Functional Conservation of Coenzyme Q Biosynthetic Genes among Yeasts, Plants, and Humans

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Introduction

Coenzyme Q (CoQ), also known as ubiquinone, is an isoprenoid quinone that is distributed widely in almost all living organisms [1–3]. CoQ is a component of the respiratory chain in the inner mitochondrial membrane of eukaryotes and functions primarily as an electron transporter during aerobic respiration and oxidative phosphorylation. CoQ serves as the electron transporter of the NADH dehydrogenase and succinate dehydrogenase complexes to form CoQ:cytochrome c reductase and thus is an essential component of the ATP synthesis pathway. CoQ also functions as a lipid-soluble antioxidant that scavenges reactive oxygen species in cellular biomembranes [4]. Additional roles of CoQ include disulfide bond formation [5], sulfide oxidation [6], and pyrimidine metabolism [7,8].

In living organisms, CoQ exists in a number of different forms with differing isoprenoid side chain lengths. For example, in humans and the fission yeast Schizosaccharomyces pombe, the CoQ side chain comprises ten isoprene units (CoQ10), whereas those in Arabidopsis thaliana and Saccharomyces cerevisiae are composed of nine (CoQ9) and six (CoQ6) units, respectively [1]. The length of the side chain is defined by trans-polypropenyl diphosphate synthases rather than by the p-hydroxybenzoate (PHB)-polypropenyl diphosphate transferases that catalyze the condensation of PHB and polypropenyl diphosphate [9]. Synthesis of CoQ occurs in two stages: the synthesis of isoprenoid and the synthesis of quinone (Figure 1). To synthesize the isoprenoid tail, a unit of prenyl diphosphate is synthesized by polyprenyl diphosphate synthase. In S. pombe and humans, this enzyme is a heterotetramer of decaprenyl diphosphate synthase (Dps1; also known as Pdss1) and D (aspartate)-less polyprenyl diphosphate synthase (Dlp1; also known as Pdss2) [10–12]. By contrast, in budding yeast and plants, polyprenyl diphosphate synthase is homomeric (presumably a homodimer) [13–16]. Although the synthesis of isoprenoid has been characterized, the mechanisms involved in quinone synthesis in eukaryotes are less well known. In S. cerevisiae, the biosynthetic pathway that converts PHB to CoQ comprises at least eight steps that require at least seven enzymes with assigned roles [1,2,17]; these steps include the condensation and transfer of the isoprenoid side chain to PHB, followed by methylation, decarboxylation and hydroxylations (Figure 1). Para-aminobenzoic acid (pABA) is also a precursor of CoQ biosynthesis in budding yeast [18]. PHB-polyprenyl diphosphate transferase, known as Coq2 in budding yeast [19] or Ppt1 in fission yeast [20], catalyzes the condensation of PHB and polyprenyl diphosphate [21,22]. Coq4 is absolutely required for CoQ biosynthesis but its enzymatic function remains unknown [23]. Coq5 catalyzes the only C-methylation
step in the pathway [24]. Coq6 is a flavin-dependent monoxygenase responsible for adding the hydroxy group to polyprenyl-PHB at the C5 position [25,26]. The mono-oxygenase Coq7 is involved in the penultimate step of CoQ biosynthesis [22]. Coq8 functions as a protein kinase that phosphorylates some Coq proteins and stabilizes the protein complex [27,28]. Coq9 is required for CoQ biosynthesis but its enzymatic function is unknown [29]. Coq10 is a binding protein of CoQ [30,31], and indirectly affects but is not required for CoQ synthesis [32].

Although the CoQ biosynthetic pathway has been examined in bacteria and yeasts, little is known about the pathway in higher eukaryotes. Understanding the CoQ biosynthetic pathway in humans is important because CoQ is essential for energy production and is the only endogenously synthesized lipid-soluble antioxidant. Furthermore, CoQ deficiency can lead to the development of severe diseases such as Leigh syndrome [33].

Here, we identified ten genes (dps1, dip1, ppt1, and coq3–9), including some that have been reported previously, that are related to CoQ biosynthesis in S. pombe. Deletion strains were constructed for all of these genes; all of the CoQ10-deficient mutants were sensitive to oxidative stress, produced a large quantity of sulfide, required an antioxidant to grow on minimal medium, and did not survive in the stationary phase. The biosynthetic pathway of CoQ in higher eukaryotes was also investigated by performing functional complementation analyses of the S. pombe coq deletion mutants using human and A. thaliana homologues.

**Materials and Methods**

**Strains, media, and genetic manipulation**

The S. pombe strains used in this study are listed in Table 1. Standard yeast culture media and genetic manipulations were used. The S. pombe strains were grown in complete YES medium (0.5% yeast extract, 3% glucose, 225 mg/l adenine, 225 mg/l leucine, 225 mg/l uracil, 225 mg/l histidine, and 225 mg/l lysine hydrochloride) or EMM medium (0.3% potassium hydrogen phthalate, 0.56% sodium phosphate, 0.5% ammonium chloride, 2% glucose, vitamins, minerals, and salts) [34]. The appropriate auxotrophic supplements were added as necessary (75 mg/l...
determined by the dideoxynucleotide chain-termination method.

Construction of the deletion strains

were followed [35]. The restriction enzymes (TOYOBO, Takara, NEB) were used as the host strain for all plasmid manipulations and was grown in LB medium (1% bactotryptone, 0.5% yeast extract, and 1% NaCl; pH 7.0). Standard molecular biology techniques were followed [35]. The restriction enzymes (BanHI, BglII, Xhol, NdeI, NotI and SalI) were used according to the suppliers’ recommendations (TOYOBO, Takara, NEB). Nucleotide sequences were determined by the dideoxynucleotide chain-termination method using an Applied Biosystems 3500 Genetic Analyzer.

adenoine, 75 mg/l leucine, 75 mg/l uracil, 75 mg/l histidine, 75 mg/l lysine, or 400 mg/l cysteine). Escherichia coli DH5α was used as the host strain for all plasmid manipulations and was grown in LB medium (1% bactotryptone, 0.5% yeast extract, and 1% NaCl; pH 7.0). Standard molecular biology techniques were followed [35]. The restriction enzymes (BanHI, BglII, Xhol, NdeI, NotI and SalI) were used according to the suppliers’ recommendations (TOYOBO, Takara, NEB). Nucleotide sequences were determined by the dideoxynucleotide chain-termination method using an Applied Biosystems 3500 Genetic Analyzer.

Table 1. Strains used in this study.

| Strain | Genotype | Resource |
|--------|----------|----------|
| L972   | h         | Lab stock |
| PR110  | h ura4-18 | Lab stock |
| LJ1030 | h ura4-18 | [6]       |
| RM19   | h ura4-18 | [22]      |
| LA1    | h ura4-18 | [6]       |
| KH2    | h ura4-18 | This study |
| KH3    | h ura4-18 | [22]      |
| KH4    | h ura4-18 | This study |
| KH5    | h ura4-18 | This study |
| KH6    | h ura4-18 | This study |
| KH7    | h ura4-18 | [22]      |
| KH8    | h ura4-18 | This study |
| KH9    | h ura4-18 | This study |

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Plasmid construction

The S. pombe deletion strains were constructed by replacing the coq genes with a selectable marker; chromosomal genes were disrupted by homologous recombination using fragments generated by polymerase chain reaction (PCR) [36] (Figure S1). The 1.6 kb kanMX6 module was amplified with flanking sequences corresponding to the 5' and 3' ends of the target genes. Resistant colonies were selected on YES plates containing 100 mg/L G418 (Sigma) and disruption of the gene of interest was verified by colony PCR. DNA fragments of 400–300 bp corresponding to the 5' or 3' regions of the pp1, coq4, coq5, coq6, coq7, or coq9 gene were amplified by PCR using the pp1/coq4/5/6/8/9-x and pp1/coq4/5/6/8/9-z primer pairs (Table S1). For each gene, the 5' and 3' amplified fragments were fused to the ends of the kanMX6 module by PCR. The wild type PR110 strain (Table 1) was transformed with the resulting pp1::kanMX6, coq4::kanMX6, coq5::kanMX6, coq6::kanMX6, coq7::kanMX6, and coq9::kanMX6 fragments to form the deletion strains (Table 1). Transformants were selected using 100 mg/L G418. To confirm the chromosomal deletion of the pp1, coq4, coq5, coq6, coq7, and coq9 genes, PCR was performed using the nh2 and pp1/coq4/5/6/8/9-x primers (Table S1); the resulting deletion strains were designated KH2 (∆pp1), KH4 (∆coq4), KH5 (∆coq5), KH6 (∆coq6), KH8 (∆coq8), and KH9 (∆coq9), respectively. The Δdp1 strain and the Δps1-dp1 double deletion strain were constructed as described previously (Zhang et al., 2008). The ∆dp1, ∆coq3 and ∆coq7 strains were also constructed as described previously (Miki et al., 2008).

Table 2. Genes involved in CoQ synthesis in S. cerevisiae, S. pombe, humans, and A. thaliana.

| S. cerevisiae | S. pombe | H. sapiens (accession #) | Arabidopsis (systematic name) | Enzyme (function) |
|---------------|----------|--------------------------|-----------------------------|------------------|
| COQ1          | dp1-dp1  | HSPOSTS1(AI90245)-HSPOSTS2(BII571760) | ATPS32 (At2g34630) | Polypropenyl diphosphate synthase |
| COQ2          | pp1/coq2 | HSPOSTQ2 (BF026793)       | ATPPT (At4g23600) | Polypropenylpyrophosphate transferase |
| COQ3          | coq3     | HSPOSTQ3 (AF193016)       | ATCOQ3 (At2g30920) | O-Methyltransferase |
| COQ4          | coq4     | HSPOSTQ4 (BQ685658)       | ATCOQ4 (At2g36090) | Unknown |
| COQ5          | coq5     | HSPOSTQ5 (BC004916)       | ATCOQ5 (At5g57300) | C-Methyltransferase |
| COQ6          | coq6     | HSPOSTQ6 (BQ688512)       | ATCOQ6 (At2g24200) | C5-Hydroxylation |
| COQ7          | coq7     | HSPOSTQ7 (AF1532900)      | ATCOQ7 (At1g19140) | CS-Hydroxylation |
| COQ8/ABC1     | coq8     | HSPOSTQ8/ADCK3 (BC005171) | ATCOQ8 (At1g19140) | Protein kinase |
| COQ9          | coq9     | HSPOSTQ9 (BC064946)       | ATCOQ9 (At1g19140) | Unknown |

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(Table 2) were obtained from the Arabidopsis Biological Resource Center (ABRC) or Riken BRC. The plasmids were constructed in the same manner as those expressing the human COQ3 genes. For expression of AtCOQ6, the mitochondrial targeting signal of coq6 from *S. pombe* was fused to the AtCOQ6 gene sequence (Figure S5). The genes amplified by PCR were verified by DNA sequencing.

**Extraction and measurement of CoQ from *S. pombe***

CoQ was extracted from *S. pombe* as described previously [31]. Briefly, crude lipid extracts were analyzed by normal phase thin layer chromatography using authentic CoQ10 (as the standard) and benzene on a Kieselgel 60 F254 plate. Following UV visualization, the band containing CoQ10 was collected from the plate and extracted with chloroform/methanol (1:1 v/v). The samples were dried and resolved in ethanol. Purified CoQ was analyzed further by high-performance liquid chromatography (HPLC), with ethanol as the solvent.

**Measurement of extracellular sulfide**

Extracellular sulfide was quantified using the methylene blue method [6]. Briefly, *S. pombe* was grown to late log phase in YES or EMM medium (50 ml). The cultures (0.5 ml) were mixed with 0.1 ml of 0.1% dimethylphenylenediamine (in 5.5 N HCl) and 0.1 ml of 23 mM FeCl₃ (in 1.2 N HCl) and then incubated at 37°C for 5 min. Following centrifugation at 12,000 g for 5 min, the supernatant was removed and absorbance was measured at 670 nm. The blank consisted of reagents alone.

**Mitochondrial staining and fluorescence microscopy**

Mitochondria were stained using MitoTracker Red FM dye (Invitrogen). The cells were suspended in 10 mM HEPES (pH 7.4) containing 5% glucose and MitoTracker Red FM was added to a final concentration of 50 nM. After incubation at room temperature for 15 min, the cells were visualized at 1000× magnification using a BX51 fluorescent microscope (Olympus, Tokyo, Japan). The fluorescence of GFP<sub>S65A</sub> was observed at an excitation...
Figure 3. Mitochondrial localization of *S. pombe* Coq proteins. The indicated Coq proteins were fused with GFP and their localization was observed under a fluorescence microscope. The plasmids used in this experiment are shown in Figure S2B. (A) A bright-field image and the GFP and MitoTracker Red signals in the Δdlp1 strain expressing the Dlp1-GFP fusion protein. The merged GFP and MitoTracker Red signals are also shown. (B–J) GFP signals of the Dps1-GFP fusion expressed in Δdps1 cells (B), the Ppt1(Coq2)-GFP fusion expressed in Δppt1 cells (C), the Coq3-GFP fusion expressed in Δcoq3 cells (D), the Coq4-GFP fusion expressed in Δcoq4 cells (E), the Coq5-GFP fusion expressed in Δcoq5 cells (F), the Coq6-GFP fusion expressed in Δcoq6 cells (G), the Coq7-GFP fusion expressed in Δcoq7 cells (H), the Coq8-GFP fusion expressed in Δcoq8 cells (I), and the Coq9-GFP fusion expressed in Δcoq9 cells (J). All cells were stained with MitoTracker Red to verify the localization of the Coq proteins to mitochondria (data not shown).
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wavelength of 485 nm. Fluorescent images were obtained using a digital camera (DP70, Olympus) connected to the microscope.

Results

Construction and phenotypes of the S. pombe coq deletion strains

The biosynthetic pathway of CoQ has been well characterized in S. cerevisiae [2]. Here, we examined the involvement of ten S. pombe coq genes (which are homologous to S. cerevisiae COQ genes) (Figure 1 and Table 2) in CoQ biosynthesis. We previously reported the construction and characterization of S. pombe Ddps1 [6,38], Ddlp1 [11], Ddps1-Ddlp1 (Zhang et al., 2008), Dppt1/coq2 [20], Dcoq7 [22], Dcoq8 [27] strains, which are unable to synthesize CoQ. Here, we constructed four new S. pombe deletion strains in which the coq4 (SPAC1687.12c), coq5 (SPCC4G3.04c), coq6 (SPBC146.12), or coq9 (SPAC19G12.11) gene was replaced with the kan marker (Figure S1 and Table 1). S. pombe ppt1 or coq8 deletion strain containing the kan marker was also generated to equalize a background of strains. The phenotypes of eleven S. pombe deletion strains (Ddps1, Ddlp1, Ddps1-Ddlp1, Dppt1/coq2, Dcoq7, Dcoq8, Dcoq6, Dcoq5, Dcoq3, Dcoq4, and Dcoq9; Table 1) were determined. Unlike the wild type strain (PR110), all of the deletion strains required cysteine to grow on minimal medium and were sensitive to CuSO4 when grown on YES medium (Figure 2A). In addition, the ten single deletion strains (the Ddps1-Ddlp1 strain was not tested) were unable to grow on YES medium containing yeast extract supplemented with ethanol and glycerol (Figure 2B) and did not survive in the stationary phase (data not shown; [22]). The inability to grow on YES medium containing yeast extract supplemented with ethanol and glycerol and the sensitivity to CuSO4 are symptoms of respiration defective and oxidative stress-sensitive phenotypes, respectively. As reported previously for the S. pombe Dcoq7 strain [22], all ten single coq deletion strains were also sensitive to H2O2 (data not shown). Overall, these results suggest that the ten single coq deletion strains share common phenotypes.

Localization of the S. pombe Coq proteins

The cellular localizations of Ppt1/Coq2 and Coq7 from S. pombe have been reported previously [20]. GFP-fusions were constructed to examine the localization of these and other S. pombe Coq proteins (Figure S3). As shown in Figure 3A, the fluorescent signal generated by the Dlp1-GFP fusion protein expressed in the Ddlp1 strain overlapped with that of mitochondria stained using MitoTracker Red FM dye. The Dps1, Ppt1/Coq2, Coq3, Coq4, Coq5, Coq6, Coq7, Coq8, and Coq9 GFP fusion proteins also localized to the mitochondria (Figures 3B–J).

Complementation of the S. pombe coq deletion strains by human COQ genes

In an HPLC analysis, a lipid extract from the wild type S. pombe strain (PR110) yielded a major peak at 10 min, which was consistent with that of authentic CoQ10. To compare the biosynthetic pathways of CoQ in S. pombe and higher organisms, we determined whether defective CoQ10 production by S. pombe coq deletion strains could be functionally complemented by human genes encoding CoQ biosynthetic enzymes. Plasmids containing

Figure 4. Functional complementation of the S. pombe Adps1 and Adlp1 strains by HsDPS1 and HsDLP1. HPLC analyses of lipid extracts from the RM19 (Adlp1) strain expressing S. pombe dlp1 or HsDLP1 and the LA1 (Adps1-Adlp1) strain expressing S. pombe dps1 and dlp1 or HsDPS1 and HsDLP1.
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HsCOQ genes (which were identified on the basis of their sequence similarity to known *S. pombe* coq genes) (Figure 1 and Table 2) under the control of the *nmt1* promoter were generated (Figure S4). For some human COQ genes, the use of a weaker promoter (*nmt1*) was required to avoid growth inhibition caused by high levels of expression. The plasmids were expressed in the corresponding *S. pombe* deletion strains and lipid extracts were analyzed by HPLC.

In a previous study, HsDPS1/PDSS1 was able to complement an *S. pombe* *dps1* mutant, but HsDLP1/PDSS2 was not able to complement an *S. pombe* *dlp1* mutant [12]. Here, the *S. pombe* *dlp1* strain expressing HsDLP1 containing a mitochondrial targeting signal from *dlp1* did not produce CoQ10 (Figure 4), indicating that the failure of complementation by this human gene is not due to mislocalization of the protein. By contrast, the *S. pombe* *dps1-dlp1* double deletion strain expressing HsDPS1 and HsDLP1 was able to produce CoQ10 (Figure 4), indicating that HsDPS1 and HsDLP1 can form a functional complex in *S. pombe*. These results suggest that, in both human and *S. pombe*, Dps1 and Dlp1 are necessary to constitute a heterotetrameric decaprenyl diphosphate synthase.

Four putative start codons were identified in the HsCOQ2 sequence; therefore, four plasmids that included a different putative start codon (1st–4th) of the gene were generated (Figure 5A). All of the HsCOQ2 constructs restored the growth of the *S. pombe* * Aptt1* strain (data not shown) and recovered its defective production of CoQ10 (Figure 5B). Thus, the 4th methionine is sufficient for functional expression in the *Aptt1* strain. Similar results were obtained when HsCOQ4 and HsCOQ6 were expressed in the *Acoq4* and *Acoq6* strains, respectively (Figures 6A and 6B). By contrast, HsCOQ2 (Figure 6C) and HsCOQ7 (Figure 7A) failed to complement their respective *S. pombe* deletion strains. Since all *S. pombe* Coq proteins localize to the mitochondria (Figure 3), the HsCOQ2 and HsCOQ7 constructs were modified to include a promoter region containing the mitochondrial targeting signal from the *S. pombe* *coq3* and *coq7* genes, respectively. Targeting of HsCOQ2 and HsCOQ7 to the mitochondria successfully recovered defective CoQ10 production by the *S. pombe* *Acoq3* (Figure 6C) and *Acoq7* (Figure 7A) strains. An HPLC peak that we previously identified as demethoxyubiquinone (DMQ10) [22] was detected in the *Acoq7* strain; this peak was fully converted to CoQ10 by expression of *S. pombe* *coq7* and partly

**Figure 5. Functional complementation of the *S. pombe* *Aptt1* strain by HsCOQ2.** (A) Schematic illustration of the four HsCOQ2 plasmids that included different 5’ ends of the gene in pREP1. M, start codon; aa, amino acid. (B) HPLC analyses of lipid extracts from the KH2 (*Aptt1*) strain expressing *S. pombe* *ppt1* or the four different HsCOQ2 constructs.
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converted by expression of HsCOQ7 containing a mitochondrial targeting signal (Figure 7A).

Unidentified peaks, which may represent intermediates, were identified in the S. pombe Δcoq5 strain (Figure 7B, asterisks); these peaks did not merge with the CoQ10 standard (data not shown). When the S. pombe coq5 gene was expressed in the Δcoq5 strain, the intermediate peaks disappeared and a CoQ10 peak was observed on the HPLC trace (Figure 7B). By contrast, expression of the HsCOQ5 gene in this strain failed to recover a clear CoQ10 production (Figure 7B); however, when the lipid extract from the HsCOQ5-complemented Δcoq5 strain was mixed with CoQ10, one of the peaks merged with that of CoQ10, indicating that a small amount of CoQ10 was in fact produced (Figure 7B). Similarly, CoQ10 production by the S. pombe Δcoq8 strain was fully recovered by expression of S. pombe coq8 but only moderately recovered by expression of HsCOQ8/ADCK3 (Figure 7C). Expression of

Figure 6. Complementation of the S. pombe Δcoq4, Δcoq6 and Δcoq3 strains by HsCOQ3, HsCOQ4 and HsCOQ6. (A, B) HPLC analyses of lipid extracts from the KH4 (Δcoq4) strain expressing S. pombe coq4 or HsCOQ4 and the KH6 (Δcoq6) strain expressing S. pombe coq6 or HsCOQ6. Growth on minimal media (EMM) plates is also shown. (C) HPLC analyses of lipid extracts from the KH3 (Δcoq3) strain expressing S. pombe coq3, HsCOQ3, or HsCOQ3 fused with a mitochondrial targeting sequence (Mt-signal).

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HsCOQ7 containing a mitochondrial targeting signal from coq7 fully restored the growth of the Δcoq7 strain, and HsCOQ5 and HsCOQ8/ADCK3 partially restored growth of the Δcoq5 and Δcoq8 strains, respectively; the doubling times of these complemented strains were approximately 0.7 times that of the wild type strain (Figure 7D). Despite the inclusion of a mitochondrial targeting signal within its sequence, HsCOQ9 did not complement the S. pombe Δcoq9 mutant (data not shown). Overall, these data indicate that, like the S. pombe Δdps1, Δdlp1, Δppt1/Δcoq2, Δcoq3, Δcoq7, and Δcoq8 strains described previously, the Δcoq4, Δcoq5, Δcoq6, and Δcoq9 strains are also unable to synthesize CoQ. Furthermore, with the exception of COQ9, human COQ biosynthetic genes can functionally complement their corresponding S. pombe coq mutants.

Sulfide and CoQ production

We next measured sulfide production to further determine the efficiency of complementation of the S. pombe coq deletion strains by human COQ genes. Sulfide production in the S. pombe coq deletion strains is known to be higher than that in the wild type strain [6]. Expression of HsDPS1, HsCOQ2, HsCOQ4, and HsCOQ6 in the corresponding deletion strains reversed this increase (Figure 8). Expression of HsCOQ2 and HsCOQ8 (ADCK3) also partially reversed the increased sulfide production (Figure 8). Expression of HsCOQ9 failed to reverse increased sulfide production by the Δcoq9 strain. Similarly, expression of HsCOQ3 and HsCOQ7 failed to reverse increased sulfide production by the Δcoq3 and Δcoq7 strains, respectively; however, the addition of a mitochondrial targeting signal to these human genes successfully recovered sulfide production to a level similar to that of the wild type strain (Figure 8). These results are consistent with the levels of CoQ10 production in the human gene-complemented coq deletion strains, indicating that the restoration of CoQ10 production and sulfide production was correlated.

Complementation of S. pombe coq deletion strains by A. thaliana genes

Next, we examined the ability of A. thaliana (At) COQ genes (Table 2) to complement the S. pombe coq deletion mutants.
Although AtSPS1 and AtSPS2, which encode two solanesyl diphasphate synthases in *A. thaliana*, complement the fission yeast *Adps1* mutant [13], recent studies show that Act2g34630 (named here AtSPS3) is most likely responsible for CoQ synthesis in *A. thaliana* [39]. Here, the abilities of the AtCoQ3, AtCoQ4, AtCoQ5, AtCoQ6, and AtCoQ9 genes to complement the corresponding *S. pombe* mutants were determined. Expression of AtCoQ3, AtCoQ4, and AtCoQ5 rescued CoQ10 production by the *S. pombe* Δcoq3, Δcoq4 and Δcoq5 strains, respectively (Figures 9A–C). By contrast, expression of the AtCoQ6 gene did not complement the Δcoq6 strain; however, the addition of a mitochondrial targeting signal to AtCoQ6 did enable recovery of CoQ10 production when expressed in the Δcoq6 strain (Figure 9D). *A. thaliana* does not contain an ortholog of Coq7, and both Coq6 and Coq7 are involved in the hydroxylation step of CoQ biosynthesis; however, AtCoQ6 was unable to recover CoQ10 production by the *S. pombe* Δcoq7 strain (data not shown). It is still not clear what kind of enzyme is responsible for this hydroxylation step in *A. thaliana*. Unlike HsCoQ8/ADCK3, the AtCoQ8 gene was able to functionally complement the *S. pombe* Δcoq8 strain (Figure 9E); however, expression of AtCoQ9 did not recover CoQ10 production by the Δcoq9 strain (data not shown).

**Discussion**

Ten coq deletion strains of the fission yeast *S. pombe*, including six that were reported previously (Uchida, et al., 2000, Saiki, et al., 2003, Miki, et al., 2008), were created. The ten deletion strains were phenotypically indistinguishable; they did not produce CoQ10 (were respiration defective), were sensitive to oxidative stress, produced a large amount of sulfide, required an antioxidant to grow on minimal medium, were sensitive to Cu2+, and did not survive long at the stationary phase. In recent large scale deletion library screening studies, a number of coq deletion strains were sensitive to Cd2+ and doxorubicin, an inhibitor of topoisomerase 2 [40,41]. As a consequence of the various roles of CoQ, coq deletion strains show pleiotropic phenotypes. CoQ is an essential component of the electron transfer system; therefore, coq deletion strains grew slowly under non-fermentation conditions and failed to grow on medium containing glycerol and ethanol as carbon sources. Since CoQ functions as an antioxidant and couples sulfide oxidation and cysteine metabolism, the *S. pombe* coq deletion strains were sensitive to H2O2 and produced large amounts of sulfide. CoQ is also required for de novo UMP synthesis, resulting in poor growth of the coq deletion strains on minimal medium [7]. We did not see the growth recovery of CoQ deficient *S. pombe* cells by supplementation of CoQ10 (Figure S6), whereas supplementation of CoQ6 generally restored the growth of *S. cerevisiae* coq deletion mutants on non-fermentable carbon source [42]. The slow growth of a coq deficient mutant was partly rescued by supplementation of CoQ10 encapsulated by γ-cyclodextrin, but its respiration was not restored [43]. It is not easy to test the effect of CoQ10 supplementation in *S. pombe*. In *S. cerevisiae*, the two essential genes, *ARH1* and *YAH1*, are involved in CoQ synthesis through the regulation of Coq6 [26]. Although *S. pombe* contains orthologs of these genes, it will be difficult to determine their functions in CoQ biosynthesis via deletion analyses because they are essential for growth.

All *S. pombe* coq deletion strains failed to synthesize CoQ10, intermediate peaks were observed on HPLC traces of lipid extracts from the Δcoq5 and Δcoq7 strains. The peak identified in the Δcoq7 extract was previously determined to be DMQ10 [22], while those identified in the Δcoq5 extract are currently unknown. At least two peaks were detected in the Δcoq5 extract, which may indicate the existence of two pathways originating from the precursors PHB and pABA, as reported in *S. cerevisiae* [18]; however, additional studies are required to confirm this hypothesis.

The results presented here demonstrate that all of the *S. pombe* Coq proteins localize to mitochondria, indicating that CoQ biosynthesis occurs in these cellular compartments. These results are consistent with those of a previous study [44] that examined the localization of 4,431 *S. pombe* proteins, although Coq6 was not included. Coq proteins in *S. cerevisiae* also localize to mitochondria, where they form a complex [23]. A recent study showed that biosynthesis of CoQ in humans also occurs in the Golgi [45]; however, one of the CoQ biosynthetic steps, PHB-polypropenyl...
Figure 9. Complementation of the *S. pombe* coq deletion strains by *A. thaliana* COQ genes. (A–E) HPLC analyses of lipid extracts from the KH3 (*Δcoq3*) strain expressing *S. pombe* coq3 or AtCOQ3 (A); the KH4 (*Δcoq4*) strain expressing *S. pombe* coq4 or AtCOQ4 (B); the KH5 (*Δcoq5*) strain expressing *S. pombe* coq3 or AtCOQ5 (C); the KH6 (*Δcoq6*) strain expressing *S. pombe* coq6, AtCOQ6, or AtCOQ6 fused with a mitochondrial targeting sequence (Mt-Signal) (D); and the KH8 (*Δcoq8*) strain expressing *S. pombe* coq8 or AtCOQ8 (E). DMQ10, demethoxyubiquinone.
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condensation, is mediated by the enzyme UBLAD1. Because this enzyme is absent in yeasts, it is likely that CoQ is synthesized predominantly in mitochondria in S. cerevisiae and S. pombe.

As mentioned above, HsDPS1/PDSS1 is able to complement an S. pombe Adp1 mutant, but HsDLP1/PDSS2 is not able to complement an S. pombe Adp1 mutant [12]. This suggests that the S. pombe Dps1 protein cannot form a complex with the HsDLP1 protein, even though they share sequence similarity (28% identity; see Table 3). When HsDPS1 and HsDLP1 genes were expressed in a Adp1 Adp1 strain, CoQ10 was predominantly produced, while S. pombe naturally produce CoQ10 with a lesser amount of CoQ9 (Fig. 4). This result indicates decaprenyl diphosphate synthase from humans strictly produces 10 isoprene units of prenyl diphosphate, which is consistent with the observation that CoQ10 is predominantly found in human bodies.

Homologs of all of the S. pombe coq genes exist in humans and homologues of all except coq7 exist in A. thaliana. In most cases, functional complementation of the S. pombe coq deletion strains by human or A. thaliana genes was successful (Figure 10); however, in some cases (HsCOQ2, HsCOQ7 and AtCOQ8), the addition of an N-terminal mitochondrial targeting sequence from the corresponding S. pombe coq gene was required for complementation. One of reasons may be a shortage of targeting sequences in cases of HsCOQ7 and HsCOQ9, as there are longer length transcriptional variants in these genes. The other reason may be incapability of working mitochondrial targeting sequence of humans or A. thaliana genes in S. pombe cells. The sequences of the N-terminal regions of the coq/coq genes in S. pombe, humans, and A. thaliana are rather diverged, and we were unable to find any apparent rules that govern how the N-terminal sequences affect mitochondrial targeting.

Although some complementation analyses of S. cerevisiae coq deletion strains expressing human genes have been conducted, a comprehensive analysis has not yet been performed. Studies performed to date show that expression of HsCOQ2 [46], HsCOQ3 [21], HsCOQ4 [47], and HsCOQ6 [48] functionally complement their corresponding S. cerevisiae coq mutants. HsCOQ8 (ADCK3) also complements the S. cerevisiae Acoq8 mutant weakly [49], which is consistent with the results presented here for S. pombe. To our knowledge, functional complementation of S. cerevisiae Acoq5 and Acoq7 mutants by human COQ5 or COQ7, respectively, has not yet been tested. AtSPS3, which encodes solanesyl diphosphate synthase, and AtPPT1, which encodes PHB-polypropenyl diphosphate transferase, complement the S. cerevisiae Acoq1 and Acoq2 mutants, respectively [9,13].

HsCOQ9 and AtCOQ9 failed to complement the S. pombe Acoq9 strain. Similarly, in a previous study, HsCOQ9 failed to complement the S. cerevisiae Acoq9 mutant [50]. Although its exact function is still unknown, Coq9 is absolutely required for the biosynthesis of CoQ in S. cerevisiae and, as shown here, S. pombe. Genetic disorders of CoQ biosynthesis related to mutation of the HsCOQ9 gene [51] suggest that this enzyme is also involved in CoQ synthesis in humans. However, the function of COQ9 may not be conserved between humans, A. thaliana, and yeast because these Coq9 proteins share only 28–30% amino acid identity (Table 3).

Complementation analyses of plant genes encoding CoQ biosynthetic enzymes are scarce. Based on an analysis of the AtPPT1 gene, CoQ appears to be essential for seed formation in A. thaliana [9]. AtSPS1 is functional when expressed in S. pombe [13] but the protein may not be involved in CoQ synthesis; on the other hand, AtSPS3 (Atg34630) is responsible for CoQ synthase in this species [39]. Since A. thaliana does not contain a COQ7 gene, we tested the ability of the other COQ genes in this species (AtCOQ3, AtCOQ4, AtCOQ5, AtCOQ6, AtCOQ8, and AtCOQ9) to recover CoQ10 production in S. pombe coq deletion strains. With
the exception of AtCOQ9, the functions of all other \textit{A. thaliana} COQ proteins were conserved. In knockdown analyses of \textit{Caenorhabditis elegans}, the \textit{coq7} deletion strain was able to survive for long periods, unlike other \textit{coq} deletion strains. No apparent \textit{Coq9} ortholog was found in \textit{C. elegans}, which might be relevant to the fact this organism produces rhodoquinone. In conclusion, this study describes the functional conservation of \textit{CoQ} biosynthetic genes in humans, plants, and the fission yeast \textit{S. pombe}. It also demonstrates that the functions of the human \textit{COQ} genes can be determined by expression in \textit{S. pombe} deletion mutants. Determining the function of \textit{CoQ} biosynthetic genes may be useful for understanding genetic disorders caused by human \textit{CoQ} biosynthetic deficiencies.

Supporting Information

Figure S1 Construction of the \textit{S. pombe} \textit{ppt1}, \textit{coq4}, \textit{coq5}, \textit{coq6}, \textit{coq8}, and \textit{coq9} deletion strains. One step homologous recombination was used to delete the \textit{S. pombe} \textit{coq} genes. The \textit{coq4} deletion strategy is shown as an example. The \textit{coq4-x} and \textit{coq4-y} primers were homologous to the flanking regions of the \textit{coq4} and \textit{kan} resistance genes. The \textit{coq4-w} and \textit{coq4-z} primers were homologous to regions located approximately 500 bp downstream and upstream of the \textit{coq4} gene. The \textit{nb2} and \textit{coq4-c} primers were used to verify the replacement of \textit{coq4} by \textit{kan}. All other deletion strains were constructed similarly. (TIFF)

Figure S2 Construction of the plasmids to express \textit{S. pombe} \textit{coq} genes. The \textit{S. pombe} \textit{coq} genes were inserted into the pREP1 or pREP2 vector under the control of the \textit{urnt1} promoter. (TIFF)

Figure S3 Construction of the plasmids to express \textit{S. pombe} \textit{coq-GFP}. To determine the cellular localization of \textit{Coq} proteins, GFP-fusions were generated by inserting the \textit{coq} genes into the pSFL272L-GFP\textsubscript{SGA} vector (used in most cases). (TIFF)

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