Development of CoQ10-enriched rice from giant embryo lines

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Coenzyme Q (CoQ), also called ubiquinone, is an electron transfer molecule in the respiratory chain; it is also a lipid-soluble antioxidant. Most cereal crops produce mainly CoQ9, which has nine isoprene units, whereas humans produce mainly CoQ10, with 10 isoprene units. CoQ10 is a very popular food supplement. Using the cultivar Nipponbare, we previously produced CoQ10-enriched rice plants (Nipponbare-type CoQ10-enriched rice plants) by introduction of the gene for decaprenyl diphosphate synthase (DdsA). In Nipponbare-type CoQ10-enriched rice plants, seed CoQ10 content per weight was increased to up to 10 times that of wild-type rice. However, seed CoQ10 level of those rice plants is still insufficient for practical use, and further 10-times increase in CoQ10 content have been expected. Here, we confirmed preferential accumulation of CoQ in bran and germ of rice seed, and produced improved CoQ10-enriched rice plants by using 2 kinds of giant embryo rice lines, Haiibuki and Chukei-toku 70 (Toku 70). In giant embryo line-type CoQ10-enriched rice plants, seed CoQ10 content per weight was increased to up to 1.4 times (Haiibuki-type) or 1.8 times (Toku 70-type) that in Nipponbare-type CoQ10-enriched rice plants, demonstrating that use of giant embryo rice lines is effective to increase seed CoQ10 content per weight.

Key Words: CoQ10, ubiquinone, giant embryo, rice, antioxidant.

Introduction

Coenzyme Q (CoQ), also called ubiquinone, is present in a wide variety of organisms. CoQ plays an indispensable role as a component of the respiratory chain in mitochondria, and the reduced form of CoQ shows strong anti-oxidation ability. CoQ consists of an isoprenoid side chain and a benzoquinone frame; the length of the side chain varies naturally depending on the organism (Kawamukai 2002). For example, humans produce mainly CoQ10, which has 10 isoprene units, whereas most cereal crops produce mainly CoQ9, which has 9 isoprene units (Kamei et al. 1986). CoQ10 has been commercially sold as a food supplement and a cosmetic. The CoQ biosynthesis pathway has been intensively studied in Escherichia coli and Saccharomyces cerevisiae. The major part of CoQ biosynthesis begins with the transfer of the isoprenoid side chain to the benzoquinone frame, and 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 (Kawamukai 2002). Most of CoQ biosynthetic pathway in plants remains unclear. Because genes homologous to those engaged in yeast CoQ biosynthesis have been found in Arabidopsis (Kawamukai 2002), the pathway in plant is presumed to be similar to that of yeast. In addition to a main CoQ species, many organisms contain minor portion of distinct CoQ species, however, the function of such minor CoQ is unknown.

Using the cultivar Nipponbare, we previously produced CoQ10-enriched rice plants (Nipponbare-type CoQ10-enriched rice plants) by introduction of the gene for decaprenyl diphosphate synthase (DdsA), the enzyme responsible for the synthesis of 10 isoprene units of the CoQ side chain, isolated from Gluconobacter suboxydans (Okada et al. 1998). In Nipponbare-type CoQ10-enriched rice plants, CoQ10 mainly accumulated instead of CoQ9, and seed CoQ10 content of CoQ10-enriched rice plants was up to 10 times that of wild-type rice (Takahashi et al. 2006). However, seed CoQ10 level of those rice plants is still insufficient for practical use, and further 10-times increase in CoQ10 content have been expected.
We previously reported very low CoQ9 content of milled rice (Takahashi et al. 2006), whereas Kamei et al. (1986) reported high CoQ9 content of rice bran and wheat germ. These data suggest that a large portion of the CoQ9 in rice seed is localized in bran and germ. Several rice lines with giant embryos (giant embryo rice line) have been produced (Maeda et al. 2001, Matsushita et al. 2008). Embryos of these rice lines are 2 to 3 times the size of those of normal rice. Our working hypothesis was that seed CoQ10 content could be increased by use of giant embryo rice lines. Here, we confirm preferential accumulation of CoQ in the bran and germ of rice seeds; we also report our production of giant embryo line-type CoQ10-enriched rice plants.

Materials and Methods

Plant materials

We used 2 rice (Oryza sativa L.) lines with giant embryos: the cultivar Haibuki (Matsushita et al. 2008) and the breeding line Chukei-toku 70 (Toku 70). Toku 70 has been produced by crossing Haiminori, a cultivar with a giant embryo (Maeda et al. 2001), and Chugoku 152, a breeding line with small grain (S. Iida, personal communication). Nipponbare, with normal-sized embryos, was used as a reference. We previously reported the production of Nipponbare-type CoQ10-enriched rice plants (Nipponbare-type S14:ddsA plants; N-S14:ddsA plants) (Takahashi et al. 2006). In this study we used T0 plants and their self-pollinated seeds. Plants were grown in a naturally illuminated greenhouse at 27°C.

Measurement of CoQ

In the old method, CoQ was extracted as described in Takahashi et al. (2006) except for omitting of TLC development. 100-mg of dehulled seeds were ground with mortar and pestle into fine powder and pre-treated with 2 ml of 3% H2S and 20 µl of 250 µg/ml CoQ6 (an internal control) at 120°C, 1 atom for 30 min, and then 4 ml of 14% NaOH at 20°C, 1 atom for 40 min. After pre-treatment, CoQ9 was extracted once by 3 ml of n-Hexane:isopropyl (5:1, v/v), and 1.5 ml of 2.5 ml n-Hexane layer was recovered. In the old method without pre-treatment steps, 100-mg of ground seed was suspended with 6 ml of H2O and 20 µl of 250 µg/ml CoQ6. CoQ was extracted once by 3 ml of n-Hexane:isopropyl (5:1, v/v), and 1.5 ml of 2.5 ml n-Hexane layer was recovered. In the new method, CoQ was extracted from 10- to 20-mg of sample as follows; the ground seed was incubated at 37°C for 16–18 h with 1 ml of 0.1 M acetate buffer (pH 5.0) containing 20–100 U of Cellulase (from Aspergillus niger, SIGMA-Aldrich, St. Louis, US). After incubation, 20 µl of 250 µg/ml CoQ6 or 50 µl of 0.22 mM vitamin k (Vk) was added as an internal control; then CoQ was extracted 3 times by 3 ml of n-Hexane. In the all method, the extract was evaporated and then dissolved in 1 ml of methanol:acetoneitrile (1:1, v/v). The extracted CoQ was analyzed by HPLC (LC-20A system, Shimadzu, Kyoto, Japan; column, CAPCELL PAK C8 UG120, Shiseido Fine Chemicals, Tokyo, Japan, 4.6 mm i.d. × 250 mm; solvent system, methanol:acetoneitrile (4:1, v/v); temperature 40°C; flow rate, 1 ml min⁻¹; and detection, 275 nm with a SPD-20A UV/VIS detector, Shimadzu). For the measurement of rice bran and rice germ, dehulled seeds were polished for 40 s with a rice polisher (Pearlest, Kett Electric Laboratory, Tokyo, Japan), and the collected bran and germ were separated by sieving. In the analyses of brown rice and milled rice, there was a noise peak overlapping the CoQ9 peak. The size of the noise peak was corresponded to about 0.2 µg/g of CoQ9, so we set the quantitative detection limit of CoQ9 to 2 µg/g (except for the data shown in Table 1). Based on this criterion, the CoQ9 content of the brown rice samples showing CoQ10-preferential accumulation (including N-S14:ddsA #3-12 and #91-1) and all milled rice samples were regarded as under the detection limit.

<table>
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<tr>
<th>Table 1.</th>
<th>Recovery of an internal control and calculated CoQ content in CoQ extraction by the old, old-pre, or new method</th>
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a Mean values were calculated from 2 experiments and standard deviations are shown.
b The old method without pre-treatment steps.
° The value was corrected with the ratio (0.6) of recovery volume per total volume of the n-hexane layer.
d The recovery rate of internal control was assumed to be 100%.
We performed Agrobacterium-mediated transformation of these rice materials according to the methods of Toki (1997), with the following modifications. At the steps of selection and regeneration, the media were replaced with those described by Fukuoka et al. (2000). Transgenic rice plants were selected on medium containing hygromycin (50 mg l\(^{-1}\)). After selection of 450 Haibuki and 1400 Toku 70 Agrobacterium-infected calli, we obtained 6 regenerated shoots of Haibuki and 5 of Toku 70; among these, 5 of shoots each cultivar showed ddsA gene integration (confirmed by PCR amplification, data not shown). The primary transformants (T\(_0\) plants) were transplanted into soil and grown in a naturally illuminated greenhouse at 27°C. T\(_0\) plants and T\(_1\) seeds were used for the experiments.

Protein blot analysis

The leaves from transgenic rice plants were ground to a fine powder in 8-fold volumes of extraction buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 6% 2-mercaptoethanol, 10% glycerol). The suspended samples were centrifuged at 13,000 \(\times\) g for 5 min at 4°C, and proteins in the supernatants were collected and 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. Antiserum against DdsA protein was prepared as described previously (Takahashi et al. 2006).

Results and Discussion

In our previous study, we used the ‘old’ CoQ extraction method which requires at least 100-mg samples (corresponding to 5 to 10 rice seeds), so we evaluated the seed CoQ content of each N-S14:ddsA plant using T\(_1\) seeds of the ddsA-homozygous T\(_1\) plants (Takahashi et al. 2006). In this study, we developed a new small-scale CoQ extraction method which requires only 10- to 20-mg samples (corresponding to a single rice seed), so that we could measure the CoQ content of a single T\(_1\) seed in which ddsA gene was carried as homo, hemi or null. Comparison of the old and the new CoQ extraction method is shown in Table 1. In CoQ extraction from 100-mg of Nipponbare seed by the old method, the recovery rate of CoQ6 was 97.7 ± 1.9% (Table 1, experiment #5), showing a large (over 30%) variation in the recovery rate of CoQ6 depend on sample in extraction by the old method. When CoQ was extracted from 10-mg of Nipponbare seed by the old method, the recovery rate of CoQ6 was below 10%, and obtained CoQ9 content was much higher than that from 100-mg scale extraction by the same method (data not shown), showing the old method was inappropriate to the small scale CoQ extraction. While, in CoQ extraction from 10-mg of Nipponbare or N-S14:ddsA seed according to the new method, the recovery rate of CoQ6 were about 90 to 100%, and the calculated CoQ9 and CoQ10 contents were similar to those from 100-mg scale extraction by the old method if the recovery rate of CoQ6 was assumed to be nearly 100% (Table 1, experiment #3 vs. #1 in parenthesis, and experiment #6 vs. #5), showing usefulness of the new method for the small scale CoQ extraction. If vitamin k\(_1\) (Vk\(_1\)) was used as an internal control in the small scale CoQ extraction by the new method, nearly 100% of Vk\(_1\) was recovered and the calculated CoQ9 and CoQ10 contents were very similar to that from the same (i.e. new) method using CoQ6 as an internal control (Table 1, experiment #4 vs. #3, and experiment #7 vs. #6). Because of the better separation of the Vk\(_1\) peak than the CoQ6 peak from noise peaks, we used the small scale CoQ extraction by the new method with Vk\(_1\) in the following analysis. In our previous paper, we reported that the seed CoQ10 content of N-S14:ddsA #91-1 was about 18 µg/g, whereas here we found the CoQ10 content of the seeds from the same plant was about 9 µg/g (Table 1, experiment #7). The difference probably arose from the overestimation of the CoQ content reported in the previous paper.

To define CoQ distribution in rice seed, CoQ9 and CoQ10 contents were measured in brown rice (whole grain), bran, and germ of Nipponbara, a cultivar with normal-sized embryos (Fig. 1A). We also tried to measure the CoQ9 and CoQ10 contents of milled rice, but they were under the detection limit. In Nipponbare seed, CoQ9 mainly accumulated; the CoQ9 content of brown rice was 3.6 ± 0.2 µg/g. The CoQ9 contents of bran and germ were 19.0 ± 1.3 µg/g and 57.9 ± 6.4 µg/g, respectively; these values were 5 and 16 times, respectively, that of brown rice (Fig. 1A). In our previous paper we reported the low CoQ9 content of milled rice (Takahashi et al. 2006), and the high CoQ9 content of bran has been reported by Kamei et al. (1986). Here, we show that germ, as well as bran, has a high CoQ9 content. Then we estimated the CoQ9 amount distributed to bran or germ of 1-g brown seeds. Generally, for milled rice commercially sold for food in Japan, the weight ratio of milled rice to brown rice is 90%–91% (Hagiwara 2001); in the sample analyzed here, it was 90%. Since the weight ratio of germ (=embryo)
The weight ratio of bran to brown rice was calculated to be 6%–7%. Taking account of the weight ratio of bran to brown rice, the CoQ9 amount distributed to bran and germ of 1-g brown seeds was estimated to be 1.3 ± 0.1 µg and 1.7 ± 0.2 µg, respectively (Fig. 1B); these values corresponded to 37% and 48%, respectively, of the total CoQ9 amount of 1-g brown rice. Thus, preferential accumulation of CoQ in the bran and germ was confirmed in Nipponbare seed. CoQ distribution was also analyzed in seeds of Nipponbare-type CoQ10-enriched rice plants (N-S14:ddsA plants) (Takahashi et al. 2006). Here we measured CoQ9 and CoQ10 contents in brown rice, bran, and germ of 2 homozygous N-S14:ddsA lines (#3-12 and #91-1) (Fig. 1A). Seeds of both homozygous lines accumulated mainly CoQ10; the CoQ10 contents of brown rice from these homozygous lines were 11.0 ± 0.4 µg/g (#3-12) and 7.7 ± 0.9 µg/g (#91-1). In N-S14:ddsA #3-12, the CoQ10 contents of bran and germ were 63.3 ± 3.6 µg/g and 180.2 ± 8.1 µg/g, respectively (Fig. 1A); these values were 6 and 16 times, respectively, that of brown rice of that line. Similar to N-S14:ddsA #3-12, the CoQ10 content of bran and germ of N-S14:ddsA #91-1 were 7 and 17 times, respectively, that of brown rice of that line (Fig. 1A). These results show that germ and bran of N-S14:ddsA plants have a high CoQ10 content. Then the CoQ10 amounts distributed to bran or germ of 1-g brown seeds of N-S14:ddsA lines were calculated in the same way as for Nipponbare. In N-S14:ddsA #3-12, among 11.0 µg of CoQ10 contained in 1-g brown seed, 4.4 ± 0.2 µg (40%) and 5.4 ± 0.2 µg (49%) of the CoQ10 was estimated to be distributed to bran and germ, respectively (Fig. 1B). In N-S14:ddsA #91-1, among 7.7 µg of CoQ10 contained in 1-g brown seed, 3.9 ± 0.4 µg (51%) and 3.8 ± 0.2 µg (50%) of the CoQ10 was estimated to be distributed to bran and germ, respectively (Fig. 1B). Thus, as in Nipponbare, preferential accumulation of CoQ in the bran and germ was confirmed in N-S14:ddsA seed, although CoQ10 mainly accumulated instead of CoQ9 in N-S14:ddsA.

The brown rice of the 2 giant embryo lines (Haibuki and Toku 70) and Nipponbare are shown in Fig. 2A. The weight ratio of germ to brown rice in Haibuki is 9.4% (Matsushita et al. 2008); which is about 3 times that in Nipponbare. The embryo of Toku 70 is as large as that of Haibuki (Fig. 2A); moreover, the weight of Toku 70 brown rice grains is 55%–57% of those of Nipponbare or Haibuki brown rice (Fig. 2B). The weight ratio of germ to brown rice of Toku 70 is presumed to be higher than that of Haibuki. Seed CoQ9 contents of Haibuki and Toku 70 were 1.9 times or 2.8 times, respectively, that of Nipponbare, demonstrating an increased seed CoQ9 content of giant embryo lines (Fig. 2C). The high seed CoQ9 content in giant embryo lines encouraged us to produce giant embryo line-type CoQ10-enriched rice plants.

The chimeric gene construct S14:ddsA (Takahashi et al. 2006) was introduced into Haibuki and Toku 70 using Agrobacterium-mediated transformation. We obtained 5 Haiibuki-type S14:ddsA (Hi-S14:ddsA) primary transformed (T₀) plants and 5 Toku 70-type S14:ddsA (T₇₀-S14:ddsA) T₀ plants. Transformation efficiency (transformed shoot carrying ddsA/selected callus) was 1.3% for Haibuki and 0.4% for Toku 70. In S14:ddsA construct, a constitutive CaMV35S promoter was used to drive a ddsA gene, and in N-S14:ddsA plant, DdsA accumulated in all parts of plant analyzed including leaf, root, stem, and seed (for accumulation in leaf, Takahashi et al. 2006). Therefore we performed protein blot analysis to select 2 Hi-S14:ddsA and 2 T₇₀-S14:ddsA T₀ plants accumulating the DdsA protein in their leaves (Fig. 3A). In N-S14:ddsA plants analyzed, 1 to 3 copy of ddsA gene were integrated, and there was no obvious correlation among the integrated ddsA copy number, the amount of DdsA protein in leaves, nor the seed CoQ10 content (data not shown). So we next analyzed the
CoQ10 content of the self-pollinated seeds (T1 seeds) in all Hi-S14:ddsA and T70-S14:ddsA plants accumulating DdsA protein.

We measured CoQ10 content of T1 seeds in 4 N-S14:ddsA, 2 Hi-S14:ddsA, and 2 T70-S14:ddsA plants (Fig. 3B). For N-S14:ddsA plant, the data of 4 out of 24 plants showing the highest CoQ10 content in the preliminary experiment are shown. Since T1 seeds could carry ddsA gene as homo, hemi or null, the presence of ddsA gene was checked by PCR amplification for each single T1 seed (data not shown), and the seeds carrying ddsA gene were subjected to measurement of CoQ. All ddsA positive T1 seeds showed predominant CoQ10 accumulation. The average seed CoQ10 content of each transgenic plant was calculated. The CoQ10 content of ddsA-homozygous seeds might be higher than that of ddsA-hemizygous seeds, however, the average CoQ10 content of T2 seeds of 2 N-S14:ddsA homozygous lines (#3-12 and #91-1; both lines carrying one copy of ddsA gene) were very similar (118% or 104%, respectively) to that of the T1 seeds of their lines (data not shown), suggesting small difference in CoQ10 content between ddsA-homozygous and -hemizygous seeds. Seed CoQ10 content varied among plants; Only 5 out of 24 plants showed relatively high seed CoQ10 content in the preliminary experiment (estimated to be >10 µg/g, data not shown). If we can produce more giant embryo line-type CoQ10-enriched rice plants, then we expect that we will obtain plants with even higher CoQ10 accumulation.

In Japan, when CoQ10 is used as a drug for heart disease, 30 mg/day intake is prescribed. The optimal intake of CoQ10 for use as a food supplement remains to be elucidated. In principle, a CoQ10 intake of less than 30 mg/day is recommended, and the safety of CoQ10 intake exceeding that level is under consideration by the Food Safety Commission in Japan. Therefore, we set a target value of CoQ10 intake from CoQ10-enriched rice seed of 20–30 mg/day. Since the average dietary intake of rice (seed) of Japanese people is 61 kg/year (=167 g/day in 2006; Ministry of Agriculture, Forestry and Fisheries 2007), we calculated the target seed CoQ10 content to be 120–180 µg/g. The maximum seed CoQ10 content of newly produced giant embryo line-type CoQ10-enriched rice plants was only 12%–18% of the desirable level. However, the same 167-g portion of giant embryo line-type CoQ10-enriched rice seeds (T70-S14:ddsA #1) would provide 3.7 mg of CoQ10, which corresponds to 82% of the 4.5 mg/day dietary CoQ10 intake in the Japanese population (Kubo et al. 2008). From this point of view, it can be thought that giant embryo line-type CoQ10-

Fig. 2. (A) Pictures of brown rice (dehulled rice) of Nipponbare and the 2 giant embryo lines used in this study. (B) Weight of seed (brown rice). (C) CoQ9 and CoQ10 contents of seed. CoQ was extracted from a single seed. Mean values were calculated from 4 independent measurements, and standard deviations are shown.

Fig. 3. Analysis of giant embryo line-type S14:ddsA rice plants. (A) Protein blot analysis of transgenic plants. About 10 µg of soluble proteins from leaves of T0 plants of N-S14:ddsA #91 (as a positive control), Hi-S14:ddsA, and T70-S14:ddsA, and corresponding wild-type plants (Nipponbare, Haiibuki, and Toku 70) were analyzed using an anti-DdsA antibody. (B) CoQ10 content of T1 seed of Nipponbare- and giant embryo line-type S14:ddsA plants. Four or 5 seeds from each plant were analyzed, and each closed circle represents the CoQ10 content of a single seed. Numbers and types of transgenic plants are shown below the graph.
enriched rice seed contains a significant level of CoQ10. We also think that the seed CoQ10 content of giant embryo rice lines may be further increased by other strategies, such as modification of the promoter driving the \( ddsA \) gene.

In this study, we produced giant embryo line-type CoQ10-enriched rice plants in order to further increase seed CoQ10 content. In giant embryo line-type CoQ10-enriched rice plants, seed CoQ10 content was up to 1.4 (Haiibuki-type) or 1.8 times (Toku 70-type) that of Nipponbare-type CoQ10-enriched rice plants, demonstrating that use of giant embryo rice lines is an effective way to increase seed CoQ10 content. Of the 2 giant embryo rice lines used in this study, Haiibuki is already commercially used in Japan as partly milled rice but retaining its giant embryo. Our results showed that Haiibuki is a suitable host cultivar for developing a practical CoQ10-enriched rice for food.

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Literature Cited


