High specificity in *C. elegans* innate immune responses

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Contents

Declaration

I, **Alejandra Zárate-Potes**, declare that:

Apart from my supervisors' guidance the content and design of the thesis is all my own work;

Specific aspects of my thesis were supported by colleagues; their contribution is specified in detail in the following section "Authors' Contributions";

The thesis has not already been submitted neither partially nor wholly as part of a doctoral degree to another examining body. Apart from the included published paper (Chapter 2, High Innate Immune Specificity through Diversified C-type Lectin-like Domain Proteins in Invertebrates. Journal of Innate Immunity) no other part of the thesis has been published nor submitted for publishing;

The thesis has been prepared subject to the Rules of Good Scientific Practice of the German Research Foundation (DFG).

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Authors' Contributions

This PhD thesis consists of three chapters, each represented by a publication, or unpublished manuscripts. Alejandra Zárate-Potes developed original ideas, performed the majority of experiments, analysed and interpreted results and wrote the manuscripts with major contribution for chapters 1 and 3 and on collaborative basis for chapter 2.

Chapter 1

The GATA transcription factor ELT-2 mediates *Caenorhabditis elegans* strain-specific interaction with the natural pathogen *Bacillus thuringiensis*

Alejandra Zárate-Potes, Wentao Yang, Barbara Pees, Sabrina Butze, Rania Nakad, Philipp Segler, Rebecca Schalkowski, Philip Rosenstiel, Hinrich Schulenburg, Katja Dierking

AZP, HS and KD jointly conceived the idea.
AZP, BP, SB, RN, PS, RS, and KD performed the experiments.
PR provided next generation sequencing platform.
WY analysed transcriptomic data.
AZP analysed other experimental data.
AZP, HS and KD interpreted data and wrote manuscript.
HS and KD have joint senior authorship.

Chapter 2

High Innate Immune Specificity through Diversified C-type Lectin-like Domain Proteins in Invertebrates. *J Innate Immun*, 2015.

Barbara Pees, Wentao Yang, Alejandra Zárate-Potes, Hinrich Schulenburg, Katja Dierking

BP, WY, AZP, HS, and KD jointly conceived the idea, reviewed the relevant literature and wrote the manuscript.
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Chapter 3

The involvement of nested miRNAs in the defence of the nematode *Caenorhabditis elegans* against pathogenic *Bacillus thuringiensis*

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WY analysed the transcriptomic data.
AZP and BA analysed other experimental data.
AZP, KD and HS wrote the manuscript.

Hiermit bestätige ich als Betreuer die obenstehenden Angaben.

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Summary

Invertebrate immune systems, previously understood as lacking specificity, are now known to be able to mount specific responses to different pathogen species and even to different strains of the same pathogen species. This is not so surprising, as pathogens exert a strong selective pressure over their hosts. Therefore, evolution of specific, and possibly energetically economic, immune responses would be selected for, upon encounter with pathogen strains with which a natural history is shared. The molecular mechanisms that confer invertebrate immune systems the ability to mount specific responses remain mostly unknown. Gaining a more detailed understanding of host-pathogen interactions at the molecular level has the potential to change the way in which we interpret and treat infectious disease. The genetically tractable nematode model *Caenorhabditis elegans,* has the potential to reveal insights into the immune systems of other metazoans, including humans. There is already phenotypic evidence of specificity in the interaction of this nematode host with the Gram-positive, spore-forming, Crystal Pore-Forming Toxin (Cry PFT)-producing bacterium *Bacillus thuringiensis* (BT), for which numerous nematocidal strains have been isolated.

Here we found that *C. elegans* is able to mount specific transcriptomic responses to the BT strains MYBT18247 (BT247) and MYBT18679 (BT679), corresponding to 9% of all differentially expressed (DE) genes upon infection. We found that the GATA Transcription Factor (TF) ELT-2 is a central regulator of the strain-specific response. It positively regulates resistance to BT679 by promoting higher survival rate through the expression of putative immune effectors. ELT-2 also negatively regulates tolerance to BT247, as *elt-2* knockdown leads to an increase in survival rate, without affecting the pathogen load. In our results we observed that the main BT virulence factors, the different Cry PFTs, are likely to be responsible for eliciting specific responses. This may be due to particular modes of action or kinds of damage that characterize each PFT. BT247 and BT679 produce very different PFTs, what could explain the strain-specific responses. From this observation, we concluded that the immune responses mounted by *C. elegans* upon BT infection might even be virulence factor-specific. We hypothesize that cooperation with immune signalling pathways and additional TFs drives the specificity of the ELT-2-mediated response to BT.

Although we were able to characterize some of the molecular mechanisms downstream of ELT-2 leading to positive regulation of resistance to BT679 or negative regulation of tolerance to BT247, we couldn't find the upstream molecules that recognize the distinct pathogen signals. This thesis includes a review of the functional evidence available of the involvement of C-Type Lectin-like Domain (CTLD)-containing proteins in immune responses of invertebrate taxa, including nematodes. This diversified protein family (283 genes in *C. elegans*) has the

potential of contributing to immune specificity, with its members acting either as recognition receptors or as immune effectors. However, more experimental functional evidence needs to be gathered, before we can know if this is, in fact, the case. Apart from the molecules responsible of recognition, which remain unknown in the *C. elegans*-BT system, we are still missing some insight into how the intricate signal transduction and regulation network within the cell performs the decision-making process that leads to a specific response. In the end of this thesis, the focus is on the microRNA (miRNA)-mediated regulation of immune responses, particularly miRNAs found in nested genomic arrangements within protein-coding genes. We found that co-expression of miRNA and host genes found in genomic arrangements is not coupled with a shared function in response to BT infection. An example of this is *mir-58.1*, which negatively regulates immune responses to BT while its protein-coding host gene Y67D8A.2 does not.

The *C. elegans* response to BT infection is thus, specific, being able to mount particular responses even upon encounter with different virulence factors produced by this bacterial pathogen. This suggests that the specificity seems to be driven by a functional interaction with the pathogen and not by pathogen taxonomy.

Zusammenfassung

Obwohl die Immunsysteme von Invertebraten bisher als unspezifisch angesehen wurden, gibt es mittlerweile Nachweise dafür, dass es sehr wohl spezifisch auf verschiedene Pathogen Arten und –Stämme antworten kann. Diese Nachweise überraschen nicht, da Pathogene einen starken Selektionsdruck auf ihren jeweiligen Wirt ausüben, welches die Entwicklung spezifischer und daher energetisch deutlich ökonomischer Immunantworten favorisieren würde, wenn Wirt und Parasit eine gemeinsame natürliche Ökologie teilen. Allerdings, sind die molekularen Mechanismen, die dem wirbellosen Immunsystem die Fähigkeit verleihen spezifische Antworten zu geben, größtenteils unbekannt. Ein besseres Verständnis dieser Wirt-Pathogen-Interaktion auf molekularer Ebene könnte die Art und Weise, wie wir Infektionskrankheiten interpretieren und behandeln, verändern. Der genetisch manipulierbare Fadenwurm *Caenorhabditis elegans* hat das Potenzial, uns Einblicke in das Immunsystem anderer Metazoen, einschließlich des Menschen, zu geben. *C. elegans* eignet sich auch sehr da es hier bereits einen phänotypischen Nachweis der Spezifität in der Interaktion mit dem Gram-positiven, sporenbildenden, Toxin-produzierenden Bakterium *Bacillus thuringiensis* (BT), für das zahlreiche nematozide Stämme isoliert worden sind.

In dieser Arbeit fanden wir heraus, dass *C. elegans* in der Lage ist, mit spezifischen transkriptomischen Antworten auf die BT-Stämme MYBT18247 (BT247) und MYBT18679 (BT679) zu reagieren, was 9 % aller anders exprimierten (differentially expressed - DE) Gene nach Infektion entspricht. Wir fanden heraus, dass der GATA-Transkriptionsfaktor (TF) ELT-2 ein zentraler Regulator der stammspezifischen Antwort ist. Dieser reguliert die Resistenz gegen BT679 positiv, indem er zu einer höheren Überlebensrate durch die Expression potentieller Immuneffektoren führt. ELT-2 reguliert auch die Toleranz gegenüber BT247, hier allerdings negativ, da ein *elt-2*-Knockdown zu einer Erhöhung der Überlebensrate führt, ohne die Pathogen Belastung zu beeinflussen. In unseren Ergebnissen beobachteten wir, dass die wichtigsten BT-Virulenz Faktoren, die verschiedenen Toxine, wahrscheinlich für die Auslösung spezifischer Reaktionen verantwortlich sind, da diese charakteristisch für spezifische Toxine sein können. Die beiden pathogenen Stämme BT247 und BT679 produzieren sehr unterschiedliche Toxine, was die stammspezifischen Reaktionen erklären könnte. Aus diesen Beobachtungen schlussfolgerten wir, dass die Immunantworten die wir hier beobachten können Virulenz Faktor spezifisch sein könnten. Wir vermuten, dass die Kooperation mit Immunsignalwegen und zusätzlichen TFs die Spezifität der ELT-2-vermittelten Antwort auf BT beeinflusst.

Obwohl wir in der Lage waren, einige der molekularen Mechanismen stromabwärts von ELT-2 zu charakterisieren, die entweder zu Resistenz gegenüber BT679 oder Toleranz gegenüber BT247 führten, konnten wir die stromaufwärts gelegenen

Moleküle, welche die unterschiedlichen Pathogen Signale erkennen, bisher nicht identifizieren. Diese Arbeit beinhaltet eine Übersicht über die funktionellen Beweise für die Beteiligung von Proteinen mit einer C-Typ Lektin-ähnlichen Domäne in Immunantworten von wirbellosen Taxa, einschließlich Nematoden. Diese diversifizierte Proteinfamilie (283 Gene in *C. elegans*) hat das Potenzial zur Immunspezifität beizutragen, wobei ihre Mitglieder entweder als Erkennungsrezeptoren oder als Immuneffektoren fungieren könnten. Allerdings müssen mehr experimentelle funktionelle Beweise gesammelt werden, bevor wir dies nachweisen können. Abgesehen von den für die Erkennung verantwortlichen Molekülen, die im *C. elegans*-BT-System unbekannt sind, fehlen uns noch Erkenntnisse darüber, wie das komplizierte Signaltransduktions- und Regulationsnetzwerk innerhalb der Zelle den Entscheidungsprozess durchführt, der zu einer spezifischen Antwort führt. Zusätzlich liegt ein Fokus dieser Arbeit auf der microRNA (miRNA)-vermittelten Regulation von Immunantworten, insbesondere miRNAs, die sich in verschachtelten (*nested*) genomischen Anordnungen innerhalb von proteinkodierenden Genen befinden. Wir fanden heraus, dass die Koexpression von miRNA- und Wirtsgenen in genomischen Anordnungen nicht mit einer gemeinsamen Funktion als Antwort auf eine BT-Infektion gekoppelt ist. Ein Beispiel dafür ist *mir-58.1*, das die Immunantworten auf BT negativ reguliert, während das proteinkodierende Wirts Gen Y67D8A.2 dies nicht tut.

Die Antwort von *C. elegans* auf die BT-Infektion ist daher spezifisch, sogar bei Konfrontation mit verschiedenen von diesen bakteriellen Pathogenen erzeugten Virulenz Faktoren. Dies deutet darauf hin, dass die Spezifität durch eine funktionelle Interaktion mit dem Pathogen und nicht durch eine Pathogen Taxonomie entsteht.

INTRODUCTION

This PhD thesis attempts to answer the question of whether or not the invertebrate host *Caenorhabditis elegans* is able to mount high immune specific responses upon exposure to different strains of the Gram-positive pathogen *Bacillus thuringiensis*. If so, this thesis additionally seeks to uncover the underlying molecular mechanisms for high immune specificity in this invertebrate host. In this section, I will start with an introduction to the topic of immune specificity in invertebrates and why this is a relevant topic of study. Then, I will introduce the invertebrate genetically tractable host *Caenorhabditis elegans*, followed by a summary of what is known about the immune system of this organism. Finally, I will comprehensively describe what is known about the molecular basis of the *Caenorhabditis elegans-Bacillus thuringiensis* interaction. After reading this introduction, the reader should be able to understand the relevance of the research question and the significance of the results described further on.

Immune specificity in invertebrates

Immune systems are a feature of all living organisms and their role is to maintain homeostasis and ensure evolutionary fitness (understood as reproductive success), by mediating the interactions of the host organism with the abiotic environment, associated microbes and pathogens (Chen et al., 2013; Hooper et al., 2012). On the other hand, the pathogens' fitness depends on their ability to exploit the host's resources by infecting and causing damage, what is known as virulence (Casadevall and Pirofski, 2001). Immune systems are subject to strong selective pressures, particularly from their interaction with an unpredictable biotic environment filled with potential pathogens (Schulenburg and Félix, 2017). Therefore, they show an extreme taxon-specific evolution, even though it is also true that several modules of the immune system are conserved across taxa (Bailey et al., 2013). A hallmark function of the immune system is to carry out the distinction between harmful or harmless and self or non-self, while orchestrating overall responses leading to resistance (elimination of the pathogen) or tolerance (enhanced fitness without elimination of the pathogen), accordingly (Bailey et al., 2013; Janeway and Medzhitov, 2002; Medzhitov et al., 2012). To achieve its function, the immune system works at different scales spanning from the molecular, to the cellular and up to the organismal level (Chen et al., 2013). Traditionally, immune systems have been classified as innate or adaptive according to diagnostic characteristics. Innate immunity is general and produces the same responses independent of the challenge faced. It is widely accepted that all living organisms possess innate immunity (Hoffmann et al., 1999). On the other hand, adaptive immunity is traditionally considered as specific. It achieves different overall responses depending on the challenge faced and displays learning and memory characteristics. These characteristics make it able to better react during

the second encounter with the same pathogen. Adaptive immunity has been mechanistically described in the lymphocyte receptor-based system observed in jawed vertebrates (Brehélin and Roch, 2008).

In this thesis, I am particularly interested in immune specificity, which is the ability to distinguish between different species, strains or virulence factors of a given pathogen and mount custom-made responses, accordingly. Specificity in immune responses is selected for, in cases in which a fast and energetically-economic response can be mounted against pathogens with which hosts share a natural history, resulting in a fitness advantage for the host (Pees et al., 2016; Schulenburg et al., 2008). Breakthrough phenotypic discoveries in past years showed the presence of immune specificity in invertebrates (Kurtz and Franz, 2003; Little et al., 2006; Schulenburg and Ewbank, 2004; Schulenburg and Müller, 2004). Today this is still a major topic of interest in the field of study of host-pathogen interactions, because much remains to be elucidated about the extent and about the mechanisms behind immune specificity in invertebrates.

Evidence of immune specificity has been observed in a variety of invertebrate model systems. One of the major phenomena supporting this, is the ability of some invertebrate taxa to mount faster, and more specific responses, against a pathogen that is encountered for the second time. This is known as immune priming or acquired immunity (Cooper and Eleftherianos, 2017; Kurtz and Franz, 2003; Rimer et al., 2014; Sadd and Schmid-Hempel, 2006). Due to intensive research in the past years, some of the molecular mechanisms behind immune priming in different host-pathogen pairs have been uncovered. Here I name two examples. (1) In the fruit fly *Drosophila melanogaster,* phagocyte activation is faster and more specific, upon a second encounter with the Gram-positive pathogen *Streptococcus pneumoniae.* It has been shown that priming is regulated by the canonical innate immune signalling pathway Toll, which was first described in *Drosophila* (Lemaitre et al., 1996) and is now known to be conserved across several metazoan taxa (Ausubel, 2005; Medzhitov et al., 1997). The priming with *S. pneumoniae* is not efficient against a second encounter with any other alternative tested pathogen, demonstrating priming specificity (Figure 1A) (Pham et al., 2007). (2) Similarly, in the lophotrochozoan snail host *Biomphalaria glabrata*, priming with a given strain of the trematode pathogen *Schistosoma mansoni* confers 100% protection against a second encounter with the same pathogen strain, but is not as efficient when it encounters different strains or a sister species of the pathogen (Figure 1B) (Portela et al., 2013). The mechanism through which the memory of the first encounter is stored, is not yet known. However, it has been described that upon the first encounter with the pathogen the host's response is carried out by innate immune cells that phagocytize, isolate and eliminate the pathogen in a process called encapsulation. However, upon a second encounter with the pathogen, the recognition and elimination are mediated exclusively by soluble proteins in the snail haemolymph. Recognition occurs through a repertoire of Fibrinogen-RElated

Proteins (FREPs) and elimination occurs through the antimicrobial agent Biomphalysin (Pinaud et al., 2016).

Additional phenotypes that evidence immune specificity in invertebrates, without necessarily involving immune priming, are genotype-genotype interactions between hosts and pathogens. In this case, different strains of the host show variation in their susceptibility to a given strain of the pathogen, or inversely, a given strain of the host shows variation in the susceptibility to infection with different strains of the same pathogen. This was observed for the crustacean *Daphnia magna* and its bacterial Gram-positive pathogen *Pasteuria ramosa,* (Figure 1C) (Auld et al., 2013; Little et al., 2006) to name one example. All the previously mentioned cases, suggest that there are different mechanisms and degrees of immune specificity according to the animal host taxon assessed. This implies that the larger number of taxa that are studied, the larger the diversity of immune specificities that will be uncovered. This diversity is expected, as each taxon has had the same amount of time to evolve under the particular selection pressures exerted by their associated pathogens and environment. The examples mentioned also make evident that the mechanistic features behind immune specificity are yet to be elucidated in most of these taxa, and their understanding may reveal general principals behind animal immune system evolution and insights to the way in which our own immune system works.

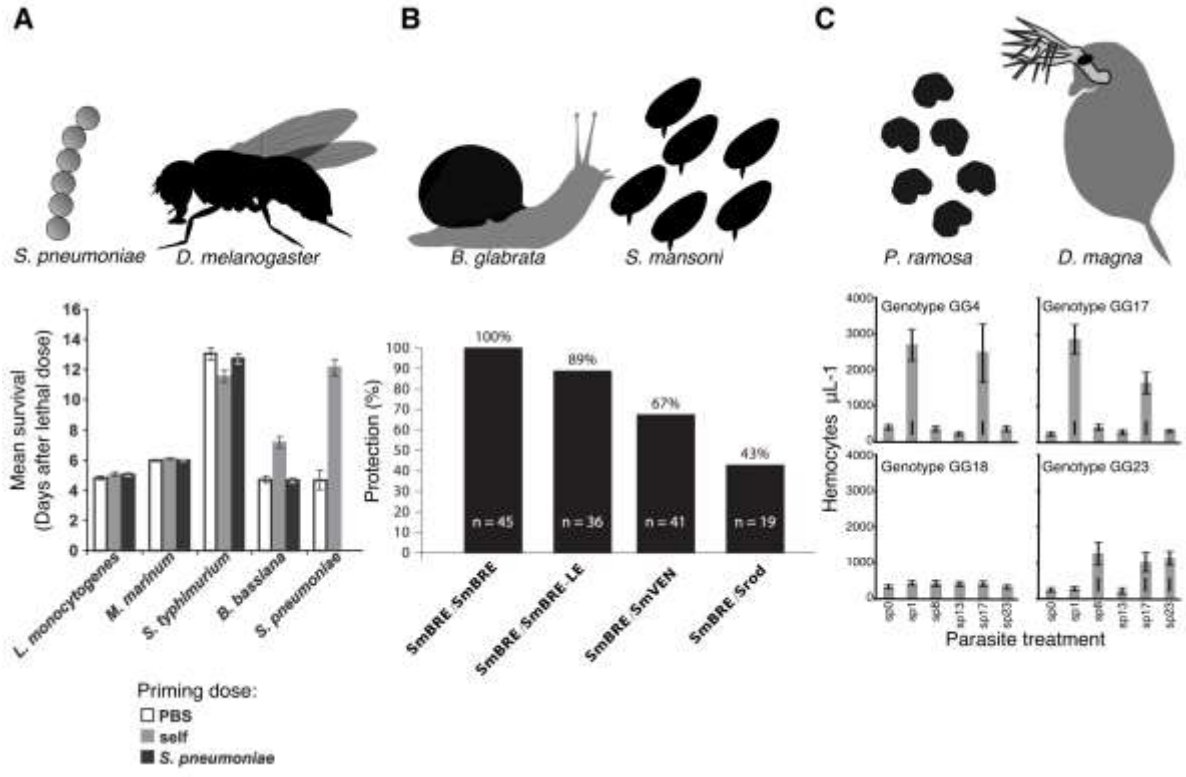

Figure 1. Phenotypic evidence of host immune specificity in (A) the fruit fly *D. melanogaster* infection with Gram-positive bacterium *S. pneumoniae* (B) the snail *B. glabrata* infection with the trematode parasite *S. mansoni* and (C) the crustacean

***D. magna* infection with the Gram-positive bacterium *P. ramosa*. (A)** Plot taken from (Pham et al., 2007) shows mean host survival and Standard Error of the Mean (SEM) of flies primed with treatment shown below the plot and then challenged with microbes listed in the x-axis. Only priming with *B. bassiana* and *S. pneumoniae* protect against a second challenge with the same pathogen, but not a different pathogen. **(B)** Plot taken from (Portela et al., 2013) shows percentage of protected hosts primed with exposure to the first trematode strain in the x-axis (SmBRE) and then exposed to the second trematode strain in the x-axis. SmBRE-LE, different strain from smBRE isolated in the same country, smVEN is a different strain from a different country and Srod is a different trematode species. (C) Plot taken form (Auld et al., 2013) shown number of circulating host haemocytes (± 1 SEM) for different host genotypes shown at the top of the plot compared to different pathogen genotypes denoted by the sp labels in the x-axis. Successful infections are marked with I inside the bar.

Caenorhabditis elegans as a model organism to study immune specificity

To uncover some of the molecular mechanisms leading to host-pathogen specific interactions, we need a genetically tractable metazoan host model. The bacterivorous, rotting-plant-material dwelling nematode *C. elegans* is a suitable choice. This is, because it has become an important subject for the study of innate immunity, with several infection models established under laboratory conditions (Figure 2A) (Aballay and Ausubel, 2002; Ermolaeva and Schumacher, 2014; Kurz and Ewbank, 2003; Marsh and May, 2012; Troemel et al., 2006).

More importantly, *C. elegans* is a metazoan host that shows phenotypic evidence of specific interaction with different kinds of pathogens, such as: virus, microsporidia, fungi and bacteria. In the following I will give three examples. (1) Several species of microsporidia parasites have been isolated from nematodes worldwide. Microsporidian host range has been shown to vary from being generalist, infecting all different nematode species and strains tested, to being strictly host strain-specific (Figure 2B) (Zhang et al., 2016b). The *C. elegans* Hawaii-isolated strain CB4856 is resistant to infection (i.e., it has an increased survival and reduced pathogen load) with the main microsporidian parasite studied so far, *Nematocida parisii*, compared to the standard lab *C. elegans* strain N2. Some genetic loci have been linked to this phenotype, but no further mechanistic explanation of it has been reported. More interestingly, this resistance is only evident when worms are infected at the early L1 larval stage (Figure 2C). This adds an age-dependent level of complexity to host immune specificity (Balla et al., 2015).

(2) *C. elegans* also displays natural variation in its susceptibility to the positive-strand RNA virus Orsay. Increased susceptibility to the virus is dependent on a 159 bp deletion in the Retinoic acid Inducible Gene I (RIG-1)-like helicase *drh-1*, which is found at intermediate frequency in *C. elegans* populations worldwide (Figure 2D). *drh-1* is involved in the direct recognition of viral RNA both in *C. elegans* and mammals (Ashe et al., 2013). The reason for the high prevalence in *C. elegans*

populations of an allele that results deleterious upon virus encounter is not understood. On the other hand, the Orsay virus is the only virus that has been co-isolated with the worm, suggesting that *C. elegans* is particularly immune to viral infection (Ashe et al., 2013).

(3) Further phenotypic variation in host-pathogen genotype-genotype interactions was described for the Gram-negative bacterium *Serratia marcescens*. Host survival upon pathogen exposure varies depending on the pathogen strain faced (Schulenburg and Ewbank, 2004). *C. elegans* also displays natural variation in the olfactory preference to the pathogen *S. marcescens*. The conventional laboratory strain N2 shows higher attraction towards this pathogen than the Hawaiian isolate, CB4856. Although, several genomic loci have been linked to natural variation in this trait, the exact mechanism that governs it has not yet been uncovered (Glater et al., 2013). Yet another phenotypic trait that evidences specificity in the *C. elegans*-*S. marcescens* interaction involves avoidance behaviour. The worm is able to specifically avoid the *S. marcescens* hallmark surfactant serrawetin W2 (Pradel et al., 2007).

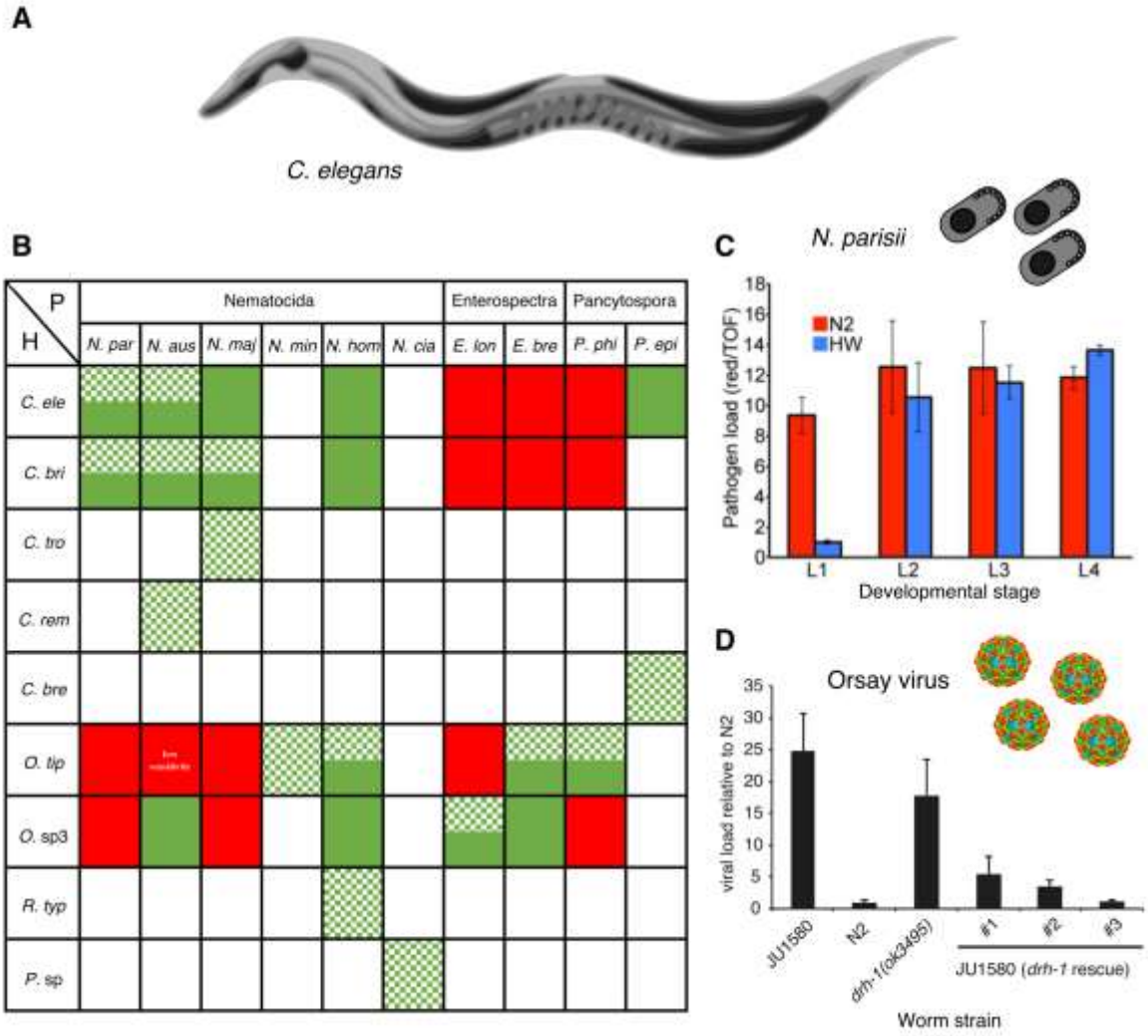

Figure 2. The invertebrate model host *C. elegans* (A), species-specific host-pathogen interactions with microsporidia (B), natural variation of *C. elegans* strains N2 and CB4856 (HW) in pathogen load of microsporidian *N. parisii* (C) and natural variation of *C. elegans* strains N2 and JU1580 in Orsay virus load due to an allele of *drh-1* (D). (B) Plot taken from (Zhang et al., 2016b) summarizes the interactions between rhabditid nematodes and microsporidia in the wild and under laboratory conditions. Solid green means successful infection in the laboratory, mosaic green represents observed natural infections, solid red are unsuccessful infections in the lab, white means infection not tested. P: pathogen, H: host. Hosts and pathogens denoted by first letter of genus and three first letters of species names. **(C)** Plot taken from (Balla et al., 2015) showing developmental stage-dependent natural variation in *N. parisii* pathogen load, assessed by using a pathogen-specific FISH probe normalized by worm size. Worm size was estimated as time of flight (TOF) in a COPAS biosort. Bars represent SD. **(D)** Plot taken from (Ashe et al., 2013) shows viral load in indicated host strains assessed by RT-qPCR of the Orsay virus RNA1 genome after 4 days of infection. *drh-1*(*ok3495*) is a knockout mutant of *drh-1* with N2 genetic background. JU1580 *drh-1* rescue refers to injection of the N2 allele in JU1580 and integration in the genome using x-rays. Virus Cryo-electron microscopy structure of capsid assembly (Guo et al., 2014) is shown.

Apart from all previously mentioned phenotypic and mechanistic evidence of specific interactions with pathogens, *C. elegans* also mounts distinct inducible transcriptomic responses upon exposure to different pathogenic and non-pathogenic microbes (Figure 3B) (Nakad et al., 2016; Yang et al., 2016). This has been studied in detail by comparing all available transcriptomic data for *C. elegans* upon exposure with the Orsay virus, the microsporidian *N. parisii*, fungi, Gram-positive and Gram-negative bacteria, including non-pathogens. Certain microbe-specific transcriptomic response was observed in all datasets assessed. For gut pathogens, the GATA motif was found to be enriched in the promoter regions of these pathogen-specific responsive genes. This suggests that GATA transcription factors (TFs) regulate gut pathogen-specific transcriptomic responses (Yang et al., 2016). Infection-specific transcriptomic signatures give further mechanistic support to *C. elegans* immune specificity.

Another of the *C. elegans* pathogen models for which there is phenotypic data available on host-pathogen specific interactions is the Gram-positive spore-forming bacterium *Bacillus thuringiensis* (BT). BT is characterized by the presence of plasmids encoding crystal pore-forming toxins (PFTs) that are determinants, and also define the host specificity of a given BT strain (Vilas-Bôas et al., 2007). This bacterium has the advantage that there is a wide variety of strains that are known to be pathogenic to worms, allowing us to address the question of invertebrate immune specificity at the pathogen strain level (Borgonie et al., 1995; Borgonie et al.; Wei et al., 2003). For the studies presented in the current thesis, we chose to work with the strains MYBT18247 (BT247) and MYBT18679 (BT679), because valuable aspects of the specific interaction of *C. elegans* with these strains have been characterized (see also further sections of Introduction). These include: variation in behavioural defences and variation in physiological defences

evidenced as changes in genetic and life history traits upon experimental evolution. These are explained in more detail below.

Different strains of *C. elegans* show natural variation in pathogen avoidance behaviour and cessation of feeding upon exposure to the strain BT247 (Hasshoff et al., 2007; Nakad et al., 2016; Schulenburg and Müller, 2004) and also variation in avoidance behaviour upon exposure to BT679 (Figure 3A) (Nakad et al., 2016). The variation in this trait is at least partially due to allelic diversity in the homolog of the mammalian neuropeptide Y receptor gene *npr-1* (Nakad et al., 2016). During experimental coevolution of *C. elegans* and BT there was a reciprocal increase in virulence on the pathogen side, coupled with an increase in resistance or tolerance on the host side. This was often linked to variation in fitness or growth rate on both sides (Figure 3C) (Schulte et al., 2010). It has also been shown that coevolution with BT drives the increase of diversity across host populations, leading sometimes to pathogen or host local adaptation (Schulte et al., 2010, 2011). Additionally, it has been reported, at least for BT679, that coevolution with the host selects for virulence factors and the maintenance of pathogenicity, while one-sided bacterial evolution leads to loss of virulence (Masri et al., 2015).

Thus, strong phenotypic and genetic evidence for specificity is available in this host-pathogen model system. Now is the time to spend research effort in dissecting the underlying molecular mechanisms responsible for the phenotypes described. Host adaptations involving the immune system are the first logical candidates to mediate pathogen strain-specific interactions. Therefore, in the following section we introduce what is known about the *C. elegans* immune system.

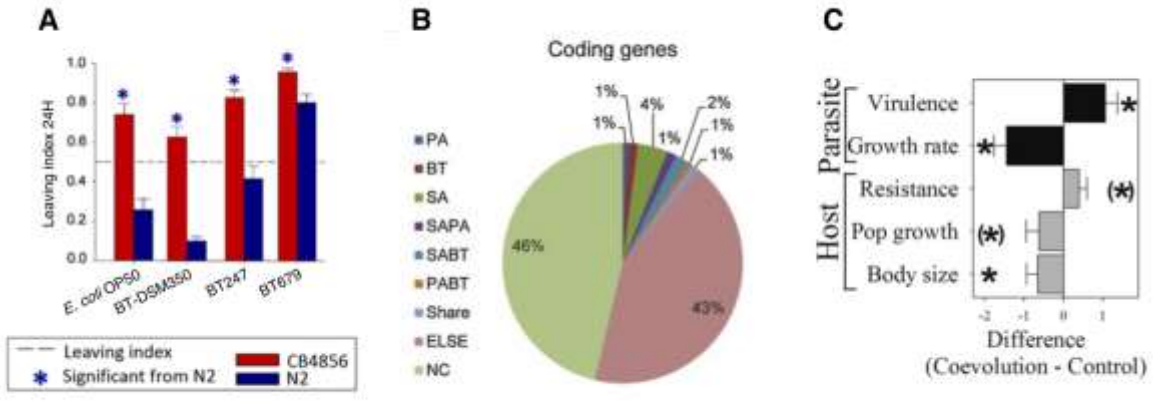

Figure 3. Phenotypic evidence of natural variation in bacterial lawn avoidance between *C. elegans* strains N2 and CB4856 (A), pathogen-specific transcriptomic signatures (B) and phenotypic consequences of 48 host generations of experimental evolution (C). (A) Plot taken from (Nakad et al., 2016) showing host natural variation in lawn leaving behaviour for indicated bacterial strains. Leaving index refers to the number of worms that left the lawn compared to the total number of worms in the

experiment. Asterisks denote significant differences (p-value<0.05) between worm strains in a Kruskall-Wallis test, Bonferroni corrected. **(B)** Plot taken form (Yang et al., 2016) shows the *C. elegans* coding genes that are responsive to pathogens in percentage. PA: *Pseudomonas aeruginosa*; BT: *Bacillus thuringiensis*; SA: *Staphylococcus aureus*; Share: overlap between PA, BT and SA; NC: non-changed by pathogen exposure. Additional abbreviations denote overlaps among the previously mentioned pathogens. **(C)** Plot taken from (Schulte et al., 2010). Bars represent Mean and SEM. Asterisks denote significance using paired *t*-test, asterisks in parenthesis denote trends (p-value <0.1).

The *C. elegans* immune system as main candidate to regulate specific interactions with pathogens

The physiological immunity of *C. elegans* has overlaps but is also distinct from that described in other metazoans. Lacking professional immune cells, *C. elegans* relies primarily on the response mounted by epithelial cells (Ewbank and Pujol, 2016; Schulenburg et al., 2004). The immune response of a cell can be understood as the sum of three components: the recognition module, the signalling module and the effector module (Hoffmann et al., 1999). Our knowledge for these three components in *C. elegans* is further described below.

The recognition module of the immune system is composed of receptor molecules that are able to identify the presence of a pathogen. This is achieved by direct interaction of the receptors with the pathogen or its derived products, which are identified as non-self (Brunet, 1941). What these receptors sense is known as Microbe-Associated Molecular Patterns (MAMPs) and the receptors themselves are known as Pattern Recognition Receptors (PRRs). Alternatively, recognition receptors can indirectly sense the damage caused by the pathogen (altered self), which is considered a danger signal (Matzinger, 1994). What these receptors sense is known as Danger-Associated Molecular Patterns (DAMPs). Homologs for most PRRs present in other animals have not been found in *C. elegans*, or they don't fulfil a function in immunity, except for the previously mentioned homologue of the mammalian RIG-1 receptor involved in the recognition of viruses (Ashe et al., 2013; Lu et al., 2009). The exact receptors responsible for bacterial and fungal recognition, whether in the context of immunity or identification of food sources, remain unknown. However, there is evidence of the importance of the recognition of DAMPs in *C. elegans* as a major component of its recognition module. There is evidence that *C. elegans* identifies when core cellular activities are disrupted by RNAi, which is administered through the food bacteria. The response of the worm involves aversive learning and avoidance of the detrimental food source (Melo and Ruvkun, 2012). It is also possible that *C. elegans* can recognize DAMPs product of abnormal cell death (caused by disease or violence) (Kono and Rock, 2008), but the receptors for these DAMPs remain unknown. Only one DAMP receptor has been identified in *C. elegans*. The G-protein coupled DihydroCaffeic Acid Receptor-1 (DCAR-1) detects an endogenous damage signal, the tyrosine derivate 4-HydroxyPhenylLactic Acid (HPLA), which accumulates in the epidermis after

fungal infection or wounding, and mounts defensive responses accordingly (Zugasti et al., 2014).

The signalling module of the immune system consists of signalling cascades that transfer the information acquired by the recognition module to a downstream target that triggers a response. The targets of the different signalling cascades are generally a TF or a network of TFs. The combinatorial action of these TFs fine tunes the immune response towards the identified pathogen threat, by inducing specific changes in the regulation of genes that belong to the effector module (Ewbank, 2006). This is, so far, the best-known module of the *C. elegans* immune system. The Toll pathway leading to the activation of the NF-kβ family of TFs, one of the main signalling pathways associated to innate immunity in other metazoans, seems not to function in the context of immunity in *C. elegans.* There are, additionally, no NF-kβ TFs found in this organism (Pujol et al., 2001). However, it has been shown that, the p38 Mitogen-Activated Protein Kinase (MAPK), the JNK MAPK, the ERK MAPK, the Insulin-Like Receptor (DAF-2/ILR), the SMA/TGF-β and the RNAi pathways are involved in mounting immune responses against pathogens (Ewbank, 2006; Irazoqui et al., 2010; Schulenburg et al., 2008). Each of these pathways is associated with the downstream activation or repression of TFs. It has been proposed that the GATA TF ELT-2 plays a central role in the regulation of inducible immune responses in the *C. elegans* intestine, and that additional TFs might interact with ELT-2 to generate more specific responses (Block and Shapira, 2015; Yang et al., 2016). The signals that are responsible for its activation, or for driving its functional specificity, have not yet been fully elucidated (Block and Shapira, 2015; Yang et al., 2016). However, there is evidence that ELT-2 coordinates its action with the p38 MAPK pathway to mount defensive responses (Block et al., 2015; Head et al., 2016). The basic Helix-loop-Helix (bHLH) TF HLH-30 (homologous to the mammalian TFEB) might be a good candidate to cooperate with ELT-2 in the generation of specific immune responses. HLH-30 is a major regulator of immune responses against *Staphylococcus aureus* (Visvikis et al., 2014). Like ELT-2, HLH-30 binds to GATA motifs in the promoter regions of its target genes (Yang et al., 2016).

The effector module is the branch of the immune system which carries out an action that generally leads to the destruction of the pathogen or its virulence factors. The effector module uses protective cellular responses such as autophagy, the production of reactive oxygen species (ROS), and the production of antimicrobial effector proteins to neutralize pathogens (Dierking et al., 2016; Schulenburg et al., 2004). Putative antimicrobial effectors include caenopores expressed mainly in the gut, lysozymes expressed throughout the body, AntiBacterial Factor (ABF) peptides, Neuropeptide-Like Peptides (NLPs) and caenacins (CNCs) (Ewbank and Zugasti, 2011; Roeder et al., 2010). These mechanisms have been reviewed in (Dierking et al., 2016).

Components belonging to all three branches of the immune response participate in the *C. elegans*-BT host-pathogen system. In the following section I will give a comprehensive list of molecular components that conform our current knowledge of the response of *C. elegans* to BT infection at the molecular level. I will also give more information about the BT virulence factors that elicit the *C: elegans* responses described to date.

The molecular mechanisms behind *C. elegans*-BT strain-specific interactions

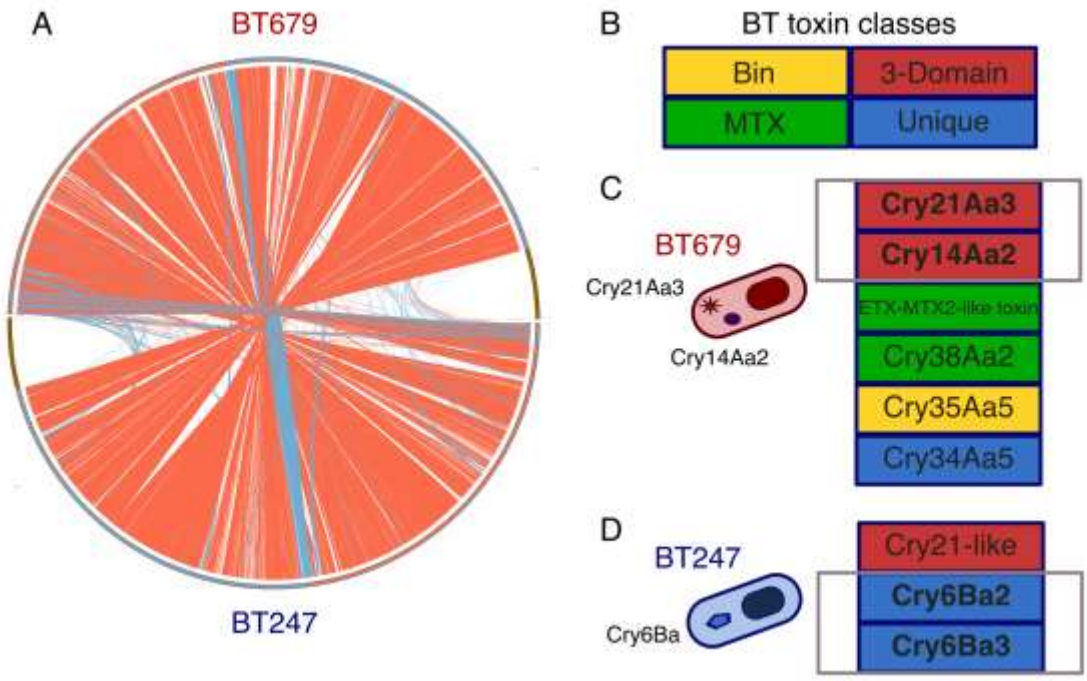

Figure 4. Genomic comparison of BT679 and BT247 (A), classification of BT Cry PFTs (B), PFT content of BT679 (C) and PFT content of BT247 (D). The genome alignment of the concatenated chromosomal and plasmid sequences of both BT247 and BT679 in (A) was produced by Cynthia Chibani using Circos (Krzywinski et al., 2009). Red represents conserved regions in the same orientation on both genomes, blue represent conserved regions in opposite orientation. White represents non-conserved regions. The brown region represents the extrachromosomal plasmids. (B) Classification of BT PFT classes (Crickmore et al., 1998). PFT content of BT679 (C) (Masri et al., 2015) and BT247 (D) (Hollensteiner et al., 2017). Boxes designate the PFTs mainly responsible for nematicidal activity.

As it was introduced before, we have chosen the BT strains BT247 and BT679 to directly ask **the question if *C. elegans* is able to mount specific responses to these two pathogen strains and, if so, what are the underlying molecular mechanisms behind the specificity**. The two BT strains that we have selected are different from each other at the genomic level (Figure 4A). Most of the chromosomal genome is shared between the two strains either in the same (red inner shading figure 4A) or in opposite (blue inner shading Figure 4A) orientation (Hollensteiner et al., 2017; Masri et al., 2015). Some regions in the chromosome are not shared (white inner shading figure 5A). However, the greatest genomic difference lays in the extrachromosomal plasmids which harbour the Cry PFTs (Brown outer shading Figure 4A). There are four major Cry PFT classes: Bin, three-domain (3d), MTX and unique (Figure 4B), of which the most commonly used in agriculture is the 3d class of Cry PFT (Crickmore et al., 1998). The main Cry PFT toxins are Cry34Aa5, Cry35Aa5, Cry38Aa2, ETX-MTX2-like, Cry21Aa3 and

Cry14Aa2 for BT679; and Cry6Ba2, Cry6Ba3 and Cry21-like for BT247 (Figure 4C and 4D). There is experimental evidence of nematode mortality upon exposure to Cry21Aa3, Cry14Aa2, Cry6Ba2 and Cry6Ba3 (Figure 4). This means that the main virulence factors responsible for host mortality in both of these BT strains are very different, and therefore the potential to encounter specific responses is great, making this experimental setup ideal to answer our research questions.

BT and its Cry PFTs are important in agriculture as pest control strategies. Genetically modified crops expressing transgenic Cry toxins are rendered resistant to various insect pests. However, pest resistance has emerged, making it necessary to study the molecular mechanisms behind metazoan defence and acquired resistance to PFTs (Marroquin et al., 2000). For the past couple of decades, *C. elegans* has been an essential whole-animal model system to study host defences against BT infection. BT Cry PFTs Cry5B, Cry6A, Cry6B, Cry12A, Cry14A, Cry21A and Cry55A are the ones described so far, that have nematocidal activity (Guo et al., 2008; Wei et al., 2003). To study the inducible protective response of *C. elegans* towards BT infection, either purified PFTs, PFTs heterologously expressed in *E. coli* or BT sporulated cultures are commonly used. The most studied toxins are Cry5B, Cry14A and Cry21A with a characteristic 3d structure; and Cry6A and Cry6B which share a similar unique structure, different from that of the 3d Cry PFTs.

All the molecular components known to participate in the *C. elegans* response to BT infection are summarized in Figure 5 with white background. The components of the physiological protective responses against BT characterized so far are: the p38 (Huffman et al., 2004) and JNK MAPK (Kao et al., 2011; Treitz et al., 2015; Yang et al., 2015) pathways, the DAF-2/ILR pathway (Chen et al., 2010; Hasshoff et al., 2007; Wang et al., 2012; Yang et al., 2015), the hypoxia pathway (Bellier et al., 2009), the Unfolded Protein Response (UPR) response (Bischof et al., 2008), autophagy (Chen et al., 2016) and vesicle trafficking (Los et al., 2011). Some putative effector molecules have been found to contribute to host defence against BT infection, including the saposin-like caenopore *spp-12* (Hoeckendorf and Leippe, 2012), the lysozymes *lys-2, lys-5* and *lys-7* (Boehnisch et al., 2011) and the galectins *lec-6* (Wang et al., 2017) and *lec-8* (Ideo et al., 2009). Apart from the physiological responses mentioned above, behavioural responses of worms against BT infection or Cry PFT exposure include cessation of feeding at least partially regulated by the G-protein subunit *goa-1* (Los et al., 2013).

There are host endogenous factors that contribute to sensitivity to BT infection, because when they are removed by either knockdown or knockout the worms display a higher survival rate upon infection. These factors are represented with grey background in Figure 5. The host factors that contribute to sensitivity to BT strongly depend on the PFT or bacterial strain analysed, suggesting once more, specificity in the interaction. Host glycolipids synthesized by the pathway formed

by the gene products of *bre-2, bre-3, bre-4* and *bre-5* serve as receptors of the PFT Cry5B, but not Cry14A nor Cry21A, and therefore lead to sensitivity to Cry5B exclusively (Griffitts et al., 2005). The necrosis pathway, including the aspartic protease *asp-1,* confers susceptibility to Cry6Aa exposure in a calcium–dependent manner, but not to Cry5B (Zhang et al., 2016a). Finally, it has been shown that the miRNA processing pathway, including the RNAse III *dcr-1,* negatively regulates target genes that would otherwise increase survival rates when facing an infection with BT DB27 (Iatsenko et al., 2013). Two negative regulators of pathogen avoidance towards BT247 have been described, namely the membrane bound C-type lectin-like C54G4.4 (Pees et al., 2017) and the G-protein coupled neuropeptide receptor *npr-1* (Nakad et al., 2016).

This diverse response characterized by using only few BT strains and PFTs already shows great potential for immune specificity mounted by the host. It especially does so, when taking into account the host factors that lead to susceptibility to BT, which are specific depending on the strain and PFT assessed, suggesting that there is specificity in each host-BT strain and even every host-PFT interaction.

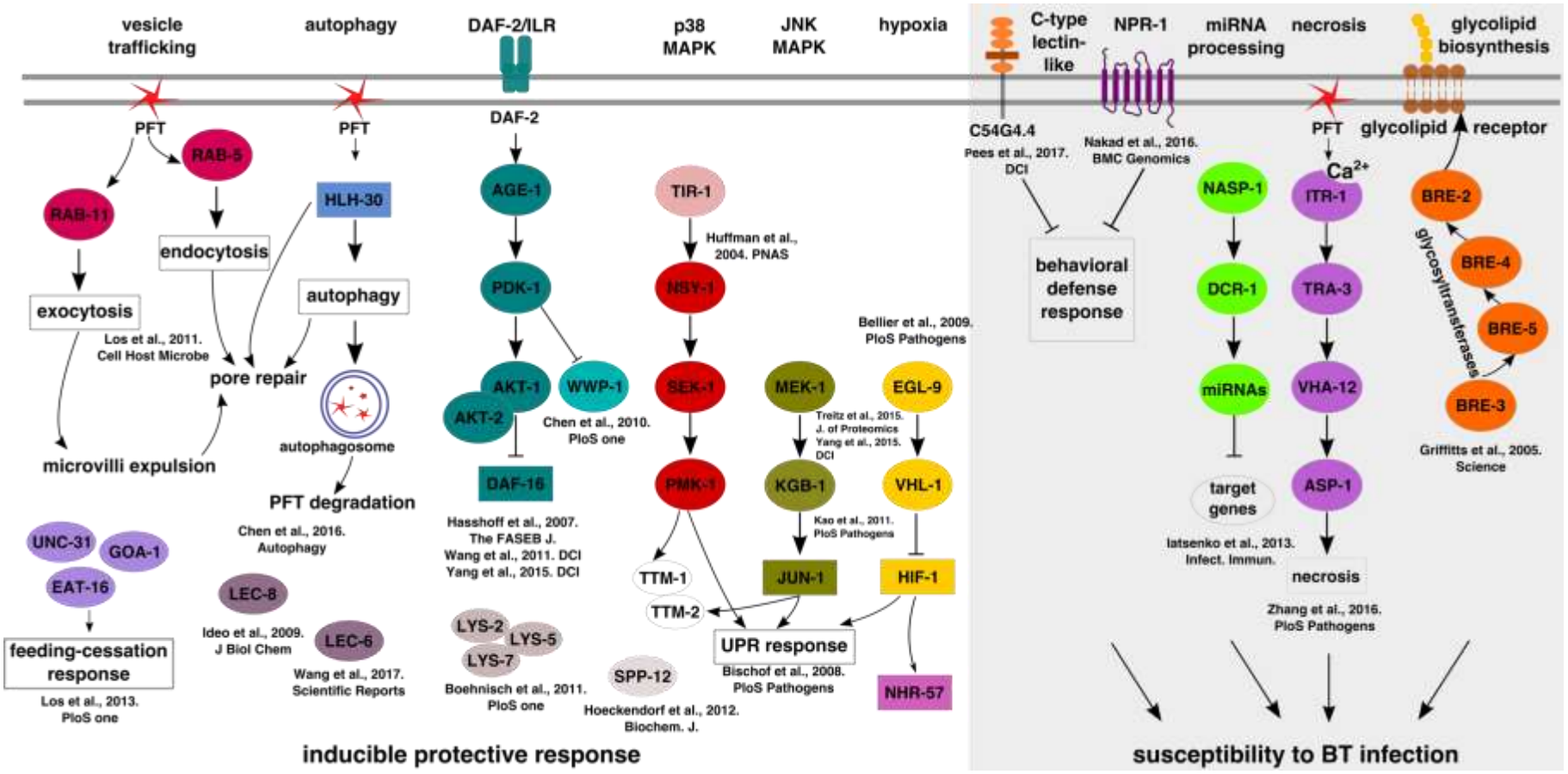
vesicle trafficking
autophagy
DAF-2/ILR
p38 MAPK
JNK MAPK
hypoxia
C-type lectin-like
NPR-1
miRNA processing
necrosis
glycolipid biosynthesis
PFT
RAB-5
RAB-11
endocytosis
exocytosis
Los et al., 2011. Cell Host Microbe
microvilli expulsion
pore repair
HLH-30
autophagy
autophagosome
PFT degradation
Chen et al., 2016. Autophagy
UNC-31
GOA-1
EAT-16
feeding-cessation response
Los et al., 2013. PloS one
LEC-8
Ideo et al., 2009. J Biol Chem
LEC-6
Wang et al., 2017. Scientific Reports
DAF-2
AGE-1
PDK-1
AKT-1
AKT-2
WWP-1
Chen et al., 2010. PloS one
DAF-16
Hasshoff et al., 2007. The FASEB J.
Wang et al., 2011. DCI
Yang et al., 2015. DCI
LYS-2
LYS-5
LYS-7
Boehnisch et al., 2011. PloS one
TIR-1
Huffman et al., 2004. PNAS
NSY-1
SEK-1
PMK-1
TTM-1
TTM-2
SPP-12
Hoeckendorf et al., 2012. Biochem. J.
Bellier et al., 2009. PloS Pathogens
MEK-1
EGL-9
Treitz et al., 2015. J. of Proteomics
Yang et al., 2015. DCI
KGB-1
VHL-1
Kao et al., 2011. PloS Pathogens
JUN-1
HIF-1
UPR response
Bischof et al., 2008. PloS Pathogens
NHR-57
inducible protective response
C54G4.4
Pees et al., 2017. DCI
Nakad et al., 2016. BMC Genomics
behavioral defense response
NASP-1
DCR-1
miRNAs
target genes
Iatsenko et al., 2013. Infect. Immun.
PFT
Ca2+
ITR-1
TRA-3
VHA-12
ASP-1
necrosis
Zhang et al., 2016. PloS Pathogens
glycolipid
receptor
BRE-2
BRE-4
BRE-5
BRE-3
glycosyltransferases
Griffitts et al., 2005. Science
susceptibility to BT infection

Figure 5. Diagram showing reported host protective mechanisms against BT infection (white background) and host factors that lead to susceptibility to BT infection (grey background).

PhD thesis content

This PhD thesis explores to what extent the response mounted by the invertebrate host *C. elegans* upon infection with BT is specific. Additionally, it uncovers some of the molecular mechanisms behind immune specificity in this host-pathogen system. In the following paragraphs I will describe the content of the different chapters of this thesis, which are all product of collaborative research.

Chapter 1 presents unpublished work titled "The GATA transcription factor ELT-2 mediates *Caenorhabditis elegans* strain-specific interaction with the natural pathogen *Bacillus thuringiensis*". We compared the transcriptomes of N2 worms with two pathogenic strains BT247 or BT679 and found strain-specific responses. Using further transcriptomics and genetic analysis, we found that the GATA TF ELT-2 is the central regulator of *C. elegans* strain-specific immune responses against BT247 and BT679. ELT-2 acts in parallel to the p38 MAPK pathway and cooperates with the bZip TF *skn-1* to promote resistance to BT679. Resistance is likely mediated by the expression of immune effectors, which are positively regulated by ELT-2. On the other hand, ELT-2 negatively regulates tolerance to BT247 infection. We present evidence suggesting that the enzyme-encoding genes: *cdr-2*, *poml-3*, *dhs-30* and *tre-3* are negatively regulated by ELT-2 and positively influence survival rate upon BT247 infection in *elt-2*(RNAi) worms, without affecting the pathogen load. According to the putative functions of these enzymes, we suggest that tolerance to BT247 is likely energetically fuelled by lipid metabolism and mediated by detoxification systems. Additional TFs that we identified to positively regulate tolerance to BT247 infection include: FOXO *daf-16*, bZip *zip-2*, *nhr-99* and *nhr-193*. In this chapter we uncovered several molecular aspects of strain-specific immune responses in this specific host-pathogen interaction. Our contribution mainly provides more information about the signalling, the regulation through different TFs and major mechanisms of the effector module, like resistance and tolerance. However, the molecular mechanisms behind the recognition that leads to the described strain-specific responses, remain unknown.

Chapter 2 is a review titled "High innate immune specificity through diversified C-type lectin-like domain proteins in invertebrates", which was published in 2015 in the Journal of Innate Immunity. This chapter presents the knowledge, available until publication, of the involvement of C-Type Lectin-like Domain (CTLD)-containing proteins, or the genes encoding them, in invertebrate immunity. This protein domain is interesting because it has particular biochemical characteristics that allow it to bind stably to a variety of macromolecules, particularly sugars. Some CTLD-containing proteins have roles as PRRs and also as immune effectors in

vertebrates, giving them the potential to fulfil similar roles in invertebrates. Initially the review deals with the information gathered from insect and crustacean taxa, where much functional information at the protein level exists, liking them to immunity. Then the review focuses on nematode taxa, where the CTLD gene family is largely expanded, giving it great potential for regulating immune specificity, perhaps even at the level of recognition. However, there is very little functional evidence linking CTLDs to immune functions in nematodes. Evidence is specially lacking at the protein level and genetic analysis using gene knockout or knockdown very often leads to no visible phenotype, possibly because of functional redundancy. Therefore, the review proposes to focus further research effort in comprehensive approaches to uncover the function of this diverse protein family in nematodes.

Chapter 3 is an unpublished manuscript titled: "The involvement of nested miRNAs in the defence of the nematode *Caenorhabditis elegans* against pathogenic *Bacillus thuringiensis*". In this chapter we address the topic of specificity of gene regulation during infection. For this, we focused on microRNAs (miRNAs), which are small non-coding RNA-molecules that silence the expression of protein-coding target genes. They achieve this by binding to the messenger RNA (mRNA) produced from the target gene and triggering its degradation. It is common for miRNAs to be found in the genome in nested arrangements of miRNAs encoded within larger, protein-coding, host genes. Since genes found in nested arrangements are commonly transcribed together, we hypothesized that the function of nested miRNAs and host genes could be linked. Upon infection with BT247 and BT679 all differentially expressed (DE) miRNAs were downregulated, and for those in nested arrangements, the host genes were also downregulated. This hints that miRNAs are, in general, negatively regulating target genes that positively influence defensive responses against BT infection. We performed genetic analyses and found that although nested miRNAs and host genes are co-transcribed during infection, generally only genetic disruption of host genes affects the survival rate of worms upon pathogen exposure, but not genetic disruption of miRNAs. This further supports that post-transcriptional regulation of gene expression is indeed crucial to fine-tune cellular responses and co-transcription doesn't necessarily imply co-function. Also, different miRNAs may share the same target genes. This redundant function might make it difficult to observe changes in a phenotype like survival rate. We additionally found that knockout of *mir-58.1* promotes enhanced survival rate upon exposure to both BT247 and BT679. We hypothesize that *mir-58.1* negatively regulates targets involved in general immune responses against BT.

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Chapter 1

The GATA transcription factor ELT-2 mediates *Caenorhabditis elegans* strain-specific interaction with the natural pathogen *Bacillus thuringiensis*

Original Research

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SUMMARY

In the past decade, the paradigm which claimed that invertebrate immune systems lack specificity has been reconsidered. Accumulating evidence supports that invertebrate immune systems are able to mount specific responses to the pathogen species-, and even to the pathogen strain-level. However, the underlying molecular mechanisms behind invertebrate immune specificity remain mostly unknown. Studying the molecular basis of invertebrate immune specificity in a genetically tractable model, such as the nematode *Caenorhabditis elegans,* has the potential to reveal insights into the immune systems of other metazoans, including humans. We chose to study the mechanisms of specific immune responses of the worm to two different pathogenic strains of the Gram-positive bacterium *Bacillus thuringiensis* (MYBY18247 and MYBT18679), because there is phenotypic evidence of specific genotype-genotype interactions between this host-pathogen pair. We did an initial RNA-Seq experiment upon pathogen exposure and found that 9% of the differentially expressed genes change their expression in different ways when comparing the two pathogen strains. Through promoter region motif enrichment analysis, we found the GATA transcription factor ELT-2 is responsible for the pathogen strain-specific transcriptomic response. Upon *elt-2* knockdown worms exposed to MYBT18679 display lower survival rate coupled with higher intestinal damage than non-infected controls. Additionally, by performing further genetic analysis using gene knockdown and knockout, we found that the p38 MAPK pathway acts likely in parallel to *elt-2* and the transcription factor *skn-1* cooperates with *elt-2* to promote resistance to MYBT18679. On the other hand, *elt-2* knockdown leads to a substantially higher survival rate, together with lower intestinal tissue damage compared to control worms, upon exposure to MYBT18247, another pathogenic *Bacillus thuringiensis* strain. The MYBT18247 pathogen load of *elt-2*(RNAi) worms compared to control worms remained unchanged, suggesting the *elt-2* negatively regulates tolerance towards MYBT18247. We found that tolerance to MYBT18247 was positively regulated by the transcription factors: FOXO *daf-16*, bZip *zip-2*, *nhr-99* and *nhr-193*. To identify *elt-2* negatively-regulated downstream targets that could promote tolerance to MYBT18247, we performed a second RNA-Seq experiment, this time including *elt-2*(RNAi) worms exposed to both pathogenic strains. We found four genes negatively regulated by *elt-2*: *cdr-2*, *poml-3*, *dhs-30* and *tre-3*, with putative function in detoxification and lipid metabolism, which can mediate tolerance to MYBT18247. We conclude that ELT-2 coordinates strain-specific immune responses in this invertebrate host and promotes resistance upon exposure to MYBT18679, while it negatively regulates tolerance to MYBT18247. The response is likely to be specific to the crystal pore-forming toxins produced by this pathogen.

INTRODUCTION

Pathogens and also varying microbial communities exert strong and highly dynamic selection pressure on hosts. The ability to recognize a particular pathogenic threat and mount specific, and therefore energetically economic, responses to control infection should be selectively favoured, especially when facing a pathogen with which a common natural history is shared. Classically, it was believed that invertebrates, which lack the vertebrate T- and B-Cell-based immunity and only rely on innate immunity, are only capable of mounting general defence responses against pathogens (Loker et al., 2004). However, two main lines of evidence suggest that invertebrates can display high specificity in their immune responses. (1) The ability of some invertebrates to specifically mount a faster response upon the second encounter with the same pathogen, a phenomenon known as immune priming, (Cooper and Eleftherianos, 2017; Kurtz and Franz, 2003; Pham et al., 2007; Pinaud et al., 2016; Portela et al., 2013; Rimer et al., 2014; Roth et al., 2009; Sadd and Schmid-Hempel, 2006) and (2) genotype-genotype interactions between host and pathogen strains (Auld et al., 2013; Little et al., 2006; Schulenburg and Ewbank, 2004a). The molecular mechanisms underlying immune specificity in invertebrates are largely unknown. Closing this knowledge gap is of great importance, because although the paths that evolution of immunity has followed are taxon-specific, the evolution of immune systems in all organisms is ruled by common principles (Rimer et al., 2014). This means that it is likely that the mechanistic understanding of invertebrate immune systems can provide new insights into human or another animal immunity. In consideration of its advantages as a tractable genetic system, the invertebrate model organism *Caenorhabditis elegans* may help to dissect the involved molecular processes.

In its natural environment, the nematode *C. elegans* colonizes microbe-rich habitats such as rotting fruits and decaying plant material where it feeds on bacteria and yeast (Frézal and Félix, 2015; Schulenburg and Félix, 2017). Some of the microorganisms that share their habitat with *C. elegans* can be infectious pathogens leading to tissue damage and death of the worm. Thus, *C. elegans* had to evolve defence mechanisms in order to coordinate its interactions with microbes. The defence system of *C. elegans* has three main components: First, there are behavioural adaptations that prevent or reduce the exposure to bacteria with potential detrimental effects. Second, there are physical barriers such as the thick extracellular cuticle layer that surrounds the body and the pharyngeal grinder that crushes ingested particles, prevent microbial penetration of the tissues. Third, there is a complex network of physiological immune responses (Ewbank and Pujol, 2016; Schulenburg et al., 2004). Based on this defence system, *C. elegans* displays food preference and can distinguish between different bacterial species and strains, to choose particularly attractive food sources over those that are non-attractive or repulsive (Burlinson et al., 2013). It is not known exactly what role the physiological immune system plays in food choice or if it cooperates with other

processes (such as chemosensation and/or mechanosensation) to evaluate food source quality (Clark and Hodgkin, 2014).

One of the open research questions remaining regarding *C. elegans* defence system is how specific the responses are. At the phenotypic level, a high degree of specificity to identify bacteria or bacterial products and mount particular responses was demonstrated. The underlying molecular mechanisms for most cases yet remain unknown. *C. elegans* mounts distinct transcriptomic responses (Irazoqui et al., 2010a) and expresses different sets of effector genes (Alper et al., 2007) according to the pathogen species it encounters (Yang et al., 2016a). It is known that *C. elegans* is able to detect *Pseudomonas aeruginosa* homoserine lactone autoinducers and secondary metabolites (Beale et al., 2006; Meisel et al., 2014) and *Serratia marcescens* biosurfactants (Pradel et al., 2007), which elicit a pathogen avoidance response in the host. Similar behavioural avoidance responses have also been shown towards nematocidal strains of *Bacillus thuringiensis* (BT) in *C. elegans* (Hasshoff et al., 2007a; Nakad et al., 2016; Schulenburg and Müller, 2004). *C. elegans* also harbours natural variation in resistance to infection with BT (Masri et al., 2015; Schulenburg and Müller, 2004; Schulte et al., 2010, 2011), the microsporidian parasite *Nematocida parisii* (Balla et al., 2015), the positive-strand RNA virus Orsay (Ashe et al., 2013) and the Gram-negative opportunistic pathogen *Serratia marcescens* (Glater et al., 2013; Pradel et al., 2007; Schulenburg and Ewbank, 2004b). Natural variation in the resistance to infection with pathogens suggests the presence of genotype-genotype interactions between host and pathogen, which may rely on high immune specificity on the host side (Bose and Schulte, 2014; Schulenburg and Ewbank, 2004b).

One of the pathogens engaged in specific interactions with *C. elegans* is BT. This Gram-positive bacterium forms spores under adverse conditions and produces parasporal crystal pore-forming toxins (Cry PFTs) called δ-endotoxins that are associated to the spores. Lethality occurs by oral uptake of spore-toxin mixtures, followed by toxin solubilisation, proteolytic activation and binding to specific intestinal cell receptors where the pores are formed (Borgonie et al., 1995; Borgonie et al.; Guo et al., 2008; Wei et al., 2003). Cry PFTs do not conform only one family of homologous proteins, but they are an aggrupation that encompasses different unrelated lineages, that all form parasporal crystal inclusions. These lineages include: the three-domain (3d) PFTs, which is the largest group; the binary (Bin) PFTs; the ETX_MTX2 family of PFTs and the group with unique structure that only contains the Cry6 PFTs (Berry and Crickmore, 2017; Crickmore et al., 1998; Palma et al., 2014). The 3d PFT Cry5B has been the most widely studied in the context of *C. elegans* response to PFTs (Bellier et al., 2009; Bischof et al., 2008; Chen et al., 2010, 2016; Huffman et al., 2004; Ideo et al., 2009; Kao et al., 2011; Los et al., 2011, 2013). It is now known that glycolipids serve as receptors for Cry5B, but not for 3d Cry21A, 3d Cry14A or the unique structure PFT Cry6A (Chen

et al., 2010; Griffitts et al., 2005; Yu et al., 2014). The nematocidal BT strains MYBT18247 (BT247) and MYBT18679 (BT679) vary in their pathogenicity because they produce different Cry PFTs and likely vary in the expression of additional virulence factors (Hollensteiner et al., 2017a; Masri et al., 2015; Schulte et al., 2010, 2011). The main toxins responsible for nematode killing in BT679 are the 3d PFTs Cry21Aa3 and Cry14Aa2 (Masri et al., 2015), whereas the main nematocidal toxins of BT247 are the unique-structure PFTs Cry6Ba2 and Cry6Ba3 (Hollensteiner et al., 2017a). Both strains additionally show differences in the exact pattern of nematode infection; BT679 is more virulent at lower dose and generally requires host killing before it colonizes the intestine, while BT247 shows a faster colonization of the intestinal lumen in the living host (Wang et al., 2012a).

Based on the previous observations of highly specific interactions between BT and *C. elegans*, we have chosen this model to explore the underlying molecular mechanisms of highly specific immune responses. We first used a transcriptomic approach to assess if *C. elegans* is able to mount specific transcriptomic responses to the BT strains BT247 and BT679 and to identify possible candidate processes for specificity. We then performed genetic analyses of candidate genes and used further RNA-Seq, Transmission Electron Microscopy (TEM), pathogen load estimation by Colony Forming Unit (CFU) counts and fluorescence microscopy, to characterize in detail the molecular basis of the specific response.

RESULTS

Distinct pathogenesis/cytopathology of *C. elegans* infection with BT247 and BT679

The two nematocidal BT strains BT247 and BT679 produce different Cry PFTs upon sporulation, which result in differences in the infection patterns of the two strains (Wang et al., 2012a). To determine differences in the cytopathology of infection with the two different strains of *B. thuringiensis*, we used TEM to assess damage in the anterior intestinal epithelium (Figure 1 A) at 2h, 6h, 12h, and 24h hours post infection (p.i). For all treatments, only worms that were still alive at the respective time point were used for imaging, except for worms exposed to BT679 at 24h p.i. The same spore dilution was used, which resulted in ~50% survival of wildtype (N2) worms after 24h of exposure to BT679 and ~88% survival of N2 worms after 24h of exposure to BT247 (data not shown). This confirms the previously reported variation in virulence between the two strains (Schulte et al., 2010; Wang et al., 2012a). The non-pathogenic BT strain BT407 (Figure 1 D, H, L, P) that does not produce any PFTs and *E. coli* OP50 (Figure 1 E, I, M, Q) were used as controls. In worms exposed to BT247 the intestinal lumen was distended and microvilli were shortened and disorganized already at 6h p.i (Figure 1 B-I). These phenotypes became more severe through the course of the infection, but the terminal web always remained intact. At later time points spores were observable in the gut lumen but never vegetative cells. An undetermined substance accumulated in the intestinal lumen (Figure 1 B, F, J, N). Worms exposed to BT679 showed more severe infection phenotypes, with gut lumen widening and shortening/disorganization of microvilli happening already at 2h p.i (Figure 1 C). At 6h p.i spores were visible inside the intestinal lumen, but the terminal web remained intact. At later time points the apical membrane was disrupted, the microvilli had disappeared, and vegetative cells were visible in the intestinal lumen (Figure 1 G, K). At 24h p.i the worm tissues were not visible anymore and only vegetative cells appeared inside the worm's body (Figure 1 O). These results indicate a clear difference in the infection pattern of both BT strains. Infection with BT679 leads to an early spore germination and fast colonization of the gut lumen, leading to severe tissue damage already at early time points. In contrast, infection with BT247 leads to gradually increasing damage and accumulation of spores over time. BT247 vegetative cells were not seen at any time point. These observations oppose the low bacterial load data upon BT679 infection reported by (Wang et al., 2012a). Both studies used pathogen doses leading to similar mortality rates. However, here only TEM pictures were assessed and actual bacterial load cannot be accurately estimated from these data. Next, we asked if the differences observed in cytopathology between the two BT strains could also translate to different transcriptomic responses by the worm, elicited by the different pathogenic BT strains.

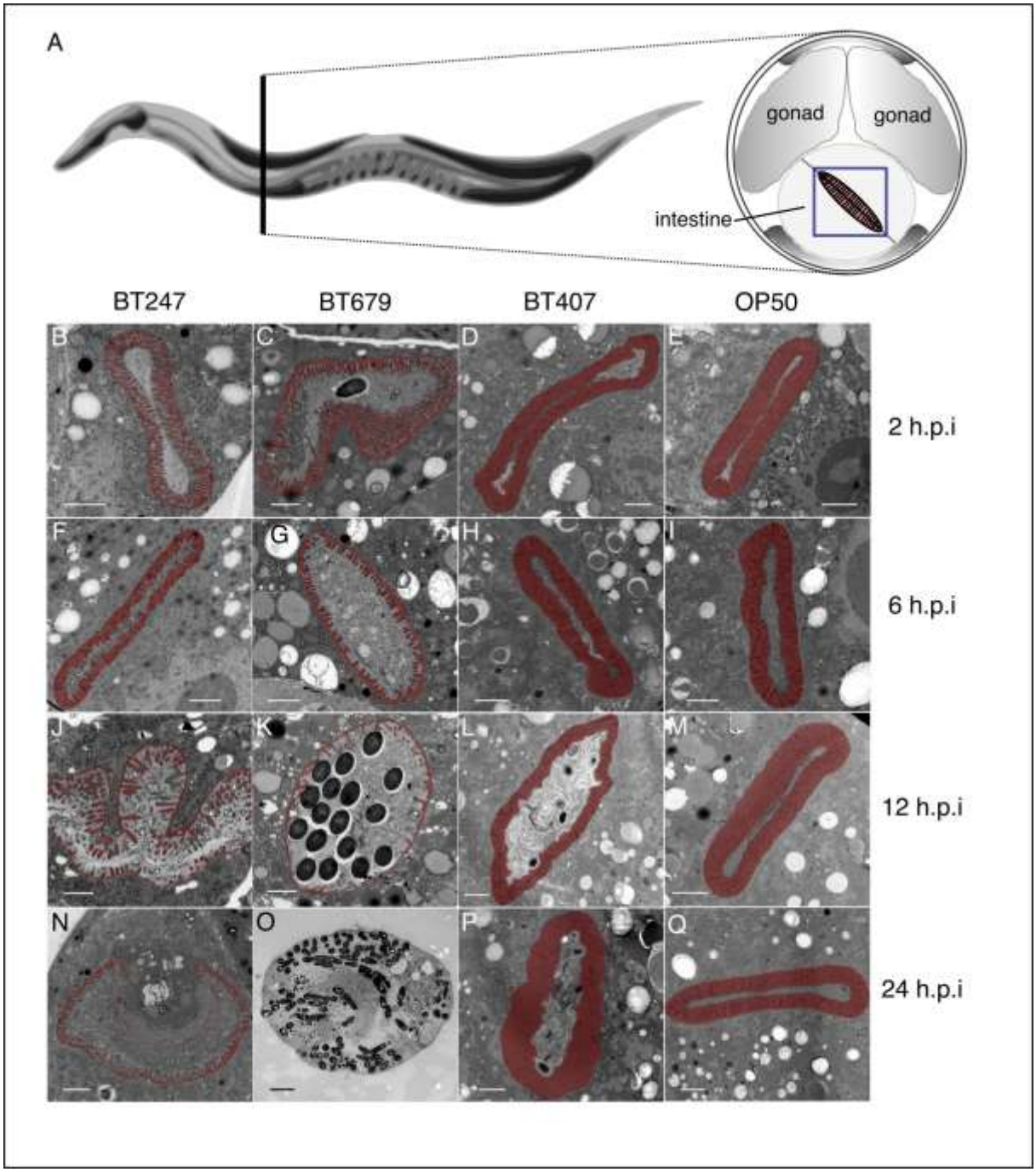

Figure 1. BT247 infection is characterized by gradual increase in host intestinal tissue damage, while BT679 infection leads to early spore germination (12h p.i), fast colonization of the intestinal lumen and severe tissue damage. TEM pictures of transversal cuts of the *C. elegans* anterior intestinal epithelium (A) 2h, 6h, 12h and 24h p.i to different bacterial strains (B-Q). Microvilli are artificially coloured in red to point out to their integrity during infection. Scale bar represents 1 μm except for panel O where scale bar represents 5 μm. Section delimited by a blue square in diagram in (A) indicates area shown in pictures (B-N, P-Q).

C. elegans strain-specific transcriptomic responses to BT are mediated by GATA transcription factors (TFs)

We assessed N2 transcriptomic responses to BT247 and BT679 by performing whole transcriptome sequencing to determine if the *C. elegans* response to BT247 and BT679 infection differs. To our knowledge, this is the first time that the transcriptomic response of *C. elegans* to different pathogenic strains of the same bacterial species is examined, to determine specific responses. Worms were exposed to a comparable dose of spores of the pathogenic BT strains BT247 and BT679, and the non-pathogenic controls BT407 and *E. coli* OP50, as detailed in the experimental procedures. RNA was extracted at 2h, 6h, 12h, and 24h p.i (same run as for Figure 1 A). Survival rates of the pathogen exposures at 24h p.i were the same as in the TEM experiment (data not shown).

We found 5477 differentially expressed (DE) genes between infection treatments (BT247 and BT679) and the non-pathogenic control (BT407). DE genes were clustered according to common expression pattern changes (Figure 2 A). GO enrichment analyses and EASE analysis using WormExp (Yang et al., 2016b) were performed for every cluster (Figure 2 B). Clusters 1, 3-7, 9 and 10 show a common transcriptional response (either up- (46% of all DE genes) or down-regulation (45% of all DE genes)) to both BT strains and contain the genes that compose the general response to BT infection (Figure 2 A). Clusters 1 (325 genes) and 3 (516 genes) are of particular interest because they contain the genes that are up-regulated already at early time points p.i. In cluster 3 these genes remain up-regulated throughout the infection (Figure 2 A). Both clusters contain genes encoding known mediators and effectors of the *C. elegans* response to *E. coli* expressing the BT crystal PFT Cry5B or to *B. thuringiensis*: Cluster 1 includes the mitogen-activated protein kinase kinase (MAP2K) gene *sek-1*, a component of the p38 MAPK pathway (Huffman et al., 2004). Cluster 3 contains *jun-1*, a terminal TF of the JNK MAPK signalling pathway (Kao et al., 2011; Treitz et al., 2015; Yang et al., 2015), and the galectin gene *lec-8* (Ideo et al., 2009).

More important for our research question on immune specificity are clusters 2 (234 genes) and 8 (275 genes), accounting for approximately 9% of the DE genes. These clusters show variation in gene expression changes upon exposure to either BT247 or BT679, suggesting the presence of a strain-specific response module. Cluster 2 contains genes that are up-regulated on BT247 but do not change upon BT679 exposure (Figure 2 A). The effector genes: *lec-6*, known to be responsive to the PFT Cry6Aa (Wang et al., 2017) and the lysozyme *lys-2*, important for resistance against BT247 (Boehnisch et al., 2011), were part of cluster 2. Cluster 8 contains genes, whose expression is down-regulated at early time points on BT247 and BT679, but up-regulated upon exposure to BT247 and down-regulated upon exposure to BT679 at later time points. In this cluster, we found the necrosis terminal aspartic protease gene *asp*-1, which was found to lead to susceptibility to

the PFT Cry6Aa (Zhang et al., 2016). EASE analyses with WormExp (Yang et al., 2016b) on clusters 2 and 8 revealed an enrichment in targets of both the p38 MAPK and ILR/DAF-2 signalling pathways, both known to play a role in *C. elegans* immune defences (Kim et al., 2002; Schulenburg et al., 2004; Troemel et al., 2006).

Our RNA-Seq experiment allowed us to uncover pathogen strain-specific signatures in the *C. elegans* transcriptomic response to the two pathogenic BT strains BT247 and BT679. We then sought to identify TF binding sites that govern the gene expression changes upon BT infection. For this, we performed promoter region motif enrichment analysis of the individual gene expression clusters using an Automated Motif Discovery (AMD) tool (Shi et al., 2011) (Figure 2 B).

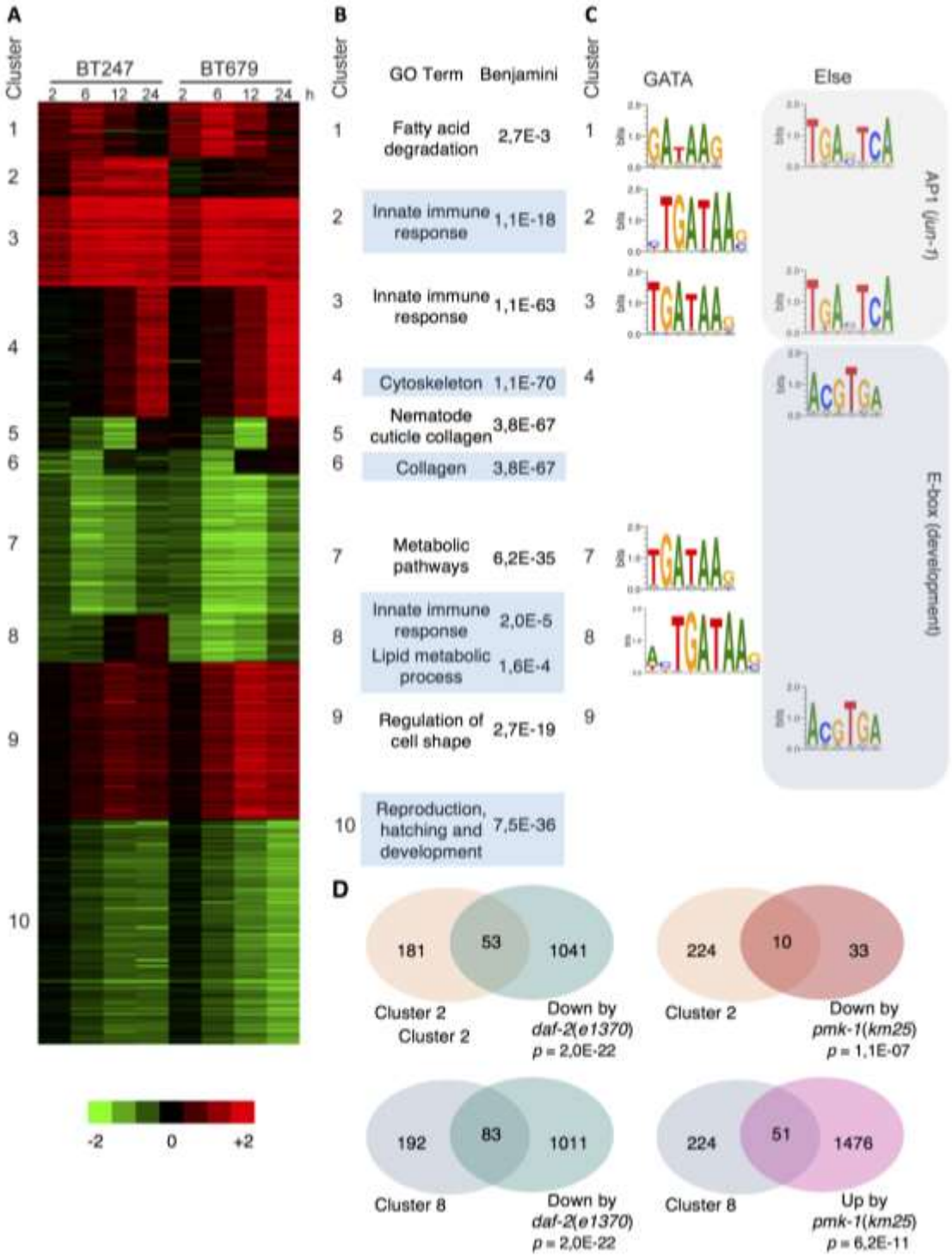

Figure 2. In total 9% of the *C. elegans* DE genes upon BT infection are pathogen strain-specific, their promoter regions are enriched in GATA motifs and they significantly overlap with regulation targets of the p38 MAPK and DAF-2/ILR immune pathways. (A) Heatmap representing all DE genes upon exposure to either BT247 or BT679 compared to BT407. 2h, 6h, 12h and 24h of exposure were assessed. Co-expressed genes based on K-means clustering yielded ten clusters. (B) GO functional enrichment analysis (EASE) per cluster. (C) TF binding-motifs enrichment per cluster inferred with AMD (Shi et al., 2011). (D) Example of the top hits of enriched functional categories in clusters 2 and 8, assessed with WormExp (Yang et al., 2016b). Numbers in

Venn diagrams correspond to gene counts and the number below the hit population (i.e., gene sets) corresponds to p-values, Bonferroni corrected, including hit populations for *daf-2*(*e1370*) (Chen et al., 2013); *pmk-1*(*km25*) (McEwan et al., 2016) enriched in cluster 2 and *pmk-1*(*km25*) (Mertenskötter et al., 2013) enriched in cluster 8.

Common early induced gene expression response requires AP-1 while strain-specific gene expression response is mediated by the GATA TF ELT-2

The binding motif of the Activator Protein-1 TF complex (AP-1; c-jun, c-fos) was enriched in the promoter regions of clusters 1 and 3 (Figure 2 A), containing genes commonly upregulated early (2h-12h) after infection with both BT247 and BT679 (Figure 2 B). The *C. elegans* JNK-like MAPK pathway (Figure 3 A), which can activate AP-1-dependent transcription, is important for defences against *E. coli* expressing the BT crystal PFT Cry5B (Kao et al., 2011). To test if AP-1(JUN-1/FOS-1) is required for resistance to the BT strains used in this study, we assessed the survival rate of *jun-1*(*gk551*) knockout mutant worms to different BT-spore dilutions at 24 hours p.i. *jun-1*(*gk551*) mutants were highly susceptible to both BT strains (Figure 3 A), suggesting that JUN-1 and the AP-1 complex regulate the common/general early transcriptional response to BT infection.

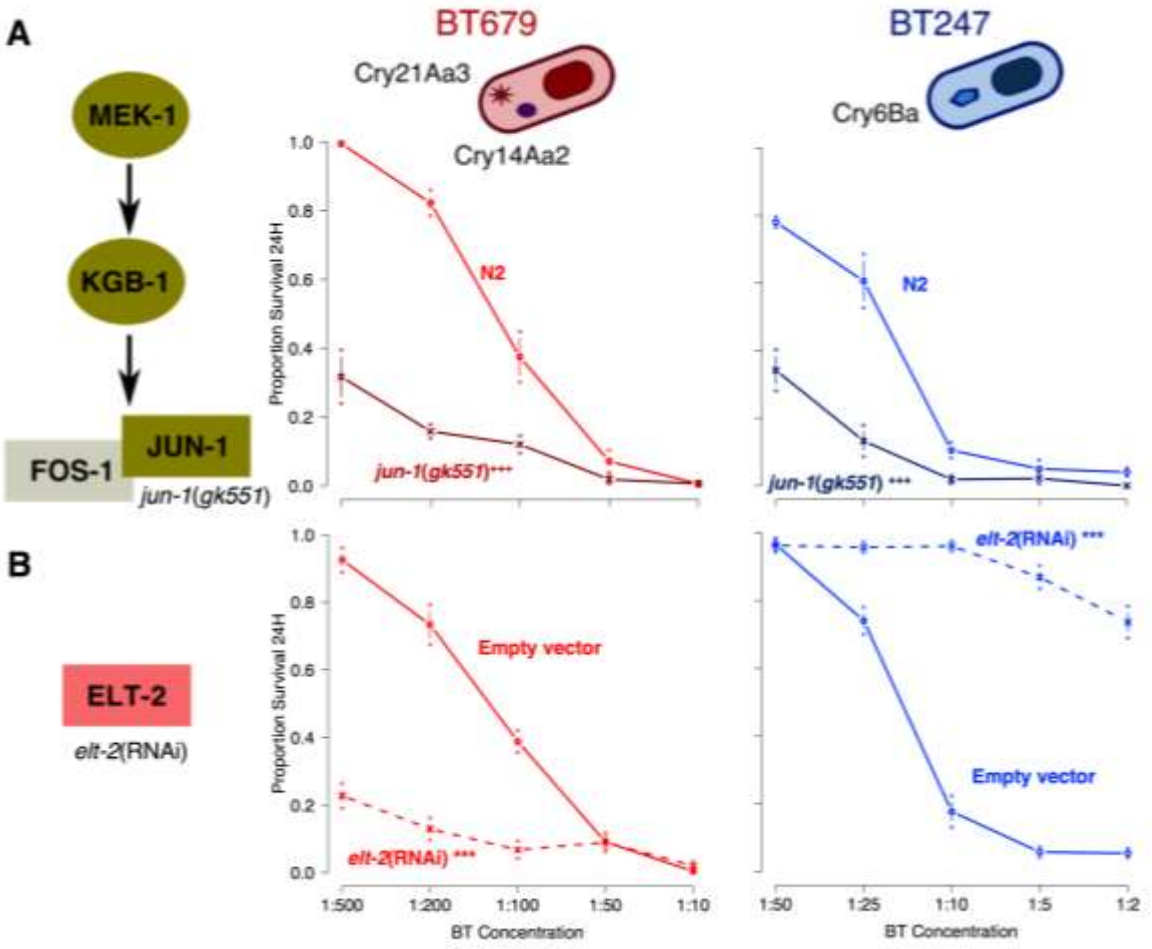

Figure 3. Knockdown of the GATA TF *elt-2* leads to reduced survival rate on BT679 and increased survival rate on BT247 compared to empty vector. Survival of (A) the AP-1 component *jun-1*(*gk551*) mutant and (B) GATA TF *elt-2*(RNAi) worms after 24h infection with either BT679 (left and in red) or BT247 (right and in blue). Left shows the genes tested, for (A) within the context of the JNK signalling cascade. The right panels show the proportion of surviving worms per nematode strain and treatment across different BT concentrations. Mean and standard error of the mean (SEM) are shown, N=5 plates with 30 worms each. GLM of the binomial family was performed followed by a Tukey Test (see experimental procedures section), where mutant or knockdown worm strains were compared to control strains. Plus signs show significant differences for all bacterial concentrations between knockout mutant and N2 (A). Asterisks show significant differences for all bacterial concentrations between knockdown treatment and Empty Vector control (B). *** or $^{+++}$ indicate p-value < 0.001, Bonferroni adjusted. Results are representative of at least 3 independent runs.

In contrast, the binding motif for GATA TFs was enriched in promoter regions of clusters 2 and 8 (Figure 2 A), containing genes differentially regulated upon exposure to the pathogenic strains BT247 and BT679 (Figure 2 B). Three zinc-finger GATA-type TFs bind to GATA motifs and are active in the *C. elegans* adult

intestine, namely, ELT-2, ELT-4 and ELT-7 (McGhee et al., 2007). ELT-4 is believed to be non-functional (Wiesenfahrt et al., 2016), while ELT-7 and ELT-2 have a synergistic action in endoderm differentiation during development (Sommermann et al., 2010). ELT-2 is likely to be the only essential GATA TF regulating gene expression in the *C. elegans* adult intestine (McGhee et al., 2007, 2009a) and is a homolog of Drosophila SERPENT (Senger et al., 2006) and vertebrate GATA4-6 (Block et al., 2015). To test the involvement of intestinal GATA TFs in resistance to BT247 and BT679, we analysed the survival rate of *elt-7*(*ok835*) and *elt*-7(*tm840*) mutant worms and of worms, in which *elt-2* expression was silenced by RNAi (*elt-2*(RNAi), efficient knockdown shown in Figure S1). As expected, *elt-2*(RNAi) resulted in lower worm survival rate after BT679 infection. Surprisingly, however, it also led to significantly higher worm survival rate after BT247 infection (Figure 3B). While *elt-7*(*ok835*) mutants also showed the opposite survival rate phenotype, as *elt-2*(RNAi) worms upon infection with BT247 and BT679, the effect was much less pronounced and not visible in *elt-7*(*tm840*) mutants (Figure 4 C and D)).

Importantly, we also observed an opposite effect of *elt-2*(RNAi) on the survival rate of worms exposed the two BT strains, when *C. elegans* was infected with cultures of BT247 and BT679 that exhibit the same virulence levels (Figure 4 A). This indicates that the effect of *elt-2*(RNAi) is not primarily determined by the difference in virulence between the two BT strains. Moreover, we first performed *elt-2*(RNAi) from the L1/L2 larval stage onwards and found worms to be viable and show similar survival rates as control worms after 24h of exposure to the non-pathogenic control BT407 (Figure S2; see also (Kerry et al., 2006)). Nevertheless, ELT-2 regulates the expression of more than 80% of all intestinal genes during *C. elegans* development and adulthood (McGhee et al., 2009a). Therefore, to disentangle its function in intestinal development from its function in the defence against BT infection, we repeated the survival assay with worms treated with *elt-2*(RNAi) only from the L4 stage onwards (as in (Shapira et al., 2006)). These additional analyses similarly revealed the opposite survival phenotype upon *elt-2*(RNAi) (Figure 4 B). This result strongly suggests that *elt-2* function in BT defence is distinct from its function in development. We conclude that ELT-2 is involved in mounting distinct inducible responses to the two BT pathogen strains, thereby demonstrating the ability of *C. elegans* to express pathogen strain-specific defence.

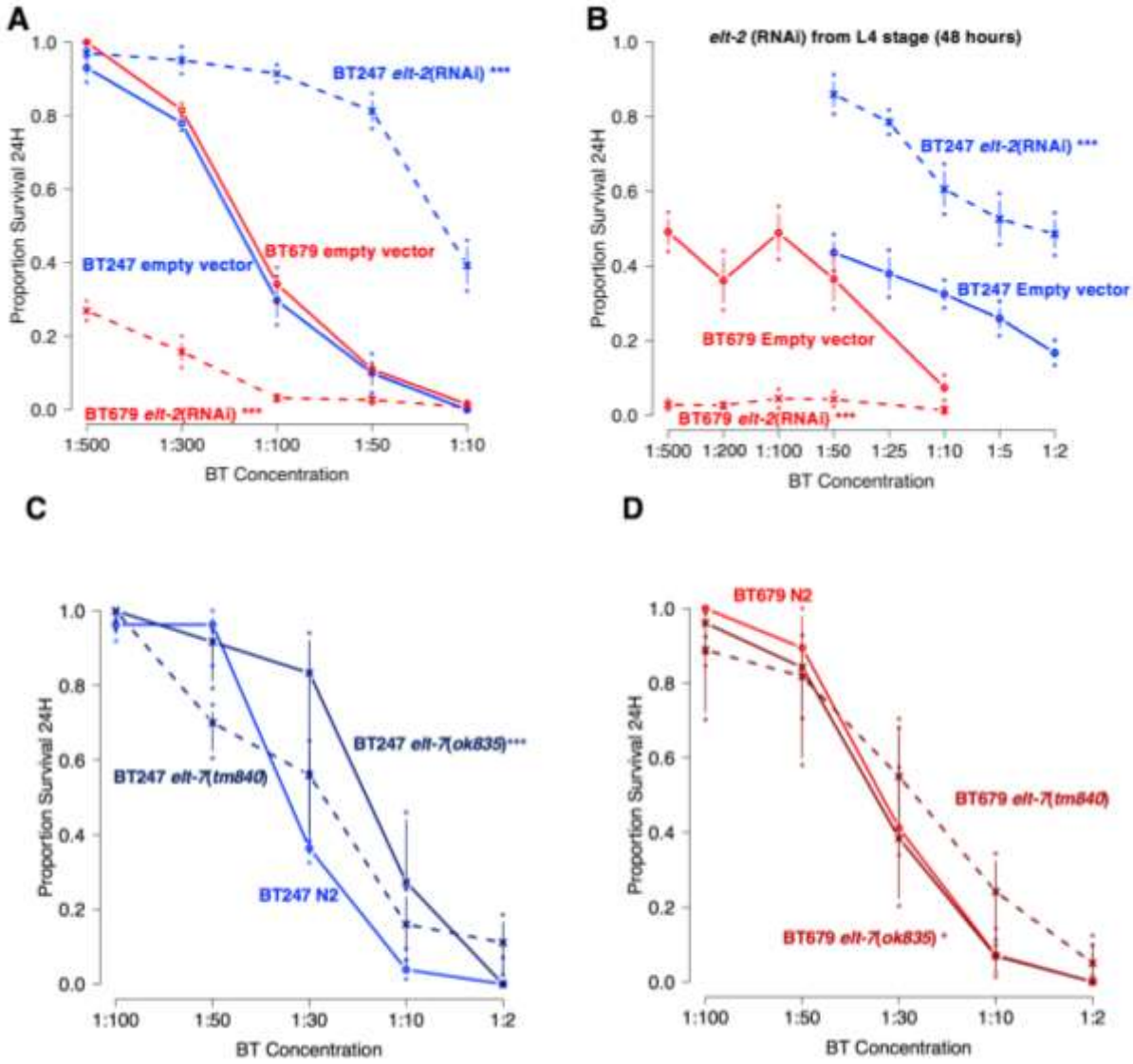

Figure 4. Opposite survival phenotype of *elt-2*(RNAi) worms is robust to changes in virulence levels of BT247 and BT679 cultures and is also robust to the developmental stage when knockdown treatment starts. (A) Survival of *elt-2*(RNAi) worms on BT247 and BT679 cultures with the same level of virulence, and (B) on BT247 and BT679 in 2-day old adults, in which *elt-2* was knocked down only at the L4 stage for 48 hours. Survival of *elt-7*(*tm840*) and *elt-7*(*ok835*) mutants on (C) BT247 and (D) BT679. Each curve represents the proportion of surviving worms of each worm strain and treatment combination. Mean and SEM are shown, N=5 plates with 30 worms each. Statistics as in figure 3. *** shows p-value < 0.001 comparing RNAi to empty vector, $^{+++}$ shows p-value < 0.001 comparing mutant to N2, Bonferroni adjusted. Results are representative of at least 3 independent runs.

Resistance phenotype of *elt-2*(RNAi) worms is unique among nematocidal BT strains

ELT-2 has previously been implicated in the immune response of *C. elegans* against several microbial pathogens including *Salmonella enterica*, *Enterococcus faecalis*, *Cryptococcus neoformans* (Kerry et al., 2006), *Pseudomonas aeruginosa* (Block et al., 2015; Kerry et al., 2006; Shapira et al., 2006) and *Burkholderia pseudomallei* (Lee et al., 2013). *elt-2*(RNAi) worms display higher susceptibility than wild type worms to infection by all of these pathogens, similar to the phenotype that we observe upon exposure to BT679. It has additionally been shown that *B. pseudomallei* manipulates the host immune system by inducing the degradation of the ELT-2 protein through the endogenous ubiquitin-proteasome system (Lee et al., 2013). These findings strongly support the idea of an important function of ELT-2 in *C. elegans* immune defences. In fact, ELT-2 has been recently suggested as a central regulator of inducible immune gene expression in the adult intestine against all pathogens studied to date (Block and Shapira, 2015; Yang et al., 2016a). We thus tested the survival of *elt-2*(RNAi) worms on three additional nematocidal BT strains (MYBT18243 (BT243), MYBT18245 (BT245) and MYBT18246 (BT246)), to see which of the two phenotypes (lower or higher survival rate than controls) is predominant among pathogenic BT strains. *elt-2*(RNAi) worms showed reduced survival rate with all three additional infections (Figure 5 A), including BT246, which is very similar to BT247 at the genomic level, but which contains plasmids encoding different 3d Cry PFTs (Hollensteiner et al., 2017b, 2017a). The resistance phenotype of *elt-2*(RNAi) worms exposed to BT247 is thus unique among all tested pathogens.

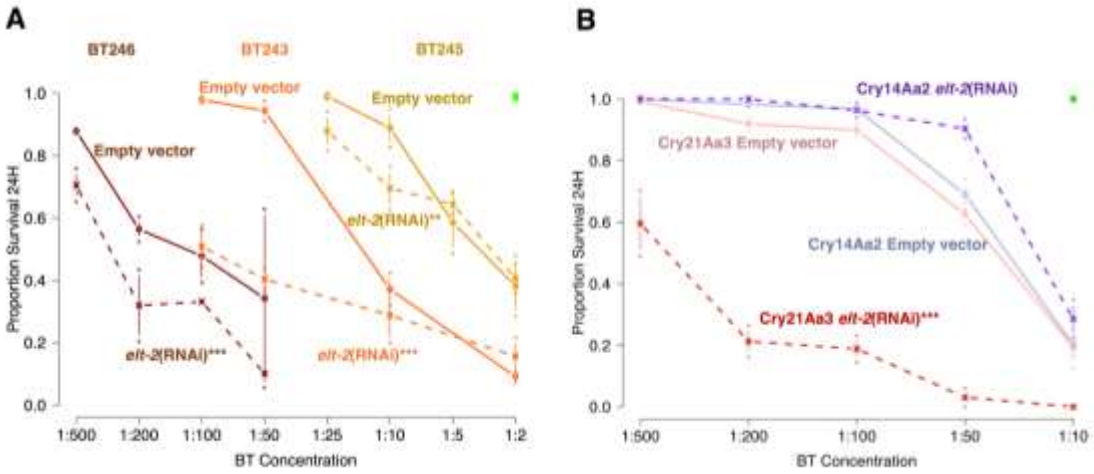

Figure 5. *elt-2*(RNAi) worms are more susceptible to all other BT strains tested and also to BT679 expressing only the PFT Cry21Aa3 but not when only the PFT Cry14Aa2 is expressed. Survival of (A) *elt-2*(RNAi) worms exposed to BT243, BT245 and BT246; and survival of (B) *elt-2*(RNAi) worms exposed BT679 Cry- (+ Cry14Aa2) and BT679 Cry- (+Cry21Aa3). Each curve represents the proportion of surviving worms of each worm strain and treatment combination. Mean and SEM are shown, N=5 plates with

30 worms each. Statistics as in figure 3. *** shows p-value < 0.001 comparing RNAi to empty vector, Bonferroni adjusted. Green dot in (A) represents survival of worms exposed to BT407 and in (B) survival of worms exposed to BT679 Cry- (+ RFP).

The *elt-2*-dependent response to BT679 is elicited specifically by the Cry21Aa3 3d PFT but not by the Cry14Aa2 3d PFT

BT produces different virulence factors to attack the host in its different stages of infection. However, the main virulence factors responsible for early infection in hosts, including nematodes, are the crystal PFTs (Borgonie et al., 1995; Borgonie et al.; Guo et al., 2008; Wei et al., 2003). The main crystal PFT contributing to pathogenicity against nematodes in BT247 is Cry6Ba (Hollensteiner et al., 2017a), while BT679 produces two nematicidal toxins, Cry21Aa3 and Cry14Aa2, encoded by the same plasmid (Masri et al., 2015). In the coevolution experiment performed by Masri et al. (2015) a non-pathogenic BT679 Cry- strain was generated and pathogenicity was rescued by the separate reintroduction of the toxins Cry21Aa3 and Cry14Aa2 with the help of an expression vector (Masri et al., 2015). We thus asked next if *elt-2* is required for the response to each of the two BT679 crystal PFTs. *elt*-2(RNAi) worms were more susceptible to BT679 Cry- expressing Cry21Aa3 (strain BT679 Cry- (+Cry21Aa3) but not Cry14Aa2 (strain BT679 Cry- (+Cry14Aa2; Figure 5B). This result suggests that only the Cry21Aa3 toxin is sufficient to elicit the ELT-2-dependent response to BT679, but not Cry14Aa2, confirming that the level of specificity in the nematode's response to BT that can be mediated by single toxins. This is confirmed by previously reported results in which defence responses to a given PFT (e.g., Cry5B) are different from those to other studied PFTs (e.g., Cry14A, Cry21A and Cry6A) (Bellier et al., 2009; Chen et al., 2013; Griffitts et al., 2005; Zhang et al., 2016).

The p38 MAPK pathway is required for defence against BT679, but dispensable for defence against BT247

In our search for the molecular mechanisms behind *C. elegans* specific immune responses to BT679 and BT247, we wondered whether *elt-2* interacts with the known Cry toxin/BT defence pathways, such as the p38 MAPK and ILR/DAF-2 pathways. These pathways coordinate *C. elegans* immune responses against a variety of pathogens (Kim et al., 2002; Pujol et al., 2008; Schulenburg et al., 2004; Troemel et al., 2006; Ziegler et al., 2009), including BT or pore-forming toxins (Hasshoff et al., 2007a; Huffman et al., 2004; Wang et al., 2012a; Yang et al., 2015). Moreover, ELT-2 cooperates with the p38 MAPK pathway in the response against infection with *Pseudomonas aeruginosa* and *Salmonella enterica* and in the subsequent recovery from these infections (Block et al., 2015; Head and Aballay, 2014; Head et al., 2016). Our enrichment analysis of the specificity clusters 2 and 8 (Figure 2 A) now revealed an overrepresentation of the targets of these two pathways (Figure 2 C, Figure 6 A), suggesting an interaction between ELT-2 and the pathways in mediating specificity. To test this idea, we first exposed *pmk-1*(*km25*) and *sek-1*(*km4*) mutants to pathogenic BT. *pmk-1* (Figure 6 B and C) and *sek-1* (Figure S3) mutants were more susceptible than the wild type to killing by BT679, while they were as resistant as wildtype worms on BT247. This suggests that the p38 MAPK pathway contributes to the defence against BT679, but not BT247.

To further assess the importance of the interaction between ELT-2 and the p38 MAPK pathway, we knocked down *elt-2* by RNAi in *pmk-1*(*km25*) and *sek-1*(*km4*) mutants. *pmk-1*(*km25*);*elt-2*(RNAi) animals were significantly more susceptible than *elt-2*(RNAi) worms alone, indicating that both ELT-2 and the p38 MAPK pathway are required for mounting a response against BT679, likely acting in parallel (Figure 6, Figure S3). We then asked if the p38 MAPK pathway is required for defence against both nematicidal BT679 Cry toxins. *pmk-1*(*km25*) and *sek-1*(*km4*) mutants were more susceptible to both BT679 Cry-(+ Cry21Aa3) and BT679 Cry- (+ Cry14Aa2) (Figure 6 D), thus in contrast to *elt-2*(RNAi) worms, which were only susceptible to BT679 Cry-(+ Cry21Aa3). This result indicates that the p38 MAPK pathway constitutes a general response module to BT679 toxins while *elt-2* specifically interacts only with one of the single toxins.

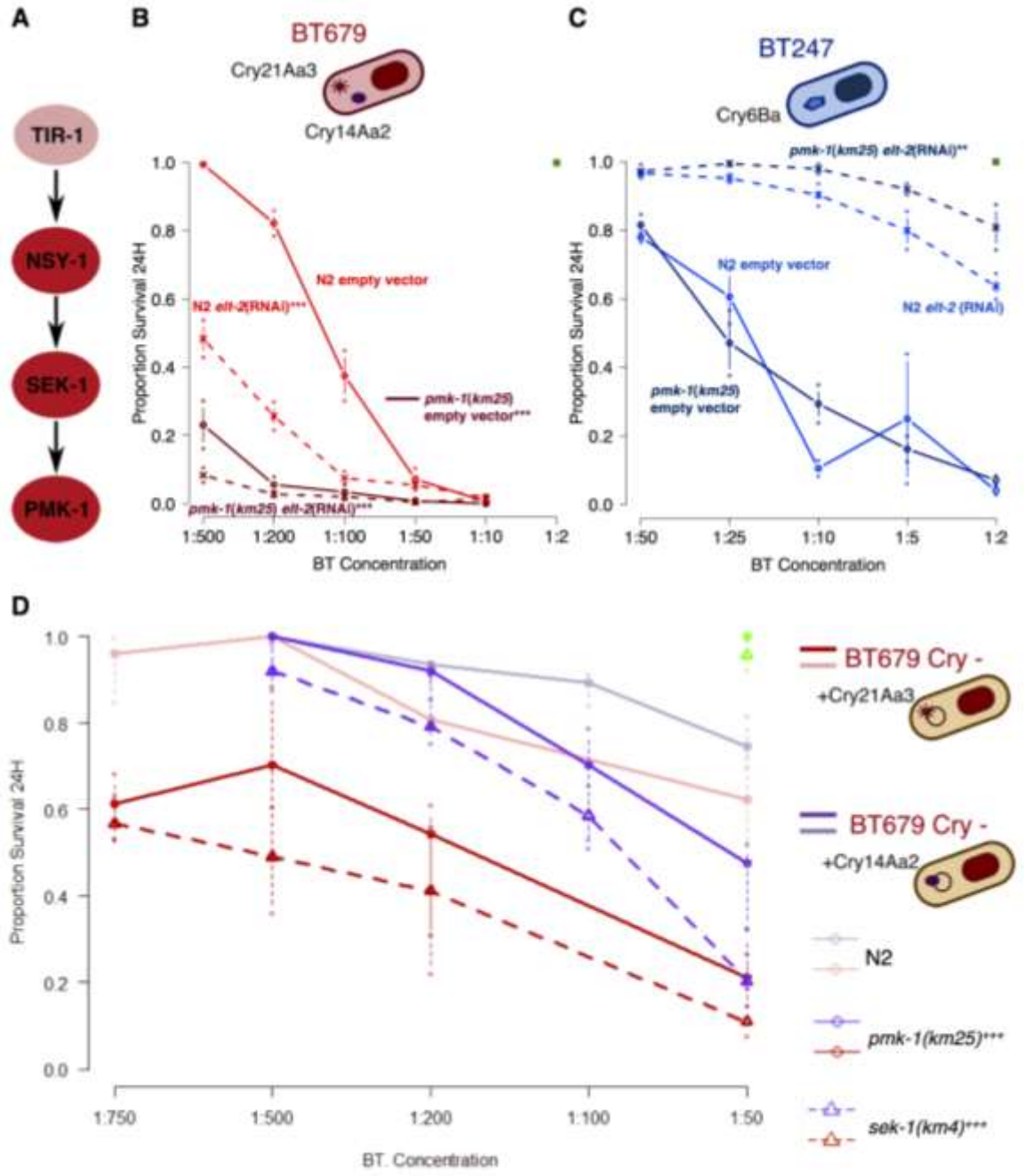

Figure 6. The p38 MAPK pathway acts together with *elt-2* to enhance the survival rate of worms upon BT679 infection but has no effect in the survival rate of worms infected with BT247. Diagram of (A) the p38 MAPK pathway in *C. elegans* and survival of (B) *pmk-1*(km25) mutants exposed to BT679 and (C) BT247; and survival of (D) p38 MAPK mutants *pmk-1*(km25) and *sek-1*(km4) exposed BT679 Cry- (+ Cry14Aa2) and BT679 Cry- (+Cry21Aa3). Each curve represents the proportion of surviving worms of each worm strain and treatment combination. In (A) and (B) dashed lines represent worms with *elt-2*(RNAi). Mean and SEM are shown, N=5 plates with 30 worms each. Statistics as in figure 3. *** shows p-value < 0.001 comparing RNAi to empty vector, $^{+++}$ shows p-value < 0.001 comparing mutant to N2. Bonferroni adjusted. Green dots in (B-C) represent survival of worms exposed to BT407 and in (D) survival of worms exposed to BT679 Cry- (+ RFP). Results are representative of at least 3 independent runs.

The ILR/DAF-2 contributes to a strain-specific response to BT247 and BT679

Targets of the ILR/DAF-2 pathway were also enriched in clusters 2 and 8 (Figure 2 A and 2 C). To test whether the ILR pathway interacts with *elt-2* in regulating the defence against BT, we knocked down *elt-2* by RNAi in *daf-16*(*mgDf50*) and *daf-2*(*e1370*) mutants and the *daf-16*(*mgDf47*)/*daf-2*(*e1370*) double mutant. *daf-2*(*e1370*) mutants reared on *E. coli* OP50 were more resistant than N2 when exposed to both BT679 and BT247 (Figure S4), as previously described (Hasshoff et al., 2007b; Wang et al., 2012a). Epistasis analysis of *daf-2* with *elt-2* revealed new interactions: *daf-2*(*e1370*);*elt-2*(RNAi) animals exposed to BT679 were as resistant as wildtype (Figure 7 B-C). This indicates that in the absence of DAF-2, which negatively regulates DAF-16 activation (Figure 7 A) and the immune response to BT679 infection, the hypersusceptibility phenotype of *elt-2*(RNAi) animals is fully rescued. This happened in a DAF-16-dependent manner, as was evidenced by the same susceptibility of *elt-2*(RNAi) worms and the *daf-16*(*mgDf47*)/*daf-2*(*e2370*) double mutant with *elt-2*(RNAi) treatment (Figure S5 A). This suggests that upon exposure to BT679, ELT-2 acts upstream or independently of ILR/DAF-2 pathway, as already reported in the context of *Salmonella enterica* and *Cryptococcus neoformans* infection (Kerry et al., 2006). DAF-16 responds to pathogenicity by BT247 and BT679 in a magnitude proportional to the killing rate of the pathogen and independent of the bacterial load (Wang et al., 2012b). The *daf-16*(*mgDf50*) mutant shows the same survival rate as N2 on BT679 and an increase in susceptibility of *elt-2*(RNAi) worms normally resistant to killing by BT247 (Figure 7 D, 7 E, Figure S4). Together, these results show that the TF *daf-16* is responsible for mounting responses promoting resistance to both pathogenic BT strains. It acts either downstream or in parallel to *elt-2* to jointly promote resistance to BT679, while it genetically interacts with *elt-2* to oppose its function and promote resistance to BT247.

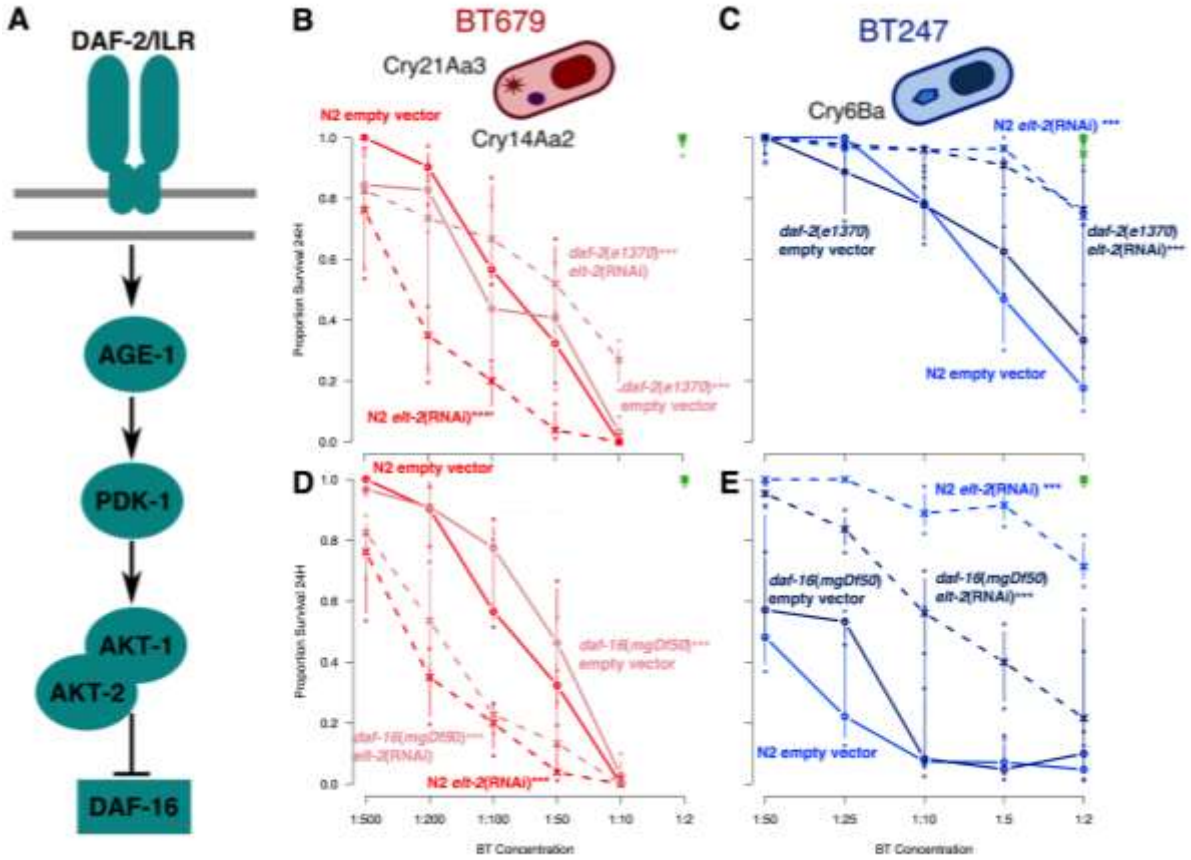

Figure 7. The DAF-2/ILR pathway interacts with *elt-2* to mount strains-specific responses to BT. Diagram of (A) ILR/DAF-2 pathway and survival of (B-C) *daf-2*(*e1370*) and (D-E) *daf-16*(*mgDf50*) mutants exposed to BT679 and BT247. Each curve represents the proportion of surviving worms of each worm strain and treatment combination. Mean and SEM are shown, N=5 plates with 30 worms each. Statistics as in figure 3. *** shows p-value < 0.001 comparing RNAi to empty vector, $^{+++}$ shows p-value < 0.001 comparing mutant to N2. Bonferroni adjusted. Green dots in (A) and (B) represent survival rate of worms exposed to BT407. Results are representative of at least 3 independent runs.

Higher worm survival to BT247 is not mediated by host factors known to contribute to Cry toxin susceptibility

What makes *elt*-2(RNAi) worms so resistant to BT247 infection? This question of particular interest because all previous work with a variety of pathogens suggest that *elt-2*(RNAi) rather reduces nematode resistance (Kerry et al., 2006; Lee et al., 2013; Shapira et al., 2006). One explanation could be that ELT-2 is required for the expression of an intrinsic host factor that is essential for binding or activation of the BT247 Cry toxin in the adult intestine. It was previously shown that *C. elegans* glycosylation-deficient mutants are resistant to *E. coli* expressing the BT 3d PFT Cry5Ba, because they lack the intestinal surface glycolipids, to which the toxin binds (Figure 8 A) (Griffitts et al., 2005). To investigate if these BT toxin-resistant (*bre*) mutants are also resistant to the BT strains we used in this study,

we exposed *bre* and additional glycosylation-deficient mutants to BT679 (containing 3d PFTs Cry21Aa3 and Cry14Aa2) and BT247 (containing the unique-domain PFT Cry6Ba). All tested mutants were susceptible to killing by both pathogenic BT strains (Figure 8 B).

The *bre* mutants were also found to be susceptible to killing by the BT non-3d unique domain PFT Cry6Aa (Marroquin et al., 2000). The toxin Cry6Aa induces and exploits the host's endogenous necrosis pathway by physically binding to its intestinal terminal protease ASP-1. In this way, the toxin is protected from over-degradation and provokes intestinal cell necrosis at the same time. Necrosis-deficient worms thus show increased survival upon exposure to Cry6Aa compared to wildtype worms (Zhang et al., 2016). Cry6Aa is phylogenetically the most closely related toxin to the BT247 PFT Cry6Ba (Hollensteiner et al., 2017a). To test the potential involvement of the necrosis pathway in mediating resistance to BT247, we exposed *asp-1*(*tm666*) mutants to BT247 and assessed their survival (Figure 8 C). *asp-1*(*tm666*) mutants were more resistant to BT247 than N2, however not to the extent of *elt*-2(RNAi) worms. Moreover, in combination with *elt-2* knockdown, *asp-1*(*tm666*) mutants were not as resistant to BT247 as *elt-2*(RNAi) worms and were as resistant as *asp-1*(*tm666*) mutants alone (Figure 8C). These results suggest that the necrosis pathway is necessary, but not completely responsible for the *elt-2*(RNAi)-mediated enhanced resistance to BT247.

elt-2(RNAi) reduces worm fecundity (personal observation). As sterile *C. elegans* mutants are more resistant to pathogen infection (Miyata et al., 2008), the reduced fecundity of *elt-2*(RNAi) worms could be another explanation for their increased survival rate of *elt-2*(RNAi) upon exposure to BT247. We thus tested knockout mutants of the LIN-12/Notch family of receptors member *glp-1*(*ar202*) and *glp-1*(*bn18*), which make the worms deficient in mitotic germ-line stem cells, and therefore sterile; and a knockout mutant of the RNA-dependent RNA polymerase *fer-15*(*b26*), which results in worms that possess a germline but are sperm-deficient and therefore, sterile. These mutants display general increase in survival rate upon exposure to pathogens (Miyata et al., 2008). The *fer-15*(*b26*) (Figure 8 D), *glp-1*(*ar202*), and *glp-1*(*bn18*) (Figure 8 E) mutants were indeed more resistant to BT247 infection than wildtype worms. However, silencing *elt-2* in the *fer-15*(*b26*), *glp-1*(*ar202*), and *glp-1*(*bn18*) mutant background even enhances the increased resistance observed in sterile mutants. This suggests that the mechanism responsible for the higher survival on BT247 after *elt-2*(RNAi) is different form the one observed as a consequence of sterility. Results from this and previous sections suggest that the higher survival on BT247 after *elt-2*(RNAi) in not due to the effect of a single genetic factor or signalling pathway, but it has rather a multifactorial cause.

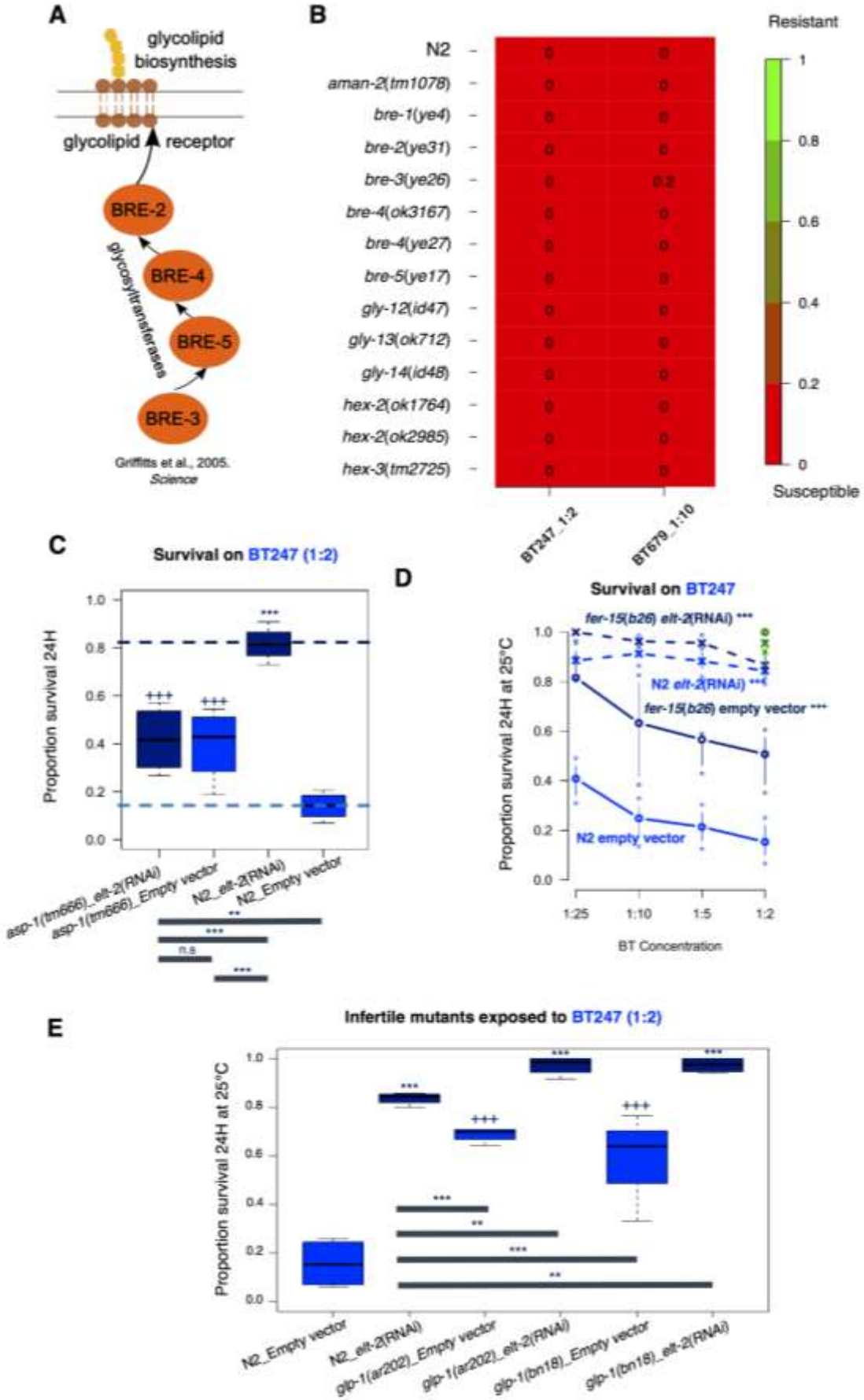
A
glycolipid biosynthesis
glycolipid receptor
BRE-2
BRE-4
BRE-5
BRE-3
glycosyltransferases
Griffitts et al., 2005. Science
B
N2
aman-2(tm1078)
bre-1(ye4)
bre-2(ye31)
bre-3(ye26)
bre-4(ok3167)
bre-4(ye27)
bre-5(ye17)
gly-12(id47)
gly-13(ok712)
gly-14(id48)
hex-2(ok1764)
hex-2(ok2985)
hex-3(tm2725)
BT247_1:2
BT679_1:10
Resistant
Susceptible
C
Survival on BT247 (1:2)
Proportion survival 24H
asp-1(tm666)_elt-2(RNAi)
asp-1(tm666)_Empty vector
N2_elt-2(RNAi)
N2_Empty vector
D
Survival on BT247
fer-15(b26) elt-2(RNAi) ***
N2 elt-2(RNAi) ***
fer-15(b26) empty vector ***
N2 empty vector
Proportion survival 24H at 25°C
BT Concentration
E
Infertile mutants exposed to BT247 (1:2)
Proportion survival 24H at 25°C
N2_Empty vector
N2_elt-2(RNAi)
glp-1(ar202)_Empty vector
glp-1(ar202)_elt-2(RNAi)
glp-1(bn18)_Empty vector
glp-1(bn18)_elt-2(RNAi)

Figure 8. Increased survival rate of *elt-2*(RNAi) worms on BT247 is not entirely explained by sterility, glycosylation-deficiency or *asp-1*-dependent necrosis deficiency. (A) Glycolipid biosynthesis pathway dependent on *bre* glycosyltransferases. Survival of (B) glycosylation-deficient mutants on BT247 and BT679; and survival of the (C) necrosis terminal aspartic protease mutant *asp-1*(*tm666*) and the sterile mutants (D) *fer-15*(*b26*) and (E) *glp-1*(*ar202*) and *glp-1*(*bn18*). In (B) a binary score considered resistance as >70% survival of ~300 worms on plate. N=5. Each curve in (D) represents the proportion of surviving worms of each worm strain and treatment combination. Mean and SEM are shown, N=5 plates with 30 worms each. Green dots in (D) represent survival of worms exposed to BT407. Statistics for (C-E) as in figure 3. *** shows p-value < 0.001 comparing RNAi to empty vector, or as indicated by bar. $^{+++}$ shows p-value < 0.001 comparing mutant to N2 in the same treatment combination, Bonferroni adjusted.

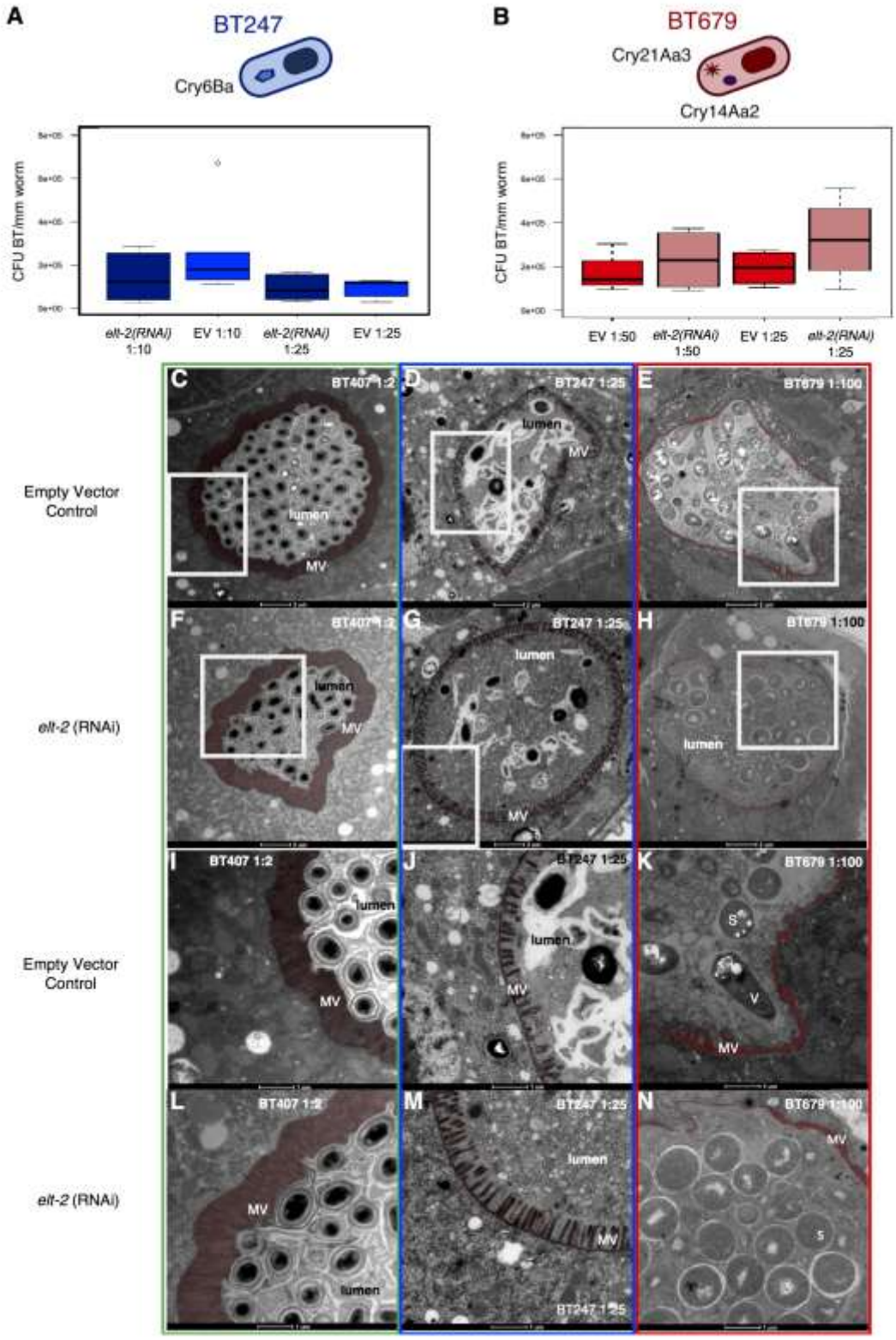

Figure 9. *elt-2*(RNAi) worms have the same bacterial load on BT247 but tend to have higher bacterial load on BT679 compared to the empty vector control. On both

pathogen strains, worm survival rate is inversely proportional to intestinal epithelium damage. Bacterial load of *elt-2*(RNAi) and control worms exposed to (A) BT247 and (B) BT679; and TEM pictures of transversal sections of the anterior intestine of *elt-2*(RNAi) and control worms exposed to different BT strains (C-N). Pictures corresponding to each BT treatment are surrounded by a green box in the case of BT407, a blue box in the case of BT247 and a red box in the case of BT679. Microvilli (MV) are artificially coloured in red to indicate their integrity during infection. Scale bar represents 2 μm in panels C-H and 1 μm in panels I-N. White boxes in panels C-H show area that is zoomed-in in panels I-N.

elt-2 positively regulates resistance to BT679 and negatively regulates tolerance to BT247

To further explore the *elt-2*-mediated contrasting defence phenotypes, we assessed infection patterns in *elt-2*(RNAi) nematodes, as these may yield insights into the underlying processes mediating resistance. Previous work demonstrated that active *elt-2* contributes to the reduction of bacterial load (Kerry et al., 2006), presumably because it positively regulates the expression of putative effector genes (Shapira et al., 2006). Our new analysis revealed that the bacterial load of *elt-2*(RNAi) worms and empty vector worms exposed to BT247 did not differ (Figure 9 A), despite the significantly higher survival rate of *elt-2*(RNAi) worms upon BT247 exposure (Figure 3 B). On BT679, the bacterial load of *elt-2*(RNAi) worms tended to be higher (p-value = 0.09) than that of the empty vector controls, but this difference was not significant (Figure 9 B). These results suggest that the mechanism through which *elt-2*(RNAi) enhances host survival on BT247 involves enhanced tolerance to pathogen infection, which we here assume to be defined as the ability to withstand the same bacterial load with unaffected or increased fitness/health (Ferrandon, 2009; Medzhitov et al., 2012). This response is distinct from resistance, which we assume to be characterized as increased survival due to decreased bacterial load, usually as a consequence of immune effector activity.

Resistance is likely to underlie the pattern observed for BT679. *elt-2*(RNAi) worms have a reduced survival when exposed to BT679 compared to empty vector controls and display a trend for higher bacterial load. Moreover, *elt-2* is known to regulate the expression of putative effector genes (McGhee et al., 2009a), which could help to eliminate or at least inactivate pathogens. To verify this idea, we searched our RNA-Seq data for putative effector genes that are upregulated upon BT679 infection in an *elt-2*-dependent manner. Cluster 9 contains genes that are upregulated upon exposure to both BT247 and BT679, but some, like the invertebrate lysozyme *ilys-3*, are more highly upregulated towards BT679. *ilys-3* is upregulated upon infection with other pathogens and the protein possesses lytic activity against Gram-positive cell wall components (Gravato-Nobre et al., 2016). The survival rate of the *ilys-3*(*ok3222*) mutant was not significantly different from N2, although a trend for lower survival rate was seen (Figure S6 B). As a control,

we tested the saposin-like protein *spp-8* gene which was found in cluster 8, containing genes that are downregulated on BT679 and slightly upregulated on BT247 at late time points. No difference in survival rate form controls was seen on any of the two pathogens (Figure S6).

We next explored the *elt-2* mediated tolerance phenotype towards BT247. We first assessed whether tolerance is associated, as usually assumed, with increased health. As an indicator of health, we inspected the integrity of the intestinal epithelium, which is usually considered essential for digestive function of the gut (Gelino et al., 2016; Irazoqui et al., 2010a). After 12 hours of infection, we found that intestinal damage is inversely proportional to the survival rate of the worms (Figure 9 C-N). The intestinal epithelium in *elt-2*(RNAi) worms exposed to BT247 showed clear signs of damage, including distension of the intestinal lumen, shortening of the microvilli, and gaps in the intestinal brush border (Fig 9 G and M). Importantly, the damage occurred to a lesser degree than in empty vector worms exposed to BT247 (Figure 9 D and J). These observations suggest that *elt-2*(RNAi) reduces but does not abolish the damaging effects of BT247 toxin. The increased survival on BT247 may directly result from the reduced damage and/or additional processes that help the worm to cope with the remaining damage. The opposite was seen on BT679, where *elt-2*(RNAi) worms had lost the intestinal brush border (Figure 9 H and N) to a more dramatic extent than empty vector controls (Figure 9 E and K).

We validated our findings by studying intestinal epithelial integrity in the transgenic fluorescent reporter strain BJ49, which contains a CFP-tagged intermediate filament protein (IFB-2::CFP). IFB-2 is a structural component of the intestinal terminal web. The CFP tag surrounds the intestinal lumen and is seen as two parallel thin continuous lines when healthy worms are inspected under the fluorescence microscope (Hüsken et al., 2008). Damage in the intestinal epithelium can be observed when these continuous thin lines produce gaps, twists, blur or collapse into one line (Stutz et al., 2015). *elt-2*(RNAi) worms on BT247 (Figure 10 K and L) display the same intestinal tissue integrity as non-pathogenic controls (Figure 10 C, D, G, H). In contrast, *elt-2*(RNAi) worms on BT679 display collapsed lines or gaps (Figure 10 O and P), similar to the damage phenotypes observed for empty vector worms exposed to BT247 (Figure 10 I and J) and BT679 (Figure M and N). Our results confirm that increased worm survival coincides with reduced damage to their intestinal brush border and terminal web (Figure 10 A-P). The overall results could be further verified through an additional independent approach, based on the intestinal leaking assay (smurf) with blue food dye (Gelino et al., 2016). In this case, *elt-2*(RNAi) worms on BT247 showed again substantially less damage at the intestinal epithelium than *elt-2*(RNAi) worms on BT679 (Figure S7).

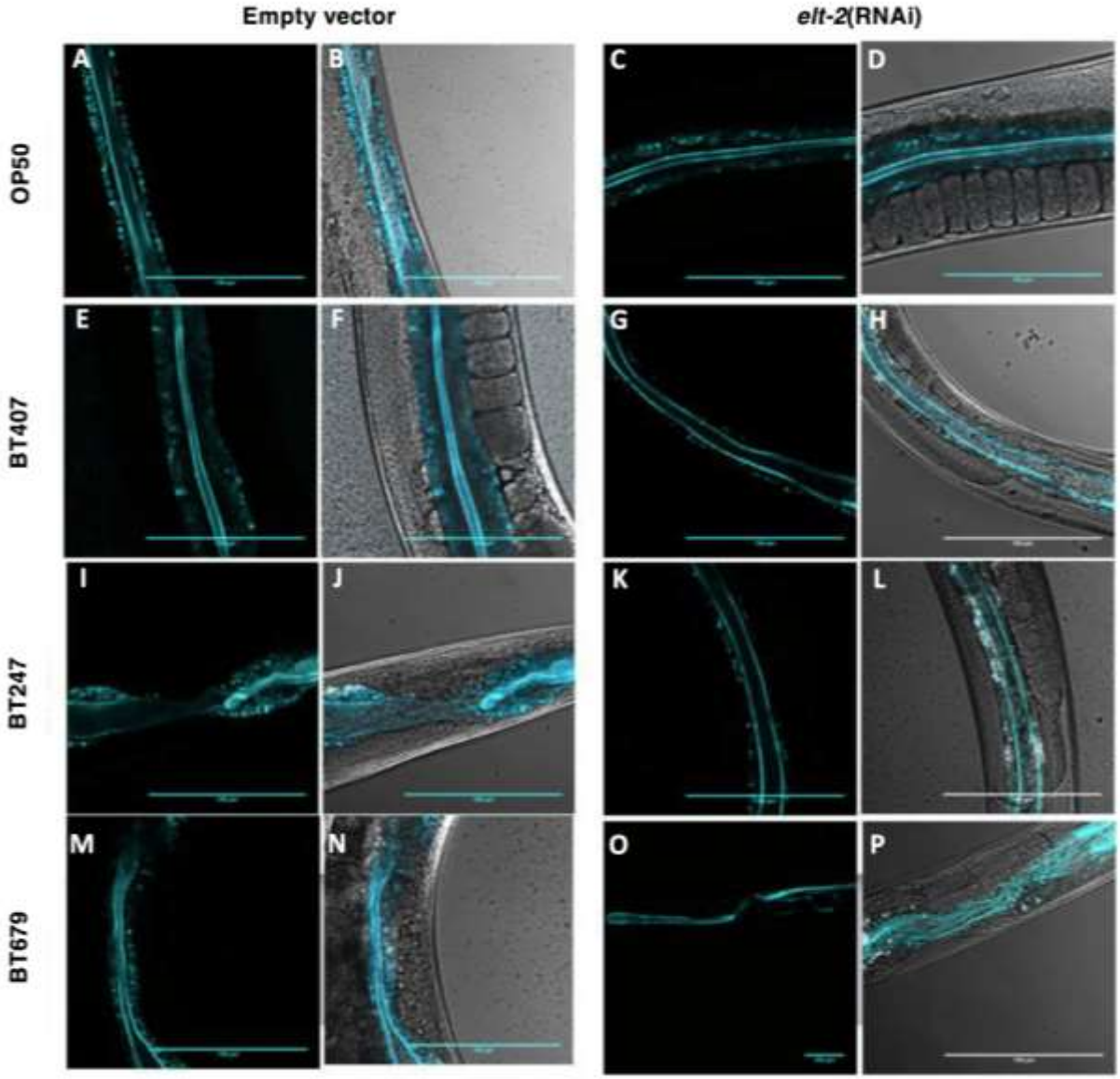

Figure 10. The intestinal epithelium integrity of *elt-2*(RNAi) BJ49 fluorescent reporter worms on BT247 is the same as that of non-pathogenic control worms. Fluorescence images and fluorescence merged with bright field images of the BJ49 strain carrying the IFB-2::CFP transgene 24 hours after exposure to (A-D) *E. coli* OP50 (E-H) BT407 (I-L) BT247, and (M-P) BT679. Scale bars represent 100 μm.

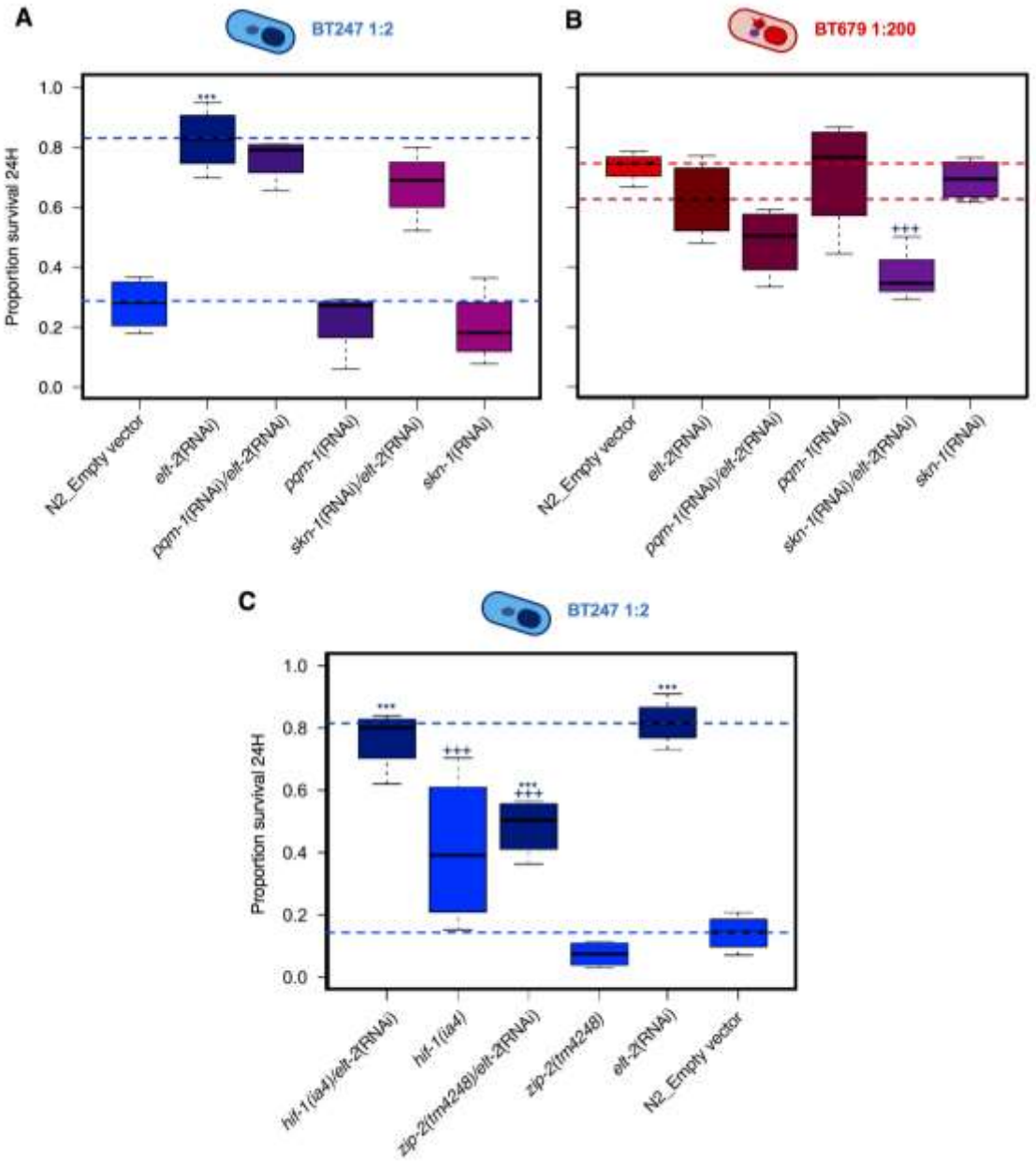

Figure 11. The skinhead bZIP TF *skn-1* contributes to resistance to BT679, while the hypoxia inducible factor *hif-1* contributes to resistance to BT247. The bZIP TF *zip-2* partially rescues *elt-2*(RNAi) worm survival rate increase on BT247. Survival of empty vector, *elt-2*(RNAi), *pqm-1*(RNAi)/*elt-2*(RNAi), *pqm-1*(RNAi), *skn-1*(RNAi)/ *elt-2*(RNAi), *skn-1*(RNAi) worms exposed to (A) BT247 and (B) BT679 and (C) *hif-1*(*ia4*) and *zip-2*(*tm4248*) mutants exposed to BT247. Each boxplot represents the proportion of surviving worms of each treatment combination. N=4 plates with 30 worms each. Statistics as in figure 3. *** shows p-value < 0.001 comparing RNAi to empty vector, $^{+++}$ shows p-value < 0.001 comparing mutant to N2, Bonferroni adjusted. Horizontal dashed lines indicate the median of N2 empty vector or N2 *elt-2*(RNAi) worms.

BT-strain specific responses mediated by *elt-2* are achieved via interactions with other TFs

We next explored the interaction of *elt-2* with other TFs, because the identification of such interacting TFs and assessment of their function(s) may further help us to understand the molecular processes underlying enhanced tolerance to BT247. Block and Shapira have suggested that ELT-2 regulates the response to *Pseudomonas aeruginosa* PA14 infection in cooperation with the p38 MAPK-activated TFs ATF-7 and SKN-1 (Block and Shapira, 2015; Block et al., 2015). We have previously shown that GATA-dependent transcription dominates the *C. elegans* immune response against gut pathogens, but that additional TF binding motifs likely regulate more specific responses, either acting alone or jointly with GATA TFs (Yang et al., 2016a). The interaction with alternative TFs may thus drive the specificity of the transcriptional program controlled by ELT-2. We began by assessing genetic interactions between *elt-2* and genes encoding TFs with a known function in *C. elegans* immunity or stress response. We used gene knockouts (*daf-16*(*e1370*), *hif-1*(*ia4*) and *zip-2*(*tm4248*)) and RNAi clones from the Ahringer library (*skn-1* and *pqm-1*) (Kamath and Ahringer, 2003; Kamath et al., 2003) (see methods). We assessed the survival rates of the TF knockout/knockdown worms in the response to BT infection, and also their genetic interaction with *elt-2*.

The skinhead bZIP TF *skn-1* was previously found to regulate immune gene expression in the defence against *P. aeruginosa* PA14 together with *atf-7* and *elt-2* (Block et al., 2015). *skn-1*(RNAi) worms were as susceptible as control worms on BT679 and BT247 (Figure 11). Interestingly, *elt*-2;*skn-1*(RNAi) worms were even more susceptible to infection with BT679 than *elt-2*(RNAi) worms (Figure 11 B), indicating that RNAi of *elt-2* and *skn-1* has a synergistic effect on worm survival. In the context of BT247 infection *elt-2*;*skn-1*(RNAi) worms were as tolerant as *elt-2(*RNAi) worms (Figure 11 A), indicating no effect by *skn-1*.

We previously found that the FOXO TF *daf-16* undergoes nuclear translocation and is activated upon BT247 and BT679 infection (Wang et al., 2012a). *daf-16* also plays a part in the positive regulation of tolerance to BT247 infection in *elt-2*(RNAi) worms (Figure 7 B, E). We have found that *elt-2* antagonizes this function by negatively regulating tolerance to BT247 infection. It was previously reported that *pqm-1* and *daf-16* both bind to the same regulatory elements in promoter regions of intestinal genes and are mutually antagonistic in at least their ageing-related functions (Tepper et al., 2013). We now found that *pqm-1* does not interact with *daf-16* to influence BT247 infection, as *pqm-1*(RNAi) did not differ from the corresponding controls (Figure 11 A, B).

The bZIP TF *zip-2* is involved in the *C. elegans* response to infection by *P. aeruginosa* PA14 (Dunbar et al., 2012). We analysed the *zip-2*(*tm4248*) mutant

and found that, upon BT247 infection, this TF has the same survival rate as N2 (Figure 11 C). However, when studying its interaction with *elt-2* we find that *zip-2*(*tm4248*)/*elt-2*(RNAi) worms have reduced survival rate compared to the highly tolerant *elt-2*(RNAi) worms upon BT247 infection (Figure 11 C). We conclude that, like *daf-16*, *zip*-2 antagonizes *elt-2* upon BT247 infection and positively affects the survival rate of the worms.

The terminal TF of the hypoxia pathway, *hif*-1, was previously found to enhance survival of worms upon exposure to the 3d PFTs Cry5B and Cry21A. This happens through activation of the *xbp-1*-dependent branch of the unfolded protein response (UPR) and the induction of the Nuclear Hormone Receptor (NHR) TF *nhr-57* (Bellier et al., 2009). We tested the *hif-1*(*ia4*) mutant in the context of BT247 infection and found that it behaves as N2 both alone and when treated with *elt-2*(RNAi) (Figure 11 C).

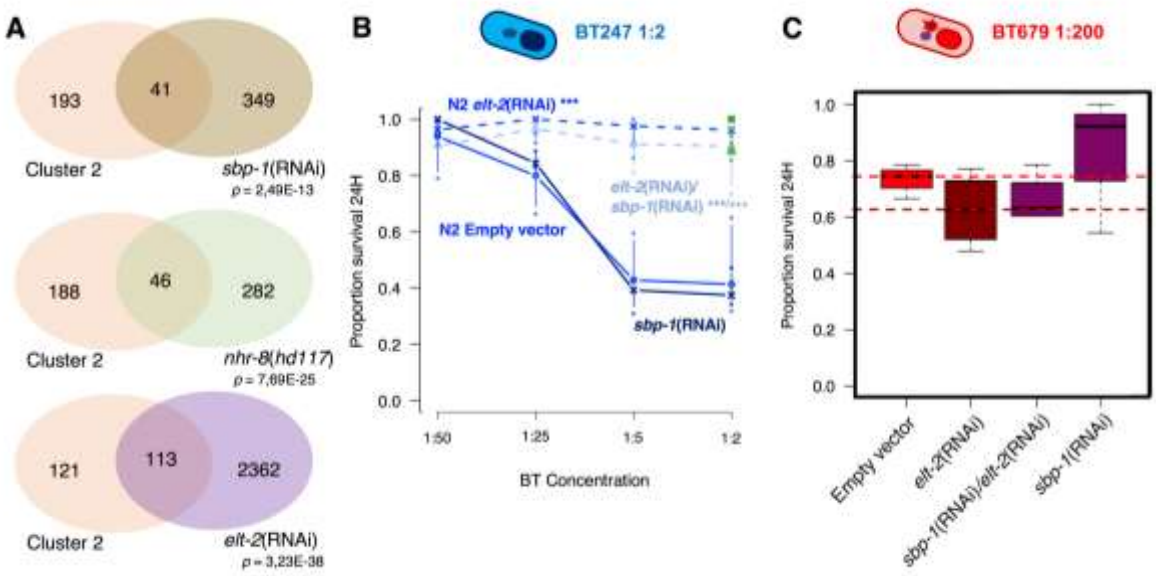

Figure 12. Genes in cluster 2 overlap with targets of the TFs *sbp-1* and *nhr-8*. *sbp-1*(RNAi) has no effect on the survival rate of worms exposed to either BT247 or BT679. (A) Venn diagram showing hits from EASE analysis of cluster 2 (from figure 2 A) using the platform WormExp (Yang et al., 2016b). Hits that were experimentally analysed in this study are shown in the figure. Numbers in Venn diagram correspond to gene counts and the number below the hit population (i.e., gene sets) corresponds to p-values, Bonferroni corrected, including hit populations for *sbp-1*(RNAi) (Ding et al., 2015); *nhr-8*(*hd117*) (Magner et al., 2013); *elt-2*(RNAi) (Mann et al., 2016). The panels show survival rates of empty vector, *elt-2*(RNAi), *sbp-1*(RNAi) and *sbp-1*(RNAi)/*elt-2*(RNAi) worms exposed to (B) BT247 and (C) BT679. In (B) mean and SEM are shown, N=5 plates with 30 worms each. Green dots represent survival of worms exposed to BT407. Boxplots in (C) represent the proportion of surviving worms. N=4 plates with 30 worms each. Statistics as in figure 3. *** shows p-value < 0.001 comparing RNAi to empty vector, $^{+++}$ shows p-value < 0.001 comparing mutant to N2, Bonferroni adjusted.

To identify additional TFs that might interact with *elt-2* in fine-tuning the *C. elegans* response to infection with BT247 and BT679, we then turned to cluster 2 of our RNA-Seq data (Figure 2 A, genes upregulated on BT247 and unchanged on BT679) and searched for enrichment of known targets of other TFs using WormExp (Yang et al., 2016b). We found a significant overlap between the genes in cluster 2 with targets of *sbp-1* (Figure 12 A). Previous studies suggested an interaction between *elt-2* and *sbp-1*, the basic helix-loop-helix (bHLH) transcription factor homologous to the mammalian Sterol Regulatory Element Binding Proteins (SREBPs) (McGhee et al., 2009a; Yang et al., 2016a), which is involved in the regulation of fat storage (Taghibiglou et al., 2009). A direct involvement of *sbp-1* in immunity has not yet been shown in *C. elegans* (Ding et al., 2015). We used an RNAi clone from the Ahringer library (Kamath and Ahringer, 2003) to study the involvement of *sbp-1* in BT infection and found no significant difference between *sbp-1*(RNAi) and control worms (Figure 12 B and C).

We also found a significant overlap between the BT247-exclusive responsive genes in cluster 2 with targets of *nhr-8* (Figure 12 A). In fact, three NHR genes (*nhr-8*, *nhr-99*, and *nhr-193*) were found in cluster 2 themselves. The multigene TF family of NHRs encompasses interesting candidates that could contribute to specification of the ELT-2 transcriptional program, because of its diversity (284 genes (Gilst et al., 2005)) and immune functions described to date. The NHRs involved in *C. elegans* immunity include, for example: DAF-12, which negatively regulates the p38 MAPK pathway through microRNA targeting; NHR-8 and NHR-48, both of which positively regulate resistance to *P. aeruginosa* PA14 (Liu et al., 2013); NHR-57, which acts downstream of the hypoxia pathway in response to PFTs (Bellier et al., 2009), and NHR-49 which positively regulates resistance to *E. faecalis* infection (Sim and Hibberd, 2016). Additionally, NHR-8 and NHR-176 are involved in the response to xenobiotic stress and detoxification, which are likely important for the clearance of pathogen toxins (Antebi, 2015; Jones et al., 2013; Taubert et al., 2011). To assess if *nhr-8*, *nhr-99*, and *nhr-193* play a role in defence against BT247 infection, we exposed knockout mutants of these genes to the pathogen. While the *nhr-99*(*gk758*) and *nhr-193*(*tm4479*) had a survival rate like N2 (Figure 13 B and C), *nhr-8*(*ok18*) worms had significantly higher survival rate than N2, although not to the extent of *elt-2*(RNAi) worms (Figure 13 A).

We also tested if *nhr-8*, *nhr-99*, and *nhr-193* regulate defence responses to BT in interaction with *elt-2*. The *nhr-99*(*gk758*);*elt-2*(RNAi) and *nhr-193*(*tm4479*);*elt-2*(RNAi) had lower survival rate on BT247 than *elt-2*(RNAi) worms (Figure 13 B and C). This suggests that both of these TFs contribute to the increase in survival rate of *elt-2*(RNAi) worms to BT247, *nhr-193* having a stronger effect, while having no effect in the response against BT679 (Figure S8). *nhr-8*(*ok186*);*elt-2*(RNAi) worms didn't show any difference to the enhanced resistance of *elt-2*(RNAi) worms when exposed to BT247. In addition to daf-16, we found that *zip-2*, *nhr-99*, and *nhr-193* are involved in the *elt-2* mediated, negative regulation of immune tolerance

to BT247, indicating that these TFs co-regulate immune tolerance together with ELT-2.

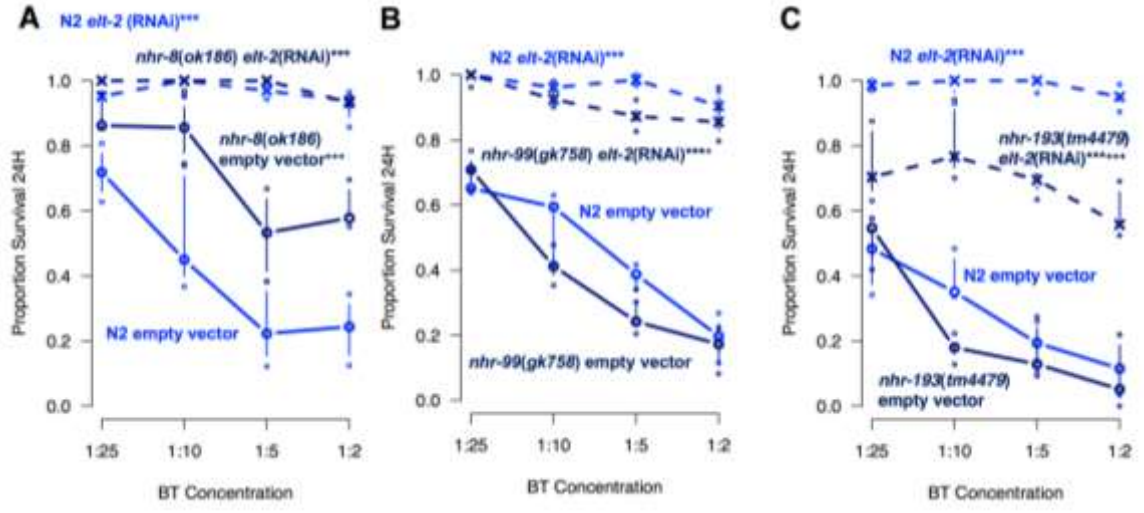

Figure 13. *nhr-8* negatively regulates tolerance to BT247. *nhr-193* and *nhr-99* are required for *elt-2*(RNAi)-enhanced tolerance to BT247. Survival of (A) *nhr-8*(*ok186*), (B) *nhr-99*(*gk758*) and (C) *nhr-193*(*tm4479*) mutants exposed to BT247. Each curve represents the proportion of surviving worms of each worm strain and treatment combination. Mean and SEM are shown, N=4 plates with 30 worms each. Statistics as in figure 3. *** shows p-value < 0.001 comparing RNAi to empty vector, $^{+++}$ shows p-value < 0.001 comparing mutant to N2, Bonferroni adjusted.

elt-2 transcriptional targets involved in the response to BT247 include putative detoxification and lipid metabolism enzymes

Most intestine-specific and intestine-enriched genes have GATA motifs in their promoter regions (McGhee et al., 2007). Therefore, ELT-2 has the potential to regulate the transcription of virtually all intestinal genes. To determine which genes are regulated by *elt-2* following an infection with our BT strains and which may thus contribute to tolerance, we performed a second RNA-Seq experiment including empty vector and *elt-2*(RNAi) worms exposed for two time-periods (6h and 12h p.i) to both BT679, BT247 and the non-pathogenic control BT407. In total, we found 6180 DE genes, whose expression is affected by *elt-2*(RNAi). When comparing the *elt-2*(RNAi) RNA-Seq dataset to the initial infection dataset, we found that the strain-specific DE patterns on BT247 and BT679 of clusters 2 and 8 had disappeared upon *elt-2* knockdown and the expression on BT247 resembled more the expression pattern on BT679 in the initial dataset (Figure 14 A). This and our previous genetic analyses support our hypothesis that the strain-specific changes in gene expression after BT infection are mainly regulated by the TF ELT-2. In our dataset, there were very few DE genes in the comparison of *elt-2*(RNAi) worms exposed to BT247 compared to BT407, suggesting that the *elt-2*(RNAi) treatment alone mimics the transcriptomic response to BT247 infection.

We then turned to analyse the genes whose expression is regulated by *elt-2* by comparing the *elt-2*(RNAi) treatment to empty vector on all three bacterial treatments (Figure 14 B). We found that 507 genes corresponding to 8% of all DE genes were downregulated on all microbes, representing the core set of genes positively regulated by *elt-2* (Figure 14 B, cluster 6). 698 DE genes, corresponding to 11% of all DE genes were upregulated in all microbes for this comparison, which suggests that these genes are usually negatively regulated by *elt-2* (Figure 14 B, cluster 9). To find genes that contribute to the enhanced tolerance and increased survival rate of *elt-2*(RNAi) worms to BT247 infection, we decided to focus on the DE genes in the comparison of *elt-2*(RNAi) worms exposed to BT247 versus empty vector worms exposed to BT247 (Figure 14B, shaded background in heading title). Enrichment analysis using the EASE tool (Ashburner et al., 2000) and the WormExp (Yang et al., 2016b) platform showed that the set of downregulated genes in this comparison has an overrepresentation of genes putatively involved in fatty acid degradation, detoxification and xenobiotic metabolism, C-type lectins, collagen and glycosyl-transferases (Figure 14D). The list of upregulated genes was also enriched with the putative functions of detoxification and fatty acid metabolism (Figure 14D).

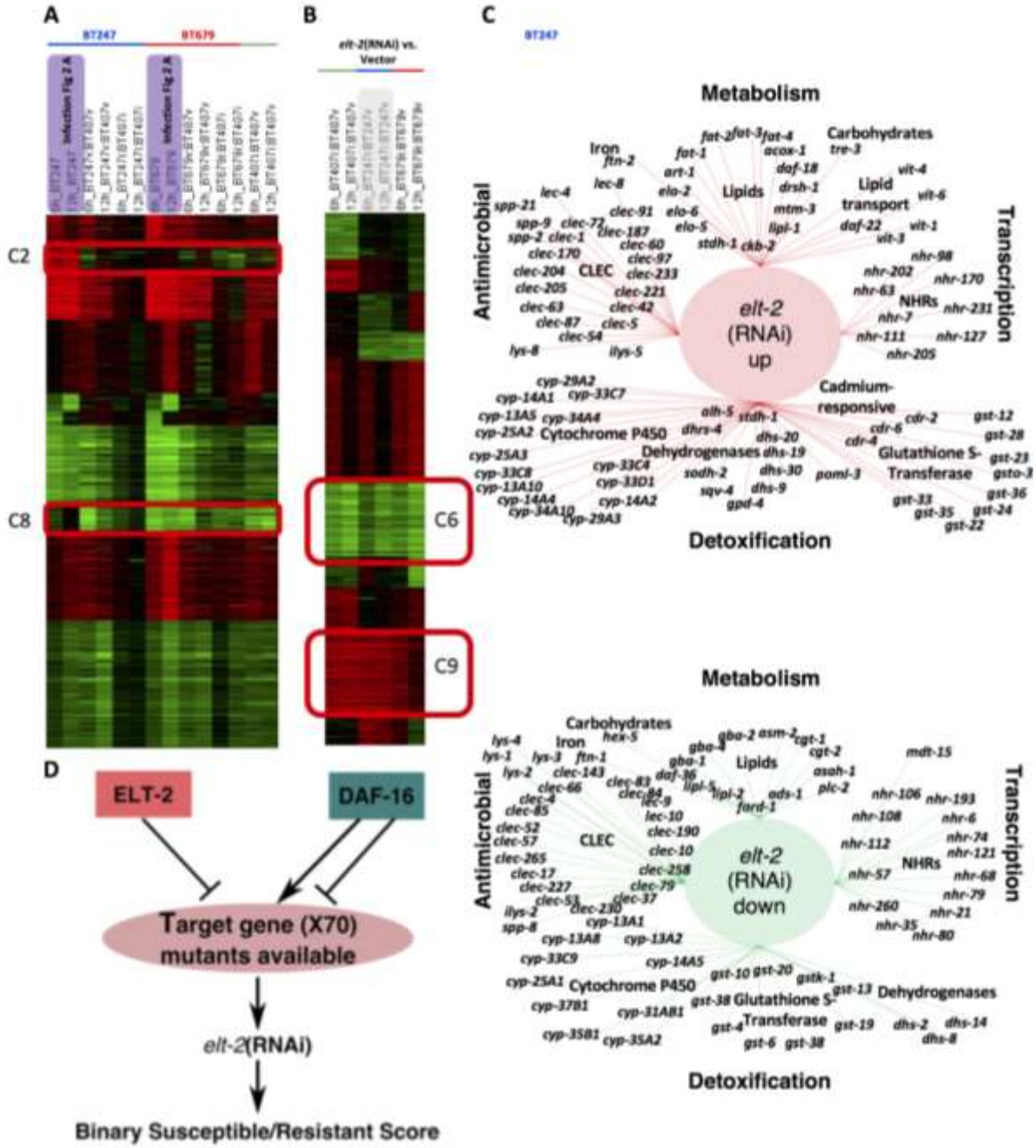

Figure 14. Clusters 2 and 8 disappear upon *elt-2*(RNAi) and there are changes in the expression of genes putatively involved in lipid metabolism, detoxification, transcription and antimicrobial function. Heatmaps showing differentially expressed genes (A) 6h and 12h p.i in the original infection RNA-Seq experiment (EXP1) compared to the RNA-Seq experiment on *elt-2*(RNAi) worms; and in (B) the DE genes in the comparison of *elt-2*(RNAi) treatment versus empty vector on each microbe. In (C) enrichment of the commonly up and down regulated clusters in B (6 and 9). (D) Strategy for small-scale suppressor screen to find an *elt-2* downstream target necessary for increased survival rate on BT247. In (A-B) differential gene expression results from the comparison of pathogen versus non-pathogen or RNAi versus empty vector. i = *elt-2*(RNAi), v = empty vector. 6h and 12h refers to hours p.i when RNA samples were taken. BT247 and BT679 refer to the same shown in Figure 2, compared to BT407.

Putative detoxification enzymes *cdr-2*, *dhs-30*, *poml-3* and *tre-3* contribute to enhanced tolerance of *elt-2*(RNAi) worms to BT247 infection

We then used the strong and reliable resistance phenotype of BT247 infected *elt-2*(RNAi) worms to conduct a small-scale candidate suppressor screen to identify genes that act downstream of *elt-2*. We focused on the genes that were upregulated in *elt-2*(RNAi) worms infected with BT247 (compared to empty vector controls) (Figure 14 B-C, shaded background), whose gene products, we hypothesize, are found in higher amount in *elt-2*(RNAi) worms. If any of these genes would contribute to enhanced tolerance, then its knockout might thus suppress the high survival rate of *elt-2*(RNAi) worms to BT247 (Figure 14 D). We focused on *daf-16* target genes for two reasons: 1) *daf-16*(mgDf50)/*elt-2*(RNAi) worms show a partial suppression of the tolerance phenotype of *elt-2*(RNAi) worms (Figure 7 E); and 2) there is evidence of a genetic interaction between *daf-16* and *elt-2*. (McGhee et al., 2009a) propose that ELT-2 acts in combination with DAF-16 to control downstream genes or that ELT-2 directly regulates transcription of DAF-16 isoforms. We thus used the platform WormExp (Yang et al., 2016b) to identify *daf-16*-regulated genes within the genes that were upregulated in the comparison *elt-2*(RNAi) on BT247 versus empty vector on BT247. In total, we found 130 genes that are negatively regulated by *elt-2* upon BT247 exposure and whose expression is also affected by *daf-16* (McElwee et al., 2003) (Figure 14 D). Of these 130 genes, 70 mutants were available (Table S1), which were then used in a small-scale screen on highly concentrated BT247 as shown in (Figure 14 D). We identified 20 candidate mutants in the initial screen, of which four were confirmed in a second validation screen to suppress the enhanced resistance of *elt-2*(RNAi) worms to BT247. These genes are the transmembrane, cadmium-responsive, glutathione-s-transferase domain-containing *cdr-2*; the dehydrogenase *dhs-30*; the orthologue of the human paraoxonase 3 (PON3) *poml-3*, and one of the four putative trehalases in *C. elegans tre-3* (Wormbase version WS262) (Figure 15). Although the exact function of these genes has not yet been determined, their protein-domain content strongly suggests a role in detoxification and/or lipid metabolism. These results suggest that the *elt-2*(RNAi)-dependent tolerance may be mediated by changes in detoxification and/or lipid metabolism.

In this work we have characterized the *elt-2*-mediated strain-specific responses to BT at the molecular level. In the case of BT679, *elt-2* cooperates with the p38 MAPK pathway and the skinhead bZip TF *skn-1* to reduce tissue damage, possibly through the induction of multiple effector genes such as the invertebrate lysozyme *ilys-3*. We uncovered that *elt-2* only promotes higher survival rate to the BT679 PFT Cry21Aa3 and not Cry14Aa2. In the case of BT247, *elt-2* negatively regulates tolerance, which we evidenced by higher survival rate and unchanged pathogen load in *elt-2*(RNAi) worms compared to empty vector. Tolerance to BT247 infection

is positively regulated by the TFs bZip *zip-2*, *nhr-193*, *nhr-99* and FOXO *daf-16*. Upon BT247 infection *daf-16* positively regulates the expression of putative detoxification and lipid metabolism genes *poml-3*, *cdr-2*, *tre-3* and *dhs-30*, which promote tolerance to this pathogen.

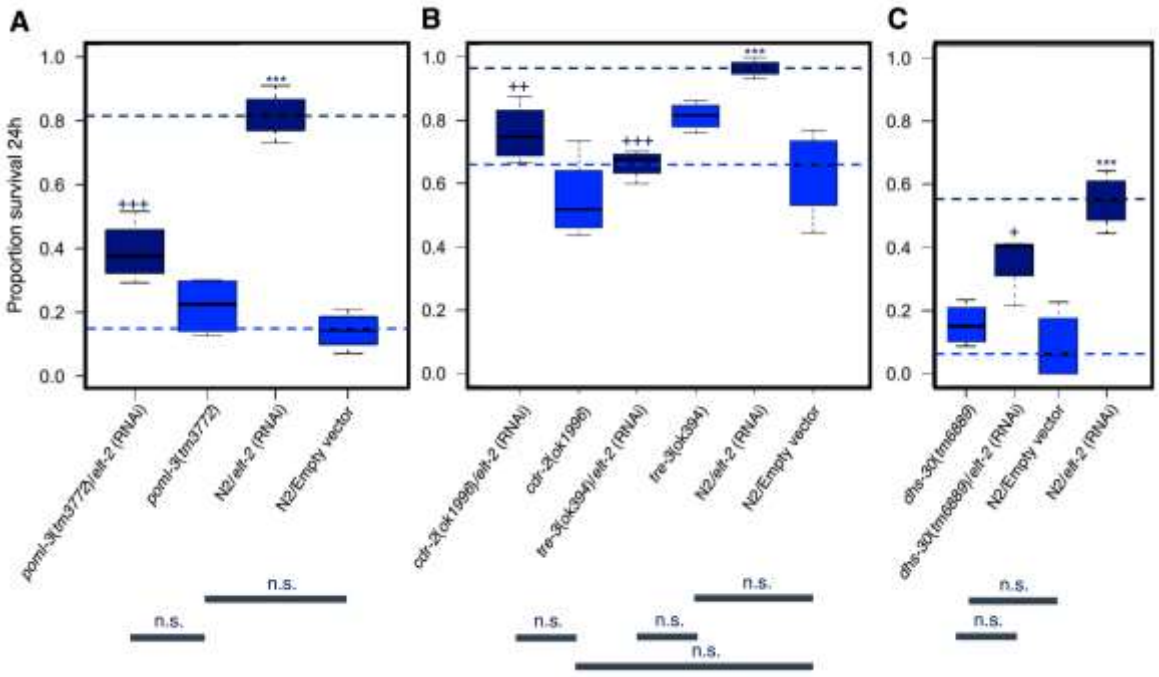

Figure 15. The (A) *poml-3*(*ok3772*), (B) *cdr-2*(*ok1996*), *tre-3*(*ok394*), and (C) *dhs-30*(*tm6889*) mutants have reduced survival rate compared with N2 on BT247 upon *elt-2*(RNAi). Each boxplot represents the proportion of surviving worms of each treatment combination. N=5 plates with 30 worms each. Statistics as in figure 3. *** shows p-value < 0.001 comparing RNAi to empty vector, $^{+++}$ shows p-value < 0.001, $^{++}$ shows p-value < 0.01, $^{+}$ shows p-value < 0.05; comparing mutant to N2, Bonferroni adjusted.

DISCUSSION

C. elegans mounts (in part) a strain-specific response to BT247 and BT679

The immune system of the invertebrate host *C. elegans* was once believed to be only capable of mounting a general response to the enormous diversity of encountered pathogens (Loker et al., 2004). The work from the last decade already highlighted that *C. elegans* can respond specifically to different species of pathogens or their derived products (Alper et al., 2007; Beale et al., 2006; Irazoqui et al., 2010a; Meisel et al., 2014; Pradel et al., 2007). In this work, we now explored whether *C. elegans* can mount a highly specific response towards different strains of the same pathogen species using BT as a model pathogen.

We initially performed an RNA-Seq experiment comparing N2 worms infected with either pathogenic BT247 or BT679 to the non-pathogenic strain BT407. We found that 91% of the differentially expressed (DE) genes (both upregulated and downregulated) changed their expression in the same way upon exposure to either BT247 or BT679 (Figure 2 A, clusters 1, 3-7, 9-10). These genes comprise what we called the *C. elegans* general transcriptomic response to BT. However, we also found that 9% of all DE genes changed their expression in different ways when the worms were exposed to BT247 compared to BT679 (Figure 2 A, clusters 2 and 8). Cluster 2 grouped genes that were upregulated upon exposure to BT247, but showed no expression change when exposed to BT679. In cluster 8 we found genes that were slightly upregulated at 24h p.i with BT247 but were strongly downregulated on BT679. These DE genes are evidence of a pathogen strain-specific inducible response of *C. elegans*. To our knowledge, this is the first time that the specificity of *C. elegans* transcriptomic responses is assessed at the pathogen strain-specific level, since all previous studies have only focused on species-specific responses (e.g., (Alper et al., 2007; Bogaerts et al., 2010; Engelmann et al., 2011; Irazoqui et al., 2010a; Nakad et al., 2016; Pees et al., 2017; Yang et al., 2016a)).

We searched for TF binding-motif enrichment in the promoter regions of genes from clusters 2 and 8 and identified the binding motif of the intestinal GATA TF, ELT-2 (Figure 2 B). *elt-2*(RNAi) worms displayed a lower survival rate on BT679 and a higher survival rate on BT247 compared to control worms (Figure 3 A and B). The opposite effect of *elt-2*(RNAi) on the survival rate of worms exposed to BT247 and BT679 represents new evidence that *C. elegans* is able to mount strain-specific responses to BT247 and BT679. In the past *elt-2*(RNAi) had only been associated to lower survival rates of worms infected with *S. enterica*, *E. faecalis*, *C. neoformans* (Kerry et al., 2006), *P. aeruginosa* (Block et al., 2015; Kerry et al., 2006; Shapira et al., 2006) and *B. pseudomallei* (Lee et al., 2013). We assessed survival rate of *elt-2*(RNAi) worms exposed to additional strains BT243, BT245 and BT246, observing lower survival rates than controls in every case (Figure 5 A). The

elt-2(RNAi)-dependent higher survival rate on BT247 is, thus, unique. It is a pathogen strain-specific phenotype, which is independent of the virulence level of the pathogen (Figure 4 A). It is also not a developmental artefact of the RNAi treatment, as it is observed both when *elt-2*(RNAi) is applied on worms at the L1 (Figures 2 B and 3 A) or L4 (Figure 3 B) larval stages. We also could not attribute the higher survival rate to any of the host factors previously reported to confer resistance to BT infection, such as glycosylation-deficiency (Figure 8 B) (Griffitts et al., 2005), necrosis pathway-deficiency (Figure 8 C) (Zhang et al., 2016), or sterility (Figure 8 D and E) (Miyata et al., 2008).

Another way of assessing pathogen strain-specific responses is to test if known host immune factors respond specifically to different pathogen strains. The signalling branch of the *C. elegans* immune system has been described in detail (Ewbank and Pujol, 2016; Schulenburg et al., 2008). Two of the signalling pathways involved in defence against BT are p38 MAPK and ILR/DAF-2 (Hasshoff et al., 2007a; Huffman et al., 2004; Wang et al., 2012a; Yang et al., 2015). The p38 MAPK pathway has been repeatedly linked to positive regulation of defence responses against infection with pathogens (Irazoqui et al., 2010b; Kim et al., 2002; Yang et al., 2015). It is known to cooperate with *elt-2* in response to (Block et al., 2015), and also during the recovery from (Head et al., 2016) *P. aeruginosa* infection. We infected p38 MAPK mutants *pmk-1*(*km25*) and *sek-1*(*km4*) and found that they had lower survival rates than control worms when exposed to BT679 (Figures 6 B and S3 B), but no difference in survival rate compared to controls when exposed to BT247 (Figures 6 C and S3 C). When analysing the genetic interaction of the p38 MAPK pathway with *elt-2*, we found that *pmk-1*(*km25*) and *sek-1*(*km4*) worms with *elt-2*(RNAi) have an even lower survival rate than the p38 MAPK mutants and the *elt-2*(RNAi) worms alone, suggesting that both components of the response against BT679 act in parallel (Figures 6 B and S3 B). It remains unknown, which cues lead to the activation of the ELT-2 and p38 MAPK transcriptional programs during BT679 infection. In contrast, we found no indication of genetic interaction between the p38 MAPK and *elt-2* in the context of BT247 infection (Figures 6 C and S3 C).

The involvement of the p38 MAPK in response to BT679 and not BT247 provides further evidence of the pathogen strain-specific response of *C. elegans*. This was not the case for the ILR/DAF-2 pathway, as we found that its terminal TF *daf-16* promotes resistance to BT679 in synergistic interaction with *elt-2* (Figure 7 B) and promotes resistance to BT247 in antagonistic interaction with *elt-2* (Figure 7 E). Our results for the *daf-16*(*mgDf50*) and *daf-2*(*e1370*) mutants do not fully agree with our previously published results (Hasshoff et al., 2007a; Wang et al., 2012a). Differences in the survival phenotype of worms depending on the bacterial food source have been observed by us in the past. For example, in the *daf-16*(*mgDf47*);*daf-2*(*e1370*) double mutant when reared either on *E. coli* HT115 or *E. coli* OP50 (Figure S5 A compared to Figure S5 B). This has been reported to be

due, at least in specific cases, to the nutritional quality of the bacteria and the media used (Clark and Hodgkin, 2014; Revtovich et al., 2017).

The BT side: What is different between BT247 and BT679?

We next asked which factors on the pathogen side elicit the specific *C. elegans* response to BT247 and BT679. BT247 and BT679 differ in virulence and infection dynamics (Figure 1). BT679 spores are more efficient at accumulating in the intestine, producing epithelial damage, germinating and killing the host than BT247 (Figure 1). The differences in cytopathology could be due to variation in virulence or in the virulence factors produced by both strains. However, we also demonstrated that the general variation in virulence levels between strains is not responsible for the difference in the *elt-2*-mediated responses (Figure 4 A).

BT247 and BT679 produce PFTs, which are very different in their amino acid sequence and likely in their mode of action. BT679 produces three-domain (3d) PFTs Cry21Aa3 and Cry14Aa2, while BT247 only produces the unique domain PFT Cry6Ba (Hollensteiner et al., 2017a; Masri et al., 2015). The case of BT679 is similar to other pathogens in that ELT-2 is essential for host defence. We studied the isolated effects of the BT679 Cry PFTs by expressing the toxins individually in a BT679 Cry- genetic background (Masri et al., 2015). We could show that ELT-2 is mainly conferring resistance to only one toxin, the 3d PFT Cry21Aa3 (Figure 5 B). The second 3d PFT in BT679, Cry14Aa2, elicits responses mediated by the p38 MAPK pathway independent of *elt-2* (Figures 6 D). So far, our attempts to study the individual effect of the BT247 PFT Cry6Ba by either purification or heterologous expression were not successful.

Although it is widely accepted that the general mode of action of Cry PFTs consists of epithelial intestinal cell lysis by pore formation (Bravo et al., 2007), the host cellular responses to different Cry PFTs can vary greatly. For example, the *C. elegans bre* mutants, which are resistant to the 3d PFT Cry5Ba, are susceptible to the unique-domain PFT Cry6Aa (Marroquin et al., 2000). Conversely, the necrosis pathway mutant *asp-1*(*tm666*), which is resistant to Cry6Aa, is susceptible to Cry5Ba (Zhang et al., 2016), showing already toxin-specific responses in the host. Moreover, the hypoxia terminal TF *hif-1* was previously found to be more important for the intrinsic cellular defence against Cry5B than Cry21A (Bellier et al., 2009). In this work, we showed that *C. elegans bre* and *asp-1* mutants, resistant to Cry5Ba and Cry6Aa, respectively, are susceptible to BT247 and BT679 infection (Figures 8 B and C). These findings highlight the value of studying the nematode's interaction with whole bacterial strains, which may express a range of different virulence factors, thereby shaping the interaction between these antagonists in nature.

Mechanisms of immune specificity: Resistance to BT679 vs tolerance to BT247

The classical understanding of immune function is based on an initial recognition of pathogens or altered-self, followed by signalling of the detected threat and activation of effector mechanisms to avoid (behavioural defence) or eliminate (physiological defence) the pathogens. Under this scenario, immune function leads to pathogen resistance, including reduction of pathogen load if not their complete removal to increase host fitness (Medzhitov et al., 2012). As an alternative, the response to pathogens may also lead to immune tolerance, in which hosts are able to maintain high fitness and health through different mechanisms (e.g., tissue repair and reduction of damage caused by the immune response alone) without affecting the pathogen load (Ferrandon, 2009; Medzhitov et al., 2012).

In the past, the immune role of ELT-2 has been linked to resistance, as *elt-2*(RNAi) normally leads to reduced health and fitness, as indicated by reduced survival rate upon infection (Figure 5 A) (Block et al., 2015; Kerry et al., 2006; Shapira et al., 2006). Active ELT-2 contributes to resistance by positively regulating the expression of putative immune effector genes like *clec-67* upon *S. enterica* infection (Kerry et al., 2006) or *lys-2* upon *P. aeruginosa* (Block et al., 2015; Shapira et al., 2006) and BT247 (Boehnisch et al., 2011) infection. Although we were not able to detect a higher bacterial load in *elt-2*(RNAi) worms exposed to BT679 (Figure 9 B), the concomitant reduction in survival under these conditions suggests that active *elt-2* mediates resistance to BT679 infection. However, we were so far not able to identify individual ELT-2-regulated effector molecules that are specifically required for resistance to BT679 (Figure S6). To find effector molecules against BT679 is something that could be further explored in the future, taking advantage of the generated RNA-Seq data.

Conversely, *elt-2*(RNAi) causes higher nematode survival upon BT247 infection, but also increased pathogen load according to CFU analysis. These observations strongly suggest that inactive ELT-2 causes tolerance towards this pathogen strain. Interestingly, the highest concentration with BT247 causes some mortality and tissue damage to *elt-2*(RNAi) worms, as indicated by TEM analysis (Figure 9 G and M), the IFB-2::CFP intestinal tissue integrity fluorescent reporter (Figure 10), and the intestinal leaking smurf assay (Figure S 7). Although BT247 pathogenesis is clearly reduced in *elt-2*(RNAi) worms, it is not completely abolished, and the worms appear to be able to cope with the infection (rather than resist it), possibly through activation of detoxification programs (Figures 14 C and 15). Detoxification processes have recently received more attention in the *C. elegans* immunity field for their possible role in tolerance and recovery (Govindan et al., 2015; Head and Aballay, 2014; van der Hoeven et al., 2011; Pukkila-Worley et al., 2014).

Underlying mechanisms of ELT-2-dependent strain-specific response

Taken together, our data support a central role of ELT-2 in mounting highly specific and distinct *C. elegans* defence responses to infection by the pathogenic BT strains BT679 (likely immune resistance) and BT247 (likely immune tolerance). What are the molecular processes that ELT-2 controls to produce these contrasting responses? ELT-2 regulates the transcription of a major portion of genes expressed in the intestine, which in turn are involved in all intestinal functions. Intuitively, this apparently leaves little opportunity for ELT-2 to be involved in specific responses. However, such specificity may be achieved through interaction with other TFs and cofactors (Block and Shapira, 2015; McGhee et al., 2009b; Yang et al., 2016c). Our results provide support to this hypothesis. We focused our analysis of possible ELT-2 interactions with other TFs known to be important for the worm's response to infection (*daf-16*, *skn-1*, *pqm-1*, *zip-2*, *hif-1*), or whose targets overlapped with the strain-specific responses observed in our first RNA-Seq experiment (*nhrs* and *sbp-1*). Some of the tested TFs indeed contributed to the response against BT679 (*skn-1* and *daf-16*) (Figures 6 B and 7 B) or BT247 (*zip-2*, *nhr-99*, *nhr-193* and *daf-16*). For BT679 we found that the TF gene *skn-1* (possibly downstream of p38 MAPK signalling) contributes to resistance in cooperation with *elt-2* (Figure 11 B), consistent with a similar previous finding for the defence response against *P. aeruginosa* (Block et al., 2015). For BT247, the knockout of the FOXO/*daf-16*, *nhr-99*, *nhr-193* and the bZIP *zip-2* TF genes caused decreased survival rate of the *elt-2*(RNAi) worms (Figures 7 E; 11 C; and 13 C), suggesting this time an antagonistic interaction between the respective TFs. Overall, these findings suggest that environmental challenges such as pathogen infection is met by *C. elegans* by the coordinated action/interaction of several TFs, either in cooperation or in antagonizing form. Our results point to the *elt-2* gene as a master regulator or at least a highly connected nod in the complex network of TF interactions.

We further sought to clarify the molecular mechanisms behind immune specificity to BT247 and BT679 by performing a second RNA-Seq experiment, this time using *elt-2*(RNAi) worms (Figure 14). When comparing the first infection RNA-Seq dataset with the second, *elt-2*(RNAi) dataset, we saw that clusters 2 and 8 did no longer produce strain-specific DE patterns (Figure 14 A). Thus, this result confirms the particular role of *elt-2* in mediating the observed strain-specific transcriptomic response. When analysing the expression pattern of the strain-specific-response genes in cluster 2 in the *elt-2*(RNAi) RNA-Seq experiment, we observed that their expression changes in *elt-2*(RNAi) worms on BT247 in the same way as in *elt-2*(RNAi) worms on BT679 (they become part of the general response to BT). This is surprising, because the survival rates of *elt-2*(RNAi) worms on these two pathogen strains are opposite. This incongruence between transcriptomics and survival rate phenotype, is an example that highlights the complications of using transcriptomic DE profiles to explain and analyse other phenotypes, like survival

rate. Additionally, it points to how a given transcriptional response pattern can lead to very different fitness outcomes depending on the environment faced, what justifies why transcriptional responses would evolve to be tightly regulated, context-specific and display genotype-environment interactions (Grishkevich et al., 2012). We further observed that in our *elt-2*(RNAi) RNA-Seq experiment the DE pattern of cluster 2 from the initial infection RNA-Seq experiment did not appear in empty vector worms, without *elt-2*(RNAi). We attribute this discrepancy between RNA-Seq experiments to the bacteria on which the worms were grown before pathogen exposure. In the infection RNA-Seq experiment worms were grown on *E. coli* OP50 and in the *elt-2*(RNAi) RNA-Seq experiment worms were grown on *E. coli* HT115, which is commonly used for RNAi treatment in *C. elegans* (Kamath and Ahringer, 2003). It is known that the differences in the nutritional values of these bacteria can alter the interactions with pathogens and the outcome of phenotype measurement in genetic studies (Figure S5) (Revtovich et al., 2017).

Interestingly, gene expression comparison of *elt-2*(RNAi) worms on the non-pathogenic BT407 and exposed to BT247 yielded very few differences (Figure 14 A), suggesting that *elt-2*(RNAi) alone already triggers a defence response similar to that triggered by BT247 infection. Such a defensive response caused by disruption of major homeostasis regulators has previously been described in the literature (Melo and Ruvkun, 2012) and strongly supports the activation of defensive immune responses by danger or damage signals instead of direct pathogen recognition. Alternatively, since *elt-2*(RNAi) worms display higher survival rates, the lack of DE genes in the comparison of *elt-2*(RNAi) worms on BT247 and BT407 could also be interpreted as a maladaptation, or lack of adaptation, of worms in their transcriptional response to BT247. Apparent maladaptation could happen when the adaptation to have higher survival rates upon exposure to BT247 leads to a trade-off with other life history traits, e.g. reduction of fitness. Lack of adaptation of worms in their transcriptional response to BT247 can happen if the worms have not coexisted long enough with this pathogen strain to evolve an adaptive response. We cannot, as yet, exclude a third explanation, which involves the possibility of pathogen manipulation of the host's immune system. Such manipulation was shown in *C. elegans* by, for example, the pathogen *B. pseudomallei* (Lee et al., 2013), which degrades the host's ELT-2 to impair the response to infection.

We hypothesized that the worms could increase their survival rate either because of the absence of factors that are detrimental under regular infection conditions (these would be found in the list of downregulated genes in the comparison *elt-2*(RNAi) worms vs. empty vector worms exposed to BT247) or because of the new presence of normally downregulated factors that increase survival upon infection (found in the group of upregulated genes of the above-mentioned comparison). The interaction of *C. elegans* and BT247 and its mediation by ELT-2 remains incipiently understood, although its cooperation with the ILR/DAF-2 pathway was

shown here (Figure 7 E). It was previously reported that both BT679 and BT247 are able to induce nuclear translocation and activation of the FOXO/DAF-16 TF (Wang et al., 2012b). We thus, focused our search for potential *elt-2* downstream targets that could be responsible for the increased survival rate phenotype of *elt-2*(RNAi) worms to BT247 on loci under the transcriptional control of DAF-16. Through a small-scale screen of 70 *elt-2* downregulated genes, we found that the transmembrane cadmium-responsive gene *cdr-2*, the trehalase *tre-3*, the short-chain dehydrogenases/reductase *dhs-30*, and the PON (paraoxonase) and MEC-6-Like protein-encoding gene *poml-3* contribute to the enhanced resistance of *elt-2*(RNAi) worms to BT247 (Figure 15). Although their exact contribution is not entirely clear, yet their protein-domain content suggests that these genes could work in lipid degradation or detoxification. Both of these functions could contribute to tolerance to this pathogen, which attacks through the action of the unique domain PFT Cry6Ba. In the future, studies with the purified toxin could confirm if the increased survival rate of *elt-2*(RNAi) happens exclusively because of the action of this toxin. If so, it would be very interesting to study the exact mechanism of action of this toxin, as it could be different from the other previously characterized Cry PFTs.

This work provides experimental support of a role of ELT-2 in generating pathogen-strain immune specificity and its coordinated action with different transcription factors (Yang et al., 2016c). It also provides further evidence of the role of ELT-2 during intestinal infection in cooperation with SKN-1 and the p38 MAPK pathway (Block et al., 2015). The exact underlying molecular processes that mediate tolerance are still not entirely clear. They are likely based on the interaction of ELT-2 with other TFs or regulators, such as SKN-1, ZIP-2, NHR-99, NHR-193, p38 MAPK and ILR/DAF-2. Tolerance itself may be mediated through detoxification or changes in lipid metabolism. Our work establishes *C. elegans* as a model for dissecting strain-specific immune defence that can be explored in the future to assess in more detail the involved molecular processes.

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EXPERIMENTAL PROCEDURES

C. elegans and bacterial strains

Worm strains were maintained in the lab at 20°C and fed with *E. coli* OP50 as described in (Stiernagle, 2006). Mutant strains used were: CB1370 (*daf-2*(e1370)III.), GR1307 (*daf-16*(mgDf50)I.), GR1309 (*daf-16*(mgDf47) I; *daf-2*(e1370) III.), KU25 (*pmk-1*(km25)IV.), KU4 (*sek-1*(km4)X.), VC1184 (*jun-1*(gk551)II.), DH26 (*fer-15*(b26)II.), GC833 (*glp-1*(ar202)III.), DG2389 (*glp-1*(bn18)III.), AE501 (*nhr-8*(ok186)IV.), VC1629 (*nhr-99*(gk758)V.), FX04479 (*nhr-193*(tm4479)V.), ZG31 (*hif-1*(ia4)V.), FX04248 (*zip-2*(tm4248)III.) and FX00666 (*asp-1*(tm666)V.). Strain BJ49 (IFB-2::CFP) (Hüsken et al., 2008) was used to assess intestinal barrier integrity. Worm strains included in the small-scale suppressor screen are listed in Table S1. All worm strains were obtained from the Caenorhabditis Genetics Center (CGC) or the Japanese National BioResource Project (NBRP), except otherwise stated in the acknowledgements section. The two pathogenic BT strains MYBT18247 (BT247) and MYBT18679 (BT679) are available in the Schulenburg laboratory and were isolated from a single colony derived from strains NRRL-B18247 and NRRL-B18679 obtained from the Agriculture Research Service Patent Culture Collection of Microorganisms and Cell Cultures (United States Department of Agriculture, Peoria, Illinois, USA). The non-pathogenic strain BT407 was obtained from Christina Nielsen-LeRoux (INRA, France). BT spore solutions were obtained by culturing BT in liquid BT medium with high salt concentration for seven days to induce sporulation and subsequently harvested and stored, as previously described (Hasshoff et al., 2007a; Masri et al., 2015; Schulte et al., 2010).

BT survival assays and gene knockdown

To assess survival of wild type (N2) and mutant strains, worm populations were synchronized and raised on 9 cm diameter Nematode Growth Medium (NGM) plates seeded with 700 µl of an overnight liquid culture of *E. coli* OP50 in LB medium. When worms reached L4 larval stage they were put in 6 cm diameter peptone-free NGM (PFM) inoculated with 100 µl of different concentrations of pathogenic BT247 and BT679 spore solutions mixed with *E. coli* OP50 adjusted to an optical density of 5 with PBS buffer. Non-nematocidal BT strain BT407 Cry- (BT407) lacks pore-forming toxins and is used as infection treatment control only at the highest concentration in which pathogens were tested (Sheppard et al., 2013). Survival was scored 24h p.i. Approximately 30 worms were exposed per plate. For *elt-2* knockdown by feeding a clone of *E. coli* HT115 expressing double stranded *elt-2* stored in position X-5I11 from the publically available Source Bioscience Ahringer RNAi library was used (Kamath and Ahringer, 2003). Additionally, knockdown of *skn-1* (IV-2N18), *sbp-1* (III-6C01) and *pqm-1* (II-7N05), using clones from the same library, was performed. Eggs were synchronized

overnight in M9 buffer until hatched and 300 L1 larvae were put on 6 cm plates seeded with 100 µl of concentrated RNAi bacteria for 48 hours. As treatment control we used *E. coli* HT115 transformed with the empty vector. Survival assessment on BT lawn was done as described before. ELT-2 is a major regulator of intestinal development. To verify that the changes in the survival phenotype of *elt-2*(RNAi) worms exposed to pathogenic BT were not exclusively due to an abnormal development we performed *elt-2* knockdown starting at the L4 larval stage for 48 hours. *elt-2*(RNAi) adult worms were exposed to BT and survival was scored as described before. The assessment of resistance to BT of glycosylation-deficient mutants and the small-scale suppressor screen were performed in the same setup as the survival assays but resistance or susceptibility were score in a binary fashion (resistant/susceptible) after short inspection of worms on the plates. Resistance was scored when >75% of the worms on plates survived. Treatment combinations of the different experiments were evaluated in parallel and observer bias was prevented by coding of samples. Analysis of survival experiments was done using R (R Development Core Team, 2011).
The model fitted was glm(formula = cbind(Worms Alive, Worms Dead) ~ worm treatment and/or worm strain*BT concentration, family = binomial). Statistical test applied was a multiple comparison of means: Tukey contrasts and p-values were Bonferroni corrected.

BT exposure, RNA extractions and differential expression analysis

elt-2 knockdown worms treated at the L1 larval stage were exposed to BT as described before in 9 cm diameter PFM plates inoculated with 250 µl of BT spore solution diluted with *E. coli* OP50 OD5 at 600 nm. After 2h, 6h, 12h and 24h p.i worms were washed off the plates using PBS supplemented with Tween-20 to a final concentration of 0,3%. Worms were centrifuged and washed in M9 buffer 3 times and then transferred to QIAzol lysis Reagent™ (QIAGEN). Worms were cyclically thawed and frozen 4 times using liquid nitrogen and a heating block at 37°C to allow the QIAzol reagent to enter the cells and better preserve the RNA. RNA was extracted using the NucleoSpin® RNA purification kit (Macherey-Nagel) and stored at -80°C. Messenger RNA was sequenced using Illumina HiSeq technology with a coverage of 20 million reads per sample where only 5% of reads were discarded by quality. Bioinformatics analyses done used the following software: Alignment of reads: Tophat2 (Ghosh and Chan, 2016) (https://ccb.jhu.edu/software/tophat/index.shtml), differential expression analysis: Cuffdiff (Ghosh and Chan, 2016) (http://cole-trapnell-lab.github.io/cufflinks/cuffdiff/) and ABSSeq (Yang et al., 2016d), clustering: Cluster3.0 (de Hoon et al., 2004) (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm). Promoter region motif enrichment analysis was done with AMD (Shi et al., 2011), EASE analysis: EASE (Hosack et al., 2003) and WormExp (Yang et al., 2016b) (http://wormexp.zoologie.uni-kiel.de/wormexp/).

Bacterial load assessment by CFU counts

Bacterial load was estimated by exposing the worms as described for the survival assays and after 12 hours of bacterial exposure five worms were picked to 50μl M9+ 25 mM Tetramisole (TM buffer) and washed two times with TM buffer + 100μl/ml Gentamicin and two additional times with M9 buffer. After washing worms were resuspended in 150μl PBS+1% Triton X-100 and ground using a SPEX SamplePrep 2000 GenoGrinder Tissue Homogenizer at 1200 strokes/min for 3 minutes. Serial dilutions of worm lysate were done in PBS and the dilutions 1:100 and 1:1000 were plated on LB agar in triplicate for colony counting. The buffer from the last wash was also plated in triplicate to assess washing efficiency. Five worms from each treatment were also mounted onto microscope slides and their length was measured using a Leica M165 FC Fluorescence Stereomicroscope and the LAS software. Worm length was used to normalize the bacterial load per mm of worm as *elt-2*(RNAi) and empty vector worms have different sizes.

Fluorescence, Light Microscopy and TEM

For fluorescence microscopy *elt-2*(RNAi) worms were exposed to BT in 6 cm diameter plates and at each time point 10 worms were transferred to a microscope slide with an agar pad, immobilized with 50 mM Sodium Azide and observed with a Carl Zeiss HXP 120 C inverted fluorescence microscope or the confocal Carl Zeiss LSM 700. For light microscopy samples were observed under a Carl Zeiss HXP 120 C inverted fluorescence microscope equipped with a colour camera. For TEM worms were exposed to BT as described for RNA extractions and washed off plates 12 hours post-infection directly with fixation solution (2.5% glutaraldehyde, 2% paraformaldehyde, 0,1 M cacodylate buffer pH 7,2). Worms were cut in half to ensure penetration of the fixation solution and prepared as described in (Hall et al., 2012) for visualization with the Tecnai 2 electron microscope.

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SUPPLEMENTARY MATERIAL

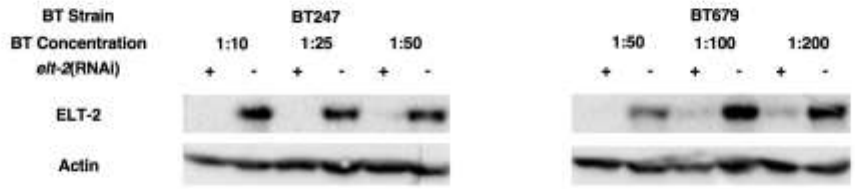

Figure S1. *elt-2*(RNAi) efficiently depletes ELT-2 protein levels in worms exposed to BT247 and BT679. ELT-2 and Actin protein detection upon *elt-2* knockdown and exposure to the pathogens BT247 and BT679. Worms were exposed to the different pathogen concentrations and total protein was extracted after 6 hours of infection.

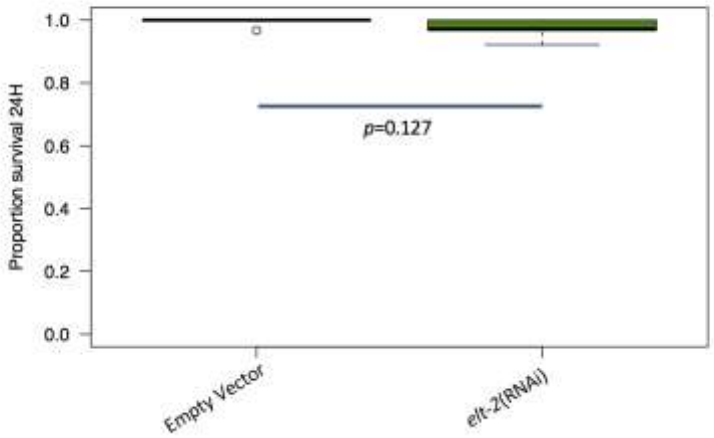

Figure S2. *elt-2*(RNAi) does not affect worm survival rate on the non-pathogenic strain BT407 after 24 hours of exposure compared to empty vector. Proportion survival of empty vector and *elt-2*(RNAi) worms after 24h of exposure to non-pathogenic BT407. Boxplots show proportion of surviving worms of each worm strain and treatment combination. N=5 plates with 30 worms each. Results are representative of at least 30 independent experiments. GLM of the binomial family was performed followed by a Tukey Test (see experimental procedures section), where mutant or knockdown worm strains were compared to control strains. p-value is Bonferroni adjusted.

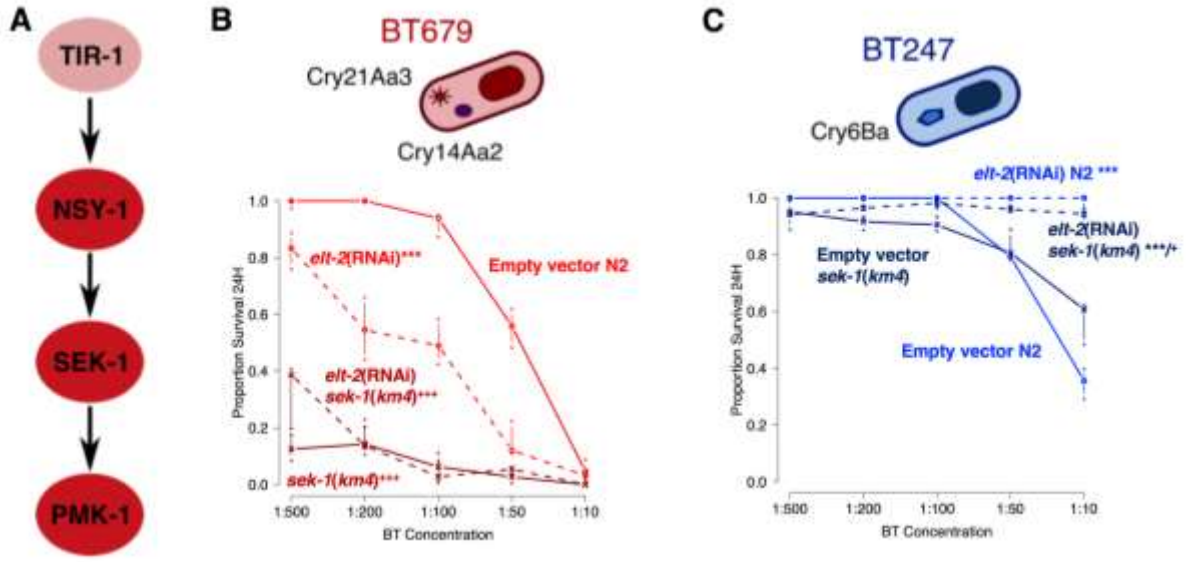

Figure S3. *sek-1*(*km4*) mutants are more susceptible to BT679 than *elt-2*(RNAi) worms but have the same survival rate as control worms on BT247. Survival of *sek-1*(*km4*) mutants from the p38 MAPK pathway (A) exposed to (B) BT679 and (C) BT247. Worms were exposed to the different pathogen concentrations and survival was assessed after 24 hours. Each curve represents the proportion of surviving worms of each worm strain and treatment combination. Median and range are shown, N=5 plates with 30 worms each. Statistics as in figure 3. *** shows p-value < 0.001 comparing RNAi to empty vector, $^{+++}$ shows p-value < 0.001 comparing mutant to N2, Bonferroni adjusted. On BT679 *elt-2*(RNAi) in the *sek-1*(*km4*) background is not significantly different from *sek-1*(*km4*) Empty vector. Results are representative of at least 3 independent runs.

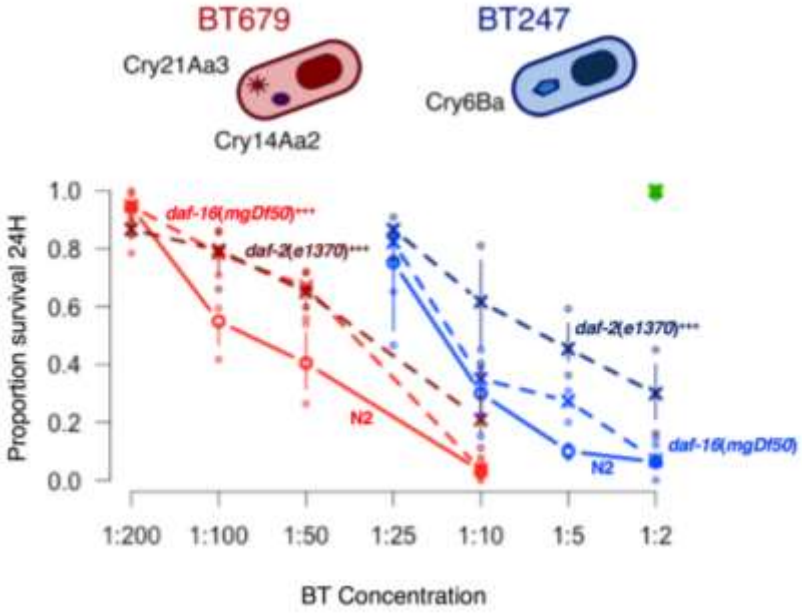

Figure S4. *daf-2*(*e1370*) mutants reared on *E. coli* OP50 are more resistant than N2 on both BT247 and BT679. Survival of *daf-16*(*mgDf50*) and *daf-2*(*e1370*) mutants exposed to BT247 and BT679. Worms were grown on *E. coli* OP50 and exposed to the different pathogen concentrations and survival was assessed after 24 hours. Each curve

represents the proportion of surviving worms of each worm strain and treatment combination. Median and Range are shown, N=5 plates with 30 worms each. Statistics as in figure 3. $^{+++}$ shows p-value < 0.001 comparing mutant to N2, Bonferroni adjusted. Green dots represent survival rate of worms exposed to BT407. Results are representative of 2 independent runs.

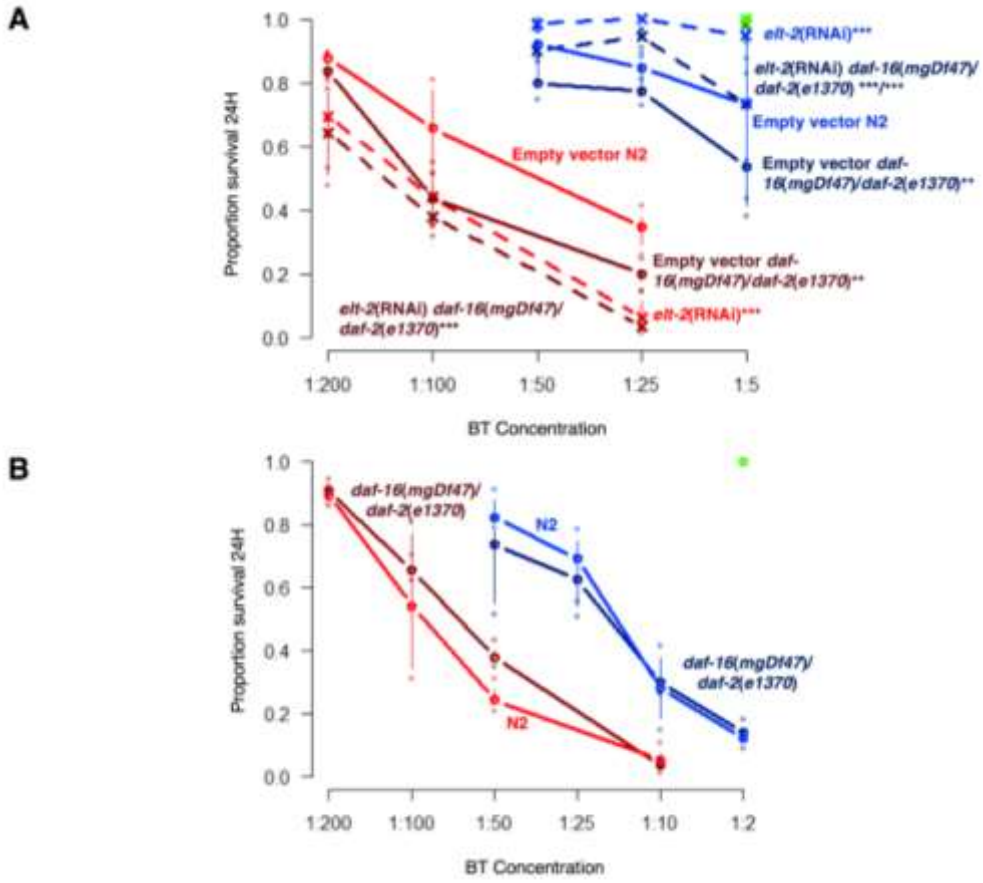

Figure S5. *daf-2*(*e1370*) mutant increase in survival rate on BT is *daf-16*-dependent. Survival of *daf-2*(e1370)/*daf-16*(mgDf50) double mutants exposed to BT247 and BT679 when grown on (A) *E. coli* HT115 with either *elt-2*(RNAi) or empty vector, or (B) *E. coli* OP50 before pathogen exposure. Worms were exposed to the different pathogen concentrations and survival was assessed after 24 hours. Each curve represents the proportion of surviving worms of each worm strain and treatment combination. Green points represent survival rate on the non-pathogenic control BT407. Median and range are shown, N=5 plates with 30 worms each. Statistics as in figure 3. $^{+++}$ shows p-value < 0.001, $^{++}$ represents p-value < 0.01. Plus sign represents comparison of mutant to N2, asterisk represents comparison of *elt-2*(RNAi) to empty vector. p-values are Bonferroni adjusted.

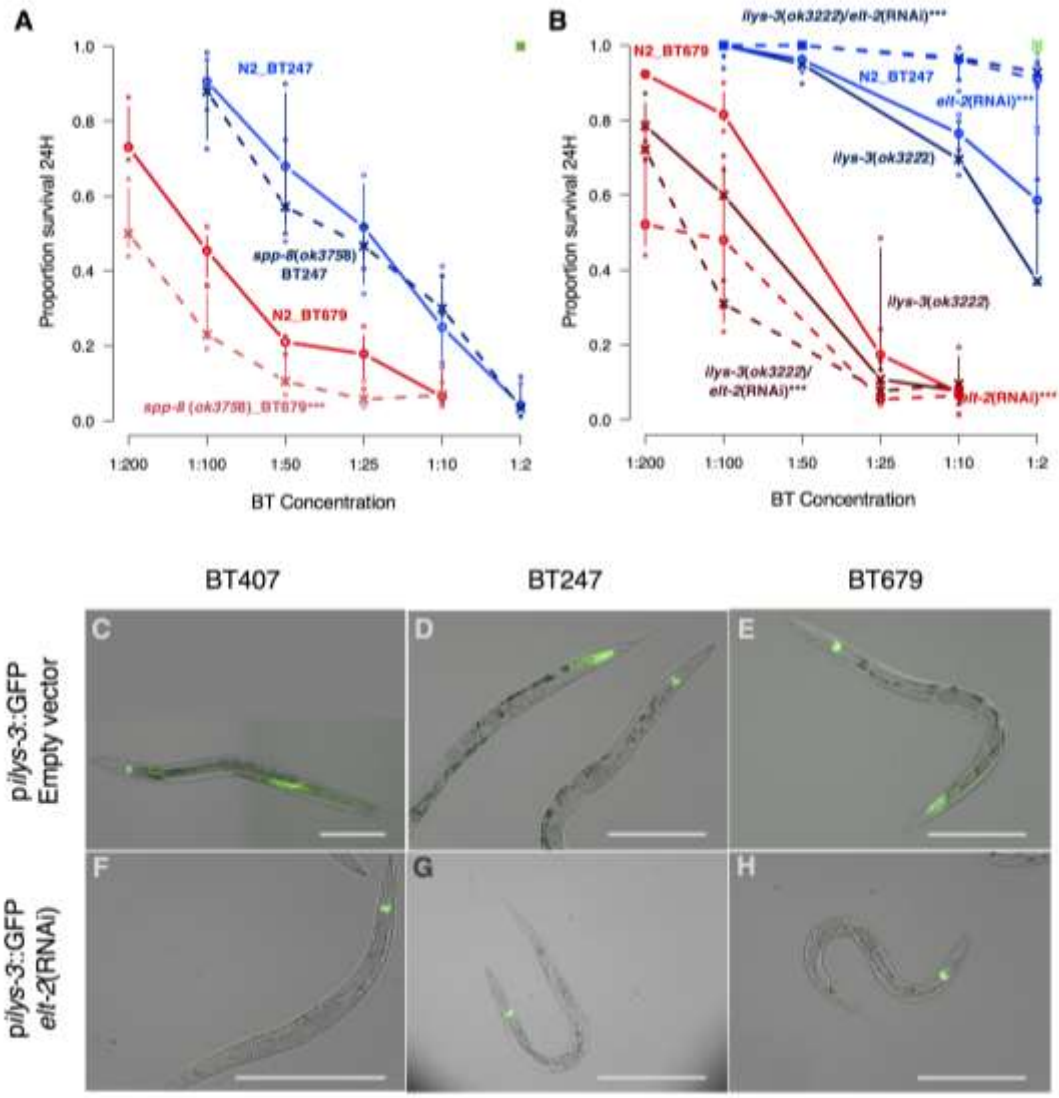

Figure S6. The mutant *spp-8*(*ok3758*) has reduced survival rate upon BT679 infection but has the same survival as wild type on BT247. *ilys-3*(*ok3222*) mutants have the same survival rate as controls upon exposure with both pathogen strains, its expression in the *C. elegans* intestine is regulated by *elt-2*. Survival of (A) *spp-8*(*ok3758*) and (B) *ilys-3*(*okk322*) and *ilys-3*(*okk322*)/*elt-2*(RNAi) on BT247 (blue) and BT679 (red). Worms were exposed to the different pathogen concentrations and survival was assessed after 24 hours. Each curve represents the proportion of surviving worms of each worm strain and treatment combination. Green points represent proportion survival on the non-pathogenic control BT407. Median and Range are shown, N=5 plates with 30 worms each. Statistics as in figure 3. $^{+++/***}$ shows p-value < 0.001, Plus sign represents comparison of mutant to N2, asterisk represents comparison of *elt-2*(RNAi) to empty vector. p-values are Bonferroni adjusted. (C-H) Overlap of DIC and GFP pictures of reporter strain CB7163 p*ilys-3*::GFP with indicated treatment combinations. Scale bar represents 250 μm.

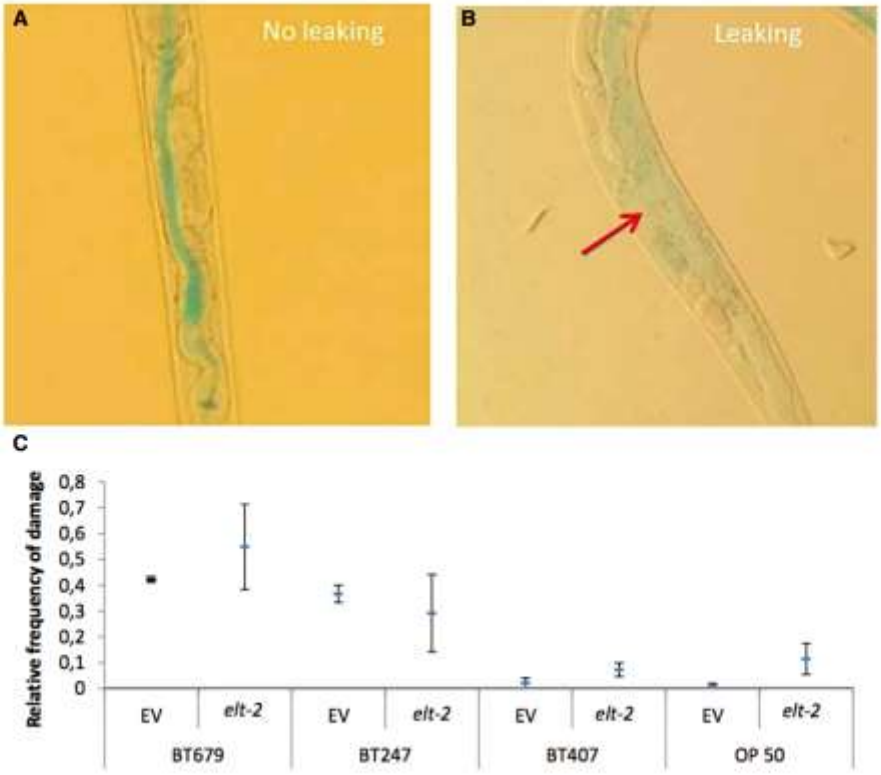

Figure S7. *elt-2*(RNAi) worms exposed to BT247 display little intestinal leaking, comparable with non-infected controls. Smurf worm assay to assess intestinal epithelium integrity show in (A) a non-leaking intestine (integral), (B) a leaking intestine (loss of integrity or damage) and (C) the relative frequency of intestinal damage of worms exposed to BT679, BT247 and bacterial controls. Worms were exposed to the different bacterial treatments for 24 hours and then for 3 hours to *E. coli* OP50 with blue food dye. Red arrow in (B) points to intestinal damage. Damage was scored as a binary observation (present/absent) of blue food dye in the body cavity. 30 worms per treatment were observed and the relative frequency was calculated as the number of worms presenting damage over the total number of worms per treatment.

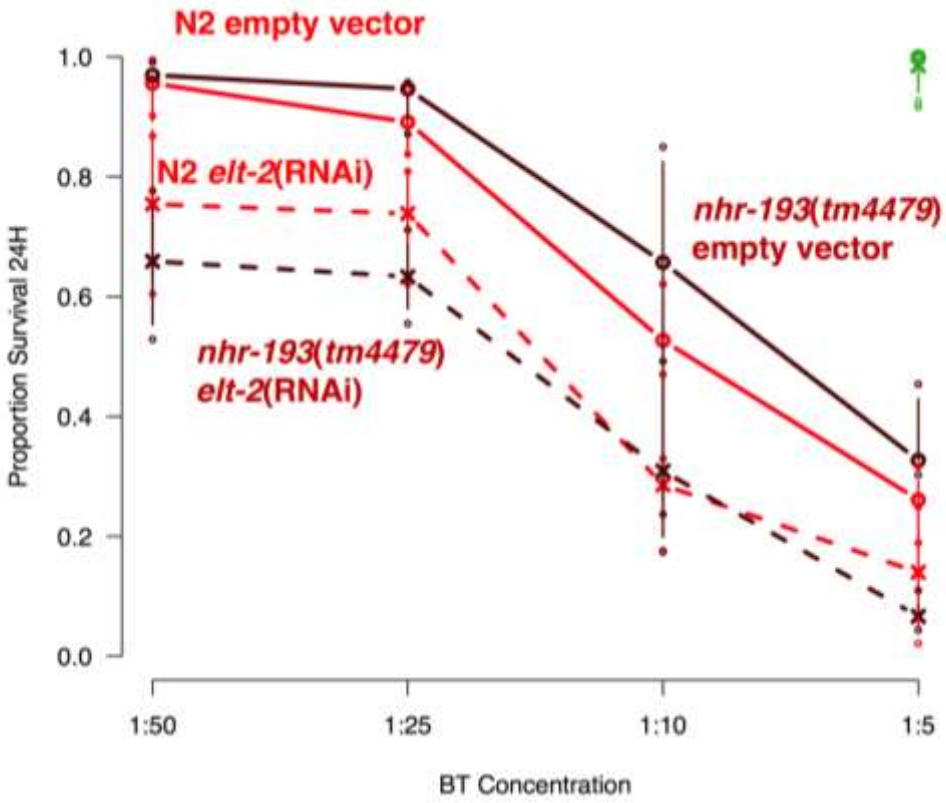

Figure S8. *nhr-193*(*tm4479*) mutants display no difference in survival rate compared to N2 upon exposure to BT679 either alone or in interaction with *elt-2*. Survival of *nhr-193*(*tm4479*) mutants exposed to BT679. Worms were exposed to the different pathogen concentrations and survival was assessed after 24 hours. Each curve represents the proportion of surviving worms of each worm strain and treatment combination. Median and Range are shown, N=5 plates with 30 worms each. Green dots represent survival of worms exposed to BT407.

#	Strain	Genotype	# (cont…)	Strain (cont…)	Genotype (cont…)
1	VC40032	K03A11.5(*gk960736*)	36	VC40260	*cyp-33C4*(*gk541234*)
2	VC40265	*vap-2*(*gk544863*)	37	RB732	*cpz-1*(*ok497*)
3	VC20763	*fbxc-7*(*gk963066*)	38	VC40981	*tre-3*(*ok394*)
4	RB1791	*amt-1*(*ok2318*)	39	VC322	*cpl-1*(*ok360*)
5	VC40679	*skr-4*(*gk759439*)	40	VC1749	F55G11.8(*gk3130*)
6	VC40415	*xtr-1*(*gk626376*)	41	RB671	*fmo-1*(*ok405*)
7	VC30103	T20D4.9(*gk414026*)	42	VC40112	*nlp-25*(*gk468461*)
8	VC40101	*kin-16*(*gk462323*)	43	VC20076	Y41C4A.11(*gk186803*)
9	VC3025	*abhd-5.1*(*ok3722*)	44	VC40229	H06H21.8(*gk524709*)
10	VC20025	ZC196.2(*gk242000*)	45	RB2169	*hpo-34*(*ok2936*)
11	RB1966	*pck-2*(*ok2586*)	46	VC20215	C05D12.3(*gk315446*)
12	RB1634	F32A5.4(*ok2011*)	47	FX01154	C44F1.3(*tm1154*)
13	VC40602	*cyp-25A2*(*gk715215*)	48	FX04124	R09D1.12(*tm4124*)
14	RB1623	*cdr-2*(*ok1996*)	49	FX01992	T14F9.3(*tm1992*)
15	RB1493	*nkb-3*(*ok1758*)	50	FX02004	T04H1.9(*tm2004*)
16	VC40816	Y6E2A.5(*gk828033*)	51	FX05127	W02H5.8(*tm5127*)
17	VC3130	*poml-3*(*ok3772*)	52	FX06243	K05F6.5(*tm6243*)
18	VC40618	CC8.2(*gk725534*)	53	FX01801	T24H7.5a(*tm1801*)
19	VC40318	*fbxa-54*(*gk570814*)	54	FX03154	*tat-4*(*tm3154*)
20	VC40517	F15B9.6(*gk675436*)	55	FX03013	*nas-5*(*tm3013*)
21	VC1147	*far-3*(*ok313*)	56	FX02318	*hsp-70*(*tm2318*)
22	VC40905	F47B8.2(*gk875363*)	57	FX06889	*dhs-30*(*tm6889*)
23	VC40041	*cutl-24*(*gk191483*)	58	FX04867	*gnrr-2*(*tm4867*)
24	VC30074	*tth-1*(*gk409144*)	59	FX05081	F09C8.1(*tm5081*)
25	VC30174	F46H5.7(*gk951450*)	60	FX05013	F52E1.7(*tm5013*)
26	VC30038	*cyp-29A2*(*gk247016*)	61	FX00981	C24G7.2(*tm981*)
27	VC40645	M04C3.1(*gk741853*)	62	FX06436	Y32F6A.4(*tm6436*)
28	VC1003	*vha-3*(*ok1501*)	63	FX06957	*tba-4*(*tm6957*)
29	VC40793	T28B4.4(*gk815609*)	64	FX04627	*haao-1*(*tm4627*)
30	RB1868	*hum-8*(*ok2414*)	65	FX06273	F58G6.7(*tm6273*)
31	WU209	*cdf-1*(*n2527*)	66	FX14770	*ppfr-1*(*tm2180*)/hT2
32	VC40835	*pcp-4*(*gk961685*)	67	FX03171	F39B1.1(*tm3171*)
33	VC40756	*ugt-47*(*gk796710*)	68	FX01743	F55A11.3(*tm1743*)
34	VC20306	Y39B6A.1(*gk960053*)	69	FX03267	*gale-1*(*tm3267*)/+
35	VC40563	*clec-204*(*gk695507*)	70	FX01006	*nhr-63*(*tm1006*)/+

Table S1. List of knockout or change of function mutants of negatively-regulated *elt-2* downstream targets.

SUPPLEMENTARY METHODS

Western Blot

Worms were prepared and exposed to bacteria as described for survival assays except that it was done in 9 cm NGM plates and 1000 worms were put onto plates. After 6 hours of infection worms were washed for protein extraction using standard methods (Chan et al., 2015). Samples were probed with anti-ELT-2 monoclonal antibody provided by James McGhee, University of Calgary, AB, Canada, together with goat anti-mouse-IgG (H+L) HorseRadish Peroxidase (HRP) conjugate. Samples were visualized using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific product #34080).

Intestinal Integrity Assay (Smurf)

For the smurf worm protocol *C. elegans* Bristol strain N2 was used to assess intestinal damage upon infection. In this approach the nematodes were placed in a liquid *E. coli* OP50 culture, stained with blue food dye (Spectrum FD&C Blue #1 PD110; 0.05g/ml) for 3 hours after infection (Gelino et al., 2016). The blue food dye was visible within the transparent nematodes and leaking due to intestinal damage into the body cavity.

Chapter 2

High innate immune specificity through diversified C-type lectin-like domain proteins in invertebrates

Review

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Review

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High Innate Immune Specificity through Diversified C-Type Lectin-Like Domain Proteins in Invertebrates

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Key Words
C-type lectin-like domain proteins · Immune specificity · Innate immunity · Insects · Crustaceans · *Caenorhabditis elegans*

Abstract
A key question in current immunity research is how the innate immune system can generate high levels of specificity. Evidence is accumulating that invertebrates, which exclusively rely on innate defense mechanisms, can differentiate between pathogens on the species and even strain level. In this review, we identify and discuss the particular potential of C-type lectin-like domain (CTLD) proteins to generate high immune specificity. Whilst several CTLD proteins are known to act as pattern recognition receptors in the vertebrate innate immune system, the exact role of CTLD proteins in invertebrate immunity is much less understood. We show that CTLD genes are highly abundant in most metazoan genomes and summarize the current state of knowledge on CTLD protein function in insect, crustacean and nematode immune systems. We then demonstrate extreme CTLD gene diversification in the genomes of *Caenorhabditis* nematodes and provide an update of data from CTLD gene function studies in *C. elegans*, which indicate that the diversity of CTLD genes could contribute to immune specificity. In spite of recent achievements, the exact functions of the diversified invertebrate CTLD genes are still largely unknown. Our review therefore specifically discusses promising research approaches to rectify this knowledge gap.

Introduction

Animals depend on the specificity of their immune reaction to efficiently and economically protect themselves against pathogens. For a long time, the generation of a highly specific immune response was considered a hallmark of adaptive immunity, based on its immense diversity of T and B cell receptors generated by somatic recombination and hypermutation. There is, however, accumulating evidence that invertebrates, which solely rely on innate defense mechanisms and lack T and B cells, can differentiate between pathogens on the species and even strain level [1–3]. Whether and how such high levels of specificity can be generated by the innate immune system is one of the main questions in current immunity research.

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A prerequisite for the generation of a pathogen-specific immune reaction is a repertoire of diversified receptors, which enables the organism to specifically recognize and respond to different immune challenges. In vertebrates the well-characterized pattern recognition receptors (PRRs) of the innate system, such as the Toll-like receptors (TLRs), retinoic acid-inducible gene-I-like receptors (RLRs), and NOD-like receptors (NLRs), recognize structures that are conserved among microbial species and mediate a broader pattern of specificity (i.e. on the level of a particular pathogen group, such as Gram-negative or Gram-positive bacteria). The diversity of ligand binding can be expanded by the formation of receptor homo- or heterodimers and/or the interaction with other PRR coreceptors, as is well described for mammalian TLRs. However, in invertebrates, high levels of specificity (on the species and strain level) are unlikely mediated by TLRs, NLRs or RLRs, as these are too few in number in most, although not all (see below), animal species.

There are few examples of the diversification of alternative putative immune receptors in invertebrates. The hypervariable protein Down syndrome cell adhesion molecule (Dscam), which is generated by alternative somatic splicing, was shown to be implicated in the immune response of insects and crustaceans [4, 5], and suggested to act as a pathogen-specific recognition molecule. Further experimental evidence to support this hypothesis is, however, still scarce [6]. A high level of genetic polymorphism was observed in the immunoglobulin (Ig)-type variable (V) region-containing chitin-binding proteins (VCBPs) of the cephalochordate *Branchiostoma floridae* [7], and in the Ig domain of fibrinogen-related proteins (FREPs) of the snail *Biomphalaria glabrata* [8]. However, the molecular mechanisms underlying the diversification of VCBPs and FREPs and their functional significance for immune specificity are not well understood. Yet another example of diversification of putative innate immune system proteins is the Sp185/333 system in echinoderms, such as the purple sea urchin *Strongylocentrotus purpuratus*. The members of this highly diverse, novel gene family are not homologous to any other known genes, they are highly upregulated after immune challenge and expressed in the phagocyte class of sea urchin coelomocytes. The function of Sp185/333 proteins is unknown. The high diversification of Sp185/333 genes and proteins is achieved through different mechanisms, including maintenance of high polymorphism, gene recombination and modifications at the RNA and protein level [9]. Similar multigene expansions, usually based on repeated gene duplications that potentially produce high-sequence diversification, were observed for TLR and NLR gene families in the genomes of the sea urchin *S. purpuratus* [10] and the lancelet *B. floridae* [11], but the functional consequence of this diversified repertoire of potential receptors remains elusive. Here, we argue that another group of known, yet neglected, PRRs – the C-type lectins – show surprisingly high diversity in most metazoan genomes and have the potential to generate immune specificity.

C-type lectins are a family of both soluble and membrane-bound proteins with a characteristic carbohydrate-recognition domain (CRD) that mediates ligand binding. The CRD has a distinct protein fold determined by highly conserved residues that form a characteristic sequence motif [12]. It was first described in Ca^{2+}-dependent ('C-type') carbohydrate binding animal proteins ('lectins'), but has subsequently been found in many other proteins that do not bind sugar and/or calcium. Thus, we here use the term C-type lectin-like domain (CTLD) in its broadest sense, as proposed previously [13], and use sequence similarity to identify genes containing CTLDs in different metazoan genomes. CTLD proteins have a wide range of functions and bind a wide variety of ligands, such as sugars, proteins, lipids and inorganic compounds [13].

In vertebrates, CTLD proteins are known to fulfill important tasks in the immune system by acting as PRRs [for a review, see 14], as well as effector proteins with bactericidal activity [15]. As we will demonstrate further below, CTLD genes are highly abundant in many metazoan genomes. Yet, our knowledge on CTLD protein function in immunity mainly derives from the few well-studied vertebrate CTLD proteins, while the function of the majority of CTLD proteins is unknown. In invertebrates, it has repeatedly been suggested that the diversified CTLD proteins contribute to immunity, yet exact functional genetic information is largely absent and the few exceptions mainly focus on the assessment of single CTLD proteins. Moreover, the particular role of CTLD proteins in generating immune specificity is completely unexplored. Therefore, we require a systematic research program that aims at elucidating the role of diversified CTLD proteins in immune specificity.

Here, we highlight that CTLD genes constitute expanded gene families in many metazoan taxa and summarize the available evidence for a role of CTLD proteins in invertebrate immunity, mainly inferred from studies on crustaceans and insects. We then turn to the nematode genus *Caenorhabditis*, in which several complete genomes are available, to demonstrate extreme CTLD gene diversification, followed by a summary of data from *C. elegans* that strongly indicate a role of the diversified CTLD proteins in immune specificity. Finally, we empha-

Table 1. Number of CTLD genes (proteins) in different metazoan genomes

Species	Taxon	Genome size, Mb	Total coding genes	Total InterPro[1] genes (proteins), n	Total Pfam[1] genes (proteins), n	Immune function[2]	Ref.	Genome assembly
H. sapiens	Mammalia	3,209.29	20,805	100 (277)	94 (268)	e.g. DC-SIGN, DECTIN-1, DECTIN-2, MINCLE, Langerin (CD207), BDCA-2, MBL2, COLEC11, MRC-1, MRC-2, SELP, SELE, SELL, CLEC5A, CLEC5F8, DCIR, CLEC9A, DEC-205, and others	reviewed in 14, 61, 62, 73	GRCh37
M. musculus	Mammalia	2,798.79	23,148	132 (355)	129 (339)	e.g. SIGNR1, SIGNR3, SIGNR5, Dectin-1, Dectin-2, Mincle, Langerin (Cd207), MBL-A, MBL-C, Colec11, Mrc-1, Mrc-2, Selp, Sele, Sell, Clec5a, Clecsf8, Dcir, Dcir2, DNGR-1 (Clec9a), DEC-205, and others	reviewed in 14, 61, 62, 73	GRCm38
D. rerio	Teleostei	1,412.46	26,459	176 (381)	169 (360)			Zv9
B. floridae	Cephalochordata	521.90	30,601[3]	692 (694)	646 (647)	AmphiCTL1	74	v1.0
S. purpuratus	Echinodermata	936.58	28,987	293 (303)	241 (244)	SpEchinoidin	75, 76	Spur_3.1
A. gambiae	Insecta	265.03	12,810	37 (99)	33 (93)	CTL4, CTLMA2	34	AgamP3
B. mori	Insecta	481.82	14,623	21 (32)	17 (25)	BmLBP, BmMBP	21, 26, 77	ASM15162v1
D. melanogaster	Insecta	139.49	13,937	56 (96)	48 (84)	DL1, DL2, DL3	18–20	BDGP 5
C. brenneri	Nematoda	190.37	30,667	319 (320)	159 (159)			WS227
C. briggsae	Nematoda	108.48	21,936	183 (183)	111 (111)			WS224
C. elegans	Nematoda	100.29	20,541	283 (288)	166 (185)	CLEC-39, CLEC-49, CLEC-79 + 9 *clec* genes (see table 2)	see table 2	WS235
C. japonica	Nematoda	166.26	29,964	111 (139)	58 (72)			WS227
C. remanei	Nematoda	145.44	31,444	292 (292)	158 (158)			WS185
N. vectensis	Cnidaria	356.61	24,773	67 (67)	62 (62)			ASM20922v1
A. queenslandica	Porifera	166.70	30,060	2 (2)	1 (1)			Aqu1
T. adhaerens	Placozoa	105.63	11,520	45 (45)	40 (40)			ASM15027v1

The total number of CTLD genes are listed for each species.

[1] The number of genes (proteins) with at least one CTLD were obtained by automatic annotation from the InterPro (v53.0) [16] and Pfam (v28.0) databases [17]. The number of CTLD proteins is higher than the CTLD gene number as it includes different isoforms encoded by the same gene. Proteins with multiple CTLDs are counted only once.

[2] CTLD proteins for which a function in immune defense is supported by experimental evidence. The list is nonexhaustive for human and mouse CTLD genes, for which gene name synonyms are shown that are commonly used in the literature.

[3] Noncurated annotation.

size the current lack of exact understanding of the immune functions of the highly diverse CTLD proteins across metazoan taxa and define promising avenues for future research that integrate functional genetic with protein analysis approaches to rectify this knowledge gap.

CTLD Genes Are Abundant in Metazoan Genomes and Mediate Immune Responses in a Variety of Invertebrate Taxa

While CTLD proteins have been shown to fulfill diverse and fundamental tasks in the vertebrate immune system, their function in invertebrate immunity is much less understood. This lack of data on CTLD function in invertebrates is surprising given the fact that genes encoding CTLD proteins are present in all invertebrate genomes sequenced to date and in some cases in very large numbers. In table 1 we list the available genome sequence data for some selected species as representatives of the major invertebrate groups and show the number of CTLD genes and proteins as provided by the InterPro v53.0 [16] and Pfam v28.0 databases [17]. CTLD genes are highly abundant in many metazoan genomes, including, for example, 132 members in *Mus musculus* (the 30th most abundant gene family in *M. musculus*) or 283 gene family members in the nematode *C. elegans* (the 7th most numerous gene family in *C. elegans*). The CTLD encod-

ing gene repertoire in the analyzed invertebrate species varies from two in the sponge *Amphimedon queenslandica* to more than 600 in the cephalochordate *B. floridae* [11]. We also looked for the total number of CTLD genes in the genomes of nine plant species (*Arabidopsis thaliana, Glycine max, Zea mays, Oryza sativa japonica* subspp., *Populus trichocarpa, Medicago truncatula, Triticum aestivum, Hordeum vulgare* and *Sorghum bicolor*). Interestingly, the plant genomes encode only very few (1–4) CTLD genes, which further emphasizes that the expansion of CTLD genes in metazoans is remarkable. Experimental evidence of an immune function of most invertebrate CTLD-encoding genes is missing, although their role in immunity is usually assumed. Within the existing information on invertebrate CTLD proteins, the functional data is biased towards certain insect, crustacean and nematode taxa. In the following paragraphs, we will summarize the evidence of CTLD proteins functioning in the immune defenses of insects and crustaceans, before focusing on the nematode genus *Caenorhabditis*.

Insects

Although genes encoding CTLD proteins are numerous in many insect genomes (table 1), research on insect CTLD proteins has as yet been focused on assessment of single CTLD proteins from different species. For example, only 3 of the more than 50 CTLD-encoding genes in *Drosophila melanogaster* were characterized in more detail [18–20]. Several insect CTLD proteins were suggested to function as PRRs, including those found to mediate important insect immune defense mechanisms, such as hemocyte nodule formation, encapsulation, melanization and activation of phagocytosis [18, 21, 22]. Most insect CTLD proteins were first isolated from hemolymph either due to their ability to agglutinate red blood cells [19, 22], to bind to bacterial cells [21], or were selectively purified from larval plasma by carbohydrate affinity chromatography after bacterial challenge [23]. The purified CTLD proteins were subsequently characterized biochemically in vitro, for example by testing their binding properties and hemagglutination activities. These protein-level studies demonstrated CTLD proteins to possess both broad and more specific binding abilities.

While most studied insect proteins only have one CTLD, some characterized proteins from lepidopteran species, such as *Bombyx mori* [24], *Manduca sexta* [23], and the cotton bollworm *Helicoverpa armigera* [25], have two CTLDs in tandem. The *B. mori* multibinding protein (BmMBP) is an example of an insect CTLD protein with a broad binding capacity. Each of its two divergent tandem CTLDs has distinct binding specificities, enabling the molecule to bind to a large variety of microorganisms ranging from Gram-positive to Gram-negative bacteria, as well as yeasts. After binding to its target, BmMBP is able to trigger cellular immune responses, such as aggregation of hemocytes and nodule formation [26]. The expression of the four characterized *M. sexta* CTLD proteins immulectin-1 to -4 is induced upon bacterial exposure in the fat body. Immulectin-1 and -2 function in activation of the prophenoloxidase [27] and immulectin-3 and -4 act in enhancing encapsulation and melanization [28, 29]. They bind to microbe-associated molecular patterns (MAMPs) [30] with different affinities and specificities in vitro. While immulectin-1, -3 and -4 bind to a broad range of MAMPs, immulectin-2 specifically binds to lipopolysaccharide [28, 29, 31].

These and other studies provide important information on the potential immune functions of insect CTLD proteins. However, there is hardly any experimental evidence from functional genetic studies on the role of CTLD proteins in insect immune defenses in vivo. The three exceptions tested the effect of CTLD gene expression knockdown or knockout in a lepidopteran, the fruit fly and a mosquito. RNAi knockdown of the *M. sexta* CTLD gene immulectin-2 renders animals more susceptible to infection with the insect pathogen *Photorhabdus luminescens* [32]. The galactose-specific *D. melanogaster* CTLD protein DL1, whose expression is induced by injury [19] and which specifically binds to *Escherichia coli* and *Erwinia chrysanthemi*, but not to the Gram-positive *Staphylococcus aureus* or the yeast *Saccharomyces cerevisiae*, agglutinates *E. coli* and promotes the association of a *Drosophila* hemocytes-derived cell line with *E. coli* [20]. A *DL1* deletion mutant did not show any defects in antimicrobial peptide expression after pricking or infection with *E. coli*, indicating that DL1 does not participate in the humoral immune response in vivo, but might still be involved in cellular immune responses such as phagocytosis [20]. In a functional genetic screen of about 100 immune candidate genes using RNAi in the mosquito *Anopheles gambiae* (Diptera) two CTLD proteins, CTL4 and CTLMA2, were discovered to affect *Plasmodium berghei* development by acting as negative regulators of the vector's melanization response to *P. berghei* ookinetes [33]. In a subsequent genetic analysis [34], CTL4 or CTLMA2 silencing by RNAi significantly reduced the survival of adult female mosquitoes infected with *E. coli*, *Enterobacter cloacae* and Pseudomonadaceae H2.26, but not with *S. aureus*, *Micrococcus luteus* and *Enterococcus faecalis*, suggesting a specific role in defense against Gram-negative

bacteria. CTL4 and CTLMA2 expression is induced by *E. coli* and *S. aureus* infection and they both produce a heterodimer that is secreted into the mosquito hemolymph [34].

In summary, while some of the characterized insect CTLD proteins have the ability to bind to a broad range of microorganisms or MAMPs, others exhibit more specific binding properties. Insect CTLD proteins are involved in the activation of cellular immune responses rather than the humoral immune response, but also act as opsonization molecules. A direct effector function such as antimicrobial activity has not been described and their target signaling pathways are unknown. Overall, the potential immune functions of the high variety of insect CTLD proteins (table 1) are far from being understood.

Crustaceans

The second most comprehensive data set on invertebrate CTLD protein functions comes from research on crustacean species that are of economic importance, such as the Chinese white shrimp *Fenneropenaeus chinensis* or the Chinese mitten crab *Eriocheir sinensis*. CTLD protein research in these species mainly aims at the development of antiparasitic drugs, as parasitic threats are a particular concern in crustacean aquaculture. For example, the white spot syndrome virus infects most of the commercially important shrimp species and can cause major production losses [35]. Since CTLD proteins are known to participate in the immune response of other invertebrates and since many expressed sequence tags encoding lectins were identified in cDNA libraries from both healthy and immune-challenged crustaceans, characterization of immune-relevant CTLD proteins in these economically important species has been a particular focus of the current research activities. Comprehensive data sets are thus available on the molecular cloning, isolation and in vitro biochemical characterization of purified crustacean CTLD proteins (either the recombinant or the native protein), especially from the economically important shrimp species, as reviewed in depth previously [36]. Like insect CTLD proteins, crustacean CTLD proteins have repeatedly been suggested to act as PRRs in various species. They can enhance encapsulation, promote phagocytosis and induce the production of reactive oxygen intermediates in hemocytes in vitro [36]. In addition, several crustacean CTLD proteins contribute to pathogen clearance in vivo [37–39].

Interestingly, some crustacean CTLD proteins exhibit in vitro antibacterial or antiviral activity and are thus assumed to act as antimicrobials. For example, Fc-hsL from *F. chinensis* inhibits the growth of Gram-positive as well as Gram-negative bacteria and fungi, with the highest activity against Gram-positive bacteria [40]. Similarly, the two *E. sinensis* CTLD proteins EsLecA and EsLecG produce antimicrobial activity against both Gram-positive and Gram-negative bacteria in in vitro growth-inhibitory activity assays [41]. Most characterized crustacean CTLD proteins are expressed in the gills, intestine, hemocytes and hepatopancreas (the counterpart of the fat body in insects), and are likely to be secreted into the hemolymph upon immune challenge. Indeed, almost all crustacean CTLD proteins that have been studied so far show an inducible expression by either bacterial and/or viral pathogens. The majority of characterized crustacean CTLD proteins have one CTLD, the remaining have two [36]. Surprisingly, all studied crustacean CTLD proteins possess broad binding properties active towards all the bacterial strains tested. One exception might be the *Penaeus monodon* PmLec, which binds lipopolysaccharide and mainly agglutinates Gram-negative bacteria in vitro [42].

Since the genomes of these economically important crustacean species have not been fully sequenced yet, and shrimps and crabs have (as yet) not been amenable to genetic manipulation, genomic analyses and functional genetic studies on the crustacean CTLD genes are almost completely missing. The exceptions are three recent studies in which expression of crustacean CTLD proteins was genetically manipulated in vivo. Overexpression of the CTLD protein PcLec4 from the red swamp crayfish *Procambarus clarkii*, which is induced by infection with *Vibrio anguillarum*, facilitated bacterial clearance and increased survival of crayfish infected with *V. anguillarum* [39]. Knockdown of the expression of the CTLD protein MjHeCL from the kuruma shrimp *Marsupenaeus japonicus* by RNAi caused increased bacterial proliferation in the hemolymph and led to a change in expression of several antimicrobial peptides in hemocytes both in vivo and in vitro. It was thus suggested that MjHeCL plays a role in restricting the growth of the hemolymph microbiota by regulating antimicrobial peptide expression [43]. Similarly, the role of another *M. japonicus* CTLD protein, hFcLec4, and of FcLec4 from *F. chinensis* in promoting phagocytosis by acting as opsonins in hemocytes was supported by the finding that clearance of the pathogenic *V. anguillarum* from the hemolymph was delayed by RNAi knockdown of hFcLec4 and FcLec4 [44].

Interestingly, FcLec4 and hFcLec4 interact with the transmembrane protein β-integrin via their N-terminal domains and this interaction is required for their opsonic activity [44]. β-Integrins are known to participate in

phagocytosis in other invertebrates, such as flies [45]. Shrimp FcLec4 and hFcLec4 may thus act as PRRs recognizing bacteria via their CTLD and their binding to β-integrin in the membrane of hemocytes leads to cytoskeletal reorganization, which induces phagosome formation to ingest the bacteria [44]. This study is one of only two studies providing the first insight into interacting and signaling partners of invertebrate CTLD proteins. In the other study, a conserved binding motif for the transcription factor NF-κB was identified in the flanking promoter sequence of the CTLD gene LvCTL3 from *Litopenaeus vannamei*. LvCTL3 contributed to opsonization and thus marking bacteria and cells for phagocytosis. Deletion of the NF-κB binding site led to the abolishment of LvCTL3 expression, suggesting its regulation by NF-κB. This finding represents a first step towards the incorporation of CTLD proteins into the poorly understood immune signaling network in crustaceans [46].

Taken together, the functional role of several crustacean CTLD proteins was inferred at the protein level by isolating the respective proteins and biochemically analyzing their physical interaction with microorganisms or MAMPs in vitro. Almost all crustacean CTLD proteins are able to bind to a broad range of microorganism and/or MAMPs. They seem to be involved in the activation of cellular immune responses, such as encapsulation and phagocytosis, as well as in the direct elimination of pathogens by exhibiting bactericidal activity. However, the actual number of genes encoding CTLD proteins, their genomic and structural organization, and the underlying impact on the specificity of the interactions with microorganisms remains unknown due to the lack of genome sequences and further experimental analysis. Particularly, studies of the protein function in the context of the whole organism inferred from altered gene products, such as mutant or overexpression phenotypes, are rare.

CTLD Genes Are Extremely Diversified in *Caenorhabditis* Genomes

The nematode genus *Caenorhabditis* provides an impressive example of extreme CTLD gene diversification. This was previously noted for the model nematode *C. elegans* [47], which possesses 283 CTLD genes (*clec* genes) in its genome (online suppl. table S1; for all online suppl. material, see www.karger.com/doi/10.1159/000441475; note that this is an updated number and thus different from the previous report due to further improvements of the whole genome sequence). Of the 283 *clec* genes, 81% are predicted to have a signal peptide [48] and are thus likely secreted proteins, highlighting a particular role of the CTLD proteins in the pseudocoel, in the intestinal lumen and/or as components of the secreted cuticle. In contrast, 21% of the 283 *clec* genes possess membrane-spanning properties, suggesting that these function as components of cellular membranes. *clec* gene evolution is likely highly dynamic and subject to repeated duplication events, as indicated by their genomic localization in clusters (125 out of 283, equivalent to 44%; online suppl. table S1) and the high phylogenetic similarity of the genes within a cluster [47]. It is possible that the repeated duplications are favored by pathogen-mediated selection if *clec* gene duplicates allow the worm to recognize and/or eliminate a larger variety of pathogen strains [47].

In recent years, complete genome sequences became available for additional *Caenorhabditis* species, allowing cross-species comparison of *clec* gene diversification. Next to *C. elegans*, four additional species have comparatively well-annotated genomes, namely *C. briggsae*, *C. remanei*, *C. brenneri* and *C. japonica*. These five *Caenorhabditis* genomes contain between 111 *(C. japonica)* and 319 *(C. brenneri) clec* genes (online suppl. table S2). Overall, the largest group of *Caenorhabditis clec* genes are those with a single CTLD, making up half of the CTLD genes in three of the five analyzed *Caenorhabditis* species and approximately one third in the remaining two species. With one exception *(C. japonica)*, at least one third of the *Caenorhabditis clec* genes also have additional domains. These include the complement C1r/C1s Uegf Bmp1 (CUB) domain, the conserved cysteine and tryptophan residues (CW, also called PAN-3) domain, or the von Willebrand factor type A (VWA) domain. The joint presence of one or multiple CTLDs with other domains in one protein could indicate possible functions in immune signaling. Moreover, several *clec* genes (27% of all *clec* genes for *C. remanei*, 24% for *C. elegans*, 23% for *C. briggsae*, 19% for *C. brenneri* and 10% for *C. japonica*) encode proteins with more than one CTLD within the same protein. Such internal domain duplications might increase protein versatility; BmMBP from *B. mori* is one example for a tandem repetition of CTLDs that leads to extended binding abilities [26].

The majority of the *Caenorhabditis* CTLD proteins are predicted to be secreted. Besides having a putative cell-autonomous recognition function, these secreted CTLD proteins might act as highly specific immune effectors eliminating pathogens in analogy to the vertebrate lectins that are part of the complement system [49].

In summary, *clec* genes show a remarkable diversity in *Caenorhabditis* species, encoding secreted and membrane-bound proteins with one or multiple CTLDs, as well as multidomain proteins. The binding capabilities of CTLD proteins are potentially enlarged by repetitions of CTLDs within one protein. An additional functional diversification is conceivable at the protein level through dimerization or oligomerization of the CTLD proteins, as previously reported for CTLD proteins from other organisms such as CTLD protein homodimers, heterodimers and oligomers in humans and snakes [13], or the heterodimer CTL4-CTLMA2 in *A. gambiae* [34]. CTLD protein-protein interactions might have synergistic effects on CTLD binding specificity (i.e. two or multiple CTLD proteins are able to generate a higher degree of specificity if they interact with each other and with other proteins than if they act alone) and can potentially further increase the diversity available at the genetic level. Such synergistic interactions between CTLD proteins are, however, currently unexplored for the *Caenorhabditis* taxon and for invertebrates in general. Taken together, the *Caenorhabditis* CTLD proteins are particularly diverse and may thus be able to mediate immune specificity at both the level of pathogen recognition and elimination.

CTLD Proteins in *C. elegans* Show Potential for Generating High Immune Specificity

The most comprehensive data on *clec* gene function is available from the model nematode *C. elegans*. Some of the data strongly suggests that *clec* gene diversity could indeed contribute to immune specificity.

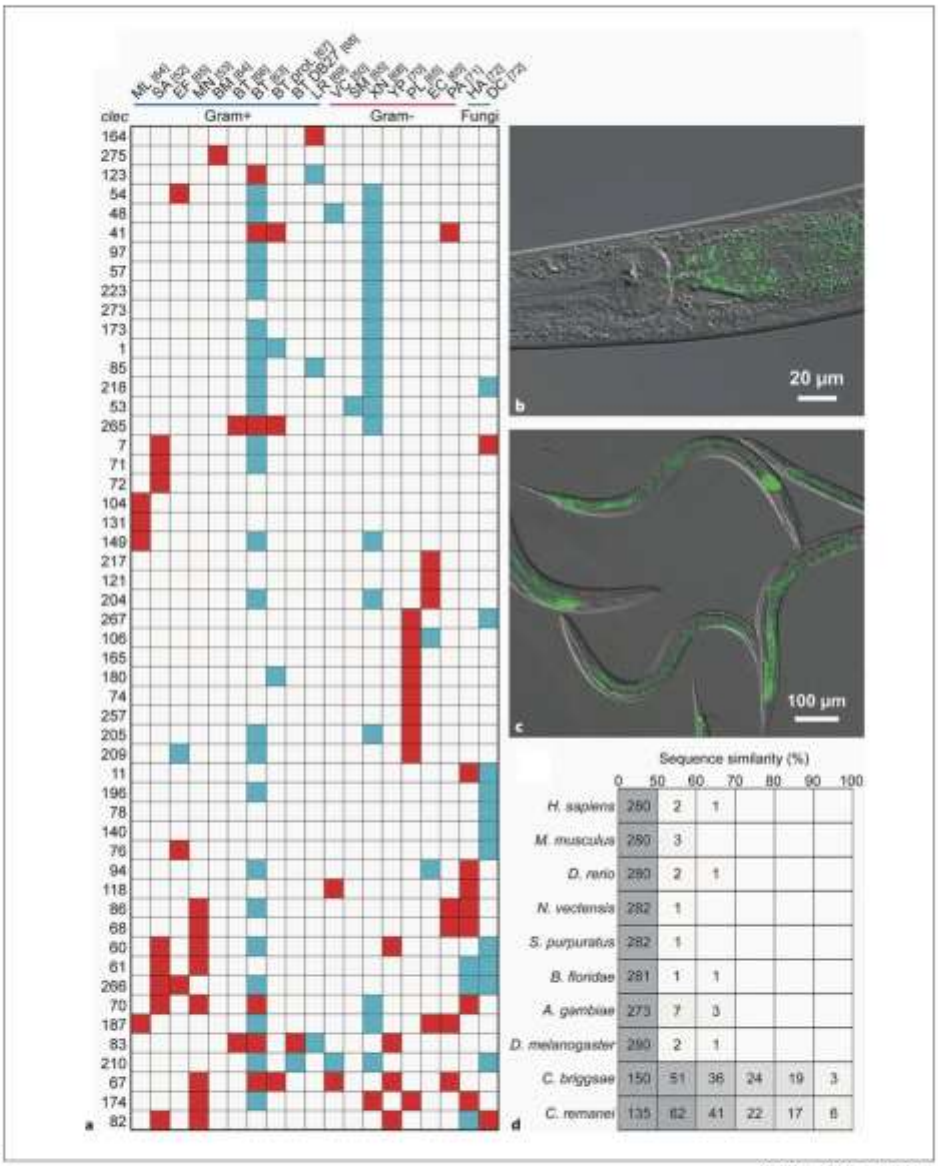
C. elegans clec Genes Are Differentially Regulated following Pathogen Infection

A large number of previous transcriptome studies in *C. elegans* demonstrate that putative immune recognition and effector gene families, such as the *clec*, lysozyme *(lys* and *ilys)* and caenopore (spp.) families, show pathogen-dependent activation upon infection [47]. Because of their extreme diversification, the *clec* genes have the highest potential to generate immune specificity. Of all *C. elegans clec* genes, 84% (237 out of 283) are differentially expressed following an infection with pathogens; 104 *clec* genes are only upregulated, 103 *clec* genes are both, up- and downregulated (online suppl. table S1), and 30 *clec* genes are only downregulated upon pathogen exposure. Importantly, some *C. elegans clec* genes are only differentially expressed in response to a specific pathogen, suggesting their highly specific regulation. In contrast, other *clec* genes are differentially expressed after infection with several pathogens. For these genes, gene induction does not appear to be influenced by general pathogen characteristics, such as the route or site of infection (e.g. the epidermis or intestine) or general pathogen surface properties (e.g. no obvious difference between Gram-negative or Gram-positive bacteria). Instead of these broader patterns of specificity expected to be common for innate immunity, a highly specific pattern of regulation is displayed (fig. 1a). For instance, *clec-67* shows upregulation upon a relatively broad range of pathogens, including both Gram-positive and Gram-negative bacteria, whereas *clec-165* is induced only by the Gram-negative *P. luminescens*. Other *clec* genes are specifically downregulated, such as *clec-273* by infection with the Gram-negative *Xenorhabdus nematophila* (fig. 1a). Interestingly, *clec* genes within a genomic cluster are not coexpressed, but seem to underlie regulation at the individual level. For example, *clec-58*, *clec-59*, *clec-60*, *clec-61* and *clec-143* lie within a cluster on chromosome II and have the same domain architecture. However, *clec-59*, *clec-60* and *clec-61* are differentially expressed after infection with several pathogens, while *clec-58* has so far not been shown to be differentially regulated upon pathogen exposure, and expression of *clec-143* is only induced following infection with *P. aeruginosa*. Different expression of *clec* genes within a genomic cluster might further indicate functional diversification.

C. elegans clec Genes Function in Pathogen Resistance

Our knowledge of the actual function of CLEC proteins in *C. elegans* is still in its infancy and it is as yet unknown why so many *clec* genes are down- or upregulated after exposure to pathogens. Few studies have tried to further elucidate the exact involvement of *clec* genes in *C. elegans* immunity by using functional genetic approaches and as yet only one study could confirm a role for CLEC proteins in pathogen binding (table 2).

The most common approach, which is taken to further analyze the function of *clec* genes, is survival analysis of either the respective *clec* mutant or *clec* RNAi-treated worms [49, 50]. In some studies, survival analysis was combined with the generation of transgenic *C. elegans* strains carrying a transcriptional *clec* gene reporter and the subsequent analysis of spatial as well as infection-induced expression patterns in vivo [51–53]. These studies demonstrated that most of the investigated *clec* genes are expressed in the intestine (fig. 1c), where most bacterial infections take place (fig. 1b), and that some *clec* genes are indeed required for resistance against infection (table 2).



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(For legend see next page.)

Table 2. Functional analyses of CTLD genes/proteins in *C. elegans*

Gene/ protein	Approach	Phenotypic measurement	Phenotype/result	Pathogen	Ref.
clec-17	RNAi knockdown	infection	hyperdeformed anal region (Dar) response, severe constipation	MN	53
clec-39	knockout mutant	survival	higher susceptibility		
clec-49		egg laying	lower egg laying		
CLEC-39	ELISA-based assay	binding assays	binding to live/dead pathogen	SM	55
CLEC-49	with recombinant proteins				
clec-52	promoter fusion reporter	fluorescence induction	expression in intestine	SA	52
	promoter fusion reporter	fluorescence induction	expression in intestine		
clec-60	RNAi knockdown	infection	hyperdeformed anal region (Dar) response, severe constipation	MN	53
	promoter fusion reporter	fluorescence induction	expression in intestine	SA	52
clec-60/61 cluster	overexpression	survival	higher susceptibility	PA	52
clec-65	RNAi knockdown	survival	higher susceptibility	EC	51
clec-67	promoter fusion reporter	fluorescence induction	expression in intestine	SE	56
clec-70	RNAi knockdown	survival	higher susceptibility	SA	52
	promoter fusion reporter	fluorescence induction	expression in intestine		
clec-70/71 cluster	overexpression	survival	higher resistance	SA	52
			higher susceptibility	PA	
CLEC-79	glycoconjugate microarray with recombinant protein	binding assay	binding to Galb1-3GalNAc		54
clec-85	promoter fusion reporter	fluorescence intensity	dependence on immune components *tir-1, nsy-1, dbl-1, daf-16*	SA PA	78
clec-86	RNAi knockdown	infection	hyperdeformed anal region (Dar) response, severe constipation	MN	53
clec-174	RNAi knockdown	survival	higher susceptibility	VC	50

EC = *E. coli* strain LF82; SE = *S. enterica*. For other abbreviations, see the legend to figure 1.

Fig. 1. The highly specific pattern of *clec* gene regulation through pathogen exposure, intestinal expression of *C. elegans clec-66*, and analysis of protein sequence similarity between *C. elegans* CTLD proteins and CTLD proteins from 10 other taxa. **a** Hierarchical clustering of differential expressions of randomly selected *C. elegans clec* genes (rows) after exposure to different microbes (columns), grouped in Gram-positive bacteria (blue), Gram-negative bacteria (pink) and fungi (green). Each column represents *clec* up- (red) or downregulation (blue) according to published transcriptome studies. DB27 and I-3690 are strains of *B. thuringiensis* and *Lactobacillus rhamnosus*, respectively. *clec* expression after infection with *B. thuringiensis* [63] represents merged data from two *B. thuringiensis* concentrations and two time points. BM = *B. megaterium*; BT = *B. thuringiensis*; DC = *D. coniospora*; EC = *E. carotovora*; EF = *E. faecalis*; HA = *Harposporium* spp.; LR = *L. rhamnosus*; ML = *M. luteus*; MN = *M. nematophilum*; PA = *P. aeruginosa*; PL = *P. luminescens*; SA = *S. aureus*; SM = *S. marcescens*; VC = *V. cholerae*; XN = *X. nematophilus*; YP = *Y. pestis*. **b** GFP-labeled spores of the pathogenic *B. thuringiensis* strain MYBT18247 accumulate in the *C. elegans* anterior intestine at an early time point after infection. **c** Animals carrying a *pclec-66*::GFP reporter that is expressed throughout the intestine with highest expression in the anterior and posterior intestine. The coinjection marker *ptts-3*::RFP (red) is expressed in head neurons. **d** The number of *C. elegans* CTLD proteins showing different levels of sequence similarity to the CTLD protein repertoire of other vertebrates and invertebrates. Sequence similarity was assessed with BLAST and a comparison of the 283 *C. elegans* CTLD proteins to those of 10 selected vertebrate and invertebrate taxa. The matrix shows the number of proteins falling into different percentage similarity categories. The observed variation is further highlighted by different shades of gray (dark gray for large numbers, light gray for small numbers).

There have been only two studies investigating the function of CLEC proteins on the protein level. One of these provided experimental evidence for the sugar-binding qualities of CLEC-79 [54], though not in the context of an immune response to infection, while the other demonstrated that CLEC-39 and CLEC-49 were able to bind live and dead *Serratia marcescens* in a Ca^{2+}-independent manner [55]. Both *clec-39* and *clec-49* seem to be required for nematode fitness in the course of an encounter with *S. marcescens* since knockout mutants produced lower reproductive and survival rates. The authors reasoned that due to a lack of bactericidal activity these CLEC proteins could act as PRRs, inducing downstream immune pathways after recognition of the pathogenic bacteria.

There is thus accumulating evidence that *clec* genes function in *C. elegans* resistance to pathogen infection. However, the exact role of the vast majority of CTLD proteins in *C. elegans* immunity remains unclear.

Expression of C. elegans clec Genes Is Regulated by Stress and Immunity Pathways

In *C. elegans* several highly conserved signaling pathways (e.g. p38 MAPK, insulin signaling and TGFβ) are required for the induction of immune effector molecules and, ultimately, host survival. The expression of numerous *clec* genes was shown to be under the control of these *C. elegans* immunity pathways and other known regulators of immunity, such as the intestine-specific GATA transcription factor ELT-2 [56] or the E-box transcription factor HLH-30 [57].

Taken together, CTLD proteins are highly diversified in the *C. elegans* genome with a potential further extension of binding capabilities through oligomerization and internal domain duplications. Experimental evidence demonstrates a *clec* gene function in *C. elegans* innate immunity, their regulation by known immunity pathways, and their highly specific response to different pathogens. Especially the latter emphasizes the particular potential of *C. elegans* CTLD proteins to generate immune specificity.

Similarity of *C. elegans* CTLD Proteins to Those from Other Taxa

Although CTLD proteins from the various taxa are related through the presence of the conserved CTL domain, it is not clear how similar the complete protein sequences are to each other. To address this point, we used the *C. elegans* CTLD protein repertoire as an example and determined their sequence similarity to the CTLD proteins from a selected range of 10 invertebrate and vertebrate taxa, including *Homo sapiens*, *M. musculus*, *D. melanogaster* and *Nematostella vectensis*. This analysis revealed that similarities above 70% are only found for comparisons within the same genus (fig. 1d). In comparisons to distinct taxonomic groups, similarity scores for the large majority of proteins are below 50% and none of the comparisons revealed a value of more than 60%. Interestingly, even in the comparisons to the other *Caenorhabditis* taxa, approximately half of the proteins produce similarity scores of less than 50%.

These results highlight that: (i) CTLD proteins are primarily related through the presence of the CTL domain and a likely function in immunity (at least indicated for numerous CTLD proteins), but otherwise seem to show high diversification in the various phylogenetic lineages, (ii) CTLD proteins thus appear to comprise a highly dynamic, fast-evolving gene family, possibly in response to selective pressure from pathogens, and (iii) information on the function of a certain CTLD protein from one taxon, for example *C. elegans*, cannot be directly transferred onto a specific CTLD protein from a different taxon, for example humans, even though general functional similarities can be deduced through a comparative approach. These findings reinforce our notion that CTLD proteins represent a gene family that is of particular interest to our understanding of the evolution of innate and invertebrate immune systems, and especially their dynamic nature within different phylogenetic lineages.

Current Limitations in Our Understanding of Invertebrate CTLD Proteins

The recently described, surprisingly high degree of specificity of innate immune systems must be based on as yet unknown underlying molecular mechanisms. One of the possibilities may rely on genomically diversified gene families, such as those of the CTLD genes. These genes are numerous in many invertebrate species (table 1) and possess several additional characteristics that support a role in high immune specificity. In the model nematode *C. elegans*, the expression of many *clec* genes is regulated through pathogen exposure in a highly specific form (fig. 1a) [47]. CTLD proteins play an important role in the innate immune system of various invertebrate taxa by activating cellular, and in one case humoral [43], immune responses. Several insect CTLD proteins were found to specifically bind to Gram-negative [18, 20, 31, 34] or Gram-positive bacteria [25]. On the protein level, further

functional diversity of CTLD proteins might be achieved through oligomerization and internal domain duplications.

To date, however, the exact role of invertebrate CTLD proteins in generating immune specificity is completely unexplored. In most cases, only single CTLD proteins from a particular invertebrate species have been studied, but not a larger set of the gene family. On the protein level, some invertebrate CTLD proteins were found to bind to either a broad range of microorganisms (both Gram-positive and Gram-negative bacteria) or alternatively only to a particular pathogen group (Gram-negative or Gram-positive bacteria), already indicating a certain level of immune specificity. At the moment, none of the studied CTLD proteins specifically bind to a single pathogen species. The reasons for this might be that most, if not all, binding analyses on CTLD proteins are performed in vitro and might not reflect what is happening during pathogen infection in vivo. Moreover, they usually do not consider the pathogen variants, which coexist and coevolve with the host in nature and against which high immune specificity may thus have evolved across time. In the future, a larger variety of CTLD proteins should be studied for a particular species. For these studies, it is important that CTLD gene function is analyzed both in vitro and in vivo. The latter is particularly important (yet also challenging) because it can reveal to what extent interactions with other proteins, dimerization and oligomerization might influence the binding properties of CTLD proteins. In addition, the identification of highly specific host-pathogen interactions requires a study system in which the host is infected in a natural way with a natural pathogen and obviously with different strains of the same pathogen species, a setup that is so far not available for studies in *C. elegans*, crustacean and insect models.

Moreover, at the moment, the general functions of the majority of invertebrate CTLD proteins have also not been characterized. While the studies on single CTLD proteins in invertebrates provide important evidence on their role in innate immunity, an understanding of their exact properties and overall functions is far from complete. For example, we lack detailed information regarding how the expression of CTLD proteins is regulated and controlled, or their exact binding properties and targets. In *C. elegans*, CTLD proteins have repeatedly been suggested to be involved in immune defense by acting as antimicrobial effectors, and signaling pathways regulating CTLD gene expression have been identified, whereas in insects and crustaceans CTLD proteins are mainly assumed to act as PRRs activating cellular immune responses. This discrepancy might be due to the fact that in *C. elegans* comprehensive genomic and transcriptomic data sets are available, which revealed that the majority of *C. elegans* CTLD proteins are induced upon pathogen infection and also contain a signal peptide and are thus likely to be secreted. In addition, several *C. elegans* CTLD gene knockout or knockdown animals have been phenotypically characterized, supporting the view that CTLD proteins are important for host resistance to infection. Nevertheless, for this nematode, we currently lack data on CTLD protein interactions with other proteins, the possible downstream molecular signaling pathways, and also data from biochemical assays to infer CTLD binding properties on the protein level.

In contrast, such analyses at the protein level are available for different crustaceans and insects. In particular, in vitro studies using recombinant or native proteins revealed the binding characteristics of several CTLD proteins and provided information on the involvement of CTLD proteins in activating cellular immune defenses. However, in these taxa information from functional genetic studies to confirm a role of CTLD proteins in immune defense in vivo are extremely scarce. Such functional genetic analyses may represent a particular challenge for such large gene families because of functional redundancy among paralogs. Thus, a single gene knockout or knockdown may not necessarily result in a visible phenotype even if the gene is of functional importance. Two approaches have been successfully used to target functionally redundant genes and may prove useful in the future: the simultaneous introduction of mutations in several or all members of a gene family using the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system [58, 59] and the simultaneous knockdown of several genes using RNAi-based approaches [60].

To conclude, it remains unclear for most invertebrate CTLD proteins if they either act as PRRs, directly recognizing pathogens by binding to MAMPs, as signaling mediators or as antimicrobial effectors. To identify mechanisms of CTLD protein action biochemical analyses on the purified native or recombinant proteins to identify binding capabilities and interacting proteins need to be combined with assays on bactericidal effects and also in vivo functional genetic analyses in the respective host taxa. The study by Wang et al. [44] represents a first example of such an integrated approach.

Even though our review focused on invertebrates, we would like to note that similar CTLD protein diversifica-

tion within and across species is also found in vertebrates. The potential role of CTLD proteins in contributing to immune specificity may thus also be of relevance in vertebrate taxa. Consistent with this idea, Drickamer and Taylor [61] proposed in a recent review that mammalian CTLD proteins, which bind to endogenous or self-glycans, are generally conserved, while those which bind to MAMPs are generally divergent and vary in number among species. Examples of such species-specific differences are: (i) two SIGN molecules expressed in humans (DC-SIGN and DC-SIGNR) compared to eight SIGN molecules expressed in mice, (ii) two loci coding for functional mannose-binding lectin in mice compared to only one in humans [61], (iii) one BDCA-2 gene in humans with no reported homologue in mice and (iv) one DCIR-coding locus in humans compared to four (Dcir1–4) in mice [62]. The CTLD proteins in mice and humans do not only differ in number but also in their binding specificities, suggesting that they could indeed contribute to specific responses to pathogens and that their diversification may be a consequence of an arms race with specific coevolving pathogens enabling the host to meet the particular challenges imposed by rapidly evolving pathogens.

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Supplementary Tables

Table S1. Diversity of CTLD proteins in the *C. elegans* genome.

Class	Protein domains[a]	Total[b]	Secreted/membr.-bound[c]			Genomic clusters[d]		Pathogen-induction[e]	
		#	# S	# M	# S + M	#	Cluster names	#	Pathogens
I	1 CTLD 	144	115	8	13	65	I.1, I.2, I.3, II.2, II.3, II.4, III.1, IV.1, IV.2, IV.3, IV.4, IV.5, V.1, V.3, V.6, V.7, V.8	99	BM, BT, LR, SM, PL, EC, HA, EF, DC, ML, VC, SA, MN, YP, PA, XN
II	2-3 CTLD 	44	23	17	1	26	II.2, II.3, IV.4, IV.5, V.4, V.5	40	SM, EF, PL, HA, EC, ML, SA, DC, BT, PA
III	1-3 CTLD + 1-3 CUB 	31	22	1	7	9	II.1, V.2, V.6	25	MN, PL, PA, DC, EF, SM, HA, BT, XN, SA
IV	1-2 CTLD + 1-2 CW 	35	24	4	0	14	I.2, I.4, II.3, IV.4, V.8, V.9	20	HA, EF, SM, PL, DC, PA
V	1 CTLD + 1-2 VWA 	14	14	0	0	10	II.5, II.6	12	DC, EF, SM, PL, PA, HA, SA, MN, YP, BT, EC

Class	Protein domains[a]	Total[b]	Secreted/membr.-bound[c]			Genomic clusters[d]		Pathogen-induction[e]	
		#	# S	# M	# S + M	#	Cluster names	#	Pathogens
VI	Complex structure 	15	4	2	8	1	I.3	11	EF, PL, SM, DC, HA, YP, BM
Sum		283	202	32	29	125		207	

[a] Number of protein domains present. Domain abbreviations: CTLD, C-type lectin-like domain; CUB, Complement C1r/C1s Uegf Bmp1 domain; CW, conserved cysteine and tryptophan residues, Caenorhabditis-specific domain; VWA, Von Willebrand factor type A domain; Complex structure, complex domain structure, where CTLDs might not determine the actual function of the protein.
[b] Total number (#) of CTLD proteins per class.
[c] Number (#) of CTLD proteins with either signal sequence (S, secreted), transmembrane-spanning region (M, membr.-bound) or both according to SignalP 4.0 [48].
[d] Number (#) of clec genes within genomic clusters and their respective names. A genomic clusters is defined as at least three genes within a continuous 20 kb fragment. Roman and Arabic numbers of the cluster names refer to the chromosome and cluster position within each chromosome, respectively.
[e] Number of microorganism-induced clec genes per CTLD class taken from previous gene expression studies [50,52,53,64–66,68–72,79–82]. Abbreviations for microorganisms as explained in figure 1

Table S2. Diversity of CTLD genes in *Caenorhabditis* genomes.

Species	*C. remanei*				*C. japonica*				*C. briggsae*				*C. brenneri*			
Protein domains[a]	Total[b]	Secreted/membr.-bound[c]			Total[b]	Secreted/membr.-bound[c]			Total[b]	Secreted/membr.-bound[c]			Total[b]	Secreted/membr.-bound[c]		
	#	# S	# M	# S + M	#	# S	# M	# S + M	#	# S	# M	# S + M	#	# S	# M	# S + M
1 CTLD	107	79	5	7	71	35	3	7	85	54	6	6	166	98	7	15
2-3 CTLD	48	30	8	5	9	4	1	1	21	15	2	0	26	16	3	2
1-3 CTLD + 1-3 CUB	39	25	1	5	9	6	0	1	28	22	0	4	44	32	0	6
1-2 CTLD + 1-2 CW	52	26	2	3	14	8	1	1	19	10	1	1	56	26	1	2
1 CTLD + 1-2 VWA	33	28	1	0	4	1	0	1	18	13	0	1	15	12	0	1
Complex Structure	13	6	1	3	6	4	0	0	11	5	2	2	11	6	3	2
Sum	292	194	18	23	113	58	5	11	182	119	11	14	318	190	14	28

[a] Number of protein domains present. Domain abbreviations as explained in table S1.
[b] Total number (#) of CTLD proteins per class.
[c] Number (#) of CTLD proteins with either signal sequence (S, secreted), transmembrane-spanning region (M, membr.-bound) or both according to SignalP 4.0 [50].

Chapter 3

The involvement of nested miRNAs in the defence of the nematode *Caenorhabditis elegans* against pathogenic *Bacillus thuringiensis*

Short Communication

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ABSTRACT

microRNAs (miRNAs) are small non-coding RNA-molecules that influence translation by binding to the target gene's mRNA causing its subsequent degradation, in a process known as gene silencing. Many miRNAs are found in nested arrangements within introns, or sometimes exons, of larger protein-coding host genes. miRNA and host genes in a nested arrangement can be encoded either in the same, or in opposite DNA strands. Often genes in a nested arrangement are transcribed simultaneously. This led us to the hypothesis that miRNA found in nested arrangements might share a common function with their host genes. We explored the role of nested miRNA and their host genes in the immune response of the nematode *Caenorhabditis elegans* (*C. elegans*) to infection with a Gram-positive pathogen: *Bacillus thuringiensis* (BT). We performed whole transcriptome sequencing of *C. elegans* upon BT infection and found that 12% of the known *C. elegans* miRNAs were differentially regulated upon exposure to BT, all were downregulated. Half of the differentially expressed miRNAs were found in nested arrangements and, in five cases, the host gene had the same change in expression pattern upon infection as its nested miRNA. We infected knockout or, when not available, knockdown worms of either the miRNA or host gene in four nested arrangements and found that in two cases the host genes but not the miRNAs were required for resistance against infection. In the case of *mir-58.1* knockout, we observed an increase in survival rate of worms exposed to BT, but we did not observe the same for Y67D8A.2, the *mir-58.1* host gene. This suggests that *mir-58.1* acts as a negative regulator of targets directly involved in immune responses against BT, which may include the p38 MAPK *pmk-2*, the DBL-1/TGF- ß-like pathway or other host factors that remain, as yet, unknown.

KEYWORDS

C. elegans; *Bacillus thuringiensis*; nested miRNAs; host genes, immune defences

INTRODUCTION

The invertebrate model *C. elegans* played a central role in the discovery of microRNAs (miRNAs), and in turn, miRNAs are now known to be central for the regulation of diverse biological processes in this nematode (Kaufman and Miska, 2010). miRNA-mediated RNA silencing of target protein-coding transcripts is a conserved process in both animals and plants (Axtell et al., 2011). miRNA regulation leads to decreased abundance of the protein product of the target transcript in the cell. Because miRNAs are cellular tools to fine-tune protein levels, the relationships between miRNAs and their targets are predicted to be established and conserved by natural selection (Bartel and Chen, 2004). Thus, the expression of miRNA genes must be tightly regulated. One such consequence that may conserve stable gene regulation may indeed be the nested arrangement of a miRNA within their targeted host gene. Consistent with this concept, miRNAs in both plants and animals are particularly abundant in nested genomic arrangements and thus within the transcribed part of a particular host gene, usually within introns of protein-coding genes (Axtell et al., 2011; Godnic et al., 2013; Jovelin and Cutter, 2016; Lee and Chang, 2013). Moreover, joint transcription is found more often for miRNAs and genes in close proximity, including pairs with nested miRNA arrangement and irrespective of whether the pairs are transcribed from the same or opposite strands (Baskerville and Bartel, 2005; Chen and Stein, 2006). Since miRNA transcription is coupled to the transcription of host genes (in miRNAs that display a nested arrangement), it could also be that the function of both miRNA and host genes is related, even though the gene products might not directly interact.

miRNAs also contribute to regulation of immune responses and tolerance towards microorganisms (Chen et al., 2013). Yet, to date, immune responses in animals have usually been characterized with a focus on protein-coding genes. The regulation of immune responses by miRNAs adds a new layer of complexity, influencing post-transcriptional regulation, translational efficiency, and stability of the response (Chen et al., 2013). microRNAs can have both positive and negative roles in the regulation of immune responses by directly reducing levels of effector proteins (leading in some cases to tolerance and controlling autoimmunity), or by negatively regulating protein immune-inhibitors (thus activating immunity) (Chen et al., 2013). Although the role of some miRNA genes in animal, and particularly *C. elegans*, immune responses has been elucidated, high redundancy and lack of knowledge of the targets have limited a more detailed understanding of their functions (Chen et al., 2013). *C. elegans* has been established as a model for studying invertebrate immunity (Ewbank and Pujol, 2016; Irazoqui et al., 2010). The role of miRNAs has been shown in the *C. elegans* response to pathogens either by regulating targets with putative effector functions (Iatsenko et al., 2013), or by directing the neuron-specific expression of the *pmk-2* MAPK, which contributes to pathogen avoidance behaviour (Pagano et al., 2015). However, the

extent of the contribution of microRNAs to pathogen defence in *C. elegans* still remains largely uncharacterized.

The aim of the current study is to explore the involvement of miRNAs in the immune response of *C. elegans* to the Gram-positive pathogen *Bacillus thuringiensis* (BT). The interaction between *C. elegans* and this pathogen has been assessed in a variety of studies. These studies have revealed the molecular underpinnings of the worm's defence, consisting for example of (i) resistance through alterations of intestinal membrane glycolipids, to which the BT crystal toxins bind, (ii) inducible defences mediated by MAPK and insulin-like signalling, and (iii) avoidance behaviours (Boehnisch et al., 2011; Griffitts et al., 2005; Hasshoff et al., 2007; Kao et al., 2011; Nakad et al., 2016; Wang et al., 2012; Yang et al., 2015; Zhang et al., 2016). Moreover, evolution experiments demonstrated fast and complex co-adaptations between interacting *C. elegans* and BT populations (Masri et al., 2015; Schulenburg and Müller, 2004; Schulte et al., 2010, 2011). The importance of miRNAs was demonstrated for the worm's interaction with the highly virulent BT strain DB27. In particular, the mutations in the miRNA-processing deficient RNAse III enzyme dicer (*dcr-1*) increase resistance to DB27 (Iatsenko et al., 2013). A possible explanation for this could be, that miRNAs processed by *dcr-1* mediate negative regulation of targets that have a role in the resistance to BT DB27. We here explore the role of miRNAs in the response to two other distinct BT strains, MYBT18247 (BT247) and MYBT18679 (BT679), which harbour clearly distinct sets of cry toxins, which vary in pathogenesis kinetics, and which elicit distinct responses in the worm (Hollensteiner et al., 2017; Masri et al., 2015; Schulte et al., 2010; Wang et al., 2012) (also Zárate-Potes et al., unpublished results (See Chapter 1 of this thesis)). We decided to use the host-pathogen system of *C. elegans* and BT, together with a transcriptomic approach coupled to genetic analysis, to study the miRNA gene involvement in responses against infection and if the nested genomic architecture of these miRNAs implies that the host genes are also involved in pathogen responses.

MATERIALS AND METHODS

Worm and Bacterial Strains

Worm strains were maintained at 20°C and fed with *Escherichia coli* OP50 (*E. coli* OP50) using standard protocols (Stiernagle, 2006). Next to the canonical *C. elegans* laboratory strain N2, the following mutants were used: MT12979 (*mir-70*(*n4110*)V.), VT1362 (*pmp-5*;*mir-70*(*n4109*)V.), MT14875 (*sel-11*;*mir-250*;*mir-61*(*nDf59*)V.), (*sel-11*(*tm1743*)V.), *ppfr-1*(*tm2180*)/hT2)I., MT12993 (*mir-71*(*n4115*)I.) VT1361 (*mir-2*(*n4108*)I.), MT15024 (*mir-58.1*(*n4640*)IV.). All worm strains were obtained from the Caenorhabditis Genetics Center (CGC) or the Japanese National Bio Resource Project (NBRP). For RNA interference- (RNAi-) mediated gene silencing, worms were fed with clones of *E. coli* HT115 from the Ahringer library expressing double stranded transcripts of the following genes: Y67D8A.2 (IV-8D06), Y67D8A.2 (IV-8D04) and *dcr-1* (III-4C08), all available through Source Bioscience (Kamath et al., 2000). All plasmid inserts were confirmed by sequencing. The two pathogenic BT strains MYBT18247 (BT247) and MYBT18679 (BT679) are available in the Schulenburg laboratory and were originally derived from strains NRRL-B18247 and NRRL-B18679 obtained from the Agriculture Research Service Patent Culture Collection of Microorganisms and Cell Cultures (United States Department of Agriculture, Peoria, Illinois, USA). The non-pathogenic strain BT407 was obtained from Christina Nielsen-LeRoux (INRA, France). BT spore solutions were obtained by culturing BT in liquid BT medium with high salt concentration for seven days to induce sporulation and subsequently harvested and stored, as previously described (Hasshoff et al., 2007; Masri et al., 2015; Schulte et al., 2010).

Survival assay and RNAi

To assess survival of wild type (N2) and mutant strains, worm populations were synchronized and raised on 9 cm diameter Nematode Growth Medium (NGM) plates seeded with 700 µl of an overnight liquid culture of *E. coli* OP50 in LB medium. Fourth-instar larvae (L4) were transferred onto 6 cm diameter peptone-free NGM (PFM) inoculated with 100 µl of different concentrations of BT spore solutions mixed with *E. coli* OP50 as food source, which was adjusted to an optical density of 5 measured at 600 nm, with phosphate buffered saline (PBS) buffer. Survival was scored 24 hours post-infection (p.i). Approximately 30 worms were exposed per plate. For gene knockdown, eggs were synchronized overnight in M9 buffer until hatched. 300 L1 larvae were allowed to feed on 100 µl of concentrated RNAi bacteria for 48 hours on 6 cm NGM plates. As treatment control we used *E. coli* HT115 transformed with an empty vector. Analysis of survival experiments GLM of the binomial family was performed followed by a multiple comparison of means: Tukey contrasts, where mutant worm strains were compared to control

strains. Bonferroni adjusted p-values are reported. Statistical analyses were done using R (R Development Core Team, 2011).

RNA isolation, RNA sequencing, Differential Expression and Statistical Analyses

N2 worm populations were synchronized and, when reaching the L4 larval stage, were exposed to the different BT strains in 9 cm PFM plates inoculated with 250 µl of BT spore solution diluted with *E. coli* OP50 OD 5. 2, 6, 12 and 24 hours p.i. worms were washed off the plates using PBS supplemented with 0.3% Tween-20. Worms were centrifuged and washed in M9 buffer three times and then transferred to QIAzol lysis Reagent™ (QIAGEN). Worms were cyclically thawed and frozen four times using liquid nitrogen and a heating block at 37°C to allow the QIAzol reagent to enter the cells and better preserve the RNA. Messenger RNA (mRNA) and miRNA were simultaneously extracted using the NucleoSpin® miRNA purification kit (Macherey-Nagel) and stored at -80°C. miRNAs were sequenced using Illumina HiSeq technology with a coverage of 20 million reads per sample where only 5% of reads were discarded due to insufficient quality. The reads were aligned to the reference *C. elegans* genome with Tophat2 (Ghosh and Chan, 2016) (https://ccb.jhu.edu/software/tophat/index.shtml). Differential gene expression was assessed with Cuffdiff (Ghosh and Chan, 2016) (http://cole-trapnell-lab.github.io/cufflinks/cuffdiff/) and ABSSeq (Yang et al., 2016). Clustering of genes with similar change in expression patterns was performed with Cluster3.0 (de Hoon et al., 2004) (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm).

RESULTS AND DISCUSSION

BT infection leads to downregulation of miRNAs

Exposure to the two pathogenic BT strains BT247 and BT679 led to differential expression of 11,3% of the known *C. elegans* miRNAs, when compared to the non-pathogenic control BT407 (Figure 1A). 12% of the known *C. elegans* miRNAs responded to the pathogen strains in comparison to *E. coli* OP50 (Figure 1B) (two additional nested miRNAs: *mir-58* and *mir-70*). At 2h p.i, most of the miRNAs showed no change in expression or only a slight upregulation. At 6h and 12h p.i, most miRNAs were significantly downregulated on both pathogens (Figure 1A). The only exception to this expression change pattern was *mir-63,* which was induced on BT247, but unchanged or slightly downregulated on BT679. *mir-63* may thus be an interesting candidate for future analysis of pathogen strain specific immune responses. The general downregulation of miRNAs upon BT infection suggests that the miRNA machinery negatively regulates the defence response to BT. Therefore, it is possible that the targets of these miRNAs directly contribute to resistance against BT, for example as immune effectors or positive regulators of immune cascades. Assessment of this prediction is at this point difficult, because the exact targets of most *C. elegans* miRNAs are still unknown and their bioinformatic prediction is subject to high errors (Chen et al., 2013).

Occasional common transcriptional regulation of nested miRNAs and their host genes

Approximately 45% percent of the known *C. elegans* miRNAs display a nested genomic arrangement, being mostly encoded in intronic, but also exonic regions of larger protein-coding host genes (Jovelin and Cutter, 2016). Consistent with a common function of the genes nested arrangement, many nested miRNAs and their respective host genes overlap in their inducible gene expression (Baskerville and Bartel, 2005; Chen and Stein, 2006; Godnic et al., 2013). In our infection study, around 50% of the differentially expressed miRNAs display this nested arrangement and around half of these miRNAs were encoded on the same strand as their host genes (Examples are given in Figure 1D, 1F and 1G). In 5/17 cases, host gene expression showed overlapping expression pattern changes with their nested miRNAs upon BT infection (Two examples are shown in Figure 1C). This common expression regulation pattern of miRNAs and their host genes supports the hypothesis of simultaneous transcription of the nested arrangements. This was particularly observed, when both genes are encoded in the same strand (Figure 1A-C, blue shading background), although there is one example of *mir-250* and *mir-61*, which are encoded in the opposite strand to their host gene: *sel-11* (Figure 1E). For nested arrangements where the expression change pattern of its genes did not overlap, generally the host gene was not differentially regulated. The common downregulation of both miRNAs and their host genes suggests that the

host genes are most likely not targets of their nested miRNAs. If this would have been the case, we would have observed opposite regulation of the host genes in comparison to the miRNAs, because miRNAs are by definition negative regulators of their target genes.

We hypothesized that the observed joint regulation of the nested miRNAs and their host genes could have biological significance in response to BT infection. This physical proximity would allow the fast, simultaneous, and uncostly transcription of two genes with related function. Such effective regulatory control could be favoured by natural selection, under conditions where a fast and accurate response needs to be mounted, such as during pathogen infection. Therefore, we decided to assess an immune function of nested miRNAs and their host genes that overlapped in BT-mediated expression. Following (Osokine et al., 2008), we specifically tested four cases, for which the availability of mutants and/or RNAi clones allowed us to disentangle the separate effects of inactivating either the miRNA and/or its respective host gene (Figure 1D-G).

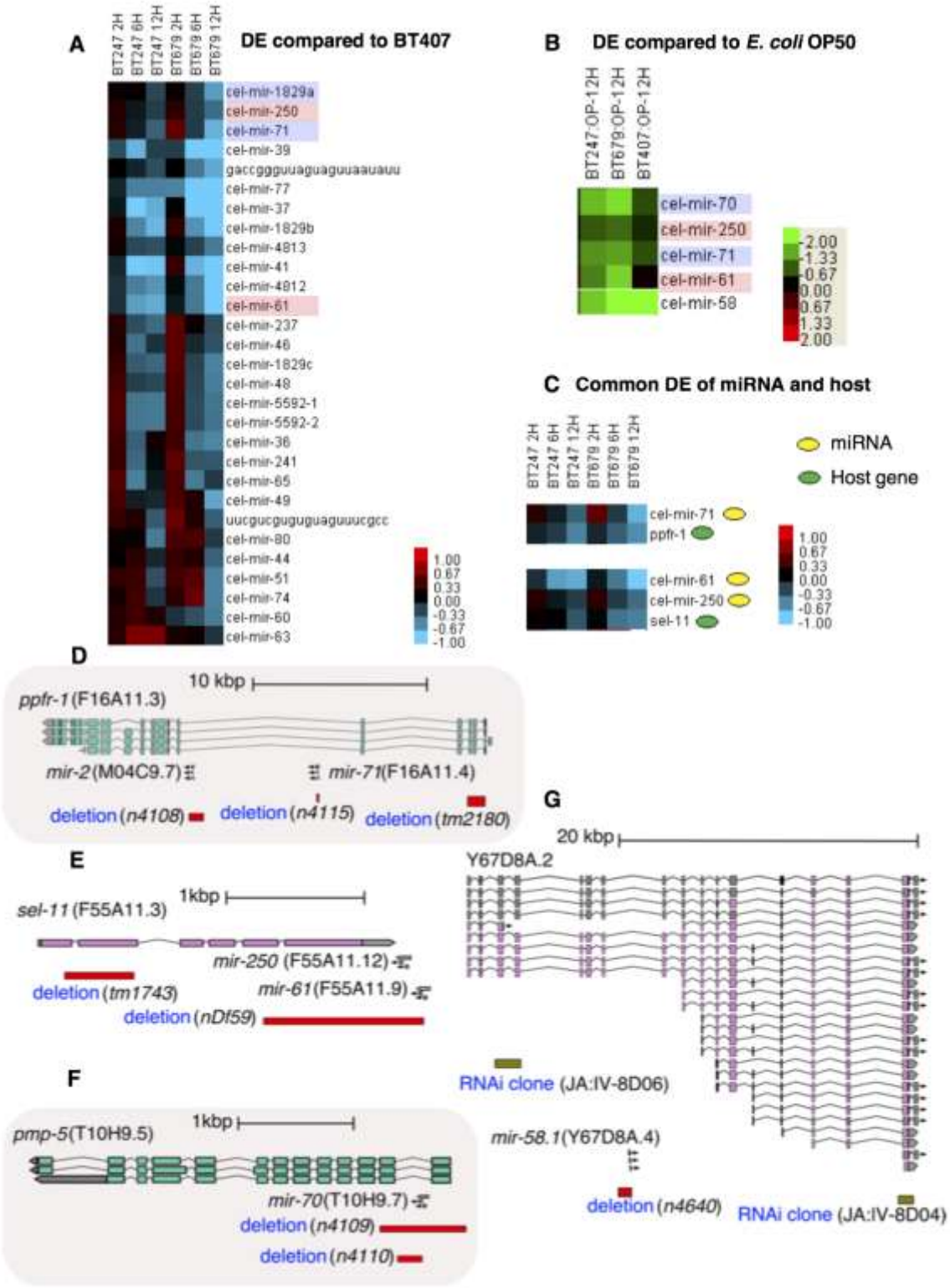

Figure 1. Nested miRNAs and their host genes are downregulated upon BT247 and BT679 infection. (A) Differential expression (DE) of *C. elegans* miRNAs after 2h, 6h, and 12h p.i with two pathogenic BT strains compared to the non-pathogenic strain BT407. Background shading of miRNA name represents common change in expression patter on

for miRNA and host gene. Blue background shading represents that miRNA and host gene stand on the same strand and red represent nested pairs standing on opposite strands. (B) *C. elegans* miRNAs that displayed differential expression after 12h p.i with two pathogenic BT strains compared to *E. coli* OP50, two of them, *mir-58* and *mir-70*, showed no differential expression in the comparison with the non-pathogenic BT407. (C) Illustration of two examples where nested miRNAs (indicated in yellow) show overlapping expression patterns with their respective host genes (green). In (A-C) red indicates upregulation and blue/green downregulation. (D-G) Four examples of the architecture of miRNAs nested within their host genes. The gene diagrams were obtained from Wormbase, version WS262 (www.wormbase.org). Exon colour code and arrows represent the strand on which genes are encoded. The position of deletions in mutants and of RNAi clones are shown.

miRNA	Host gene	Strand	miRNA targets (Wormbase)$	Mutant allele/ RNAi clone	Effect on survival rate upon BT infection
mir-70	*pmp-5*	S	--	*mir-70*(*n4110*)	n4110: none
				mir-70; *pmp-5*(*n4109*)&	**n4109:** more susceptible to both BT strains
mir-250	*sel-11*	O	--	*sel-11*;*mir-61*; *mir-250*(*nDf59*)&	nDf59: none
				sel-11(*tm1743*)	tm1743: none
mir-61	*sel-11*	O	*vav-1*	*sel-11*;*mir-61*; *mir-250*(*nDf59*)&	nDf69: none
				sel-11(*tm1743*)	tm1743: none
mir-71	*ppfr-1*	S	*sod-3, cdc-25.1, tir-1, daf-2, lipl-4, lin-42, hbl-1, unc-31, age-1, pha-4*	*mir-71*(*n4115*)	n4115: none
				ppfr-1 (tm2180/hT2)	**tm2180:** more susceptible to BT247
mir-2	*ppfr-1*	S	--	*mir-2*(*n4108*)	n4108: none
				ppfr-1 (tm2180/hT2)	**tm2180:** more susceptible to BT247
mir-58.1	Y67D8A.2	S	*pmk-2, dbl-1, sma-6, daf-7, daf-1, daf-4*	*mir-58.1*(*n4640*)	**n4640:** more resistant to both strains
				Y67D8A.2 (IV-8D06, IV-8D04)	Y67D8A.2*: none

Table 1. Summary of survival of miRNA and host gene mutants upon infection with BT247 and BT679. miRNA-host gene pairs are shown. The Strand column indicates whether miRNA and host gene are on the same strand (S) or on the opposite strand (O). The miRNA targets were previously identified and deposited on Wormbase, ($) version WS262 or are as yet unknown. Tested mutant alleles or RNAi clones are shown. The column Effect on survival rate upon BT infection indicates whether the survival on pathogenic BT is significantly different between mutant/RNAi knockdown and wild type

using GLM of the binomial family followed by a multiple comparison of means: Tukey contrasts, and Bonferroni adjustment of p-values. *Two RNAi clones for gene Y67D8A.2 were assessed. As shown in Figure 1G, the two different clones target different subpopulations of alternatively spliced transcripts. & represents alleles where the deletion also removes part of the host gene.

mir-58.1 contributes to defence against BT

Gene knockout or knockdown changed survival upon BT infection in three of the four cases of nested arrangements studied. In two of these three cases, a difference in survival was only observed when the mutant allele or RNAi clone affected the putative host gene (Fig. 2A, 2D) or both host gene and nested miRNA (Fig. 2B, 2E), but not when the miRNA alone was inactivated (Table 1, Figure S1). A lack of phenotype in miRNA knockout mutants has been previously reported in the literature and may be explained, in part, by high functional redundancy (Miska et al., 2007). To discard the effect of miRNA redundancy, we performed survival analysis of *dcr-1*(RNAi) worms, which are impaired in pre-miRNA processing, upon BT infection, but found no difference to controls (Figure S2). These results suggest that the host genes function in *C. elegans* defence against BT infection, while the respective miRNAs do not. The host genes themselves do influence defence against BT, a positive effect on survival rate upon BT infection, which has not yet been reported. As *pmp-5* and *ppfr-1* knockout (for deletion see Figure 1D and F) enhances susceptibility to BT infection, their activity seems to contribute directly to immunity. The *pmp-5* gene encodes a member of the ATP-binding cassette proteins, which influence ATP-dependent membrane transport (Lee et al., 2014). It could thus, increase immunity by supporting either detoxification processes (i.e., cellular removal of the BT toxins) and/or enhancing secretion of antibacterial effectors. The second gene, *ppfr-1,* encodes a protein phosphatase regulatory subunit that is generally essential for worm survival, and therefore, it could only be studied here by the help of a gene balancer (Gomes et al., 2013; Han et al., 2009). In this case, the observed increased pathogen susceptibility may be due to a general misbalance in cellular homeostasis rather than a failure in pathogen responsive mechanisms.

In the third case, the miRNA mutant *mir-58.1*(*n4640*) showed increased resistance to both pathogens compared to control worms (Figure 2C and 2F), although its host gene did not show a difference in phenotype upon RNAi-mediating silencing (Table 1). This suggests that *mir-58.1* functions in *C. elegans* defence against BT, while its host gene, Y67D8A.2, does not. In fact, *mir-58.1* was previously demonstrated to negatively regulate members of the DBL-1/TGF-ß-like pathway, which play a role in animal growth, development, resistance to stress (de Lucas et al., 2015), innate immunity (Roberts et al., 2010; Zugasti and Ewbank, 2009), and aversive olfactory learning behaviour (Zhang and Zhang, 2012). It also plays a role in determining the spatial localization of the p38 MAPK PMK-2 specifically to neurons, which in turn influences the behavioural response to pathogenic

Pseudomonas aeruginosa (Pagano et al., 2015). Consequently, *mir-58.1* may mediate general defence to BT infection through either of these two pathways or both. Neither the DBL-1/TGF- ß-like pathway, nor PMK-2 have as yet been implicated in the *C. elegans* response to BT infection. As the knockout of *mir-58.1* enhances resistance, this miRNA is likely to be involved in negative regulation of the defence response. Next to these three cases, a fourth host gene, *sel-11*, and its two nested miRNAs was assessed. In this case, genetic manipulation of miRNAs or host gene did not lead to variation in survival, suggesting that these genes do not contribute to the nematode's defence against pathogenic BT.

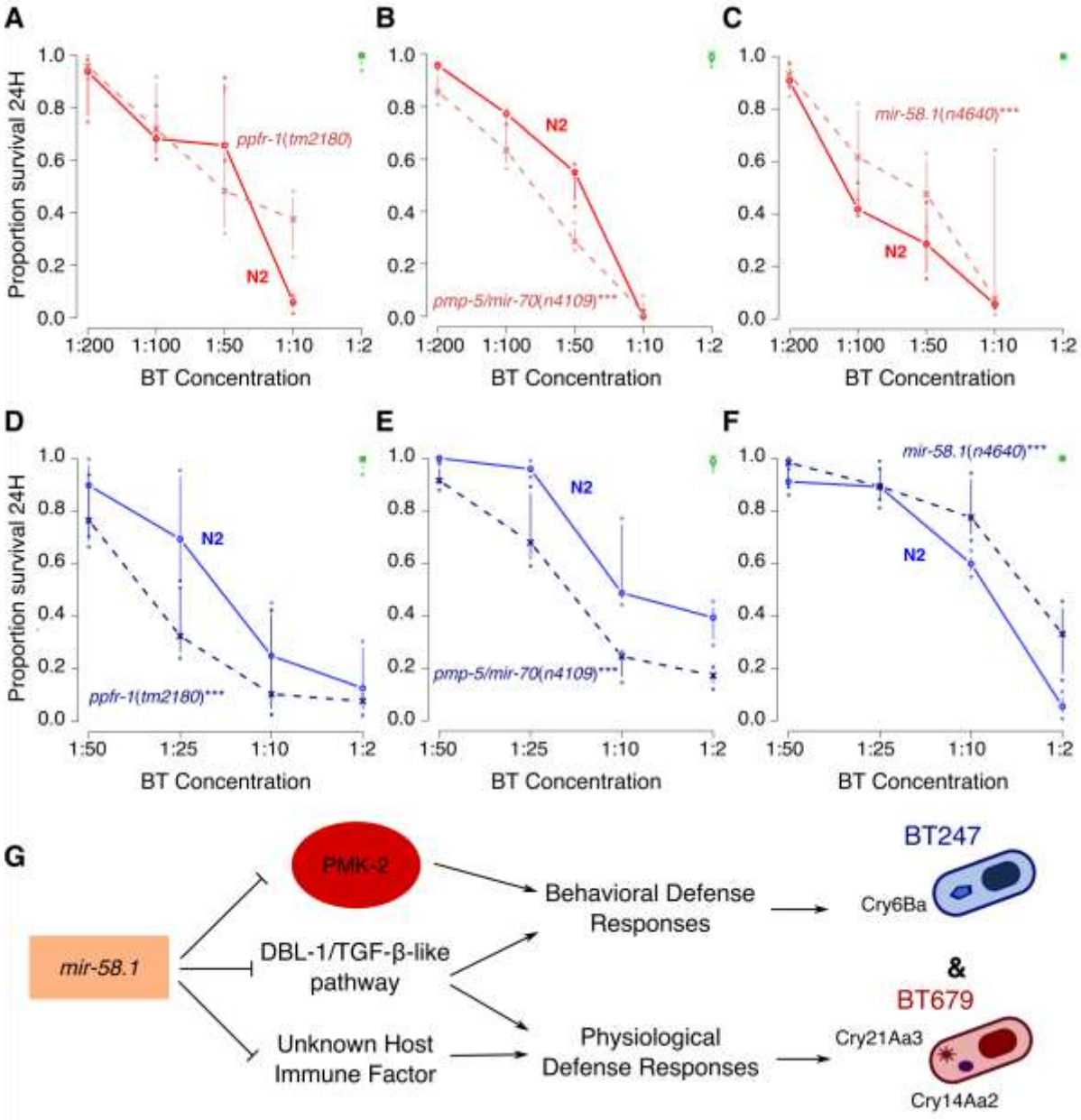

Figure 2. Survival rate of *ppfr-1*(*tm2180*), *pmp-5/mir-70*(*n4109*) and *mir-58.1*(*n4640*) exposed to BT679 (A-C) and BT247 (D-F) and a model of the putative function of *mir-58.1* in BT defence. Each curve represents the proportion of surviving worms of each *C. elegans* strain (Mean and SEM, 5 replicates with 30 worms each). GLM of the binomial family was performed followed by a Tukey Test, where mutant worm strains were

compared to control strains. Asterisks show significant differences for all bacterial concentrations between knockout and wildtype. *** $p < 0.001$, Bonferroni adjusted. Results are representative of at least 3 independent runs. Green dots in (A-F) represent survival of worm strains exposed to BT407. (G) Model of the putative contribution of *mir-58.1* to the nematode's defence against BT679 and BT247.

CONCLUSIONS

We performed a transcriptome study of the *C. elegans* miRNA response to BT exposure and found that all differentially regulated miRNA genes are downregulated upon infection, suggesting that they act as negative regulators of the general immune responses to BT. Around 50% of the differentially regulated miRNAs were found in nested genomic arrangements and in 5 cases, both host gene and associated miRNA overlapped in the transcriptomic response, possibly indicating that the host genes share a common function with the nested miRNA. We tested this hypothesis in four examples through functional analysis of mutants or RNAi-mediating gene silencing. In two cases, the host genes but not the corresponding miRNAs influenced survival upon pathogen infection. In only one case, inactivation of a miRNA, *mir-58.1*, enhanced resistance to BT. This effect was not observed for the *mir-58.1* host gene. We conclude that *mir-58.1* is a negative regulator of the defence response against BT infection, yet functions independently of the associated host gene.

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SUPPLEMENTARY MATERIAL

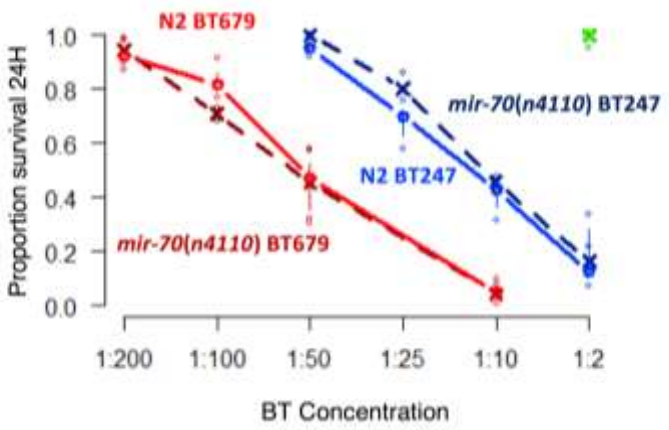

Figure S1. Survival of *mir-70*(*n4110*) mutants on BT247 and BT679 is as wild type. Each curve represents the proportion of surviving worms of each worm strain. Mean and SEM are shown, N=5 plates with 30 worms each. GLM of the binomial family was performed followed by a Tukey Test, where mutant worm strains were compared to control strains. Results are representative of at least 3 independent runs. Green dots represent survival of worm strains exposed to BT407.

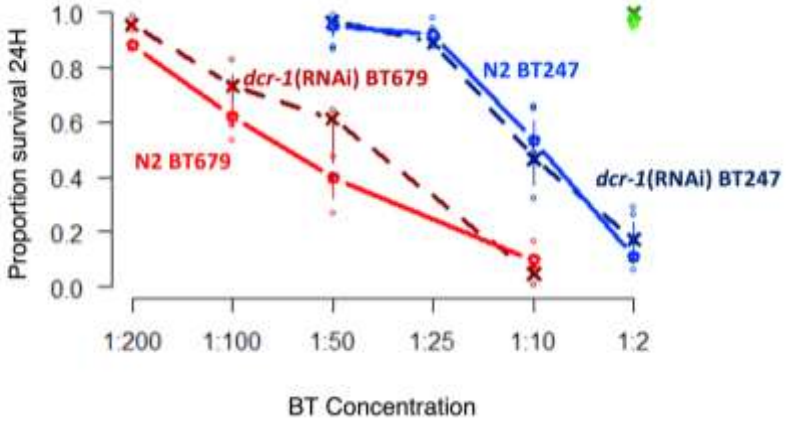

Figure S2. Survival of *dcr-1*(RNAi) mutants on BT247 and BT679 is as wild type. Each curve represents the proportion of surviving worms of each worm strain. Mean and SEM are shown, N=5 plates with 30 worms each. GLM of the binomial family was performed followed by a Tukey Test, where RNAi worms were compared to empty vector controls. Results are representative of at least 3 independent runs. Green dots represent survival of worm strains exposed to BT407.

GENERAL DISCUSSION

Changing classical immunology paradigms

In classical immunology, jawed vertebrate immune responses are the only ones considered as adaptive because of the presence of T- and B-cell receptors. These molecules with a highly diverse repertoire, mediate antigen-specific responses (specificity to the molecular level) and preserve up to life-long lasting memory of previous encounters with threats, to make further encounters harmless. The high diversity and specificity of these receptors is achieved by random rearrangements of genomic segments that encode the antigen-binding region. This diversity anticipates future threats. All of these characteristics are considered the hallmarks of adaptive immune systems (Brehélin and Roch, 2008). On the other hand, all non-jawed vertebrates and invertebrates, which lack T- and B-cell receptor-mediated immune responses are considered to only possess innate immune systems. These are characterized by: general responses independent of the threat faced, lack of memory of previous encounters and absence of diversified repertoires of anticipatory recognition receptors (Bailey et al., 2013; Brehélin and Roch, 2008; Hoffmann et al., 1999). The classical view of innate immune systems has dramatically changed in recent years, as forms of immune memory called immune priming (Cooper and Eleftherianos, 2017; Kurtz and Franz, 2003; Rimer et al., 2014; Sadd and Schmid-Hempel, 2006), diversified recognition receptor repertoires (reviewed in (Bailey et al., 2013)) and variable degrees of specificity in immune responses (Auld et al., 2013; Little et al., 2006; Pham et al., 2007; Pinaud et al., 2016; Portela et al., 2013) have been identified in non-jawed vertebrate and invertebrate metazoan taxa. These findings challenge the classical distinction between innate and adaptive immunity.

To further support the current change in the way we understand the function and evolution of metazoan immune responses, **I wanted to broaden our understanding of the extent to which invertebrate immune systems are able to mount specific responses, by asking if such immune responses can be pathogen strain-specific**. That is, if they are able to mount customized responses according to the pathogen strain faced, as opposed to mounting a unique generalized response to any possible strain or pathogen encountered, as it was believed to be in the past. I also wanted to go one step further and **elucidate the molecular mechanisms behind such pathogen strain-specific immune responses in invertebrates**, as only by doing this we can fully comprehend the way in which diverse metazoan immune systems work and the different evolutionary trajectories they have followed. This research question on invertebrate immune specificity is of relevance in evolutionary biology, because all animal taxa existing today have had the same time to evolve their immune system under high pathogen selection pressure. Therefore, all have the same potential to

have generated specificity following different evolutionary paths (Bailey et al., 2013). Hypothetically, all living organisms should display various levels of immune specificity, since mounting immune responses is costly and the diversity of threats encountered is great. However, our understanding of the extent of the specificity of invertebrate immune systems and the molecular mechanisms that govern them is still rudimentary.

In the following sections I will illustrate how the results presented in this PhD thesis contribute to our understanding of three important aspects of the *Caenorhabditis elegans* response to infection with different *Bacillus thuringiensis* (BT) strains, namely: (1) that there are identifiable strain-specific responses upon exposure to strains MYBT18247 (BT247) and MYBT18679 (BT679), (2) that a large part of the response mounted by *C. elegans* upon infection with these two BT strains is common, and (3) that likely what is driving the specificity of the *C. elegans* response to BT is the identity of the strain's main virulence factors: the Crystal Pore-Forming Toxins (Cry PFTs). This implies that specificity in the immune response is determined by the functional interaction between host and pathogen and not by the taxonomic relatedness that pathogen strains may share.

Results from a study of immune specificity in an invertebrate

To be able to answer our research question we turned to the nematode host *C. elegans*, which is amenable to genetic research due to: its short life cycle, the self-fertilization of hermaphrodites that leads to the easy establishment of isogenic lines, the fully sequenced and well annotated genome and the various tools and resources available to perform genetic analysis (Wormbase version WS262). These resources for genetic analysis include, among others: genome-wide RNAi libraries (e.g. (Kamath and Ahringer, 2003)), large collections of available mutants and reporter strains (CGC and NBRP) and standardized methods for generation of transgenic animals (Rieckher and Tavernarakis, 2017). More importantly, there is accumulating phenotypic evidence supporting the ability of *C. elegans* to mount pathogen strain-specific behavioural and physiologic immune responses against, among others, the Gram-positive pathogen, BT (Hasshoff et al., 2007; Schulenburg and Müller, 2004; Wang et al., 2012). Genotype-genotype interactions with this pathogen have also been observed (Schulenburg and Müller, 2004).

In **Chapter 1** of this thesis, we began with an RNA-Seq experiment to assess if there are specific transcriptomic responses to the pathogenic strains BT247 and BT679, compared to the non-pathogenic strain BT407. We found that 9% of the BT responsive genes change their expression in different ways when exposed to BT247 compared to BT679 exposure. The remaining 91% of the differentially expressed (DE) genes changed expression in the same way upon exposure to both pathogenic strains. The expression of the strain-specific DE genes is, at least

in part, regulated by the GATA transcription factor (TF) ELT-2. *elt-2*(RNAi) worms show enhanced susceptibility to BT679 and an increase in survival rate upon BT247 infection. **We consider this as a compelling first line of evidence of the ability of *C. elegans* to mount pathogen strain-specific responses to BT247 and BT679.** We then proceeded to elucidate the mechanisms behind the *elt-2*-mediated specific responses.

The next question we asked was: **how can a TF with a single isoform and constant localization in the cell nucleus, lead to different transcriptomic responses depending on the pathogen strain faced?** There is transcriptomic and functional genetic evidence form our lab and others, supporting that specific transcriptomic programs are achieved through the joint action of master transcription regulators, such as ELT-2, and additional signalling pathways and transcription factors and cofactors (Block and Shapira, 2015; Block et al., 2015; Yang et al., 2016). This would be similar to the transcriptional regulation networks involved in the action of other metazoan TF families, that have a large number of activating signals and, consistently, an extensive number of targets (Oeckinghaus and Ghosh, 2009; Stefanovic and Christoffels, 2015). In these cases, the regulation and fine-tuning of the transcriptional response is highly complex. Here I present two examples.

First, the NF-κβ family of TFs is important in many metazoan taxa for inflammation, immunity, cell proliferation and differentiation. This family has five proteins that form functional homo- and heterodimers through combinatorial association and this also leads to functional differentiation and specification (Oeckinghaus and Ghosh, 2009). Additionally, in many cell-types the inactive TF complex is sequestered in the cytoplasm by inhibitors that have to be degraded before nuclear translocation occurs. However, once inside the nucleus, there are other mechanisms in place that participate in the regulation of the transcriptomic response to a stimulus. The specificity of the DNA binding sites of this TF family doesn't rely on the DNA sequence alone, but also on the presence of other TFs, cofactors and repressors at this site. Additionally, post-transcriptional modifications of the TF complex are also important to constrain their action to the required transcriptional response (Oeckinghaus and Ghosh, 2009). The NF-κβ family of TFs is not found in *C. elegans* (Pujol et al., 2001). Additionally, ELT-2 generally already has nuclear localization and doesn't need to conform functional homo- or heterodimers (McGhee et al., 2009). However, the interaction with other protein complexes at DNA binding sites and post-translational modifications might contribute to context-specific transcriptional outcomes.

Second, what is known for the GATA4-6 TFs in mammals, to which ELT-2 is homologous (Block et al., 2015), provides the basis for a more directly translatable model of how ELT-2 transcriptional specification might occur in *C. elegans*.

GATA4-6 are important in cardiac development, among other processes (Stefanovic and Christoffels, 2015). In mammals, although a vast number of genes possess GATA motifs in their promoter or enhancer regions, only a subset of them are occupied by GATA TFs per tissue (Stefanovic and Christoffels, 2015). It is believed that the occupation of a GATA motif is determined by the local chromatin environment, neighbouring TF binding motifs and, as yet unknown, intrinsic features of the GATA motifs themselves (Stefanovic and Christoffels, 2015). These factors play a role in the decision-making process of what genes are expressed in a given moment. Additionally, the GATA TF DNA-binding domains are two zinc-fingers that can have two distinct DNA binding conformations: (1) a more stable one wrapping a palindromic GATA domain with both zinc-fingers and (2) a less stable one, where a single zinc-finger bridges two distant fragments of DNA. According to the DNA binding conformation, the GATA TF-dependent regulation of transcription is very different (Stefanovic and Christoffels, 2015). Lastly, it has also been evidenced for GATA4-6 that interaction with additional TFs, cofactors, chromatin-remodelling enzymes and post-translational modifications of the GATA TFs themselves, affect the set of genes that are transcribed in a given context (Stefanovic and Christoffels, 2015).

We believe that a multicomponent transcription regulation network might also be controlling *elt-2*-mediated strain-specific responses to BT247 and BT679. We searched for possible TFs that could interact with ELT-2 to drive the response specificity upon infection. We also proceeded to do follow-up phenotypic measurements, such as: pathogen load and intestinal tissue damage. Additionally, we performed a second RNA-Seq experiment, this time with *elt-2*(RNAi) worms exposed to both pathogenic strains, to identify *elt-2* downstream targets involved in specific responses to pathogens (**Chapter 1**). We analysed transcriptional changes of non-coding RNAs, particularly miRNAs, upon infection with BT247 and BT679 (**Chapter 3**). We then performed further genetic analyses with candidate genes drawn from transcriptomic analyses or form the literature, accounting for around 120 mutant strains and around five fluorescent reporter strains (**Chapter 1**). In the end, we present a model of interactions between different host factors that contribute to general and strain-specific responses to BT.

A summary of the host's molecular responses found in this thesis is given in Figure 1. In our current working model, derived from the results presented in this thesis, we propose that the GATA TF ELT-2 is a central regulator of the strain-specific response to BT247 and BT679. ELT-2 positively regulates resistance to BT679 by promoting higher survival rate through the expression of putative immune effectors. ELT-2 also negatively regulates tolerance to BT247, as *elt-2* knockdown leads to an increase in survival rate, without affecting the pathogen load. We further propose that ELT-2 regulates these strain-specific responses through a complex transcription regulatory network, that involves, at least, other transcription factors, which are different in the context of BT679 and BT247 infection. I will discuss in

more detail what we know about the ELT-2-mediated strain-specific responses in the following sections, starting with BT679 then continuing with BT247.

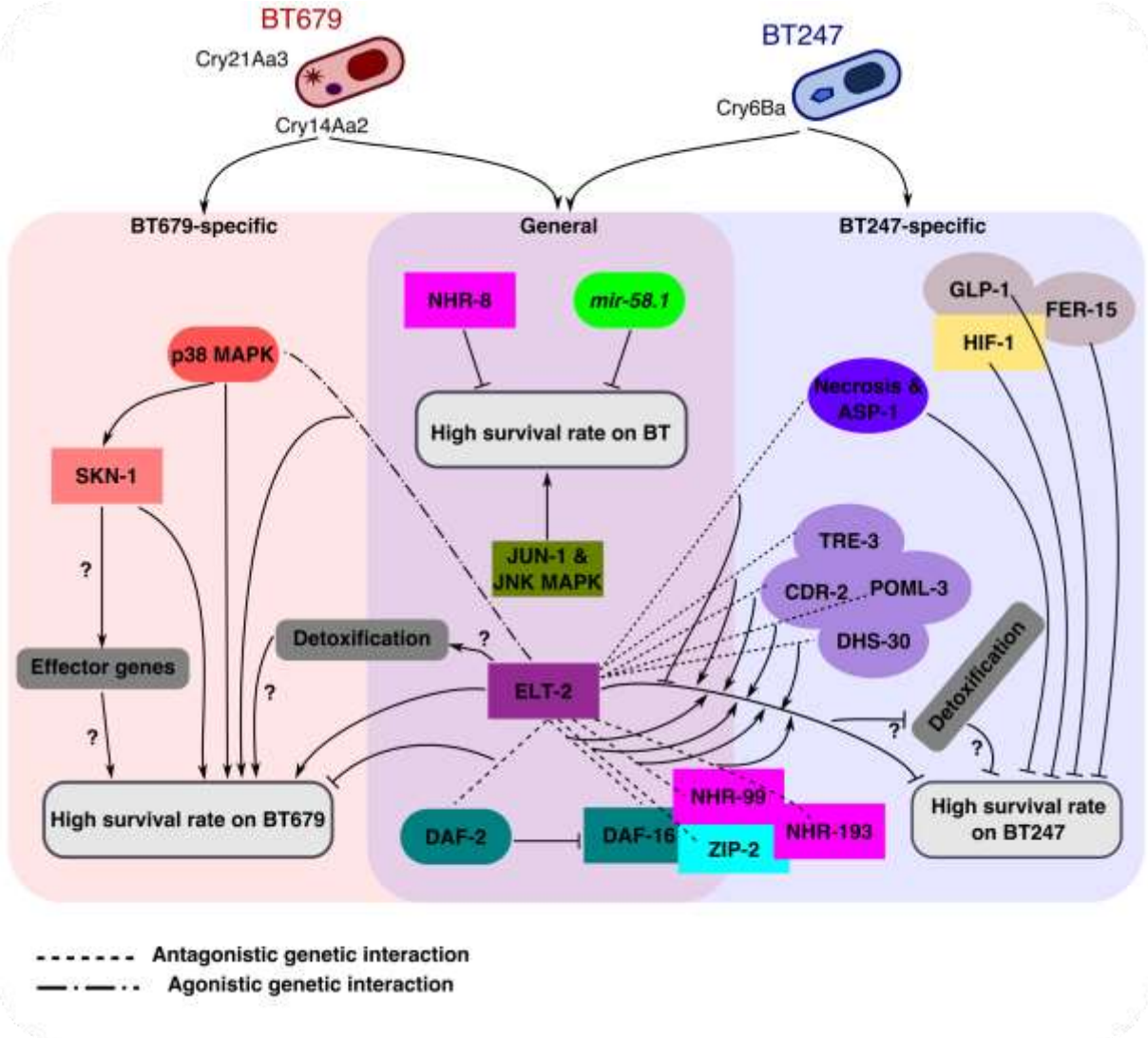

Figure 1. *C. elegans* general and strain-specific molecular responses described in this thesis. Antagonistic genetic interaction refers to two-gene interactions (in which one of the genes is always *elt-2*) that have opposite effects on survival rate. Agonistic genetic interaction refers to two-gene interactions (in which one of the genes is always *elt-2*) that have an effect on survival rate in the same direction. Arrowheads denote positive regulation and bars denote negative regulation. Arrows starting from a line representing a genetic interaction illustrate the effect of the double knockout/knockdown on the single *elt-2*(RNAi) phenotype. Question mark represents an interaction for which there is no empirical evidence as yet.

C. elegans mounts, in part, specific responses to BT679

We argue, based on our findings and what is reported in the literature, that, in the case of BT679, *elt-2* mediates worm resistance to the pathogen through the expression of effector genes that lead to reduced pathogen load and reduced

tissue damage. This happens through the joint action of *elt-2* and the *skn-1* TF, possibly acting downstream of the p38 MAPK pathway as it has been suggested for *P. aeruginosa* (Block et al., 2015). *elt-2*(RNAi) in the genetic background of the insulin-like receptor (ILR)/ *daf-2*(*e1370*) mutant leads to rescue of the susceptibility to BT679 (like it was described for other pathogens in (Kerry et al., 2006)), suggesting that some *elt-2*-positively regulated components that lead to resistance to BT679 are also negatively regulated by *daf-2*. This is evidence of a complex transcriptional regulation network upon infection.

Although we tested the survival rate of knockout mutants for several candidate effector genes, we were not able to identify immune effectors downstream of *elt-2*, the p38 MAPK pathway or *skn-1*, that lead to resistance to BT679. Searching for *elt-2*-induced effectors that promote resistance to BT679 remains an interesting topic for future studies. The lack of an immunocompromised phenotype in knockout mutants of effector genes to this point means that alternative, non-mutually exclusive explanations for the contribution of *elt-2* to increased survival rate of worms on BT679, are still plausible. (1) A lack of phenotype of knockout mutants of putative *elt-2*-induced effector genes could be explained by functional redundancy between immune effectors. To further test if this is the case, multiple knockouts of co-induced, *elt-2*-dependent, putative effector genes could be made and survival rate upon BT679 infection could be tested. The generation of strains with multiple gene-knockouts is possible now thanks to the precision genome editing technology CRISPR-Cas9 (Paix et al., 2017).

(2) *elt-2* could contribute through the modulation of targets that positively regulate feeding cessation or also pathogen avoidance behaviour, which has been shown to contribute to higher survival rate upon BT679 exposure (Nakad et al., 2016). In the future, this explanation could be studied by specifically measuring behavioural responses (such as avoidance or feeding cessation) upon infection. This could also be done for single- or multiple-gene knockout mutants of *elt-2* downstream targets, to observe how behavioural responses are affected.

(3) In an *elt-2*(RNAi) RNA-Seq experiment upon BT247 and BT679 exposure, we observed that *elt-2* positively regulates many putative detoxification genes in the context of BT679 infection. This suggests that *elt-2* could play a role in toxin clearance, tissue repair and recovery from infection; as it has been shown for recovery form infection with the pathogens *P. aeruginosa* (Head et al., 2016) and *S. enterica* (Head and Aballay, 2014). To further learn about this, we need to observe the *C. elegans*-BT679 interaction at the cellular level at more frequent time points using microscopy to better assess tissue integrity and repair. This can be achieved with the help of intestinal integrity reporter strains, like the ones used in this thesis (Hüsken et al., 2008), or the DNA dye Propidium Iodide (PI) (like used in (Zhang et al., 2016)) that can bind to DNA of damaged cells. The combination of microscopy techniques with the use of knockouts of *elt-2*-regulated genes,

putatively involved in detoxification and tissue repair, would shed light to the role of this mechanisms in the *C. elegans* defence against BT679.

C. elegans mounts, in part, specific responses to BT247

elt-2(RNAi) results in a substantial increase in survival rate upon exposure to BT247. This suggests that *elt-2* contributes to the response to BT247 in a different way compared to the *elt-2*-dependent response to BT679; and the response to other BT strains, as we have shown in this thesis (**Chapter 1**), or to other bacterial pathogens (Block et al., 2015; Kerry et al., 2006; Shapira et al., 2006). We first investigated if *elt-2* was involved in the same pathway as other host factors that increased the survival rate of the worms upon infection, like glycosylation-deficiency (Griffitts et al., 2005), or sterility (Miyata et al., 2008). Then we performed an *elt-2*(RNAi) RNA-Seq experiment in order to find the downstream transcriptional targets affected by *elt-2* knockdown upon BT247 infection. Knockouts of selected genes that were upregulated upon *elt-2*(RNAi) were tested to find out how they affected the survival rate of *elt-2*(RNAi) worms infected with BT247.

We couldn't find any other single known host factor contributing to susceptibility to BT (i.e. negative regulation of immune response by miRNAs (**Chapter 3**) or the presence of glycolipid receptors synthesized by glycosyl-transferases (**Chapter 1**)) that could recapitulate the increased survival rate of worms on BT247 to the extent that *elt-2*(RNAi) did. We found, however, that the genetic manipulations of three additional host factors lead to increased survival rate on BT247: 1) Knockout of the LIN-12/Notch family of receptors member *glp-1*(*ar202*) and *glp-1*(*bn18*), which make the worms deficient in mitotic germ-line stem cells, and therefore make the worms sterile; a knockout mutation in the RNA-dependent RNA polymerase *fer-15*(*b26*), which results in worms that possess a germline but are sperm-deficient and therefore, sterile. 2) Knockout of the hypoxia-induced factor *hif-1*(*ia4*). *elt-2*(RNAi) on the mentioned mutant backgrounds leads to further increase in the survival rate of worms, suggesting parallel molecular mechanisms that lead to enhanced survival rate on BT247. 3) Knockout of the necrosis pathway mediated by aspartic protease *asp*-1 in the context of BT247 infection was a very interesting case of study. It has been shown that knockout in *asp-1*(*tm666*) leads to higher survival rate upon exposure to BT Crystal (Cry) Pore-Forming Toxin (PFT) Cry6Aa compared to wildtype controls, because the Cry6Aa toxin physically interacts with ASP-1 to induce necrosis signalling in detriment of the host and to protect the Cry6Aa toxin from degradation (Zhang et al., 2016). Cry6Aa is the closest related protein known to Cry6Ba, which is the main nematocidal factor produced by BT247, although they only share around 50% of protein sequence identity (Wei et al., 2003). *asp-1*(*tm666*) leads to enhanced survival rate on BT247 compared to wildtype controls and additional *elt-2*(RNAi) treatment on the *asp-1*(*tm666*) genetic background doesn't lead to yet increased survival rate. This suggests that the *elt-*

2(RNAi)-dependent increase in survival rate on BT247 is *asp-1* and necrosis pathway-dependent.

We were interested in the molecular mechanisms that lead to an increase in survival rate of *elt-2*(RNAi) upon BT247 infection. For that we focused on finding additional genes that when knocked out with simultaneous *elt-2*(RNAi) treatment led to a reduced survival rate on BT247, compared to *elt-2*(RNAi) worms alone. We found several different host factors that partially contribute to a decrease in survival rate of *elt-2*(RNAi) worms on BT247. This implies that the increase in survival rate of *elt-2*(RNAi) worms on BT247 is most likely not due to a single gene whose transcription regulation is affected by *elt-2*(RNAi), but several. This leads us to believe that the increased survival rate of *elt-2*(RNAi) worms on BT247 is a complex, multifactorial, phenotype. The factors that reduce the survival rate of *elt-2*(RNAi) on BT247 include: the FOXO TF *daf-16*, the Nuclear Hormone Receptor (NHR) TFs *nhr-99* and *nhr-193*, the bZIP TF *zip-2* and the *elt-2*-negatively regulated downstream targets *poml-3*, *cdr-2*, *tre-3* and *dhs-30*, whose functions putatively involve lipid metabolism or detoxification. The putative involvement of these factors in detoxification, the fact that *elt-2*(RNAi) worms don't have reduced pathogen loads compared to controls, and the observation that they exhibit reduced intestinal tissue damage compared to controls; led us to hypothesize that *elt-2*(RNAi) leads, at least in part, to increased tolerance to BT247 infection.

We can't entirely rule out the possible explanation that *elt-2* leads to reduced resistance through the negative regulation of immune effectors in the context of BT247 infection. The argument against effector-mediated immunity is the high pathogen load of *elt-2*(RNAi) worms, which suggests that the worms are not reducing the BT population density. However, we know of the putative effector lysozyme *lys-2*, which has been shown to contribute to resistance against BT247 (Boehnisch et al., 2011). In our *elt-2*(RNAi) RNA-Seq dataset we observed that the expression of *lys-2* is negatively-regulated by *elt-2* upon BT247 infection. To further explore if increased expression of *lys-2* after *elt-2*(RNAi) leads to higher survival rate, we would need to show an effector function of LYS-2 against BT247.

Our *elt-2*(RNAi) results on BT247 are counterintuitive, because worms show increased survival when *elt-2* is silenced. This means that an endogenous host factor (ELT-2) regulates the response to BT247 infection in a non-optimal way, which prevents the worms from reaching their best performance. The following question arises: if silencing of *elt-2* results in highly tolerant worms, why is *elt-2* not down-regulated in non-manipulated worms upon infection conditions? There are four explanations that could account for this. (1) *elt-2* downregulation, (and through that the upregulation of detoxification mechanisms), may lead to trade-offs with other life-history traits, such as fitness, since *elt-2*(RNAi) leads to reduced progeny. (2) The Cry6Ba toxin is very rare in nature. We, and others, hypothesize that it has a special mechanism of action compared to other Cry PFTs, because of its distinct

protein structure and because its purified crystal protein is non-toxic to worms (Wei et al., 2003), suggesting that additional factors are necessary for its pathogenicity. Therefore, it might be possible that *C. elegans* has not had enough time to adapt its transcriptomic responses to this threat. (3) A completely opposite explanation to this last one would be, that the apparent maladaptive response of *C. elegans* to BT247 would actually be the product of pathogen manipulation of the host's immune system. (4) Yet another explanation is that, although keeping high *elt-2* levels during infection leads to reduced survival rate after 24h of infection, high levels of *elt-2* could result in faster recovery form infection, possibly after the worms are no longer exposed to the toxin. This results a very good explanation, as in nature it is unlikely that *C. elegans* is ever exposed to the high dosages of BT247 spores that we supply in the lab. To further explore this last possibility, we need to study if *elt-2* actually contributes to recovery form the damage caused by BT247. Similarly as for BT679, microscopy of intestinal integrity reporter strains (Hüsken et al., 2008), or the DNA dye PI (Zhang et al., 2016) , can be used to study the contribution of *elt-2* in repair from damage caused by BT247.

BT virulence factors, particularly Cry PFTs, might be elicitors of specific immune responses

BT679 kills the worm through the production of two distinct Cry three-domain (3d) PFTs: Cry21Aa3 and Cry14Aa2, which belong to the structural group of most commonly found Cry PFTs in pathogenic BT strains and are used in agriculture as pest control agents (Wei et al., 2003). All 3d Cry PFTs share a common ancestry (Wei et al., 2003). Our results on BT679 also show that *elt-2* positively regulates resistance to the PFT Cry21Aa3 and not Cry14Aa2, demonstrating that the host responses can be virulence-factor specific. A similar example has been shown before in the case of the contribution of the hypoxia pathway, mediated by the terminal TF *hif-1,* to the intrinsic cellular defences against the 3d PFT Cry5B but not the PFT Cry21A (both toxins share around 40% of protein identity (Wei et al., 2003)), although increased activity of HIF-1 leads to increased survival rate of worms on both toxins. The authors argued that Cry5B is the most potent toxin and this is why the loss of the hypoxia intrinsic cellular defence pathway is more easily seen in a phenotypic readout, such as survival rate (Bellier et al., 2009). *hif-1* is one of the host factors that when knocked out leads to increased survival rate on BT247, as mentioned in the previous section. Yet another example includes the study in which the glycolipid receptors for the PFT Cry5B were identified (Griffitts et al., 2005). In this study, glycolipid-synthesizing enzymes (*bre* genes) are required for Cry5B, and to a lesser extent Cry14A pathogenicity. The authors suggest that this is because Cry5B seems to exclusively have glycolipids as receptors, while Cry14A may have additional protein receptors other than glycolipids (Griffitts et al., 2005). In a separate study, it was also revealed that knockdown of the glycosyl-transferase *bre-3* conferred full protection to Cry5B but not Cry21A, suggesting that the 3d PFT Cry21A also has additional receptors in

the host (Chen et al., 2010). Our data confirms these results as a panel of glycosyl-transferase mutants was completely susceptible to BT679 and BT247 (**Chapter 1**).

All the previously mentioned Cry PFTs belong to the 3d family and have common ancestry, this makes finding differences in the elicited host protective responses remarkable. It suggests that evolutionary closely related toxins are likely to have highly divergent receptor specificities and mechanisms of action. It is possible that such differences in host responses to Cry toxins are also present in the unique domain family of Cry PFTs, which includes Cry6Aa and Cry6Ba. For virulence of the members of this Cry PFT family, as I discussed in the previous section, the host's aspartic protease *asp-1* plays an important role. It was previously reported that the host's endogenous necrosis pathway activated by aspartic protease *asp-1* negatively influences survival rate upon Cry6Aa exposure, by directly interacting with the toxin and preventing its degradation (Zhang et al., 2016). Knockout mutant *asp-1*(*tm666*) also had a higher survival rate upon exposure to BT247, a strain whose main nematocidal virulence factor is the PFT Cry6Ba (Hollensteiner et al., 2017). Possibly the mechanism of action through which *asp-1* contributes to susceptibility to Cry6Aa is the same for its contribution to Cry6Ba susceptibility. However, *elt-2*(RNAi) on *asp-1*(*tm666*) yielded no increase in the survival rate of worms beyond of what is seen on *asp-1*(*tm666*) mutants alone. This result strongly suggests that the increase in survival rate of *elt-2*(RNAi) is dependent on the *asp-1*-mediated necrosis pathway (**Chapter 1**). An important experiment to see how similar the responses to these two Cry PFTs are, would be to assess survival rate of *elt-2*(RNAi) worms upon exposure to Cry6Aa and see if the result is similar to what is observed upon BT247. It still remains to be confirmed if the increase in survival rate observed for *elt-2*(RNAi) worms on BT247 is exclusively due to the Cry6Ba toxin, or if additional virulence factors play a role. For this, the Cry6Ba toxin should be heterologously expressed either in *E. coli*, in a non-pathogenic BT strain like BT407 or in a strain derived from BT247 that has lost the Cry6Ba toxin and is no longer pathogenic, like it has been done for the BT679 Cry toxins (Masri et al., 2015). We have confirmed that when BT247 loses the Cry5Ba-encoding plasmids during experimental evolution, the bacterium is no longer able to kill *C. elegans* (personal communication Andrei Papkou). The function of *elt-2* in the defence against the Cry6Aa toxin still needs to be elucidated. It is very likely that differences between the host responses to Cry6Aa and Cry6Ba will be evidenced after more research effort has been spent, to discover more general and specific host cellular responses to these two toxins. It was already shown that the *asp-1*-mediated necrosis pathway plays no role in the defence against the 3d PFT Cry5Ba (Zhang et al., 2016).

This thesis and previous published studies make clear that *C. elegans'* responses to Cry PFTs are very diverse and in most cases toxin-specific. Every Cry PFT studied so far elicits a specific set of cellular responses. A single BT strain may contain an extensive cocktail of Cry PFTs, as is the case for the strain used in this

thesis, BT679 (Cry14A, Cry21A and Cry35A (Masri et al., 2015)), or strain YBT1518 (Cry5Ba2, Cry6Aa2 and Cry55Aa1 (Crickmore et al., 1998)). Therefore, in the future, the most sensible expectation is that the host's responses against any particular BT strain will be (strain-)specific, to the stresses generated by the Cry PFTs and additional virulence factors in the repertoire of the strain. This statement makes our original research question obsolete. Under our new model it is more likely that the host's responses to two divergent pathogen strains with virulence factors that share a common mechanism of action would be more similar, than the responses to two very closely related strains that have virulence factors with divergent mechanisms.

The available data makes evident that the question about high immune specificity should be asked at the virulence factor functional level, instead of the taxonomic level. In the past, PFTs have been perceived as unsophisticated and generalist virulence factors. However, they are widespread throughout life and have highly diverse mechanisms of action, pore architecture/permeability and receptors with which they interact in the target cell membrane (Dal Peraro and van der Goot, 2016; Jimenez et al., 2014). Multiple combinations of all these PFT characteristics have the potential to elicit cellular responses that are equally PFT-specific. This is, without mentioning the possibility of emerging attributes, product of multi-PFT interactions or interactions of PFTs with other virulence factors present simultaneously in a given pathogen model. The adjacent concentration of PFTs is an additional, quantitative factor, that might greatly influence the nature of cellular responses to a given PFT stress situation, which hasn't been studied in thorough detail (Dal Peraro and van der Goot, 2016). Cellular responses to PFTs have been considered to be very general. However, the molecular mechanisms of very few PFT-cellular response pairs have been characterized with enough detail, to allow the assessment of their specificity. The *C. elegans* detoxification machinery, which we repetitively found to be enriched in our infection transcriptomic datasets, might be much more complex than expected. Some of its components comprise expanded gene families, such as Cytochrome P450, with 81 genes, or Glutathione S-transferase, with 44 genes (Wormbase version WS262). For most of these genes a function has not yet been identified (Lindblom and Dodd, 2006).

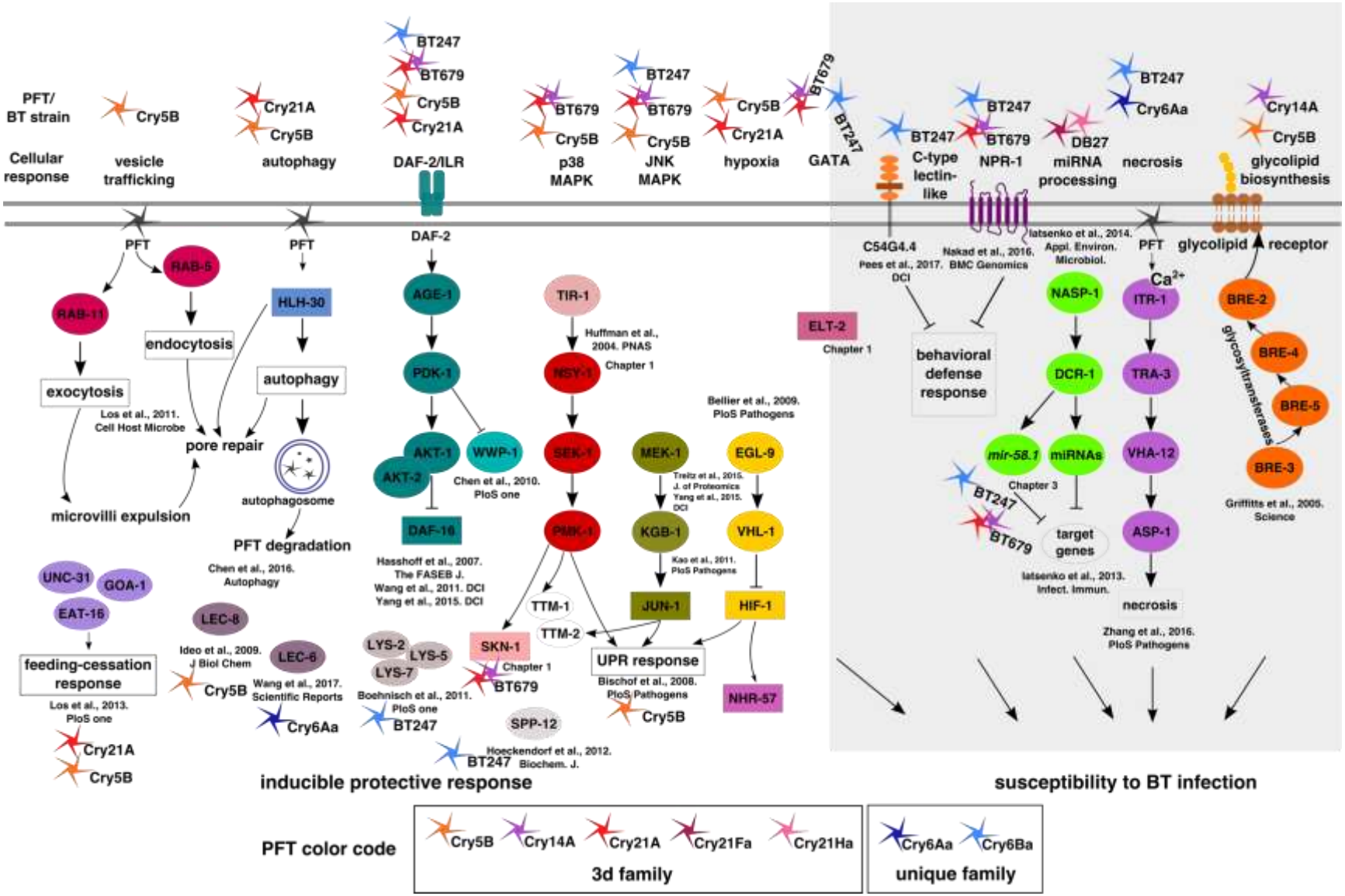
PFT/
BT strain
Cellular
response
Cry5B
vesicle
trafficking
Cry21A
Cry5B
autophagy
BT247
BT679
Cry5B
Cry21A
DAF-2/ILR
BT679
Cry5B
p38
MAPK
BT247
BT679
Cry5B
JNK
MAPK
Cry5B
Cry21A
hypoxia
BT679
BT247
GATA
BT247
C-type
lectin-
like
BT247
BT679
NPR-1
DB27
miRNA
processing
BT247
Cry6Aa
necrosis
Cry14A
Cry5B
glycolipid
biosynthesis
PFT
RAB-5
RAB-11
exocytosis
endocytosis
HLH-30
autophagy
pore repair
autophagosome
microvilli expulsion
PFT degradation
Los et al., 2011.
Cell Host Microbe
Chen et al., 2016.
Autophagy
PFT
DAF-2
AGE-1
PDK-1
AKT-1
AKT-2
WWP-1
DAF-16
Chen et al., 2010.
PloS one
Hasshoff et al., 2007.
The FASEB J.
Wang et al., 2011. DCI
Yang et al., 2015. DCI
TIR-1
Huffman et al.,
2004. PNAS
NSY-1
Chapter 1
SEK-1
PMK-1
Bellier et al., 2009.
PloS Pathogens
MEK-1
EGL-9
Treitz et al., 2015.
J. of Proteomics
Yang et al., 2015.
DCI
KGB-1
VHL-1
Kao et al., 2011.
PloS Pathogens
JUN-1
HIF-1
TTM-1
TTM-2
SKN-1
Chapter 1
UPR response
Bischof et al., 2008.
PloS Pathogens
NHR-57
LYS-2
LYS-5
LYS-7
Boehnisch et al., 2011.
PloS one
SPP-12
Hoeckendorf et al., 2012.
Biochem. J.
C54G4.4
Pees et al., 2017.
DCI
Nakad et al., 2016.
BMC Genomics
Iatsenko et al., 2014.
Appl. Environ.
Microbiol.
ELT-2
Chapter 1
behavioral
defense
response
PFT
glycolipid
receptor
NASP-1
DCR-1
mir-58.1
miRNAs
Chapter 3
target
genes
Iatsenko et al., 2013.
Infect. Immun.
Ca2+
ITR-1
TRA-3
VHA-12
ASP-1
necrosis
Zhang et al., 2016.
PloS Pathogens
BRE-2
BRE-4
BRE-5
BRE-3
glycosyltransferases
Griffitts et al., 2005.
Science
UNC-31
GOA-1
EAT-16
feeding-cessation
response
Los et al., 2013.
PloS one
LEC-8
Ideo et al., 2009.
J Biol Chem
LEC-6
Wang et al., 2017.
Scientific Reports
Cry5B
Cry6Aa
Cry21A
Cry5B
BT247
BT679
BT247
inducible protective response
susceptibility to BT infection
PFT color code
Cry5B
Cry14A
Cry21A
Cry21Fa
Cry21Ha
3d family
Cry6Aa
Cry6Ba
unique family

Figure 2. Host protective mechanisms against BT infection (white background) and host factors that lead to susceptibility to BT infection (grey background. Cry PFTs that elicit each response are marked next to/ on top of the molecule or pathway. Unfolded protein response (UPR). BT DB27 PFTs reported in (Iatsenko et al., 2014).

The missing immune recognition receptors that specifically detect the pathogen threat encountered

It has generally been assumed that a prerequisite for immune specificity involves the presence of a repertoire of diversified immune receptors (Little et al., 2006; Roth et al., 2009; Sadd and Schmid-Hempel, 2006). We can only fully explain *C. elegans* high immune specificity if we understand the way in which pathogenic threats are sensed to then elicit specific responses. In our current working model of the *C. elegans* specific immune response to infection with Cry PFTs, no Microbe-Associated Molecular Pattern (MAMP) receptors or Danger-Associated Molecular Pattern (DAMP) receptors have so far been described. Although in this thesis we have unveiled part of the complicated network of factors that are regulating specific transcriptomic responses to BT247 and BT679, we still don't know what signalling pathways and receptor molecules act upstream of *elt-2* in this host-pathogen system. *C. elegans* is an organism for which we have remarkably poor knowledge of the molecules that work as immune recognition receptors. Here, we explored the possibility of the diversified family (283 genes) of C-type lectin-like-domain-containing proteins (CTLDs) in *C. elegans* to fulfil such a role (**Chapter 2**) (Pees et al., 2016).

We found abundant transcriptomic data suggesting that CTLD-containing proteins could specifically respond to diverse pathogen threats, including Gram-positive bacteria, Gram-negative bacteria and fungi (**Chapter 2**) (Pees et al., 2016). Many CTLD-encoding genes are expressed in the intestine (Wormbase version WS262) (**Chapter 2**) (Pees et al., 2016), the tissue of BT infection and where *elt-2* is active. In fact, *elt-2* both positively and negatively regulates the expression of many CTLD-encoding genes, as is suggested by our *elt-2*(RNAi) data (*clec* genes) (**Chapter 1**). It is thus, very likely that a subset of CTLD-containing proteins plays a role in the general and specific responses against BT infection. However, to date our knowledge of the involvement of CTLD-encoding genes in the response against BT infection remains incipient. We only know that the transmembrane CTLD-containing protein C54G4.4 negatively regulates behavioural avoidance of BT247 (Figure 2) (Pees et al., 2017).

The difficulty of studying the involvement of CTLD-containing proteins in *C. elegans* immune responses is the general lack of phenotype upon gene knockout or knockdown. It is likely that the lack of phenotype is due to functional redundancy, in a gene family with such a large number of members. Functional analyses of whole gene clusters, or entire groups of paralogues, coupled with functional

studies at the protein level are still necessary to show the involvement of this gene family in immune responses. These would be possible thanks to the CRISPR-Cas9 genome editing technology (Paix et al., 2017).

Similar efforts must be undertaken to fully dissect the recognition role of other multigene families, which, like the CTLDs, have few members already identified as participating in immune responses. These multigene families include: the G-Protein Coupled Receptors (GPCRs, ~1300 genes) (Reboul and Ewbank, 2016) and the Nuclear Hormone Receptors (NHRs, ~284 genes) (**Chapter 1**) (Antebi, 2015; Liu et al., 2013) (Wormbase version WS262). Additional efforts to identify recognition receptors could include binding assays to find interactions between proteins obtained from whole worm lysates and pathogen cells, or pathogen derived virulence factors, such as Cry PFTs.

C. elegans mounts in part general responses to BT679 and BT247

Our research question focused on strain-specific responses, and therefore we have discussed in detail all host responses that we found to be different between BT247 and BT679. However, most of the transcriptomic response (91% of the DE genes) was common between the two strains. Among the response modules that are conserved we found that the JNK MAPK pathway and its downstream activator protein component *jun-1,* lead to protection against BT infection (**Chapter 1**, reported previously by (Kao et al., 2011)). Similarly, it has been reported that autophagy is necessary for general defence against bacterial PFTs (Chen et al., 2016), although these results were derived only from responses to Cry5B and Cry21A and no members of the unique domain Cry6 PFT family have been tested for this pathway. Additionally, miRNA-mediated negative regulation of immune effectors happens in the context of infection with both BT247 and BT679 (also reported for BT DB27 (Iatsenko et al., 2013)), as knockout worms *mir-58.1*(*n4640*) are more resistant to both BT strains (**Chapter 3**). This PhD thesis shows a new layer of complexity in the regulation of transcriptional responses to BT infection mediated by the fast-response miRNAs. We also found that knockout of the NHR *nhr-8*(*ok186*) leads to a moderate but significant increase in survival rate of worms exposed to both BT247 and BT679, representing a new host factor that leads to general susceptibility to BT (**Chapter 1**). The *nhr-8* function has been linked to regulation of steroid hormone signalling, detoxification, lipid metabolism and immunity (Liu et al., 2013; Magner et al., 2013). As discussed for *elt-2*, there may be many explanations for the “maladaptation” in the response of *C. elegans* mediated by *nhr-8* towards the optimal survival rate possible upon infection. What results interesting is that, in this case, the effect is common to both strains.

New overview of immune specificity across the metazoan tree and inter-taxa translational possibilities

Immune solutions to threats are tightly linked to the environment and the evolutionary history of each species. However, the principles that have driven the evolution of immune systems in all organisms are common. Therefore, the mechanistic understanding of other immune systems is likely to provide new insights into human immunity or that of commercially important species (Rimer et al., 2014). In this thesis, we conclude that **high immune specificity should be considered at the functional level of the sum of the virulence factors present in a given context**. Before we can thoroughly assess the level to which host responses are specific, we need to characterize in more detail the mechanism of action of PFTs and the cellular responses that they elicit. This includes understanding the cellular "decision-making" process for the resolution of multiple context-sensing input signals into a given output or response, and the responses themselves.

It would seem that the high complexity of the cellular responses to a given PFT-mediated infection context is almost impossible to comprehend. However, there are approaches that will help us understand complex transcription regulation networks, such as the one in which *elt-2* is a central node to respond to BT infection (Stefanovic and Christoffels, 2015). Such approaches include: (1) chromosome conformation capture technology, for the identification of long range genomic interactions (Sati and Cavalli, 2017); (2) ChIP-Seq technology to identify TF-TF, TF-cofactor, TF-chromatin-remodelling enzyme and TF-DNA interactions at specific genomic sites, like the GATA motif (Raha et al., 2010); (3) cell-sorting based transcriptomics that allow individual tissue- and cell-specific analysis and (4) genome-wide chromatin occupancy databases to gather all experimental data, like the ENCODE database for humans (The ENCODE Project Consortium, 2012). Additionally, in order to develop a comprehensible model of the complex transcription regulatory network of *C. elegans* immune responses to BT that has *elt-2* at its centre, a system biology approach is required (Stefanovic and Christoffels, 2015). Now, more than ever, we are able to study this complexity by integrating multiple forms of bioinformatics and empirical data into new host-pathogen models with great detail and resolution at the molecular level.

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List of Abbreviations

3d	three-domain
ABF	AntiBacterial Factor
AMD	Automated Motif Discovery
AP1	Activator Protein 1
ASP-1	Aspartyl Protease 1
BIN	BINary
BMP	Bone Morphogenetic Proteins
BRE	*Bacillus thuringiensis* toxin REsistant
BT	*Bacillus thuringiensis*
BT243	*Bacillus thuringiensis* MYBT18243
BT245	*Bacillus thuringiensis* MYBT18245
BT246	*Bacillus thuringiensis* MYBT18246
BT247	*Bacillus thuringiensis* MYBT18247
BT679	Bacillus thuringiensis MYBT18679
CAS-9	CRISPR-ASsociated protein 9
CDR	CaDmium Responsive
CFP	Cyan Fluorescent Protein
CFU	Colony Forming Units
CGC	Caenorhabditis Genetics Center
ChIP-Seq	Chromatin Immunoprecipitation Sequencing
CLEC	C-type LECtin
CNC	Caenacin
CRISPR	Clustered Regulatory Interspaced Short Palindromic Repeats
Cry PFT	Crystal Pore-Forming Toxin
CTLD	C-Type Lectin-Like Domain
CYP	Cytochrome P450 family
DAF	abnormal Dauer Formation
DAMP	Danger-Associated Molecular Pattern
DBL-1	DPP/BMP-Like
DCAR-1	DihydroCaffeic Acid Receptor
DCR-1	DiCer Related Helicase
DDP	DecaPentaPlegic (TGF-Beta family)
DE	Differentially Expressed
DFG	Deutsche Forschungsgemeinschaft-German Research Foundation
DHS	DeHydrogenases, Short chain
DIC	Differential Interface Contrast
DRH-1	Dicer Related Helicase
EASE	Expression Analysis Systematic Explorer
ELT-2	Erythroid-Like Transcription factor family
ENCODE	ENCyclopedia of DNA Elements

ERK	Extracellular-signal-Regulated Kinase
FER-15	Synonym of RRF-3 (RNA-dependent RNA polymerase Family)
FOXO	Forkhead boX O
FREP	Fibrinogen-RElated Protein
GFP	Green Fluorescent Protein
GLM	Generalized Linear Model
GLP-1	abnormal Germ Line Proliferation
GLY	GLYcosilation-related
GO	Gene Ontology
GOA-1	G protein, O, Alpha Subunit
GPCR	G-Protein-Coupled Receptor
GST	Glutathione S-Transferase
HEX	HEXosaminidase
HIF-1	Hypoxia Inducible Factor 1
HLH-30	basic Helix-loop-Helix 30
HPLA	4-HydroxyPhenylLactic Acid
HRP	HorseRadish Peroxidase
IFB-2	Intermediate Filament B
ILR	Insulin-Like Receptor
ILYS	Invertebrate LYSozyme
JNK	Jun amino-terminal Kinases
L4	fourth instar Larvae
LEC	gaLECtin
LIN-12	abnormal cell LINeage 12
LYS	LYSozyme
MAMP	Microbe-Associated Molecular Pattern
MAPK	Mitogen-Activated Protein Kinase
MIR	MIcroRna
miRNA	microRNA
mRNA	messenger RNA
MV	Microvilli
NBRP	National BioResource Project
NGM	Nematode Growth Medium
NHR	Nuclear Hormone Receptor
NLP	Neuropeptide-Like Peptide
NPR-1	NeuroPeptide Receptor family 1
p.i	post infection
PBS	Phosphate Buffered Saline
PFM	Peptone-free NGM
PI	Propidium Iodide
PMK	P38 Map Kinase family
PMP-5	Peroxisomal Membrane Protein related
POML	PON (paraoxonase) and MEC-6 Like

PPFR-1	Protein Phosphatase Four Regulatory subunit
PQM-1	ParaQuat (Methylviologen) responsive
PRR	Pattern Recognition Receptor
RFP	Red Fluorescent Protein
RIG-1	Retinoic acid Inducible Gene 1
RNA-Seq	RNA Sequencing
RNAi	RNA interference
ROS	Reactive Oxygen Species
SAPK	Stress-Activated Protein Kinases
SBP-1	Sterol regulatory element Binding Protein (in *C. elegans*)
SEK-1	SAPK/ERK kinase
SEL-11	Suppressor/Enhancer of Lin-12
SEM	Standard Error of the Mean
SKN-1	SKiNhead 1
SPP	SaPosin-like Protein family
SREBP	Sterol Regulatory Element Binding Protein (in Human)
TEM	Transmission Electron Microscopy
TF	Transcription Factor
TFEB	Transcription Factor E-Box
TGF-β	Transforming Growth Factor Beta
TM buffer	TetraMisole buffer
TOF	Time of Flight
TRE	TREhalase
UPR	Unfolded Protein Response
XBP-1	X-Box-Binding Protein
ZIP	bZIP transcription factor family

List of Equipment

Equipment	Brand	Setting/Specification
dissecting scope	Leica S6E	Leica KL 200 LED (Light source)
Cleanbench	Heraeus	Heraeus LaminAir HBB2472 K GS
Thermostatschrank	Lovibond	25°C
Spectrophotometer	Jenway 6300	
Trans-Blot Turbo Transfer System	Biorad	
GyroMini™ Nutating Mixer		
Microcentrifuge 5415 R	Eppendorf	
Mini-centrifuge	IKA mini G S000	
Vortexer SU 1900	sunlab	
dissecting scope	Leica S6E	Leica KL 200 LED (Light source)
Vortexer Reax 1 D	Heidolph	
Stereomicroscope Leica M80	Leica	Leica KL 1500 compact (Light source)
Stereomicroscope Leica M80	Leica	Leica KL 1500 LCD (Light source)
Microscope Standard 25	ZEISS	
Shaker (Falcon tubes)	GFL 3006	
Vortex-Genie 2	Vortex	
Autoclave	Systec VX-120	Series Number 2791
Memmert Incubator	80°C	
Laboratory dish washer Miele G 7883 Professional		
Centrifuge 5810R	Eppendorf	
Fine balance PCB	Kern	2000-1
IKA RET basic IKAMAG		Magnetic stirrer with heating plate
RH basic 2 IKAMAG®	VWR	Magnetic stirrer with heating plate
pH meter HI 221	Hanna Instruments	
Thermomixer	ditabis	Heiz-ThermoMixer MHR 11
Thermomixer	Eppendorf	F2.0
Cleanbench	Kojair	Kojair BW-130
Rumed Incubator	Rumed	20°C
BIOTEK plate reader	Eon	
Centrifuge	Hettich GmbH & Co. KG	Universal 320
dissecting scope	Leica S6E	Leica KL 200 LED (Light source)
Mini-centrifuge	Rotilabo	
Vortex Mixer	Stuart SA8	
Mini-centrifuge	IKA mini G S000	

Microscope (Microinjection)	ZEISS Axio Observer	Software Axio Vision
CLSM	ZEISS Axio Observer	Software Zen black
Leica fluorescence dissecting scope	Leica M205FA	HXP R 120W (Light source), Cam DFC 420C
Panasonic Microwave	NN-SD 456W	
Power source 300V	VWR	
ChemiDoc™ Touch Imaging System	Biorad	
Thermal Printer	Mitsubishi	
Mini ReadySub-Cell™ GT Horizontal Electrophoresis Cell	Biorad	
Mini-PROTEAN® Tetra Cell Systems	Biorad	
PCR Cycler	SensoQuest Labcycler	with and without temperature gradient
Sartorius CERTOMAT IS	Sartorius	Shaking incubator
Incubator	Binder	37°C
Thermostatschrank	Lovibond	20°C
Rumed Incubator	Rumed	15°C
minus 80°C freezer	Scala	
Tissue shredder	Geno / Grinder	120 strokes per minute

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Curriculum Vitae

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