

Transcriptome analysis of the *xrep-1* (*RNAi*) phenocopy in *C. elegans*Koichi Hasegawa^{1)*} and Johji Miwa^{1), 2)}¹⁾College of Bioscience & Biotechnology, Chubu University,²⁾Institute for Biological Function, Chubu University

Abstract

Phase II enzymes play a major protective role against xenobiotics. Little is known, however, about molecular mechanisms operating in the regulation of these enzymes against xenobiotics. To dissect a signaling pathway from a starting-gate where xenobiotics meet their target to a terminal where phase II enzymes are induced, we have isolated many *Caenorhabditis elegans* mutants in the xenobiotics response pathways (*xrep*). One of the genes we have identified so far is *xrep-1*. It encodes a nematode homolog of WD repeat-containing proteins. The transcriptional profile for *xrep-1* loss-of-function mutants is similar to that for *xrep-1*(*RNAi*) animals and also N2 wild-type animals treated with acrylamide, an ideal model xenobiotic compound. The present transcriptome analysis demonstrates that XREP-1 regulates negatively a large number of phase II as well as some phase I enzymes.

Key words: *Caenorhabditis elegans*, xenobiotics, *xrep-1* gene, WD repeat protein, Glutathione S-transferases, phase II enzyme.

Introduction

In studying actions in and effects on the model organism *C. elegans* by acrylamide (AA), once thought to be merely a hazardous industrial chemical and now considered to be one of the most prevalent and widely discussed food contaminants, we have gradually realized that it is not simply a harmful chemical but could be a beneficial one dependent upon concentrations. That is, we found that, physiologically, low (0.5 to 50 µg/L) and high (≥500 mg/L) AA concentrations shortened the animal's lifespan, whereas AA concentrations between them (especially 5 to 50 mg/L) caused no deleterious effect on the animal's lifespan (Hasegawa et al., 2004). Biochemically, at the low AA concentrations no phase II enzymes were upregulated, whereas a large number of them, glutathione S-transferases (GSTs) in

particular, were induced and upregulated at intermediate or higher concentrations (Hasegawa et al., 2008). We reason that this biphasic response of lifespan against AA concentration is caused by AA's own property of inducing phase II enzymes, which in turn function to eliminate AA itself from the animal's body. Thus, we think this is the mechanism behind the so-called "hormetic" response.

We have recently applied the above reasoning to an essential component of *Wasabi*, allyl isothiocyanate (AITC), as an inducer of phase II enzymes, and found that AITC indeed confers resistance on animals against oxidative stressors like juglone and paraquat (Hasegawa et al., 2010).

Xenobiotics, such as harmful food substances like AA, environmental contaminants, and exotoxins from infected microorganisms, together with endogenous

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toxicants, such as reactive oxygen species or free radicals from energy metabolic by-products, are now recognized to be direct or indirect threats to our lives, which eventually lead to aging and age-related diseases, including cancers (Friedman, 2003; Dybing et al., 2008; Amzal et al., 2009; Joseph, 2009; Tan et al., 2009). At the same time, many xenobiotics are also very good inducers of phase II enzymes. Therefore, some of those that are not very toxic themselves are potentially useful substances to slow down aging or to prevent diseases because of their hormetic action.

From a toxicological point of view, AA is a fascinating chemical, for it is an excellent xenobiotic stressor inducing numerous phase II enzymes and confers resistance against oxidative stresses and bacterial infection (unpublished results; Miwa, S. et al., 2009, personal communication). Thus, we have decided to use AA as a model xenobiotic.

Amongst all phase II enzymes, a family of glutathione S-transferases (GSTs) is found in almost every organism from prokaryotic bacteria to humans (Vuilleumier & Pagi, 2002; Hayes et al., 2005; Lindblom & Dodd, 2006) and recognized to be a major player for protecting organisms against a variety of xenobiotics and endogenous toxicants (Gao et al., 2001; Nguen et al., 2003; Hayes et al., 2005; Hasegawa et al., 2008).

Thus, to dissect genetically a xenobiotic response pathway, which we define as a pathway from the starting point where xenobiotics find a target to the terminal point where phase II enzymes are expressed, we have been isolating *Caenorhabditis elegans* mutants in the xenobiotics response pathways (*xrep*) that exhibit abnormal GST regulation against AA. We have so far identified four different genes from 24 *Xrep* mutants and at molecular and cellular levels analyzed one such gene, *xrep-1*: it encodes a nematode homolog of the WD repeat-containing protein and

negatively regulates the phase II enzyme GST expression (Hasegawa and Miwa, manuscript in preparation). Here in this report we present the *C. elegans* whole genome expression profile obtained from the *xrep-1* gene knockdown animal, that is, from the *xrep-1 (RNAi)* phenocopy.

Materials and Methods

Nematode culturing and strains

Nematode culturing and handling were carried out as described by Brenner (1974). Strains used in the present experiments were: N2 (*C. elegans* ver. Bristol), MJC003 {*kEx3[gst-7::gfp, pRF4]*}, MJC009 {*unc-119(ed3) III; kEx09[gst-38::gfp, pDP#MM016B]*}, MJC012 {*unc-119(ed3) III; kEx12[gsto-2c::gfp, DP#MM016B]*}, MJC017 {*xrep-1(k1017) I; unc-119(ed3) III; kIs17[gst-4::gfp, pDP#MM016B] X*}, MJC028 {*unc-119(ed3) III; kEx28[gsto-2b::cfp, pDP#MM016B]*}, MJC058 {*unc-119(ed3) III, kIs15[gst-4::rfp, gst-2::gfp, pDP#MM016B] IV*}.

RNAi

Gene fragments of *xrep-1* cDNA was prepared by PCR amplification of *C. elegans* N2 cDNA with the following primers: *xrep-1_exon4-11_For* 5' – AGG GAA CAA CAT ATT GCA TTT AGT – 3'; *xrep-1_exon4-11_Rev*EcoRI 5' – GGG AAT TCT TGG GAT GAT CGT ATG GTG CAA – 3'. Each PCR fragment was digested with *EcoRI* and cloned into the *EcoRI* restriction site of the RNAi vector pPD129.36 (kindly provided by Fire, A., Stanford University). The PCR fragment-ligated plasmid or the blank vector pPD129.36 was used to transform *E. coli* HT115 (Kamath et al., 2001).

Microarray

Three independent lines of synchronized L1-stage animals were prepared by treatment of egg-containing

adults with sodium hypochlorite (Stiernagle, 1999) and grown for 48 hours at 20°C on 500 mg/L acrylamide or control (without acrylamide) plates (both containing 50 µg/mL ampicillin and 12.5 µg/mL tetracycline) seeded with *E. coli* HT115 transformed with either *xrep-1* containing or blank plasmids. By this time, the animals on each plate were at late L4 stage. The animals were isolated and washed with M9 buffer, and then frozen in liquid nitrogen until all samples were collected. Total RNAs were isolated from each of the culture lines with the Total RNA Isolation Kit (Fujifilm, Japan) according to the manufacturer's instructions, and biotin-labeled aRNAs were prepared with the Affymetrix GeneChip 3' IVT Expression Kit (Affymetrix). Biotinylated aRNAs were fragmented and hybridized to the GeneChip *C. elegans* array, according to the Affymetrix instruction protocol. Washing, staining, and scanning were also performed with Affymetrix Fluidic Station 450 and GeneChip scanner 3000 (Affymetrix).

Microarray image data were analyzed by Affymetrix Expression Console software version 1.1. The signal values from each probe were first normalized and then calculated with the MAS 5.0 algorithm. Triplicate data were analyzed with SAM (Significance analysis of microarray) software (Tusher et al., 2001) to identify significant differences in RNA expression levels. Functional annotation of the significantly expressed genes was performed with the program DAVID 2008 (Huang et al., 2009).

Construction of transgenic animals with *xrep-1(k1007)*

By standard genetic methods, the *xrep-1(k1007)* was transferred into MJCU058 $\{(kIs15) IV\}$ to construct MJCU059 $\{xrep-1(k1007) I; (kIs15) IV\}$. MJCU059 was crossed with MJCU003 $\{kEx3$

$[gst-7::gfp, pRF4]\}$, MJCU009 $\{kEx9[gst-38::gfp, pDP\#MM016B]\}$, MJCU012 $\{kEx12[gsto-2c::gfp, pDP\#MM016b]\}$, or MJCU028 $\{kEx28[gsto-2b::cfp, pDP\#MM016B]\}$ to make the strains MJCU069 $\{xrep-1(k1007) I; kIs15 IV; kEx12\}$, MJCU070 $\{xrep-1(k1007) I; kIs15 IV; kEx28\}$, MJCU071 $\{xrep-1(k1007) I; kIs15 IV; kEx3\}$, MJCU072 $\{xrep-1(k1007) I; kIs15 IV; kEx9\}$. Fluorescence expression patterns were observed with a Nikon SMZ800 dissection microscope equipped with a fluorescence filter.

Results and Discussion

By genetic screening we have isolated many *xrep* mutations that exhibited abnormal GST regulation against the model xenobiotic acrylamide (AA). One of these mutations, *xrep-1*, is recessive and constitutively expresses GST over the whole body in the absence of AA. The *xrep-1* gene cloning and sequencing revealed that it encodes a nematode homolog of the WD repeat-containing protein, which negatively regulates GST-4 and GST-30 expressions (Hasegawa & Miwa, manuscript in preparation). We investigated gene expression changes in animals lacking the *xrep-1* function.

Of the 22,500 genes examined, 90 genes were upregulated whereas only three were downregulated in the *xrep-1(RNAi)* animal (Table 1). These genes were classified according to their biological function by DAVID (Huang et al., 2009). Many of the upregulated genes corresponded to phase I and II detoxification enzymes, which include 16 members of glutathione S-transferases (*gst*), 3 members of UDP-glucuronosyl/glucosyl transferases (*ugt*), and 5 short-chain type dehydrogenases (*sdr*) (Table 1). Genes that encode enzymes necessary for glutathione metabolism, thioredoxin, and oxidoreductase were

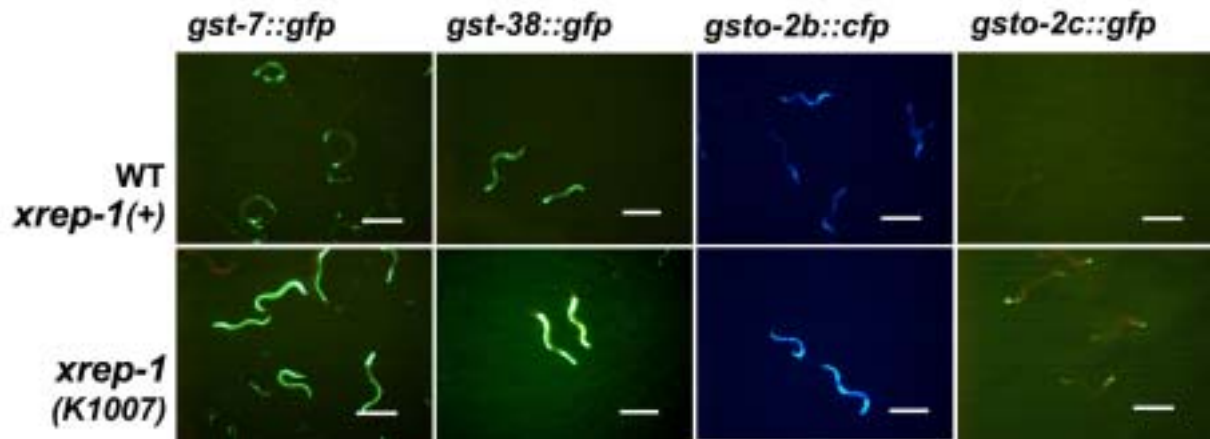


Figure 1. The *xrep-1(k1007)* mutation affects the expression of several acrylamide-responsive *gst* genes. In the *xrep-1(+)* background (upper panels), MJCU003 (*gst-7::gfp*), MJCU009 (*gst-38::gfp*), MJCU012 (*gsto-2c::gfp*) (C02D5.3c), and MJCU028 (*gsto-2b::cfp*) (C02D5.3b) constitutively express their GST (upper), whereas their expression is induced in mutants homozygous for *xrep-1(k1007)* (lower panels).

also distinctively upregulated (Table 1). This expression feature was very similar to that for acrylamide-treated N2 animals (Hasegawa et al., 2008). We further confirmed the *xrep-1(k1007)* mutant, which constitutively expressed GST-4 and GST-30 from the L1 stage animals in the absence of AA (Hasegawa & Miwa, in preparation), by crossing it with transgenic animals that carry extrachromosomal xenobiotics-responsive *gst::gfp* or *gst::cfp* fusion genes, MJCU003 (*gst-7::gfp*), MJCU009 (*gst-38::gfp*), MJCU012 (*gsto-2c::gfp*), and MJCU028 (*gsto-2b::cfp*) (Hasegawa et al., 2008). The constitutive expression patterns of these transgenics differed from one another, and AA treatment compounded their inductive expressions onto the constitutive expressions (Hasegawa et al., 2008). The similar compounded expressions were observed in the transgenics harboring the mutation *xrep-1(k1007)* (Figure 1). In *C. elegans*, the Cap-N-Collar family of the transcription factor SKN-1 functions similarly to the mammalian NF-E2-related factor Nrf2, which induces phase II enzyme

expression in response to xenobiotics (An & Blackwell, 2003; An et al., 2005; Hasegawa et al., 2008; Tullet et al., 2008; Oliveria et al., 2009; Park et al., 2009). Constitutive GST expression caused by the *xrep-1* mutation was also under the SKN-1 control (Hasegawa & Miwa, manuscript in preparation).

The negatively-regulating gene *xrep-1* encodes a WD repeat-containing protein, predicted to form a β -propeller structure, which provides a site for interaction with other proteins (Hudson and Cooley, 2008). In mammals, a role similar to that for XREP-1 is played by the oxidative stress sensor Keap1 (Kobayashi et al., 2006), whose Kelch-repeat domains are also predicted to form the β -propeller structure (Hudson and Cooley, 2008). Thus in *C. elegans*, XREP-1 might control SKN-1 similarly to the mammalian Keap1 for Nrf2.

We continue to study the three remaining *xrep* genes, as we continue seeking for new groups of *xrep* mutants to understand the whole of the xenobiotic response pathways.

Table 1 Genes upregulated or downregulated in the *xrep-1* (RNAi) animal

	Gene	Gene title	Fold change
Phase II enzyme, UP-regulated			
1	<i>gst-1</i>	Glutathione S-transferase, pi class	1.2
2	<i>gst-4</i>	Glutathione S-transferase, sigma class	2.3
3	<i>gst-5</i>	Glutathione S-transferase, sigma class	1.4
4	<i>gst-6</i>	Glutathione S-transferase, sigma class	2
5	<i>gst-7</i>	Glutathione S-transferase, sigma class	2.1
6	<i>gst-8</i>	Glutathione S-transferase, sigma class	2.7
7	<i>gst-10</i>	Glutathione S-transferase, pi class	1.6
8	<i>gst-13</i>	Glutathione S-transferase, sigma class	1.8
9	<i>gst-16</i>	Glutathione S-transferase, sigma class	3.3
10	<i>gst-20</i>	Glutathione S-transferase, sigma class	1.6
11	<i>gst-25</i>	Glutathione S-transferase, pi class	6.4
12	<i>gst-30</i>	Glutathione S-transferase, sigma class	5.7
13	<i>gst-38</i>	Glutathione S-transferase, sigma class	4.5
14	<i>gst-39</i>	Glutathione S-transferase, sigma class	1.9
15	<i>gst-40</i>	Glutathione S-transferase, sigma class	6.4
16	<i>gst-2</i>	Glutathione S-transferase, omega class	2.3
17	<i>ugt-10</i>	UDP-glucuronosyl /glucosyl transferase	3.3
18	<i>ugt-13</i>	UDP-glucuronosyl /glucosyl transferase	2.9
19	<i>ugt-48</i>	UDP-glucuronosyl /glucosyl transferase	2.3
Phase I enzyme, UP-regulated			
20	<i>sdz-8</i>	Short chain-type dehydrogenase	3.8
21	<i>dhs-8</i>	Short chain-type dehydrogenase	3.7
22	C55A6.6	Short chain-type dehydrogenase	2.2
23	C55A6.7	Short chain-type dehydrogenase	1.3
24	F20G2.2	Short chain-type dehydrogenase	1.2
Thioredoxin, UP-regulated			
25	C35B1.5	Thioredoxin	1.4
26	F26E4.12	Glutathione peroxidase	1.5
27	Y52E8A.3	Thioredoxin	5.3
28	<i>cdr-1</i>	Glutathione S-transferase-like protein	0.7
Glutathione metabolic process, UP-regulated			
29	<i>gcs-1</i>	Gamma-glutamylcysteine synthetase	2
30	C46F11.2	Pyridine nucleotide oxidoreductase	1
31	M176.2	Glutathione synthase	1.3
Oxidoreductase activity, UP-regulated			
32	W06H8.7	NADH:flavin oxidoreductase	5
33	F17A9.4	NADH:flavin oxidoreductase	2.2
34	F56D5.3	NADH:flavin oxidoreductase	4.2
35	F20D6.11	FAD-binding oxidoreductase	2.3
36	C46H11.2	Flavin-containing monooxygenase	1.1
37	<i>sod-1</i>	Superoxide dismutase	0.8
38	<i>alh-1</i>	Aldehyde dehydrogenase	0.6
39	F55G1.9	Pyrroline-5-carboxylate reductase	3
Metabolism related, UP-regulated			
40	C12C8.2	Cystathionine beta-lyases	1.9
41	Y32F6.4	Amino acid transporter	0.8
42	<i>fil-1</i>	Triacylglycerol lipase	1.2
43	K10C2.3	Aspartyl protease	3.1
44	<i>msra-1</i>	Methionine sulfoxide-S-reductase	1.6
45	C52E4.5	Glycosyl hydrolases	0.7
46	<i>ptps-1</i>	6-pyruvoyl tetrahydrobiopterin synthase	1.7
47	<i>pcp-2</i>	Hydrolytic enzymes	1.6

48	T12A2.8	5'-3' exonucleases	0.8
49	C37H5.6	Adenylosuccinate synthase	0.7
50	C50B6.7	Alpha-amylase	0.6
51	Y32F6A.5	Serine carboxypeptidase	0.9
52	<i>fce-1</i>	Metalloprotease	0.4
53	F37H8.3	Hydrolase	1.4
54	<i>ssp-8</i>	Prosaposin	0.3
55	<i>ssp-10</i>	Prosaposin	0.6
56	<i>tag-196</i>	Cysteine proteinase cathepsin F	0.5
Others, UP-regulated			
57	<i>cllec-173</i>	C-type lectin	0.9
58	<i>cllec-3</i>	C-type lectin	6.8
59	<i>cllec-66</i>	C-type lectin	1.7
60	<i>hel-1</i>	ATP-dependent RNA helicase	0.3
61	F49E2.1	Molybdenum cofactor biosynthesis protein	0.9
62	Y1910A.4	Permease	0.8
63	F01D5.2	Secreted surface proteins	3.1
64	D2045.8	Polymerase delta-interacting protein	2
65	F56D10.1	Carboxypeptidase	1.3
66	Y42G9A.3	Leucin rich repeat	2.8
67	F14D7.6	Transmembrane protein	1.3
68	<i>mab-21</i>	Cell fate specification proteins	1.3
69	<i>fbxa-51</i>	F-box protein	1.1
70	C07D8.6	Aldo/keto reductase	0.8
71	<i>aqp-11</i>	Aquaporin	1.3
72	Y40B10A.2	O-methyltransferase	1.5
73	<i>oac-54</i>	O-acyltransferase	1.4
74	T09F3.2	Mitochondrial carrier protein	0.6
75	M04B2.4	Oxidoreductase	0.7
76	F55G11.8	CUB domain	2.2
77	H20E11.2	CUB domain	2.9
78	F55G11.2	CUB domain	5
79	H20E11.3	CUB domain	2.8
80	C32H11.4	CUB domain	4.5
81	ZK896.4	CUB domain	1.5
82	<i>dod-17</i>	Unknown	3.3
83	<i>dod-24</i>	Unknown	3.6
84	E02H9.3	Unknown	1.1
85	C18H9.6	Unknown	1.7
86	F18E3.11	Unknown	1.6
87	F55G11.4	Unknown	1.3
88	R102.4	Unknown	1.6
89	C38D4.1	Unknown	0.7
90	F53A9.1	Unknown	1
Down-regulated			
91	R12C12.1	Glycine dehydrogenase	- 0.3
92	F28A12.4	Aspartyl protease	- 0.9
93	R07E5.1	RNA binding protein	- 0.3

Microarray analysis was repeated three times.

Numbers represent fold-change of log₂ ratio, compared with *xrep-1* (+) without acrylamide.

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