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Biosynthesis of Chloroplastidic Isoprenoids in Liverworts: Uptake of Farnesyl Diphosphate by the Chloroplasts of *Heteroscyphus planus* and *Ptychanthus striatus*

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ABSTRACT. *A research was carried out to study the uptake of isoprenyl diphosphates by two liverwort species, Heteroscyphus planus and Ptychanthus striatus. Previous studies have shown that the farnesyl diphosphate (FPP) derived portion of the phytyl side-chain of chlorophyll a is labeled preferentially when exogenous ^{13}C-labeled mevalonate (MVA) was fed to the cultured cells of the above two liverwort species. The terminal isopentenyl diphosphate (IPP) portion is labeled with less intensity. The objective of this research was to elucidate the mechanism of this preferential labeling of the phytyl side-chain. It was shown that FPP, which is synthesized in the cytoplasm via MVA pathway, was preferentially taken into the chloroplasts and condensed to GGPP using IPP synthesized within the chloroplasts. The results of this study suggest a possible mechanism for the non-equivalent labeling observed in the biosynthesis of phytyl side-chain of the chlorophyll a in suspension cultured cells of liverworts. This research further demonstrates the exchange of metabolites between different cell compartments of liverworts.*

INTRODUCTION

The isoprenoids make up the largest family of natural products comprising more than 22,000 known compounds (Connoly and Hill, 1991). Isoprenoids or isoprenoid-derived compounds play a vital role in all living organisms. They include various primary metabolites, such as, sterols, carotenoids, growth regulators and quinones. Polymers (rubber, chicle), pharmaceuticals (artemisinin, taxol) and agrochemicals (pyrethrins, azadirachtin) are some of the industrially important terpenoids. However, functions of the vast majority of the known isoprenoids are still largely undiscovered.

The basic building block of all isoprenoid natural products is isopentenyl diphosphate (IPP). In plant cells, two pathways exist for the IPP biosynthesis in different cell compartments. The mevalonate (MVA) pathway functions mainly functioning in the cytoplasm for the biosynthesis of sterols (Cvejic and Rohmer, 2000). The MVA-independent deoxyxylulose (DXP) pathway (Rohmer *et al*., 1993) is localized to the plastids for the formation of hemiterpenoids, monoterpenoids, phytol (Barlow *et al*., 2001), carotenoids (Schwender *et al*., 1996) and other diterpenoids (Eisenreich, *et al*., 1999). However, many instances of exchange of metabolites between the two compartments have been reported. Labels from cytosolic isopentenyl diphosphate were

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shown to be incorporated into various plasitidic isoprenoids (Kreuz and Kleinig, 1981; Kreuz and Kleinig, 1984; Soler *et al*., 1993).

Studies on the biosynthesis of phytyl side-chain of cholorophyll *a* in the liverworts have shown that the phytyl side-chain of the chlorophyll *a* of the liverworts, *Heteroscyphus planus*, *Locopholea heterophylla* and *Ptychanthus striatus*, is labeled non-equivalently when ^{13}C labeled MVA was fed to the cultured cells (Nabeta *et al*., 1995(a); Nabeta *et al*., 1995(b); Nabeta *et al*., 1997; Karunagoda *et al*., 2001). The FPP-derived portion is labeled preferentially with the exogenous MVA, while the terminal IPP portion is labeled with less intensity (Fig. 1).

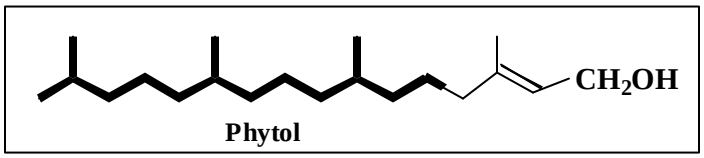

Fig. 1. Non-equivalent labeling of the phytyl side-chain of chlorophyll a in *H. planus* (Nabeta *et al*., 1997) and *P. striatus* (Karunagoda *et al*., 2001) by exogenous MVA (The ^{13}C-labeled FPP-derived portion is drawn in heavy lines).

This labeling pattern suggests that the FPP-derived portion of the phytyl side-chain of chlorophyll *a* is derived from exogenously supplied MVA. It was suggested that liverwort chloroplasts import either IPP or FPP into the chloroplasts and use them for biosynthesis of the phytyl side-chain. The objective was to elucidate the mechanism of the non-equivalent labeling of the phytyl side-chain in liverworts.

MATERIALS AND METHODS

General experimental procedures

[4-^{14}C]-IPP (57.5 mCi/mmol) was purchased from NEN^{TM} Life Science Products, Inc, Boston and [1-^{3}H]-GGPP (14Ci/mmol) from Amersham Pharmacia Biotech UK Ltd. [1,2-^{14}C]-FPP (55 mCi/mmol) and [1-^{3}H]-geranyl diphosphate (GPP) (20 Ci/mmol) were purchased from American Radiolabeled Chemicals Inc. USA. All the other chemicals were commercially purchased.

Plant material

The origin of intact plants, induction and subcultures of suspension cultured cells of *H. planus* and *P. striatus* have been reported previously (Nabeta, *et al*., 1993; Nabeta, *et al*., 1998). Each of the cultures of *P. striatus* and *H. planus* were grown in 75 ml of MSK-4 medium (Takeda and Katoh, 1981) and grown on a rotatory shaker at 90 rpm at 25^0C under the light intensity of 2500 lux. Intact chloroplasts were isolated from fresh cells of *P. stratus* and *H. planus* of 25-28 day-old suspension cultures according to a previously described method (Walker, 1980). The intactness of the chloroplasts was assayed using ferricyanide as Hill oxidant (Lilley *et al*., 1975). Total chlorophyll of the

isolated chloroplast suspensions was measured according to the method of Arnon (1949).

Assay for chloroplastidic GGPP synthase

Isolated chloroplasts from 19g of 25-day-old *H. planus* suspension cells were resuspended in 400 ml of grinding buffer (50 mM HEPES-NaOH, pH 7.7; 1 mM EDTA; 2 mM DTT; 0.1 mM PMSF) and crushed in liquid N_2 to rupture the intact chloroplasts. The broken chloroplasts with liquid N_2 were kept at 4^0C until the ruptured chloroplasts were thawed. The broken chloroplast suspension was used for the assay for prenyltransferase. A portion of the broken chloroplast suspension was kept at 100^0C for 5 min to inactivate the enzyme and used as a control reaction. The assay for prenyltransferases using [4-^{14}C] IPP (57.5 mCi/mmol) was carried out according to Sagami and co-workers (1994). The incubation reaction contained of 1.25 µl of 1 M HEPES-NaOH (pH 7.5), 1.25 µl of 0.1 M $MgCl_2$, 1.25 µl of 0.1 M DTT, 1.25 µl of 0.1 M NaF, 1.25 µl of 0.5 mM FPP, 1 µl of [4-^{14}C] IPP (353840 Gdpm) and 18.5µl chloroplast preparation in a total volume of 25µl. The reaction was incubated at 30^0C for 24 hours. The products were extracted in hexane and hydrolysis of the diphosphates were carried out using potato acid phosphatase enzyme (2 units/mg, grade II, Boehringer) according to the method reported by Fuji and others (1982). The hydrolysed products were co-chromatographed on a reverse phase TLC system (Whatman KC18F silica gel 60A, solvent-acetone/H_2O 7:1) with authentic GGOH and phytol. Authentic GGOH and phytol were detected by exposing the plates to I_2 vapour and the radioactivity of the corresponding positions was determined by scintillation counting of the scraped GGOH and phytol positions.

Feeding of prenyl diphosphate precursors:

Administration of the prenyl diphosphate precursors was carried out according to Kreuz and Kleinig (1984). After 3 hours of incubation, the reaction mixtures were centrifuged at 3000g for 1 min (x 2) to stop the reaction and separate the chloroplast pellet and the medium. The resultant chloroplast pellets were suspended in 15 ml methanol separately and stirred overnight at room temperature to extract the prenyl diphosphates. The radioactivity of the methanol extract was determined using aliquots from each precursor-fed sample separately in a liquid scintillation counter (Aloka 5200) in ACSII Liquid Scintillation Cocktail.

Hydrolysis of the diphosphates:

Each methanol extract was divided into 2 equal fractions. These two fractions were hydrolysed separately by HCl to yield isoprenols and Cs_2CO_3 to yield phytol. HCl hydrolysis was carried out by concentrating the Methanol extract *in vacuo* at 35^0C and hydrolysing with 1N HCl and 10mM EDTA in a total volume of 5 ml. The reaction mixture was stirred vigorously for 5 min with 2.5 ml ether, at room temperature, the ether fraction was separated and the isoprenols in the H_2O fraction was again extracted with 4 ml ether (x 2). The ether fractions were combined and dried with dry Na_2SO_4 overnight at room temperature. Cs_2CO_3 hydrolysis was performed by adding aq. Cs_2CO_3 (75 µl of 0.61 mM) to the methanol extract and stirring at room temperature for 2 hours. Another 150 µl of 0.61 mM aq. CS_2CO_3 was added, and after further stirring overnight, the reaction solution was extracted with 6 ml of pentane (X 3). The pentane

extracts were combined, dried overnight with dry Na_2SO_4 at room temperature. Separation of the hydrolysates was carried out using TLC (on separate 2cm X 20cm reverse-phase TLC plates; Whatman, KC 18F silica gel 60A, solvent; acetone/H_2O 7:1). The hydrolysed samples were concentrated *in vacuo* and co-chromatographed with authentic farnesol (FOH) (R_f - 0.63), geranylgeraniol (GGOH) (R_f - 0.50) and phytol (R_f - 0.32). The positions of isoprenols and phytol were observed using I_2 vapour. Radioactivity of isoprenols and phytol was determined by scintillation counting.

RESULTS AND DISCUSSION

Use of cell suspension cultures for biosynthetic studies provides an excellent opportunity to obtain a homogenous population of cells for easy application of exogenous precursors. Studying of biosynthetic pathways in chloroplasts of higher plants using cell suspension cultures, however, has certain limitations as most cultured cells of higher plants lack active chloroplasts. Liverworts, in this regard, have an advantage over higher plants, as they possess active chloroplasts under cultured conditions (Katoh, 1988). As the liverworts are evolutionarily placed between the algae and higher plants, they can easily represent the biosynthesis of chloroplastidic isoprenoids in higher plants (Asakawa, 2001).

As the first step of elucidation of the non-equivalent labeling, presence of the GGPP synthase in the liverwort chloroplasts was examined (Fig. 2).

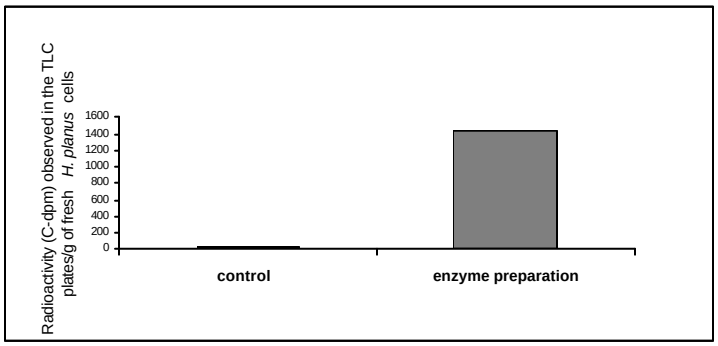

Fig. 2. Radioactivity in GGOH formed by chloroplastidic enzyme preparation of *H. planus* fed with [4-^{14}C] IPP.
Note: The control experiment represents the inactivated enzyme.

A high radioactivity of 1430 C-dpm was observed in GGOH (obtained by hydrolysis of GGPP) in the enzyme preparation, showing that GGPP has been biosynthesized from the applied radioactive IPP. The control reaction, which had the inactivated enzyme, demonstrated a very low activity (30 C-dpm), confirming the presence of GGPP synthase in the chloroplasts of *H. planus*. These results are in agreement with the studies of higher plant chloroplasts, which show an incorporation of ^{14}C-IPP into the chloroplastidic prenyllipids (Keruz and Klening, 1984). Isolated chromoplasts of the *Narcissus pesudonarcissus* flowers also incorporated [1-^{14}C]IPP into GGOH and carotenes efficiently (Keruz and Klening, 1981). The incorporation of

radioactivity was, however, remarkably low in *H. planus* (only 0.35% of the administered radioactivity was incorporated into GGOH) when compared with previous similar studies using the plastids of higher plants. A significant amount of ^{14}C-IPP (26.8%) was incorporated into the GGOH fraction in spinach chloroplasts (Kreuz and Kleinig, 1981). The results of the present study suggest the low activity of GGPP synthase in liverwort chloroplasts.

The incorporation experiments were carried out by administering equal amount of (6 nmol) IPP, GPP and FPP to the intact chloroplasts of *P. striatus* and *H. planus.* The radioactivity of the methanol extracts, which represents the precursor incorporated into the chloroplasts is shown in Table 1.

Table 1 Radioactivity (C-dpm or H-dpm) observed in methanol extract of the intact chloroplasts equivalent to 160mg of chlorophyll of *P. striatus* and *H. planus* fed with radiolabeled prenyl diphosphates

Precursor	Radioactivity in *P. striatus* chloroplasts	Radioactivity in *H. planus* chloroplasts
IPP (C-dpm)	3971.3 (0.06%)	4567.5 (0.07%)
GPP (H-dpm)	10702.5 (0.79%)	7616.3 (0.56%)
FPP (C-dpm)	23801.3 (0.87%)	35077.5(1.28%)

Note: Activity in the methanol extract as a percentage of the administered radioactivity is shown within brackets.

The highest radioactivity was shown by the FPP-fed samples in both liverworts, *P. striatus* and *H. planus*, whereas, IPP-fed samples showed the lowest. This indicates that FPP is preferentially taken into the chloroplasts, while the IPP incorporation is very low. The methanol extracts were hydrolysed and analyzed by TLC and the radioactivity was measured in each spot corresponding to FOH, GGOH and phytol (Table 2).

Table 2. Radioactivity (C-dpm or H-dpm) in FOH, GGOH and phytol obtained by bioconversion of radioactive isoprenyl diphosphates by the intact chloroplasts of *P. striatus* and *H. planus*.

	Radioactivity in *P. striatus*			Radioactivity in *H. planus*		
Precursor	FOH	GGOH	Phytol	FOH	GGOH	Phytol
IPP (C-dpm)	73.2	37.2	35.1	33.8	37.8	38.3
GPP (H-dpm)	204.5	138.2	91.3	336.5	285.5	131.2
FPP (C-dpm)	3764.9	251.0	49.9	1513.4	738.1	68.9

IPP-fed samples have always shown a very low radioactivity in all the fractions in both liverworts, suggesting a markedly low permeability of the liverwort chloroplast membrane to IPP. These results were not expected, as cytosolic IPP has been shown to be a very good precursor for chloroplastidic isoprenoids in higher plants (Kreuz and Kleinig, 1984). The low incorporation of cytosolic IPP into GGOH or its derivatives in liverwort chloroplasts may imply that the isoprenoid biosynthetic pattern of liverwort chloroplasts is different to that of the higher plants. However, the intact chloroplast membrane of the unicellular green alga, *Acetabularia mediterrania* was shown to be impermeable to IPP, although IPP has been a good precursor for chloroplastidic isoprenoids in osmotically lysed chloroplasts of *Acetabularia* (Bauerle *et al*., 1990). Liverworts show the same behaviour, intact chloroplast membrane being impermeable to IPP. The ruptured chloroplasts of *H planus*, however, have shown an incorporation of exogenous labeled IPP into GGOH (Fig.1). Being closer evolutionary relatives, the liverworts and algae may possess chloroplasts, which share certain features in incorporating exogenous prenyl diphosphates into their chloroplasts.

The GPP-fed sample of *P. striatus* showed a higher activity in the FOH region (204.5 H-dpm) and a lower, but prominent activity in the GGOH region (138.2 H-dpm, Table 1). *H. planus* fed with GPP showed high activities in the FOH and GGOH regions (336.5 H-dpm and 285.5 H-dpm respectively). This indicates that exogenous GPP has been incorporated into the chloroplasts and condensed to FPP and GGPP, presumably using IPP synthesized *via* the MVA-independent pathway. High activity in the FPP region following administration of GPP implies the presence of FPP synthase within the chloroplasts. Sanmiya and co-workers (1999) also reported on the localization of FPP synthase in rice, wheat and tobacco chloroplasts by immunoassay.

The FPP incorporated chloroplasts showed a remarkably high radioactivity in the FOH region (3764.9 C-dpm in *P. striatus* and 1513.4 C-dpm in *H. planus*) and also a high count in the GGOH region (251.0 C-dpm in *P. striatus* and 738.1 C-dpm in *H. planus*, Table 2). This shows that FPP has been preferentially incorporated into the chloroplasts and condensed with IPP to yield GGPP. The much higher count of FOH relative to GGOH implies that exogenous FPP is present either attached to the chloroplast envelope or as an endogenous FPP pool apart from condensation to GGPP. At this stage, it is difficult to say whether the radioactivity observed in FOH is due to the endogenous FPP pool formed as a consequence of incorporation into the chloroplasts or due to FPP adhering to the chloroplast envelope. The activity shown in GGOH region can be regarded as an index for the actual incorporation of FPP as this GGPP is exclusively synthesized from exogenous FPP.

So far, there have been no reports on the uptake of FPP into intact chloroplasts. Some authors suggested a low permeability of FPP through the chloroplast envelope (Kreuz and Klening, 1984), although, no evidence has been provided in this regard. Some indirect evidence for preferential incorporation of FPP into the chloroplasts has been reported in the biosynthesis of Ginkgolide in *Ginkgo biloba* chloroplasts (Schwarz and Arigoni, 1999).

A possible explanation for the non-equivalent labeling of the phytyl side-chain is suggested in this study (Fig. 3). FPP, which is synthesized *via* the MVA pathway, is incorporated into the chloroplasts from the cytoplasm and condensed with endogenous IPP to form GGPP, using chloroplastidic GGPP synthase. Since we observed that cytoplasmic IPP is not effectively incorporated to the liverwort chloroplasts, FPP may

have used endogenous IPP (synthesized *via* the MVA-independent pathway) for the biosynthesis of GGPP, to synthesize phytyl side chain of chlorophyll *a*.

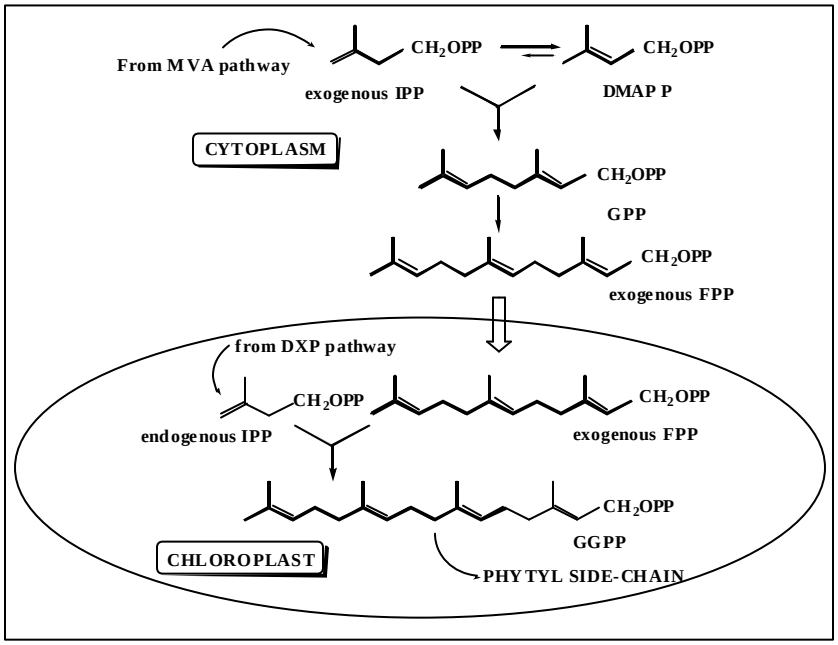

Fig. 3. The proposed mechanism for the non-equivalent labeling of the phytyl side-chain of liverworts

CONCLUSIONS

This study provides evidence for the preferential uptake of FPP into the liverwort chloroplasts. FPP is biosynthesized in the cytosol, *via* the MVA pathway, and is channeled, at least partly, to the chloroplast for the biosynthesis of the phytyl side-chain. GGPP is synthesized from this incorporated FPP by condensation with endogenous IPP, synthesized *via* the MVA-independent pathway. The phytyl side-chain in the liverworts *H. planus* and *P. straitus* can be biosynthesized from this GGPP. Furthermore, this study provides evidence for exchange of metabolites between different cell compartments of plants and the evolution of chloroplast membrane for the uptake of different precursors for prenyllipid synthesis.

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