FLR-4, a Novel Serine/Threonine Protein Kinase, Regulates Defecation Rhythm in *Caenorhabditis elegans*

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The defecation behavior of the nematode *Caenorhabditis elegans* is controlled by a 45-s ultradian rhythm. An essential component of the clock that regulates the rhythm is the inositol trisphosphate receptor in the intestine, but other components remain to be discovered. Here, we show that the *flr-4* gene, whose mutants exhibit very short defecation cycle periods, encodes a novel serine/threonine protein kinase with a carboxyl terminal hydrophobic region. The expression of functional *flr-4::GFP* was detected in the intestine, part of pharyngeal muscles and a pair of neurons, but expression of *flr-4* in the intestine was sufficient for the wild-type phenotype. Furthermore, laser killing of the *flr-4*-expressing neurons did not change the defecation phenotypes of wild-type and *flr-4* mutant animals. Temperature-shift experiments with a temperature-sensitive *flr-4* mutant suggested that FLR-4 acts in a cell-functional rather than developmental aspect in the regulation of defecation rhythms. The function of FLR-4 was impaired by missense mutations in the kinase domain and near the hydrophobic region, where the latter allele seemed to be a weak antimorph. Thus, a novel protein kinase with a unique structural feature acts in the intestine to increase the length of defecation cycle periods.

INTRODUCTION

Many biological functions are associated with rhythms that are genetically controlled. Circadian rhythms, which change the metabolism and behavior of animals, plants and micro-organisms with periods of ~24 h, are generated by the transcriptional oscillation of special genes (Young and Kay, 2001; Stanewsky, 2003). These rhythms are said to have innate clocks, based on the following criteria: 1) even in the absence of environmental cues, the rhythm continues at the same period; 2) the period remains constant over a wide range of temperatures; and 3) the phase of oscillations can be reset by certain external stimuli. Rhythms other than circadian rhythms are also known. Some of them are infradian rhythms, which have periods longer than a day, like reproductive cycles of primate females. Others are ultradian rhythms, which have periods shorter than a day.

Ultradian rhythms are found in diverse aspects of many living organisms (Hall, 1995; Iwasaki and Thomas, 1997; Calabrese, 1998). They include heart beat rhythms (Attali, 1996), rhythms of locomotion (Grillner, 1985), the courtship song of *Drosophila* (Hall and Rosbach, 1988), and the cycle of rapid eye movement (REM) and non-REM sleep (Weitzman, 1981). These rhythms have periods ranging from seconds to

hours and seem to be based on diverse mechanisms, unlike circadian rhythms. How these rhythms are regulated remains to be elucidated in most cases.

The defecation behavior of *Caenorhabditis elegans*, which repeats with a period of ~45 s, is a typical example of an ultradian rhythm (Thomas, 1990). The unit of behavior consists of three sequential motor steps: posterior body-wall muscle contraction (pBoc), anterior body-wall muscle contraction (aBoc), and expulsion (Exp). Behavioral analysis showed that the defecation rhythm, like circadian rhythms, is controlled by an endogenous clock (Liu and Thomas, 1994). First, the rhythm is independent of the motor steps. Animals stop exhibiting defecation behavior when they move away from food. Even in such conditions, the rhythm continues at the same period, and, after returning to the food, animals resume defecation behavior depending on this rhythm. Second, the defecation cycle period remains almost constant over a temperature range of 19–30°C. Finally, the phase of defecation rhythm can be reset to time zero, i.e., just after the expulsion, by lightly touching the body of animals.

The cells and genes that control the motor steps have been investigated. Analysis of mutants showed that there are genes that specifically control pBoc, aBoc, and Exp as well as genes that control both aBoc and Exp (Aex phenotype) (Thomas, 1990). Based on these results, a model for the control of defecation behavior was postulated, in which the defecation clock or cycle generator controls aBoc and Exp independently of pBoc. In agreement with the model, laser ablation experiments revealed that two GABAergic neurons, AVL and DVB, are required for aBoc and Exp but not for pBoc (McIntire *et al.*, 1993). Attempts to identify neurons that are required for pBoc, however, have resulted in failure (E. M. Jorgensen and H. R. Horvitz, cited in Dal Santo *et al.*, 1999). The study on the inositol trisphosphate receptor gene (Dal Santo *et al.*, 1999), which is explained below, suggests

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that the signal for pBoc may be generated in the intestine and transmitted directly to body wall muscles.

Genes that regulate defecation rhythms have been identified by mutations (Dec mutations) that change defecation cycle periods, which map to at least 13 genes and that are classified into two categories: Dec-short and Dec-long (Iwasaki *et al.*, 1995). Of the latter genes, *itr-1* (= *dec-4*) gene encodes the IP₃ receptor, and its mutants show long defecation cycle periods (hypomorph) or no defecation behavior (amorph), whereas overexpression of this gene results in short defecation cycle periods (Dal Santo *et al.*, 1999). Genetic mosaic analysis revealed that expression of the wild-type *itr-1* gene in the intestinal cells is sufficient for the wild-type phenotype. Furthermore, *itr-1* gene also controls the periods of Ca²⁺ oscillation in the intestinal cells, where the defecation rhythm has the same periods as Ca²⁺ oscillation, with pBoc being preceded by the peaks of calcium concentration. Thus, the IP₃ receptor in the intestinal cells seems to play an essential role in the defecation rhythm.

Mutants in *flr-1*, *flr-3*, and *flr-4* are unique among Dec mutants in that they show very short defecation periods (Iwasaki *et al.*, 1995). Furthermore, they show other phenotypes such as resistance to fluoride ions, slow growth, abnormal regulation of dauer larva formation (Katsura *et al.*, 1994), and frequent skip of Exp (Take-uchi *et al.*, 1998). One of these genes, *flr-1*, has been cloned and shown to encode an ion channel of the DEG/ENaC superfamily that is expressed only in the intestine (Take-uchi *et al.*, 1998). However, to elucidate the regulatory system that prolongs defecation cycle periods, it is necessary to clone *flr-3* and *flr-4* genes and investigate detailed properties of their mutants. Here, we report our analysis of *flr-4* genes, which revealed that a novel Ser/Thr kinase is involved in the regulation of defecation cycle periods.

MATERIALS AND METHODS

Strains and Nomenclature

All the strains used in this study derive from the wild-type *C. elegans* var. Bristol N2. The mutations used were *flr-1(ut11)* X, *flr-4(n2259, sa201, ut3, ut7)* X, *flr-3(ut9)* IV, *unc-3(e151)* X, *mnDf19* X, and *mnDp1(X;V)*. The deficiency *mnDf19* deletes *flr-4* gene (Katsura *et al.*, 1994), whereas the duplication *mnDp1*, which is homozygous sterile, covers *mnDf19* (Herman *et al.*, 1979). Genetic nomenclature was as described previously (Horvitz *et al.*, 1979; Hodgkin, 1997). Animals were grown at 20°C as described previously (Brenner, 1974), except where otherwise noted.

Assays of Phenotypes

Defecation behavior was observed at 23°C essentially as described (Liu and Thomas, 1994), except that the temperature was 20 or 25°C in temperature-shift experiments. The defecation motor steps of *flr* mutants sometimes became too weak to observe even the pBoc step, and the animals looked as if they ceased defecation behavior for a long time. Hence, we did not include defecation interval lengths longer than 100 s in the statistics. For statistical analysis, we first calculated a mean defecation cycle period for each animal and then a mean and SEM of these results. This method was chosen, because the variation of defecation cycle periods among different animals was often larger than that within the same animal. Dauer formation (Thomas *et al.*, 1993), growth rate and fluoride sensitivity (Katsura *et al.*, 1994) were measured as described.

Cloning of *flr-4* Gene and cDNA

Mutations in the *flr-4* gene were genetically mapped in a region between *unc-3* and *unc-7*, covered by the *mnDp25* duplication and not detected by the *mnDf20* deficiency, on the linkage group X (Katsura *et al.*, 1994). By microinjection of genomic clones in this region into the gonad of *flr-4* mutants, we found that the cosmid F09B12 (courtesy of the *C. elegans* genome project) could rescue the phenotypes. We then subcloned several DNA fragments from F09B12 and tested them for the rescuing activity. A clone containing an 8.0-kb *KpnI-PstI* fragment, pMT11-2, was found to rescue the phenotypes of *flr-4* mutants. The sequence of F09B12 by the *C. elegans* genome consortium revealed that only one complete open reading frame (ORF), which codes for

a predicted protein kinase, is present in the 8.0-kb *KpnI-PstI* region. We cloned the cDNA that encodes the predicted protein kinase by RT-PCR, 5'- and 3'-RACE methods. We think the cDNA is full length, since the SL1 sequence is present at the 5' end and since a single band corresponding to the expected size of the mRNA was detected by Northern analysis.

Reporter Fusions

The plasmid pMTG7-11 is comprised of the *flr-4* rescuing construct with the carboxy terminus tagged with GFP through a linker sequence. It was constructed by the following procedures. 1) A 420-base pair fragment, corresponding to the 3' part of *flr-4* gene, was amplified from pMT11-2 (minimal rescuing genomic DNA clone) using the primers f4-PK522 (CTCTGCAGGG-TACCCACCTCATTTATAGTCGGC) and f4-BG320 (GGGATCCACCGTTT-TCTTCATTGTCTGGTCCAGACG), and cloned into a T-vector. The primer f4-PK522 contained a *PstI* site and a *KpnI* site located side by side, and f4-BG320 contained the 3' end of the *flr-4* coding sequence, a linker sequence and a *BamHI* site to connect to GFP. 2) The *PstI-BamHI* fragment of this clone was ligated to the *PstI-BamHI*-digested pQE9 vector (QIAGEN, Chatsworth, CA). 3) The *KpnI-BssHII* fragment (99 base pairs) of this clone was replaced by the 7.0-kb *KpnI-BssHII* fragment of pMT11-2, which contained a 3.5-kb 5' upstream region and the coding region of *flr-4* gene, except for a short 3' part. 4) The 7.4-kb *PstI-BamHI* fragment of this clone, i.e., complete *flr-4* gene with a 3.5-kb 5' upstream region and a 3' linker sequence, was ligated into the *PstI-BamHI*-digested pPD95.77E1 to make pMTG7-11, where pPD95.77E1 is an improved version of the *gfp* vector pPD95.77 (courtesy of A. Fire) producing a brighter GFP (F64L S65T).

GFP fluorescence was examined as described by Chalfie *et al.* (1994). The DiQ fluorescent marker 4-(*p*-dihexadecylaminostyryl)-*N*-methylquinolinium iodide (Molecular Probes, Eugene, OR) was used to help identification of neurons in the head region. The dye-filled cell types and staining method of DiQ were essentially the same as described for DiO (Starich *et al.*, 1995). A Rhodamine filter set was used to observe the fluorescence of DiQ.

Tissue-specific Expression

The plasmid pYK4-2.2 is comprised of *flr-4* cDNA driven by the intestine-specific C03F11.3 promoter (Rafaz Hoque, Roy Silverstein, and Martin Chalfie, personal communication). It was constructed by the following procedures. 1) An 822-base pair fragment containing the 5' upstream promoter sequence and the translational start point of C03F11.3 (CD36-like protein) gene was amplified from *C. elegans* genomic DNA, using the primers C03F11.3SalI: CTCGTCGACGC-CACTGTACCCAGTTGTCT and C03F11.3SphI: CTCGCATGCGTAACCTCG-GAGAAATTGAAAGTG, which contained *SalI* and *SphI* restriction sites, respectively. The PCR product, after purification and cleavage with *SalI* and *SphI*, was ligated to the *SalI-SphI*-digested pPD95.79 vector (A. Fire) and cloned. Using this clone (C03F11.3p::GFP), we confirmed that the C03F11.3 promoter drives expression only in the intestine (unpublished data). 2) *flr-4* cDNA was amplified from the full-length cDNA clone (see above), using the primers f4-NcoI-ATG: CAT-GCCATGGATGCCAATAAATTACAATCG and f4-stop-SacI: CGCAGAGCTC-CTAGTTTCTTCATTGCTG, which contained *NcoI* and *SacI* sites, respectively. The PCR product, after purification and cleavage with *NcoI* and *SacI*, was ligated to the *NcoI-SacI*-digested pPD49.26 vector (A. Fire) and cloned. 3) The C03F11.3 promoter-containing sequence was cleaved out with *SphI* and *XbaI* from the clone (1), purified, and ligated to the *SphI-XbaI* sites of the clone (2) to yield pYK4-2.2.

Transgenic Lines

Microinjection was carried out as described (Mello *et al.*, 1991). For rescue experiments, the DNA to be tested and the *rol-6(su1006)* dominant marker plasmid pRF4 (Kramer *et al.*, 1990) were coinjected into *flr-4(ut7)* animals at concentrations of 1 and 99 ng/μl, respectively. Multiple independent lines were established from each injection. A strain carrying a *flr-4::GFP* chromosomal insertion was obtained serendipitously by injecting a mixture of pMTG7-11 and pRF4 at concentrations of 95 and 5 ng/μl, respectively, to N2 animals. The chromosomal insertion containing pMTG7-11 (*flr-4::GFP*) was transferred to *flr-4(ut7)* animals by cross to test the rescue of the phenotypes. The *flr-4(ut7)* and *unc-3(e151) flr-4(ut7)* strains that express *flr-4* cDNA only in the intestine were obtained as follows. The clones of C03F11.3p::*flr-4* cDNA (pYK4-2.2), *myo-3p::GFP* (marker), and pBluescriptKS(+) (Stratagene, La Jolla, CA) were mixed to give final concentrations of 20, 2, and 78 ng/μl, respectively, and injected into N2 animals. Then, the extrachromosomal array was transferred to *flr-4(ut7)* and *unc-3(e151) flr-4(ut7)* animals by genetic cross.

Laser Microsurgery

AUA cells in anesthetized wild-type and *flr-4(n2259)* L1 larvae were killed with a 440-nm laser microbeam using a MicroPoint Laser System (Photonic Instruments, St. Charles, IL). Details were as previously described (Bargmann and Avery, 1995). After the operation, the animals were grown to young adults at 20°C, and their behavior was analyzed at 23°C.

Table 1. Phenotypes of various *flr-4* and related mutants

Genotype	Without NaF		With 400 μ g/ml NaF		Sensitivity to 400 μ g/ml NaF (larval arrest)	Generation time (days)
	Defecation cycle (sec) ^a	Exp/pBoc (%)	Defecation cycle (sec) ^a	Exp/pBoc (%)		
N2	46.9 $\pm$ 1.4 (10)	98	61.8 $\pm$ 2.4 (11)	83	Sensitive	3
<i>flr-4(n2259)</i>	26.5 $\pm$ 2.1 (9)	50	28.5 $\pm$ 2.4 (10)	48	Resistant ^b	3
<i>flr-4(sa201)</i>	21.4 $\pm$ 1.3 (10)	38	31.2 $\pm$ 2.3 (10)	44	Resistant ^b	3
<i>flr-4(ut3)</i>	37.9 $\pm$ 1.9 (11)	43	34.1 $\pm$ 2.5 (8)	33	Resistant	5–7
<i>flr-4(ut7)</i>	33.1 $\pm$ 4.0 (10)	43	29.1 $\pm$ 1.3 (10)	89	Resistant	5–7
<i>flr-1(ut11)</i>	29.8 $\pm$ 2.3 (10)	31	32.4 $\pm$ 1.3 (10)	46	Resistant	5–7
<i>flr-3(ut9)</i>	35.9 $\pm$ 3.8 (10)	52	28.0 $\pm$ 1.5 (8)	6	Resistant	5–7
<i>flr-1(ut11) flr-4(ut7)</i>	29.3 $\pm$ 1.9 (10)	37	28.7 $\pm$ 2.3 (10)	27	Resistant	5–7
<i>flr-3(ut9); flr-4(ut7)</i>	34.1 $\pm$ 2.5 (7)	35	34.6 $\pm$ 1.9 (5)	9	Resistant	5–7
<i>flr-3(ut9); flr-1(ut11) flr-4(ut7)</i>	20.5 $\pm$ 1.9 (7)	35	n.t.	27	Resistant	5–7

Defecation behavior was observed at 23°C, fluoride resistance was measured at 20 and 25°C, and generation time was measured at 20°C. All the *flr-4* mutants showed short defecation cycle periods and frequent skip of the Exp step. All the *flr-4* mutants except *n2259* and *sa201* showed slow growth. Note that the low Exp/pBoc ratio of *ut7* was suppressed by fluoride ion. The defecation motor steps of *flr* mutants sometimes became too weak to observe even the pBoc step, and the animals looked as if they ceased the defecation behavior for a long time. Hence, we did not include defecation interval lengths longer than 100 s in the statistics. The mean defecation cycle periods and SEM in this table were calculated from the mean defecation cycle periods of individual animals, each of which was an average of 4–21 defecation cycle periods.

^aValues are the period $\pm$ SEM, with the number of animals in parentheses.

^b*n2259* and *sa201* were resistant to NaF at 25°C but not at 20°C, whereas other mutants were resistant to NaF both at 20 and 25°C.

Determination of Mutation Sites by DNA Sequencing

The *flr-4* coding region was amplified by using Expand High Fidelity PCR System (Boehringer Mannheim, Mannheim, Germany) from single animals of various mutants as described (Williams *et al.*, 1992). The PCR products were cloned, and at least two independent clones for each PCR product were sequenced with a dye-terminator sequencing system (Perkin Elmer-Cetus, Foster City, CA).

Genetic Interaction between Mutations in the Kinase Domain and C-terminus

The heterozygotes were obtained as the F1 and F2 progeny of crosses between *unc-3 flr-4* hermaphrodites and *flr-4* (or N2) males or between *mnDp1/+; mnDf19* hermaphrodites, and *flr-4* males. The deficiency *mnDf19* deletes *flr-4* (Katsura *et al.*, 1994). The *unc-3* mutation, which is linked to *flr-4* and which has no effect on growth rates as a heterozygote, was used to distinguish cross-progeny from self-progeny and to estimate the genotype of F2 animals by checking the phenotypes of their progeny. To exclude the contribution of maternal effect, reciprocal crosses were performed, and the generation time of both F1 and F2 progeny was measured. *mnDf19/flr-4* could be discriminated from *mnDp1/+; mnDf19* (self progeny) and *mnDp1/+; mnDf19/flr-4* (cross-progeny), because the latter segregated *mnDp1* homozygotes, which are sterile (Herman *et al.*, 1979).

Temperature-shift Experiments

For temperature-upshift experiments, the cultivation temperature of *flr-4(n2259)* worms was raised from 20 to 25°C at various developmental stages, and their defecation behavior was observed as young adults at 25°C. For temperature-downshift experiments, the temperature was changed from 25 to 20°C at various developmental stages, and the defecation behavior was observed as young adults at 20°C.

RESULTS

Phenotypes of Various *flr-4* Mutants

To characterize the abnormality of *flr-4* mutants, we investigated the defecation behavior of four *flr-4* alleles, *n2259*, *sa201*, *ut3*, and *ut7* at 23°C (Table 1). Wild-type (N2) animals showed a mean defecation cycle period of $\sim$ 45 s, and all the motor steps (pBoc, aBoc, and Exp) could be detected in most defecation cycles, although aBoc was less obvious than pBoc and Exp. In contrast, *flr-4* mutants showed shorter mean defecation cycle periods of $\sim$ 30 s. Moreover, the Exp step was often absent in *flr-4* mutants. The aBoc step seemed to

be present at higher probability than Exp, although we could not quantify the aBoc/pBoc ratio. Two of the mutations, *n2259* (E. M. Jorgensen, personal communication) and *sa201* (Iwasaki *et al.*, 1995), were temperature-sensitive in the defecation period abnormality. Namely, they showed short defecation cycle periods at 23 or 25°C, but almost normal defecation cycle periods at 20°C (unpublished data). The Exp/pBoc ratio was higher at 20 than at 23°C, but somewhat lower than that of wild-type animals.

The mean defecation cycle periods of wild-type animals changed very little even when measurements were repeated over a long time period. However, those of *flr-4* mutants varied to a larger extent, possibly because they were more sensitive to unidentified experimental conditions. We therefore did not interpret the difference of mean defecation cycle periods among different *flr-4* alleles. The asymmetric distribution of defecation interval length, namely, very short defecation cycle periods around 20 s with occasional much longer periods (the same as that of *flr-1* mutants shown in Takeuchi *et al.* (1998)), was common to all the *flr-4* mutants and clearly differed from the symmetric distribution in wild-type animals.

It is known that exogenous fluoride ion increases the defecation cycle periods of wild-type animals but not that of *flr-1* mutants (Takeuchi *et al.*, 1998). We therefore tested the effect of fluoride ion on *flr-4* mutants. All the *flr-4* mutants, like *flr-1* mutants, did not change their defecation interval length in response to fluoride ion, with the possible exception of *sa201* (Table 1).

Surprisingly, fluoride ion suppressed the low Exp/pBoc ratio of *ut7*. This effect was confirmed by measuring the defecation cycle periods of the same animals before and 30 min after transfer the animals to plates containing NaF. The *ut7* mutant showed an almost normal Exp/pBoc ratio also on plain agar plates containing no salts (our unpublished results).

Two of the *flr-4* mutants, *ut3* and *ut7*, showed resistance to fluoride ion and slow growth rates, as reported already

Table 2. Synthetic dauer-constitutive phenotypes of various *flr-4* mutants

Genotype	% Dauer larvae	
	at 25°C ^a	at 20°C ^a
<i>flr-4(ut3)</i>	0 (193)	0 (128)
<i>flr-4(ut7)</i>	0 (156)	0 (260)
<i>flr-4(n2259)</i>	0 (208)	0 (160)
<i>flr-4(sa201)</i>	0 (171)	0 (237)
<i>unc-3(e151)</i>	0 (206)	0 (361)
<i>unc-3(e151) flr-4(ut3)</i>	57 (251)	29 (139)
<i>unc-3(e151) flr-4(ut7)</i>	85 (186)	39 (213)
<i>unc-3(e151) flr-4(n2259)</i>	24 (204)	0 (382)
<i>unc-3(e151) flr-4(sa201)</i>	3 (131)	0 (526)

The *unc-3 flr-4* double mutants formed considerable percentages of dauer larvae, if the *flr-4* allele showed the phenotype of slow growth (*ut3* or *ut7*).

^aValues are the percentages of dauer larvae grown under non-dauer-forming conditions (plenty of food and sparse population), with the number of animals scored in parentheses.

(Katsura *et al.*, 1994). The other two *flr-4* mutants, *n2259* and *sa201*, which showed temperature-dependent abnormality in defecation cycle periods, also showed temperature-dependent resistance to 400 μ g/ml NaF; namely, they were resistant to NaF at 25°C but sensitive at 20°C. However, they grew at almost normal rates at 20 and 25°C.

Some *flr-4* mutants as well as some *flr-1* and *flr-3* mutants show constitutive dauer larva formation in the *unc-3(e151)* background (Katsura *et al.*, 1994), where the dauer larva is special third-stage larvae that appears under high population density and limited food supply (Riddle and Albert, 1997), and the *unc-3* gene encodes a transcription factor of the Olf-1/EBF family, which is expressed in ASI sensory neurons (Prasad *et al.*, 1998). Because dauer larva formation is induced by environmental cues that are sensed by some head neurons, the synthetic dauer-constitutive phenotype may reflect the possible influence of *flr-4*, *flr-1*, and *flr-3* mutations on neural functions (Takeuchi *et al.*, 1998). In this study we investigated which *flr-4* alleles showed a stronger tendency to form dauer larvae. As shown in Table 2, the synthetic dauer-constitutive phenotypes were parallel to abnormality in growth rates (Table 1): the mutations *ut3* and *ut7*, which confer slow growth, showed strong dauer-constitutive phenotypes, whereas *n2259* and *sa201*, which affect growth rates very little, showed no or weak dauer-constitutive phenotypes in the *unc-3(e151)* background.

We also studied interaction between *flr-4*, *flr-1*, and *flr-3* mutations, all of which show similar phenotypes. As shown in Table 1, all the single, double, and triple mutants of *flr-1(ut11)*, *flr-3(ut9)*, and *flr-4(ut7)* showed essentially the same phenotypes in growth, fluoride-resistance, defecation cycle periods, and the Exp step on NGM plates. The results are consistent with the idea that *flr-4* acts in the same pathway as *flr-1* and *flr-3*. The suppression of low Exp/pBoc ratio of *flr-4(ut7)* by fluoride ion required the wild-type *flr-1* and *flr-3* genes. This result suggests that the *flr-4* gene acts in cooperation with or upstream of *flr-3* and *flr-1* genes.

The *flr-4* Gene Encodes a Novel Protein Kinase with Predicted Transmembrane Regions in the Carboxyl Terminus

To elucidate the molecular mechanism of FLR-4 functions, we cloned the *flr-4* gene by the transformation rescue method (See *Materials and Methods* for details). An 8.0-kb *KpnI-PstI* fragment (pMT11-2; Figure 1A) from the genomic clone F09B12 rescued all the phenotypes of *flr-4(ut7)* (Figure 1C). We also cloned the *flr-4* cDNA, which consisted of 2060 base pairs and had an SL1 splice leader sequence (Krause and Hirsh, 1987) at the 5' end and a poly(A) stretch after the consensus polyadenylation signal sequence AATAAA. Comparison with the genomic sequence in F09B12 indicated that *flr-4* gene has 12 exons (Figure 1B). The ORF, corresponding to F09B12.6, encoded a protein consisting of 570 amino acids (Figure 2A).

A homology search of the predicted amino acid sequence using the BLAST program revealed that the amino-terminal part of FLR-4 resembles many known protein serine/threonine kinases (Figure 2B). FLR-4 has all the 15 highly conserved amino acids in serine/threonine kinases (Hanks and Quinn, 1991) with the exception of Asp in subdomain VII, which is replaced by a similar residue, Asn. Moreover, the amino acid residues 217–224 (TLLYVAPE) in subdomain VIII fit with the serine/threonine kinase consensus but not the tyrosine kinase consensus (Hanks *et al.*, 1988). There is no protein kinase with very strong homology to FLR-4. Those that resemble FLR-4 relatively well, for instance, human SOK1 (Pombo *et al.*, 1996), *S. cerevisiae* CDC15 (Schweitzer and Philippsen, 1991), and *S. pombe* BYR2 (Wang *et al.*, 1991) had 28, 28, and 26% identity, respectively, to FLR-4 in the protein kinase domain (Figure 2B). Based on this homology, some researchers classified FLR-4 to the STE20 family (Plowman *et al.*, 1999). Although this is reasonable from the viewpoint of the overall homology of the kinase domain, it is also true that FLR-4 is not a typical member of the STE20 family. It lacks the STE20 signature GTP(Y/F)WMAPE in subdomain VIII (Sells and Chernoff, 1997), which plays a role in substrate recognition (Hanks and Hunter, 1995).

The C-terminal part of FLR-4 has no clear similarity to known proteins but contains a 94-amino-acid sequence rich in hydrophobic amino acids (Figure 2, A and C). Many transmembrane prediction programs, such as SOSUI (<http://sosui.proteome.bio.tuat.ac.jp/sosui/frame0.html>) and TMHMM2 (<http://www.cbs.dtu.dk/services/TMHMM2.0/>), predict two or three transmembrane regions in this sequence. Protein kinases with predicted transmembrane region(s) on the C-terminal side are relatively rare, because all the receptor-type protein kinases have their transmembrane region on the N-terminal side of the kinase domain. Examples of those relatively rare protein kinases are rat TAO2 kinase (Chen *et al.*, 1999), its human homolog PSK (prostate-derived STE20-like kinase; Mitsopoulos *et al.*, 2003), human myotonic dystrophy kinase (Waring *et al.*, 1996), and some other kinases predicted in the genome sequences of *C. elegans*, *Arabidopsis*, and many prokaryotes. Despite belonging to the same STE20 family, the ortholog of TAO2/PSK in *C. elegans* is not FLR-4 but KIN-18 (Berman *et al.*, 2001), which does not have predicted transmembrane regions. Moreover, none of SOK1, CDC15, and BYR2, which resemble FLR-4 in the kinase domain, possess predicted transmembrane regions.

flr-4::GFP Is Expressed in the Intestine, Part of the Pharynx, and AUA Neurons

To investigate the tissue and subcellular localization of FLR-4, we examined the expression of the *flr-4::GFP* fusion

Figure 1. Cloning of *flr-4* gene and cDNA. (A) Genetic and physical map around the *flr-4* gene. The cosmid clone F09B12 and its subclones pMT5-3 and pMT11-2 (but not pMT9-1) rescued the *flr-4* phenotypes by microinjection. The arrow indicates the direction of transcription of the only complete ORF contained in pMT11-2. S, *SalI* site; P, *PstI* site. (B) Top: the structure of the 8-kb genomic clone (pMT11-2) that rescued the *flr-4* phenotypes. The 12 exons are indicated by boxes. The coding region is striped. The trans-splice leader SL1 and the poly(A) tail are also shown. Bottom: the structure of pMTG7-11, which codes for a GFP(F64L S65T)-tagged FLR-4 protein. (C) Rescue of the *flr-4*(*ut7*) phenotypes by the genomic DNA fragment pMT11-2 and by *flr-4* cDNA driven by the intestine-specific C03F11.3 promoter. Various phenotypes of the wild-type (N2), *flr-4*(*ut7*), *flr-4*(*ut7*);Ex[pMT11-2] and *flr-4*(*ut7*);Ex[C03E11.3p::*flr-4* cDNA] are presented.

C

genotype	defecation cycle period $\pm$ SEM [sec] (Nr. of animals)	Exp/pBoc [%]	fluoride sensitivity	generation time [days]
N2	46.9 $\pm$ 1.4 (10)	97.5	sensitive	3
<i>flr-4</i> (<i>ut7</i>)	33.1 $\pm$ 4.0 (10)	30.9	resistant	5-7
rescue (pMT11-2)	45.7 $\pm$ 1.1 (11)	93.1	sensitive	3
rescue (intestine)	44.0 $\pm$ 2.0 (12)	89.4	sensitive	3

gene pMTG7-11 (Figure 1B) in wild-type animals. This construct fully rescued all the *flr-4* phenotypes (unpublished data), which indicates that the product was functional and present at a proper time in the cells that require the FLR-4 function for the wild-type phenotypes. Comma-stage embryos began to express the GFP-tagged protein in intestinal cells (Figure 3E), and animals retained the intestinal expression through the adult stage (Figure 3, F and D), although the intestinal expression became weaker after the L1 stage. The FLR-4::GFP fusion protein accumulated at the cell membrane of intestinal cells, especially the lateral membrane intervening the intestinal cells (Figure 3D). The fusion protein was also detected in the muscles of the pharyngeal isthmus from the threefold embryonic stage, and in a pair of head neurons, which we identified as AUA neurons, from the late L1 stage, (Figure 3D). These expression patterns were retained through the adult stage (Figure 3G).

Expression of *flr-4* in the Intestine Is Sufficient for the Wild-type Phenotype

Although the functional *flr-4*::GFP fusion gene was expressed in the intestine, part of the pharynx and AUA neurons, it does not necessarily mean that expression in all these areas is required for the *flr-4* functions investigated in this study. We therefore expressed FLR-4 only in the intestine by

using the intestine-specific promoter of C03F11.3 (CD36-like protein) gene (Rafaz Hoque, Roy Silverstein, and Martin Chalfie, personal communication) in *flr-4*(*ut7*) and *unc-3*(*e151*) *flr-4*(*ut7*) animals. The results (Figure 1C) showed that expression of FLR-4 in the intestine rescued the *flr-4*(*ut7*) mutant phenotypes for defecation cycle periods, the Exp step of defecation behavior, sensitivity to fluoride ion, and growth rates. Furthermore, the dauer-constitutive phenotype of the *unc-3*(*e151*) *flr-4*(*ut7*) mutant was also rescued by this construct. Very few (0.2%) dauer larvae were found among *unc-3*(*e151*) *flr-4*(*ut7*) animals carrying the extrachromosomal array of C03F11.3p::*flr-4* cDNA at 25°C. Thus, the expression of FLR-4 in the intestine was sufficient for all the wild-type phenotypes investigated in this study.

AUA Neurons Are Not Essential for the Control of Defecation Rhythm and Defecation Behavior

To elucidate the contribution of AUA neurons to defecation behavior and defecation cycle periods, we performed cell-killing experiments using a laser microsurgery technique. AUA neurons in six wild-type animals were killed. The AUA-killed animals showed normal defecation cycle periods (46.9 $\pm$ 2.2 s for the average $\pm$ SEM of 6 animals) and retained most of the Exp steps (Exp/pBoc = 97%). The results demonstrate that AUA neurons are not essential for

A

MPINYNRNNAV ELKLSSQLLQ WLDQRLPLGD PMRIPSIDSY
 KYIQDLGKGR FGTVCKFSNG NTFETVKKVD LTIFNHWTS
 ETKVSNRLDT FLYEFRHLHK VTNDNNRIVN FLGIYAD**ut3**SNQ
 MYIMSEYLPRGSVKDLLVKE TLGEDTAIKY LMETVEALDY
 LHNLSPPVIH RDIKAANLLI TSNDISIKLAN FGLVRDLAVD
 GFGIAIASEI TLDFRATLLY VAP**n2259 (223S)**EVLLSSAL GPGNRNAYEL
 PADIWAL**sa201 (248R)**GCT FIEMLLKRP HFEYFGHIDE IPKVLLGYAK
 SEDGKVLPT SEVLVPSSSN CVQKIVDLVF IKSP EHRPNT
 HKLRIQIKKI LDDDESEEEE TDISHPISNS NTDSSSTAISH
 NHSNDRKVGR AGTCLPIESM EYAAVRKELK KRSKPKSNNI
 MQIFVASGYY LSRILYFLNI LTRSICYLLL FLSLGITALG
 SFL LISYFVV RFVRYLIAIN CNCDLMQPQY LIISGILIVL
 MFALLFSCCM VAL**ut7 (494R)**GEYKFRM ANQTLDGSKF YLPRPQKSAV
 LCGITVITGK EDAKDTAQNM EEEIHLTPSV RRNHDDYYDY
 ESSGPANEEN

B

FLR4	(37)	-DSY	KYIQDLGKGRFGTVC	KFSNGN	-TFETVKKVDL
SOK1	(17)	-ELFTKLDRIGKGSFGEVYKGI	DNHTKEVVAIKI	IIDL	
BYR2	(391)	-IKWIRGALIGSGSFGQVY	LGMMASSGELMAVKQVIL		
CC15	(22)	-VQYHLKQVIGRGSYGVVYKA	INKHTDQVVAIKEV	VY	

FLR4	TIFN-(11)-R	LDTFLYEF	FRHLHKVTNDNNR	IVN	FLGIYADS
SOK1	EEA--	-EDEIEDIQ	ETVLSQCDSPYI	TRYFGSYLKS	
BYR2	DSVS-(8)-L	LDALAGEI	ALLQELLS--	HEHIVQYLGSNLNS	
CC15	ENDE--	-ELNDIMAEI	SLLKKNLN--	HNNIVKYHGFIRKS	

FLR4	NQMYIMS	EYLPRGSVKDLLVKE--	TLGEDTAIKYLMETVE
SOK1	TKLWIM	EYLGGSALDLLKPG--	PLEETIATTLRETLK
BYR2	DHLNIF	LEYVPGGSVAGLLTMYG-S	FEETLVKNFIKQTLK
CC15	YELYILLE	CANGSLRRLISRSSTGLS	ENESKTYVVTQTL

FLR4	ALDYHLNLSPP	VIHRDIKAANLLIT	SNDSTIKLANFGLVRD
SOK1	GLDYHSE--	RKIHRDIKAANVLLS	EQGDVKLADFGVAGQ
BYR2	GLEYLHSR--	GTVHRDIKGANILV	DNKGKIKISDFGISKK
CC15	GLKYLHGE--	GVIHRDIKAANILL	SADNTVKLADFGVSTI

FLR4	LAVDGF	GIAIASEITLDF	RATLLYVAPEVLLS-(7)-N	AYEL	
SOK1	LTDTQIKR	-----	NTFVGT	PFWMAPEVIKQ--S	AYDF
BYR2	LELNST	STKTGGAR-PS	FQGSFWM	APVVKQ--T	MHTE
CC15	VNSSA--	-----	LTLAGTLN	WMAPEILGN--R	CAST

FLR4	PADIWALG	CTFIEMLL-(14)-	IPKVLLGYAKS	EDGKVL	PYT
SOK1	KADIWSL	GLITAIELAK--	-GEP	PNSDLHPMRVL	FLIPKN
BYR2	KTDIWSL	GC	LVIEMLT--	-SKH	PYPNCDQMQAIFRIGEN
CC15	LSDIWSL	GA	TVVEMLT--	-KNP	PYHNLTDANIYYAVEN

FLR4	SEVL	VPS-S	SNCVQKIVDLVF	IKSPEH	RPNT	HKLR-(246)
SOK1	SPPT	LEGQHSK	PFKEFVEACLNK	DPFR	FRPTAKELL-(161)	
BYR2	ILPEF	P	SNISSAIDFLEKT	FATDCNLR	PTASELL-(6)	
CC15	DTYYP	P	SSFSEPLKDFLSKC	FVKNMYK	RPTADQLL-(707)	

C

Figure 2. *flr-4* encodes a novel Ser/Thr protein kinase with a C-terminal hydrophobic region. (A) Amino acid sequence of FLR-4. The kinase domain and the sequence rich in hydrophobic amino acids are shown by lines with arrowheads. The mutation sites of *ut3*, *ut7*, *n2259*, and *sa201* are indicated by bold letters with the allele name and the resultant amino acid substitution. The mutation site of *ut3* is located at a splice acceptor site (AG to AA). The cDNA sequence has been deposited in the DDBJ/EMBL/GenBank database (accession no. AB012700). (B) Comparison of the amino acid sequences between FLR-4 and Ser/Thr protein kinases resembling FLR-4, i.e., human SOK1 (Pombo *et al.*, 1996), *S. pombe* BYR2 (Wang *et al.*, 1991) and *S. cerevisiae* CDC15 (Schweitzer and Philippsen, 1991). Similar residues in three or four of them are boxed. The numbers in parentheses show those of amino acids omitted. The horizontal line indicates the STE20 signature GTP(Y/F)WMAPE. (C) Hydrophobicity plot (Hopp and Woods, 1981), showing a hydrophobic sequence consisting of 94 amino acids (horizontal line) in the C-terminal part (amino acid residues No. 400–493) of FLR-4.

Figure 3. Expression of GFP-tagged FLR-4 protein. (A–C) Nomarski images and (D–F) epifluorescence images of the corresponding animals of N2 carrying pMTG7–11 (GFP-tagged *flr-4*). (A and D) Early L1 larva. (B and E) Comma stage embryo. (C and F) Three-fold stage embryo. (G) Ventral view of an adult head region, showing that the fluorescence of FLR-4::GFP is detected in AUA neurons as well as the muscles of the pharyngeal isthmus. The DiQ fluorescent marker, which penetrates into some neurons (not including AUA), is shown in red. Scale bar, 50 μ m.

normal defecation behavior and defecation cycle periods in wild-type animals. We also killed AUA neurons in *flr-4(n2259)* animals. Even after the laser surgery, the animals exhibited short defecation cycle periods (27.2 ± 7.4 s for the average $\pm$ SEM of 4 animals) and frequently skipped the Exp step (Exp/pBoc = 30%). These findings show that defecation abnormalities of the *flr-4* mutant are not caused by the abnormal activity of AUA neurons.

Mutations in the Kinase Domain and near the Hydrophobic Region Impair Normal Defecation Rhythms

To study the structure-function relationship of the FLR-4 protein, we identified mutational changes in four independently derived *flr-4* alleles: *n2259*, *sa201*, *ut3*, and *ut7* (Figure 2A). Two

temperature-sensitive alleles, *n2259* and *sa201*, had missense mutations in the kinase domain (Figure 2A). The mutation *n2259* was located at a Proresidue in the activation loop of the protein kinase domain (P223S). This Proresidue is in the highly conserved consensus triplet, Ala-ProGlu, in the subdomain VIII of protein kinases (Hanks *et al.*, 1988) and seems to be important for the three-dimensional structure. The neighboring Glu residue probably forms an ion pair with the invariant Arg residue in subdomain XI and stabilizes protein conformation (Knighton *et al.*, 1991). The mutation *sa201* was a substitution of Arg for Gly at residue 248 (G248R) in subdomain IX. This Gly residue is nearly invariant in protein kinases (Hanks *et al.*, 1988). The large volume increase from Gly to Arg may cause instability in the protein structure.

Table 3. The allele *ut7* is antimorphic

Genotype	Generation time (days)
Wild type (N2)	3
<i>flr-4(ut3)</i>	5–7
<i>flr-4(ut7)</i>	5–7
<i>flr-4(n2259)</i>	3
<i>flr-4(sa201)</i>	3
<i>unc-3(e151) flr-4(ut3)/flr-4(n2259)</i>	3
<i>unc-3(e151) flr-4(ut3)/flr-4(sa201)</i>	3
<i>unc-3(e151) flr-4(n2259)/flr-4(ut3)</i>	3
<i>unc-3(e151) flr-4(sa201)/flr-4(ut3)</i>	3
<i>unc-3(e151) flr-4(ut7)/flr-4(n2259)</i>	5–7
<i>unc-3(e151) flr-4(ut7)/flr-4(sa201)</i>	5–7
<i>unc-3(e151) flr-4(n2259)/flr-4(ut7)</i>	5–7
<i>unc-3(e151) flr-4(sa201)/flr-4(ut7)</i>	5–7
<i>unc-3(e151) flr-4(ut7)/flr-4(ut3)</i>	5–7
<i>unc-3(e151) flr-4(ut3)/flr-4(ut7)</i>	5–7
<i>unc-3(e151) flr-4(ut3)/+</i>	3
<i>unc-3(e151) flr-4(ut7)/+</i>	3
<i>mnDf19/flr-4(n2259)</i>	3
<i>mnDf19/flr-4(sa201)</i>	3
<i>mnDf19/flr-4(ut3)</i>	5–7
<i>mnDf19/flr-4(ut7)</i>	5–7

Generation time was measured at 20°C. The deficiency *mnDf19* deletes *flr-4* gene. The *unc-3* mutation, which was used as a marker for strain construction, had no effect on growth rates as heterozygotes. To exclude the possibility that some *flr-4* mutations may show maternal effects, reciprocal crosses (such as *unc-3 flr-4(ut7)* hermaphrodites with *flr-4(n2259)* males and *unc-3 flr-4(n2259)* hermaphrodites with *flr-4(ut7)* males) were carried out, and both F1 and F2 heterozygotes were examined for the measurements.

The mutation *ut7* was a substitution of Arg for Gly at residue 494 (G494R), just after the stretch of 94 hydrophobic amino acid residues. This allele seems to be a weak antimorph, as shown in the next section.

The mutation *ut3* changed a splice acceptor sequence AG to AA, which is located within the DNA sequence for the kinase domain (Figure 2A). The nucleotide change is in agreement with the prediction that this mutation is null or hypomorphic, which is based on the results that *ut3/Df* shows almost the same phenotype as *ut3/ut3* (Katsura *et al.*, 1994).

ut7, a Missense Mutation in the C-terminus, Is a Weak Antimorph

To learn about the nature of *ut7*, a missense mutation in the C-terminus, we investigated the phenotypes of the heterozygotes *ut7/n2259* and *ut7/sa201*, where *n2259* and *sa201* are weak missense alleles that show normal growth. As controls, we used *ut3*, an allele with a nucleotide change in a splice-acceptor sequence, and *mnDf19*, a deficiency deleting the entire *flr-4* gene. Although both *ut3* and *ut7* showed slow growth as homozygotes (Tables 1), they showed distinct phenotypes concerning growth rates when they were placed *in trans* to the weak alleles, *n2259* and *sa201* (Table 3). The heterozygotes *ut3/n2259* and *ut3/sa201* grew at normal rates, but the heterozygotes *ut7/n2259* and *ut7/sa201* grew slowly. The result indicates that *ut7* is antimorphic, because the latter heterozygotes show stronger phenotypes than *mnDf19/n2259* and *mnDf19/sa201*, which grow at normal rates. We conclude that *ut7* is a weak antimorph, whose effect is apparent only in heterozygotes *ut7/n2259* or *ut7/sa201* but not in *ut7/+*.

Figure 4. Temperature-shift experiments of the *flr-4 (n2259ts)* mutant concerning defecation cycle periods. For temperature-upshift experiments, the cultivation temperature of *flr-4(n2259)* worms was raised from 20 to 25°C at various developmental stages, and their defecation behavior was observed as young adults at 25°C. For temperature-downshift experiments, the temperature was changed from 25 to 20°C at various developmental stages, and the defecation behavior was observed as young adults at 20°C. The open and closed circles represent the mean defecation cycle periods of young adult animals after temperature upshift and downshift, respectively, at the developmental stage indicated on the abscissa. “Control” indicates experiments with no temperature-shift. Data are mean $\pm$ SEM. The mean and SEM values were calculated from the mean defecation cycle periods of 3–11 animals, each of which was an average of 7–22 defecation cycle periods.

FLR-4 Function Is Required for the Control of Defecation Cycle Periods at the Time of Behavioral Assay

To estimate the developmental stage at which the *flr-4* function is required, we performed temperature-shift experiments using *n2259*, an allele that exhibits temperature-sensitive abnormality in defecation cycle periods. As shown in Figure 4, when the temperature was shifted upward during larval development, the animals had short defecation cycle periods as young adults, regardless of the time of the temperature shift during the development. Short defecation cycles were observed as soon as 30 min after shift to 25°C (unpublished data). In contrast, when the temperature was downshifted, the animals had normal defecation cycle periods, unless the time of the temperature shift was just before the behavioral assay. From these results we conclude that the FLR-4 function is required at the time of the behavioral assay, which was performed at the adult stage. This suggests that FLR-4 is involved in a cell-functional aspect of defecation rhythm regulation rather than a developmental aspect, for instance, the generation or differentiation of the cells required for the regulation.

DISCUSSION

FLR-4, a Novel Ser/Thr Protein Kinase, Regulates Defecation Rhythm in Intestinal Cells

The *flr-4* gene, whose mutants show very short defecation cycle periods, encodes a novel serine/threonine protein kinase. This suggests that the defecation rhythm is regulated by protein phosphorylation. Examples of protein phosphorylation that regulates ultradian rhythms have been reported. Mutants in the *Drosophila dco* gene, which encodes the major catalytic subunit of protein kinase A, display arrhythmic locomotor activity (Majercak *et al.*, 1997). Phosphorylation of the metabotropic glutamate receptor 5a by protein kinase C

evokes Ca^{2+} oscillations in HEK293 cells transfected with the glutamate receptor (Kawabata *et al.*, 1996).

Although the functional *flr-4::GFP* fusion gene was expressed in the intestine, part of pharyngeal muscles and AUA neurons, expression at all these sites may not be necessary for the functions of FLR-4. Experiments with an extrinsic promoter revealed that expression in the intestinal cells was sufficient for the wild-type phenotypes in defecation rhythm, the Exp step of defecation behavior, growth, dauer larva regulation, and fluoride sensitivity. Furthermore, laser ablation of AUA neurons did not change the characteristics of defecation rhythm and defecation behavior of wild-type and *flr-4* animals. Thus, the expression of FLR-4 in the intestine is apparently sufficient for all the functions investigated in this study.

The temperature-shift experiments of the *flr-4(n2259ts)* mutant showed that a functional *flr-4* gene product is required at the time of defecation measurements at the adult stage. The result suggests that the *flr-4* gene has a function in differentiated cells, and not in the generation or differentiation of cells involved in the regulation of defecation rhythm.

Taking these arguments into consideration, we conclude that FLR-4 acts in the intestine at the time of defecation behavior to increase the length of defecation cycle periods.

Roles of the Kinase Domain and Hydrophobic Region in the Functions of FLR-4

The kinase domain of FLR-4 appears to be important for defecation rhythm. FLR-4 contains essentially all the conserved amino acid residues of protein kinases, except that the DFG motif in subdomain VII is changed to a closely related sequence NFG. Two mutations, *n2259* and *sa201*, are located at nearly invariant residues in the kinase domain. The results suggest that the kinase domain is essential at least for the FLR-4 functions that are impaired by these kinase domain mutations, i.e., normal defecation rhythms, the Exp step of defecation behavior, and sensitivity to fluoride ion. The three-dimensional structures of protein kinases (Knighton *et al.*, 1991; DeBondt *et al.*, 1993) suggest that the amino acid residues altered by these mutations may play a role in stabilizing the structure of the kinase domain. This is consistent with the temperature-sensitive phenotype on the defecation cycle abnormality of these mutations.

The allele *flr-4(ut7)*, which has a mutation adjacent to the hydrophobic region outside the kinase domain, appears to be a weak antimorph. This study revealed that the heterozygotes *ut7/n2259* and *ut7/sa201* grow slowly, whereas *Df/n2259* and *Df/sa201* grow almost normally. The antimorphic effect was clear only in heterozygotes *ut7/n2259* or *ut7/sa201* but not in *ut7/+*. The reason may be that *flr-4(n2259)* and *flr-4(sa201)* genes, probably supply a smaller number of active FLR-4 protein molecules than the wild-type *flr-4* gene, due to protein instability or protein folding abnormality. The antimorphic property suggests that FLR-4(*ut7*) participates in molecular interactions required for the FLR-4 function but hinders at least one of the interactions, resulting in a poisonous effect. The amino acid substituted by the *ut7* mutation, which is adjacent to the C-terminal hydrophobic region, may be located at or near the interaction site. Alternatively, the *ut7* mutation may change the overall protein conformation and damage the interaction site that is distantly located from the substituted amino acid.

The function of the predicted transmembrane regions in the C-terminal part is intriguing in relation to the unique structural feature of FLR-4. Only a few protein kinases have predicted transmembrane regions on the C-terminal side of the kinase domain, like FLR-4. Of the predicted transmem-

brane regions of such exceptional protein kinases, those of prostate-derived STE20-like kinase (PSK) and myotonic dystrophy kinase are actually not transmembrane regions. The former are required for localization to microtubules (Mitsopoulos *et al.*, 2003), whereas the latter play a role in the formation of a large self-associated complex that favors peripheral association with membranes (Waring *et al.*, 1996). Like these cases, the C-terminal part of FLR-4 may play a role in the interaction with other proteins. Alternatively, it may contain membrane-spanning regions that anchor the FLR-4 molecule to the cell membrane. Unfortunately, it is difficult to study the function of the predicted transmembrane regions by detecting the localization of FLR-4. FLR-4::GFP without the C-terminal part is already localized at the cell membrane, like full-length FLR-4::GFP (unpublished data).

Relationship among Various FLR-4 Functions

flr-4 mutants show diverse phenotypes: resistance to fluoride ion, slow growth, tendency to form dauer larvae, and defects in the Exp step of defecation, in addition to short defecation cycle periods (Katsura *et al.*, 1994; Iwasaki *et al.*, 1995). This study revealed that they also show a severe decrease in Exp/pBoc ratio. The result suggests that *flr-4* has a role in the signaling downstream of the defecation cycle clock in addition to its role in regulating the defecation cycle period. The Exp step is controlled by the DVB neuron, which innervates rectal muscles, whereas expression of *flr-4* in the intestine is sufficient for normal Exp. Hence, probably there are signal molecules that are controlled by *flr-4* gene and that transmit the signal of Exp from the intestine to the DVB neuron and/or AVL neuron, which is also required for Exp. Other molecules that transmit a signal from the intestine to neurons may be present for the regulation of dauer larva formation by *flr-4* gene. Because *ut3* and *ut7* mutants exhibit a starved appearance as well as slow growth, this signal may be induced by malnutrition due to functional defects of the *flr-4* mutant intestine. Investigation of the relationship among those diverse functions may be useful also for elucidating the mechanism of defecation rhythm regulation by *flr-4* gene.

Of the phenotypes of *flr-4* mutants, short defecation cycle periods, frequent skip of Exp, and resistance to fluoride ion can be separated from slow growth rates and tendency to form dauer larvae. The mutants *flr-4(n2259)* and *flr-4(sa201)*, as well as the double mutants *flr-4(ut3 or ut7); flr-2* and *flr-4(ut3 or ut7); flr-5*, showed only the former and not the latter phenotypes, except that *flr-4(n2259)* produced some dauer larvae in the *unc-3(e151)* background (this study; Katsura *et al.*, 1994; our unpublished results). The results indicate either that *flr-4* gene acts in different pathways for the two groups of phenotypes or that the threshold of the FLR-4 activity between the mutant and wild-type phenotype is different for the two groups.

The *flr-4* gene regulates both defecation rhythm and Exp, although they are controlled in general by distinct genes (Thomas, 1990; Iwasaki *et al.*, 1995). The results of this study revealed that the Exp phenotype but not the Dec-short phenotype of *flr-4(ut7)* is suppressed by fluoride ion or by low osmotic pressure. The suppression is specific, because slow growth, which is suppressed relatively easily as discussed above, is not suppressed in this case. These results suggest that FLR-4 may control defecation rhythm and Exp in different pathways.

Fluoride ion kills wild-type larvae and elongates the defecation interval length of wild-type adults. In contrast, it does not kill *flr-4* mutant larvae efficiently, nor does it elon-

gate the defecation interval length of *flr-4* adults. Namely, the effects of fluoride ion require an intact *flr-4* gene. Although the target of fluoride ion for these effects has not been identified, we suspect that a protein phosphatase or a trimeric G-protein may be the target. It is known that fluoride inhibits the activity of some protein phosphatases (Ballou and Fischer, 1986), and, as aluminum fluoride, fixes trimeric G-proteins to the active state (Gilman, 1984). The FLR-4 kinase may act in the same pathway as a G-protein or a protein phosphatase, although there is so far no evidence. Identification of the target, therefore, may help in elucidating the mechanism of defecation rhythm regulation.

The flr-4 Gene, Together with flr-1 and flr-3, May Constitute a Regulatory System that Controls Defecation Rhythm

The *flr-4* gene appears to regulate defecation rhythm in the same pathway as two other genes, *flr-1* and *flr-3*. Mutants in these genes as well as double and triple mutants of *flr-1*, *flr-3* and *flr-4* show essentially the same phenotypes as those of *flr-4* (Katsura *et al.*, 1994; Iwasaki *et al.*, 1995; Table 1 of this article). *flr-1* and *flr-3* are expressed only in the intestine (Take-uchi *et al.*, 1998; Kawakami, Ishihara, and Katsura, unpublished results). Furthermore, the expression of *flr-4* in the intestine is sufficient for its functions, although it is expressed in part of the pharynx and AUA neurons in addition to the intestine. These results support our hypothesis that *flr-1*, *flr-3*, and *flr-4* constitute an intestinal regulatory system that controls defecation rhythm (Take-uchi *et al.*, 1998).

How do *flr-1*, *flr-3*, and *flr-4* interact with each other to achieve their functions? The FLR-4 protein kinase may activate the FLR-1 ion channel by phosphorylation, or FLR-1 may activate FLR-4 by changing the intracellular concentration of a certain ion. The former possibility was suggested by the presence of many putative phosphorylation sites in the intracellular domain of the FLR-1 ion channel (Take-uchi *et al.*, 1998). However, further studies are necessary to prove the hypothesis.

Dal Santo *et al.* (1999) demonstrated that the calcium concentration in the intestinal cells oscillates with peak calcium levels just before the pBoc steps of defecation and that the activity of the IP₃ receptor in the intestine regulates both defecation rhythm and calcium oscillation frequency. Thus, calcium oscillations in the intestine seem to be an essential part of defecation rhythms. On the basis of these results, an important issue that remains to be resolved is how *flr-1*, *flr-3*, and *flr-4* genes modulate the period of calcium oscillations in the intestine.

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