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The *AtPPT1* gene encoding 4-hydroxybenzoate polyprenyl diphosphate transferase in ubiquinone biosynthesis is required for embryo development in *Arabidopsis thaliana*

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Abstract

4-Hydroxybenzoate polyprenyl diphosphate transferase (4HPT) is the key enzyme that transfers the prenyl side chain to the benzoquione frame in ubiquinone (UQ) biosynthesis. The *Arabidopsis AtPPT1* cDNA encoding 4HPT was cloned by reverse transcription-polymerase chain reaction (RT-PCR) based on the information of the *Arabidopsis* genomic sequence, and the function of the gene was determined. Heterologous expression of the *AtPPT1* gene enabled restoration of the respiratory ability and UQ synthesis in a yeast mutant that was defective in 4HPT activity. The mitochondrial fraction that was prepared from the yeast mutant, which expressed the *AtPPT1* gene, exhibited 4HPT enzymatic activity with geranyl diphosphate (GPP) as the prenyl substrate. This indicated that the *AtPPT1* gene encodes active 4HPT with a broad substrate specificity in terms of the prenyl donor. The *AtPPT1* mRNA was predominantly expressed in the flower cluster, and the green fluorescent protein (GFP) fused with the signal peptide of AtPPT1 was translocated into the mitochondria. T-DNA insertion mutation that disrupts the *AtPPT1* gene in *Arabidopsis* resulted in the arrest of embryo development at an early stage of zygotic embryogenesis. These results demonstrate that the *AtPPT1* gene involved in the biosynthesis of mitochondrial UQ plays an essential role in embryo development in *Arabidopsis*.

Introduction

Ubiquinone (UQ), a lipid-soluble compound that is widely distributed in living organisms, is generally known to function as an electron carrier in the respiratory chain (Grunler *et al*., 1994; Ernster and Dallner, 1995). Living organisms possess different species of UQ depending on the length of the side chain, which is produced by polyprenyl diphosphate synthase (PDS). For example, humans and *Schizosaccharomyces pombe* produce UQ-10, mice and *Zea mays* produce UQ-9, *Escherichia coli* produces UQ-8, and *Saccharomyces cerevisiae* produces UQ-6 (Collins and Jones, 1981; Kawamukai, 2002). 4-Hydroxybenzoate polyprenyl diphosphate transferase (4HPT) is an important enzyme in UQ biosynthesis that transfers the isoprenoid side chain to the benzoquione frame, and

it has a broad specificity in terms of the prenyl substrate in bacteria and yeast (Meganathan, 2001). *E. coli* UbiA protein can accept geranyl diphosphate (GPP) (C_{10}), farnesyl diphosphate (C_{15}), and solanesyl diphosphate (C_{45}), in addition to octaprenyl diphosphate (C_{40}), as prenyl substrates for transfer to the benzoquinone frame (Melzer and Heide, 1994). This broad acceptance of prenyl substrates enables various UQ species, which are not originally produced in the host strain, to be synthesized in both yeast and *E. coli* by simply expressing PDS that produces a defined length of the isoprenoid tail (Okada *et al*., 1996). Thus, it has been demonstrated that the species of UQ in UQ biosynthesis is determined by PDS; however, the reason why different organisms produce various species of UQ with different tail lengths remains unknown. It has been demonstrated that the growth of yeast, which are engineered to have distinct types of UQ, is not greatly affected by differences in the UQ tail lengths (Okada *et al*., 1998).

The biological roles of UQ have been studied based on the phenotypic analysis of mutants that are defective in the enzyme responsible for UQ biosynthesis. *E. coli ubiA* mutant and *S. cerevisiae coq2* mutant, both of which are defective in 4HPT activity, can only grow on a medium containing a fermentable carbon source. This is because they lack UQ, which is necessary for respiration (Ashby *et al*., 1992; Suzuki *et al*., 1994). Among higher organisms, *Caenorhabditis elegans* has been extensively studied as a representative phenotype of a UQ-less animal. The *C. elegans clk-1* mutant that is defective in monooxygenase, which is responsible for the penultimate step of UQ biosynthesis, exhibits delayed embryonic and postembryonic development, a prolonged life span, and low egg production (Ewbank *et al*., 1997). The *C. elegans coq3* mutant that lacks methyltransferase has been generated as the UQ-null mutant. This mutant displays delayed development and sterile phenotype at the first homozygote, and these are lethal at the embryonic stage in the next generation (Hihi *et al*., 2002). In addition, such lethal phenotypes have also been reported in the analysis of a mouse *clk-1* mutant, indicating that a defect in UQ fatally affects the embryo development in mammals (Nakai *et al*., 2001; Rodriguez-Aguilera *et al*., 2003).

These mutant analyses suggest that UQ has a novel function other than that of being a component of the respiratory chain in the mitochondria. The mouse *clk-1* mutant cells were unable to complete embryo development, despite having relatively high mitochondrial respiration ability. Thus, embryogenesis in a mouse appears to require UQ at non-mitochondrial sites, in addition to the respiratory chain in the mitochondria (Levavasseuer *et al*., 2001). Recent evidence shows that UQ at the non-mitochondrial membrane has various biological functions, including disulfide bond formation (Bader *et al*., 1999), functioning as a lipid soluble antioxidant (Frei *et al*., 1990), and as a component of sulfide metabolism (Uchida *et al*., 2000). Therefore, the pleiotropic functions of non-mitochondrial UQ may be necessary for complete embryo development in living organisms.

In plants, the biosynthesis pathway of UQ appears to be similar to the pathway in yeast. Several genes, which appear to be responsible for UQ synthesis, are found in the *Arabidopsis* genome sequence (Lange and Ghassemian, 2003). However, as far as we know, only two genes have been characterized as UQ biosynthetic genes in plant species. One is the *AtCOQ3* gene encoding dihydroxypolyprenylbenzoate methyltransferase (Avelange-Macherel and Joyard, 1998). The other gene is the *At-SPS* gene encoding solanesyl diphosphate synthase, which produces the C_{45} isoprenoid tail and can be used for UQ-9 or plastoquinone-9 biosynthesis (Hirooka *et al*., 2003). Thus, many genes involved in UQ biosynthesis in plants remain to be characterized. To further understand the biosynthetic pathway and the biological roles of UQ in plants, we characterized the *AtPPT1* gene encoding 4HPT and the T-DNA insertion mutant that has defective 4HPT activity in *Arabidopsis*. This is the first report, which demonstrates that UQ plays an essential role in the embryo development in plants, similar to that in mammals.

Results

Cloning of full-length AtPPT1 *cDNA from* Arabidopsis

As shown in Figure 1, in the *Arabidopsis* genome databases, we found a putative gene that exhibits a

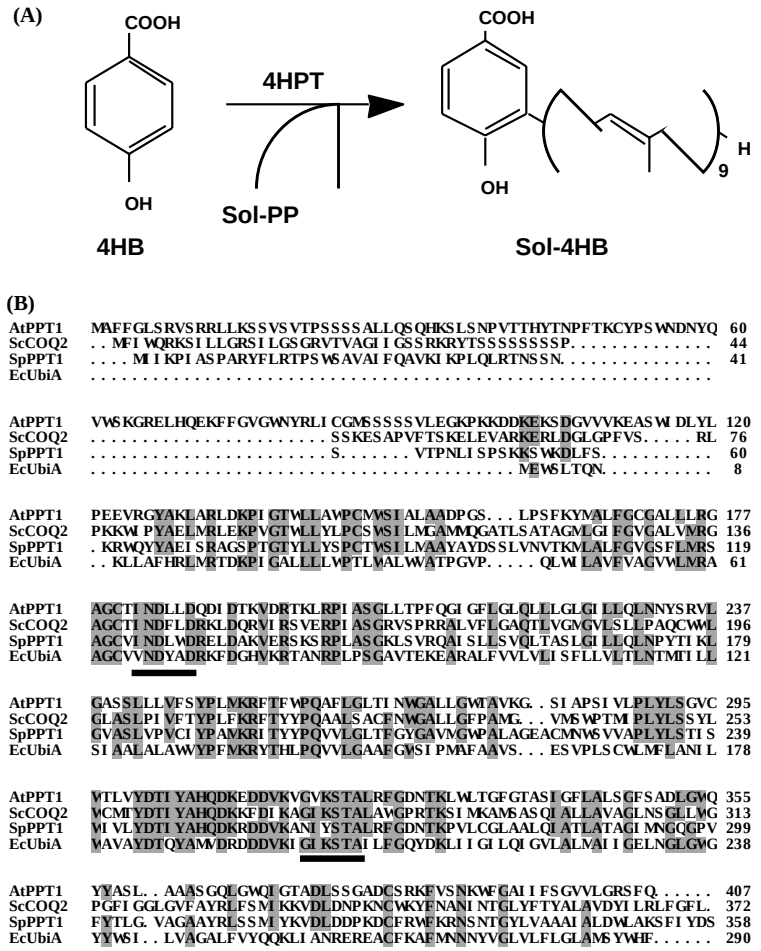

Figure 1. Enzymatic reaction and alignment of 4-hydroxybenzoate polyprenyl diphosphate transferase (4HPT) from various organisms. A. 4HPT catalyzes transfer of polyprenyldiphosphate to 4-hydroxybenzoate to yield 3-polyprenyl-4-hydroxybenzoate. In *Arabidopsis*, solanesyl diphosphate (Sol-PP) is used for the polyprenyl side chain and 3-solanesyl-4-hydroxybenzoate is produced in the reaction. B. Multiple alignment of *Arabidopsis* AtPPT1 protein with 4HPT from other *E. coli* and Yeast. EcUbiA, *E. coli* (P26601); ScCOQ2, *S. cerevisiae* (M81698); SpPPT1, *S. chizosaccharomyces pombe* (Z69728-4). Highly conserved amino acids among organisms are indicated by shaded areas. Underlines show the putative prenyl diphosphate binding site consisting of NDXXD and the GX (K/Y)STAL motif.

high similarity in amino acids level compared to *S. pombe* Ppt1 protein encoding 4HPT. This gene product exhibited 43.7% and 44.7% similarity in amino acids compared to *S. pombe* Ppt1 (Uchida *et al*., 2000) and *S. cerevisiae* Coq2 (Ashby *et al*., 1992), respectively; we designated this gene as *AtPPT1*. The *orf* of the *AtPPT1* gene was isolated by RT-PCR, and the full-length *AtPPT1* cDNA was obtained by subsequent 5′ and 3′ rapid amplification of cDNA ends. We obtained *AtPPT1* cDNA that was 1835 bp in length and included a 21 bp poly(A) tail, and it encoded a protein of 407 amino acids with an estimated molecular mass of 44 600 Da (accession no. AB052553). Recently, *AtPPT1* cDNA was also deposited in the RIKEN *Arabidopsis* full-length (RAFL) cDNA database (accession no. AY059864). It was revealed that the *AtPPT1* cDNA we cloned possesses a 153 bp and a 229 bp extended sequence at the 5′ and 3′ ends, respectively, compared with the RAFL clone, whereas the sequence of the *orf* was identical in the two cDNA sequences. The amino acid sequence of the AtPPT1 protein had an extended *N*-terminal sequence, which was predicted to be a mitochondrial targeting signal according to the PSORT

WWW server (http://psort.nibb.ac.jp/). The *AtPPT1* gene appeared to be a single copy gene in *Arabidopsis* because there was no other homolog displaying high similarity to the AtPPT1 protein in the *Arabidopsis* genomic sequence.

AtPPT1 protein is the active 4HPT that has broad specificity in terms of prenyl substrate

A yeast complementation system demonstrating recovery of normal growth of a 4HPT-defective mutant was used to determine whether the *AtPPT1* gene encodes the active enzyme of 4HPT. It has been demonstrated that the disruptants for the *S. cerevisiae COQ2* gene (W303Δ*coq2*) are UQ-less yeast as a result of defects in 4HPT activity, and these require glucose as the carbon source for normal growth on minimum media (Ashby *et al.*, 1992). *AtPPT1* cDNA were subcloned into the yeast expression vector pDR196 (Rentsch *et al.*, 1995) to yield pDR-AtPPT1, and the construct was heterologously expressed in the W303Δ*coq2* strain. The growth defect of the mutant was successfully complemented by expression of *AtPPT1* and they grew normally, similar to wild-type yeast, whereas the mutant harboring the vector alone was unable to grow on the same media (Figure 2A). This result suggested that *AtPPT1* gene product was active 4HPT. This can substitute 4HPT in the function as a yeast ortholog. HPLC analysis demonstrated that UQ-6, which normally exists in wild-type *S. cerevisiae*, was detected in the complemented yeast (W303Δ*coq2*/pDR-AtPPT1) grown on minimum medium containing glucose as the carbon source. This indicated that the recovery of the respiration ability in the mutant is due to a recovery in its ability to produce UQ-6 (Figure 2B). In addition, our complementation study that uses a fission yeast *S. pombe* Ppt1 defective mutant (NU609) suggests that the AtPPT1 protein can also function as 4HPT in *S. pombe*, and the production of UQ-10 in the complemented mutant was confirmed by HPLC (data not shown).

In vitro 4HPT enzyme assay was conducted to directly demonstrate the enzymatic activity of the AtPPT1 protein. Yeast W303Δ*coq2* expressing pDR-AtPPT1 was cultured on synthetic defined (SD) medium without uracil, and the mitochondrial fraction was prepared from the harvested yeast. Yeast harboring pDR196 empty vector was used as the negative control. The prepared mitochondrial fractions were incubated with both GPP as a prenyl substrate and 4-hydroxybenzoate as a benzoquinone moiety. Subsequently, the reaction product, 3-geranyl-4-hydoroxybenzoic acid, was detected by HPLC, as described earlier (Yazaki *et al.*, 2002). The mitochondrial fraction of the transformant harboring pDR-AtPPT1 exhibited apparent enzymatic activity of 23 nmol/h/mg protein, whereas the negative control with an empty vector did not exhibit detectable activity (Figure 2C). This result again indicated that

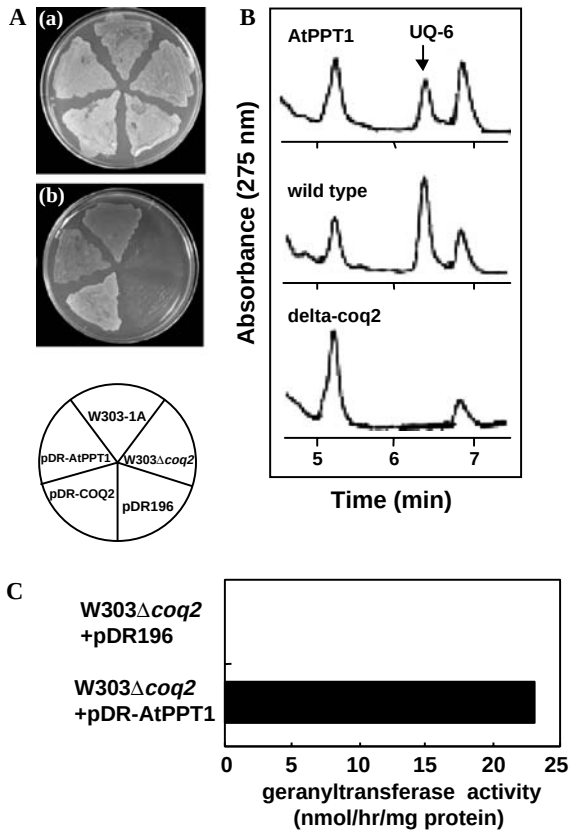

Figure 2. Functional analysis of *Arabidopsis AtPPT1* gene product. A. Complementation of yeast *coq2* disruptant with *Arabidopsis AtPPT1* gene. Yeast strains were plated on SD-medium containing glucose (a) or glycerol (b) as a carbon source. W303-1A, wild-type; W303Δ*coq2*, *coq2* mutant; pDR-COQ2, W303Δ*coq2* expressing *COQ2* gene; pDR-AtPPT1, W303Δ*coq2* expressing *AtPPT1* gene; pDR196, control empty vector. B. Reversed phase HPLC of UQ prepared from the wild-type, *coq2* mutant, and *coq2* mutant complemented by *AtPPT1*. Elution position of UQ-6 is indicated by an arrow. C. Prenyltransferase activity in the mitochondrial fraction of *coq2* mutant that has pDR196 empty vector or *AtPPT1* gene were measured using 4-hydroxybenzoate and geranyl diphosphate as the prenyl acceptor and donor, respectively.

AtPPT1 protein has 4HPT enzymatic activity, and suggested that this enzyme has a broad specificity in terms of prenyl substrates, ranging from GPP (C_{10}) to decaprenyl diphosphate (C_{50}).

Expression of AtPPT1 *mRNA and subcellular localization of the protein*

The expression pattern of *AtPPT1* mRNA in *Arabidopsis* was determined by quantitative RT-PCR using TaqMan technology. Four-week-old *Arabidopsis* was grown on Murashige-Skoog (MS) solid medium to obtain roots, rosette leaves, cauline leaves, stems, and flower clusters that include young buds, flowers, and young ovary with developing embryo. Total RNA prepared from these organs was subjected to reverse transcription. The expression level of the *AtPPT1* gene was represented as the relative amount of *AtPPT1* mRNA normalized with respect to the expression level of 18S rRNA. As shown in Figure 3B, *AtPPT1* mRNA was predominantly accumulated in the flower cluster, and it was demonstrated that other organs (stems, leaves, and roots) had lower expression levels than that of flowers. Northern blot analysis also revealed that *AtPPT1* mRNA was predominantly expressed in the flower cluster and slightly expressed in roots, but not in other parts, whereas the *AtGGPS1* gene, encoding plastidial geranylgeranyl diphosphate synthase (Okada *et al*., 2000), was expressed uniformly through the organs. This result also indicated that the expression of the *AtPPT1* gene was relatively abundant in flowers, at least in four-week-old adult plants (Figure 3A).

The subcellular localization of AtPPT1 protein was predicted using the program on the PSORT WWW server. The *N*-terminal peptide of 100 amino acids in the AtPPT1 protein was presumed to be a mitochondrial targeting signal, which translocates the protein into the mitochondria. To confirm this, a construct was prepared in which the green fluorescent protein (GFP) was fused to 100 amino acids of the *N*-terminal region of the AtPPT1 protein and the fusion construct was under the CaMV 35S promoter in the pTH121 vector (Zhu *et al*., 1997). Transgenic plants expressing *35S::AtPPT1-GFP* gene were produced by the *Agrobacterium*-mediated floral dipping method (Clough and Bent, 1998), and homozygous transgenic plants were selected in the T3 generation. Wild-type plants treated with $DiOC_6$ reagent,

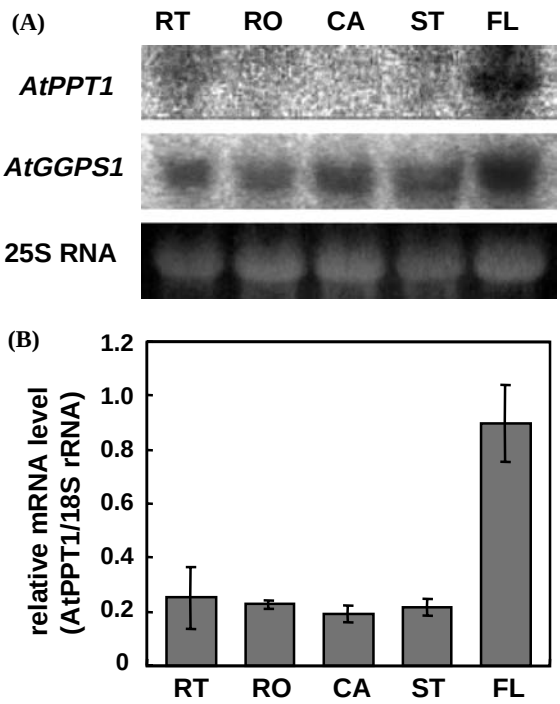

Figure 3. Expression pattern of *AtPPT1* mRNA. Total RNA prepared from root (RT), rosette leaves (RO), cauline leaves (CA), stems (ST), and flower cluster (FL) were subjected to northern blot (A) and QRT-PCR (B) analyses. A. 10 μg of total RNAs were loaded on each lane. ^{32}P labeled full-length *AtPPT1* cDNA (top) or *AtGGPS1* cDNA (middle) were used as a probe. Bottom lane shows 25S rRNA in the formaldehyde-agarose gel containing ethidium bromide. B. 2 μg of total RNAs were used for reverse transcription. Expression level of the *AtPPT1* mRNA was normalized by the 18S rRNA value. Values are means with standard errors from three measurements.

which can specifically stain mitochondria, were microscopically studied along with the *35S::AtPPT1-GFP* transgenic plants. When two-week-old transgenic plants were observed by a confocal laser scan microscope, the GFP signal was detected as a small particle in the roots, hypocotyl, and guard cell in true leaf. In wild-type plants treated with $DiOC_6$, the same signal patterns were observed (Figure 4). These results suggested that the AtPPT1 protein is probably localized in the mitochondria where UQ biosynthesis occurred.

The AtPPT1 *gene is essential for embryo development in* Arabidopsis

There has been no report on the isolation of a plant mutant that is defective in 4HPT activity and which

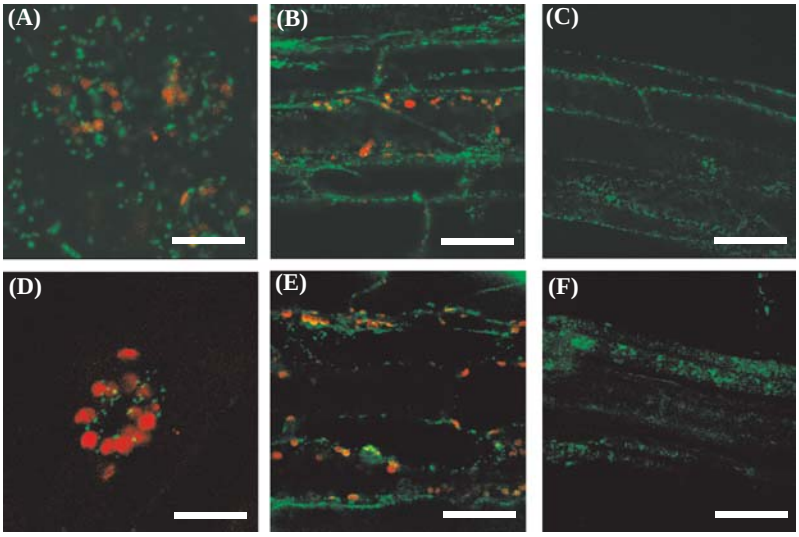

Figure 4. Subcellular localization of AtPPT1-sGFP fusion protein in *Arabidopsis*. Fourteen-day-old AtPPT1-GFP transgenic plants (A, B, C) and wild-type plants stained by DiOC6 (D, E, F) were studied by confocal laser scan microscopy. Green signal (from GFP or DiOC6) and red signal (chlorophyll autofluorescence) were superimposed in each image. (A) and (D), guard cell in true leaves; (B) and (E), hypocotyls; (C) and (F), roots. Bars indicate 15 μm (A, D) or 50 μm (B, C, E, F).

results in a defect in UQ. To gain more insight into the physiological features of UQ-less plants, we screened the *Arabidopsis atppt1* mutant from the 6000 Feldman T-DNA-tagged lines in the population of Ws-2 (Wassilewskija) by PCR (McKinney *et al.*, 1995). After the 3rd PCR screening, the *AtPPT1* disrupted mutant that had a T-DNA insertion was found, and the sequence analysis showed that the T-DNA of *AtPPT1* was inserted in the 5th intron (Figure 5A). The mutant (seed stock no. CS10238) was obtained from the *Arabidopsis* Biological Resource Center (ABRC) and backcrossed once to wild-type Ws-2 using the mutant line as the pollen donor. The F1 progeny plants selected for resistance to kanamycin (km) were checked to have a T-DNA insertion on the *AtPPT1* gene locus. The mutant appeared to contain a second insertion of T-DNA on the locus other than the *AtPPT1* gene, because a km-resistant plant, which revealed no insertion on the *AtPPT1* locus, could be segregated. Thus, the mutant possessing a T-DNA insertion only at the *AtPPT1* gene locus was characterized further. The *atppt1* F2 plants segregated approximately 2:1 for km resistance vs. sensitivity. We were unable to identify any progeny homozygous mutant when heterozygous F2 plants were selfed, suggesting that the *AtPPT1* gene is essential. The segregation ratio for km resistant vs. sensitive in one of the progeny lines at F3 genera-

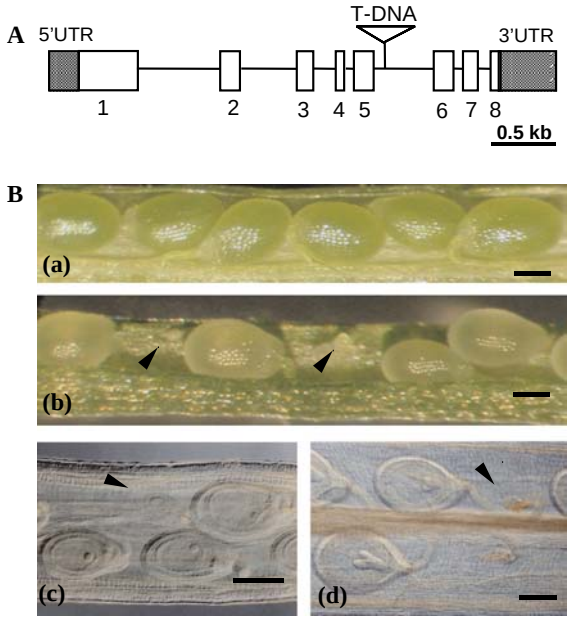

Figure 5. Seed and embryo phenotype of T-DNA insertion *atppt1* mutant. A. Genomic gene structure of *AtPPT1* locus. Untranslated regions (UTR) at 5′ and 3′ ends are shown as solid boxes, and numbered boxes (open) show 8 exons in the *AtPPT1* gene. T-DNA is inserted into the position between exon 5 and exon 6. B. Immature siliques containing seed from selfed wild-type plants (a) and selfed heterozygous *atppt1* mutant (b). Shrunken seeds in the *atppt1* mutant are indicated by arrowheads. Developing embryo at globular stage (c) and heart stage (d) in the *atppt1* mutant. Arrowheads indicate embryos that arrested its development. Bars indicate 200 μm.

Table 1. Segregation analysis. Number of F3 progeny segregated to kanamycin resistant or sensitive was counted. Line unrelated was segregated as kanamycin resistant plant with *atppt1* from parental line CS10238.

Line	F3 progeny	Kanamycin resistance		Ratio (Km +/−)
		Resistant (+)	Sensitive (−)	
atppt1	298	204	94	2.17
unrelated	290	225	65	3.46

tion was shown to converge on 2:1 again, whereas *atppt1*-unrelated segregant (unrelated) showed approximately 3:1 segregation ratio with regard to km resistance (Table 1). These results suggest that the homozygous *atppt1* mutation is lethal, probably because development of zygotic embryogenesis was incomplete after fertilization. In addition, pollen viability in the heterozygous mutant is likely to be maintained enough to fertilize, because the mutation of *atppt1* could be transmitted to the next generation through the pollen in the mutant.

To investigate this phenomenon in more detail, we observed the seeds in each silique from the self-crossed heterozygous *atppt1* mutant. In the *atppt1* mutant, we frequently observed defects in the mature seed in all the silique, whereas this seed loss was hardly detected in the silique of wild-type plants (Figure 5B). The average defection of the seeds in the silique of *atppt1* mutant was approximately 30% (data not shown). The development of a zygotic embryo in the silique of the *atppt1* mutant was studied further by microscopy using cleared silique samples. The development of the zygotic embryo, which appeared to be derived from homozygous *atppt1* mutant, was already delayed at the globular stage, and was completely arrested at the heart stage compared with the normal embryo (Figure 5B). Thus, besides the segregation test, microscopic observation also suggested that the *AtPPT1* gene is essential for embryo development in *Arabidopsis*.

Discussion

In this report, we have demonstrated the enzymatic activity of 4HPT encoded by the *Arabidopsis AtPPT1* gene, which is highly homologous to yeast Coq2 and Ppt1 proteins. A yeast *coq2* mutant expressing *AtPPT1* gene recovered its respiratory capacity to grow on a medium containing a non-fermentable carbon source. We have also demonstrated that UQ-6 was produced when the *coq2* mutant was complemented by the expression of the *AtPPT1* gene. These results indicated that the AtPPT1 protein can substitute the function of yeast Coq2 protein and is responsible for the UQ biosynthesis in *Arabidopsis*. Since *Arabidopsis* produces UQ-9, and the *At-SPS* gene encoding solanesyl diphosphate synthase has been identified in *Arabidopsis* (Hirooka *et al*., 2003), the prenyl substrate of AtPPT1 protein should specifically be solanesyl diphosphate. *In vitro* prenyltransferase assay with GPP as a prenyl substrate demonstrated that the AtPPT1 protein could transfer GPP to benzoquinone moiety, suggesting that the enzyme has a broad substrate specificity in terms of the prenyl donor. A common feature of 4HPT appears to be that it can accept various lengths of prenyldiphosphate since yeast 4HPT catalyzes prenyl donors ranging from GPP (C_{10}) to decaprenyl diphosphate (C_{50}) (Okada *et al*., 1998; Yazaki *et al*., 2002).

The subcellular compartment of UQ biosynthesis in plants has not yet been clarified. Lutke-Brinkhaus *et al*. (1984) reported that the mitochondria prepared from potato tubers have several enzymatic activities in UQ biosynthesis. In contrast, Swiezewska *et al*. (1993) proposed that UQ and plastoquinone-9 biosynthesis occur in the endoplasmic reticulum-Golgi system, because nonaprenyl-4-hydroxybenzoate transferase activity was found in microsomal and Golgi preparation. More recently, Avelange-Macherel and Joyard (1998) demonstrated that the *Arabidopsis AtCOQ3* gene product, dihydroxy prenylbenzoquinone methyltransferase, was localized within mitochondrial membranes. Our localization analysis indicated that the AtPPT1 protein was probably translocated into the mitochondria. From these results, we propose that mitochondria are one of the main organelles for synthesizing UQ in plants. These observations also suggested the possibility that the UQ transport system between mitochondria and other cellular compartments exists in plants cells (Wanke *et al*., 2000).

A quantitative RT-PCR analysis demonstrated that *AtPPT1* mRNA was expressed three-fold higher at the flower cluster compared with other

parts. The northern blot analysis also demonstrated the same expression pattern, that is, it was predominant at flower parts and hardly detected at other parts. This expression pattern may suggest that UQ biosynthesis is active at flower parts in order to obtain the large amount of energy required for the flowering events. It has been reported that the expression level of the nuclear encoded mitochondrial gene encoding Rieske iron-sulfur protein was high in floral organs in which mitochondrion number increased compared to other organs (Huang *et al*., 1994). However, to the best of our knowledge, the UQ content among plants organs has not been studied. In this respect, our result on the observation of the *atppt1* mutant supports the idea that UQ is particularly abundant and required at the flowering stage, because the homozygous *atppt1* mutant is embryonic lethal. Therefore, such an expression pattern was in good agreement with the fact that the *atppt1* mutant, which seems to lack UQ in the reproductive organs, exhibited a defect in the embryo development after fertilization in flowers.

Our mutant analysis strongly suggested that UQ is essential for precise embryonic development in *Arabidopsis*. This is the first observation upon analyzing the phenotype of a plant mutant that lacks UQ biosynthetic enzyme activity. The same phenomena of embryonic lethality have been observed in *C. elegans* and *M. musculus*, both of which are defective in the UQ biosynthetic enzyme activity (Levavasseur *et al*., 2001; Rodriguez-Aguilera *et al*., 2003). In the case of mammals, an arrest in embryo development is observed at the midgestation stage in mice, and some progeny were generated in nematodes, whereas embryo development in *atppt1* mutant seemed to be arrested at a stage earlier than the globular stage. This suggests that the effect of UQ depletion could be more severely expressed in plant species than in mammals. A possible explanation may be that the content of UQ that is carried over from maternal cells is limited to a very low level in plants, and embryos in a homozygous *atppt1* mutant, which is the UQ-null phenotype, never develop normally. In contrast, UQ is not necessarily required for the basal growth of bacteria and yeast when these organisms are grown on a medium containing fermentable sugar (Ashby *et al*., 1992; Suzuki *et al*., 1994; Okada *et al*., 1998). It appears that unlike bacteria and yeasts, higher organisms have a distinct dependence on UQ for their embryonic development.

Several reports demonstrate the pleiotropic effects of UQ in non-mitochondrial cells (Saiki *et al*., 2003). Therefore, besides depletion of mitochondrial UQ, depletion of non-mitochondrial UQ might be the reason for the arrest of embryo development in the homozygous *atppt1* mutant, as observed in mammals. Because of the lethality in the *atppt1* mutant, we were unable to investigate how the endogenous level of UQ affects embryogenesis in *Arabidopsis*. To support these observations, the UQ level in the heterozygous *atppt1* mutant was maintained at the same level as that of the wild-type plants, and the heterozygous mutant was able to grow normally, similar to wild-type plants (data not shown). To understand the relationship between the UQ level and its effect on plants, transgenic *Arabidopsis* are being generated, in which the expression of *AtPPT1* gene is modified. Such transformants that have elevated or decreased UQ levels would provide a clue toward understanding the biological roles of UQ at mitochondrial and non-mitochondrial sites in plant cells.

Materials and methods

Plant materials and growth conditions

Arabidopsis ecotype Wassilewskija (Ws-2) was used throughout the study. Plants were grown under continuous light at 22 °C on MS medium (GIBCO-BRL, Cleveland) or on soil. Mutant plants were selected on MS plate containing 100 μg/ml of kanamycin, then transferred to soil for phenotypic observation. For RNA preparation, roots, rosette leaves, cauline leaves, stems, and flowers were harvested separately from plants grown on MS medium for 4 weeks.

cDNA cloning and plasmids constructions

An amount of 1 μg of total RNA prepared from 4-week-old plants were used for reverse transcription by SuperscriptII reverse transcriptase (GIBCO BRL) with poly-T oligo primer, and obtained 1st strand cDNA was subjected to PCR with oligonucleotide primers of AtCOQ2-S (5′-ATGGCGT

TTTTTGGGCTCTCCCGTGTTTC-3′) and ATC OQ2-A (5′-TTATTGAGTACCGAGATCTGCA CTGAATCC-3′). The amplified fragment was directly cloned into pT7 blue-T vector (Novagen, Madison, WI) and sequenced. To determine 5′-untranslated region (UTR) and 3′-UTR of the cDNA obtained as above, 5′ and 3′ rapid amplification of cDNA ends (RACE) were performed using Marathon cDNA Amprification Kit (Clontech, Palo Alto, CA) according to manufacture's instruction. For 5′-RACE, oligonucleotide primers of PPT1RACE-1 (5′-CCAACGCAATCGACCA-CAT-3′) and PPT1RACE-2 (5′-CCACGCAAGC AACCAAGTTCCAATG-3′) were used for synthesizing 1st strand cDNA and nested PCR, respectively. Full length *AtPPT1* cDNA was amplified by RT-PCR with oligonucleotide primers of AtPPT1-S2 (5′-TCCCTGAGAGATTAGA AACGCC-3′) and AtPPT1-GK (5′-TAGTTATT AACATCCTTTAGG-3′), cloned into pT7 blue-T and sequenced. The resulting plasmid was designated as pT7ATPPT1. *Eco*RI and *Xho*I fragment prepared from pT7ATPPT1 was cloned into the same site of the pDR196 vector to yield pDR-AtPPT1, which was used for heterologous expression in yeast. For construction of GFP-fusion transgene (AtPPT1LS-GFP), *Bam*HI-*Sal*I fragment of *AtPPT1* cDNA were prepared by PCR using oligonucleotide primers of PPT1FLS-Bam (5′-GGATCCGCGTTTTTTGGGCTCTC-3′) PPT1FLA-Sal (5′-GTCGACTTATTGAAAA CTTCTTCCAAG-3′), and cloned into the same site of the pTH121 vector (Zhu *et al*., 1997) to yield pTH-ATPPT1. AtPPT1LS-GFP fragment prepared from pTH-ATPPT1 was transferred to binary vector pPZPY122 (Yamamoto *et al*., 1998), and the resulting construct pPZ-ATPPT1 was used for transformation of *Arabidopsis* by *Agrobacterium*-mediated floral dipping method (Clough and Bent, 1998).

Functional analysis of AtPPT1 *gene by yeast complementation system*

Function of *AtPPT1* gene was confirmed on yeast complementation using *S. cerevisiae coq2* disruptant (W303Δ*coq2*). The pDR-AtPPT1 plasmid was heterologously expressed in W303Δ*coq2*, and functional complementation of the mutant by *AtPPT1* gene was evaluated by recovery of its growing ability on the medium containing non-fermentable carbon source as described earlier (Yazaki *et al*., 2002). Geranyltransferase assay was conducted using the mitochondrial membrane prepared from complemented yeast (W303Δ*coq2*/pDR-AtPPT1) with 4-hydroxybenzoate and geranyl diphosphate as substrates. The reaction product, 3-geranyl-4-hydroxybenzoic acid, was quantitatively detected by high performance liquid chromatography as described before (Yazaki *et al*., 2002).

Northern and QRT-PCR analysis

Total RNA was extracted from separated organs (flower, stem, cauline leaves, rosette leaves, and root) in 4-week-old plants using RNeasy plant total RNA isolation kit (Qiagen, Valencia, CA). Ten micrograms of total RNAs were separated on a 1.2% (w/v) agarose gel containing 2.2 M formaldehyde and transferred to a Hybond^{+} membrane (Amersham-Pharmacia Biotech, Uppsala), then hybridized with a ^{32}P-labeled cDNA probe of *AtPPT1*(Sambrook *et al*., 1989). The membrane was washed and exposed to the imaging plate, then detected by the image analyzer (BAS2500, Fuji Photo Film, Tokyo).

QRT-PCR using Taq-Man technology (Holland *et al*., 1991) was employed to determine transcript levels. Total RNA (2 μg) was treated with DNase I (RQ1 RNase-free DNase; Promega, Madison, WI) and used as a template to synthesize first-strand cDNA with random hexamer using a SuperScript first-strand synthesis system according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Quantitative real-time PCR was performed using the first-strand cDNA as a template on a sequence detector system (model7700; Applied Biosystems, Foster City, CA). To calculate mRNA level of the *AtPPT1* gene, the copy number of the *AtPPT1* mRNA was determined by generating standard curve using a series of known concentration of the target sequence. For normalization across samples, 18S rRNA was used as an internal standard. The value at 4×10^{-15} M target RNA in the reaction mixture (25 μl) using 125 ng of total RNA was arbitrarily set as 1.0. For each sample, the mean value from triplicate real-time PCRs was adapted to calculate the transcript abundance. The measurements were repeated using two independent plant materials.

Microscopic observation

Seven-day-old *Arabidopsis* seedlings transformed with AtPPT1LS-GFP transgene were examined for visualization of both the GFP signal and chlorophyll autofluorescence using an confocal lazer scan microscope (maicro-Radiance 2000, Bio-Rad) equipped with epifluorescence. Plant materials from at least 20 independently obtained T3 seedlings were put on the glass slide and covered with a coverslip in 50 mM potassium phosphate buffer. Guard cells of stoma in true leaves, hypocotyl epidermis and roots of transgenic plants were used for observation of these fluorescent signals. 175 nM $DiOC_6$ solution (Molecular Probes Inc., Oregon USA) was used for staining mitochondria in 7-day-old wild-type plants. Silique of the mutant plants were fixed and cleared according to the methods of Tsugeki *et al*. (1996) with slight modification, and visualized with Nomarski optics on a microscope (model BX60F5, Olympus Optical, Tokyo).

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