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Origin of two isoprenoid units in a lavandulyl moiety of sophoraflavanone G from *Sophora flavescens* cultured cells

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Abstract

Cell suspension cultures of *Sophora flavescens* produced large amounts of sophoraflavanone G, an 8-lavandulylated flavanone and lupalbigenin, a 6,3′-di-dimethylallylated isoflavone, by the simultaneous addition of cork tissues and methyl jasmonate. The labeling pattern of the isoprene units resulting after administration of [1-^{13}C] glucose into the cell cultures in the presence of the above additives revealed that two isoprene units in the lavandulyl group of sophoraflavanone G and two dimethylallyl groups of lupalbigenin were biosynthesized via the 1-deoxy-D-xylulose-5-phosphate pathway.

Keywords: *Sophora flavescens*; Leguminosae; Cell suspension cultures; Isoprenoid biosynthesis; 1-Deoxy-D-xylulose-5-phosphate pathway; Prenylated flavonoids; Sophoraflavanone G; Lupalbigenin

1. Introduction

Many prenylated aromatic secondary metabolites such as coumarins, flavonoids, quinones and xanthones are widely distributed in the plant kingdom. In many of these compounds, 3,3-dimethylallyl groups, the simplest C_5 units, are bound to aromatic moieties, although other larger prenyl groups such as geranyl and farnesyl groups conjugated to aromatic rings are known as well (Barron and Ibrahim, 1996; Matern et al., 1988; Peres and Nagem, 1997; Tahara and Ibrahim, 1995). In general, these regular prenyl groups are presumed to be formed prior to their addition to aromatics (Barron and Ibrahim, 1996), as in the case of geranylation of *p*-hydroxybenzoic acid (Heide and Tabata, 1987; Mühlenweg et al., 1998) and olivetolate (Fellermeier and Zenk, 1998).

It has long been believed that these prenyl groups were biosynthesized via the mevalonate pathway. Recently, isopentenyl diphosphate (IPP), a common building block of diverse isoprenoid derivatives, was also reported to be derived from 1-deoxy-D-xylulose-5-phosphate (DOXP; Rohmer et al., 1993, 1996; Schwender et al., 1996). This finding led to re-investigation of the origin of prenyl moieties in prenylated aromatics (Adam et al., 1998; Disch et al., 1998; Eisenreich et al., 1997; Lichtenthaler et al., 1997; Turner et al., 1999). Incorporation studies using ^{13}C-labeled glucose or ^{2}H-labeled deoxyxylulose showed that dimethylallyl groups bound to aromatic compounds such as the prenylated flavanone in *Glycyrrhiza glabra* (Asada et al., 2000), humulone in *Humulus lupulus* (Goese et al., 1999) and furanocoumarins in *Apium graveolens* (Stanjek et al., 1999) were biosynthesized via the DOXP pathway. In addition, the geranyl moiety of cannabinoids (Fellermeier et al., 2001) as well as the hemi- and mono-terpenoid moieties of isopentenyl phenyl ethers (Barlow et al., 2001) also originated from DOXP. In contrast, the geranyl group of *m*-geranyl-*p*-hydroxybenzoic acid, a key intermediate in shikonin biosynthesis, was derived from mevalonate (Li et al., 1998). These findings demonstrate that the origin of aromatics-linked prenyl units varies with compounds.

In addition to flavonoids with the above regular prenyl side chains, *Sophora flavescens*, a leguminous plant, produced diverse flavanones with lavandulyl chains, irregular monoterpenoid groups, such as kurarinone and sophoraflavanone G (Hatayama and Komatsu,

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1971; Kang et al., 2000; Kuroyanagi et al., 1999; Wu et al., 1985a, b, c, 1986). It is structurally characteristic of these flavanones to possess a hydroxy group at C-2′ together with a lavandulyl group at C-8 or C-6. We have recently shown that sophoraflavanone G was biosynthesized by a two-step dimethylallylation of flavanone between which 2′-hydroxylation occurs (Yamamoto et al., 2000). That is, the first dimethylallylation takes place on the flavanone nucleus and the second on the prenyl side chain of the resulting prenylated 2′-hydroxyflavanone. It was of interest to investigate the origin of these two isoprene units, especially the second dimethylallyl group in the lavandulyl group of sophoraflavanone G, which is part of an unique aromatic-bound prenyl group elongation reaction.

In the present study, we administered [1-^{13}C] glucose to *S. flavescens* cells in order to investigate the biosynthetic origin of the lavandulyl group of sophoraflavanone G and two dimethylallyl groups of lupalbigenin. The resulting labeling patterns indicated that the DOXP pathway was involved in the formation of each of the isoprene units in both compounds.

2. Results and discussion

2.1. Stimulation of prenylated flavonoids production in *S. flavescens* cells

For the effective incorporation of [1-^{13}C] glucose into prenylated flavonoids, we re-investigated the effects of several additives on the production of prenylated flavonoids in *S. flavescens* cells (Fig. 1). As previously reported (Yamamoto et al., 1996, 1999, 2001a), cork tissues stimulated the production of sophoraflavanone G when added to the medium (nearly three times higher compared with the control cells). Methyl jasmonate, a well-known strong secondary metabolism stimulator

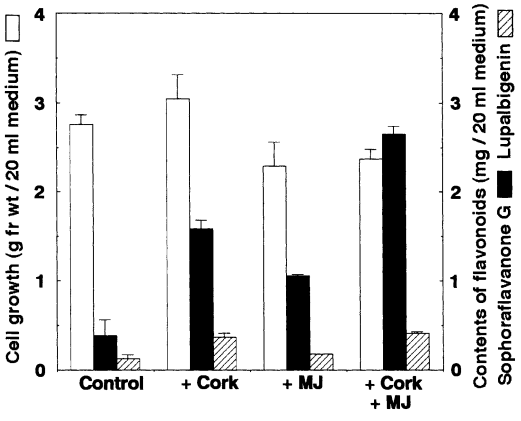

Fig. 1. Effects of cork tissues and methyl jasmonate on the production of prenylated flavonoids in *Sophora flavescens* cell suspension cultures. Cork tissues (50 mg) and / or methyl jasmonate (100 μM) were added to the medium. Bars represent SE ($n=3$).

(Hahlbrock and Scheel, 1989; Gundlach et al., 1992; Mueller et al., 1993), also stimulated the production of sophoraflavanone G (twice as high as the control cells). When cork tissues and methyl jasmonate were simultaneously added to the medium, the amount of sophoraflavanone G reached up to 2.7 mg/20 ml medium (five times higher than that in the control cells), suggesting that stimulatory effects of these additives would be regulated in different manners. The detailed mechanism of stimulatory effect by cork tissues is to be further investigated, but preliminary work showed that polysaccharides in hemicellulose B fraction from cork tissues stimulated sophoraflavanone G production (Yamamoto et al., 2001a). Simultaneous addition of cork tissues and methyl jasmonate also increased the production of lupalbigenin.

2.2. Incorporation of [1-^{13}C] glucose into prenylated flavonoids

[1-^{13}C] Glucose was administered to aseptic cultures of *S. flavescens* containing cork tissues and methyl jasmonate. After cultivation for 2 weeks, the cells and cork tissues were harvested together and the compounds, sophoraflavanone G and lupalbigenin, were isolated as described in Section 3. The ^{13}C NMR spectra of natural abundance and enriched samples were obtained under identical conditions. The relative ^{13}C abundance of individual carbon atoms was calculated from the comparison of the integrals of labeled isolates with natural abundance samples. The values were referenced to 1.1% for the carbon with the lowest ^{13}C enrichment.

Glucose is utilized as carbon source for isoprenoid biosynthesis either via the mevalonate pathway or via the DOXP pathway. These two pathways, however, are easily differentiated by analysis of the labeling patterns in isoprenoids derived from [1-^{13}C] glucose. The label from [1-^{13}C] glucose should be incorporated into C-1 and C-5 of IPP/DMAPP formed through DOXP from C-3 labeled triose phosphate converted by glycolysis, whereas into C-2, C-4 and C-5 of IPP/DMAPP formed via mevalonate from C-2 labeled acetyl CoA (Fig. 2). In the ^{13}C NMR spectrum of ^{13}C-enriched sophoraflavanone G, clear increases of the signals of C-1″, C-3″, C-7″ and C-10″ in the lavandulyl group were observed (Table 1), giving proof for the origin of two isoprene units of lavandulyl group being C-1 and C-5 of IPP, and hence for the operation of DOXP pathway. C-6″ and C-9″ of sophoraflavanone G were also labeled to a lesser extent than the four above-mentioned carbons, presumably due to some imperfect stereochemical course in the DMAPP/IPP isomerase reaction (Arigoni et al., 1997; Li et al., 1998; Asada et al., 2000). In addition to the incorporation of ^{13}C into the prenyl side chain of sophoraflavanone G, incorporation of the label into C-2, C-2′ C-6, C-6′ and C-8 of flavonoidal skeleton

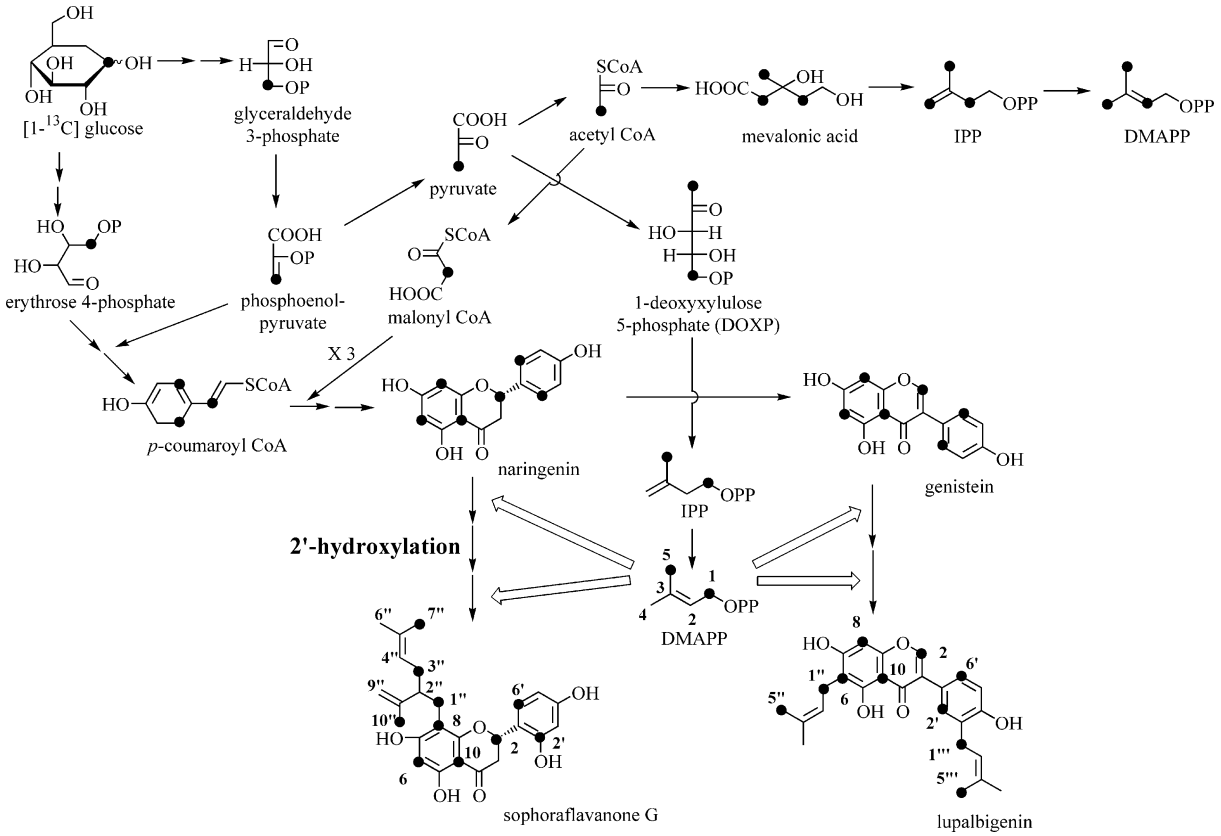

Fig. 2. Incorporation of [1-^{13}C] glucose into sophoraflavanone G and lupalbigenin.

resulting from the complex turnover of the glucose into flavonoids (Asada et al., 2000) was also observed.

Similarly, analysis of the ^{13}C NMR spectra of labeled lupalbigenin revealed that the labeling pattern (C-1″, C-1‴, C-5″ and C-5‴) of the two dimethylallyl groups was in accordance with the operation of the DOXP pathway (Table 1). In addition, incorporation of the label into C-2, C-2′, C-6, C-6′ and C-8 of isoflavone skeleton were also observed.

The present study established that the lavandulyl residue, an irregular monoterpenoid unit, of sophoraflavanone G was generated via the operation of DOXP pathway. The biosynthesis of IPP/DMAPP from DOXP is restricted to plastids (Mandel et al., 1996; Lichtenthaler et al., 1997), suggesting that the two discontinuous dimethylallylation steps for lavandulyl group formation take place in the same organelle, like other aromatics-dimethylallyltransferases (Dhillon and Brown, 1976; Biggs et al., 1990). On the other hand, the 2′-hydroxylation, which is required for the second dimethylallylation in sophoraflavanone G biosynthesis, was catalyzed by a cytochrome P450 monooxygenase (Yamamoto et al., 2001b), which may be associated with the endoplasmic reticulum (von Wachenferdt and Johnson, 1995). These findings might indicate close cooperation between plastids and endoplasmic reticulum, which were sites of two dimethylallylations and 2′-hydroxylation, respectively, as well as the presence of transport systems which shuttle the intermediates to the corresponding reaction sites. Further understanding of sophoraflavanone G biosynthesis requires clarification of subcellular localization of enzymes responsible for the formation of the lavandulyl group, namely naringenin 8-dimethylallyltransferase (Yamamoto et al., 2000), 8-dimethylallylnaringenin 2′-hydroxylase (Yamamoto et al., 2001b) and the second dimethylallyltransferase which have not yet been characterized (Yamamoto et al., 2000).

3. Experimental

3.1. General

D-[1-^{13}C] Glucose (99% isotopic abundance) was purchased from Isotec Inc., USA. The ^{1}H and ^{13}C NMR spectra were obtained with a Varian UNITY plus 500 spectrometer. ^{1}H-decoupled ^{13}C NMR spectra of natural abundance and ^{13}C-labeled samples were measured under identical conditions (125 MHz; 27 °C; repetition time 2.00 s; 30 ° pulse angle) using standard Varian software.

Table 1
^{13}C Abundance in sophoraflavanone G and lupalbigenin after feeding of [1-^{13}C] glucose

Sophora flavanone G			Lupalbigenin		
Carbon	δ[a]	Abundance	Carbon	δ	Abundance
C-2	75.3	**23.9**[b]	C-2	153.9	**6.6**
C-3	42.8	2.5	C-3	123.1	1.1
C-4	198.2	3.3	C-4	181.7	1.2
C-5	163.0	3.3	C-5	160.6	1.1
C-6	96.2	**18.3**	C-6	112.4	**6.2**
C-7	165.3	2.3	C-7	162.6	1.2
C-8	107.8	**8.7**	C-8	93.7	**6.6**
C-9	162.1	3.9	C-9	155.9	1.5
C-10	103.2	**19.7**	C-10	106.0	**6.9**
C-1′	117.8	1.8	C-1′	123.3	1.1
C-2′	156.1	**14.0**	C-2′	131.2	**7.2**
C-3′	103.4	2.3	C-3′	124.2	1.2
C-4′	159.4	3.2	C-4′	156.8	1.2
C-5′	107.6	1.1	C-5′	115.5	1.2
C-6′	128.6	**22.3**	C-6′	128.5	**7.4**
C-1″	27.8	**22.4**	C-1″	29.1	**5.4**
C-2″	47.8	2.3	C-2″	123.1	1.1
C-3″	31.9	**21.3**	C-3″	132.4	1.7
C-4″	124.4	2.8	C-4″	25.8	2.2
C-5″	131.6	5.4	C-5″	17.9	**5.6**
C-6″	25.8	6.9	C-1‴	22.0	**5.2**
C-7″	17.8	**20.5**	C-2‴	123.6	1.1
C-8″	149.1	4.1	C-3‴	131.6	1.5
C-9″	111.2	7.6	C-4‴	25.9	2.2
C-10″	19.1	**21.6**	C-5‴	17.8	**5.8**

[a] Referenced to the acetone-d_6 centerline at 29.8 ppm.
[b] The numbers in bold type represent significant ^{13}C incorporation from [1-^{13}C] glucose.

3.2. Plant material and culture method

The origin and subculturing of callus cultures (Yamamoto et al., 1991) and the establishment of cell suspension cultures (Yamamoto et al., 1996) of *S. flavescens* were described in previous papers. Cells (1 g) were subcultured in 20 ml MS liquid medium (Murashige and Skoog, 1962) containing 1 μM 2,4-D and 1 μM kinetin (100 ml flasks) for 2 weeks, and then inoculated into the fresh liquid medium containing additives and agitated on a rotary shaker at a speed of 100 rpm at 23 °C in the dark for 2 weeks. Cork tissues (50 mg) were added to the medium (20 ml) before autoclaving (Yamamoto et al., 1996). Filter-sterilized 100 mM methyl jasmonate solution in DMSO (20 μl) was aseptically added to the medium (final conc. 100 μM) after autoclaving. For the metabolism experiment, [1-^{13}C] glucose (1 g) dissolved in 10 ml of MS liquid medium containing 1 μM 2,4-D and 1 μM kinetin were filter-sterilized and administered to five fresh media (20 ml) containing cork tissues (50 mg) and 100 μM methyl jasmonate.

3.3. Extraction and isolation of prenylated flavonoids

After culturing for 2 weeks, cells and cork tissues were harvested together by filtration. The mixture (18 g fresh cells and 0.25 g cork tissues) were extracted with MeOH (3×300 ml) by ultrasonication (90 min). The extract was partitioned between *n*-BuOH and H_2O. The *n*-BuOH fraction evaporated in vacuo (515 mg) was then subjected to Toyopearl HW-40 column chromatography (Tosoh Co. Ltd., Tokyo, Japan) eluting with EtOH, to give a fraction containing sophoraflavanone G and lupalbigenin. This fraction was further purified by repeated reversed-phase HPLC to afford sophoraflavanone G (7 mg) and lupalbigenin (4 mg), respectively. HPLC conditions was as follows: column, Hikarisil C18–2E (5 μm, 20×250 mm, Showa Denko Co. LTD., Japan); solvent system, MeOH/H_2O/HCOOH = 65/35/0.1; flow rate, 5 ml/min; oven temp., 40 °C; detection, 294 nm. The purity of ^{13}C-labeled sophoraflavanone G and lupalbigenin were confirmed by TLC, HPLC and ^1H and ^{13}C NMR spectra, respectively.

3.4. Quantitative analyses of prenylated flavonoids

Harvested fresh cells (3 g) were extracted with 10 ml MeOH by ultrasonication (90 min). Quantitative analyses of prenylated flavonoids were carried out as described (Yamamoto et al., 2001a).

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