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Investigation of tocotrienol biosynthesis in rice (*Oryza sativa* L.)

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ABSTRACT

Rice tocotrienol (T3) has gained attention due to its physiological activities (e.g., antiangiogenesis). However, the biosynthetic pathway for T3 production in rice grain has not been well studied. We hypothesized that T3 biosynthesis enzymes and/or precursors play an important role in T3 production in whole grain. This proposal was evaluated in rice (*Oryza sativa* L.) by PCR and HPLC techniques. Grain tocopherol as well as flag leaf vitamin E levels were also investigated for comparison. For rice samples 14 days after flowering, grain was abundant in T3, but not in flag leaf. Expression of a gene encoding homogentisate geranylgeranyltransferase (HGGT, which has long been believed to be important for T3 production) differed significantly between grain and flag leaf. We then investigated rice samples during the grain maturation period, and found that grain T3 and HGGT levels increased in the early stage and then reached a plateau. T3 precursors such as homogentisate and geranylgeranyl pyrophosphate decreased during maturation. No increase in grain T3 from the middle to late stages of maturation and a decrease in T3 precursors during maturation suggest that HGGT would be an essential, but not limiting factor for T3 biosynthesis, and T3 precursors could regulate the T3 level in grain. The results of this study would be useful for nutraceutical purposes (e.g., development of T3-overproducing rice for the prevention of angiogenic disorders).

1. Introduction

Recently, some studies showed that rice (*Oryza sativa* L.) varieties have differences in composition of functional components (Chatha, Anwar, Manzoor, & Bajwa, 2006; Storck, Silva, & Fagundes, 2005). For instance, Pakistani Basmati cultivars are reported to be good sources of valuable compounds such as tocopherol (Toc), phytosterols, and γ-oryzanol (Zubair, Anwar, Ashraf, & Uddin, 2012). From the results of these studies, increasing attention has been given to the rice rich in nutraceutical compounds, which may increase the nutritional and commercial value of rice.

Among above bioactive compounds of rice, tocotrienol (T3), an unsaturated form of vitamin E with three double bonds in its isoprenoid side chain (Fig. 1), is present in especially high concentration in rice grain (Iqbal, Bhanger, & Anwar, 2005; Rohrer & Siebenmorgen, 2004). T3 has recently received considerable attention for its various biological properties (Theriault, Chao, Wang, Gapor, & Adeli, 1999). T3 shows better antioxidative (Ghosh et al., 2009), antihypercholesterolemic (Qureshi, Sami, Salser, & Khan, 2002), anticancer (Goh, Hew, Norhanom, & Yadav, 1994; Wada et al., 2005), and neuroprotective activity (Khanna, et al., 2005) than Toc. In addition, we have found that T3 suppresses pathological angiogenesis (Miyazawa et al., 2009; Nakagawa, et al., 2007; Shibata, Nakagawa, Sookwong, Tsuzuki, Oikawa, & Miyazawa, 2008a; Shibata et al., 2008b), which is an important stage in the progression of disorders including diabetic retinopathy, rheumatoid arthritis, and cancers. These findings suggest that T3 has a wide range of physiological functions, and developing a rice variety that could biosynthesize high concentrations of T3 would be useful for nutraceutical applications. Thus, we have determined the T3 content in 250 varieties of rice samples (Sookwong, Nakagawa, Murata, Kojima, & Miyazawa, 2007) and have crossed T3 rich varieties with Koshihikari (the most popular rice variety in Japan), resulting in more self-pollinated progenies with significantly higher T3 levels (Sookwong et al., 2009). However, the biosynthetic pathway for T3 in rice grain has not been well studied.

In general, plant biosynthesis of vitamin E is believed to occur as shown in Fig. 1. The initial step of T3 biosynthesis is condensation of geranylgeranyl pyrophosphate (GGPP) with homogentisate

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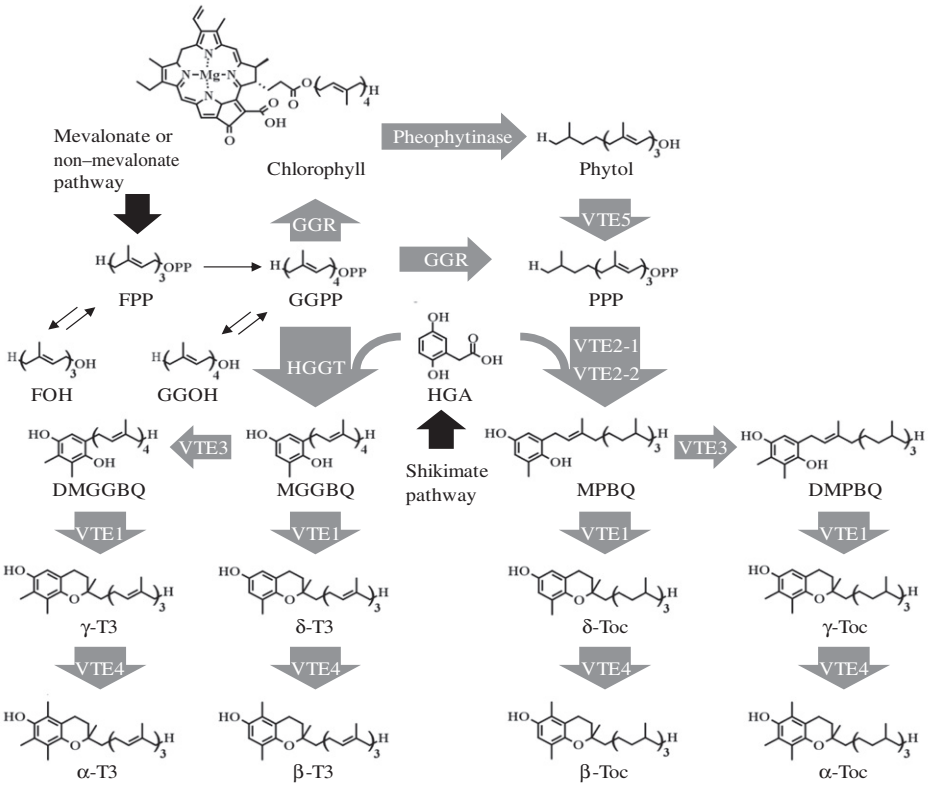

Fig. 1. General biosynthetic pathway for vitamin E in plants. Abbreviations refer to T3, tocotrienol; Toc, tocopherol; FOH, farnesol; GGOH, geranylgeraniol; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; PPP, phytyl pyrophosphate; HGA, homogentisic acid; MGGBQ, 2-methyl-6-geranylgeranylbenzoquinol; DMGGBQ, 2,3-dimethyl-6-geranylgeranylbenzoquinol; MPBQ, 2-methyl-6-phytylbenzoquinol; DMPBQ, 2,3-dimethyl-6-phytylbenzoquinol. Enzymes including HGGT, GGR, VTE2-1,2, T3/Toc methyltransferase (VTE3 and VTE4), T3/Toc cyclase (VTE1), pheophytinase, and phytol kinase (VTE5) are believed to be involved in vitamin E biosynthesis.

(HGA), catalyzed by homogentisate geranylgeranyltransferase (HGGT) (Cahoon, Hall, Ripp, Ganzke, Hitz, & Coughlan, 2003). This reaction is followed by methylation or cyclization of the chroman ring to yield T3, which accumulates in plant cells (Bergmuller, Porfirova, & Dormann, 2003; Kumar et al., 2005; Shintani, Cheng, & DellaPenna, 2002). When GGPP is reduced to phytyl pyrophosphate (PPP) by geranylgeranyl reductase (GGR), PPP (instead of GGPP) combines with HGA by homogentisate phytyltransferase (VTE2-1, 2-2) (Collakova & DellaPenna, 2001; Keller, Bouvier, d'Harlingue, & Camara, 1998; Mene-Saffrane & DellaPenna, 2010a; Sadre, Gruber, & Frentzen, 2006; Savidge et al., 2002; Schelbert et al., 2009; Schledz, Seidler, Beyer, & Neuhaus, 2001; Shpilyov, Zinchenko, Shestakov, Grimm, & Lokstein, 2005; Valentin & Qi, 2005b; Valentin et al., 2005a) to be finally converted into Toc (Bergmuller et al., 2003; Kumar et al., 2005; Shintani et al., 2002). Therefore, we speculated that T3 synthesis enzymes (e.g., HGGT) and/or precursors (e.g., GGPP) play an important role in the pathway utilized by rice to produce T3 in grain. This possibility has not been previously addressed.

In this study, we investigated the T3 and Toc content and gene expression encoding their synthetic enzymes in whole grain of rice (*O. sativa* L.) during maturation. Since grain (bran) is reproductive tissue, vegetative tissue (flag leaf, the uppermost leaf on the stem) was also evaluated as a comparison. Furthermore, T3 precursors in grains and flag leaves were analyzed by LC-MS/MS. From the obtained results, it was found that HGGT is an essential, but not limiting factor for T3 biosynthesis, and T3 precursors (e.g., HGA, GGPP) regulate the T3 level in rice grain. Such knowledge (understanding of T3 biosynthesis) would be highly useful for the development of T3-overproducing rice varieties for nutraceutical purposes.

2. Materials and methods

2.1. Materials

Japonica rice plants (*O. sativa* L. cv. Koshihikari) were grown in a greenhouse under a 26/20 °C (day/night) temperature cycle. Twenty seeds were planted in each pot (18 cm diameter × 20 cm height). All tillers other than the main culm were cut to normalize the plant growth rate. Whole grains and flag leaves were harvested during maturation (0–50 days after flowering, DAF). The rice samples (grains and flag leaves) were frozen and powdered, and each powdered sample was stored at −80 °C until analyzed.

Oligonucleotide primers for PCR were purchased from Invitrogen (Carlsbad, CA, USA), and the sequences are described in Supplementary Table 1. PPP was purchased from Funakoshi (Tokyo, Japan), and other T3 precursors were obtained from Sigma–Aldrich (St. Louis, MO, USA). Four isomers of T3 and Toc were a gift from Eisai (Tokyo, Japan). Other chemicals were obtained from Wako (Osaka, Japan). All reagents used were of analytical grade.

2.2. Vitamin E determination

T3 and Toc concentrations in rice grains and flag leaves were determined by HPLC with fluorescence detection (FL) (Sookwong et al., 2007). Rice samples (grains and flag leaves, 50 mg of powder) were homogenized in 3 mL of 2-propanol containing 0.025% (w/v) butylated hydroxytoluene, and the mixture was sonicated for 10 min. Following mixing and centrifugation at 3200 rpm for 10 min, the supernatant was collected and filtered through a 0.45 μm PTFE filter (Millipore, Bedford, MA, USA). 200 μL of the