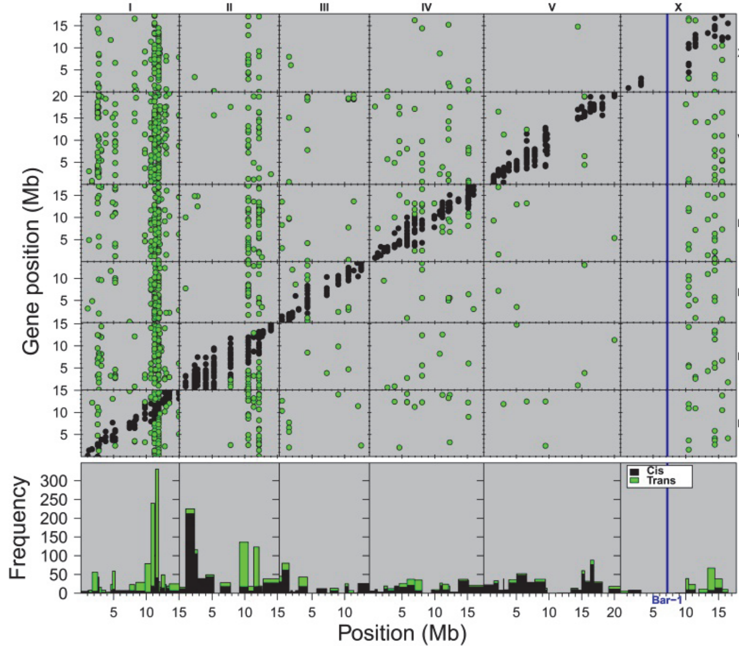

Figure 6. Genome wide overview of eQTL peaks per gene transcript. On the x-axis the position of the makers is shown. On the y-axis of the lower panel the number of significant eQTLs per marker is given. The y-axis of the upper panel shows the position of the gene transcripts (with a significant eQTL). In green the *trans*-eQTLs and in black the *cis*-eQTLs. The blue vertical line shows the position of *bar-1*, which has the loss-of-function *ga80* mutation in the population used for mapping these eQTLs. Chromosomes are indicated at the top and right side of the figure.

Confirmation of eQTLs through miNILs

We confirmed the eQTLs found on chromosome *I* and *II*. We generated *bar-1* miNILs carrying the mutation together with a CB4856 locus. The *bar-1* induced mutant introgression lines spanned a region slightly smaller than 300 Kbp limited by the genomic regions 10,740,532 bp (~10,740Kbp) and 10,443,852 bp (~10,444Kbp) harboring 100 genes approximately on chromosome *I,* and a 55 Kbp region on chromosome *II* mapped between the genomic regions 2,852,907 bp (~2,853Kbp) and 2,907,665 bp (~2,908Kbp) where we harbored nearly 15 described genes.

Each selected line was phenotyped for vulva morphology and therefore classified as normal vulva (wild type), Pvl or Exv. The miNILs were also genotyped using the SNPs on the two ends of the introgression (Table 1). We observed a more severe phenotype in the lines containing the introgression than in *bar-1 (ga80)* alone (Figure 7).

Using Wormbase database version WS238 and investigating the polymorphic genes between N2 and CB4856 that belong to the introgressed region, we found candidate modifiers for the Wnt pathway. These loci are placed within the regions of interest on chromosome *I* and *II*. Following the previously mentioned criteria we found the following genes as most promising candidate as canonical Wnt pathway modifiers: *mus-101* (on chromosome *I*), which has a crucial role during germ line proliferation and embryonic development and *sqv-2* (chromosome *II*) that acts in cytokinesis of one-cell embryos and in vulval morphogenesis [39].

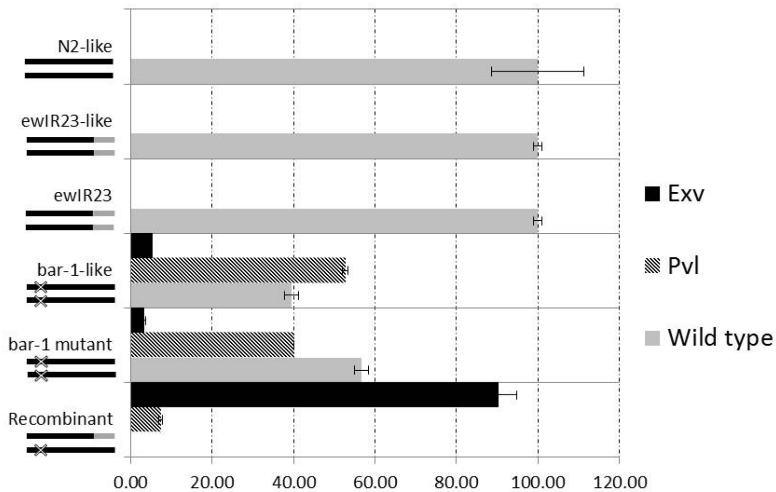

Figure 7. Percentage Vulva morphology. Populations of 20-30 nematodes from the different genotypes were phenotyped for vulval morphology. In blue the % of nematodes showing normal wild type vulval morphology, in red the % of protruding vulva (Pvl) and in green the % of exploding vulva within the studied populations. N2-, ewIR23- and *bar-1*-like refer to offspring lines proceeding as a result of the crossing between *bar-1(ga80)* and ewIR23 that present the genotype from only one of the parents (ewIR23-like and *bar-1*-like) or that did not integrate the introgression and neither the mutation (N2-like).

Discussion

Natural variation

Our genotypes are genetic mosaics of divergent *C. elegans* strains containing the *bar-1 (ga80)* loss of function. In this study we explored the genetic mechanisms behind vulva development and the quest for novel effectors of the Wnt signalling pathway. On the basis of well-known *C. elegans* genotypes and phenotypes of Bristol N2, CB4856 and *bar-1 (ga80)*, and combining them together, we attempted to unravel new pathway effectors. The eQTL results revealed the loci, which were affected by the *bar-1* mutation and the genetic polymorphisms existing between CB4856 and N2/*bar-1(ga80)*. The genes mainly affected by the genetic polymorphisms described *cis*-eQTLs, while the genes affected by the *bar-1* mutation have a *trans*-eQTL when the effect of the *bar-1* mutation is modified by the CB4856 background.

To get more insight in the biological processes affected by the *bar-1* mutation on gene expression level and modified by a polymorphic modifier between N2 and CB4856, an enrichment study was performed on genes with an eQTL. We used 5 groups: i) all genes with an eQTL, ii) all genes with a *cis* eQTL, iii) all genes with a *trans* eQTL, iv) genes with a *trans* eQTL mapping to the major *trans*-band on chromosome *I*, and *v*) genes with a *trans* eQTL mapping to the major *trans*-band on chromosome *II* (Figure 6).

β-catenins in *C. elegans*

C. elegans has 4 genes encoding β-catenin: *bar-1*, *hmp-2*, *sys-1* and *wrm-1* (this is quite unique among sequenced metazoans). Despite they are homologs, it is well described that they regulate Wnt signalling in different cellular functions. WRM-1 acts during embryogenesis, HMP-2 intervenes in cell adhesion, SYS-1 during gonadogenesis and BAR-1 mediates in cell fate and cell migration [11]. BAR-1 is the only β-catenin that interacts with the transcription factor POP-1 and both proteins together activate the expression of the Hox genes such as *lin-39* [40, 41]. The involvement of this gene in vulval development and the clear phenotype revealed in loss-of-function mutant *bar-1(ga80)* pointed at *bar-1(ga80)* as a very suitable Wnt pathway member to be used for our purpose.

Wnt pathway related to complex human diseases

Wnt pathway is well conserved among metazoans and plays an essential role in tissue homeostasis. The malfunction of this signalling pathway may result in several human diseases [24] including different cancer types [42]. Deciphering the effectors that play a direct or indirect role in such a crucial biological mechanism may help for a better understanding of the complete pathway and interacting modifiers that are related to it as well as in the development of therapeutics to counteract the associated disease phenotypes.

More specifically in the case of the canonical Wnt pathway, in which β-catenin is the main player, it is well described that β-catenin is closely associated with complex human diseases such as cancer and neurodegenerative diseases [43, 44]. A recent model in *C. elegans* shows how *bar-1* and *sir-2.1* trigger the ability of *daf-16* to activate *upc-4* expression, a homolog of UPC2 (mitochondrial uncoupling protein), which is an effector in Huntington (htt) toxicity in mammals [29].

Regulatory regions

From the genes with a *trans* eQTL mapping to the major trans-band on chromosome *II* (Table 2), we focused on the ones that were polymorphic between N2 and CB4856 and therefore potential candidates that contributed to the genetic variation in Wnt pathway signaling between these two *C. elegans* strains. Following this criterion and after narrowing down the regulatory region in chromosome *II* to 55Kb using miNILs, the results pointed at the galactosyltransferase II gene *sqv-2* as a most suitable candidate gene to be a modifier of canonical Wnt pathway signaling.
The *C. elegans* gene *sqv-2* encodes a glycosaminoglycan galactosyltransferase II that is required for cytokinesis of one-cell embryos and for vulval morphogenesis. We suggest this gene as an external effector of canonical Wnt pathway in *C. elegans*. Preliminary results showed a strongly enhanced vulva defective phenotype in double loss-of-function mutants *bar-1/sqv-2* compared to *bar-1* defective mutants (data not shown). The human homolog of

sqv-2 is galactosyltransferase II (β3GalT6), encoded by B3GALT6 [45]. Recent studies described mutations in the B3GALT6 gene identified in connective tissue disorders [46]. Also recently some assays in therapeutics are focusing in Wnt pathway as therapy in the treatment of human disease [47].

Hwang *et al*. [39] described *sqv-2* as an effector in *C. elegans* vulva invagination morphogenesis by acting in the biosynthesis of chondroitin and heparan sulphate. They showed that epithelial invagination during vulval development in *C. elegans* is strictly regulated by the biosynthesis of glycosinaminoglycans (GAGs), in which SQV-2 catalyzed the addition of galactose to a disaccharide acceptor, Galβ1,4Xylβ-O-benzyl.
Additionally, other studies have been conducted showing the utilization of modifiers of the Wnt pathway in stem cells for regeneration of tissues in injury repair [22, 48, 49].

Further research focusing on *sqv-2* and its functional mechanisms might open up a new path in diagnostics and therapeutics of Wnt associated human diseases [24, 27].

Genetical genomics plus *bar-1*: advantages of mutation introgression

The combination of QTL mapping based on *C. elegans* natural variants together with the insertion of a mutation turned out to be a promising approach to uncover novel regulatory regions and pathway modifiers. The development and utilization of this method have allowed us to detect novel polymorphic modifiers and to uncover cryptic variation in two genetic backgrounds. By generating a wide-range set of miRILs and mapping their gene expression QTLs we designed a new way, which allows for describing genetic networks of complex pathways as well as their genetic variation. Additionally, the use of ILs helped us to confirm experimentally the loci of polymorphic modifiers.

Therefore the current genetic variation between the strains resulted in phenotypic variation in the case of a mutation integrated into a different background. The use of RILs and ILs allowed us to identify and define the region responsible for the modification of the phenotype and thus the pathway we are studying. In addition we have proven in this study how including mutations such as *bar-1(ga80)* (usually introgressed into a typical Bristol N2 background) into the CB4856 genetic background emerges as a powerful tool in order to uncover cryptic genetic variation that might be associated with pathways, such as Wnt signalling in *C. elegans.*

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Supplementary data

Supplementary table S1. Markers used for SNPs genotyping. *Primer orient.* stands for primer orientation. F means forward primer and R stands for reverse primer.

<table>
<tr><th>Line</th><th>Polymorphism</th><th>Primer orientation</th><th>Oligonucleotides</th><th>Restriction Enzyme</th><th>Restriction Lengh in N2 (bp)</th><th>Restriction length CB4856 (bp)</th></tr>
<tr><td rowspan="4">ewIR20</td><td rowspan="2">pkP2101</td><td>F</td><td>aagaggtgttcttctgcagc</td><td rowspan="2">Dra I</td><td rowspan="2">217, 402</td><td rowspan="2">619</td></tr>
<tr><td>R</td><td>accatacacgcagttcattc</td></tr>
<tr><td rowspan="2">pkP2114</td><td>F</td><td>gcgatttgacctctgtaagg</td><td rowspan="2">Alu I</td><td rowspan="2">647</td><td rowspan="2">274, 373</td></tr>
<tr><td>R</td><td>tccatctccagttgtcgttc</td></tr>
<tr><td rowspan="4">ewIR21</td><td rowspan="2">pkP2101</td><td>F</td><td>aagaggtgttcttctgcagc</td><td rowspan="2">Dra I</td><td rowspan="2">217, 402</td><td rowspan="2">619</td></tr>
<tr><td>R</td><td>accatacacgcagttcattc</td></tr>
<tr><td rowspan="2">egPF202</td><td>F</td><td>ggaaccattgatggagttcg</td><td rowspan="2">Kpn I</td><td rowspan="2">104, 145</td><td rowspan="2">249</td></tr>
<tr><td>R</td><td>atactaccctcacctcgtag</td></tr>
<tr><td rowspan="4">ewIR23</td><td rowspan="2">egPE201</td><td>F</td><td>aggagtttgccgtactactg</td><td rowspan="2">Alu I</td><td rowspan="2">69, 114, 319</td><td rowspan="2">183, 319</td></tr>
<tr><td>R</td><td>taaaaggcattctccggtgg</td></tr>
<tr><td rowspan="2">pkP2124</td><td>F</td><td>tagcaaacaaagtgttctcgg</td><td rowspan="2">Apa LI</td><td rowspan="2">13, 155, 262</td><td rowspan="2">13, 417</td></tr>
<tr><td>R</td><td>gtgattccgtgcacataccg</td></tr>
<tr><td rowspan="2">bar-1</td><td rowspan="2">bar-1(ga80)</td><td>F</td><td>agatagacacacacacacaa</td><td rowspan="2">Mse I</td><td rowspan="2">450</td><td rowspan="2">100, 100, 250</td></tr>
<tr><td>R</td><td>gagcattgttgcatgttgga</td></tr>
</table>

4

Genetic variation for stress-response hormesis in *C. elegans* life span

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Abstract

Increased life span can be associated with greater resistance to many different stressors, most notably thermal stress. Such hormetic effects have also been found in *C. elegans* where short-term exposure to heat lengthens the life span. Genetic investigations have been carried out using mutation perturbations in a single genotype, the wild type Bristol N2. Yet, induced mutations do not yield insight regarding the natural genetic variation of thermal tolerance and life span. We investigated the genetic variation of heat-shock recovery, i.e. hormetic effects on life span and associated quantitative trait loci (QTL) in *C. elegans*. Heat-shock resulted in an 18% life span increase in wild type CB4856 whereas N2 did not show a life span elongation. Using recombinant inbred lines (RILs) derived from a cross between wild types N2 and CB4856 we found natural variation in stress-response hormesis in life span. Approx. 28% of the RILs displayed a hormesis effect in life span. We did not find any hormesis effects for total offspring. Across the RILs there was no relation between life span and offspring. The ability to recover from heat-shock mapped to a significant QTL on chromosome *II*, which overlapped with a QTL for offspring under heat-shock conditions. The QTL was confirmed by introgressing relatively small CB4856 regions into chromosome *II* of N2. Our observations show that there is natural variation in hormetic effects on *C. elegans* life span for heat-shock and that this variation is genetically determined.

Introduction

Hormesis is defined as a favourable biological response to low level exposure to toxins and other stressors. Already reported in 1888 for the effect of toxic compounds in yeast [1], hormesis was formally defined in 1943 [2] as reviewed by Calabrese *et al*., (1999) [3] for numerous toxicants. Over the past decade, the concept of hormesis found its way into aging and life span research [4, 5]. Increased life span can be associated with greater resistance to many different stressors, most notably thermal stress. Temperature elevation has a pervasive effect on almost all developmental, cellular and molecular processes. Following a sudden increase in temperature all cells exhibit a heat shock response, a highly conserved program of stress-inducible gene expression, to prevent cellular degeneration and increase thermal tolerance. Thermal tolerance and life span are intrinsically related and much research has been carried out in the nematode worm *Caenorhabditis elegans*, which serves as an important model in aging and life span research [6]. Short-term exposure to heat lengthens the life span of *C. elegans* [5, 7-11] and there is a strong genetic component involved in life span increase [6]. Studies on the mechanistic relationship between stress response and altered life span was reported for the insulin like signaling (ILS) pathway and the induction of heat shock proteins (HSP), which rescue miss-folded native proteins [12]. Also in other species sudden stress factors, like a brief heat shock, can have hormetic effects and extend life span by induction of intrinsic stress responses for an example in *Drosophila* spp. [13].

Most research on hormetic effects of thermal stress on life span in *C. elegans* has been carried out in induced mutants. Yet, induced mutations do not yield insight regarding the natural genetic variation of thermal tolerance and life span [14]. Moreover, life span is a complex trait, controlled by multiple genes or loci. These can be detected and identified using recombinant inbred lines (RILs) for genetic analysis of longevity and thermo tolerance phenotypes, and RILs can be used to identify quantitative trait loci (QTL) for life span under experimentally controlled thermal environments [15].

Here we investigated the genetic variation of heat-shock recovery, i.e. hormetic effects on life span and offspring, and associated loci in *C. elegans*. We also measured life span in relation to offspring because it is widely recorded, that these two traits are tightly linked. For many species it is shown that more offspring often decreases life span and that increased life span comes with the cost of reduced offspring [16]. Over the past decade *C. elegans* has increasingly been used as a model organism for quantitative genetic studies [17, 18]. In particular, the quantitative genetic control of responses to abiotic environmental cues has received a great deal of attention. For instance Gutteling *et al*., (2007a,b), Kammenga *et al*., (2007) and Li *et al*., (2006) studied the influence of temperature and pesticides [19-24] on the genetic response in a range of different traits, ranging from life-history traits to gene expression profiles.

To gain insight into the subtle effects of natural variants and the genetics of heat-shock recovery and life span and offspring, we have performed an experiment combining genotypic and phenotypic effects of heat-stress in *C. elegans* RILs. These RILs were derived from a cross between parental wild types N2 (Bristol) and CB4856 (Hawaii), the two most genetically divergent strains in *C. elegans*. The cross was described by Li *et al*., (2006; 2010), Viñuela & Snoek *et al*., (2010), Viñuela *et al*. (2012) and Elvin & Snoek *et*

al., (2011) [22, 24-27]. We used a subset of 58 RILs to study the genetic variation of the heat-shock. Our observations show that there is natural variation in hormetic effects on life span for heat-shock and that this variation leads to modulation of life span and offspring. We detected a QTL affecting the ability to recover from heat-shock on chromosome *II*.

Materials and Methods

Nematode cultures and RILs

[22] generated approx. 1500 RILs of which 80 were randomly chosen and genotyped with 121 SNPs, evenly distributed across the genome [22]. Another 120 were randomly chosen from the set of 1000 and genotyped with 96 SNPs. From these 200 RILs, we selected 58 of the most divergent ones. From Li *et al*., (2006) [22] we used 33 strains WN1, WN11, WN12, WN13, WN14, WN15, WN16, WN17, WN18, WN19, WN2, WN20, WN21, WN3, WN30, WN33, WN34, WN35, WN36, WN37, WN4, WN43, WN49, WN5, WN6, WN62, WN7, WN70, WN72, WN73, WN8, WN80, and WN9. From Elvin & Snoek *et al.*, (2011) [25] we used WN111, WN185 and WN186. The 22 newly added strains are WN118, WN119, WN120, WN122, WN127, WN136, WN137, WN144, WN146, WN149, WN150, WN151, WN152, WN164, WN179, WN197, WN82, WN88, WN91, WN94, WN95, and WN96. The genotypes of these 22 new RILs are reported in this study. All strains were grown in nematode growth medium (NGM) with *Escherichia coli* strain OP50 as a food source [28]. Nematode populations were maintained at 12°C. Prior to each experiment, the strain cultures were age-synchronized by bleaching [29] to obtain fourth larval stage nematodes (L4).

Genotyping the new RILs

The new recombinant inbred lines were genotyped for 96 SNP by Illumina “Golden gate” SNP genotyping [30] (Supplemental Table S1). SNPs correspond to previously used SNPs to genotype 80 RILs [22].

Selecting the exposure time for heat shock

We reasoned that the heat shock period should be long enough to have an effect on life span and short enough so that not all worms would be killed. Therefore, we first carried out a pilot experiment with N2 and CB4856. We decided to expose the worms during the L4 stage because at this stage the full gene expression machinery is active and most genes are differentially expressed between N2 and CB4856 [31]. To make sure we exposed all worms at the right stage N2 and CB4856 strains were synchronized to L4 stage at 16°C. At this temperature development does not proceed too fast so that worms can be staged reliably.

Nematodes were synchronized by bleaching to reach L4 stage. The dishes were rinsed with 1 mL M9 buffer [28] to a 1.5 mL Eppendorf tube and centrifuged (20,817 G, 20 sec.). The supernatant was discarded and 800 µl bleach solution (2 mL NaClO, 0.5 mL NaOH (10 M), 7.5 mL MQ) was added. The solution was mixed for three minutes and after centrifugation, the supernatant was discarded and immediately 1 mL of M9 buffer was added. Samples

were centrifuged (20,817 G, 20 sec.) and the pellet containing the eggs was placed in a fresh 6 cm petri dish with NGM and *E. coli* OP50.

L4 larvae were transferred individually into twelve-well dishes (Greiner bio-one, product No. 665180) with NGM and *E. coli* OP50. Several replicates from each strain were transferred (12 individuals per strain and treatment) and exposed to 35°C for 4, 6 and 10 hours at heat stress conditions (after which the worms were placed at 20°C to recover) and to 20°C during their whole life as control treatment [11]. Survival and total number of eggs were measured daily. Supplemental Table S1 shows that a heat shock period of 4 hrs. was sufficient to have a strong effect on life span and offspring. Longer exposure periods caused a very high mortality (average of 40% at 6 hrs. and 92% at 10 hrs. for both strains). This was in agreement with survival assays by Wiegant *et al*. (2009) [32] who used a 35°C, 3 hrs. heat-shock.

Heat shock and recovery experiment

Experiments with 58 RILs, CB4856 and N2 were started by picking 12 nematodes at the first or second larval stage (L1 or L2) and subsequently placing these individually in a 12 wells plate with NGM and *E. coli* OP50 [28]. The nematodes were incubated at 20°C and allowed to grow. After laying eggs, the hermaphrodites were transferred to freshly made Petri dishes with NGM and *E. coli* OP50 and the wells containing males were discarded. The set of 58 RILs was divided into 6 batches and one of the parental strains (N2 or CB4856). These were bleached at 16°C (see above) to obtain L4 larvae.

Life span and offspring assay and defining hormesis

Both life span and offspring were measured for the same individual worm for each RIL and the parents in order to relate these two traits within the same individual rather than longitudinally. L4 were exposed to 35°C for 4 hours and the control population was incubated at 20°C. Stress treated worms were then permitted to recover at 20°C (Supplemental Figure S1). We tested: 58 x 12 wells x 2 treatments = 1392 RILs, 3 batches x 12 x 2 = 72 N2 worms and 3 batches x 12 x 2 = 72 CB4856 worms.

Life span and offspring (offspring = juveniles + unhatched eggs. Unhatched eggs are eggs that failed to hatch) were measured daily after the heat exposure. Our experiments were set up in such a way that we did not find vital egg stages, only juveniles and unhatched eggs. The unhatched eggs could be due to heat-shock induced egg mortality or a failure of egg self-fertilization. The nematodes were categorized as dead when they failed to respond to gentle prodding from a wire. Offspring was measured by transferring the adults to fresh multi-well plates and the juveniles and non-hatched eggs were counted. Lost, desiccated and bagged worms were excluded.

We defined hormesis as a prolongation of life span after heat-shock, compared to the control. For offspring we defined it as increased offspring after heat-shock, compared to the control.

Statistical analysis and QTL mapping

Differences between treatments and parental strains and RILs were tested with a 2-tailed *t*-test for samples of unequal variance. The scored phenotypes were averaged per genotype. These mean values were used in QTL mapping. QTL mapping was done by a linear model in which the 121 (96) genetic markers were used one by one to explain the variation between the RILs. We used backwards selection on 30 equally spaced initial cofactors (five per chromosome), the maximum number of co-factors in the final model was 6 and the threshold for excluding a background marker each round was $p > 0.05$. After cofactor selection a genome-wide QTL profile was calculated by excluding the cofactors within a 6 marker window of the marker under study. We used 1000 permutations to determine the thresholds for the mapped QTLs. The phenotypic values were randomly distributed over the genotypes at each permutation. Heritability for the different traits was H^2, the broad-sense heritability that was computed as the ratio of among-RIL variance component (VG) divided by the total phenotypic variance (VG+VE).

Results

Heat-shock effects on life span

Figure 1 shows the effect of heat-shock on life span in the parent strains N2 and CB4856. Heat shock treatment significantly impaired life span with 32% in CB4856 but not in N2. The life span of those CB4856 worms that survived the heat shock, i.e. the recovered worms, was 18% longer when compared to the control treatment ($p < 0.05$). The N2 worms did not show a significant elongation of life span after heat shock. This response was different between N2 and CB4856 ($p = 0.025$)

Table 1 shows the heat-shock effect across all RILs. The mean life span of the control worms (20°C) was ~16 days whereas the heat-shocked worms only lived ~14 days. We noticed however that the majority of worms that died after heat-shock did so in a small number of days just after the heat-shock (Figure 2). When we compared the mean life span of the control and heat-shock group, the mean life span of the two groups become equal after worms that died before 6 days have been excluded (Supplemental Figure 2). This means that for the heat-shock effect on life span the recovery period is 3 days (or 6 days after hatching). We took this measure of the recovery period to further dissect the effects of heat-stress.

Supplemental Figure 3 shows the variation among the RILs for life span in the control and recovered worms. In the recovered worms, ~28% displayed a hormesis effect in life span ($p < 0.05$). Life span in the RILs ranged from 7.1 to 22.5 days in the recovered worms at 20°C and from 11.1 to 32.4 days in the recovered worms after the heat-shock. The RIL with the maximum mean life span lived ~2 times longer than N2, the wild type strain in which almost all life span research in *C. elegans* has been conducted so far. These values are within the range of previously reported life span assays in an N2 x Bergerac RIL population [33, 34]. The mean life span in the RILs was not different than either of the two parents, suggesting that no selection during the construction of the RILs had taken place. This is in contrast to the reports of Shook and Johnson (1999) [35] who found evidence for selection

during the generation of RILs derived from a cross between wild-type strains Bristol (N2) and Bergerac (BO).

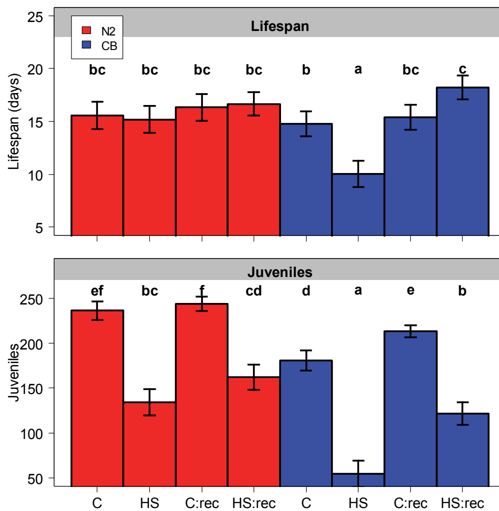

Figure 1. Life span and offspring of CB4856 (blue) and N2 (red). Letters indicate statistically different groups ($p<0.05$). Error bar show standard error. Labels on the x-axis indicate the different groups: C for control, HS for heat-shock, C:rec for the control RILs that lived beyond the recovery period and HS:rec for the heat-shocked worms that lived beyond the recovery period.

Table 1. Differences in life span and reproduction between control and heat-shocked worms from all RILs.

	Control			Heat-shock				Control (rec)			Heat-shock (rec)			
Trait	Mean	SD	SE	Mean	SD	SE	p	Mean	SD	SE	Mean	SD	SE	p
Mean Life-span (days)	16.2	6.45	0.29	13.8	7.80	0.33	<4e-8	16.7	6.14	0.28	17.3	6.34	0.31	0.22
Juveniles	201	85.9	3.13	96	83.3	3.07	<3e-16	223	68.2	2.71	134	72.8	3.2	<3e-16
Un-hatched eggs	48.8	57.8	2.1	41.3	55.0	2.0	=0.01	59.7	59.4	2.4	62.6	58.4	2.6	0.42
% Hatched eggs	83.1	15.6	0.57	75.5	23.0	0.85	<5e-12	80.8	15.1	0.60	70.0	22.0	0.97	<3e-16
Total eggs	250	114.4	4.17	137	116.8	4.30	<3e-16	282	93.2	3.71	196	98.1	4.32	<3e-16

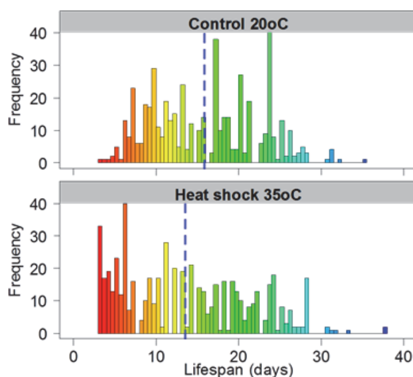

Figure 2. Distribution of life span. Upper panel control worms at 20°C, lower panel heat-shocked worms (4h at 35°C). Bar colours can be used to compare the different distributions. Blue dotted vertical lines indicated the mean life span.

Heat-shock effects on offspring

The number of juveniles in CB4856 was lower compared to N2 in the control and after heat shock. CB4856 showed a 70% reduction in juveniles after heat shock whereas N2 showed a 40% reduction after heat shock (Figure 1). In the RILs a ~50% reduction was found, the control worms produced ~200 on average, whereas a significant reduction was found in the heat-shocked worms with only ~100 juveniles on average (Table 1). For recovered worms, the average number of juveniles was increased. The recovered control worms produced ~230 juveniles over all RILs and the recovered heat-shocked worms ~130. The average number of juveniles per RIL ranged from ~91 to ~374 in the recovered worms at 20°C and from ~38 to ~214 in the recovered worms after the heat-shock. The number of juveniles per individual worm has a normal distribution under control condition whereas a more uniform distribution is found after heat-shock conditions (Figure 3). This might indicate that after heat-shock reproduction becomes a more stochastic process.

Overall, the number of unhatched eggs was larger in the control worms (~50) than the heat-shocked worms (~40; $p=0.01$). Yet, for the worms that recovered no difference was found. Relatively, however, the heat-shocked worms had more unhatched eggs (~25%) than the control worms (~17%; $p<5e-12$). When we correct for recovery this difference increases (~30% vs. 20%; $p<3e-16$). So in general, in contrast to life span, with regards to offspring, worms cannot fully recover from a heat-shock and have a lower number of viable offspring. Not only did heat-shock affect the number of offspring also the timing at which eggs were deposited was altered (Figure 4). The heat-shock worms were slower or started later with egg-laying, after the recovery period control worms deposited ~71% of total eggs whereas the heat-shock worms only deposited ~62% ($p<1e-8$). For all the eggs this is 63% for the control worms and ~54% for the heat-shocked worms ($p<3e-10$; Figure 4).

In summary, heat-shock has a negative effect on the total number of eggs, the relative number of hatching eggs and egg deposit time. Overall we did not find any hormesis effects for offspring.

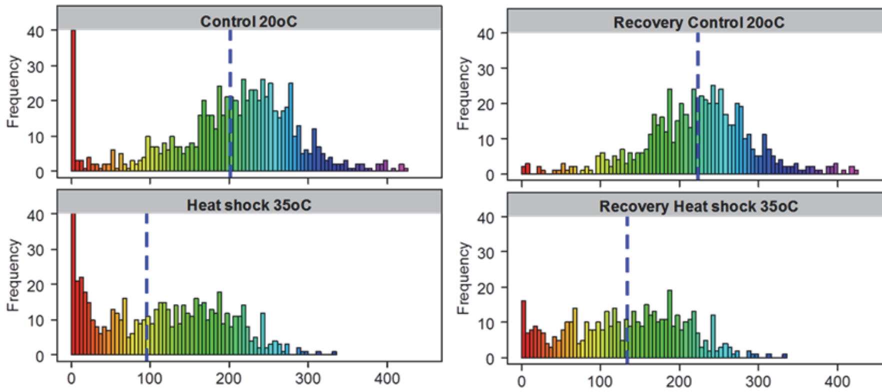

Figure 3. Distribution of number of juveniles produced by individual worms.

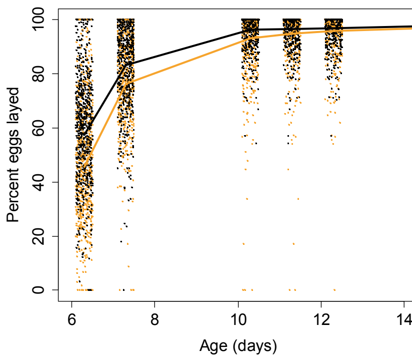

Figure 4. Timing of egg laying. The percentage of the total number of eggs for the individual worms at five different ages. In orange the heat-shocked worms and in black the control worms. Averages per age are shown as lines.

Relation between life span and reproduction

Overall we did not find a strong relation between life span and offspring, except for the recovery period (Figure 5). The recovery period overlaps with the reproductive period and worms that died within that period had therefore less offspring (Figure 5A) and less total eggs (Figure 5C). After the recovery period this relation was not present anymore. This is not the case for unhatched eggs, in which longer living worms seemed to have a larger absolute number and percentage of unhatched eggs (Figure 5B and 5D). This is probably the result of non-hatching eggs being mainly produced at the end of the reproduction period. The heat-shocked worms had a lower number of juveniles, due to a lower number of total eggs and a higher percentage of eggs that did not hatch.

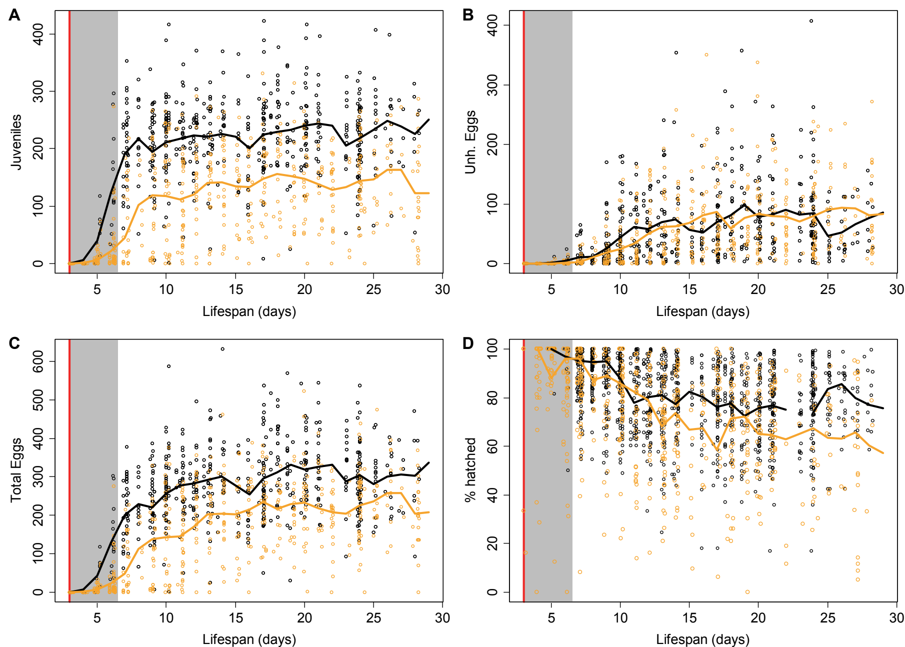

Figure 5. Relation between life span and reproduction. Control shown in black and heat-shock shown in orange. Individuals shows as dots, 3-day moving average shown as lines, Red vertical lines shows time of heat-shock and the grey area shows the recovery period. Life span in days against: A) Number of juveniles, B) Number of unhatched eggs, C) Total number of eggs, D) Percent eggs that hatched.

QTL for recovery and number of juveniles

We conducted QTL mapping for recovery and number of juveniles of the recovered worms in the control and heat-shock treatment (Figure 6). Data used can be found in supplemental table S1. A significant QTL was detected on chromosome *II and* chromosome *IV* for juveniles in heat-shocked worms. When we defined recovery as a binary trait for calculations we found a heritability of 0.64 and 0.78 at 20°C and 35°C, respectively. We detected a significant recovery QTL on chromosome *II* at the same position as for juveniles. We did not detect a significant QTL for life span in either treatment group, nor for offspring number in the control group. Shook *et al*., (1996) and Shook and Johnson (1999) [35, 36] reported QTLs for mean life span and temperature-sensitive fertility on chromosomes *II*. To verify this QTL we recorded recovery in introgression lines (ILs) previously developed by Doroszuk *et al*., (2009) [17]. We selected 3 ILs harboring a CB4856 segment on chromosome *II* in an otherwise complete N2 background: ewIR24 (SNP egPP206), ewIR25 (SNP egPQ207) and ewIR26 (region of 604 Kbp between egPQ207 and pkP2116). We tested 3 replicate dishes with worm numbers ranging from 56-74 in the control, and 52-78 in the heat-shock treatment. Figure 7 shows that ILs ewIR25 and ewIR26 were affected by the heat-shock and displayed the same phenotype as wild type CB4856 in contrast to N2.

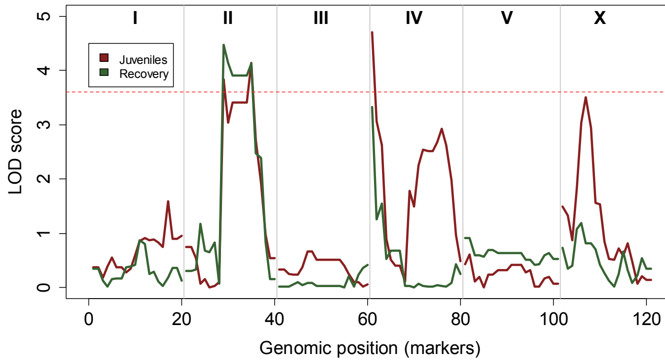

Figure 6. QTL profiles of recovery (green) and number of juveniles (red) after heat-shock.

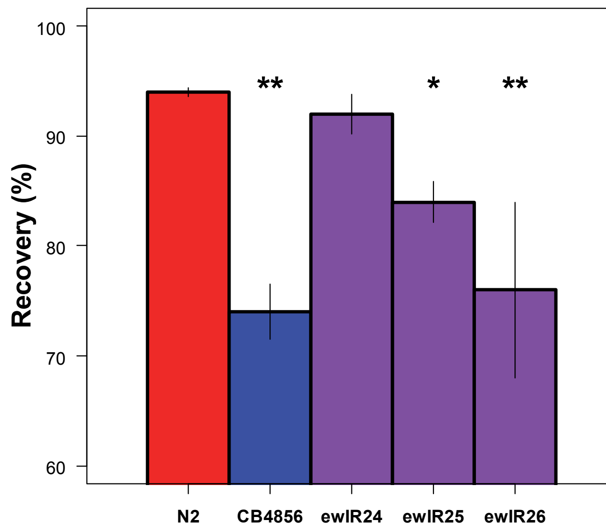

Figure 7. Recovery (%), 72 hours after heat-shock (4h at 35°C). N2 in red, CB4856 in blue, introgression lines in purple. Stars above bars indicate the lines significantly different from N2 (* < 0.05; ** < 0.01). Error bars show standard error.

Discussion

We found genetic variation for stress response hormesis in *C. elegans* life span. The parental wild types differed strongly in this respect. The life span of CB4856 increased after heat-shock whereas in N2 there was no hormesis effect. Our results for N2 can be compared to those reported by Olsen *et al*., (2006) [37]. They reported a life span extension following a mild hormetic heat treatments of 33°C for 4 hrs in young adult worms after 4 and 8 days. Other studies also report life span extension following a single hormetic heat treatment early in life. Worms normally reared at 20°C were exposed to a range of temperatures (30–35°C) and periods (1–13 h) [7, 9, 11, 38-41]. Both CB4856 and N2 have been maintained under laboratory conditions for decades (McGrath *et al*., 2009; Weber *et al*. 2010). N2 has been kept in the laboratory since the late 50's and CB4856 since 1972. It can be speculated that these differences in maintenance might have resulted in divergent heat tolerance between the two strains. Anderson *et al*., (2007) [42] studied variation in thermal preference between the two wildtypes and found that CB4856 had a clear preference for cool temperatures (~17°C). Glauser *et al*., (2011) [43] compared the divergence between the two wildtypes in their threshold for heat avoidance (the thermal barrier assay and the ability to avoid noxious temperature extremes) and their results indicated that CB4856 tolerates higher temperatures. The latter results are in agreement with our findings, namely that recovered CB4856 worms after heat shock lived longer than N2.

We did not find any hormetic effects of heat-shock on offspring in N2 and CB4856, which is in agreement with the reports of Lithgow *et al*., (1994, 1995) on N2 [38, 44]. Across all RILs, our results show that heat-shock strongly diminished egg hatching rate. Very little is known about the effect of thermal, or any kind of stress on egg hatching success in *C. elegans*. Skantar *et al*., (2005) [45] reported that egg hatching success increased at relatively low levels of chemical stress. They postulated that this might be due to Hsp90-mediated hormesis. *C. elegans daf-21*/Hsp90 is involved in thermo tolerance and upregulated by DAF-16 [46], which further supports involvement of Hsp90 in a hormetic biological switch involved in stress responses, including heat-shock [45]. Yet we did not find evidence for such hormesis effects. Perhaps our heat-shock was too rapid or the temperature was too high.
To our knowledge this is the first study dealing with *natural variation* in hormetic stress response and life span in *C. elegans*. In the recovered RILs we found genotypes that had longer life spans after heat-shock. This is in agreement with data from Defais *et al*., (2011) [47] in *Drosophila* who reported hormetic effects on longevity induced by heat-stress in several RILs. The QTL that we detected for recovery on chromosome *II* was confirmed by the introgression lines and can be regarded as a hormesis-QTL because it maps the recovery after heat-shock. This cannot be directly compared to the QTL mapping of Defais *et al*., (2011) who identified QTL explaining either positive or negative changes in longevity induced by heat stress at young ages. Our results on hormesis effects are corroborated by other studies on *Drosophila*. Heat-shock treatment prolonged average life span in flies [48] and an inverse relationship between life span and temperature was also found by Zwaan *et al*., (1995) [49] and Lithgow *et al*., (1995) [38].

We have not been able to detect significant QTL for life span. Most likely, the variation in life span measurements was too large to enable QTL detection in this limited set of RILs.

Ebert *et al*. (1993) [50] were among the first to map QTL for life span in *C. elegans* in an N2-by-Bergerac derived recombinant inbred panel. The markers used in these studies were transposable elements (Tc1), which did not equally cover the full genome. Ayyadevara *et al*., (2001) [51] used a Bergerac by RC301 recombinant inbred population also with Tc1 markers to map loci associated with life span. The significant QTL on chromosome *II* for heat-shock recovery are in agreement with a QTL detected on this chromosome in an N2-by-Bergerac study by Ebert *et al*., (1996) [52]. They exposed the RILs to acute toxic levels of hydrogen peroxide. Genotyping of hydrogen-peroxide-resistant worms indicated the existence of a locus associated with longevity on chromosome *II*. Superoxide dismutase and catalase levels, enzymes involved in hydrogen-peroxide-resistance, confirmed polymorphisms in the corresponding genes as inferred from the chromosome *II* mapping [52]. Vertino *et al*., (2011) [53] studied life span QTL in a Bergerac-BO x CL2a RIL population. They did not record recovery but performed life span assays and studied the difference in survival curves.

The heat-shock hormesis results in *C. elegans* are supported by many artificially induced mutants known to confer life span extension in *C. elegans* (*age-1, daf-2*, *spe-26* and *clk-1*) and are also more resistant to a variety of environmental insults, including oxidative stress [54, 55], elevated temperature [38] and UV irradiation [56]. The hormesis QTL on chromosome *II* harbours *age-1* as a polymorphic gene. Iser *et al*., (2011) [57] suggested that *hsf-1* may participate in the pathways mediating activities of *age-1* in *C. elegans*. The detection of a heat-stress recovery QTL on chromosome *II* are consistent with a model in which *age-1* mutations exhibit thermo tolerance and extended life span as a result of elevated levels of molecular chaperones. Walker *et al*., (2001) [58] showed elevated levels of heat shock protein 16 (HSP16) in the *age-1* mutant during and after heat stress. Mutation of the *age-1* gene, encoding a phosphatidylinositol 3-kinase catalytic subunit, results in both extended life span and increased intrinsic thermo tolerance in adult hermaphrodites.

We did not find a correlation between life span and number of juveniles, unhatched eggs or total offspring and we have not found evidence for loci exhibiting opposing effects on life span and offspring. There are a number of reasons that could explain why we did not find a correlation and associated loci. Firstly, because the power of our setup was too limited to detect small effects of multiple loci. An increased RIL population could lead to the detection of such loci. But Shook *et al*., (1996) [36] also reported no significant correlation between mean life span and mean fertility in a *C. elegans* RIL set. Secondly, it could well be that we could not observe any correlations because of the environment used. By studying the same RIL population in different environments we previously found correlations between various life-history traits. Depending on the environment, we detected pleiotropic or closely linked QTL, which supported the negative correlation between egg size and egg number, the positive correlation across temperatures for body mass, and the positive correlation between body mass and egg size. These results indicate that specific loci control the co-variation in these life-history traits and that locus control is prone to environmental conditions [19, 20]. Finally, we may be unable to detect QTLs just because loci are not closely linked to the markers used in our study. Knight *et al*., (2001) [59] studied the genetic relationship between maternal body size and fertility. They found two body size and three fertility QTLs. One of the fertility QTLs co-localized with the two body size QTLs. They showed that these QTLs were genetically separable, hence none of the five body size or fertility QTLs identified displayed detectable pleiotropy for the assayed traits.

Acknowledgments

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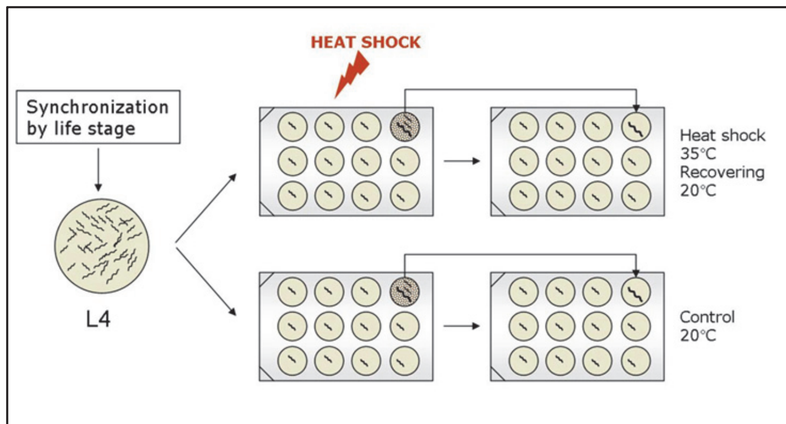
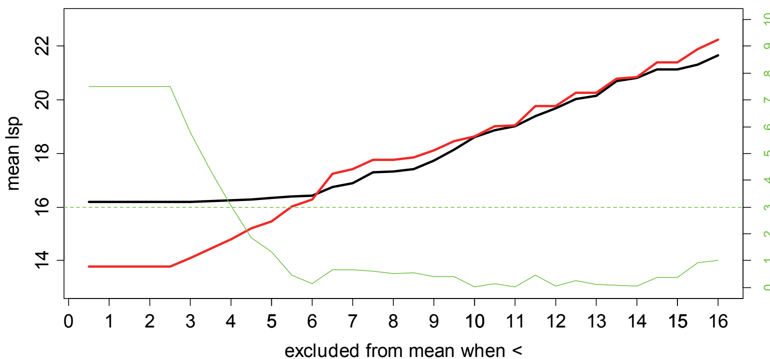
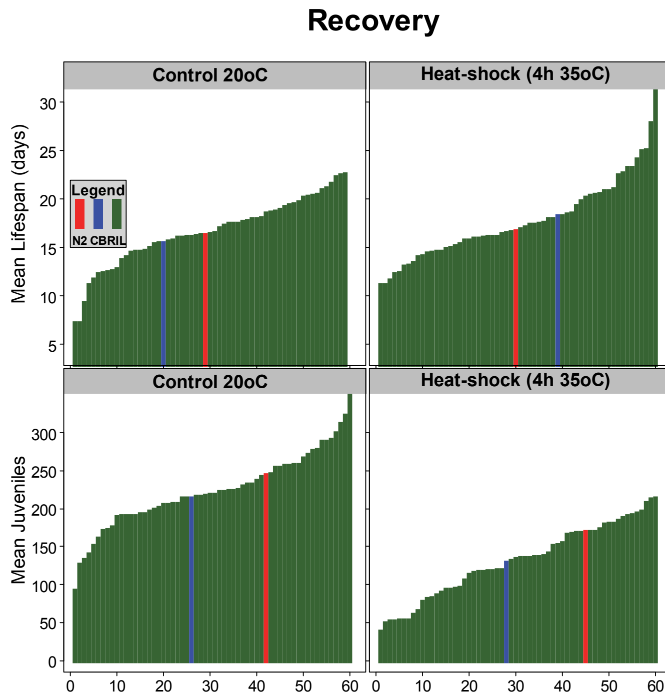
Supplementary data



Supplemental Figure 1. Life span experiment**,** RILs were exposed to 35°C for 4 hours and to 20°C (control population). Heat shocked worms were then permitted to recover at 20°C for the rest of their lives . Survival, offspring and number of eggs were recorded daily after the heat exposure. Offspring was measured by transferring the adults to fresh multiwell plates.



Supplemental Figure 2. Mean life span of worms still alive (y-axis) after a number of days (x-axis) of control (black line) and heat-shocked (red line). In green the significance (in –log10 p) of the difference in mean life span between control and heat-shocked worms when excluding worms that died before a certain age.



Supplemental Figure 3. Distribution of the RIL phenotypes. The mean per RILs was calculated on the recovered worms. Upper panels show mean life span per RIL. Lower panels show the mean number of juveniles per RIL. Heat-shock on the right and control on the left. In red the phenotypes of N2 and in blue CB4856.

Supplemental Table S1: Pilot experiment for life span and offspring. A heat-shock of 6 and 10 hrs had too strong effects on offspring and life span (life span is the number of days after the onset of reproduction)

N2															
N=12	Control			Heat-shock 4hrs				Heat-shock 6hrs				Heat-shock 10hrs			
Trait	Mean	SD	SE	Mean	SD	SE	p (Vs control)	Mean	SD	SE	p (Vs control)	Mean	SD	SE	p (Vs control)
Mean Life span (days)	9,6	3,7	1,1	6,3	4,3	1,3	0,06101	2,9	3,7	1,2	0,34096	7,4	5,8	1,9	2,4E-05
Juveniles	279,5	40,4	12,2	119,2	74,4	23,5	3,4E-05	99,0	57,2	25,6	0,00075	0,0	0,0	0,0	5,5E-10

CB															
N=12	Control			Heat-shock 4hrs				Heat-shock 6hrs				Heat-shock 10hrs			
Trait	Mean	SD	SE	Mean	SD	SE	p (Vs control)	Mean	SD	SE	p (Vs control)	Mean	SD	SE	p (Vs control)
Mean Life span (days)	7,4	5,4	1,6	2,9	3,7	1,2	0,04296	2,1	0,9	0,4	0,00912	2,3	4,0	1,6	0,04696
Juveniles	224,8	31,2	10,4	20,6	30,3	10,7	4,2E-10	3,0	2,0	1,2	1,9E-08	31*	NA	NA	NA

*= only one reproductive worm left

5

Molecular confirmation of trans-regulatory eQTL in *Caenorhabditis elegans* under heat stress conditions

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Abstract

Gene expression QTL (eQTL) are regulatory loci, which are statistically associated with genetic variation of gene expression. Here we experimentally confirm the position of common stress-response-gene expression regulators represented by a *trans*-eQTL on chromosome *IV* through quantitative RT-PCR in the nematode *C. elegans*. A set of 10 genes selected from the target transcripts harbouring the eQTL were measured for their expression. Expression levels were quantified in two of the most genetically divergent strains, Bristol N2 and CB4856 Hawaii as well as in 4 introgression lines (ILs) derived from a cross between these two strains, which contain introgressed regions of CB4856 chromosome *IV* into a Bristol N2 background. We detected specific loci located on chromosome *IV* associated with the variation in heat shock expression response. We stated that the introgressed regions on chromosome *IV* ILs contain loci standing as regulators for heat stress response. We found that candidate genes directly related with stress response such as *ZK355.3*, *F08H9.4* and *sod-5* showed the most significant differences in relative mRNA levels.

Introduction

Heritable variation in gene expression regulation is the raw material for evolution on which natural selection acts. A widely used method for the detection of regulatory loci is the identification of quantitative trait loci (QTL) and the detection of the underlying quantitative trait nucleotides (QTN). So far very few QTN of large effect underlying complex traits have been reported [1, 2] across different species. The main reason for this seemingly low amount of confirmed QTL might be because most complex traits are polygenic and determined by the combined influence of small effect loci and alleles. Reported confirmations in most studies involve the detection of QTN, which are effectively Mendelian [3, 4]. There is much debate about the detection of QTN, which have been reported by experimental researchers [5]. A recent survey states that QTNs of relatively large effect may be uninformative about evolutionary issues if large-effect variants are unrepresentative of the alleles 'that matter' [4]. Despite these considerations, QTN are meaningful from the viewpoint of understanding genetic pathways and gene networks, in particular the identification of QTL, which are associated with the genetic variation of genome wide gene expression, or eQTL, which represent regulatory loci across the genome. eQTL have been thoroughly investigated in a variety of species like humans, plants, rats, mice, worms and yeast [6-11]. eQTL studies have three advantages: firstly because a huge amount of transcripts are considered they allow for generalizations, next the transcript traits are not preselected, and finally the traits integrate over the overall phenotype of the organism. Although numerous eQTL have been detected across a range of species, very few have been mapped to QTN resolution [4].

eQTL can be *cis*- (local) or *trans*- (distant) acting regulatory loci. *Trans*-acting eQTL can provide the basis for constructing gene expression regulatory networks [9] and the detection of QTN is expected to be important for understanding genetic pathways and constructing gene networks [1]. In the pursuit of detecting the underlying QTN we report the confirmation of a *trans*-regulatory eQTL in the nematode *Caenorhabditis elegans*.

Over the past decade *C. elegans* has increasingly been studied as a model organism for natural variation studies [12]. The worm has been thoroughly studied as a model system to increase our genetic understanding of behavior and development. Moreover, *C. elegans* is increasingly applied in ecological genetics by subjecting it to different environments. This will greatly facilitate our understanding of how different *C. elegans* genotypes respond to harsh environmental conditions as a direct result of the underlying genetic architecture and molecular mechanisms of an organism [13]. The most widely studied environmental perturbations subjected the nematodes to relative extreme conditions leading to stress response [14]. Application of heat stress is one of the most widely and thoroughly studied stress factors in *C. elegans,* in which the worms are exposed to either shorter or longer periods of a sudden rise in the ambient temperature variation [14, 15]. Such a temperature increase has strong effects on nearly all cellular and molecular processes thus imprinting its response into a variety of phenotypes. Following a heat-shock, cells exhibit a heat shock response (HSR), a program of stress-inducible gene expression, to maintain cellular integrity and increase thermal tolerance [16].

Increased attention is being paid to study the heritable variation in a wide range of phenotypes under heat-shock conditions [12]. Over the past few years, studies focused on

the identification of genetic determinants of gene expression variation [17, 18], also called genetical genomics.

A typical genetical genomics study combines genetic linkage analysis with gene expression to compare genome-wide transcript abundance (from microarrays or RNA-seq platforms) in different genotypes [9, 19]. Some genetical genomics studies have been conducted to reveal polymorphic regulators of stress responses. Thus, quantitative trait loci (QTLs) can be mapped to characterize genetic regulation of a certain trait analyzing gene expression, hence by expression quantitative trait loci mapping (eQTLs). This technique represents a powerful tool to reveal up- or down-regulation of genes associated with a particular phenotype in a certain condition or treatment [20].

We recently aimed to detect polymorphic genes involved in stress resistance within the GRAPPLE project [see http://www.erasysbio.net/GRAPPLE]. Using a genetical genomics approach, we analyzed genome wide gene expression of 55 recombinant inbred lines (RILs) and 57 introgression lines (ILs) during control (20°C) and heat shock (35°C) conditions. The mapping of eQTLs revealed a regulatory hotspot on chromosome *IV* in the RILs and the ILs population. A *trans*-acting eQTL was revealed distal to chromosome *IV* under heat shock conditions. These results pointed at the presence of a 'master regulator' for heat stress response located within this genomic region affecting multiple transcripts.

The present study is based on our previous QTL mapping results where genotypic and phenotypic effects of thermo-stress were measured in *C. elegans* RILs and ILs sets.

Furthermore, in a parallel study we have performed QTL mapping combining genotypic and phenotypic effects of thermo-stress in *C. elegans* RILs and ILs sets [15]. We performed a genome-wide approach of the transcriptome in parental strains N2 and CB4856 using latest generation microarray techniques. A closer look at the data indicates the presence of natural variation for stress tolerance, which leads to modulation of reproduction rate, regulated by differential expression of metabolic effectors. The QTL mapping of this effect showed candidate loci associated with the variation in heat shock response. One of these candidate loci appeared to be located at chromosome *IV* (Figure 1).

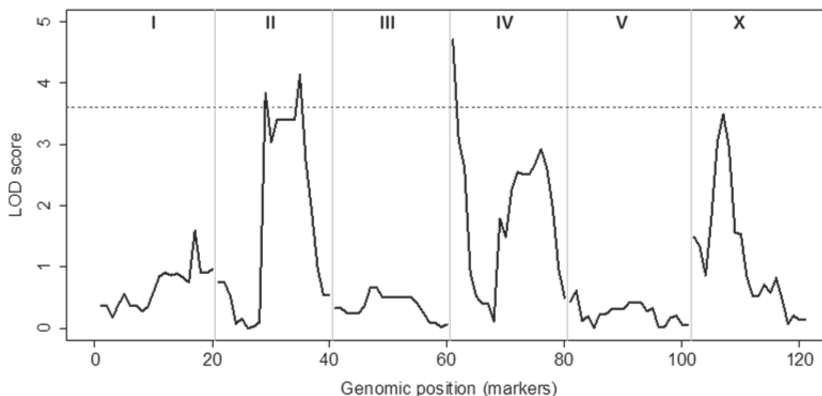

Figure 1. QTL profile for reproduction: two QTLs are selected in relation to reproduction in heat shocked RILs, they are located proximal to chromosome *II* and at the distal to chromosome *IV*.

The eQTL results suggest the existence of a key regulator between markers 61 and 65 at the beginning of chromosome *IV* (Figure 2). To experimentally confirm this *trans*-acting eQTL and to identify the putative causal regulator we aimed to molecularly confirm the locus on chromosome *IV*. We measured the gene expression by quantitative RT-PCR of a set of genes affected by the locus of study in ILs containing the introgressed fragment of interest.

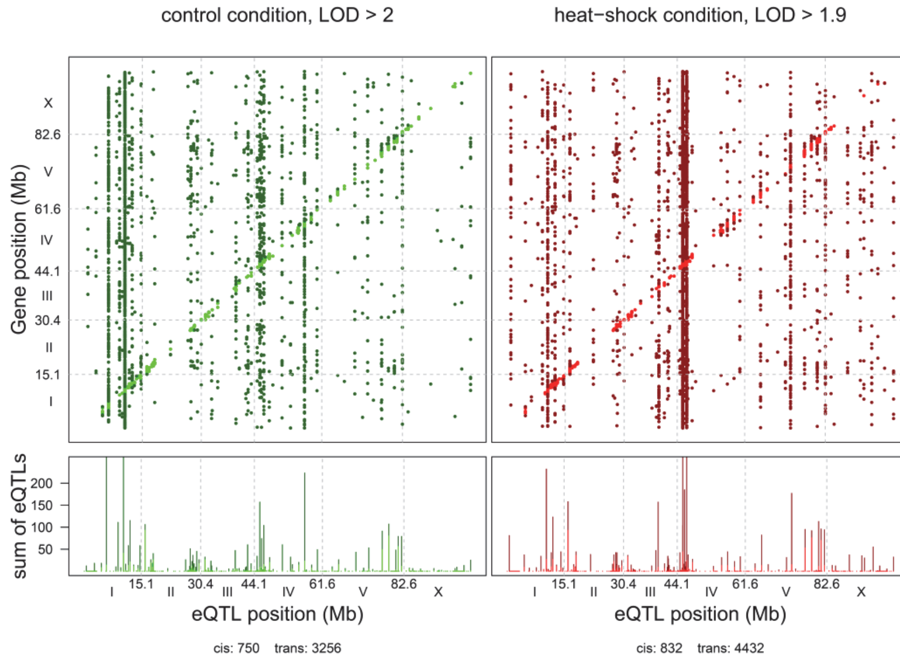

Figure 2. eQTL mapping in the ILs, FDR < 0.01. The *cis*-eQTLs are shown in lighter colours and the *trans*-eQTLs in darker colours. *Cis*-eQTLs were defined as genes that lay within 1Mb of the eQTL location. The LOD score threshold for the control condition was 2.0 and for the heat-shock condition was 1.9. In the control condition 750 *cis*-eQTLs and 3256 trans-eQTLs were found. For the heat-shock condition 832 *cis*-eQTLs and 4432 trans-eQTLs were found. In the control condition *trans*-bands can be observed on chromosome *I* and on chromosome *IV*. In the heat-shock condition the strongest *trans*-bands can be seen at the top of chromosome *IV*.

eQTL mapping techniques are statistical associations and so far a thorough confirmation of *trans*-eQTL is lacking (however see Snoek *et al*., 2012). This study is an attempt to molecularly confirm the eQTL on chromosome *IV*. For that purpose we measured gene expression by RT-PCR in selected ILs (containing introgressed regions of CB4856 into a Bristol N2 background between markers 61 and 76; see Figure 3) of a selected panel of 10 genes containing an eQTL in the *trans*-band. The selected genes were previously found within a group of genes harbouring an eQTL based on the study of transcriptome in stress conditions versus normal conditions using microarray assays. These genes together were part of a *trans*-band distal to chromosome *IV*. We studied a locus distal to chromosome *IV* previously revealed by QTL mapping of offspring in heat-shocked worms [15]. The results provide evidence of the presence of at least one locus situated on chromosome *IV* between markers 61 and 65 (genomic positions between 151,889 bp and 3,067,374 bp) regulating the heat stress gene expression response. The expression patterns of the selected set of genes in the ILs ewIR45 and ewIR48 showed significant differences in gene expression of

genes directly related with stress response in heat shock conditions versus control conditions when we compare to parental lines in identical conditions.

Materials and Methods

Nematode cultures

Introgression lines (ILs) were selected from a collection of 90 ILs previously generated [21]. Worm cultures were grown in nematode growth medium (NGM) with *Escherichia coli* strain OP50 as a food source [22]. Nematode populations were maintained at 12°C. Prior to each experiment, the cultures were age-synchronized by bleaching [23] to obtain fourth larval stage nematodes (L4).

Heat shock and recovery experiment

We used four ILs (ewIR45, ewIR48, ewIR51 and ewIR52), containing genomic introgressions on chromosome *IV* and the two parental lines (N2 and CB4856) for gene expression analysis under control and heat stress conditions (Figure 3).

Line name	Introgression (CHR, strain)	left N2 marker	left CB marker	right CB marker	right N2 marker	max CB int.	min CB int.
N2	None						
CB4856	None						
ewIR045	*VI*, CB4856	NA	61	63	64	0,00	0,00
ewIR048	*VI*, CB4857	62	63	65	66	0,00	0,00
ewIR051	*VI*, CB4858	63	64	68	69	0,00	0,00
ewIR052	*VI*, CB4859	63	64	76	77	0,00	0,00

Marker	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82
Marker name	151889	766649	1381409	2288742	3067374	3920366	4991858	5819735	6599685	7405743	8397264	9102404	10122930	10909560	11668242	12748880	13667267	14566567	14872329	15178091	16371991	17084259
Genomic posit.(bp)	egPA410	pkP4049	pkP4051	egPC402	egPD403	egPE404	pkP4071	egPG405	pkP4075	egPI406	pkP4039	pkP4080	dbP3	dbP6	pkP4043	pkP4047	pkP4048	pkP4095	pkP4045	egPR407	egPS408	egPT409

Figure 3. Selected introgression lines at chromosome *IV*. The ILs are derived from a cross between N2 and CB4856 strains, which contain introgressed regions of CB4856 chromosome *IV* (striped) into an Bristol N2 background (grey).

Nematodes were synchronized by bleaching to reach L4 stage. The dishes were rinsed with 1mL M9 buffer [22] to a 1.5 mL Eppendorf tube and centrifuged (14,000 rpm, 20 sec.). The supernatant was discarded and 800µl bleach solution (2 mL NaHCl, 0.5 mL NaOH (10 M), 7.5 mL MQ) was added. The solution was mixed for three minutes and after centrifugation, the supernatant was discarded and immediately 1 mL of M9 buffer was added. Samples were centrifuged (14,000 rpm, 20 sec.) and the pellet containing the eggs was placed in a fresh 6 cm petri dish with NGM and *E. coli* OP50. The dishes were incubated at 16ºC for 72 hours to let the nematodes grow until they reached the L4 stage.

Age synchronized populations of each line (N2, CB4856, ewIR45, ewIR48, ewIR51 and ewIR52) in L4 stage were exposed to heat stress. The populations were exposed to 35°C for 2 hours and the control populations were incubated at 20°C for exactly the same time. Immediately after the treatment the nematodes were collected in M9 buffer and the pellets were frozen in liquid nitrogen and stored at -80°C until being processed for RNA purification.

Two biological replicates of each population complied with the previously mentioned procedure.

Gene expression profile

Both control and heat shocked population lines were studied for gene expression patterns. Total RNA was isolated from treated and control nematodes. Nematode pellets were incubated at 55°C, 500 rpm shaking during 60 minutes with 1% beta-mercaptoethanol and 800μg/ml proteinase K to allow the cuticles to break preserving the RNA integrity; after this step, we used QIAGEN RNeasy® Micro kit to extract and purify total RNA following manufacturer instructions. The extracted RNA was processed for RT-PCR performance.

Target genes screening and selection

A number of predetermined characteristics were selected in order to screen for target genes. Firstly, target genes that showed a significant eQTL on chromosome *IV* were selected. For the screening method, a significance threshold was chosen of -log10(p) > 3. Secondly, target genes located on chromosome *IV* itself were discarded as we sought for *trans*-regulation, and lastly, target genes had to be observed with a clear variation in gene expression between control and heat shock conditions.

The selection of target genes was performed using data available from our eQTL mapping study (Figure 2). This included two data sets; i) heat-shock-specific mapping, which is only genotypic effect based (from worms exposed only to heat) and ii) a genotype-by-environment mapping method, which indicates a variation in expression caused by an interaction of the genotype and the environment.

Within these data-sets, we selected genes showing an eQTL on chromosome *IV*. Secondly, genes that were located on chromosome *IV*, were excluded from the potential candidate list. By these two criteria the list of genes was first narrowed down to 99 genes (Supplemental table S2). These candidate genes were individually assessed for their expression levels and their allelic variation by checking Worm Base for CB4856 polymorphisms [www.wormbase.org]. After this process 24 genes were selected, which showed allelic variation between the two *C. elegans* strains under study and were located in the same region of the *trans*-regulatory eQTL. A final selection was performed in order to decrease the number of target genes to 10 based on the strongest eQTL. To do this, two additional features were inserted for the selection: i) a functional role in biological processes or pathways for ageing, stress-resistance or disease-development (in humans) and/or ii) have a single nucleotide polymorphism (SNP) located in the target gene, based on Worm Base 225 data [WS226, development page; http://dev.wormbase.org/]. The 24 pre-selected genes were individually screened on WormBase for these features, either by gene ontology terminology, SNPs in the Hawaii strain, or common gene expression clusters based on previously published microarray data [http://dev.wormbase.org/]. Finally 14 genes were selected based on the previously mentioned features. The 10 most significant genes of the remaining candidates were finally chosen for the measurements (Supplementary Table S3).

Reference genes

The two reference genes were selected from existing microarray data of strains Bristol N2 and CB4856 Hawaii in normal and heat stress conditions (data not shown). Constitutively expressed genes in both strains with no significant genotypic or phenotypic differences both in control and heat-shock conditions were selected as reference genes. Both genes have been used as housekeeping genes for data normalization (Supplementary Figure S1).

RT-PCR primers design

Nucleotide sequences (from Bristol N2 strain) were processed in Primer3 online free software [24]. The following parameters were selected: G-C: 50-60%, range of annealing temperature between 60ºC and 65ºC, optimal annealing temperature 62ºC, no more than 3ºC difference in annealing temperature between forward and reverse primer, no more than two G or C in the last 5 bases of the 3'terminal, G or C as last 3'terminal, no repeat more than 3 times in a row, only one product and finally the product must have a length between 75 and 200 bp. All the primers were tested for specificity using NCBI BLAST. Primer efficiencies were calculated by the Pfaffl method [25]. See complete list of primers in Supplementary Table S3.

RT-PCR procedure

Biological and technical replicates of total RNA of all the samples (N2, CB4856, ewIR45, ewIR48, ewIR51 and ewIR52) were used for cDNA synthesis. Starting with 2 µg of total RNA, each sample was treated with SuperScript III reverse transcriptase (Invitrogen. Cat. No. 18080-093) following manufacturer's instructions. The resulting cDNA was diluted 20 times in nuclease-free water and 3µl of diluted cDNA were used as a template for quantitative RT-PCR. The template was added to the RT-PCR amplification mixtures (25µl) containing 7,5 µl milliQ water, 1 µl 5 mM forward primer, 1 µl 5 mM reverse primer, and 12,5µl SYBR Green Master Mix buffer (AB solute SYBR Green. Fluorescein Thermo Scientific (Catalogue # cm-225/A). Reactions were run on a Bio-Rad thermo-cycler (MyiQ. Single color. Real Time PCR detection system) and data extraction software iQ5 (version: 2.1.97.1001). The cycling conditions were 15 min at 95ºC for polymerase activation and 60 cycles of 95ºC 30 sec, 62ºC for 1 min and 72ºC for 30 sec. Every reaction was repeated 4 times, including 2 technical replicates and 2 biological replicates. All PCR-primer efficiencies were between 95% and 105%.

Data normalization and statistical analysis

The C_t values of the technical replicates were examined for any inconsistency that may have occurred. Samples were discarded that displayed a >2 unit variation. Identification of outliers between biological replicates was performed by discarding any sample that differed over 2σ from the sample mean to prevent technical errors. The data was transformed using formula 1 where Q is the relative expression of the target gene as compared to the reference gene.

$Q_{gene} = 2^{37-Ct_{gene}}$ [Formula 1]

Formula 2 was then used for normalization of the genes.

$E = \frac{Q_{gene}}{0.5*((Q_{rpl6}/\overline{Q_{rpl6}})+(Q_{Y37E3.8}/\overline{Q_{Y37E3.8}}))}$ [Formula 2]

E is the relative expression, Q is the transformed expression as previously shown in Formula 1 (*rpl-6* and *Y37E3.8* were used as reference genes). All data was normalized together to allow direct comparison. Further statistical analysis was done using R (version 2.1.15 64x).

Results

In general, all selected genes showed an expression pattern where relative expression was increased as a direct result of heat stress except for genes *djr-1.2*, *mlcd-1* and *tsp-2* (Figure 4).

We can cluster the 10 target genes panel in two groups according to the heat-shock effect in gene expression: the first group consisting of *djr-1.2*, *mlcd-1* and *tsp-2* are clustered since their expression did not significantly change after the heat-shock (Figures 4 and 5). The second group containing the remainder genes of the panel can be divided in 3 subgroups according to their differential expression after heat-shock, ranging from strong to weak responses. Thus the 3 subgroups are 2.1) *ZK355.3* and *F08H9.4* showing a strong response to heat-shock; 2.2) *lea-1*, *fipr-24, sod-5* and *mtl-1* showed a slight response to heat-shock and 2.3) with *fbxc-7* standing on its own because of its weak response to heat shock in most of the studied lines (Figure 4).

The introgression lines that showed a stronger effect in most of the expression patterns of the selected genes were ewIR45 containing a CB4856 introgression between marker 61 (egPA410, genomic position at 151889 bp) and marker 63 (pkP4051, genomic position 1381409 bp) and ewIR48 with a CB4856 introgression between marker 63 (pkP4051, genomic position 1381409 bp) and marker 65 (egPD403, genomic position at 3067374 bp).

The results show a significant higher expression of most of the selected genes after heat shock in ILs ewIR45 and ewIR48 (all genes except *mlcd-1* showed differential expression in ewIR45 control respect to heat shocked; and *sod-5*, *zk355.3*, *lea-1*, *mtl-1*, *tsp-2*, *fbxc-7*, *F08H9.4* showed different expression in ewIR45 control respect to heat shocked, two sided paired t-test, $p<0.05$). The genes showing the highest effect in gene expression were *ZK355.3*, *F08H9.4* and *sod-5* (Figure 5). The first two genes increased their expression in heat stress (~2 fold) versus control conditions in the ILs ewIR45 and ewIR48. The expression of *sod-5*, encoding a superoxide dismutase, is also highly affected by heat stress, but this overexpression is only significant in IL ewIR48.

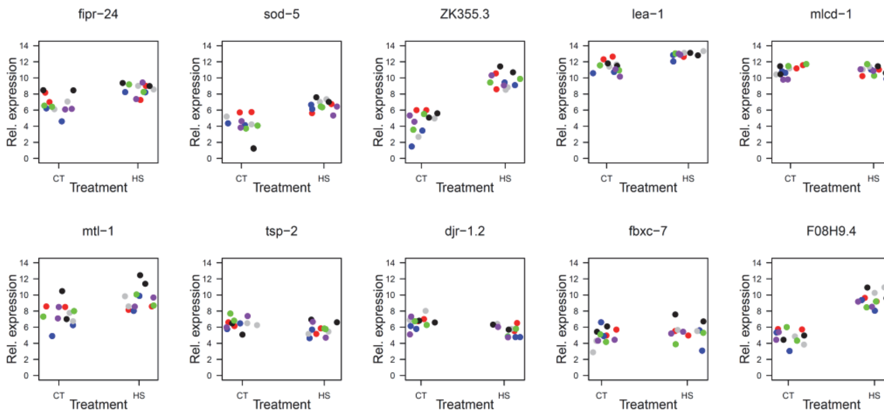

Figure 4. Relative expression of candidate genes in the different lines in control (CT) and heat stress (HS) conditions. Two biological replicates per line were measured for gene expression of the 10 selected genes in control as well as heat shock conditions.

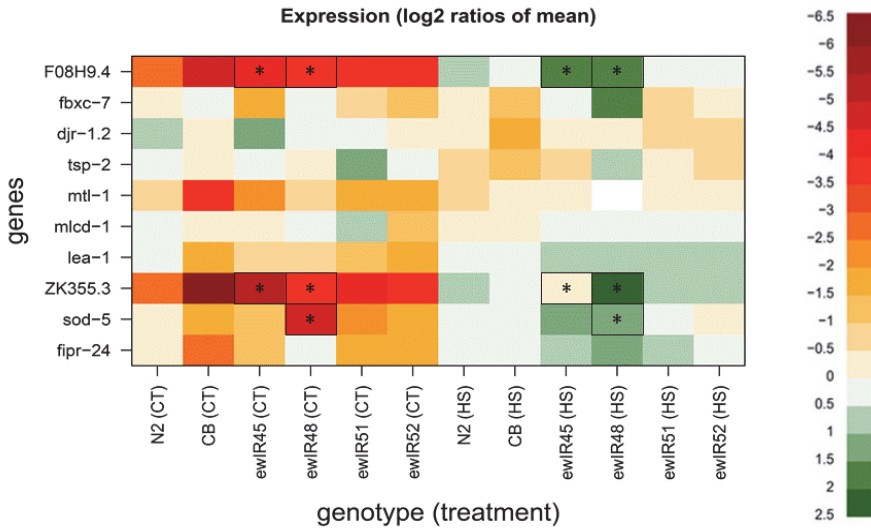

Figure 5. Differential expression heat-map. Gene expression per gene, line and treatment represented in log2 ratios of mean. Scale log2 fold up(red)/down(green). *Two sided paired t-test, $p<0.05$.

Discussion and concluding remarks

The identification of QTLs has become a useful tool for the detection of regulatory loci associated with variation of many different biological traits [26-28]. eQTLs go one step further in order to reveal information about gene expression regulatory networks.

Before one can gain insight into genetic architecture of gene expression it is important to find QTNs underlying eQTLs that may allow for a more thorough understanding of genetic networks and their heritable variation. We combined gene expression measurements with heat stress perturbation to confirm the previously described QTL distal to chromosome *IV*.

Various studies on the genetic variation of stress responses in *C. elegans* point to a region distal to chromosome *IV* and it has been suggested that this locus may provide regulators with an essential function during stress responses [29]. We recently showed a QTL distal to chromosome *IV* for total offspring in heat stressed nematodes, emphasizing the importance of this region in stress response in *C. elegans*. This QTL is consistent with results from other studies [15]. Furthermore Vertino *et al*., 2011 [30] performed life span assays and survival studies in heat and oxidative stress in a different *C. elegans* RIL population originating from a cross between strains Bergerac-BO x CL2a. Most notably, they detected a QTL for longevity and thermo tolerance that coincided on the same position on chromosome *IV*.

The RT-PCR results in this chapter confirm the position of common heat-stress-response-regulators in chromosome *IV*, by measuring gene expression of a panel of 10 genes related to stress all of which were identified previously by microarray-based eQTL mapping techniques.

Despite of the proven consistency of microarray analyses, this platform is not completely efficient for confirming a limited number of target genes. Therefore the necessity for a cost-efficient method with minimal background noise to confirm so-called *trans*-bands is a pressing issue. To our knowledge this is the first study showing the molecular confirmation of the locus in chromosome *IV* by measuring relative gene expression of a selected panel of genes that reside within the *trans*-band found by the eQTL study.

The use of introgression lines (ILs) containing a fragment of CB4856 Hawaii genome introgressed into a Bristol N2 genetic background has allowed us to increase the resolution on the effects of narrow loci previously shown in a *trans*-band as result of eQTL mapping. The introgression lines that showed a stronger effect in most of the expression of the selected genes were ewIR45 and ewIR48. These results suggest the possibility of one locus in chromosome *IV*, between marker 63 (*IV*: 1,381,409 bp) and 64 (*IV*: 2,288,742 bp). However, due to the scope of the introgression and the similar results obtained in various other ILs with nearby introgressions, the possibility of the presence of more than one regulatory locus between markers 61 (egPA410, genomic position at *IV*: 151889 bp) and 65 (egPD403, genomic position at *IV*: 3,067,374 bp) is still existent. However, it could still be that the effects seen in the other ILs are the cause of genetic interactions between these various loci (additive small effect loci).

The region overlapping ewIR45 and ewIR48 covers ~1 Mbp region at the top of chromosome *IV* consisting of 116 genes (Supplementary Table S2) between chromosome

positions ~1.3Mb (marker 63) and ~2.2Mb (marker 64). Interestingly, various genes within this region are associated with fertility and embryonic viability, such as is the case for *pbs-1* (*IV*:1,708,804), *egl-18* (*IV*:1,902,323) and *npp-20* (*IV*:1,490,656) and protein kinases like *cmk-1* (*IV*: 1,824,528) that are able to modify intermediary effectors resulting in differences in gene expression of the selected target genes [www.wormbase.org]. Taken together, these findings suggest that these genes may act as master regulators for *C. elegans* reproduction under heat stress conditions. In the present study, the issue under enquiry is the confirmation of the region distal to chromosome *IV* that was previously found by QTL mapping as responsible region for phenotypic variation in heat stress response.

Furthermore ILs ewIR45 and ewIR48 showed the highest significant effect in gene expression for *ZK355.3* and *F08H9.4* (Figure 5), the last one is reported to be related to stress response and proteostasis [31-33]. These two genes showed a higher expression in heat stress (~2 fold) versus control conditions in the ILs ewIR45 and ewIR48 when compared to control lines (N2 and CB4856) in both treatments. Currently, little is known about their function however we provide evidence for their involvement in heat stress response and suggest their involvement may even be high up in the genetic hierarchy of heat stress response regulation.

ZK355.3, located in chromosome *II*, encodes a protein of which its function remains unknown. It was earlier identified as a DAF-16 target [34] suggesting this gene to be involved in the regulation of, amongst other, life history traits, life span and stress response. Its expression in both parental lines is increased in heat shock conditions (Figure 6.A).

F08H9.4 encodes a small heat shock protein (sHSP) of the HSP16 class [31]. This gene is located on chromosome *V* of which it is known that the expression increases as a result of heat shock. It was suggested to play an important role in heat stress response but to what extent remains unknown. This gene is commonly expressed in the excretory canal and ventral nerve-cord; however under heat shock conditions *F08H9.4* expression has also been reported to be induced in the intestine [35]. The sHSPs are molecular chaperones that support/assist large heat shock proteins (HSPs) during rescuing of mis-folded proteins to prevent aggregation and thus maintain cellular proteostasis after stress [36, 37, 38]. In our previous microarray data we found similar expression of *F08H9.4* in N2 and CB4856 strains in control conditions. However, after heat shock the expression of *F08H9.4* is up-regulated in both strains, showing slightly higher regulation in Bristol N2 (Figure 6B).

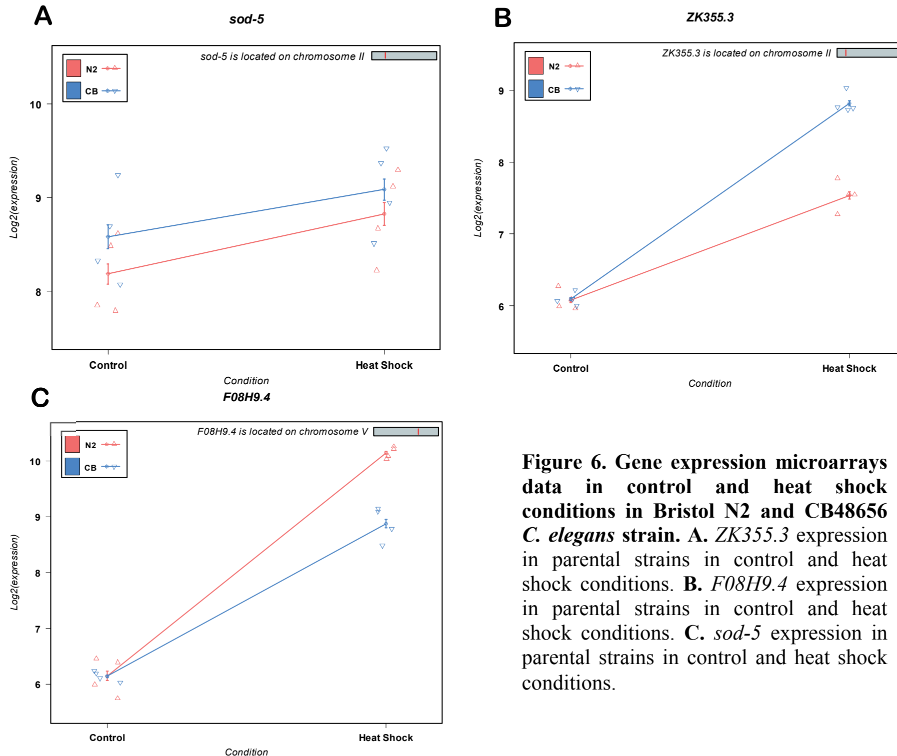

Figure 6. Gene expression microarrays data in control and heat shock conditions in Bristol N2 and CB48656 *C. elegans* strain. A. *ZK355.3* expression in parental strains in control and heat shock conditions. **B.** *F08H9.4* expression in parental strains in control and heat shock conditions. **C.** *sod-5* expression in parental strains in control and heat shock conditions.

The expression of *sod-5* (superoxide dismutase) is also highly affected by heat stress in the IL ewIR48 (Figure 4). *sod-5* was previously described as a DAF-16 target gene [39] and could therefore be closely related with stress response. Yanase *et al.* [39] described a function of *sod-5* where the role of *sod-1* is rescued by *sod-5* expression when compared *sod-1* loss-of-function mutant to wild type Bristol N2. We found no significant differences in expression patterns of *sod-5* between Bristol N2 and CB4856 strains in our previous microarray data (Figure 6C), nor in the RT-PCR data. Furthermore we observed minor up-regulation of *sod-5* in the parental lines in heat stress conditions and have obtained similar results in many of the ILs reported in this study. Interestingly, the case of ewIR48 is unique in its (expression) pattern. We observed low relative expression of *sod-5* in ewIR48 under control conditions. However after inducing the heat shock, ewIR48 expression of *sod-5* increased nearly 20-fold. These results suggest that the compensatory effect of the SOD-family enzymes may be affected by this locus distal to chromosome *IV*. Further studies focusing on SOD-family enzymes in (mutant-)ILs should be done in the future in order to confirm this hypothesis.

In this study we have confirmed the position of common heat-stress-response-regulators on chromosome *IV*, by gene expression measurements through qRT-PCR of a panel of 10 genes previously detected by eQTL mapping. We found that candidate genes directly related to stress response showed the highest variation in relative expression and that the introgression regions in ILs ewIR45 and ewIR48 may contain loci harbouring regulators for heat stress response.

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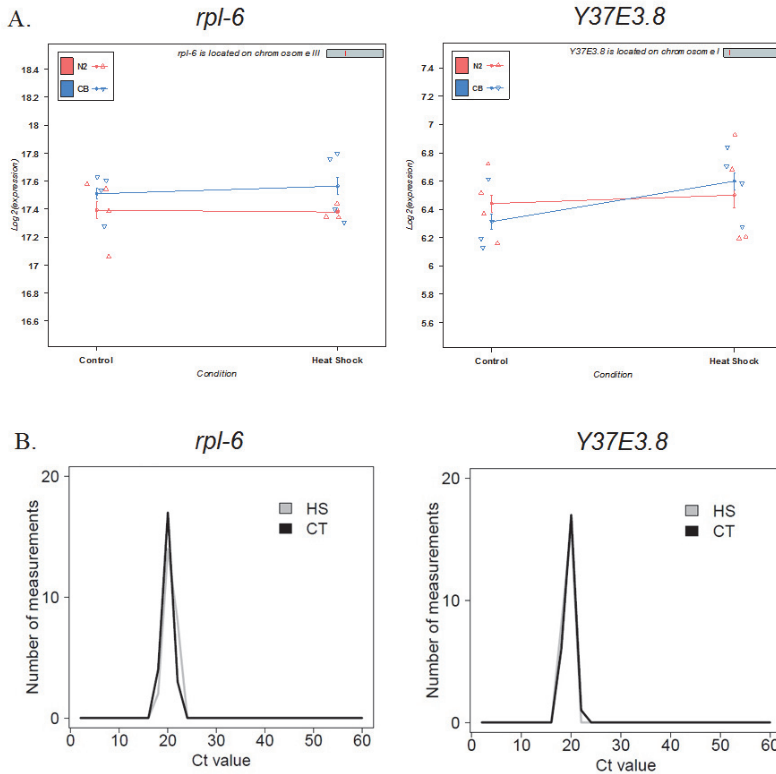
Supplementary data



Supplementary Figure S1. Reference genes: A. Expression pattern in Bristol N2 (N2) and CB4856 Hawaii (CB) in control and heat shock conditions**; B.** Average qPCR Ct value in the lines of interest for control (CT) and heat shock (HS) conditions.

Supplementary Table S2. List of preliminary candidate genes selected from microarray data on heat shock and recovery samples. Max. eQTL significance stands for the significance value of the peak of the maximum eQTL of this gene. Max. eQTL effect is the effect of the eQTL, positive values stand for the difference in gene expression means that is caused by the N2 allele, negative values show the difference in gene expression that is caused by the CB4856 allele. Max. eQTL significance is the significant value of the peak of the maximum eQTL of this gene.

							HEAT SHOCK DATA			RECOVERY EFFECT		
	Gene ID	Gene name	WormBase ID	Chromosome	Chromosomal position (bp)	Cumulative genome position (bp)	Max. eQTL significance	Marker	Max. eQTL effect	Max. eQTL significance	Marker	Max. eQTL effect
1	27359	abts-4	WBGene00019844	X	6747261	89271232	3,77318491	79	0,39138231	4,32230108	79	0,53386032
2	25618	acs-12	WBGene00009106	V	20903940	82512462	3,32583267	62	-0,32522156	3,3058358	62	-0,34330277
3	34615	acs-21	WBGene00016849	V	3089073	64697595	4,6025147	62	-0,42384252	4,69686749	62	-0,56331321
4	39004	add-2	WBGene00000073	V	12009529	73618051	4,50547399	62	-0,43792819	4,97536779	62	-0,48402809
5	30633	ant-1.2	WBGene00007057	I	6729722	6729722	5,45530051	62	-0,71102945	7,37511573	62	-0,80080616
6	25465	C03B1.13	WBGene00015381	X	6335233	88859204	3,69118774	62	-0,3759756	3,45092513	62	-0,42013455
7	18629	C06H5.6	WBGene00007394	V	17879812	79488334	3,37015813	78	0,39506493	3,48984652	79	0,56347034
8	33167	C31H5.7	WBGene00007858	I	9041366	9041366	3,88085067	62	-0,35892675	3,43670267	62	-0,41171008
9	42528	C37A5.5	WBGene00014699	I	14151855	14151855	3,38708932	62	-0,53007831	3,14192038	62	-0,61632029
10	3238	C54F6.15	WBGene00044113	V	7522013	69130535	3,39396591	62	-1,10486127	4,22357877	62	-1,35634283
11	29620	C54F6.15	WBGene00044113	V	7522013	69130535	3,76217054	62	-1,20745155	5,13548956	62	-1,58407871
12	37411	C54F6.15	WBGene00044113	V	7522013	69130535	3,73678517	62	-1,12159995	4,33199958	62	-1,27638989
13	39092	C54F6.15	WBGene00044113	V	7522013	69130535	5,15077503	62	-1,47474986	5,04207959	62	-1,55616441

14	40472	C54F6.15	WBGene00044113	V	7522013	69130535	3,66783038	62	-1,16229317	4,0591584	62	-1,26175062
15	8243	C54F6.5	WBGene00016923	V	7529098	69137620	6,16581312	62	-0,9863593	5,90690915	62	-1,06897438
16	40901	cdr-3	WBGene00008297	V	12416336	74024858	4,34787991	62	-0,7094234	4,89038554	62	-0,69336209
17	7262	cnc-11	WBGene00044900	V	1442589	63051111	4,58744959	62	-0,62861103	3,03890372	62	-0,71271402
18	9640	cnc-11	WBGene00044900	V	1442589	63051111	4,608898	62	-0,63979275	3,03339815	62	-0,72572352
19	41712	cnc-11	WBGene00044900	V	1442589	63051111	4,48269689	62	-0,6058173	3,02000482	62	-0,72504113
20	8643	cnc-9	WBGene00044548	V	7399885	69008407	4,4149218	71	-0,33095027	3,21828699	71	-0,49429003
21	30122	djr-1.2	WBGene00016789	V	4054166	65662688	5,9045756	62	-1,00934729	5,44965	63	-1,0453711
22	8290	drr-1	WBGene00009741	II	13494957	28559782	3,12423638	62	-0,1927471	3,517009	63	-0,29926845
23	25346	E01A2.7	WBGene00017089	I	4142287	4142287	4,8478641	62	-0,32262681	5,13261795	62	-0,37450823
24	18514	F08F1.4	WBGene00017258	X	8411638	90935609	4,41717516	70	-0,32615193	3,03056328	66	-0,32386681
25	42607	F08H9.3	WBGene00008591	V	14461922	76070444	3,12882685	62	-0,39187997	4,24757754	62	-0,51201034
26	12207	F08H9.4	WBGene00008592	V	14463567	76072089	7,55092513	62	-1,0976453	8,25676509	62	-1,35647223
27	10319	F14F9.2	WBGene00017465	V	5144816	66753338	3,76684819	62	-0,85177776	5,04614311	62	-1,32172489
28	21867	F25B4.8	WBGene00017771	V	5690090	67298612	3,30778249	62	-0,22795831	3,73048379	62	-0,32641008
29	4877	F26D11.6	WBGene00017823	V	7955393	69563915	4,1979866	63	-0,39112174	5,04465971	63	-0,5343855
30	7646	F32B5.6	WBGene00017979	I	2668161	2668161	3,27373415	79	0,31493491	3,68749828	79	0,46523156
31	41770	F47B8.2	WBGene00009803	V	14315839	75924361	4,58964393	62	-0,35801724	3,24826029	63	-0,33630231
32	15090	F53F10.8	WBGene00044423	I	3828252	3828252	3,41176205	78	-0,33993955	3,29272397	72	-0,42255468
33	10362	fbxc-7	WBGene00016302	II	954054	16018879	4,61172174	70	-0,55417958	3,67047572	63	-0,53310498
34	19773	fipr-13	WBGene00010183	V	10064475	71672997	3,223945	62	-0,44082164	5,41346957	62	-0,70004631
35	23130	fipr-24	WBGene00007992	I	14157615	14157615	4,73438059	62	-0,69854425	3,10709808	63	-0,69071889
36	33720	fipr-26	WBGene00009963	I	8946700	8946700	5,89912957	62	-0,99569608	5,58171592	62	-1,09795164

37	483	gln-1	WBGene00001602	X	6058214	88582185	3,07948144	75	0,21922103	3,22548778	71	0,29740367
38	43213	gst-15	WBGene00001763	II	13611681	28676506	5,30735471	62	-0,53641934	5,57365775	62	-0,62053328
39	31465	gst-29	WBGene00001777	II	15168440	30233265	3,94998485	62	-0,4785607	3,38714551	62	-0,50870263
40	8050	H39E23.3	WBGene00044201	V	14107790	75716312	5,74414746	62	-0,54948404	4,03630265	62	-0,54242577
41	9150	H39E23.3	WBGene00044201	V	14107790	75716312	5,92047789	62	-0,55087771	3,4481105	63	-0,49129559
42	17164	H39E23.3	WBGene00044201	V	14107790	75716312	5,77845061	62	-0,59022493	3,54806344	62	-0,5444836
43	29655	H39E23.3	WBGene00044201	V	14107790	75716312	5,18458273	62	-0,59492409	3,23331766	62	-0,5404292
44	22944	ins-12	WBGene00002095	II	5554590	20619415	4,51010142	71	-0,47207464	3,46795457	71	-0,55548329
45	44694	ins-12	WBGene00002095	II	5554590	20619415	3,71728187	70	-0,41571829	3,03298125	71	-0,5121361
46	16068	ins-20	WBGene00002103	II	6010804	21075629	7,25170614	66	-0,73130776	3,91813966	65	-0,66775843
47	20676	ins-20	WBGene00002103	II	6010804	21075629	7,37100074	66	-0,80859141	3,88470538	64	-0,73581222
48	23288	ins-20	WBGene00002103	II	6010804	21075629	6,55997675	66	-0,71463702	3,64031493	65	-0,67923342
49	27852	ins-20	WBGene00002103	II	6010804	21075629	7,72234256	66	-0,78438325	3,49251009	64	-0,6862324
50	27895	ins-20	WBGene00002103	II	6010804	21075629	7,47363098	66	-0,72770555	3,98134149	64	-0,70322919
51	32162	ins-20	WBGene00002103	II	6010804	21075629	6,71483834	66	-0,71669851	3,58273676	64	-0,66106219
52	38494	ins-20	WBGene00002103	II	6010804	21075629	6,51930596	66	-0,70972646	4,32739863	65	-0,72687263
53	41736	ins-20	WBGene00002103	II	6010804	21075629	4,77902022	66	-0,56723254	3,03148157	62	-0,65638134
54	44906	ins-20	WBGene00002103	II	6010804	21075629	4,91667439	65	-0,59812933	3,72176345	64	-0,70674836
55	26652	K05F6.11	WBGene00044697	II	1540934	16605759	4,12309635	62	-0,31341843	3,20554546	62	-0,3011184
56	34687	K05F6.11	WBGene00044697	II	1540934	16605759	5,50058233	62	-0,37013356	3,66116895	62	-0,40464666
57	34630	K10D3.6	WBGene00010740	I	7116096	7116096	3,38267288	62	-0,37849584	3,07653548	62	-0,50121811
58	33435	K10H10.5	WBGene00010761	II	14501656	29566481	4,93278516	62	-0,56430087	6,82867704	62	-0,65716028
59	11182	lea-1	WBGene00002263	V	10020515	71629037	5,78540809	62	-0,73964551	4,31392813	62	-0,65860707

60	39700	lea-1	WBGene00002263	V	10020515	71629037	5,65685344	62	-0,74781732	5,30311608	62	-0,77486596
61	2141	lgc-31	WBGene00008997	V	15512742	77121264	3,22303978	79	0,24988143	3,64846718	79	0,41223442
62	21932	lsm-1	WBGene00003076	II	11126874	26191699	3,23234542	77	-0,16078148	4,22265651	77	-0,24318675
63	9274	M04C9.2	WBGene00010857	I	9344715	9344715	3,36265319	62	-0,27295193	3,02791494	62	-0,357858
64	37255	M04C9.2	WBGene00010857	I	9344715	9344715	3,77011036	62	-0,30028213	3,04940909	62	-0,39740347
65	8759	M60.4	WBGene00019780	X	8232195	90756166	3,44276912	63	-0,45527099	3,26613872	62	-0,45990078
66	13489	mlcd-1	WBGene00009439	III	4566243	34905361	4,30025844	62	-0,23319072	3,44056852	62	-0,28092479
67	30106	mtl-1	WBGene00003473	V	6691352	68299874	4,45747007	62	-0,6217686	3,23041235	63	-0,69428633
68	42997	nuo-5	WBGene00021562	V	2698969	64307491	3,0144648	78	0,49060527	3,63088173	79	0,72554773
69	20322	otpl-8	WBGene00010403	X	12266835	94790806	4,89465196	62	-0,44608627	3,85815175	62	-0,5910195
70	26328	pal-1	WBGene00003912	III	4804149	35143267	3,08468188	62	-0,20402813	3,09248467	62	-0,27678392
71	31261	R07E4.1	WBGene00019935	X	5959075	88483046	4,11288003	62	0,26235399	3,55877509	62	0,34625922
72	31330	sod-3	WBGene00004932	X	17092634	99616605	7,76908022	62	-0,83023397	3,98014096	62	-0,60951009
73	15490	sod-5	WBGene00007036	II	4417674	19482499	6,92283833	62	-1,0859864	5,32591238	62	-1,01319181
74	25884	srh-195	WBGene00005408	II	2114632	17179457	3,17313056	62	-0,24113658	4,00830966	62	-0,27696687
75	30449	srh-195	WBGene00005408	II	2114632	17179457	3,57155518	62	-0,25944075	4,84535703	62	-0,30790982
76	43811	sulp-4	WBGene00010788	V	11875338	73483860	3,62183111	62	-0,32138514	3,11763504	62	-0,35876831
77	29830	T24C2.5	WBGene00011984	X	14537651	97061622	3,79091168	62	-0,44171889	3,47689715	63	-0,48217623
78	24224	T27F6.9	WBGene00014847	I	12481582	12481582	3,97047013	71	0,51405829	3,16539869	71	0,48589027
79	39045	tsp-2	WBGene00006628	III	8236838	38575956	3,91902361	77	-0,58611113	4,13961352	72	-0,74221521
80	9772	ttr-1	WBGene00010541	III	9928959	40268077	3,15214853	72	0,19626432	3,68350374	72	0,26186679
81	34888	ttr-1	WBGene00010541	III	9928959	40268077	4,40363774	65	0,17387861	3,5468938	72	0,21942184
82	6758	ugt-1	WBGene00007072	V	10395650	72004172	3,23809498	62	-0,3266686	3,55182503	62	-0,4599387

83	13639	W02D7.11	WBGene00020946	V	8302866	69911388	5,55074412	62	-0,78792208	4,63330798	63	-0,84506949
84	19343	W08F4.12	WBGene00021101	II	583078	15647903	3,73744733	63	-1,12244473	3,80283049	64	-1,18582518
85	42210	W10C8.6	WBGene00044433	I	2858618	2858618	3,4646288	62	-0,3294306	3,24433154	63	-0,38418082
86	5974	Y110A2AL.2	WBGene00022439	II	2867992	17932817	5,13244463	62	-0,60669228	6,57159244	62	-0,68562394
87	13320	Y110A2AL.2	WBGene00022439	II	2867992	17932817	5,18674205	62	-0,64821948	6,00376036	62	-0,73847276
88	16794	Y110A2AL.2	WBGene00022439	II	2867992	17932817	5,22594635	62	-0,66650304	6,23119067	62	-0,7685286
89	16833	Y110A2AL.2	WBGene00022439	II	2867992	17932817	5,16799223	62	-0,62422533	6,70764504	62	-0,72537657
90	29851	Y110A2AL.2	WBGene00022439	II	2867992	17932817	3,41337541	62	-0,59369153	5,21857564	62	-0,89066648
91	30884	Y110A2AL.2	WBGene00022439	II	2867992	17932817	5,00209145	62	-0,64578062	6,34064825	62	-0,74425428
92	4963	Y26D4A.10	WBGene00012507	I	13098675	13098675	4,21537081	71	-0,51374785	4,51963201	71	-0,6620048
93	30517	Y39H10A.1	WBGene00021478	V	3767190	65375712	6,2490723	62	-0,4872568	3,11943606	62	-0,53819315
94	9779	Y40B10A.9	WBGene00021493	V	2068679	63677201	3,40098384	75	-0,38041018	4,63660235	72	-0,64820007
95	13951	Y53C10A.1	WBGene00014915	I	11966261	11966261	3,49569703	79	0,12513482	3,60408468	79	0,20192345
96	31825	Y70C5A.3	WBGene00050904	V	16618321	78226843	4,37368831	62	-0,67097141	3,8352853	62	-0,67390245
97	38393	Y75B8A.37	WBGene00013565	III	12315363	42654481	3,675488	79	-0,42218014	5,20944524	79	-0,61778424
98	16281	ZK355.3	WBGene00022713	II	2927056	17991881	3,2068739	63	-0,52339759	4,14700937	62	-0,78797655
99	4949	ZK669.3	WBGene00014053	II	7941082	23005907	4,78431646	62	-0,4567792	3,49714378	62	-0,45352487

Supplementary table S3. Primer sequences of selected genes. QPCR reference genes are written in bold.

Gene	ID primer	Orientation	Primer Squence	Source	Primer length	Product size	%G-C	Estimated Tm (°C)
fipr-24	pP3FI-24	F	ctattcctccttgccatcct	PRIMER 3	20	146	50	62,32
		R	gtgtgtggagcttcagagt	PRIMER 3	19		55	62,89
sod-5	pP3SO-5	F	actgctgtcttcggaactg	PRIMER 3	19	103	50	63,24
		R	gaatccatgaagtcctggtga	PRIMER 3	21		50	63,19
ZK355.3	pP3ZK-3	F	gactcccaaggctcaaaatc	PRIMER 3	20	172	50	62,18
		R	agtcactgaatgggtctcc	PRIMER 3	19		55	62,01
lea-1	pP3LE-1	F	tcgagaacaaggcttcagac	PRIMER 3	20	192	50	63,32
		R	ccttgaaagagtcggctaca	PRIMER 3	20		50	63,05
mlcd-1	pP3ML-1	F	acggcgcagagttgtatag	PRIMER 3	19	117	50	63,14
		R	tgttctcgtgaaccttctcc	PRIMER 3	20		50	62,98
mtl-1	pP3MT-1	F	gcagtggagacaagtgttg	PRIMER 3	19	101	50	62,24
		R	gacagtttgcacacttgcag	PRIMER 3	19		50	62,77
tsp-2	pP3TS-2	F	cggactagatatctggggttc	PRIMER 3	21	154	52	62,53
		R	cccgtaaatgctccagtaga	PRIMER 3	20		50	62,56
djr-1.2	pP3DJ-1.2	F	tattgttccggacgtagctc	PRIMER 3	20	153	50	62,88
		R	gcaccaattagacctccaga	PRIMER 3	20		50	62,8
fbxc-7	pP3FB-7	F	cgtaggactcattggcaact	PRIMER 3	20	172	50	63,41
		R	acgaagtaggacgatgttcg	PRIMER 3	20		50	63,13
F08H9.4	pP3F08	F	gtctccaacttcaagccaga	PRIMER 3	20	81	50	63,29
		R	ctccacgtcatgttctcct	PRIMER 3	19		50	62,36
Y37E3.8	**pP3Y37**	F	gcgtttgtggtctcttgtc	PRIMER 3	19	123	50	62,58
		R	ctctgggaggagtccttttc	PRIMER 3	20		50	62,8
rpl-6	**pP3RPL-6**	F	tgtcactctccgcaagac	PRIMER 3	18	137	55	62,51
		R	tgatcttgtgtggtccagtg	PRIMER 3	20		50	63,29

6

General discussion

Miriam Rodriguez

The beauty of perturbation is exposed when you know how to overcome it when it occurs.

Perturbation

Perturbation: (*noun*) *deviation of a system, moving object, or process from its regular or normal state of path, caused by an outside influence* (definition by *Oxford Dictionaries Online*, Oxford University Press).

The response to a perturbation by a cell or organism is essential for its survival and functional characteristics. In this thesis I address how studying responses to perturbation in *C. elegans* provides insight into the genetic buffering system that is associated with genetic and cellular imbalance. The results presented in this thesis demonstrate the great importance of perturbation in the process to reveal covered effectors in vital functions.

Cryptic genetic variation: the card up the sleeve?

Despite the phenotypic similarity that genetic variants can show in normal conditions, when these variants are exposed to a certain perturbation they may respond strongly different showing a range of highly divergent phenotypes. This phenomenon is known as 'cryptic genetic variation' (CGV) [1]. CGV does not have any obvious influence on the phenotype and it is only perceptible as a reaction to perturbation. Revealing part of this CGV has become the main goal of my research and hence the central topic for this thesis.

Different genetic backgrounds may determine the diverse effects resulting from one mutation in different genetic variants [2]. It is also observable that environmental as well as genetic perturbations may have different effects when they affect different backgrounds. Recently Gidalevitz *et al*. demonstrated that natural variation plays a crucial role in the susceptibility to protein aggregation of genetically divergent *C. elegans strains [3].*

The results of my thesis point to the molecular mechanisms that enhance cellular survivorship [4-7] and suggest that these mechanisms must be robust enough to prevent unbalanced conditions in the cell in order to safeguard cell survival [8]. Thus, mild variations should be neutralized during normal conditions (i.e. non extreme conditions) in order to preserve the molecular balance. However, in the case of a severe perturbation (genetic or environmental) the molecular balance is irremediably broken at once. In this situation the cells need to use their "artillery" in order to survive. Under these extreme conditions the CGV will emerge. Using controlled critical conditions such as environmental insults or genetic unbalance like introgressed mutations and monitoring the molecular mechanisms occurring under such circumstances has helped us in revealing cryptic variation in *C. elegans*.

As a result of integrating the causal allele of the strain *bar-1(ga80)* into the CB4856 genetic background, instead of its original Bristol N2 background, cryptic genetic variation associated with Wnt signalling in *C. elegans* is exposed. Moreover, the position of responsible loci is mapped. In chapter 3 the reader can find a description of the method that finally led us to the gene *sqv-2* as a candidate regulator of canonical Wnt pathway in *C. elegans*. *sqv-2* is the *C. elegans* homolog of the human gene beta3GalT6 [9]. Recent studies described mutations in the B3GALT6 gene identified in connective tissue disorders [10]. Further research must be carried out on *sqv-2* and other candidates to demonstrate the

connection with the Wnt pathway in *C. elegans* and from *C. elegans* to mammals and humans.

With regard to human populations some individuals seem to be better “equipped” to deal with environmental or lifestyle perturbation than others. This is also the case when the perturbation has a genetic nature, i.e. when the individual carries a mutation. Over time, many studies have emerged, which presented different individuals carrying a particular mutation displaying different phenotypes [11]. What in colloquial language would be called ‘random response to a perturbation’ in scientific language we call ‘genetic background determination’.

Exhaustive studies of signalling pathways and genetic networks have been so far the best approach to understand complex human diseases. However uncovering CGV may be crucial to measure the grade of disease susceptibility, a very important value to take into consideration in research, diagnosis and treatment development of a disease. Gibson (2009) proposed that CGV determines in many cases the grade of susceptibility to a certain disease. Therefore uncovering such a CGV may explain epidemic aspects of several complex genetic diseases arising nowadays as a consequence of the abrupt changes in lifestyle of modern human societies [12].

Natural genetic variants

The biological effects of a genetic or an environmental perturbation as well as the integration of stress response are broadly studied in eukaryotes. Highly divergent organisms have been found to share physiological effects on gene expression and signalling pathways [6].

A representative case is described in chapter 3 of this thesis where we introgressed a mutation into a genomic mosaic from two highly divergent *C. elegans* strains. Many studies induced mutations in *C. elegans* in order to study gene functions in signaling pathways [13, 14]. However, the need to include natural genetic variation in order to understand genetic architecture of certain traits is becoming increasingly more accepted by researchers [15]. We found loci showing natural variation of *bar-1* mutation response. The eQTL results described *trans*-bands that indicate the effect of a concrete mutation is modified by the genetic background where that mutation is introgressed.

The need of combining different genotypes in experimental procedures emerged to understand the inherent genetic variation among individuals. There are a vast number of studies that claim the power of natural genetic variation for identifying genes underlying complex traits in *C. elegans* [16-23].
The advantage that makes *C. elegans* a relatively easy system because of its short generation time and its inbreeding ability helped us to construct large sets of RIL populations, each of them containing isogenic individuals. The polymorphic markers (genotypes) together with the phenotypic measurements were used to estimate the position of genomic regions associated to phenotypic variations as a response to the same stimulus, and finally to point at loci involved in such a variation (QTL mapping).
More concretely this thesis presents a number of loci related to stress response following different types of perturbations. Hence, introgression lines harbouring these regions were

tested and the results confirmed the significant differences in phenotypic responses under control and stress conditions. I have given evidence for new effectors of heat stress response by making use of the natural genetic variation inherent to heat-shock response. To this respect we consider the hypothesis of a locus regulating stress effectors, such as superoxide dismutase enzymes (SOD), identified by the combination of introgression lines and heat-shock experiments.

Stress in genetic variants

In this thesis the most relevant results of my research have been presented. However, some of the obtained data during my PhD could not be shown as part of the experimental chapters of this thesis since their analysis is still in process. An example of this is the general expression pattern we found for the unfolded protein response genes [24] showing that in Bristol N2 the expression of *a*ctivated in *b*locked *u*nfolded protein response genes *abu-6*, *abu-7*, *abu-8* and *abu-10* was lower than in CB4856 in control conditions and the differences in expression of these genes became higher after the heat-shock treatment when Bristol N2 showed an over-expression of the proteostasis-related genes while in CB4856 the expression patterns remained stable (Figure 1). This differential expression might explain, partly, the observed variation in hormesis between these two *C. elegans* strains that showed that recovered (after heat-shock) CB4856 nematodes lived longer than Bristol N2 and might be a key of the variation in the protection of the organism from damage caused by a perturbation in these strains.

However, the data showed other pathways involved in this response. Different genes from Insulin like/IGF-1 signalling (ILS) and TGF-ß pathway were expressed differently under heat stress conditions. Several studies showed these two pathways as responsible for stress response, resulting in dauer entry in young juveniles or elongation life span in adults [25]. In detail, ILS has been shown as the responsible pathway during early development for induction of larvae to develop into stress-resistant dauers, whereas in late developmental stages or in adults, ILS increases longevity [26].

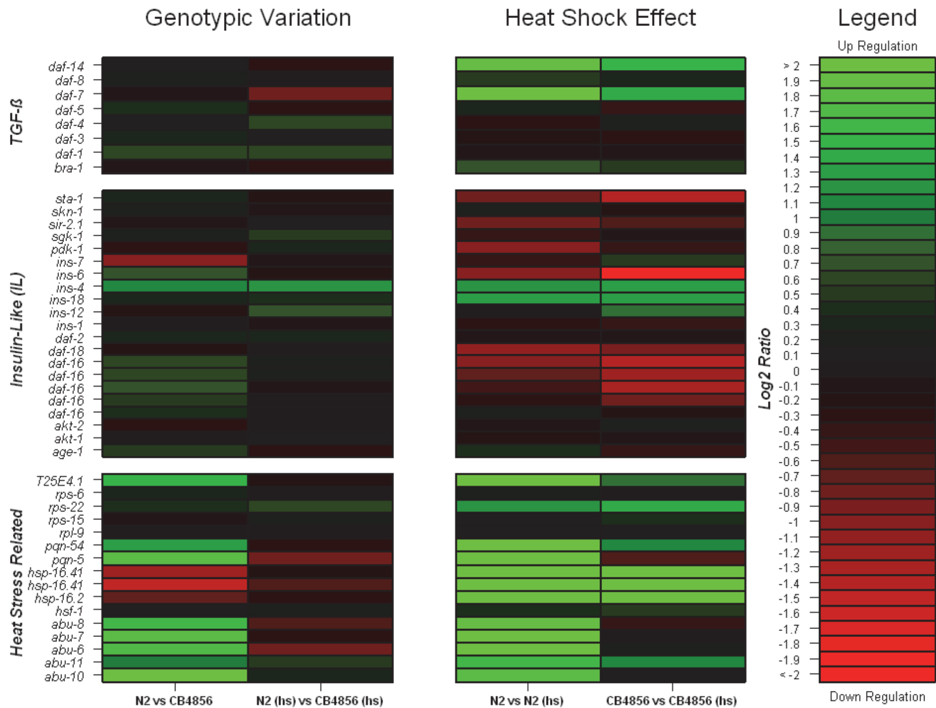


Figure 1. Heat map of transcriptome in Bristol N2 and CB4856 in control (20°C) and heat shock (35°C 2-4 hrs.) conditions.

In response to harsh environmental conditions, *C. elegans* larvae undergo dauer arrest [27]. *C. elegans* embryo and young larvae use the glyoxilate cycle to generate carbohydrates from lipid stores, but at later stages of development the nematode shifts toward aerobic respiration and exhibits a relative increase in tricarboxilic acid (TCA) cycle activity. Dauers do not undergo this shift [28]. Since our stress response studies were mostly performed in L4 pre-adult individuals, the ability to enter into dauer stage is abolished, therefore I suggest the nematodes activate a stress response intimately related with dauer entry process but with different results as it is life span elongation [29]. The different ability to express related genes for this purpose might confer a natural variation in heat stress response for these two strains and consequently for the RILs and ILs generated from their crossing.

Concluding remarks

My approach intends to detect novel genes and alleles, which affect stress response, and hence potential new candidates of human disease genes. For this purpose we used QTL mapping as our primary tool, this is no more than a statistical method to associate a certain phenotype to a genetic marker; however analyzing complex genetic traits appears much more complicated. The problem that represents the need to combine different genetic background with many different environments was approached by using RILs and ILs. These tools allowed us to create many combinations of two genomes that were exposed to different stressors, conditions and mutations. QTL mapping has been implemented in this thesis by using gene expression as a quantitative trait (eQTL). Gene expression patterns were revealed by microarrays and RT-PCR. Nevertheless I consider next generation sequencing and more concretely RNA sequencing as the next tool for transcriptomics in the way to identify new counterparts underlying complex traits.

This thesis described in detail the utilization of previous knowledge as well as the developing of new tools and finally their combination. Therefore the potential of the method has been remarkably increased in order to identify and describe novel effectors in the biology of *C. elegans*.

The CGV standing behind complex traits is becoming an essential aspect and a crucial point of research in order to understand complex genetic processes. The next efforts to understand complex traits should lead to the identification of nucleotide polymorphisms directly responsible for such a CGV.

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Summary

The genetic architecture of an organism could be considered ‘the most amazing piece of engineering’ existing in nature. Looking from a certain distance, the genetic complexity of an organism could be described as an immense jigsaw puzzle. As in a real jigsaw, the connection between two pieces will suggest or bring us to the third one and so on. The genetic responses to perturbation reveal interactions between many alleles of which effects are not that noticeable under optimal conditions.

In the general introduction of this thesis (chapter 1), the reader can find a brief overview of the model organism *Caenorhabditis elegans* and how this nematode has increasingly taken a crucial position in relevant biological studies over the last few decades. Moreover, the outline of this thesis is extensively described in this section.

My thesis brings together examples of perturbation in *C. elegans* at three different levels: phenotypic perturbation, as a result of genetic variation in Recombinant Inbred Lines (RILs), genetic perturbation, performed by genome combinations (RILs), introgressed fragments of exogenous genome in introgression lines (ILs) and induced mutations, and environmental perturbation (i.e. heat stress conditions). We have combined these perturbations in order to increase the potential of these tools in investigating genetic variation in *C. elegans*. Our RILs and ILs are combinations of two of the most divergent genomes of *C. elegans* strains these are Bristol N2 and CB4856 (Hawaii).

The translation of this knowledge to research in human disease is of great interest since many complex diseases are regulated by small effect genes that occur during stress or aging. This thesis expounds how *C. elegans* has gained a prominent position as a model organism for studying the genetic of complex disease pathways. Here I have presented different studies and concluded that environmental stress perturbation experiments in *C. elegans* represents a crucial implement in the way to increase our understanding of the genetics of human diseases such as Huntington’s disease, Parkinson’s disease, Alzheimer’s disease or cancer. We performed an exhaustive analysis of the current experiments and new insights that used *C. elegans* as a model organism for complex human diseases. This thesis contains a detailed study where we analyze the past, discuss about the present and suggest new possibilities for the future of *C. elegans* as a model organism (chapter 2).

The experimental sections of the thesis consist of quantitative genetic analyses of genomic regions associated with phenotypic variations observed under a certain perturbation. We use genotypic and phenotypic data measured in our RILs and ILs together with QTL mapping techniques to estimate the position of candidate loci, which are associated with a phenotypic variation.

In *C. elegans*, β-catenin gene *bar-1* plays an important role in vulva development whereas in humans it plays an important role in cancer progression. In our study in *bar-1* mutant RILs, we constructed different combinations of two genomes in *C. elegans* strains. The lack of β-catenin was included to the combination of the two genomes. These mutant RILs point us to the presence of polymorphic modifiers of vulva development in *C. elegans* and pave the way towards the development of a new tool to uncover cryptic variation in two genetic variants (chapter 3).

To gain insight into the subtle effects of natural variants and more concretely into the genetics of heat-shock recovery, we exposed RILs to heat-stress. We investigated here the hormesis effects on life span and offspring, and described associated loci in *C. elegans* (chapter 4). Our observations showed that there is natural variation in hormesis effects on life span for heat-shock.

As a combination of environmental and genetic perturbation, we studied the effects of heat stress in *C. elegans* combined with ILs (chapter 5). We demonstrate the importance of the chromosome *IV* and a concrete locus in heat stress response. In order to identify the role of a locus distal to chromosome *IV*, we selected a set of ILs containing a fragment of CB4856 (Hawaii) genome introgressed in a N2 (Bristol) background around this candidate locus previously described in other studies. We studied gene expression in normal and heat stress conditions by quantitative RT-PCR. Through this study we are able to confirm the role of the locus distal to chromosom*e IV* in heat stress response.

In the general discussion (chapter 6) I discuss the final results of the thesis and put them into the perspective of the current progresses in genetics studies of *C. elegans* and how stress response is associated with variations in pathways and thus in cellular and molecular process in health and/or disease.

Overall this thesis demonstrates at least a part of the incredible potential of *C. elegans* as a complex genetic model using quantitative genetic analyses, which complement more established forward genetic screens.

Samenvatting

De genetische architectuur kan beschouwd worden als een van de meest opzienbarende constructies in de natuur. Van enige afstand beschouwd, lijkt het op een immens grote puzzel waarbij het ene stukje van de puzzel naadloos past in de andere enz. De reactie van de genetische architectuur op een verstoring maakt het mogelijk om specifieke interacties tussen genen (de puzzelstukjes), of hun allelen, in kaart te brengen. Hierdoor kunnen interacties worden blootgelegd die onder normale omstandigheden niet gedetecteerd kunnen worden. Dit wordt ook wel cryptische variatie genoemd. De genetische architectuur kan goed bestudeerd worden in zgn. model organismen, soorten waarvan al heel veel bekend is zoals de DNA volgorde en de organismale ontwikkeling en fysiologie. Een belangrijk modelsoort is de nematode *Caenorhabditis elegans*.

Hoofdstuk 1 van dit proefschrift gaat dieper in op het gebruik van *C. elegans* als biologisch model door de jaren heen en voor het bestuderen van de genetische architectuur in het bijzonder. Het behandelt de verstoring van de genetische architectuur op drie verschillende niveaus: fenotypische verstoring voortkomend uit de genetische variatie die zich bevindt in recombinant inteelt lijnen; genetische verstoring voorkomend uit zgn. introgressie lijnen en kunstmatig geïnduceerde mutaties; en verstoring d.m.v. verandering van de omgeving (zoals bv. hitte stress). Deze verschillende typen verstoringen zijn in diverse combinaties bestudeerd om inzicht te krijgen in de genetische architectuur van *C. elegans*. Hierbij is uitgegaan van de genetische variatie die omvat wordt door twee van de meest genetisch divergente lijnen van *C. elegans*: Bristol N2 en CB4856 (Hawaï).

Hoofdstuk 2 gaat in op hoe kennis van de genetische architectuur kan worden gebruikt om meer inzicht te krijgen in de genetica van complex humane ziekten aangezien *C. elegans* een belangrijk model is voor de studie naar genetische pathways (gen cascade systemen) die geassocieerd zijn met disease pathways: cascadesystemen die geassocieerd zijn met de genetica van humane ziekten. Het hoofdstuk gaat in op de type stress experimenten die met *C. elegans* kunnen worden uitgevoerd om meer inzicht te krijgen in de genetica van complexe ziekten zoals de ziekte van Huntington, Parkinson, Alzheimer en kanker. Uiteengezet wordt hoe deze experimenten dienen te worden uitgevoerd, wat de implicaties zijn voor humaan onderzoek en wat de toekomstperspectieven zijn.

Hoofdstuk 3 belicht de kwantitatief genetische studie naar de genomische regio's (QTL of *Quantitative Trait Loci*) die geassocieerd zijn met fenotypische variatie onder invloed van een genetische verstoring door het uitschakelen van het gen *bar-1*. In *C. elegans* is *bar-1* van belang voor een correcte ontwikkeling van het vulvasysteem terwijl in mensen *bar-1* een belangrijke rol speelt in de ontwikkeling van bepaalde typen kanker. Door N2 x CB4856 recombinant inteelt lijnen en introgressie lijnen te kruisen met *bar-1* mutanten zijn er nieuwe genen en allelen ontdekt die een mogelijke rol spelen bij de kankerontwikkeling. De QTL zijn verder ontrafeld om potentiele kandidaatgenen aan te wijzen. Op deze manier wordt inzicht verkregen in de verborgen genetische variatie van *bar-1,* oftewel de cryptische variatie.

Hoofdstuk 4 gaat dieper in op het effect van externe verstoring d.m.v. hitte stress op de genetische architectuur van veroudering in *C. elegans*. Hiervoor werden recombinant inteelt lijnen en introgressie lijnen blootgesteld aan een korte, maar hevige hitte schok. Op deze

manier werd inzicht verkregen in de loci die geassocieerd zijn met levensduur en hitte tolerantie.

De hitte stress, in combinatie met genetische verstoring werd verder bestudeerd in hoofdstuk 5 waaruit blijkt dat een QTL op chromosoom *IV* een belangrijke rol speelt in de respons van de genetische architectuur. M.b.v. introgressie lijnen op basis van N2 en CB4856 zijn een aantal kandidaat genen getest d.m.v. kwantitatieve RT-PCR die de detectie van de QTL inderdaad valideert.

Hoofdstuk 6 besluit het proefschrift met een analyse van de bevindingen in de context van het huidige genetisch onderzoek in *C. elegans*. Hierbij wordt aangegeven hoe genetisch variatie in stress respons geassocieerd is met gen cascadesystemen en op welke wijze dit kan leiden tot meer inzicht in de genetica van humane ziekten. Samenvattend laat het de enorme mogelijkheden zien van het gebruik van genetische variatie in *C. elegans* in combinatie met mutatieanalyse.

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The truth is that every step I took in my life led me inevitably to this point. Therefore everyone I have encountered on the way has had a certain influence and contributed in one way or another to this achievement. It would be simply impossible to mention all of them in this text, otherwise the acknowledgments section would occupy more space than the thesis itself. I will try to summarize this list into some names of people that shared this great experience with me although many more have been, are and always will be present on my mind.

First of all I thank my daily supervisor and mentor Jan Kammenga whose patience, leadership and half-British refined sense of humour have helped me over all this time; this thesis is as much mine as his since without him none of this would have been possible. Also my *promotor* Jaap Bakker, who has played a very important role along the performance of this thesis, his wise advice and valuable suggestions were always of great worth, these together with his proximity and ability to keep the group together makes him an excellent professor.

During this time I had the great opportunity to be part of a project that brought together different European research groups, having worked together with brilliant scientists from whom I have learned a lot. In this matter I would like to thank the whole PANACEA project partners that I have collaborated with such as Michael Hengartner, Sabine Schrimpf and Cathy Zeng from the University of Zürich, Gino Poulin and Mark Elvin from Manchester University, Ritsert Jansen and Yang Li from University of Groningen, Jasmin Fisher and Antje Beijer from Microsoft Research Cambridge, and especially Alex Hajnal and Tobias Schmid from the Institute of Molecular Life Sciences (UZH) with whom I have closely collaborated, sharing not only priceless scientific achievements and interesting discussions but also a fun and entertaining time during meetings and congresses. I was honoured to have learned from such a brilliant group.

Back in the Netherlands, in Wageningen and concretely in Nematology it is fair to say that there was no one member of this department that has not helped me somehow. Starting by the *C. elegans* group, I especially thank Basten and Joost because they have worked actively to make this book possible. My other direct colleagues have also been a great support during this time. Mark and Rita have been my roommates for a long time and their company has always been very pleasant. My PhD predecessors in *C. elegans* group at NEMA Evert, Olga and Ana should also take an important position in my acknowledgments because their work has been a source of knowledge and inspiration during this time, I specially thank Aniezska whose legacy was crucial to perform my thesis.

The laboratory of Nematology has been a perfect working environment for me during the last 5 years, I felt part of it from the beginning and that was because of the quality of people working there who gave a familiar feeling to everyone that passed by. After these years I haven't only met co-workers but I have also treasured great friends. I will start with my dear paranymphs, Jet and Roel, they were very well chosen for this task. Jet, because she has been my faithful friend in NEMA from the beginning, we have shared good and bad

moments and she helped me and offered me her unconditional friendship all this time. Dr. Jetteke you were my paranymph since I started my PhD.
Roel came to the lab as a promising student, I could see his potential from the very first instant I met him, I trusted his work without hesitating at all and that's why his contribution resulted in a co-authorship of some of the chapters of this book, but more importantly he has an impressive human quality and we became very good friends also out of the lab, Roelious thanks for walking next to me all this time. For these reasons and a few more I can't think of anyone better than you guys to stand next to me in this important moment for my life and career. I hope that the hall in "Aula" won't be the last walk we take together.

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Sven "sunshine" this nickname is not a coincidence, people used to laugh at this but they don't know that over these years you have actually been the sunshine when I had a grey day in the lab. You really have the ability to cheer up everyone around you, to me personally you have played a very important role during my time in NEMA, you know you are my favourite colleague, but we better not say it out loud so nobody feels discriminated, *toch?*. Casper Q. your *stamppot* is the best ever no matter who may disagree, we spent great time together and I hope a lot more dinners will come. Paul, thank you for your help, not only as a lab-mate but also as a neighbour. I wish you the best in your new job, I am sure you'll do great. Rikus, I had a lot of fun with you and your peculiar laughing, you were always very

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Curriculum vitae

Miriam Rodríguez Sánchez was born on the 12th of March 1979 in Madrid, Spain. She obtained her university degree in Biology and MSc in Molecular Biology at Alcala University (Madrid) and in Immunology at Complutense University, Faculty of Medicine (Madrid). Her first experience working abroad was in the UK during her internship at the University of Hertfordshire (Hatfield, England). After some time working in the biomedical industry, she decided to return to academics and in 2009 she started her PhD at the Laboratory of Nematology at Wageningen University as part of the PANACEA European project.

Publications

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Rodriguez M., Snoek, B., de Bono, M. & Kammenga, J. E. (2013). Worms under stress: *C. elegans* stress response and its relevance to complex human disease and aging. Trends Genet. 2013 Jun; 29(6):367-74. doi: 10.1016/j.tig.2013.01.010

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HLA-G1, G2, G5, G6 and G7 transcripts are constitutively present in myeloid and plasmocytoid dendritic cells from umbilical cord blood. Jorge Martinez-Laso[1], Angela Román[1,3], Jorge Peñaloza[2], Miguel A. Herraiz[2], Jacqueline Head[1,3], **Miriam Rodriguez[1]**, Beatriz Gutierrez-Solar[1], J Vidart[2]; 1.- Cellular Immunotherapy group. Centro Nacional de Microbiología. Instituto de Salud Carlos III. Madrid, Spain; 2. Service of Ginecology & Obstetrics, Hospital Clínico de San Carlos. Madrid, Spain; 3. Microbiology department, Hospital Clínico San Carlos, Madrid, Spain. In: *Congress EFI 2008: 22nd Immunogenetics and Histocompatibility Conference; 2-5 April, 2008. Toulouse, France.*

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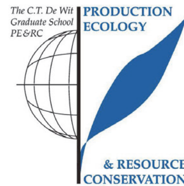
PE&RC certificate

PE&RC Training and Education Statement



With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)

- Worms under stress: *C. elegans* stress response and its relevance to complex human disease and aging

Post-graduate courses (5.6 ECTS)

- Worm Base tutorial; Online WormBase (2009)
- Basic statistics (2009)
- Introduction to R; PE&RC (2010)
- RNA seq; NIBIC (2013)
- Bioinformatics; PE&RC/EPS (2013)

Competence strengthening / skills courses (3.9 ECTS)

- PhD Competence assessment; WGS (2010)
- Techniques for writing and presenting; WGS (2010)
- Entrepreneurship boot camp for young scientist; DAFNE (2011)
- Interpersonal communication; NWO (2012)
- Last stretch of the PhD programme; WGS (2013)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.2 ECTS)

- PE&RC Day (2009-2012)

Discussion groups / local seminars / other scientific meetings (7.5 ECTS)

- Laboratory of Nematology colloquia (2009-2013)
- Ecogenomics discussion group (2009-2012)
- Ecogenomics day; Amsterdam (2010)
- Systems genetics meeting; Groningen (2010)
- Molecular mechanisms in cancer genetics; Amsterdam (2012)
- Dutch C. *elegans* meeting; Utrecht (2011-2012)

International symposia, workshops and conferences (9 ECTS)

- PANACEA Meeting (2009-2013)
- Worm meeting EBC; Hinxton, Cambridge, UK (2010)
- Model organisms to Human Biology; GSA, Washington DC., US (2012)
- 19th International *C. elegans* meeting; UCLA Los Angeles CA., US (2013)

Supervision of 2 MSc students (6 ECTS)

- Generation of a subset of introgression lines
- Analysing heat shock response of *C. elegans* natural variants

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