Localization of Farnesyl Diphosphate Synthase in Chloroplasts

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The subcellular localization of plant farnesyl diphosphate synthase (FPPS) was examined. Immunocytochemical staining using anti-FPPS1 antibody followed by electron microscopy showed that FPPS1 was localized to chloroplasts of rice mesophyll cells. Subcellular fractions from wheat leaves were examined by immunoblot analysis. FPPS was detected in the chloroplast fraction in wheat, and was protected from proteolysis following trypsin treatment of chloroplasts. FPPS was also detected in the chloroplast fraction of a dicot plant, tobacco.

Key words: Chloroplast — Farnesyl diphosphate synthase (EC 2.5.1.1/EC 2.5.1.10) — Isoprenoid — Rice (Oriza sativa L.) — Subcellular localization.

Plant isoprenoids comprise a large group of compounds which include secondary metabolites, such as monoterpenes, sesquiterpenes and diterpenes, sterols as components of membranes, plant hormones such as abscisic and gibberellic acids, pigments for photosynthesis such as carotenoids and phytols (side chains of chlorophyll), and quinones as electron carriers (Chappell 1995). IPP, the common C5 precursor into endoproducts revealed that the GAP/pyruvate pathway was involved in the synthesis of carotenoids and phytols in the plastid compartment, while the MVA pathway was involved in the synthesis of sterols in the cytoplasm/ER compartment (Arigoni et al. 1997, Lichtenthaler et al. 1997). This data is consistent with the observation that only sterols were overproduced when HMGR, which catalyzes the synthesis of MVA, was overexpressed in transformed plants (Schaller et al. 1995). The C20 compound, GGPP, is synthesized by the condensation reaction of DMAPP with three units of IPP (McGarvey and Croteau 1995). GGPP synthesis has been shown to be present in the plastids of pepper fruits (Kunz et al. 1992, Römer et al. 1993, Hugueney et al. 1996) and pea leaves (Bonk et al. 1997). The C15 compound, FPP, is synthesized by the condensation reaction of DMAPP with two units of IPP (McGarvey and Croteau 1995). FPPS (EC 2.5.1.1/EC 2.5.1.10) catalyzing the synthesis of FPP has been shown to be present in the cytoplasm of Vitis vinifera suspension cells (Feron et al. 1990) and pepper fruits (Hugueney et al. 1996).

We investigated the subcellular localization of rice FPPS (referred to as FPPS1, hereafter) to determine if it is located in the cytoplasm, as has been demonstrated in other species. We showed that FPPS1 was present in the chloroplasts of rice plants, as well as in the chloroplasts of wheat and tobacco plants.

Green leaf-specific expression of FPPS1 gene—We previously demonstrated that transcripts of FPPS1 were detected specifically in the green leaves of rice (Sanmiya et al. 1997). This organ specificity was observed only in rice plants. In other plant species, the expression of the FPPS gene was observed in the fruits of pepper (Hugueney et al. 1996), the roots of white lupin (Attucci et al. 1995), the laticifers of Hevea brasiliensis (Adiwillaga and Kush 1996), and the endosperm of maize (Li and Larkins 1996). It was also observed prominently in the flowers, stems and roots of A. thaliana (Cunillera et al. 1996).

To further investigate FPPS1 expression in rice plants, the level of FPPS1 protein in the various organs was examined by immunoblot analysis using an anti-FPPS1 antibody. SDS-PAGE was carried out by the method of Laemmli (1970) in a 13% polyacrylamide gel. Rice (Oriza sativa L. cv. Nipponbare) seeds were germinated in water at 30°C for two days and grown in synthetic soil/vermiculite (1 : 1). Antibody was raised in a rabbit using the
of each protein extract were examined by immunoblot analysis.

Fig. 1 Green leaf-specific expression of FPPS1 in rice plants. Total protein was prepared from tertiary leaf blades of 12-d-old seedlings grown in complete darkness (lane 1) or in a 14 h light/10 h dark regime (lane 2), fully expanded mature leaves of 40-d-old plants grown in a greenhouse (lane 3) and roots of hydrocultured 12-d-old seedlings (lane 4). Plant materials were homogenized in two volumes of 2x sample buffer for SDS-PAGE, and 20 µg of each protein extract were examined by immunoblot analysis.

The leaf-specific expression of the FPPS1 gene seems to be a characteristic of rice plants, since the level of FPPS mRNA was low in A. thaliana leaves (Cunillera et al. 1996). Therefore, we examined the subcellular localization of FPPS1 in rice plants by immunocytochemical staining followed by electron microscopy as described previously (Ueno 1996a, b). Segments of rice leaves were fixed in a fixative solution containing 3% (v/v) paraformaldehyde, 0.2% (v/v) glutaraldehyde, and 50 mM sodium phosphate pH 6.8. FPPS1 antibody was used as the first antibody. Labeling by gold particles indicating FPPS1 protein localization was specifically detected in the chloroplasts, especially in the thylakoids. However, distribution of gold particles in the cytoplasm and in other cellular compartments, such as mitochondria, peroxisomes, nuclei and vacuoles was barely detectable (Fig. 2B, C, D). When leaf sections were incubated in pre-immune serum, they showed evidence of only non-specific and negligible labeling with colloidal gold (Fig. 2A). These results unambiguously demonstrate the localization of FPPS1 in the chloroplasts of rice plants.

Localization of FPPS in chloroplasts of wheat plants—Because the chloroplast localization of FPPS1 was indicated immunocytochemically, we also investigated the subcellular localization biochemically. In preliminary experiments, we attempted cellular fractionation of rice leaf cells, but failed. To avoid this technical difficulty, we chose to fractionate wheat leaf cells from which protoplasts could be prepared (Edwards et al. 1978). A protein band of 36 kDa was detected in the total protein extracts from wheat leaves by immunoblot analysis, suggesting the cross-reactivity of anti-rice FPPS1 antibody with wheat FPPS. Since the FPPS1 antibody appeared to detect wheat FPPS, we investigated the subcellular localization of FPPS in wheat plants. Wheat (Triticum aestivum L. em. Thell. cv. Chihoku-komugi) seeds were provided by Genetic Resources Bank of Kihara Institute for Biological Research, Yokohama City University, stock number KT20-124. Wheat seeds were germinated and grown in vermiculite in a greenhouse. The secondary leaves were collected from 12-d-old seedlings and approximately 20 g of tissue was used for the protoplast preparation, according to the method of Edwards et al. (1978). The protoplasts were disrupted by passage through a sheet of 20 µm nylon mesh to avoid the disruption of organelles. The completeness of the disruption was checked by microscopy. The cell homogenate was subjected to differential centrifugations followed by immunoblot analysis. The pellets obtained by centrifugation at 2,000 x g for 10 min, 8,000 x g for 20 min and 100,000 x g for 40 min were used as chloroplast, mitochondrion and microsome fractions, respectively. The supernatant of the centrifugation at 100,000 x g was used as the cytoplasmic fraction. Rubisco antiserum was used to detect LSU as a chloroplastic marker. FPPS was detected in the chloroplast fraction, but not in fractions of mito-

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Fig. 2 Chloroplast localization of rice FPPS1 revealed by immunocytochemistry. Fully expanded mature leaves of 40-d-old rice plants grown in a green house were examined by immunocytochemical staining followed by electron microscopy. FPPS1 protein was detected by using an anti-FPPS1 antibody as first antibody (panels B, C and D). For the negative control, pre-immune serum was used instead of the antibody (panel A). ct, chloroplast; cy, cytoplasm; isp, intercellular space; mt, mitochondrion; n, nucleus; p, peroxisome; v, vacuole. Bar represents 1 μm.
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The presence of FPPS was observed after trypsin treatment of chloroplasts (Fig. 3B), indicating that the detection of FPPS is most likely not due to the adsorption on the surface of chloroplasts of protein derived from other cellular compartments. The observed tolerance to proteolysis might be due to the chloroplast structure, since the treatment with trypsin and Triton X-100 resulted in the cleavage of FPPS into 25 kDa and 16 kDa bands (lane 3 of Fig. 3B). This data suggests that, as in rice, FPPS in wheat is also located in chloroplasts.

Localization of FPPS in chloroplasts of tobacco plants—Because chloroplast localization of FPPS was demonstrated in rice and wheat plants, we examined whether chloroplastic FPPS is ubiquitous in higher plants. Immunoblot analysis detected a protein band of 36 kDa in the total protein extracts from tobacco leaves, suggesting the cross-reactivity of anti-rice FPPS1 antibody with tobacco FPPS. Since the protein band appeared to correspond to tobacco FPPS, we investigated its subcellular localization in the leaves of tobacco plants.

Fully expanded leaves of tobacco (Nicotiana tabacum

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**Fig. 3** Chloroplast localization of FPPS in wheat and tobacco leaves. A: Localization of FPPS in the chloroplast fraction of wheat leaves. Protoplasts were prepared from wheat leaves and cell lysates were fractionated. Localization of FPPS in the fraction of chloroplast (lane 1), mitochondrion (lane 2), microsome (lane 3) and cytoplasm (lane 4) were examined by immunoblot analysis using FPPS1 antibody (FPPS, top panel) and Rubisco antiserum (LSU, bottom panel). Ten μg of protein from each fraction were used. B: Protection of FPPS from trypsin digestion. Purified wheat chloroplasts (lane 1) were treated with trypsin (lane 2), trypsin and Triton X-100 (lane 3) or Triton X-100 alone (lane 4). Total protein from leaves of 12-d-old wheat seedlings was co-electrophoresed as a control (lane 5). Immunoblot analysis was carried out as shown in panel A. C: Localization of FPPS in the chloroplast fraction of tobacco leaves. Protoplasts from tobacco leaves were fractionated. Localization of FPPS in the fraction of chloroplast (lane 1), mitochondrion (lane 2), microsome (lane 3) and cytoplasm (lane 4) were examined by immunoblot analysis, carried out as shown in panel A except that 20 μg of protein extract was used.
L. cv. SR1) were injured by carborundum (6,000 mesh), then protoplasts were obtained according to the method of Takebe et al. (1968). Protoplasts were disrupted by passage through a sheet of 20 µm nylon mesh, the cell homogenate was subjected to cell fractionation, and each fraction was examined by immunoblot analysis using the FPPS1 antibody. FPPS was detected in the chloroplast fraction, but not in fractions of mitochondrion, microsome, or cytoplasm (Fig. 3C, top panel). LSU was detected predominantly in the chloroplast fraction (Fig. 3C, bottom panel). These results show the presence of FPPS in chloroplasts of tobacco plants. Thus, the chloroplast localization of FPPS has been demonstrated in two monocot plants, rice and wheat, and a dicot plant, tobacco. Localization of FPPS in cytoplasm and mitochondria has been shown in A. thaliana (Cunillera et al. 1997), however, it is unclear whether FPPS is localized in chloroplasts. The presence of chloroplastic FPPS in both monocot and dicot plants suggests the presence of chloroplastic FPPS in A. thaliana.

FPPS and GGPPS have been thought to be localized in cytoplasm and plastids, respectively (McGarvey and Croteau 1995). However, FPPS has been shown to be located in the chloroplasts of rice (Fig. 2), wheat and tobacco (Fig. 3), and in the mitochondria of A. thaliana (Cunillera et al. 1997). In addition, it was also shown that GGPPS was localized in the cytoplasm and the mitochondria of A. thaliana (Zhu et al. 1997), and in pea chloroplasts (Bonk et al. 1997). These findings suggest that FPP and GGPP, intermediates of isoprenoid synthesis, are produced in multiple compartments, including the cytoplasm, chloroplasts and mitochondria.

**Prediction of cytoplasmic FPPS**—We have shown that rice FPPS1 is localized to chloroplasts. Rice plants should have another FPPS regulating the synthesis of sterols in the cytoplasm. We searched for such an FPPS homologue in the rice EST database to demonstrate the presence of a cytoplasmic FPPS. A rice cDNA clone (clone ID C52647 from the Rice Genome Research Program, accession no. AB021979) was found as a putative FPPS homologue, and its nucleotide sequence was determined. Although the cDNA did not cover the entire FPPS ORF, it did contain 186 amino acids from the carboxyl terminus (data not shown). The putative amino acid sequence was highly homologous to that of rice FPPS1 (84%), moreover, it contained a putative catalytic region, domain II of FPPS, conserved in plants, yeast and rat (Anderson et al. 1989, Spear et al. 1992, Attucci et al. 1995, Cunillera et al. 1996, Hugueney et al. 1996). The cDNA appears to encode an FPPS homologue, and the rice genome appears to possess an FPPS gene family, as does the A. thaliana genome. The putative isozyme encoded by the clone was designated FPPS2.

We performed a genomic DNA blot analysis using the cDNAs for FPPS1 and FPPS2 as probes for the corresponding genes as described previously (Sanmiya et al. 1997). Total DNA from rice leaves (10 µg) was separately digested with BamHI or XbaI, and then subjected to DNA blot analysis. Both genes were suggested to be single copy, since each probe detected only a single DNA fragment in the blots of BamHI or XbaI digests (Fig. 4).

The expression of the FPPS2 gene was examined by RNA blot analysis to investigate the role of FPPS2 in rice plants as described previously (Sanmiya et al. 1997). The FPPS1 and FPPS2 cDNA probes were used to detect the corresponding transcripts, as they did not cross-react with each other (Fig. 4). Light was obtained from fluorescent lamps (0.2 mmol m⁻² s⁻¹, FLR40S-EX-N/M/36; Toshiba, Tokyo). While FPPS1 transcripts were detected primarily in green leaves under illumination, as reported previously (Sanmiya et al. 1997), FPPS2 transcripts were detected predominantly in the roots, but not in the leaves (Fig. 5). The presence of a cytoplasmic FPPS was anticipated to be required for the synthesis of sterols in rice plants, as the localization of FPPS1 is restricted to chloroplasts. FPPS2 is unlikely to be present in chloroplasts, as FPPS2 transcripts were detected predominantly in roots lacking chloroplasts (Fig. 5).

FPPS1 protein was detected in expanded leaves (Fig. 1) and shown to be located in chloroplasts of rice plants (Fig. 2). These results were obtained using an FPPS1 antibody. The antibody might recognize FPPS2, but this possibility will be excluded. The abundance of FPPS2 transcripts in roots suggests that FPPS2 protein accumulates in the roots of rice plants. However, FPPS1 anti-
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Fig. 5 Comparison of tissue-specific expression between FPPS1 and FPPS2 genes. Total rice RNA was prepared from leaves of etiolated (lane 1) and light-grown seedlings (lane 2–4), fully expanded mature leaves of 40-d-old plants grown in a green house (lane 5) and roots from hydro-cultured 12-d-old seedlings (lane 6). To examine the effects of light on the expression of the FPPS2 gene, etiolated and light-grown seedlings were treated by the following light regimes: lane 1, etiolated seedlings were grown in complete darkness for 12 days; lane 2, seedlings were grown in continuous light; lane 3, the light-grown seedlings were dark-adapted for 24 h; lane 4, the dark-adapted seedlings were re-exposed to light for 3 h. An aliquot of RNA (5 μg) from each tissue was examined by RNA blot analysis. The cDNA for FPPS2 (panel A) and FPPS1 (panel B) were used as hybridization probes. The RNA levels were examined with a rDNA probe (Takaiwa et al. 1984) in Panel C.

body did not detect any protein in the extract from roots (Fig. 1). Therefore, the FPPS1 antibody seems to specifically react with FPPS1, but not FPPS2.

Possible import of FPPS1 into chloroplasts—Although, there is no primary amino acid sequence consensus among the transit peptides of chloroplastic proteins, they share three features: (1) an N-terminal domain that is deficient in charged residues (R, K, D, E), and P, G residues; (2) a middle domain that is enriched in S, T, K, and R; and (3) a C-terminal portion that is predicted to form an amphipilic β-strand (von Heijne et al. 1989). Although the conserved N-terminal sequence was found in the rice FPPS1, sequences homologous to the middle domain and the C-terminal portion could not be found. However, FPPS1 has clearly been shown to be located in chloroplasts. This suggests that the FPPS1 precursor protein may be imported into chloroplasts by virtue of an unknown signal, whose mechanism should be clarified in the future.

Four possibilities may be postulated on the role of chloroplastic FPPS1 in isoprenoid synthesis. First, the pathway equivalent to that of the cytoplasm/ER compartment might also be present in chloroplasts, and FPPS1 might regulate the synthesis of sterols in chloroplasts. Secondly, FPP, the product of FPPS1 in chloroplasts, might be transported to the cytoplasm, then utilized for the synthesis of cytoplasmic isoprenoids. Thirdly, FPPS1 might supply FPP for the synthesis of phytools and carotenoids. The condensation reaction of FPP and IPP to produce GGPP was shown to be catalyzed by purified caster bean GGPPS in vitro (Dudley et al. 1986), and the enzyme was shown to be localized to chloroplasts of pea leaves (Bonk et al. 1997). GGPP is utilized for the synthesis of phytools and carotenoids (Kleinig 1989). Production of phytools might be a prerequisite for the synthesis of chlorophyll side chains in green leaves. Fourthly, FPPS1 might be utilized to supply FPP for protein farnesylation in chloroplasts. FPP is necessary in chloroplasts, as the activity of farnesyl protein transferase was detected in chloroplasts (Parmryd et al. 1997).

We wish to thank Dr. T. Sasaki of the Rice Genome Research Program for providing the rice EST clone, C52647. We are grateful to Dr. T. Koshiha, Dr. N. Aoki and Dr. N. Inagaki for their technical advice. This work was supported by the Special Coordination Fund for Promoting Science and Technology, Enhancement of Center-of-Excellence, to National Institute of Agrobiological Resources from the Science and Technology Agency of Japan, and in part by a fund from the Ministry of Agriculture, Forestry and Fisheries of Japan.

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(Received December 4, 1998; Accepted January 16, 1999)