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The Phosphobase Methylation Pathway in *Caenorhabditis elegans*: A New Route to Phospholipids in Animals

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Abstract

Parasitic nematodes are a major cause of human health problems with an estimated 1 billion people infected worldwide by these organisms. Identifying biochemical targets that differ between the parasite and host species is essential for finding effective new anti-parasitic molecules. The free-living nematode *Caenorhabditis elegans* is a powerful model system for experiments in genetics and developmental biology needed to achieve this goal; however, in-depth understanding of metabolic processes in this organism is limited as it still contains unexplored biochemical pathways. Eukaryotes, including nematodes and humans, share many similar metabolic pathways, which makes specific targeting of nematode parasites challenging. Recent studies suggest that *C. elegans* and other nematodes may use a plant-like pathway as the major biosynthetic route to phosphatidylcholine. In this pathway, a pair of phosphoethanolamine methyltransferases (PMT) catalyze the sequential methylation of phosphoethanolamine to phosphocholine, which can be incorporated into phosphatidylcholine. RNAi experiments demonstrate that both PMT are required for normal growth and development of *C. elegans*. Because the PMT are highly conserved across nematode parasites of humans, livestock, and plants, as well as in protozoan parasites, understanding how these enzymes function and the identification of inhibitors will aid in the development of new anti-parasite compounds of potential medical, veterinary, and agricultural value.

Keywords

Caenorhabditis elegans; nematode; phosphobase methylation; phospholipids; methyltransferase; phosphoethanolamine; phosphocholine

INTRODUCTION

Caenorhabditis elegans is a powerful model system for genetics and developmental biology, and for identifying biochemical targets for the development of drugs active against parasitic nematodes. Eukaryotes, including nematodes and humans, share many similar metabolic pathways, which makes specific targeting of parasites challenging. Recent studies suggest that *C. elegans* and other nematodes, unlike other animals, may use a plant-like pathway as the major biosynthetic route to phosphatidylcholine. In this pathway, a pair of

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phosphoethanolamine methyltransferases (PMT) catalyze the sequential methylation of phosphoethanolamine to phosphocholine, which can be incorporated into phosphatidylcholine. Although RNAi experiments demonstrate that both PMT are required for normal growth and development of *C. elegans*, the structural and molecular basis for how these enzymes function and their role in phospholipid metabolism remain to be established.

OVERVIEW OF PHOSPHOLIPID SYNTHESIS PATHWAYS IN EUKARYOTES

Phosphatidylcholine is a major phospholipid component in the cellular membranes of eukaryotes [1–2]. Phosphatidylcholine synthesis occurs through three metabolic routes in different organisms (Fig. 1). In mammals, the *de novo* choline or Kennedy pathway is the major synthetic route from choline to phosphatidylcholine [1–4]. Yeast and mammalian liver cells primarily use the Bremer-Greenberg pathway to methylate phosphatidylethanolamine to phosphatidylcholine [5–6]. In plants, metabolic flux into the Kennedy pathway occurs through a third route that involves S-adenosyl 1-methionine (SAM)-dependent methylation of phosphoethanolamine to phosphocholine [7–8]. Recent work suggests that *C. elegans* and other nematodes [9–11], along with the protozoan parasite *Plasmodium falciparum* (the causative agent of malaria), use the plant-like phosphobase pathway as the primary route to provide phosphocholine for the synthesis of phosphatidylcholine [12–13].

KNOWN ROUTES TO PHOSPHATIDYLCHOLINE IN *C. elegans*

The *C. elegans* genome contains the genes for both the *de novo* choline and Bremer-Greenberg pathways for the synthesis of phosphatidylcholine [11]; however, molecular studies suggest the presence of an alternate route to this phospholipid. In *C. elegans*, both the *de novo* choline and Bremer-Greenberg pathways have been examined to varying degrees by biochemical and high-throughput RNA interference (RNAi) approaches. These studies highlight how little is known about phospholipid synthesis in this model organism at the molecular level. RNAi screens suggest that later enzymes in the *de novo* choline pathway are important for worm growth and development, that the Bremer-Greenberg pathway plays a lesser role, and that an alternate pathway may exist in *C. elegans* to provide metabolites for the synthesis of phosphatidylcholine.

Biochemically, the best-studied parts of these pathways are the initial steps of the Kennedy pathway catalyzed by choline kinase and CTP:phosphocholine cytidyltransferase (CCT). Choline kinase catalyzes the first step in the Kennedy pathway (Fig. 1). *C. elegans* encodes seven isoforms of this enzyme, which cluster into three families A, B, and C [14]. The A-family isoforms (*cka-1* and *-2*) are 35% identical to the mammalian choline kinases, while the B-family (*ckb-1*, *-2*, *-3*, and *-4*) and C-family (*ckc-1*) share <25% sequence identity [14]. Biochemical analysis of CKA-1, CKA-2, CKB-2, and CKB-4 demonstrates a 10- to 20-fold preference for choline versus ethanolamine as a substrate [13]. The remaining isoforms are biochemically uncharacterized, but postulated to function as ethanolamine kinases [13]. In the next reaction, CCT catalyzes the synthesis of CDP-choline [1–2]. The genome of *C. elegans* contains five genes sharing roughly 60% sequence identity with mammalian CCT [14], but only one of these genes functions as a CCT [15]. The last enzyme of the pathway, diacylglycerol choline phosphotransferase, converts CDP-choline and 1,2-

diacylglycerol to CMP and phosphatidylcholine. It is a transmembrane protein that has not been purified to homogeneity from any source [1], and there are no reports describing this enzyme or its activity in *C. elegans*.

Genome-wide RNAi experiments indicate that certain isoforms of the enzymes in the *de novo* choline pathway are critical for normal growth and development [16–23]. Abnormal embryo and post-embryo developmental phenotypes were observed for *ckb-1* and *ckb-2* in some studies [17–19]; however, other genome-wide studies describe no effect resulting from silencing of any *ckb* gene [16, 20–23]. This difference may result from functional redundancy of the isoforms, alternate routes for phosphatidylcholine synthesis, or different growth conditions in these studies. It is unclear if *C. elegans* utilizes an external supply of choline for synthesis of phosphatidylcholine, as growth phenotypes require the addition of choline at concentrations (10–30 mM) far exceeding physiological levels [9–10]. Of the possible CCT isoforms, only RNAi of the verified CCT showed larva arrest in some experiments [16–17, 21], RNAi of the diacylglycerol choline phosphotransferase results in phenotypes ranging from the arrest of larva growth to embryo lethality [16–17, 21, 23]. Importantly, these studies indicate that the last two steps of the pathway are essential for normal growth and development. It should be noted that genome-wide RNAi screens are initial efforts to broadly define a role for these genes, and supporting studies are required to elucidate the contribution of various enzymes in the Kennedy pathway of nematodes.

The metabolic contribution of the Bremer-Greenberg pathway to phosphatidylcholine synthesis in nematodes appears less important for normal growth. The presence of genes encoding phosphatidylserine decarboxylase and phosphatidylethanolamine N-methyltransferase implies a functional Bremer-Greenberg pathway in *C. elegans* (Fig. 1). The putative *C. elegans* phosphatidylserine decarboxylase shares less than 40% identity with the mammalian enzyme [11]. RNAi of the decarboxylase results in abnormal development at the embryo and post-embryo stages [17–19, 21], suggesting that metabolism of ethanolamine to phosphatidylethanolamine cannot substitute for phosphatidylserine decarboxylase during development [24]. In contrast RNAi of the phosphatidylethanolamine N-methyltransferase genes in *C. elegans* yields no observable phenotype [16–17]. It is unclear if these results indicate that the methylation of phosphatidylethanolamine is not essential or if other pathways compensate for their loss.

PHOSPHOBASE METHYLATION IS REQUIRED FOR GROWTH AND DEVELOPMENT IN *C. elegans*

Unlike animals, plants use a pathway for the endogenous synthesis of phosphatidylcholine that effectively bypasses the need for either exogenous or recycled choline (Fig. 1). In the initial reaction of the phosphobase pathway, plants generate ethanolamine by direct decarboxylation of serine [25]. There is no information on the corresponding enzyme in *C. elegans*, but at least four genes encode biochemically uncharacterized proteins sharing ~40% sequence similarity with the plant serine decarboxylase.

The core of the phosphobase methylation pathway involves the sequential methylation of phosphoethanolamine into phosphocholine (Fig. 2). Early reports on the plant and

Plasmodium PMT described the presence of two hypothetical genes in *C. elegans* (ZK622.3a/b; *pmt1* and F54D11.1; *pmt2*) sharing less than 20% identity with the PMT from plants and protozoa [12, 26–28]. These observations suggested that *C. elegans* might use a plant-like phosphobase methylation pathway for phosphoethanolamine synthesis. Each of the *C. elegans* PMT are more closely related to the plant enzymes than they are to each other, as they share only 12% sequence identity [9–10].

Biochemical analysis of each potential PMT from *C. elegans* showed that each enzyme is a SAM-dependent methyltransferase with distinct reaction specificity [9–10]. CePMT1 catalyzes only the first methylation reaction (phosphoethanolamine → phosphomonomethylethanolamine; pMME) in the pathway [10], whereas, CePMT2 completes the pathway by converting pMME to phosphodimethylethanolamine (pDME) and pDME to phosphoethanolamine [9].

Targeted gene silencing of either *pmt1* or *pmt2* by RNAi yields pronounced phenotypes. Expression of each gene is required for normal growth and development of *C. elegans* at the L1 (early larva), L4 (late larva), and dauer (stasis) stages of its lifecycle [9–10]. For RNAi of *pmt1*, when the parental worm (P0) exposure began either as an L1 or dauer larva, the phenotype was complete or highly penetrant (>95%) P0 sterility [10]. When exposure began as a P0 L4 larva, the phenotype was arrested development and lethality in the progeny (F1) at the L1/L2 or the L3 larva stage. The worms show large lipid droplet accumulation in the intestines, suggesting *pmt1* is also involved in the homeostasis of triacylglycerol and phosphatidylcholine [29]. Since the *pmt1* RNAi experiments show that different larva stages were all developmentally impaired, *pmt1* is necessary at multiple stages in the life cycle. To determine if providing pathway metabolites reverses the *pmt1* RNAi phenotype, growth media were supplemented with ethanolamine, MME, DME, or choline [10]. Addition of ethanolamine did not reverse the *pmt1* phenotype for the L1, dauer, or L4 worms. Supplementing the media with MME (10 mM), DME (10 mM), or choline (30 mM) rescued the P0 sterility and F1 larval arrest. These results confirm that CePMT1 functions at a step before MME enters the pathway [10].

Similar experiments were performed using RNAi of *pmt2* [9]. L1 and dauer larva grown in the presence of *E. coli* expressing double-stranded RNA (dsRNA) from the *pmt2* gene yielded sterile progeny. Likewise, feeding RNAi to L4 larva resulted in arrested development and lethality in the L1/L2 larva [10]. Chemical complementation of the *pmt2* phenotype provides information on the biological function of the protein [10]. Growing *C. elegans* in media supplemented with choline (30 mM) reversed the *pmt2* RNAi-mediated phenotype; however, chemical rescue of the phenotype did not occur in media supplemented with ethanolamine, MME, or DME. This indicates that CePMT2 is upstream of phosphocholine entry into the Kennedy pathway and is required for the utilization of pMME and pDME [10].

The observed phenotypes of both *pmt1* and *pmt2* are similar to those reported in RNAi experiments targeting phosphatidylcholine metabolism in *C. elegans* that resulted in drastic reductions in fertility [16–23], which suggests a role of phosphatidylcholine in embryo development and worm growth. To provide insight on temporal and spatial expression

pattern of PMT, several localization studies of PMT1 have been conducted. Transgenic worms containing a *pmt1::gfp* construct showed strong expression of PMT1 in the hypodermis in the all larval stages and in seam cells in the adult stage [29]. Using wild-type worms, immunolocalization of PMT 1 suggests the presence of PMT 1 in the whole body of larva (data not shown) and in the vulva of adult worms (Fig. 3). Although RNAi experiments demonstrate a critical role for both PMT and recent localization studies examine the expression patterns of PMT1, tissue-specific expression patterns of PMT2 and co-expression patterns of both PMT in *C. elegans* remain to be examined.

THREE TYPES OF PMT IN NEMATODES, PROTOZOANS, AND PLANTS

Although organisms ranging from nematodes to protozoans to plants utilize PMT for the SAM-dependent methylation of phosphoethanolamine to phosphocholine, the physical organization of the methyltransferase domains in plants (type 1), protozoa (type 2), and nematodes (type 3) differs dramatically (Fig. 4). All of the PMT share four consensus sequence motifs (I-IV; Fig. 5) that define canonical SAM-binding domains in various methyltransferases [30].

The plant PMT contain tandem methyltransferase domains in a single polypeptide [26–28] (Fig. 4). Generation of a truncated plant PMT variant lacking the C-terminal domain resulted in an N-terminal methyltransferase domain protein that only catalyzed the initial methylation of phosphoethanolamine to pMME, suggesting that the C-terminal methyltransferase domain performs the next two reactions to yield phosphocholine [27],

In contrast to the plant PMT, the enzyme from the malarial parasite *P. falciparum* is half the length and consists of a single methyltransferase domain that accepts all three phosphobase substrates [12–13] (Fig. 4). As with *C. elegans*, the PfPMT is required for normal growth and development of this protozoan parasite [13].

The PMT in *C. elegans* reveal a third scheme for organization of the methyltransferase active sites (Fig. 4). Although the *C. elegans* PMT are distantly related to the plant enzymes, the dual methyltransferase domain architecture, as defined by the SAM-binding motifs, is not found in the *C. elegans* PMT [30]. The *C. elegans* genes encode proteins containing either the N-terminal methyltransferase domain (CePMT 1) or the C-terminal methyltransferase domain (CcPMT2). In each *C. elegans* PMT, the methyltransferase consensus motifs in the other half of each protein shows major sequence differences (Fig. 5), suggesting that the biochemical function of these domains is lost. Our published work on the steady-state kinetics of these proteins shows that CePMT 1 indeed only catalyzes the conversion of phosphoethanolamine to pMME and that CePMT2 methylates pMME to pDME and pDME to phosphocholine with each enzyme using a random bi bi kinetic mechanism [9–10]. In *C. elegans*, gene duplication and divergence into separate enzymes that perform the initial and subsequent methylation reactions, respectively, has occurred.

Although the phosphobase methylation pathway is found in three different kingdoms - animals (*C. elegans* and other nematodes), protists (*P. falciparum*), and plants (such as Arabidopsis, spinach, and wheat) - the molecular basis for the structural evolution of the

three types of PMT remains unresolved. Sequence examination of the genome of multiple parasitic nematodes of humans, including *Ascaris lumbricoides* (intestinal roundworm), *Trichuris trichiura* (whipworm), *Necator americanus* (hookworm) and *Ancylostoma duodenale* (hookworm), as well as parasites of livestock and plants, indicates that these organisms all encode highly homologous (>50% sequence identity) type 3 PMT with shared methyltransferase domain architectures [9–11]. Moreover, the type 2 PMT are also conserved (>75% sequence identity) in all four *Plasmodium* species associated with malaria [12]. Overall, the PMT are not found in humans or other mammals and are essential for normal growth and development. Thus, these proteins are new targets for the development of anti-parasitic inhibitors.

CONCLUSIONS

Parasitic nematodes are a major cause of mortality and morbidity worldwide with an estimated 1 billion people infected by each species of major nematode parasites (i.e., *Ascaris*, *Trichuris*, and hookworms) [31–32]. Moreover, increasing population levels and urbanization correlate with a rising prevalence of helminth infections [31]. Parasitic nematodes also result in considerable losses in livestock and domestic animals and more than \$125 billion in crop damage annually [32–35]. The existing pharmacopoeia targeting nematode parasites is chemically varied [11], but the primary impetus to develop new broad-spectrum anthelmintics, much like the drive for novel antibiotics, is drug resistance. Currently, reports describe resistance to all compounds of the few anthelmintic classes available [36–37]. The development of new anti-parasitic molecules requires better understanding of the biochemistry of nematodes and the identification of new pathways in these organisms that differ from the host species.

Genome science has transformed parasitology as sequence information on free-living and parasitic nematodes, including *C. elegans*, *Brugia malayi* (lymphatic filariasis), *Onchocerca volvulus* (river blindness), *N. americanus* (human hookworm), *A. lumbricoides* (human intestinal roundworm), and *Trichinella spiralis* (human-infective muscle cell nematode), and protozoans (*Babesia*, *Eimeria*, *Giardia*, *Leishmania*, *Plasmodium*, *Toxoplasma*, *Trichomonas*/Trypanosomes) allows for testing of hypothesis developed by comparative bioinformatics [32–35, 38]. Exploration of these organisms is essential to provide fundamental insights on eukaryote and pathogen biology and to open opportunities to identify and exploit novel and essential metabolic features for drug discovery targeted at neglected diseases endemic in the developing regions of Africa, Asia, and the Americas.

For example, the free-living nematode *C. elegans* serves as a useful model system for studying nematode biology and as a platform for biochemical discovery and anti-parasitic drug development [32–35]. Using *C. elegans* as a model system provides a myriad of advantages: the worms are easily cultured, rapidly and prolifically reproduce, they are transparent and allow easy visualization of molecular, cellular, and organelle structure, molecular methods are well established, the developmental lineage of each cell is known, ongoing functional annotation of the genome continues through the WormBase (www.wormbase.org) consortium, and RNAi provides a valuable tool to analyze gene function [32–35]. Moreover, *C. elegans* shares physiological and pharmacological properties

with many nematode parasites [32–35]. In contrast to the depth of developmental biology and genetic insight on *C. elegans*; however, the biochemical understanding of metabolism in *C. elegans* is limited in scope. This promises continued surprises about how this organism functions.

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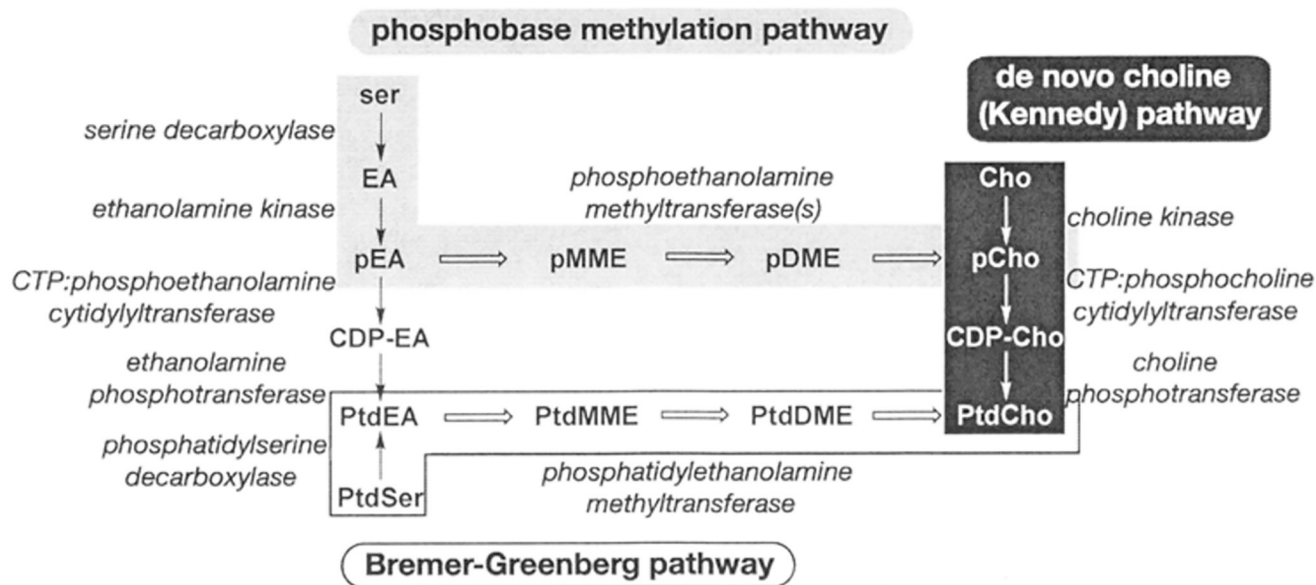


Fig. 1. Overview of Pathways.

The *de novo* choline, Bremer-Greenberg, and phosphobase methylation pathways are shown. Conversions between the base, phosphobase, CDP, and phospholipid substrates are shown as vertical arrows. Horizontal arrows correspond to SAM-dependent methylations. Names of metabolites consist of a prefix (p, phospho; CDP-, cytidine 5'-diphosphate; or Ptd, phosphatidyl) and a core name (EA, ethanolamine; MME, monomethylethanolamine; DME, dimethylcthanolamine; Cho, choline).

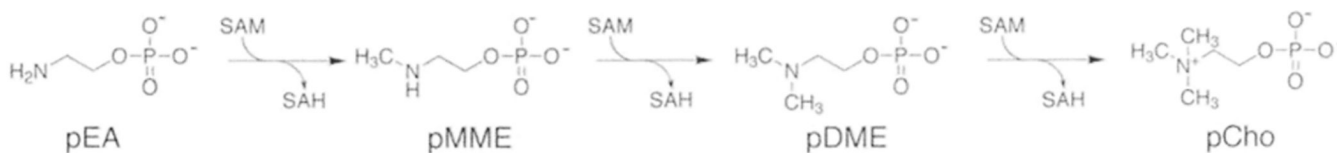


Fig. 2. The Phosphobase Methylation Pathway.

The sequential methylation of phosphoethanolamine (pEA) to phosphomonomethylethanolamine (pMME), phosphodimethylethanolamine (pDME), and phosphocholine (pCho) catalyzed by the PMT provides an entry point into the *de novo* choline pathway.

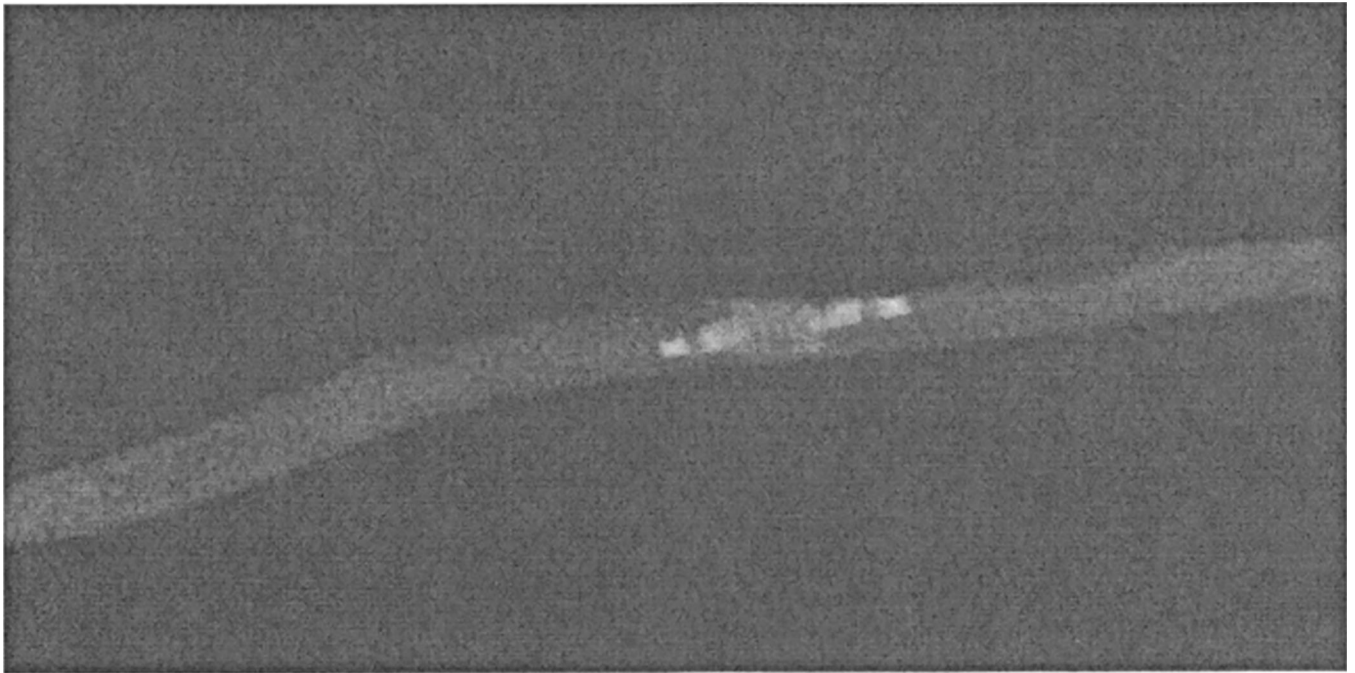


Fig. 3. Immunolocalization of PMT1 in *C. elegans*.
The permeabilized adult worms were incubated with primary PMT1 antibodies, and then with Alexa Fluor dye-conjugated anti-rabbit antibodies.

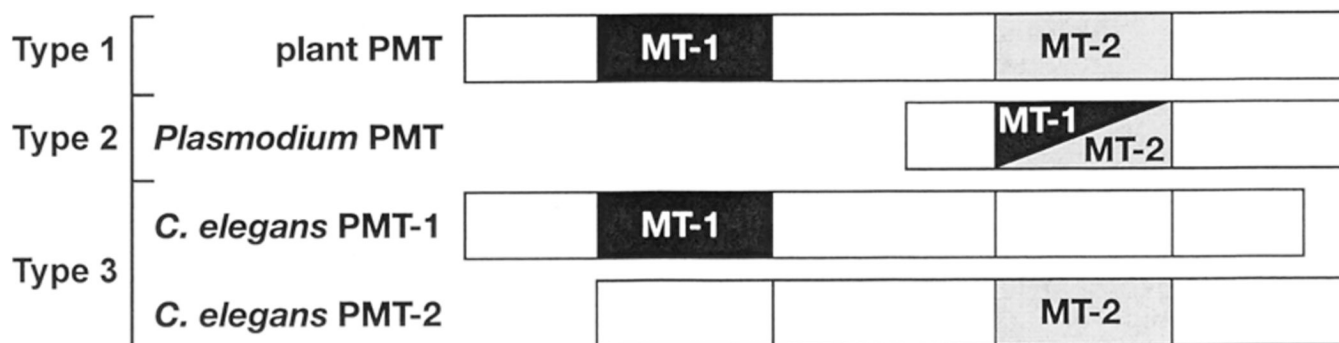


Fig. 4. Methyltransferase Domain Organization in the Three Types of PMT.

Plant PMT contain two methyltransferase domains (MT) in tandem. MT-1 (black) catalyzes the conversion of pEA to pMME. MT-2 (grey) catalyzes the methylation of pMME to pDME and pDME to pCho. *P. falciparum* PMT contains a single bifunctional domain that catalyzes all three methylation reactions. Nematodes, like *C. elegans*, encode monofunctional PMT that catalyze either the MT-1 or MT-2 reaction. Nematode PMT also contain a vestigial MT domain (white box).

		motif			
		I	II	III	IV
Domain MT-1	CePMT1	VVDIGAGIG	VLSTD	NSVDLVF	WLRSHGIVH
	SoPMT	VLELGAGIG	VIALD	NSVDIIF	WLKPGGYIF
	AtPMT	VLELGAGIG	LIALD	GSLDLIF	WIKVGGYIF
	TaPMT	VLELGAGIG	VIALD	NSVDIVF	WLKPGGHIF
	CePMT2	SL SIPRQSL	VSNVQ	NADVLI	ATAIGGTVI
		hhahGxGxG	hhxhD	NXhDhhh	hLbPGGxhh
Domain MT-2	CePMT1	TFFLYTPRT	TLAET	NQS...	..TEFLSTV
	SoPMT	VLDVGCIG	VVGID	NSFDVIY	WLKPGGKVL
	AtPMT	VLDVGCIG	VVGID	NSFDVIY	WLKPGGKVL
	TaPMT	VLDVGCIG	VLGID	NTFDVIY	WLKPGGKVL
	CePMT2	MLDIGVGIG	VHGID	NSFDYVF	ALKPGGKVL
	PfPMT	VLDIGSGLG	THGID	NNFDLIY	WLKPTGTL

Fig. 5. SAM-binding Motifs in the Two Domains of the PMT.

The canonical sequence is shown between the MT-1 (dark grey) and MT-2 (light grey) sequences (h=hydrophobic, a=acidic, b=basic, x=variable). The CePMT, SoPMT, AtPMT, TaPMT, and PfPMT are from *C. elegans*, spinach, Arabidopsis, wheat, and *P. falciparum*, respectively.