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COMMUNICATION

Caenorhabditis elegans mom-4 is required for the activation of the p38 MAPK signaling pathway in the response to *Pseudomonas aeruginosa* infection

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ABSTRACT

The p38 mitogen-activated protein kinase (MAPK) plays an evolutionarily conserved role in the cellular response to microbial infection and environmental stress. Activation of p38 is mediated through phosphorylation by upstream MAPKK, which in turn is activated by MAPKKK. In the *Caenorhabditis elegans*, the p38 MAPK (also called PMK-1) signaling pathway has been shown to be required in its resistance to bacterial infection. However, how different upstream MAP2Ks and MAP3Ks specifically contribute to the activation of PMK-1 in response to bacterial infection still is not clearly understood. By using double-stranded RNA-mediated interference (RNAi) and genetic mutants of *C. elegans*, we demonstrate that *C. elegans* MOM-4, a mammalian TAK1 homolog, is required for the resistance of *C. elegans* to a *P. aeruginosa* infection. We have also found that the MKK-4 of *C. elegans* is required for *P. aeruginosa* resistance, but not through the regulation of DLK-1. In summary, our results indicate that different upstream MAPKKKs or MAPKKs regulate the activation of PMK-1 in response to *P. aeruginosa*.

KEYWORDS *C. elegans*, MAPK, innate immunity, p38, *P. aeruginosa* PA-14, MOM-4

INTRODUCTION

Mitogen-activated protein kinase (MAPK) signaling pathways play an important role in diverse physiological processes, including development, growth and proliferation, stress responses, and immunity (Herskowitz et al.,1995; Waskiewicz et al.,1995; Schaeffer et al.,1999; Widmann et al.,1999; Kyriakis et al., 2001). MAPK signal transduction pathways are conserved from yeast to mammals (Herskowitz et al.,1995; Waskiewicz et al., 1995; Widmann et al.,1999). Based on their structural and biochemical features, three main subgroups of MAPK have been identified, including extracellular signal-regulated kinase (ERK), stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) and p38/MPK2 (Cobb et al., 1991; Dérijard et al., 1994; Han et al., 1994; Kyriakis et al., 1994; Lee et al., 1994). Mammalian p38 has been shown to play an extremely important role in the immune system (Symons et al., 2006). Activation of p38 is regulated by upstream MAP2Ks, MKK3 and MKK6 (Raingeaud et al., 1996; Wysk M et al., 1999; Tanaka et al., 2002). Recent reports from loss-of-function studies demonstrate that MKK4 is also required for ultraviolet radiation-induced activation of p38 MAPK (Brancho et al., 2003). At higher upstream levels, numerous MAP3Ks including MEKK, ASK, TAK1, Tpl-2/Cot and MLK/DAK have been implicated in the p38 activation (Yamaguchi et al., 1995; Tibbles et al.,1996; Ichijo et al.,1997; Deacon et al.,1999; Chiariello et al., 2000). Our

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previous results have pointed out the complexity of the MAP kinase signaling pathway, which is due to the fact that each individual MAP kinase can be activated by two or three upstream MAP2Ks and multiple MAP3Ks in *Drosophila* under different stimuli (Zhuang et al., 2006). Upon activation, p38 can phosphorylate and activate a number of transcription factors including ATF-2/ATF-7, CHOP/GADD153, Elk1, MEF2-C, Sap1 and CREB (Wang et al.,1996; Raingeaud et al.,1996; Han et al.,1997; Iordanov et al.,1997; Whitmarsh et al.,1997; Shivers et al., 2010), and protein kinases such as MAPKAPK2, MNK, PRAK, MSK1 and RSK-B (Stokoe et al.,1992; Fukunaga et al.,1997; Waskiewicz et al.,1997; New et al.,1998; Deak et al.,1998; Pierrat et al.,1998), thus eliciting different cellular responses.

The nematode *Caenorhabditis elegans* has been used as a model host for infection studies with the human opportunistic pathogen *Pseudomonas aeruginosa* PA-14 to identify evolutionarily conserved mechanisms of innate immune responses (Kim et al., 2005). The mammalian p38 homolog PMK-1 has been found to be required for the resistance of *C. elegans* to *P. aeruginosa* infection (Berman et al., 2001; Kim et al., 2002). Further studies showed that the NSY-1/SEK-1/PMK-1 cassette is an evolutionarily conserved module used in the defense against *P. aeruginosa* infection (Kim et al., 2002). However, whether other MAP3Ks or MAP2Ks also contribute to the activation of PMK-1, remains unknown. On the other hand, although the PMK-1 MAPK signaling pathway has been shown to play an important role in the innate immune response, little is known about whether the activation of PMK-1 is also involved in *C. elegans's* responses to other stimuli. In this study, we have used RNA interference (RNAi) and genetic mutants to suppress the expression of individual MAP kinases. Our results indicate that individual MAPKKKs and MAPKKs specifically regulate PMK-1 activation.

RESULTS

Activation of *C. elegans* p38/PMK-1 by different stimuli

In *C. elegans*, the mammalian p38 homolog PMK-1 has been found to be required for the resistance to *P. aeruginosa* infection. To analyze the signaling pathway of the *C. elegans* PMK-1/p38 activation in other stresses, we first determined whether PMK-1 can be activated by different stimuli including heat shock, osmotic stress by Sorbitol, *P. aeruginosa* infection and UV stress. Wild type *C. elegans* strain *N2* worms were exposed to different stimuli. The activation of PMK-1 was identified using p38 T^{180}/Y^{182} phospho-specific antibody. As shown in Figures 1A–D, the stimulation of *N2* worms with heat shock (35°C), sorbitol (200 mmol/L), *P. aeruginosa* infection or UV (1200 J) distinctly induced the activation of PMK-1. These results suggest that PMK-1/p38 may play a general role in *C. elegans's* response to various stimuli.

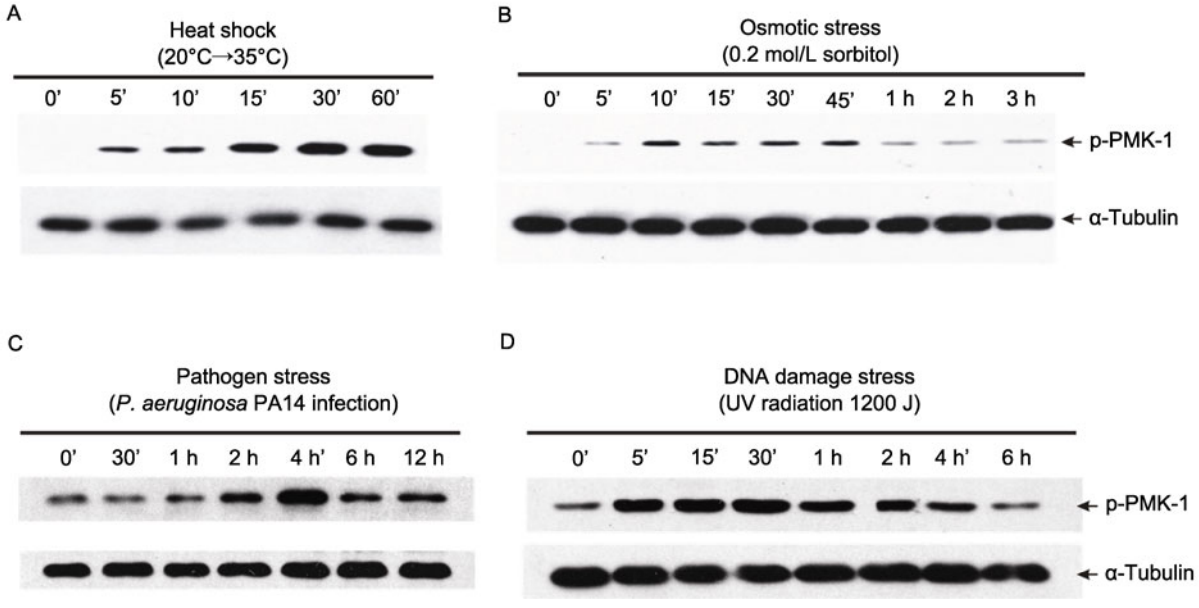

Figure 1. Activation of PMK-1 in *C. elegans* by various stimuli. (A) Heat shock. Young adult *N2* worms were washed with M9 buffer from OP50-1 NGM plates, and incubated in a 35°C water bath for the time indicated. (B) Osmotic stress. Sorbitol was added to young adult *N2* worms in M9 buffer for a final concentration of 200 mmol/L, and incubated for the time indicated. (C) Pathogenic infection. Young adult *N2* worms were placed onto plates seeded with *P. aeruginosa* for the time indicated. (D) DNA damage stress. Young adult *N2* worms were exposed to 1200 J UV, and then cultured in M9 buffer for the time indicated. Following exposure, worms were collected in M9 buffer, lysed and analyzed by western blot. Activation of PMK-1 was detected by blotting with anti-phospha-p38 antibody (upper panel), as indicated. Total proteins were measured by blotting with anti-α-tubulin (lower panel).

Activation of PMK-1 is required for resistance to *P. aeruginosa* infection

Previous studies have revealed that the activation of the NSY-1→SEK-1→PMK-1 MAP kinase cascade is essential for *C. elegans* to respond to pathogenic attacks (Kim et al., 2002).

To investigate further whether the different components of the PMK-1 signaling pathway play a role in the innate immune responses to microbial infection, we examined the survival rate of the MAP2K or MAP3K mutant strains after having *C. elegans* infected with the pathogenic *P. aeruginosa* strain *P. aeruginosa*. In consistence with previous findings, the *nsy-1*, *sek-1* and *pmk-1* mutant strains are more susceptible to *P. aeruginosa* infection (Fig. 2A). We have utilized RNAi to reduce the expression of *nsy-1*, and *sek-1*, and analyzed the susceptibility of these RNAi-treated worms to pathogenic *P. aeruginosa* infection (Fig. S1A). As shown in Fig. 2B, the RNAi of *nsy-1*, and *sek-1* make *C. elegans* more susceptible to *P. aeruginosa* infection. Consistent with these results, RNAi of *nsy-1*, and *sek-1* inhibited the activation of p38/PMK-1 in response to PA14 infection (Fig. S1B). The activation of p38/PMK-1 is also significantly reduced in *nsy-1*, *sek-1* or *pmk-1* mutants in response to PA 14, suggesting an involvement of NSY-1 and SEK-1 in regulating the activation of p38/PMK-1 (Figs. 2C and S2).

C. elegans MAPKK MEK-1 is required in the activation of PMK-1 for resistance to *P. aeruginosa* infection

Although NSY-1/SEK-1/PMK-1 cassette was known as an evolutionarily conserved module used in the defense against *P. aeruginosa* infection, whether other MAP3Ks or MAP2Ks also contribute to the activation of PMK-1, remains unclear. We found that the mutation of *mek-1* also significantly increased the susceptibility of *C. elegans* to *P. aeruginosa* infection. However, there is no significant change of susceptibility in the *jkk-1* mutants to *P. aeruginosa* infection, compared to wild type N2 worms (Fig. 2A). We have utilized RNAi to reduce the expression of *mek-1* and *jkk-1*, and analyzed the susceptibility of these RNAi-treated worms to a pathogenic *P. aeruginosa* infection. As shown in Fig. 2B, the RNAi of *mek-1*, but not of *jkk-1*, made *C. elegans* more susceptible to *P. aeruginosa* infection. As demonstrated in previous

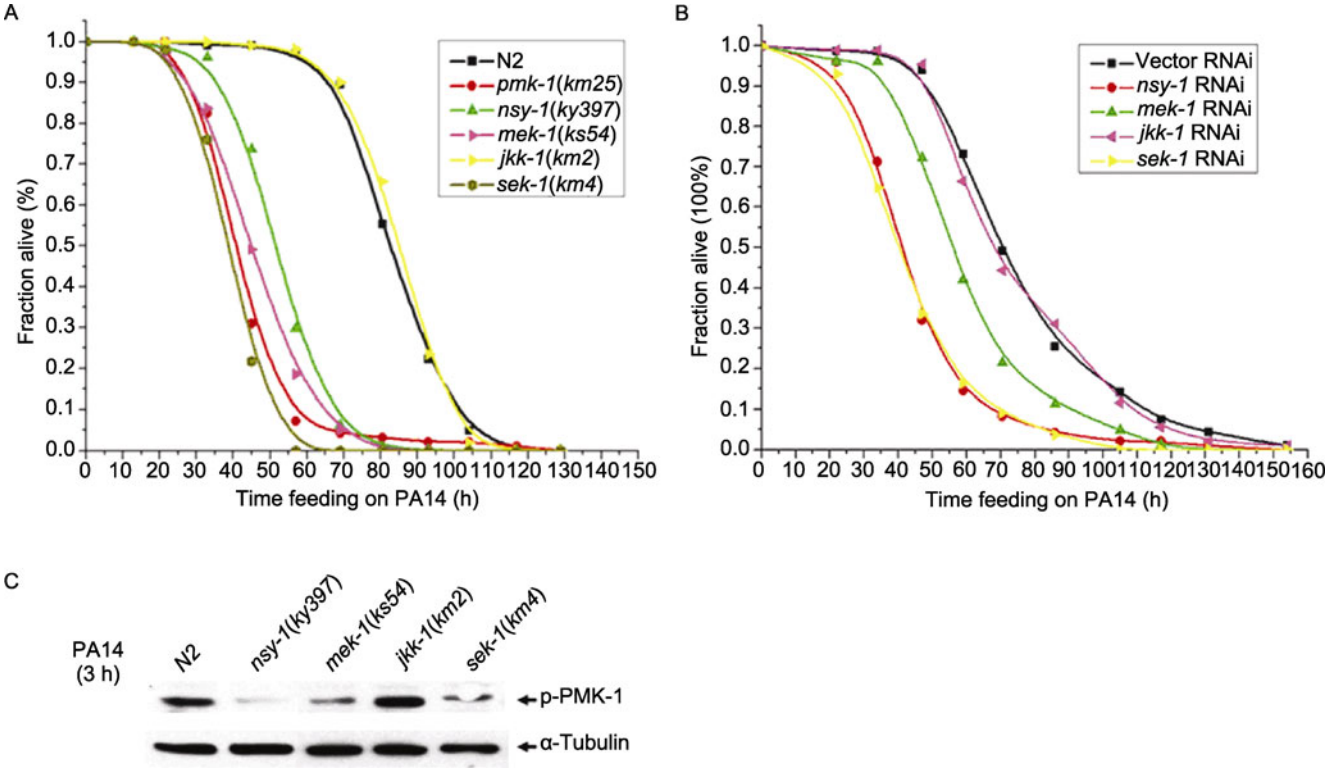

Figure 2. Activation of PMK-1 is required for resistance to *P. aeruginosa* infection. (A) Pathogen susceptibility of *C. elegans* strains N2, and mutant strains *KU25* (*pmk-1*, *km25*), *CX4998* (*nsy-1*, *ky397*), *FK171* (*mek-1*, *ks54*), *KU2* (*jkk-1*, *km2*), and *KU4* (*sek-1*, *km4*) to *P. aeruginosa* strain PA-14. Synchronized populations of young adult worms were transferred to lawns of *P. aeruginosa* strain PA-14. Assays were performed as described under methods and materials. (B) Synchronized populations of *NL2099* L1 larvae were propagated on *E. coli HT115* carrying either vector control (*L4440*), *L4440-nsy-1* positive control, or *L4440-mek-1*, *L4440-jkk-1*, *L4440-sek-1*, respectively, at 20°C. They were transferred to lawns of *P. aeruginosa* strain PA-14 at the L4 or young adult stage and counted at time indicated. *N2* and mutant worms were transferred to NGM plates seeded with *P. aeruginosa* for 3 h. (C) Worm extracts were immunoblotted with anti-phospho-p38 for the detection of PMK-1 activation (upper panel), and with anti-α-tubulin for the detection of total protein (lower panel). Data shown represent at least three independent experiments.

research, we also found that the activation of p38/PMK-1 is significantly reduced in the mek-1 mutants in response to *P. aeruginosa*, suggesting a general involvement of MEK-1 in regulating the activation of p38/PMK-1 (Fig. 2C). In contrast, the mutation of *jkk-1*, did not cause a significant change in the p38/PMK-1 activation in response to *P. aeruginosa* infection (Fig. 2C). These results indicate that MEK-1 is required in *P. aeruginosa* pathogen resistance by activation of p38/PMK-1.

C. elegans MAPKK MKK-4 is required in the activation of PMK-1 for resistance to *P. aeruginosa* infection by a DLK-1-independented manner

Genomic data of *C. elegans* was listed out the candidate components of the JNK and p38 pathways in *C. elegans* by comparison to mammalian homologs, and corresponding *C. elegans* mutants (Brenner et al., 1974; Kawasaki et al., 1999; Koga et al., 2000; Byrd et al., 2001; Sagasti et al., 2001; Sakaguchi et al., 2004; Nakata et al., 2005; Troemel et al., 2006). To confirm that jkk-1 is not involved in a response to the *P. aeruginosa* infection, and to find out whether other MAPKKKs and MAPKKs are involved in the response to pathogenic infections, we constructed RNAi plasmids for some of these candidate genes, as listed in Table 1. Worms containing these RNAi plasmids were analyzed for their susceptibility to *P. aeruginosa* infection. As shown in Fig. 3A and 3B, worms treated with RNAi mkk-4, consisting of the *mkk-4* mutant strain CZ4213 (*ju91*), showed a higher susceptibility to *P. aeruginosa* infection compared to vector treated *NL2099* worms. We next investigated whether *mkk-4* regulates the activation of PMK-1 in case of a *P. aeruginosa* infection. The *mkk-4* mutant strain CZ4213 (*ju91*) or *mkk-4* RNAi-treated worms were infected with *P. aeruginosa* and then analyzed for the activation of PMK-1. The activation of PMK-1 in the *mkk-4* mutant strain CZ4213 (*ju91*) or *mkk-4* RNAi-treated worms was markedly inhibited when infected with *P. aeruginosa* infection (Figs. 3C and S1B). However, both *jkk-1* mutant strains and *dlk-1* mutant strains died not significantly faster than the wild type worms, when infected with *P. aeruginosa* (Fig. 3A). Consequently, also *jkk-1* and *dlk-1* do not regulate the activation of PMK-1 in *P. aeruginosa* infection (Fig. 3C). These results suggest that *mkk-4* influences the resistance of *C. elegans* to *P. aeruginosa* infection, by regulating the PMK-1 activation, whereas the effect is not likely to be regulated by dlk-1.

Specific MAPKKK MOM-4 is required in the activation of PMK-1 for resistance to *P. aeruginosa* infection

A previous study revealed that mammalian specific MAPKKK's TAK1 plays a very important role in the mammalian innate immune system (Ninomiya et al., 1999). However, there still exists no direct evidence of whether MOM-4, a homolog of the mammalian TAK1, participates in the innate immune response to pathogenic infections in *C. elegans*. To investigate whether MOM-4 is also involved in the PMK-1 activation in response to pathogenic infections, we used *mom-4* mutant worms or RNAi treated worms to analyze the activation of PMK-1 in these worms, when challenged by a *P. aeruginosa* infection. As shown in Fig. 4A and 4B, *mom-4* mutant worms and RNAi-treated worms exhibit a higher susceptibility to *P. aeruginosa* infection compared to the wild

Table 1. Conserved MAPK kinases that may be involved in PMK-1 activation in *C. elegans* and corresponding *C. elegans* mutants

Class	*C. elegans* protein	*C. elegans* mutant	Mammalian homolog
MAPKKK	NSY-1	*ky393*	ASK1, ASK2
	DLK-1	*ju476*	DLK1, LZK1
	MOM-4	*or39*	TAK1
MAPKK	SEK-1	*km4*	MKK3, MKK6
	MKK-4	*ju91*	MKK4
	JKK-1	*km2*	MKK7
	MEK-1	*ks54*	MKK7
MAPK	PMK-1	*km25*	PMK1

Candidate components in this study involved in MAPK cascades and the corresponding *C. elegans* mutants listed in table 1. Among the MAPKKs identified, JKK-1 (corresponding to gene F35C8.3) and MEK-1 (corresponding to gene K08A8.1) belong to the MKK7 subgroup. SEK-1 (corresponding to gene R03G5.2) belongs to the MKK3/6 subgroup (7). MKK-4 (corresponding to gene F42G10.2), VZC374L.1 and ZC449.3 have similarities to MKK4. Members of the MAPKKK superfamily, such as MEKK, ASK and TAK are known to be activators of the JNK and p38 MAPK cascades in mammals. The *C. elegans* genome contains an ASK homolog (nsy-1: corresponding to gene F59A6.1), a DLK/LZK homolog (dlk-1: corresponding to gene F33E2.2), a TAK1 homologs (MOM-4: corresponding to gene F52F12.3 and Y105C5A.24). Obvious homologs of MEKK1, MEKK2 and MEKK3 have not been found (Sakaguchi et al., 2004).

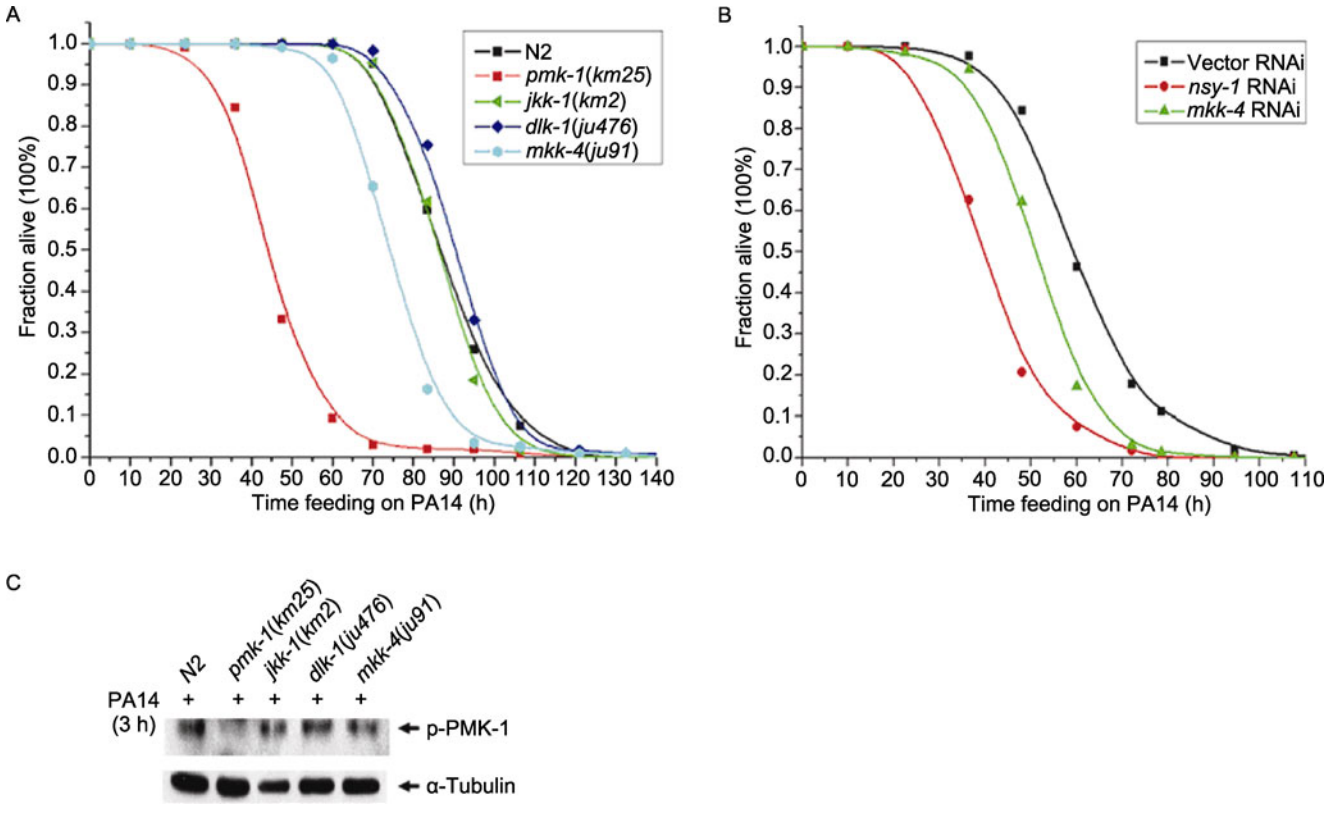

Figure 3. *C. elegans* MAPKK MKK-4 are required in activation of PMK-1 for resistance to *P. aeruginosa* infection by DLK-1-independented manner. (A) Susceptibility of *N2*, and mutant strain *pmk-1*(*km25*), *jkk-1*(*km2*), *dlk-1*(*ju476*), and *mkk-4*(*ju91*) worms to pathogenic infection (*P. aeruginosa* 3 h). (B) Synchronized populations of *NL2099* L1 larvae were propagated on *E. coli* HT115, carrying either vector control (*L4440*), *L4440- mkk-4* or *L4440-nsy-1* control, respectively, at 20°C. They were transferred to lawns of *P. aeruginosa* strain PA-14 at the L4 or young adult stage and counted at time indicated. (C) *C. elegans* strain *N2*, as well as mutant strain *pmk-1*(*km25*), *jkk-1*(*km2*), *dlk-1*(*ju476*), and *mkk-4*(*ju91*) worms were exposed to pathogenic infection (*P. aeruginosa* 3 h). Worm extracts were immunoblotted with anti-phospho-p38 for the detection of PMK-1 activation (upper panel), and with anti-α-tubulin for the detection of total protein (lower panel).

type worms. Furthermore, the *P. aeruginosa* induced activation of PMK-1 markedly reduced in the *mom-4* mutant worms compared to the wild type worms (Fig. 4C). This suggests that MOM-4 is required for the resistance of the worm to pathogenic infections, and that MOM-4 regulates response to *P. aeruginosa* infection by activation of p38/PMK-1.

DISCUSSION

The natural environment of *C. elegans* is soil, in which *C. elegans* is likely to encounter and feed on a diversity of pathogenic micro-organisms. It can be assumed, therefore, that this nematode has evolved a multifaceted immune response to potential pathogen infections. The p38 homolog PMK-1 pathway in *C. elegans* is crucial in its resistance to pathogenic infections (Berman et al., 2001; Kim et al., 2002, 2005). In this study, we have found that the PMK-1 pathway of *C. elegans* can be activated under heat, UV, Sorbitol and *P. aeruginosa* stress. We, furthermore, confirmed that the NSY-1→SEK-1→PMK-1 cascade is involved in the response to these stresses. In addition, this study revealed that the MEK-1, same as the SEK-1 upstream signaling molecular pathway of PMK-1, also regulates the PMK-1 activation in response to heat, UV, Sorbitol and *P. aeruginosa* stress (data not shown).

C. elegans is particularly suited for reverse genetic analyses, since loss-of-function RNAi phenotypes can be generated efficiently by feeding the worms with bacteria expressing double-stranded RNA (dsRNA) that is homologous to a target gene (Kamath et al., 2003). In addition, genomic RNAi clones are readily available commercially. As the homologs of MAPKs in mammals are already known from the existing genomic sequence data (Sakaguchi et al., 2004), we performed a reverse screening for new MAP kinases that could be involved in the resistance of *C. elegans* to pathogens. MKK-4, which is the homolog of mammalian MKK-4, was found to be required for *P. aeruginosa* pathogen resistance. However, the activation of PMK-1 in *mkk-4* RNAi

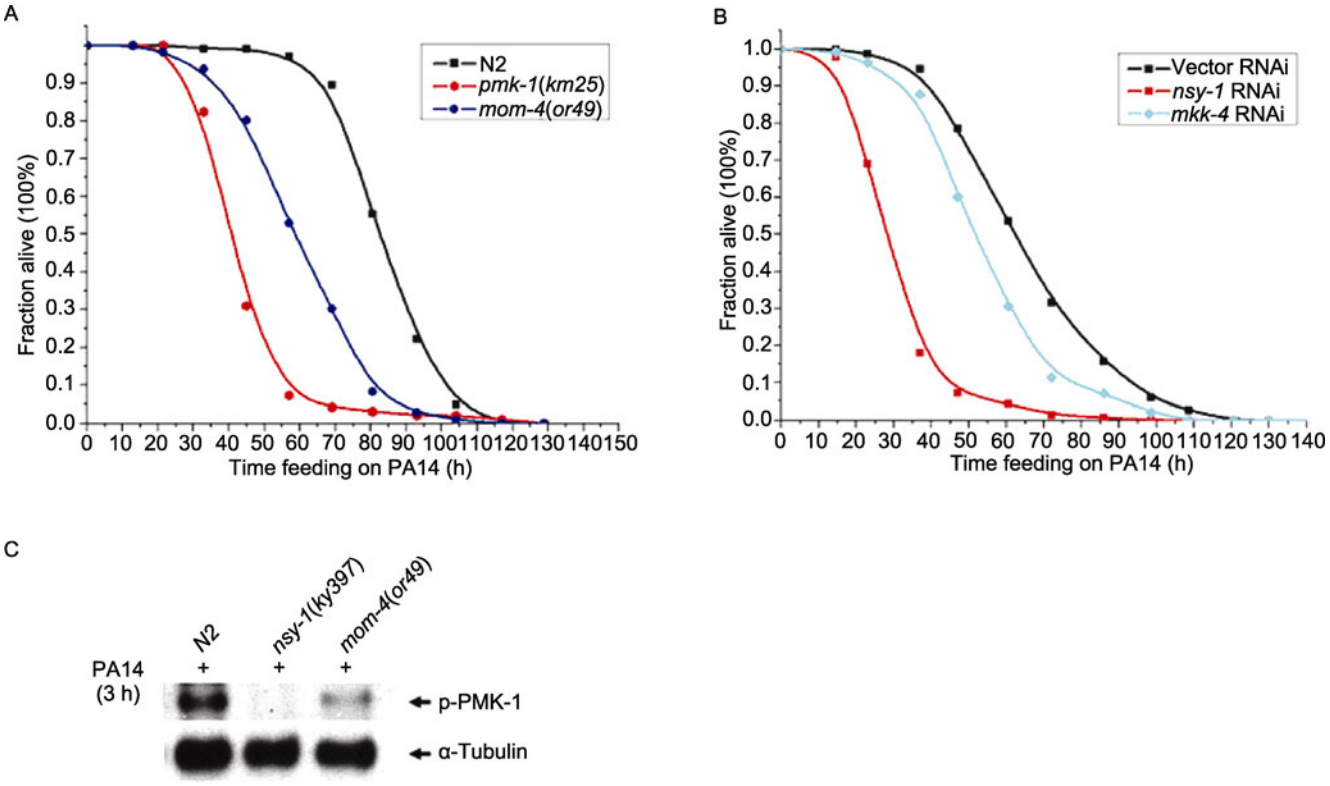

Figure 4. Specific MAPKKKs MOM-4 specifically regulate the PMK-1 activation under *P. aeruginosa* infection. *C. elegans* strain *N2*, and mutant strains *CX4998* (*nsy-1*, *ky397*), *EU446* (*mom-4*, *or49*) were treated with pathogenic infection (*P. aeruginosa* 3 h). Either *pmk-1* mutants for control (A) or synchronized populations of *NL2099* L1 larvae were propagated on *E. coli* HT115 that either carried vector control (*L4440*)*L4440-mom-4*, as well as *L4440-nsy-1* positive control (B) at 20°C. They were transferred to lawns of *P. aeruginosa* strain PA-14 at the L4 or young adult stage and counted at time indicated. (C) Worm extracts were immunoblotted with anti-phospho-p38 for the detection of PMK-1 activation (upper panel), and with anti-α-tubulin for the detection of total protein (lower panel).

treated *N2* worms was not regulated by dlk-1 when infected by *P. aeruginosa*. This suggests that in the innate immune response of *C. elegans* mkk-4 may activate PMK-1, but by DLK-1 independent signaling pathways. These results suggested that MKK4 may be involved in the resistance of *C. elegans* to pathogens by p38 pathways, but not by the JNK pathway, which concurs with previous findings (Brancho et al., 2003). Further studies are needed to substantiate the upstream MAP kinases MKK4, and how they are involved in the innate immune response to *P. aeruginosa* infection in *C. elegans*.

Our study is the first to report that the nematode homolog of TGF-β kinase-1 (TAK-1), MOM-4 is important for worm survival under *P. aeruginosa* pathogenic stress in *C. elegans*. Previous studies demonstrated that TAK1 plays important roles in immunological and developmental contexts across many species (Plowman et al., 1999; Sakaguchi et al., 2004; Wan et al., 2006; Delaney et al., 2006; Kajino et al., 2008). In mammalian tissues, TAK1 is essential for responses to a variety of inflammatory ligands, it can involve in p38 activation by MKK3/6 or in JNK activation by MKK4/7 (Kim et al., 2005). The innate immune responses of *Drosophila* require TAK1 for the expression of a number of antimicrobial peptides. Further study find that mom-4 carries out these diverse biological roles through a number of signaling pathways, such as the TGF-β/BMP, Wnt/Fz, JNK and NF-κB pathways (Delaney et al., 2006). The present study found that mom-4 regulate the PMK-1 pathway in the innate immune response to pathogenic infections in *C. elegans*, while the actual function of TAK-1 in the PMK-1 in *C. elegans* remains unexplained, TAB-1 may be involved in it (Lu et al., 2006). Despite the fact that there still is no experimental data available to prove the direct interaction between mom-4 and sek-1, killing assays of RNAi treated worms revealed that mom-4 might be functionally dependent on sek-1 in pathogenic infections (Fig. 4A and 4B).

The NSY-1→SEK-1→PMK-1 pathway plays a central role in the innate immune response to pathogenic infections. In this study we identified MOM-4 is involved in the resistance of *C. elegans*, to *P. aeruginosa* infection through regulating PMK-1 activation aside from the conserved NSY-1/SEK-1/PMK-1 cassette. MKK-4, another MAP2K, also showed similar roles. However, MEK-1 and JKK-1, both are mammalian MKK7 homolog, showed different effects, MEK-1 also regulated the PMK-1 activation in the *P. aeruginosa* infection, but JKK-1 was not functional. So we surmise that

MKK-4 could be the upstream kinase of PMK-1 in the case of a *P. aeruginosa* infection in a DLK-1-independent manner.

MATERIALS AND METHODS

Worm strains and culture

Strains *N2*, *NL2099 rrf-3* (*pk1426*) *II*, *nsy-1*(*ky397*) *II*, *CZ4213 mkk-4*(*ju91*) *X*, *CZ5730 dlk-1*(*ju476*) *I*, *mom-4*(*or39*) *I/hT2* (*I;III*) (Ninomiya et al.,1999), *FK171 mek-1*(*ks54*) *X*, *KU2 jkk-1*(*km2*) *X*, *KU4 sek-1*(*km4*) *X*, *KU25 pmk-1*(*km25*) *IV*, *and TJ1052 age-1*(*hx546*)*II* were obtained from the Caenorhabditis Genetics Center (CGC). Nematodes were handled using standard methods with a few modifications (Brenner et al., 1974). We used the *E. coli OP50* streptomycin resistant variant strain OP50-1 as the food source for the worm strains to avoid possible contamination with other microbes (Shapira et al., 2006). OP50-1 also obtained from CGC. The *P. aeruginosa* PA-14 strain was kindly provided by Dr. Ausubel's lab at the Massachusetts General Hospital, Harvard School of Medicine.

Bacteria-mediated RNAi

The bacteria-mediated RNAi process was carried out as described in references (Kamath et al., 2003; Timmons et al.,2001). Eggs from bleach synchronized gravid adult worms were hatched in M9 buffer at 20°C for 12 h. Next, the L1 worms (early larval stage) were transferred to the RNAi plates seeded with the *E. coli* strain *HT115*. *HT115* had been transformed with *L4440* vector or specific genomic DNA fragments in advance. The RNAi plates consisted of 1 mmol/L IPTG in addition to the 50 μg/mL Ampicilin in the NGM plates. The worms were grown in the RNAi plates to L4 or young adult stage at 20°C, and then used in the assays. Genomic DNA fragments of specific genes were cloned using primers named in the WormBase list of *C. elegans* genomic clones (www.wormbase.org). The primers used in this study are: *mkk-4*, F: atcgctgcagACGCAATTCAATGTCCCTTC, R: actgaagcttTCGTCGCTGTCTGGATGTAG; *mom-4*, F: atcgccatggTAAATTCAGACACCAACACAACG, R: actgctgcagAAGTCGGTCGAATATCAGCATTA; *mlk-1*, F: atcgcccgggTGCGTTGCACTTTTCATCTC, R: actgctgcagGTTAACGCTTTGCAGGAAGC; *nsy-1*, F: atcgctgcagTGAAGCAGCTTTGATGATGG, R: actgaagcttTTCGGTTACTGGATTCAGCC; *sek-1*, F: atcgctgcagCCGAGACTTAAGCAAATCGC, R: actgaagcttGCTTCTCATTTCTTGCCTGC; *jkk-1*, F: atcgctgcagTGAGATACACATCCGGACCA; R: actgaagcttGAACCAATCCGCAACTTGAT; *mek-1*, F: atcgccatggGAAGGAGCTTCAGTTCGTGG, R: actgctcgagCTCGACGTCGGTTTCAATTT; *unc-22*, F: actgctgcagCACTCTTACTGCTACCAACGCTT, R: atcgccatggAATGATCTCCCTTGTTGAGTGAA.

The DNA fragments were cloned into L4440 plasmids and transformed into HT115 for RNAi experiments as previously described (Timmons et al., 2001; Kamath et al., 2003). We used *unc-22* as the control to observe RNAi efficiency, RNAi treated worms washed with M9 buffer, and then used for subsequent assays.

Worm stress and life span assays

Slow killing *P. aeruginosa* assays were performed using the *P. aeruginosa* strain as described (Tan et al., 1999). All assays performed using L4 or young adult stage hermaphrodites, unless noted.

Briefly, *P. aeruginosa* was cultured in King's B broth, seeded on slow-kill plates, which contain NGM (0.35% instead of 0.25% peptone), and was subsequently incubated for 24 h at 37°C and then for another 24 h at 25°C before adding the worms. 100 μg/mL of 5-fluorodeoxyuridine (FUDR, Hisun Pharmaceutical Co. Ltd.) was added to the NGM killing plates to prevent progeny from hatching (Kim et al., 2002). A total of 30–50 L4 or young adult stage worms were transferred to each pathogen plate. Three plates per strain were tested for each experiment.

For the heat stress assays, young adult *N2* worms were washed with M9 buffer from OP50-1 NGM plates, and incubated in a 35°C water bath for the time indicated.

For osmotic stress assays, sorbitol was added to young adult *N2* worms in M9 buffer to a final concentration of 200 mmol/L, and incubated for the time indicated.

UV stress assays were performed using 1200 J UVB (HL-2000 HybriLinker, UVP, LLC, USA), as described (Wolff et al., 2006).

For all four modes of stress, the treated worms were counted every 6–12 h and scored by gentle prodding using a platinum wire. They were scored as dead without response after at least 3 gentle proddings. All assays were conducted at 25°C. Three plates per strain were tested for each experiment.

Life span assays

Life span assays were performed as described in references (Berman et al., 2001; Troemel et al., 2006). Gravid adult worms were synchronized by bleach method. Eggs were hatched and grown to L4 or young adult stage on NGM plates at 20°C. About 20–30 animals were then transferred to each NGM plate seeded with OP50-1. Subsequently, 100 μg/mL of 5-fluorodeoxyuridine (FUDR) was added to the NGM plates. The assays were performed at 25°C. Three plates per strain were tested for each experiment. Worms were scored every 2 days by gentle prodding using a platinum wire to test for live or dead animals. Origin7.5 software was used to calculate the killing assay and life span data, as well as to produce the graphs for this study.

SDS-PAGE and immunoblot analysis

Immunoblot analyses were performed as described in reference (Kim et al., 2002), with a few modifications. Worms subjected to various stresses were lysed in 2× Laemmli sample buffer to which cocktail inhibitors were added (Sigma). They were instantly frozen in liquid *N2*. Samples were then boiled at 100°C for 10 min. Total protein of each sample was determined by BCA kit (Beyotime Co. Ltd.). The same amount of protein for each sample was resolved by SDS-PAGE (10% gels) and transferred to nitrocellulose membranes. The membranes were blocked with 1% gelatin and subjected to antibody incubation. Antibodies that recognize doubly phosphorylated p38 (Biosource) for PMK-1 detection, and α-tubulin (Sigma) were used for total protein as loading control (Tanaka et al., 2002).

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ABBREVIATIONS

ASK-1, apoptosis signaling-regulating kinase-1; DLK-1, DAP like kinase; JKK, JNK kinase; LZK, Leucine zipper-bearing kinase; MEK-1, MAP kinase kinase or ERK kinase-1; MEKK, Mitogen-activated protein/ERK kinase kinase; MLK, Mixed lineage kinase; MKK, MAP kinase kinase; MOM-4, More of MS-4; NSY-1, neuronal symmetry-1; PMK-1, p38 MAP kinase family; SEK-1, SAPK/ERK kinase-1; TAK, Transforming growth factor beta activated kinase

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