# Characterization of Leachianone G 2′′-Dimethylallyltransferase, a Novel Prenyl Side-Chain Elongation Enzyme for the Formation of the Lavandulyl Group of Sophoraflavanone G in *Sophora flavescens* Ait. Cell Suspension Cultures

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Leachianone G (LG) 2′′-dimethylallyltransferase, a novel prenyl side-chain elongation enzyme, was identified in *Sophora flavescens* Ait. cultured cells. The enzyme transfers a dimethylallyl group to the 2′′ position of another dimethylallyl group attached at position 8 of LG to form sophoraflavanone G, a branched monoterpenoid-conjugated flavanone characteristic to this plant. This membrane-bound dimethylallyltransferase required $Mg^{2+}$ (optimum concentration was 10 mM) for the reaction and had an optimum pH of 8.8. It utilized dimethylallyl diphosphate as the sole prenyl donor, and the 2′-hydroxy function in LG was indispensable to the activity. The apparent $K_m$ values for dimethylallyl diphosphate and LG were 59 and 2.3 $\mu$M, respectively. Subcellular localization of three enzymes that participated in the formation of the lavandulyl group was also investigated by sucrose density gradient centrifugation. Two prenyltransferases, naringenin 8-dimethylallyltransferase and LG 2′′-dimethylallyltransferase, were localized in the plastids, whereas 8-dimethylallylnaringenin 2′-hydroxylase, which catalyzes the crucial step in the lavandulyl-group formation, was associated with the endoplasmic reticulum. These results suggest the close cooperation between the plastids and the endoplasmic reticulum in the formation of lavandulyl groups.

More than 30,000 isoprenoid compounds—the most chemically diverse family of metabolites—are found in nature (Eisenreich et al., 1998). The vast majority of these compounds have "regular" 1′-4 (head-to-tail) linkages between isoprenoid units formed by isoprenyl diphosphate synthases, which are types of prenyltransferase. These enzymes catalyze the prenyl diphosphate elongation reaction that results in the consecutive 1′-4 condensations of isopentenyl diphosphate (IPP), the five-carbon building unit, with allylic isoprenyl diphosphates such as dimethylallyl diphosphate (DMAPP) and geranyl diphosphate (GPP; Ogura and Koyama, 1998; Liang et al., 2002). "Irregular" (non-head-to-tail) isoprenoids are also found, of which the most prominent examples are the 1′-1 (tail-to-tail) condensed isoprenoids such as squalene and phytoene, which are important precursors of sterols and carotenoids, respectively (Poulter, 1990). The biosyntheses of these tail-to-tail condensed terpenoids have been well characterized, but little is known about other non-head-to-tail condensed terpenoids except for chrysanthemyl diphosphate synthase, which catalyzes the condensation of two molecules of DMAPP to produce chrysanthemyl diphosphate, a branched monoterpene with a c1′-2-3 linkage between two isoprenoid units (Poulter, 1990; Rivera et al., 2001). A lavandulyl group, another example of the branched monoterpenoid unit, is found in labiataeous and compositaeous plants as essential oils (de Lampasona et al., 1997; Tsuro et al., 2001; Gunawardena et al., 2002), and in leguminous and moraceous plants as diverse prenylated flavonoids (Barron and Ibrahim, 1996). Epstein and Poulter (1973) suggested very early that chrysanthemyl diphosphate might be an intermediate of lavandulyl monoterpenes. More recently, another mechanism—the direct condensation of two DMAPP molecules to form lavandulyl diphosphate—was postulated by Gunawardena et al. (2002).

*Sophora flavescens*, a leguminous plant, produces diverse flavanones with lavandulyl side chain, such as sophoraflavanone G (SFG) and kurarinone (Hatayama and Komatsu, 1971; Wu et al., 1986; Kuroyanagi et al., 1999; Kang et al., 2000). They have the

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structural characteristic of possessing a lavandulyl group at C-8 or C-6 together with a hydroxyl group at C-2′. Recent pharmaceutical studies showed that the lavandulyl side chain is essential for the antitumor activity and phospholipase-C$\gamma_1$-inhibition activity of the flavonoids isolated from this plant (Lee et al., 1997; Ko et al., 2000). We have established cultured cells of this plant, which produce SFG as a major flavonoid (Yamamoto et al., 1991), and demonstrated that the lavandulyl group of SFG was not directly transferred to the flavanone skeleton but is biosynthesized by the discontinuous two-step dimethylallylations between which the 2′-hydroxylation occurred (Fig. 1). That is, the first dimethylallylation occurs on the flavanone nucleus catalyzed by naringenin 8-dimethylallyltransferase (N8DT) that does not accept lavandulyl diphosphate as the prenyl donor to afford 8-dimethylallylnaringenin (Yamamoto et al., 2000). This intermediate is further hydroxylated to form leachianone G (LG) by 8-dimethylallylnaringenin 2′-hydroxylase (2′OH; Yamamoto et al., 2001), and the second dimethylallylation takes place on the prenyl side chain of LG catalyzed by an uncharacterized dimethylallyltransferase to give SFG. More recently, we revealed that two isoprene units in the lavandulyl group of SFG were generated via the 1-deoxy-D-xylulose-5-phosphate (DXP) pathway (Yamamoto et al., 2002), which is an alternative route for IPP biosynthesis in the plastids (Eisenreich et al., 1998), suggesting that lavandulyl group formation involves two discontinuous dimethylallylation steps that take place in the plastids. Nevertheless, no direct evidence has been reported for the subcellular localization of the enzymes responsible for SFG biosynthesis.

To elucidate the mechanism underlying lavandulyl-group formation, in the present study, we identified and characterized the second prenylation enzyme, LG 2′-dimethylallyltransferase (LGDT), from the cultured cells of *S. flavescens* (Fig. 1). To our knowledge, LGDT is the first example of a prenyltransferase that elongates the conjugated prenyl side chain to form the branched prenyl group. We also investigated subcellular localizations of three membrane-bound enzymes responsible for the lavandulyl group formation, N8DT, 2′OH, and LGDT, and found that two dimethylallyltransferases are localized in the plastids, whereas 2′OH is distributed in the endoplasmic reticulum (ER).

## RESULTS

### Detection of LGDT Activity in *S. flavescens* Cultured Cells

When crude cell-free extracts from *S. flavescens* cells were incubated with 1 mM LG, 2 mM DMAPP, and 10 mM $MgCl_2$ for 30 min at 30°C, the enzymatic formation of a new compound whose retention time and UV absorption pattern were completely identical with those of SFG was observed in HPLC-photodiode array analysis. The reaction product was isolated by preparative HPLC and identified as SFG by comparison of its electron impact mass spectrometry (EIMS) spectrum with that of authentic SFG. Most prenyltransferase activity was recovered from the microsomal fraction prepared by ultracentrifugation, and its specific activity in this fraction was about 11-fold higher than that in crude cell-free extracts, indicating that the enzyme was tightly bound to the membrane fraction of the cells (Table I). The activity was dependent on the presence of LG, DMAPP, $Mg^{2+}$, and active enzyme (data not shown).

To clarify whether the two dimethylallylations in SFG biosynthesis are catalyzed by the same or by different prenyltransferases, parallel assays on N8DT and LGDT using the microsomal membranes were performed (Table II). If one prenyltransferase cata-

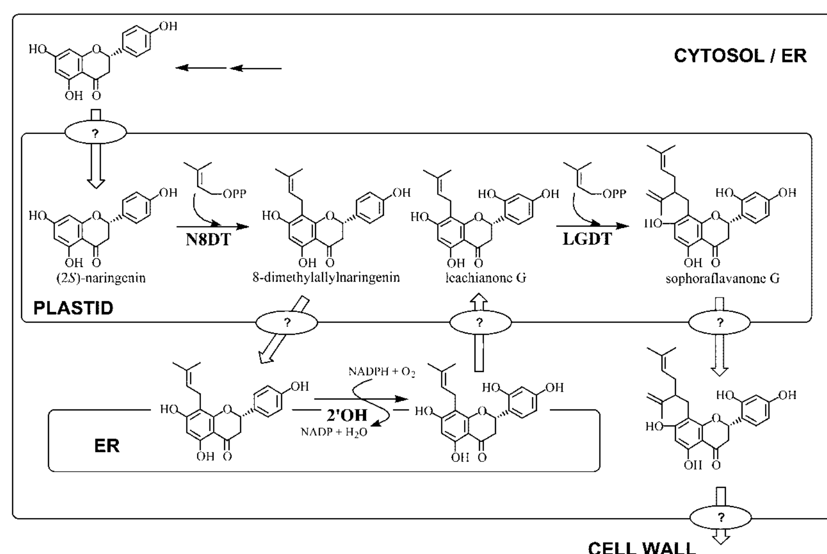



**Figure 1.** Biosynthetic route from naringenin to SFG by two discontinuous dimethylallylations and 2′-hydroxylation and transport of precursors/products in *S. flavescens* cultured cells. Ovals with question mark indicate postulated transport systems, although simple diffusion mechanisms are also explainable.

**Table I.** *Membrane association of LGDT in S. flavescens cultured cells*

Preparation of fractions and LGDT assay are described in "Materials and Methods." Data are the means of duplicate determinations ± SE.

| Enzyme Solution | Total Activity | Total Protein | Specific Activity |
|---|---|---|---|
| | *pkat g cells$^{-1}$* | *mg g cells$^{-1}$* | *pkat mg protein$^{-1}$* |
| Crude cell-free extracts | 17.08 ± 0.30 | 7.10 ± 0.49 | 2.41 ± 0.04 |
| 156,000*g* supernatant | 0.85 ± 0.45 | 5.79 ± 0.48 | 0.15 ± 0.09 |
| Microsomal fraction | 14.23 ± 0.63 | 0.52 ± 0.02 | 27.37 ± 1.21 |

lyzes two different dimethylallylation reactions, the activities of both N8DT and LGDT would be decreased in the coexistence of naringenin and LG compared with those observed with naringenin or LG as the sole prenyl acceptor due to competition between the substrates at the catalytic site. However, when naringenin and LG were co-incubated with microsomal membranes, the activities of N8DT and LGDT were virtually identical to those when the acceptors were used individually, indicating that LGDT is recognized by another prenyltransferase from N8DT.

### Biochemical Characterization of LGDT

The enzymatic reaction showed a linear dependence on protein amounts between 30 and 120 $\mu$g of microsomal protein per assay and was linear up to an incubation time of 30 min. In contrast to previously reported prenyltransferases for which the optimum pH was around 7.5 (Dhillon and Brown, 1976; Schröder et al., 1979; Biggs et al., 1987; Hamerski and Matern, 1988; Welle and Grisebach, 1991; Laflamme et al., 1993; Fellermeier and Zenk, 1998; Mühlenweg et al., 1998; Tholl et al., 2001), LGDT showed an optimum pH of 8.8 in borate buffer, with about 50% of maximum activity at pH 7.9 and 10 (Fig. 2). The activity in Tris-HCl buffer at pH 8.8 was 88% of that observed in borate buffer.

The LGDT has an absolute requirement for divalent metal ions; the activity was negligible in an assay without any divalent cations. $Mg^{2+}$ was the most effective among the divalent cations examined, and the saturation for $Mg^{2+}$ (as chloride salts) was reached at 10 mM. In the presence of $Mn^{2+}$, the activity was only 15% that of $Mg^{2+}$. The other divalent metal ions examined also gave low activities: $Ca^{2+}$ (25%), $Zn^{2+}$ (10%), $Co^{2+}$ (5%), $Ni^{2+}$ (5%), $Fe^{2+}$ (5%), and $Cu^{2+}$ (4%).

**Table II.** *Competition of prenyl acceptors in N8DT and LGDT assays*

N8DT and LGDT assay are described in "Materials and Methods." Data are the means of duplicate determinations ± SE.

| Prenyl Acceptor | Specific Activity of N8DT | Specific Activity of LGDT |
|---|---|---|
| | *pkat mg protein$^{-1}$* | |
| Naringenin | 17.32 ± 0.47 | 0 |
| LG | 0 | 4.14 ± 0.01 |
| Naringenin + LG | 15.36 ± 0.32 | 3.92 ± 0.21 |

The apparent $K_m$ values for DMAPP and LG were calculated as 59 and 2.3 $\mu$M, respectively, from the Lineweaver-Burk plot using varying concentrations (12.5–200 $\mu$M for DMAPP and 1–16 $\mu$M for LG; Fig. 3).

### Substrate Specificity of LGDT

For the investigation of substrate specificity of LGDT, the microsomal fraction was incubated with prenyl acceptor (0.3 mM), prenyl donor (1 mM), and $Mg^{2+}$ (10 mM) under the standard assay condition. In each experiment, heat-denatured enzyme-containing assay was used as the control. When the microsomal fraction was incubated with LG, $Mg^{2+}$ and IPP, or GPP, any additional peaks were not observed in their HPLC profiles, indicating that only DMAPP is utilized by LGDT as the prenyl donor (Table III, left).

The prenylation activities of LGDT for several 8-dimethylallylated flavanones were also investigated (Table III, right). LGDT did not show absolute specificity for LG. When euchrenone $a_7$ (5-deoxy derivative of LG) was used as the prenyl acceptor, the formation of lehmannin (5-deoxy derivative of SFG)

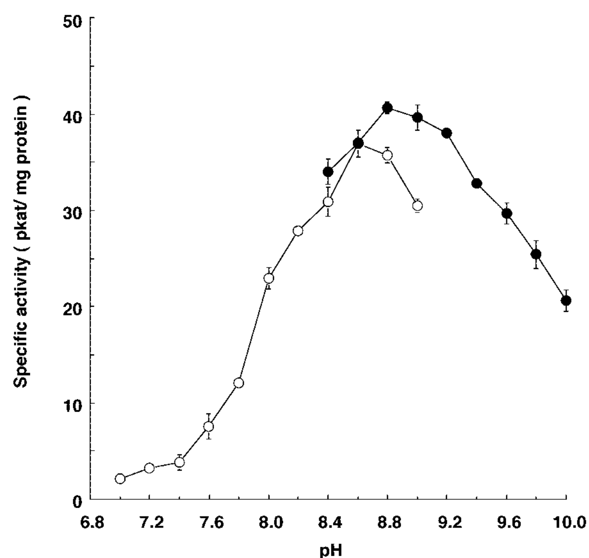



**Figure 2.** pH dependency of LGDT. ○, One hundred millimolar Tris-HCl buffer; ●, 100 mM borate buffer. Vertical bars, Ranges from two independent enzyme assays.

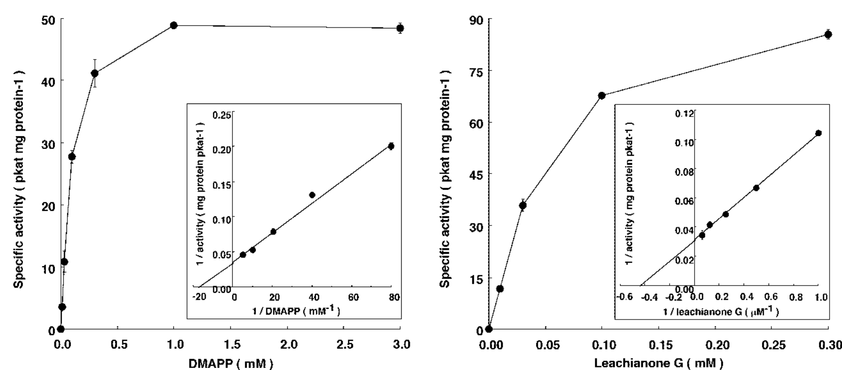



**Figure 3.** Dependency of LGDT activity on the concentration of DMAPP and LG measured with a microsomal fraction of *S. flavescens* cultured cells. Insert, Lineweaver-Burk plot with varying concentrations of DMAPP (12.5–200 $\mu$M) and of LG (1–16 $\mu$M) to calculate the apparent $K_m$ value for DMAPP and LG, respectively. Vertical bars, Ranges from two independent enzyme assays.

was observed in HPLC-photodiode array analysis. Its prenylation activity was 54% of the rate of LG. Incubation with kenusanone I, 7-methoxy derivative of LG, also afforded a new more lipophyllic compound, although its structure could not be identified in the present study. LGDT did not prenylate the 2′-hydroxyl group lacking 8-dimethylallylflavanones such as 8-dimethylallylnaringenin and isovabachin.

### Subcellular Localization of the Lavandulyl Group-Forming Enzymes

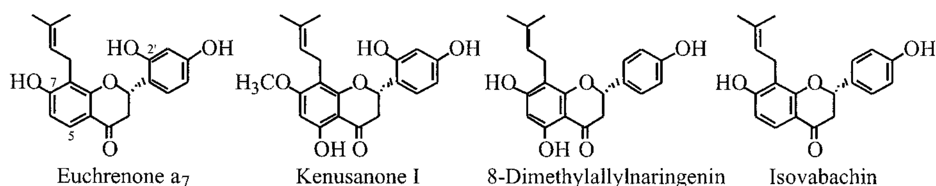
As mentioned above, three membrane-bound enzymes participate in the formation of the lavandulyl group: two prenyltransferases, N8DT and LGDT, and a cytochrome P450 monooxygenase, 2′OH. Our recent [1-$^{13}$C]Glc-feeding experiment revealed that two isoprene units in the lavandulyl group of SFG originated from a DXP pathway (Yamamoto et al., 2002), suggesting that two dimethylallylation steps were carried out in the plastids, whereas 2′OH, a cytochrome P450 monooxygenase, was thought to be localized in the ER. To confirm the subcellular localization of these enzymes, microsomal membranes were separated by Suc density gradient centrifugation. As shown in Figure 4A, both N8DT and LGDT activities were in a fraction with a density of $\rho$ = 1.07–1.08 g cm$^{-3}$ (fraction 6), coinciding with UDP-Gal:diacylglycerol galactosyltransferase activity, a well-established marker of the plastids (Douce et al., 1984), indicating that two prenyltransferases were associated with the membrane of the plastids.

On the other hand, the activities of 2′OH and cinnamate 4-hydroxylase (C4H), another cytochrome P450 monooxygenase known to exist in the ER (Ro et al., 2001), were observed in fraction 10 (Fig. 4A). The density of this fraction ($\rho$ = 1.11–1.12 g cm$^{-3}$) was in agreement with that of the ER (Robinson et al., 1994; Martinec et al., 2000). Moreover, the activity of antimycin A-insensitive NADH cytochrome *c* reductase, a marker of ER, was also found in this fraction. These results indicate that 2′OH is localized in the ER membranes, as it is in most cytochrome P450s in animals and plants.

**Table III.** *Substrate specificity of the leachianone G 2″-dimethylallyltransferase activity*

| Substrate (Donor) | Relative Activity | Substrate (Acceptor) | Relative Activity |
|---|---|---|---|
| | % | | % |
| DMAPP | 100 ± 3.0[a] | Leachianone G | 100 ± 3.0[a] |
| IPP | n.d.[b] | Euchrenone $a_7$ | 54 ± 1.5[c] |
| GPP | n.d. | Kenusanone I | n.i.[d] |
| | | 8-Dimethylallylnaringenin | n.d. |
| | | Isovabachin | n.d. |

The ultimate concentrations of acceptors and donors are 0.3 mM and 1 mM, respectively. Structures of acceptors were shown below (for leachianone G, see Fig. 1). The HPLC assay were performed according to the standard method described in "Materials and Methods". Data are means of duplicate determinations ± SE. [a] LGDT activity was 41.1 pkat mg protein-1. [b] Compared with heat-denatured control assay, any additional peaks were not detected. [c] The prenylated product was identified as lehmannin by comparison with authentic lehmannin using HPLC photodiode-array analysis. [d] The production of new compound, the elution profile of which was resemble to that of the expected product was observed (in the present HPLC condition, 8-lavandulyated product was eluted ca. 6 min later than 8-dimethylallylated substrate), though was not identified. Conversion rate of it was 11 ± 0.3 (% of control).



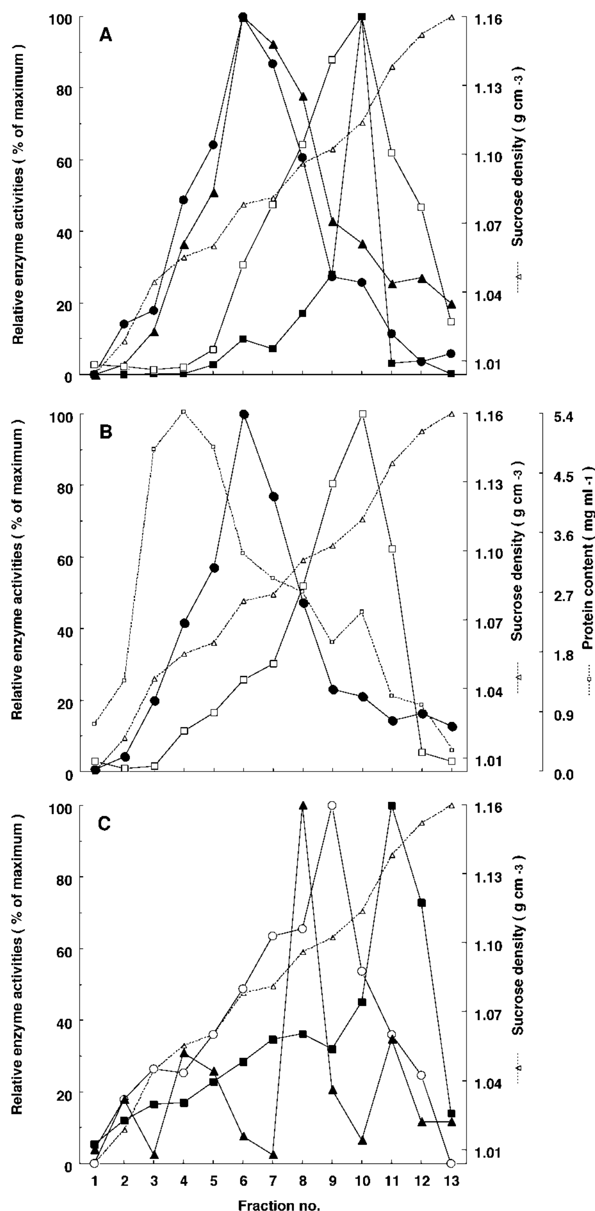



**Figure 4.** Location of the activities of LGDT, N8DT, 2′OH, cinnamate 4-hydroxylase (C4H), and marker enzymes in *S. flavescens* membranes fractionated by Suc density gradient centrifugation. Marker enzyme activities and LGDT, N8DT, 2′OH, and C4H activities were estimated in each fraction as described in "Materials and Methods." Fraction 1, Top of the gradient. Data are from a typical experiment—similar results were obtained from four other independent experiments. Activities are expressed as the percentage of the highest activity obtained in the peak fraction. A: ●, LGDT; ▲, N8DT; ■, 2′OH; □, C4H. B: ●, UDP-Gal:diacylgylcerol galactosyltransferase (plastids marker); ▫, antimycin A-insensitive NADH cytochrome *c* reductase (ER marker); ▫ (with dashed line), protein content of separated membranes. C: ○, NADPH cytochrome *c* oxidase (mitochondria marker); ▲, $KNO_3$-sensitive ATPase (tonoplast marker); ■, $Na_3VO_4$-sensitive ATPase (plasma membrane marker).

Distribution patterns of other membranes in Suc density gradient (Fig. 4C) were similar to those from *Beta vulgaris* (Bennett et al., 1984) and *Chenopoium rubrum* (Martinec et al., 2000), although Robinson et al. (1994) reported that the density of tonoplast varies with plant species and tissues.

## DISCUSSION

### LGDT Is a Novel Prenyl Side-Chain Elongation Enzyme

It is well known that many prenyl side-chain-conjugated compounds with important biological functions, such as aromatics and proteins, are widely distributed in organisms. In animals, prenylated proteins act as the modulator of cell cycle progression (Tamanoi et al., 2001), and prenylated phenolics from plants exhibit many biological activities against other organisms (Barron and Ibrahim, 1996). In these prenyl side-chain conjugation reactions, the adequate length prenyl diphosphate biosynthesized beforehand by prenyl diphosphate elongation enzymes were thought to be directly transferred to the prenyl acceptor (Fellermeier and Zenk, 1998; Mühlenweg et al., 1998; Liang et al., 2002). In the present study, we first identified a novel prenyl side-chain elongation enzyme, LGDT, from cultured cells of *S. flavescens*. In contrast to previously reported prenyl diphosphate elongation enzymes that transfer dimethylallyl group to prenyl diphosphate to form C5-elongated prenyl ones (Ogura and Koyama, 1998; Liang et al., 2002), LGDT transfers a dimethylallyl group to the dimethylallyl side chain already bound to 2′-hydroxyflavanones to afford a branched prenyl side chain. LGDT only accepted DMAPP as the prenyl donor (IPP or GPP were not transferred to the flavanone; Table III), suggesting that LGDT strictly recognizes the chain length and the position of the double bond in the prenyl donor and catalyzes the single 1′-2 condensation. The possible reaction mechanisms of LGDT and other known prenyltransferases are illustrated in Figure 5. The reaction mechanism of LGDT (Fig. 5C) seems to resemble that of the 1′-4 chain elongation prenyltransferases; for example, GPP synthase (Fig. 5A), rather than that of the prenyltransferases, which transfer the prenyl group to the aromatic compounds, such as N8DT (Fig. 5B).

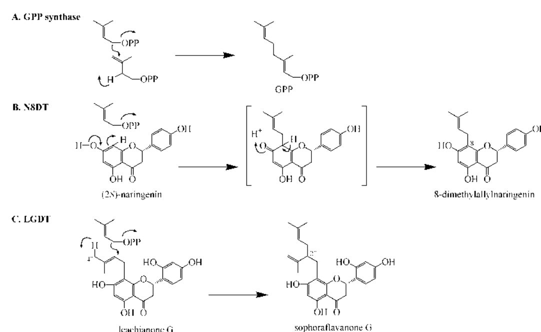



**Figure 5.** Possible prenylation mechanisms of GPP synthase (A), N8DT (B), and LGDT (C).

The reaction is started by the ionization of DMAPP, as in the case of the 1′-4 chain elongation reaction. Different from 1′-4 condensation, we suppose the deprotonation at C-4′′ and the activation of C-2′′ of LG forms a carbanion at C-2′′ that can be attacked by the DMAPP cation, resulting in the formation of the lavandulyl group in SFG. The results on substrate specificity for several 8-dimethylallylated flavanones indicated that the 2′-hydroxy function of substrates is indispensable to this chain elongation reaction (Table III). This is consistent with the fact that the 2′-hydroxy function commonly exists in most of lavandulylated flavonoid metabolites in leguminous and moraceous plants (Barron and Ibrahim, 1996).

It is noteworthy that LGDT, which is definitively a type of chain elongation prenyltransferase, is tightly bound to the membrane, similar to most prenyltransferases using aromatics as prenyl acceptor (Dhillon and Brown, 1976; Schröder et al., 1979; Biggs et al., 1987; Welle and Grisebach, 1991; Laflamme et al., 1993; Yamamoto et al., 1997, 2000; Mühlenweg et al., 1998), with an exception being olivetolate geranyltransferase (Fellermeier and Zenk, 1998), whereas previously reported prenyl diphosphate elongation enzymes are soluble proteins (Ogura and Koyama, 1998; Liang et al., 2002). Molecular biological investigation of LGDT having two unique features—a catalyzing prenyl side-chain elongation reaction and a membrane-bound chain elongation enzyme—would contribute to knowledge of the structural and functional properties of this novel prenyltransferase and provide some clues to the synthesis of novel bioactive branched prenyl side-chain-conjugated compounds.

### LGDT Is Localized in the Plastids

The plastids have long been accepted as a major subcellular site of isoprenoid metabolism, a fact underscored by the recent discovery that these organelles possess a DXP pathway for the production of the universal isoprenoid precursor IPP (Eisenreich et al., 1998). In the case of prenylated aromatics, it has been reported that the dimethylallyl group of several aromatic compounds (Goese et al., 1999; Stanjek et al., 1999; Asada et al., 2000) and the geranyl group of cannabinoids (Fellermeier et al., 2001) are derived from a DXP pathway and that the prenylations of isoflavonoids in French bean (*Phaseolus vulgaris*) and soybean (*Glycine max*; Biggs et al., 1990) and furanocoumarins in *Ruta graveolens* (Dhillon and Brown, 1976) occurred in the plastids. In contrast, in *Lithospermum erythrorhizon* cultured cells, GPP supplied from the mevalonate pathway (Li et al., 1998) in the ER/cytosol (Sommer et al., 1995) was utilized for the prenylation of *p*-hydroxybenzoic acid, which was associated with the ER (Yamaga et al., 1993).

The present subcellular localization studies clearly demonstrated that both activities of LGDT and N8PT coincided with that of UDP-Gal:diacylglycerol galactosyltransferase, the marker enzyme for the plastids (Douce et al., 1984), indicating that both prenyltransferases were localized in these organelles, whereas 2′OH, a characterized P450 monooxygenase between N8DT and LGDT in the biosynthetic pathway of SFG, is localized to the ER (Fig. 4). Because these two prenyltransferases only utilized DMAPP originated from DXP pathway (Yamamoto et al., 2002), they should be faced to inside of the plastids and not be located in the cytosolic side where the DMAPP supplied from mevalonate pathway could be used. These data indicate that a close cooperation between the plastids and the ER, which were sites of two dimethylallylations and 2′-hydroxylations, respectively, is absolute necessary for the formation of a lavandulyl group in SFG biosynthesis and may be important in regulating the production of SFG. Moreover, this result also implies that flavanone intermediates move between the membranes of these two organelles.

Figure 1 shows a model summarizing our current understanding on the formation of the lavandulyl group in SFG biosynthesis. In that, we postulate some intermediate/product transport mechanisms for the efficient production and accumulation of SFG, although simple diffusion may be also explainable. Naringenin synthesized in the cytosol/ER is transferred to the plastids for the first dimethylallylation, subsequently returned to the ER for the 2′-hydroxylation, and then transferred to the plastids again for the formation of a lavandulyl group by the second dimethylallylation. Finally, SFG as the final product accumulated in the cell wall of cultured cells (Yamamoto et al., 1996) or cork tissue of root system in intact plants (Yamamoto et al., 1992). The above-described connection between the mechanism of transport and targeting specificity is of great interest because a similar movement of intermediates/products between the plastids and the ER was also presumed in the biosynthesis of phytoalexin glyceollin and phaseollin in soybean and bean, respectively (Biggs et al., 1990).

## MATERIALS AND METHODS

### Chemicals

DMAPP was synthesized according to the method of Cornforth and Popjak (1969). IPP and GPP were a kind gift from Prof. Kazufumi Yazaki (Wood Research Institute, Kyoto University). LG, 8-dimethylallylnaringenin, euchrenone $a_7$, kenusanone I, and isovabachin were isolated from *Sophora flavescens* Ait. cells cocultured with cork tissues (Zhao et al., 2003). UDP-[$^{14}$C]Gal and antimycin A were purchased from Sigma-Aldrich (Tokyo), and cytochrome *c* was purchased from Nacalai Tesque (Kyoto). All reagents and solvents used were of analytical grade.

### Cell Cultures

The origin and subculturing of callus cultures and the establishment of cell-suspension cultures of *S. flavescens* were performed as described by Yamamoto et al. (1991, 1996, respectively).

### zEnzyme Preparation

*S. flavescens* cells were harvested by suction filtration after 6 to 8 d of cultivation in Murashige and Skoog medium (Murashige and Skoog, 1962) containing 1 $\mu$M 2,4-D and 1 $\mu$M kinetin. All further steps were performed at 4°C. The cells (5 g fresh weight) were ground in a mortar and pestle with 5 mL of 100 mM K-phosphate buffer (pH 6.5) containing 10 mM dithiothreitol (DTT), 0.5 g of polyvinylpolypyrrolidone, and sea sand. The homogenate was centrifuged at 12,000*g* for 20 min to remove cell debris, and the resulting supernatant was centrifuged at 156,000*g* for 40 min. The microsomal pellet was resuspended twice in 100 mM borate buffer (pH 8.8) containing 10 mM DTT, recentrifuged (156,000*g* for 40 min) and finally resuspended in 0.5 mL of the same buffer and used for enzyme assays.

Crude cell-free extracts were obtained by passing the 12,000*g* supernatant through a Sephadex G-25 column (PD-10, Amersham Biosciences, Tokyo) equilibrated with 100 mM borate buffer (pH 8.8) containing 10 mM DTT. The soluble fraction was also prepared by PD-10 using the 156,000*g* supernatant.

### Assay of LGDT by HPLC

The standard assay mixture contained in a total volume of 200 $\mu$L of 100 mM borate buffer (pH 8.8), 60 nmol LG (in 10 $\mu$L of ethanol), 200 nmol DMAPP, 2 $\mu$mol $MgCl_2$, and 50 $\mu$L of the microsomal fraction (approximately 60 $\mu$g of microsomal protein). In a control experiment, DMAPP was not added to the assay mixture. The reaction was initiated by the addition of LG to the mixture, and after the incubation for 30 min at 30°C, it was terminated by the addition of 50 $\mu$L of 6 N HCl. The reaction mixture was extracted with 200 $\mu$L of ethyl acetate containing 50 nmol 1-naphthaleneacetic acid as an internal standard. The amount of SFG in the ethyl acetate extract was analyzed by HPLC using a CAPCELL PAK C18 column (5 $\mu$m, 4.6 × 250 mm, Shiseido, Tokyo) in an oven at 40°C, with a methanol/water linear gradient solvent system containing 1% (v/v) acetic acid, from 54% to 90% (v/v) methanol in 20 min, at a flow rate of 0.9 mL $min^{-1}$, by monitoring the absorption at 294 nm. The quantities were calculated from the peak area at 294 nm recorded by a Chromatopac C-R4A (Shimadzu, Kyoto).

### Identification of the Reaction Product

The microsomal fraction obtained from 20 g of fresh cells was incubated with 2.4 $\mu$mol LG, 8 $\mu$mol DMAPP, and 80 $\mu$mol $MgCl_2$ in 100 mM borate buffer (pH 8.8, total volume of 8 mL) at 30°C for 2 h. The reaction was terminated by the addition of 2 mL of 6 N HCl, and the products were extracted with ethyl acetate (8 mL × 3). The organic layers were combined and concentrated in vacuo. The residue was dissolved in methanol and purified by preparative HPLC under the following conditions: column, same as above; solvent, methanol/water linear gradient solvent system containing 1% (v/v) acetic acid, from 54% to 74% (v/v) methanol in 40 min; flow rate, 0.9 mL $min^{-1}$; oven temperature, 40°C; and detection at 294 nm. The fraction around a retention time of 33 min was collected, evaporated in vacuo, and analyzed by an HPLC photodiode array system (MD-910, JASCO International, Hachioji, Japan) and EIMS (JMS DX-303, JEOL, Akishima, Japan).

SFG: UV $\lambda_{max}$ nm (log $\epsilon$, in methanol): 294 (4.5), 340sh (3.9); EIMS, *m/z*: 424 ($M_r$), 406, 301, 283, 209, 165, 136.

### Suc Density Gradient Centrifugation

For the separation of microsomal membranes, 10 mM DTT, 10 mM KCl, 1 mM $MgCl_2$, and 1 mM EDTA were added to the homogenization, resuspension, and centrifugation buffers and to the Suc gradient solution. The microsomal fractions prepared from 50 g of fresh cells were washed twice using 10 mM Tris-HCl buffer (pH 7.5, 8.6 g/100 mL Suc), resuspended in 1 mL of 10 mM Tris-HCl buffer (pH 7.5, 8.6 g/100 mL Suc), placed on the top of a Suc gradient solution (9 mL; 1.5 mL of 15%, 20%, 25%, 32%, 43%, and 50% [w/w] Suc in 10 mM Tris-HCl buffer [pH 7.5]) in a tube, and centrifuged for 90 min at 30,000 rpm in a swing-out rotor (SW40, Beckman, Japan). Equivalent fractions (0.75 mL) were removed from the top to the bottom of the gradient and analyzed for enzyme activity, Suc density, and protein content, respectively.

### Assays of LGDT, N8DT, 2′OH, and C4H by HPLC

LGDT activity was assayed as described above by using 50 $\mu$L of each gradient fraction, whereas the activities of N8DT and 2′OH were measured as described by Yamamoto et al. (2000, 2001). The C4H assay was initiated by adding NADPH at a final concentration of 1 mM to a reaction mixture (150 $\mu$L) containing 100 mM Tris-HCl buffer (pH 7.5), 1 mM trans-cinnamic acid, and 100 $\mu$L of each gradient fraction. After incubation for 1 h at 30°C, the reaction was stopped by adding 50 $\mu$L of 6 N HCl. The reaction mixture was extracted with 200 $\mu$L of ethyl acetate containing 20 $\mu$g of caffeic acid as an internal standard, and then the ethyl acetate extract was subjected to HPLC analysis.

The quantification of each enzyme assay was performed under the same HPLC conditions as in the LGDT assay except for the following modifications: N8PT, 1% (v/v) acetic acid containing acetonitrile/water linear gradient system from 40% to 70% (v/v) acetonitrile within 40 min; 2′OH, 1% (v/v) acetic acid containing acetonitrile/water gradient system from 20% to 70% (v/v) acetonitrile within 40 min; and C4H, 1% (v/v) acetic acid containing acetonitrile/water gradient system from 15% to 75% (v/v) acetonitrile within 40 min. Eluting substances were monitored spectrophotometrically at 294 nm (for N8PT and 2′OH assays) or 280 nm (for C4H assay).

### Marker Enzyme Assays

To ensure that subcellular organelle membrane fractions were successfully separated by Suc density gradient centrifugation, the following marker enzymes were assayed: plastids, UDP-Gal:diacylglycerol galactosyltransferase (Douce and Joyard, 1980); ER, antimycin A-insensitive NADH cytochrome *c* reductase (Yoshida, 1979); mitochondria, NADPH cytochrome *c* oxidase (Yoshida, 1979); tonoplast, $KNO_3$-sensitive ATPase (Uemura and Yoshida, 1986); and plasma membrane, $Na_3VO_4$-sensitive ATPase (Uemura and Yoshida, 1986).

### Protein Quantification

Protein contents were quantified using the Bradford assay (Bradford, 1976) with bovine serum albumin as the standard.

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