FEBS Letters 580 (2006) 955–959

Metabolic engineering of coenzyme Q by modification of isoprenoid side chain in plant

Sakiko Takahashi[a], Yuki Ogiyama[b], Hiroaki Kusano[a,c], Hiroaki Shimada[c,d], Makoto Kawamukai[b], Koh-ichi Kadowaki[a,*]

[a] *Department of Genetic Diversity, National Institute of Agrobiological Sciences, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8602, Japan*
[b] *Faculty of Life and Environmental Science, Shimane University, 1060 Nishikawatsu, Matsue, Shimane 690-8504, Japan*
[c] *Department of Biological Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan*
[d] *Plant Biotechnology Division, Tissue Engineering Research Center, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan*

Received 17 November 2005; revised 24 December 2005; accepted 4 January 2006

Available online 19 January 2006

Edited by Michael R. Sussman

Abstract **Coenzyme Q (CoQ), an electron transfer molecule in the respiratory chain and a lipid-soluble antioxidant, is present in almost all organisms. Most cereal crops produce CoQ9, which has nine isoprene units. CoQ10, with 10 isoprene units, is a very popular food supplement. Here, we report the genetic engineering of rice to produce CoQ10 using the gene for decaprenyl diphosphate synthase (DdsA). The production of CoQ9 was almost completely replaced with that of CoQ10, despite the presence of endogenous CoQ9 synthesis. DdsA designed to express at the mitochondria increased accumulation of total CoQ amount in seeds.**

Keywords: Metabolic engineering; CoQ10, ubiquinone; Subcellular localization; Decaprenyl diphosphate synthase; Rice

1. Introduction

Coenzyme Q (CoQ), also called ubiquinone, is an essential component of the respiratory chain, which produces ATP, and also shows strong antioxidant activity against lipid peroxides. The biosynthetic pathway for CoQ has been extensively studied in *Escherichia coli* and *Saccharomyces cerevisiae* [1–3]. The major part of its biosynthesis begins with the transfer of the isoprenoid side chain to the benzoquinone frame (Fig. 1A). The length of the side chain of CoQ is determined by polyprenyl diphosphate synthase, an enzyme involved in the synthesis of a given length of isoprenoid chain [2]. For instance, the decaprenyl diphosphate synthase (DdsA) of *Gluconobacter suboxydans* specifies the synthesis of 10 isoprene units (Fig. 1B). In addition to the native CoQ compound, some amount of CoQ10 has been produced by the expression of the *ddsA* gene from *G. suboxydans* [4] in *S. cerevisiae* and *E. coli* [4,5].

In plants, the metabolism of short-chain prenyl diphosphate (consisting from 1 to 4 isoprene units) has been well characterized [6]. However, the biosynthetic pathway for long-chain polyprenyl diphosphate (consisting from 5 to 10 isoprene units), which is also a component of CoQ, is less understood. In addition, most of CoQ biosynthetic pathway in plants remains unclear. Because genes homologous to those engaged in yeast CoQ biosynthesis have been found in *Arabidopsis* recently [2], the pathway in plant is assumed to be similar to that of yeast. All of isolated yeast genes engaged in CoQ biosynthesis have a putative mitochondrial signal [7], implying mitochondria are the place of biosynthesis of CoQ in yeast. On the contrary, biochemical analysis of spinach suggests that CoQ biosynthesis may also occur in the endoplasmic reticulum (ER)/Golgi [8].

In this study, we aimed to modify the length of side chain of CoQ from nine to 10 units by expressing DdsA to produce CoQ10 in the rice, staple food. For efficient expression, DdsA was designed to target to the mitochondria or Golgi apparatus. We show that CoQ10 is predominantly produced in the leaves and seeds of transgenic rice. Effectiveness of foreign enzyme expression at the non-native site in metabolism is discussed.

2. Materials and methods

2.1. Plasmid construction

For expression of *ddsA* gene from *G. suboxydans* in plant, the putative GTG initiation codon of *ddsA* [4] was replaced with ATG, and this modified sequence was used as *ddsA* gene in this study hereafter. A part of the rice mitochondrial *rps14* gene (amino acids 1–48 encoding the mitochondria-targeting signal, S14) [9] or a part of the tobacco *GnT1* gene (amino acids 1–77 encoding the Golgi-targeting signal, CTS) [10] was ligated in-frame upstream of the replaced ATG codon of the modified *ddsA* sequence to allow enzyme targeting. A CaMV35S promoter (35S-Ω) [11] and a NOS terminator were used to facilitate gene expression. The *Hin*dIII–*Eco*RI fragment from the resulting plasmid was inserted into the multicloning site of the binary vector pCAMBIA1301 (GenBank Accession No. AF234297).

2.2. Plant transformation and cultivation

The resultant plasmid was introduced into *Agrobacterium tumefaciens* strain EHA105 by electroporation. *Agrobacterium*-mediated transformation of rice (*Oryza sativa* L. cv. Nipponbare) was performed according to world patent WO01/06844. Transgenic plants were selected on medium containing hygromycin (50 μg/ml), and the presence of the *ddsA* gene in the genome was confirmed by PCR amplification. A primer set of DDSA-D (5′-GGTGCAGGAGTCGACAGCGCGCTAT-3′) and DDSA-G (5′-CAGTGTGGCGGTATGAATGAAC-3′),

*Corresponding author. Fax: +81 29 838 7408.
E-mail address: kadowaki@affrc.go.jp (K. Kadowaki).

Abbreviations: CoQ, coenzyme Q; DdsA, decaprenyl diphosphate synthase; ER, endoplasmic reticulum; LC/MS, liquid chromatography/mass spectrometry; PPT, *p*-hydroxybenzoate polyprenyl transferase

0014-5793/$32.00
doi:10.1016/j.febslet.2006.01.023

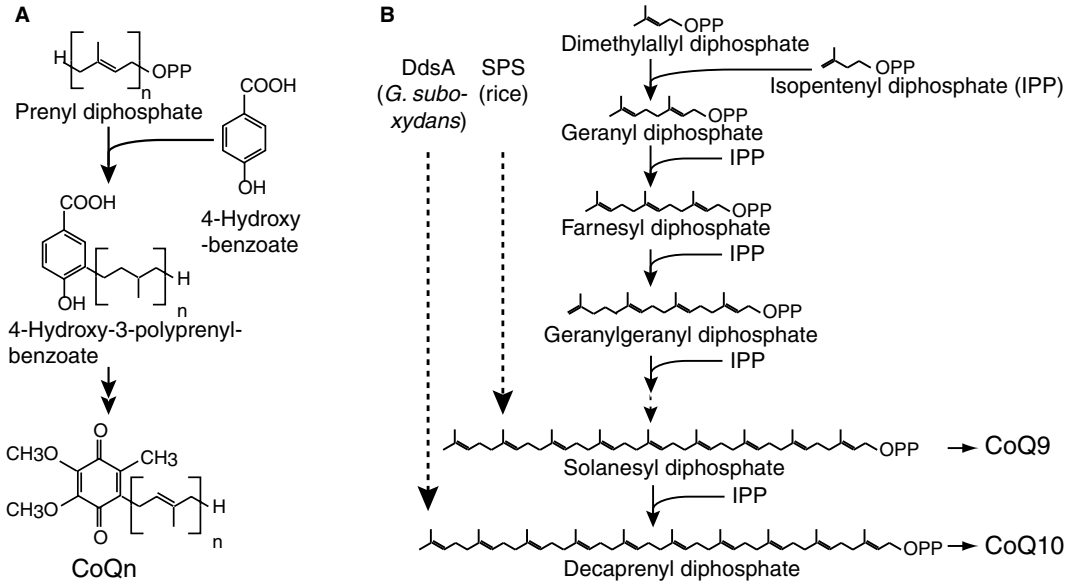

Fig. 1. Biosynthetic pathways of coenzyme Q and isoprenoid. (A) Coenzyme Q biosynthesis. (B) Isoprenoid biosynthesis. DdsA catalyzes the synthesis of decaprenyl diphosphate by sequential condensation step using geranyl diphosphate, farnesyl diphosphate, or geranylgeranyl diphosphate as allylic substrate, and isopentenyl diphosphate. DdsA, decaprenyl diphosphate synthase; SPS, solanesyl diphosphate synthase.

and 0.5 U of rTaq DNA polymerase (TOYOBO, Tokyo, Japan) were used for the PCR reaction. PCR amplification was conducted for 30 cycles where each cycle consisted of 30 s at 94 °C, 1 min at 55 °C, 1 min at 72 °C. Primary transformants (T_0 plants) were transplanted into soil and grown in a naturally illuminated green house (27 °C). T_0 plants and their self-pollinated progeny (T_1 plants and T_2 seeds) were used for the experiments.

2.3. Western blot analysis

The leaves from transgenic rice plants were ground thoroughly in eight volumes of extraction buffer (50 mM Tris–HCl [pH 6.8]; 2% SDS; 6% 2-mercaptoethanol; 10% glycerol) per gram fresh weight. The samples were centrifuged at 13,000 × *g* for 5 min at 4 °C, and the supernatants were collected. Proteins were separated on 12.5% polyacrylamide gels and transferred onto immobilon polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The ECL Plus Western Blotting Detection System (Amersham Biosciences, Tokyo, Japan) was used to detect the proteins. Antisera against DdsA protein were prepared as follows. The entire protein-coding region of *ddsA* from *G. suboxydans* was amplified by PCR, ligated into the pGEX 4T-3 vector (Amersham Biosciences) and transformed into *E. coli* JM109. The GST–DdsA fusion protein was induced by the addition of 1 mM isopropyl-β-D(−)-thiogalactopyranoside and it was purified by the method of Ausubel et al. [12]. Aliquots of purified fusion protein were injected into a rabbit six times at intervals of two weeks to raise polyclonal antibodies.

2.4. Measurement of CoQ

CoQ was extracted by the method of Okada et al. [4] with slight modifications, in the solvent used for TLC development (benzene) and CoQ elution from the TLC plate (hexane/isopropanol 1:1, v/v), with the addition of CoQ6 prior to extraction as an internal control. Fresh leaves, dehulled seeds, or polished seeds were used as materials. For the measurement of polished seeds, dehulled seeds were polished for 40 s with rice polisher (Pearlest, Kett Electric Laboratory, Tokyo, Japan). The extracted CoQ was analyzed further by HPLC on a C18 reversed-phase column (150 mm × 60 mm; YMC-Pack ODS-A, YMC, Kyoto, Japan) with ethanol as the solvent phase at a flow rate of 1 ml/min. CoQ was detected spectrophotometrically at a wavelength of 275 nm. To confirm the molecular mass of each CoQ, we performed liquid chromatography/mass spectrometry (LC/MS) using an LCT LC/MS system (Micromass, Milford, MA, USA) equipped with an atmospheric-pressure chemical ionization source (4.6 mm × 150 mm; Inertsil ODS3 column, GL Sciences, Tokyo, Japan) at a flow rate of 1 ml/min. After injection onto a column equilibrated with the mobile phase (methanol/isopropanol 3:1, v/v), the column was eluted isocratically with the mobile phase for 30 min.

3. Results and discussion

3.1. Production of transgenic rice plants expressing DdsA protein

Three kinds of chimeric gene constructs, S14:ddsA, CTS:ddsA, and ddsA, were designed to localize DdsA to the mitochondria, the Golgi apparatus, and the cytoplasm, respectively (Fig. 2A). These constructs were introduced into rice using *Agrobacterium*-mediated transformation. Gene integrations were confirmed by PCR amplification for more than 30 primary transformants (T_0 plants) per construct (data not shown). Western blot analysis of the T_0 plants using anti-DdsA antibody showed the accumulation of DdsA protein in S14:ddsA and CTS:ddsA plants, whereas no DdsA protein was detected in ddsA plants (Fig. 2B). Northern blot analysis revealed that very small amounts of *ddsA* mRNA were present in the ddsA plants (data not shown), suggesting that expression of DdsA was controlled at the transcriptional level. Apparent size of S14:DdsA and CTS:DdsA in Western blot analysis (Fig. 2B) were 41 and 45 kDa, respectively. The sizes are similar to estimated molecular size from processed S14:DdsA and CTS:DdsA proteins. Therefore, we speculate that the N-terminal cleavages happened in both proteins.

The S14:ddsA plants were fertile and look very similar to wild-type plants in phenotype although precise comparisons, such as growth rates, have not been performed. While, the CTS:ddsA plants accumulating DdsA protein at medium or high level showed obvious growth retardation (data not shown), suggesting that the expression of DdsA having Golgi targeting signal had an inhibitory effect on growth in transgenic plants.

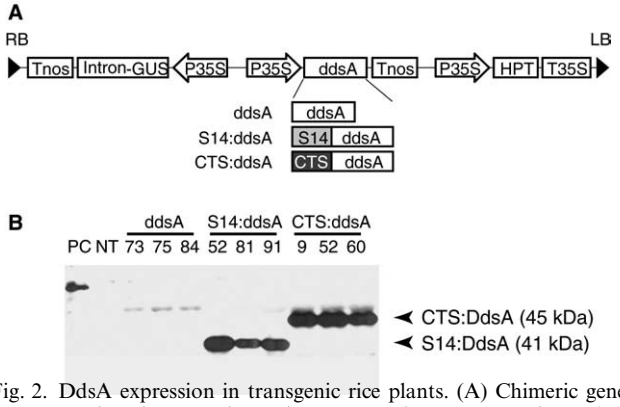

Fig. 2. DdsA expression in transgenic rice plants. (A) Chimeric gene constructs for rice transformation. Expression cassettes for DdsA were designed to contain a mitochondria-targeting signal (S14:ddsA), a Golgi-targeting signal (CTS:ddsA), or no targeting signal (ddsA). (B) Western blot analysis of transgenic rice plants. About 2.5 μg of soluble protein from leaves from T_0 plants of ddsA, S14:ddsA, CTS:ddsA, and nontransformant (NT) were analyzed using anti-DdsA antibody. Filled arrowheads indicate the positions of CTS:DdsA and S14:DdsA. PC (positive control), total protein from *E. coli* expressing GST–DdsA.

3.2. Predominant production of CoQ10 in transgenic plants

The lengths of the side chains of CoQ in leaves from S14:ddsA and CTS:ddsA plants were determined by HPLC (Figs. 3A–C). The CTS:ddsA plants produced CoQ9, like wild-type plants (Figs. 3A and B). In contrast, S14:ddsA plants produced CoQ10, with a traces of CoQ9 (Fig. 3C). The molecular masses of the CoQ molecules produced in nontransformants (Fig. 3D) and those produced in S14:ddsA plants (Fig. 3E) were determined by mass spectrometry and confirmed to be CoQ9 and CoQ10, respectively. Therefore, we successfully modified the length of the side chain of CoQ.

3.3. CoQ content in leaves and seeds from transgenic plants

The CoQ content was analyzed in detail in the progeny of independent S14:ddsA plants (Fig. 4). Five T_0 plants were selected for subsequent analysis, based on distinctive patterns on Southern blot analysis. The copy number of *ddsA* gene inserted to the genomic DNA in the selected transformants, S14:ddsA-3, 26, 52, 81, and 91, were 1, 3, 1, 2, or 1, respectively (data not shown). They were then self-pollinated to obtain T_1 seeds. The CoQ content of leaves from 1.5-month-old S14:ddsA T_1 plants was measured by HPLC (Fig. 4A). Leaves of S14:ddsA T_1 plants accumulated 40–70 μg per gram fresh weight (μg/gFW) of CoQ10, which is 11–18 times higher than the CoQ10 content of the leaves of nontransformants (Fig. 4A). These S14:ddsA T_1 plants were then self-pollinated, and homozygous S14:ddsA plants were selected. Seeds (T_2 generation) from these plants accumulated about 18 μg/gFW of CoQ10 (Fig. 4B). This is surprising because this value is two to three times higher than that of the total CoQ content (both CoQ9 and CoQ10) of the seeds of nontransformants. Thus, the total CoQ content of these seeds was significantly increased by the expression of the S14:DdsA protein.

Regarding the localization of CoQ, whole dehulled rice seeds contain 3.8 μg/gFW of CoQ9 (with standard error ±0.19, from three independent experiments), while polished rice seeds

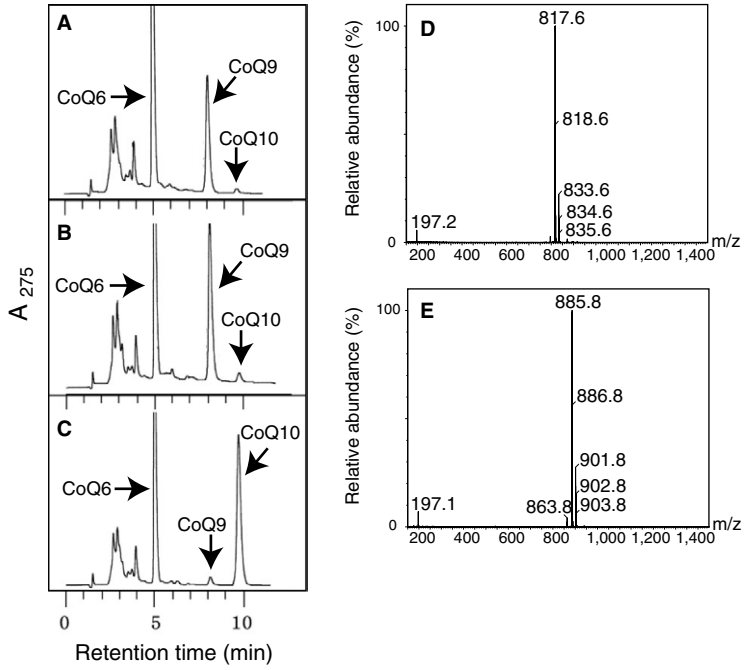

Fig. 3. Analysis of the side chain length of CoQ in rice leaves. (A–C) HPLC analysis. CoQ was extracted from wild-type plants (A), CTS:ddsA T_0 plants (B), and S14:ddsA T_0 plants (C). The same results were obtained from the analysis of the two independent CTS:ddsA T_0 plants and four independent S14:ddsA T_0 plants where DdsA protein was accumulated, and the four wild-type plants. CoQ6 (internal quantification control), CoQ9, and CoQ10 are indicated by arrows. (D, E) LC/MS analysis. The molecular masses of CoQ eluted at 8 min for (A) and at 9.7 min for (C) are shown in (D) and (E), respectively. These demonstrate that the peak at 8 min was CoQ9 [*m/z* 818 $(M + Na)^+$] and that the peak at 9.7 min was CoQ10 [*m/z* 886 $(M + Na)^+$].

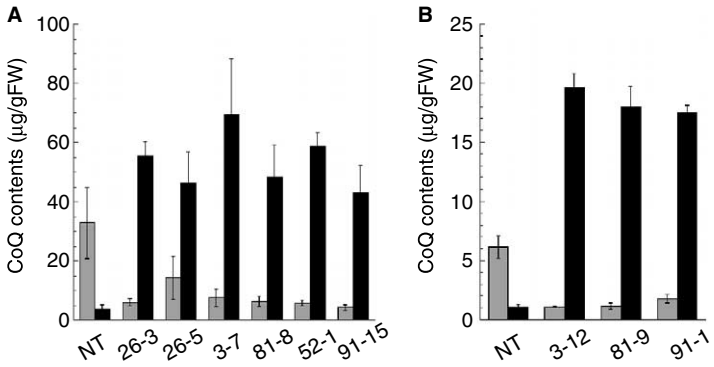

Fig. 4. CoQ9 and CoQ10 contents of rice plants. CoQ was extracted from one leaf of 1.5-month-old plants (A) or 10 dehulled seeds (B). Gray and black bars represent CoQ9 and CoQ10 contents (μg/gFW), respectively. Mean values were calculated from three measurements using independent materials and standard errors are shown by thin lines. NT indicates a nontransformant and the numbers below the graphs show independent transformants.

contain 0.3 μg/gFW of CoQ9 (with standard error ±0.03). Considering the above fact, 92% of CoQ is present in rice bran while only 8% of CoQ is present in polished rice.

3.4. Effect of intracellular localization of DdsA

Transformed *E. coli* expressing DdsA produced endogenous CoQ8 in addition to small amounts of CoQ10 and CoQ9 [4]. The previous results are great contrast to this study since we found here that S14:ddsA rice plants in which DdsA was designed to target at the mitochondria predominantly produced CoQ10 and a trace amount of CoQ9. This is noteworthy because the native pathway for CoQ9 production still operates in these plants (Fig. 3C) and we had expected the production of a mixture of CoQ9 and CoQ10 in transgenic rice, as found in *E. coli*. One of polyprenyl diphosphate synthases is found in the ER and *p*-hydroxybenzoate polyprenyl transferase (PPT), the enzyme that transfers the polyprenyl diphosphate to a benzoquinone moiety, is localized in the mitochondria in *Arabidopsis* [13]. Therefore, it is likely that polyprenyl diphosphate is synthesized in the ER and that the product is then transported to the mitochondria for the synthesis of CoQ [13]. PPT may be one of rate-limiting enzymes in the biosynthesis of CoQ because the expression of *COQ2* (encoding yeast PPT) increased the total CoQ content up to six times in transgenic tobacco leaves [14]. In the present study, the total CoQ content of S14:ddsA plant seeds increased to two to three times higher than that of nontransformants (Fig. 4B). Our results strongly suggest that the synthesis of polyprenyl diphosphate was far more effective in CoQ biosynthesis at the mitochondria than at the ER, its native site. Strangely, no accumulation of CoQ10 was observed in CTS:ddsA plants (Fig. 3B) in which DdsA was designed to target at the Golgi apparatus, even though the DdsA protein was abundantly expressed (Fig. 2B). Polyprenyl diphosphate synthesis in the CTS:ddsA plants requires further analysis.

By producing the S14:DdsA protein, which is aimed to target to the mitochondria, we successfully changed the length of the side chain of CoQ in rice, resulting in CoQ10 in rice leaves and seeds. In the transgenic rice plants, the production of CoQ9 was almost completely replaced with that of CoQ10, despite the presence of endogenous metabolic pathway in the ER. In addition, expression of the S14:DdsA protein increased accumulation of total CoQ amount in seeds. This result shows effectiveness of foreign enzyme expression at the non-native site.

Acknowledgments: We thank Kana Miyashita for technical assistance and Kazuko Ono, Naho Hara, and Dr. Seiichi Toki for production of transgenic rice plants. This work was supported by a research grant from Development of innovative transgenic plants to K.K.

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