Molecular Characterization of a Membrane-bound Prenyltransferase Specific for Isoflavone from *Sophora flavescens*<sup>ab</sup>

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Prenylated isoflavones are secondary metabolites that are mainly distributed in legume plants. They often possess divergent biological activities such as anti-bacterial, anti-fungal, and anti-oxidant activities and thus attract much attention in food, medicinal, and agricultural research fields. Prenyltransferase is the key enzyme in the biosynthesis of prenylated flavonoids by catalyzing a rate-limiting step, *i.e.* the coupling process of two major metabolic pathways, the isoprenoid pathway and shikimate/polyketide pathway. However, so far only two genes have been isolated as prenyltransferases involved in the biosynthesis of prenylated flavonoids, namely naringenin 8-dimethylallyltransferase from *Sophora flavescens* (*SfN8DT-1*) specific for some limited flavanones and glycinol 4-dimethylallyltransferase from *Glycine max* (*G4DT*), specific for pterocarpan substrate. We have in this study isolated two novel genes coding for membrane-bound flavonoid prenyltransferases from *S. flavescens*, an isoflavone-specific prenyltransferase (*SfG6DT*) responsible for the prenylation of the genistein at the 6-position and a chalcone-specific prenyltransferase designated as isoliquiritigenin dimethylallyltransferase (*SfLDT*). These prenyltransferases were enzymatically characterized using a yeast expression system. Analysis on the substrate specificity of chimeric enzymes between *SfN8DT-1* and *SfG6DT* suggested that the determinant region for the specificity of the flavonoids was the domain neighboring the fifth transmembrane α-helix of the prenyltransferases.

Prenylated flavonoids are widely distributed in plant families of Leguminosae, Moraceae, Euphorbiaceae, Guttiferae, and Umbelliferae (1, 2). They have been isolated as active compounds from various medical plants because of their divergent biological activities, such as anti-bacterial, anti-fungal, anti-oxidant, anti-tumor, anti-skin aging, and estrogenic activities, and also the regulation of blood pressure via inhibition of NO production (3, 4). Thus, prenylated flavonoids draw an interest as natural medicines and lead compounds in the medical and food industries. The addition of an isoprenoid moiety renders higher activities in the flavonoid molecule than in the parent compounds from the pharmacological point of view (5–7). One of the proposed reasons for the enhanced biological activities of prenylated flavonoids is that the prenylation of the flavonoid core increases the lipophilicity and the membrane permeability of the compound.

Out of diverse flavonoid cores, prenylated isoflavones particularly draw the attention of many researchers as notably valuable compounds due to their attractive biological activities (1, 8). For instance, anthelmintic and phytoestrogen activities have been reported in prenylated isoflavonoids isolated from *Tadzhagi triquetrum* (9). Warangalone, lupalbigenin (6,3′-di-dimethylallyl genistein), and 6,8-di-dimethylallyl genistein, which were isolated from *Derris* species, are a potent inhibitor of cyclic AMP-dependent protein kinase in rat liver, whereas non-prenylated isoflavones do not have much effect as an inhibitor (5). In addition to those activities for medicinal uses, prenylated isoflavonoids show remarkable activity to protect plants, which is important in agricultural biology fields. For example, kievitone and phaseollidin produced by *Phaseolus vulgaris* (French bean), wighteone (6-dimethylallyl genistein), and lupalbigenin in *Lupinus albus* (white lupin) are prenylated isoflavonoids that function as “phytoalexins” (10–12). These compounds have been intensively studied from the 1980s in light of interactions between plants and their pathogenic fungi. Some fungi that are pathogenic to legumes are known to enzymatically detoxify their host isoflavonoids, *e.g.* *Fusarium solani* possesses hydrolase activities for kievitone and phaseollidin, which catalyze the hydration at the dimethylallyl moiety of these prenylated isoflavones to detoxify these compounds (10, 13). This is also an example that the prenyl moiety is crucial for the anti-fungal activities. However, until very recently, the genes responsible for the prenylation reaction of flavonoids have been in a large “black box” of secondary metabolism. In our recent study, the first cDNA encoding flavonone-specific plant prenyltransferase, naringenin 8-dimethylallyltransferase (*SfN8DT-1*) from *Sophora*

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The on-line version of this article (available at http://www.jbc.org) contains supplemental experimental procedures, Tables 1 and 2, and Figs. 1–4.

The genes reported in this paper have been deposited in the DNA Data Bank of Japan (DDBJ) under accession nos. AB604222 (*SfN8DT3* cDNA), AB604223 (*SfG6DT* cDNA), and AB604224 (*SfLDT* cDNA).

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**Isoflavone-specific Prenyltransferase**

![Diagram of flavonoid metabolism]

**FIGURE 1. Biosynthesis of prenylated chalcone and isoflavone in plants.** CHI, chalcone isomerase; IFS, 2-hydroxyisoflavone synthase; HID, 2-hydroxyisoflavone dehydratase.

**Experimental Procedures**

**Plant Materials and Reagents**—Cultured cells of *S. flavescens* were maintained in Murashige and Skoog’s medium with 1 μM 2,4-dichlorophenoxyacetic acid and 1 μM kinetin as described previously (16). Dimethylallyl diphosphate (DMAPP),3 geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP) were chemically synthesized as described previously (17).

**Isolation of cDNAs Homologous to *SfN8DT-1* from *S. flavescens* Cultured Cells**—Total RNA prepared from *S. flavescens* cell cultures treated with methyl jasmonate (final concentration 0.1 mM) was extracted with an RNeasy plant mini kit (Qiagen, Valencia, CA) and reverse-transcribed using a GeneRacer kit (Invitrogen). Reverse transcription (RT) products were subjected to rapid amplification of cDNA ends according to the manufacturer’s protocol. *SfN8DT-3, SfILDT, and SfG6DT* were obtained by rapid amplification of cDNA ends using primers specific for an EST sequence of cultured *S. flavescens* cells homologous to *SfN8DT-1* (first PCR, GeneRacer 5’ primer and 1C12-5’ race2; nested PCR, GeneRacer 5’ nested primer and 1C12-5’ race2) (supplemental Table S1). Their full-length clones were re-isolated by RT-PCR using the following primer pairs: N8DT-3-Fw and N8DT-3-Rv, iLDT-Fw and iLDT-Rv, and G6DT-Fw and G6DT-Rv. These three full-length cDNAs were subcloned into pENTR1A to give pENTR-N8DT-3, pENTR-iLDT, and pENTR-G6DT. These entry vector constructs were then subjected to the GATEWAY™ system to transfer the cDNAs into the yeast shuttle vector, pDR196 (18). These EST data are available on the website of the Plant Gene Database in the Research Institute for Sustainable Humanosphere (RISH), Kyoto University.

**Construction of Expression Vectors for Chimeric Enzymes**—Chimeric enzymes between *SfN8DT-1* and *SfG6DT* were created by crossover PCR. The cDNA fragments carrying the coding region of either *SfN8DT-1* or *SfG6DT* were amplified with Phusion high fidelity DNA polymerase (Finnzymes, Espoo, Finland). The primer pairs and templates in the first PCR are shown in supplemental Tables S1 and S2. These fragments in the first PCR were fused by crossover PCR using a specific primer: i.e. N8DT-1-Fw and G6DT-1-Rv for N1G to N4G, N8DT-1-Fw and G6DT-1-Rv for G1N to G4N, N8DT-1-Fw and G6DT-1-Rv for G1N to GNG, and G6DT-1-Fw and G6DT-1-Rv for GNG. Then, these fragments obtained by crossover PCR were subcloned into the pDR196 vector using the KpnI and NotI restriction site. Sequences of all chimeric cDNAs were completely confirmed by sequencing.

**Radioactive Assay of Flavonoid Prenyltransferase with TLC**—Three candidate clones and 10 chimeric enzymes of flavonoid prenyltransferases were expressed in the yeast strain W303-1A-delta-coq2, in which the p-hydroxybenzoate prenyltransferase gene (*coq2*) was disrupted (19), by cultivating in SD (selective dropout without uracil) liquid medium (180 ml) to reach mid-log phase, and the microsomal fraction of the transformed yeast was prepared as described previously (14). The resuspended membrane fraction from each transformant was used as an enzyme solution for the flavonoid prenyltransferase assay using various flavonoids (final concentration 1 mM) and [1-14C]DMAPP (final concentration 4.5 μM, specific activity 55 mCi/mmol, American Radiolabeled Chemicals, Inc.) as sub-

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3 The abbreviations used are: DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; EST, expressed sequence tag; ESI, electrospray mass ionization; N8DT, naringenin 8-dimethylallyltransferase; G6DT, genistein 6-dimethylallyltransferase; iLDT, isoliquiritigenin dimethylallyltransferase; iLDT-Rv for iLDT to G6DT, iLDT-Fw and G6DT-Rv for G1N, G6DT-Fw and G6DT-Rv for G2N, G6DT-Fw and G6DT-Rv for G3N, G6DT-Fw and G6DT-Rv for G4N, G6DT-Fw and G6DT-Rv for GNG. Then, these fragments obtained by crossover PCR were subcloned into the pDR196 vector using the KpnI and NotI restriction site. Sequences of all chimeric cDNAs were completely confirmed by sequencing.
 Isoflavone-specific Prenyltransferase Assay of Recombinant Proteins—Candidate clones were subcloned into a yeast shuttle vector pDR196 equipped with a strong constitutive promoter PMA1 (18) and were expressed in yeast strain W303-1A-delta-coq2, in which the endogenous aromatic substrate prenyltransferase coq2 was disrupted (19). The micromoles prepared from the transgenic yeast were incubated with 14C-labeled DMAPP and various flavonoids of different groups (naringenin, leachianone G, liquiritigenin, kaempferol, apigenin, taxifolin, naringenin chalcone, isoliquiritigenin, genistin, and maackiain; supplemental Fig. S1) as substrates in the presence of Mg2+ at 30 °C for 12 h. The enzymatic reaction products were detected using autoradiography, which showed that all three clones revealed clear dimethylallyltransferase activity for either flavonoid in a Mg2+-dependent manner.

The first clone, designated Sn8NDT-3, sharing 96% amino acid identity with Sn8NDT-1, exhibited almost the same enzymatic properties as Sn8NDT-1 including the substrate preference for flavonones (naringenin and liquiritigenin). The apparent Km values of Sn8NDT-3 for both substrates were 50 and 205 μM for naringenin and DMAPP, respectively. These data suggested functional redundancy of this clone with Sn8NDT-1. In fact, several bands were detected in the genomic Southern blot using the common sequence among Sn8NDT-1, -2, and -3 as a hybridization probe (supplemental experimental procedures, supplemental Fig. S2).

**RESULTS**

**Isolation of cDNAs Homologous to Sn8NDT-1 from Cultured S. flavescens Cells**—Our previous discovery of Sn8NDT-1 indicated that plant prenyltransferases for flavonoids belong to the homogentisate prenyltransferase family involved in vitamin E and plastoquinone biosynthesis (14). Thus, the sequence information of Sn8NDT-1 was expected to provide a very powerful tool for isolating cDNAs encoding prenyltransferase that recognized other flavonoids, e.g. isoflavone and chalcone. In this study, we have searched the EST information of cultured S. flavescens cells in the Plant Gene Database in RISH of Kyoto University, and found an EST clone homologous to Sn8NDT-1 (accession number YAK03A01NGRL0017-I05). Utilizing the internal sequence, we succeeded in amplifying three more cDNAs similar to the flavonoid prenyltransferases by RT-PCR and rapid amplification of cDNA ends. These clones coded for polypeptides of 391–410 amino acids having seven or nine putative transmembrane α-helices, which were predicted by the TMHMM program. These polypeptides also possessed a conserved aspartate-rich motif of prenyltransferases, NQXX-DXXD, in the second loop (L2), adding to another characteristic sequence conserved in the flavonoid/homogentisate prenyltransferases in loop 6 (L6), KD(I)LDXE(D)GD. Only one clone contained exceptionally a different aspartate-rich motif, NEXXDXXD.

A phylogenetic tree was made with these candidates and flavonoid prenyltransferases previously reported (SfN8DT-1, Sn8NDT-2 isolated from S. flavescens, G4DT from Glycine max) (14, 15), in addition to homogentisate prenyltransferases involved in vitamin E and plastoquinone biosynthesis (Fig. 2). All three candidate clones belong to the same clade of flavonoid prenyltransferases composed of Sn8NDT-1, Sn8NDT-2, and Gm4DT, which are clearly divergent from those of homogentisate prenyltransferases for vitamin E and plastoquinone biosynthesis. Polypeptide sequences encoded by these candidate cDNA shared 64–96% identities with the S. flavescens flavonoid prenyltransferases Sn8NDT-1 and Sn8NDT-2, whereas they showed lower amino acid sequence identities with Gm4DT (~48%), with prenyltransferases for vitamin E (33–55%) (22–25), and with those for plastoquinone (~20%).

Prenyltransferase Assay of Recombinant Proteins—Candidate clones were subcloned into a yeast shuttle vector pDR196 equipped with a strong constitutive promoter PMA1 (18) and were expressed in yeast strain W303-1A-delta-coq2, in which the endogenous aromatic substrate prenyltransferase coq2 was disrupted (19). The micromoles prepared from the transgenic yeast were incubated with 14C-labeled DMAPP and various flavonoids of different groups (naringenin, leachianone G, liquiritigenin, kaempferol, apigenin, taxifolin, naringenin chalcone, isoliquiritigenin, genistin, and maackiain; supplemental Fig. S1) as substrates in the presence of Mg2+ at 30 °C for 12 h. The enzymatic reaction products were detected using autoradiography, which showed that all three clones revealed clear dimethylallyltransferase activity for either flavonoid in a Mg2+-dependent manner.

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**Enzymatic Characterization Using HPLC or LC/ESI-MS**—The cold assay mixture contained 1 mm genistein, 2 mm DMAPP, 10 mm MgCl2, and the microsomal fraction in 0.1 M Tris-HCl buffer (pH 9.0), and after incubation for 60 min at 30 °C, the reaction mixture was extracted with ethyl acetate as described above. Substrate specificity was examined using LC/ESI-MS analysis, which covered the elution period of the predicted reaction products. LC/ESI-MS was carried out on a Shimadzu model 2010A system liquid chromatograph and LC/ESI-MS analysis, which covered the elution period of the predicted reaction products. LC/ESI-MS was carried out on a Shimadzu LC-10A system: solvent, hexane-acetonitrile-water (50:20:30); column, LiChrosphere 100RP-18 4 μm, specific to brand, 250×4.6 mm (Shimadzu, Kyoto, Japan); flow rate, 0.2 ml min−1. Samples were ionized by positive-ion mode from m/z 100–600. The enzymatic reaction product of SfG6DT gave the same retention time and MS spectrum as those of standard 6-dimethylallyl genistein. The reaction product of SfG6DT gave the same retention time and MS spectrum as those of standard 6-dimethylallyl genistein. The reaction product of SfG6DT gave the same retention time and MS spectrum as those of standard 6-dimethylallyl genistein.

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The second clone, showing 64% overall amino acid identity with SnN8DT-1, possessed a NEXXDXXXD motif, which is different from the consensus sequence NDXXDXXXD. When the enzymatic activity was assayed with various flavonoids of different types as prenyl acceptors, the recombinant protein of this cDNA revealed the dimethylallyltransferase activity for a chal-

FIGURE 2. Phylogenetic relationship of plant prenyltransferases accepting aromatic substrates. A rooted phylogram was generated using a ClustalW alignment. The abbreviations are: PF, prenylated flavonoid; VE, vitamin E; PQ, plastoquinone. Species abbreviations are: Ap, Allium porrum; At, Arabidopsis thaliana; Cp, Cuphea pulcherrima; Gm, G. max; Hi, H. lupulus; Hv, Hordeum vulgare; Os, Oryza sativa; Ta, Triticum aestivum. Homogentisate phytyltransferases (VTE-1) and homogentisate geranylgeranyltransferases (HGTT) are involved in vitamin E biosynthesis, and homogentisate solanesyltransferases (VTE-2) are involved in plastoquinone biosynthesis. Accession numbers are: ApVTE2-1, DQ231057; AtVTE2-1, AY089963; AtVTE2-2, DQ231060, CpVTE2-1, DQ231058; GmG4DT, AB434690; GmVTE2-1, DQ231059; GmVTE2-2, DQ231061; HiPT1, AB543053; HvHGTT, AY222860; OsHGTT, AY222862; SfG6DT, AB604224; SfLDT, AB604223; SnN8DT1, AB325579; SnN8DT2, AB370330; SnN8DT3, AB604222; TaHGTT, AY222861.

FIGURE 3. LC/ESI-MS analysis of enzymatic reaction products. A and C, ethyl acetate-soluble portion of enzyme incubation mixture of flavonoids and DMAPP with recombinant protein expressed in yeast (A, isoliquiritigenin dimethylallyltransferase assay using recombinant SflLDT; C, genistein dimethylallyltransferase assay using recombinant SfG6DT). Upper, total ion chromatogram (TIC); middle, selected ion monitoring for substrate [M + H]+; lower, selected ion monitoring for prenylated products [M + H]+. B and D, MS spectra of dimethylallylated products formed from flavonoids by recombinant protein (B, dimethylallyl isoliquiritigenin; D, 6-dimethylallyl genistein).
cone-type flavonoid, isoliquiritigenin (Fig. 3, A and B). The substrate specificity was further analyzed with various flavonoids as prenyl acceptors where 14C-labeled DMAPP was used as the prenyl donor. However, the recombinant protein could accept only isoliquiritigenin as its substrate and no other flavonoids, i.e., flavanones, flavonols, isoflavones, and other chalcones (e.g., naringenin chalcone, 2'-hydroxychalcone, 2'-hydroxycalcone; 4-hydroxychalcone; leachianone G; kae, kaempferol; api, apigenin; tax, taxifolin; geni, genistein; bioch, biochanin A; daid, daidzein; form, formononetin; and maa, maackiai. C and D, relative enzyme activity with various prenyl diphosphates of different chain length as substrates, DMAPP, GPP, FPP, and GGPP. N.D. indicates not detected. All experiments were replicated three times, and relative activity is shown as a percentage where S.D. is shown.

The third clone, sharing 64% amino acid identity with SnN8DT-1, possessed strong enzymatic activity transferring the dimethylallyl moiety to the 6-position of the isoflavone-type flavonoid, genistein (Fig. 3, C and D). The enzymatic reaction product, 6-dimethylallyl genistein, had the identical retention time and molecular mass representing the parent ion in LC/ESI-MS analysis with a standard specimen. This clone was then designated S. flavescens genistein 6-dimethylallyltransferase, SfG6DT, and subjected to further characterization.

**Enzymatic Characterization of SfG6DT Using HPLC and LC/MS**—SfG6DT polypeptide (407 amino acids) possesses a transit peptide-like sequence at the N terminus (52 amino acids) predicted by several subcellular localization programs. In the computer prediction, the putative polypeptide of SfG6DT has a similar membrane topology as other flavonoid prenyltransferases and as p-hydroxybenzoate prenyltransferases as well, i.e., it contains 7–9 transmembrane a-helices. The truncated protein of SfG6DT, in which N-terminal sequences were removed, showed almost the same activity as the full-length clone when expressed in yeast (data not shown). The enzymatic activity was, however, very weak in demonstrating further enzymatic characterization.
Prenyl donor, and the relative activity was measured with HPLC and LC/ESI-MS analyses. Recombinant SfG6DT could accept genistein (100%, 1.71 ± 0.3 nmol h⁻¹ mg of protein⁻¹) and biochanin A (37%) as substrates, which are both isoflavones, whereas other flavonoid types, i.e., flavanones, flavonols, and flavones, were not served as the substrate of SfG6DT (Fig. 4B and supplemental Fig. S1). Interestingly, other isoflavones, e.g., daidzein and formononetin, which are 5-deoxy isomers of genistein and biochanin A, could not be prenylated by SfG6DT, suggesting the narrow substrate specificity of this enzyme.

The substrate specificity of SfG6DT for prenyl donor was analyzed with genistein as the representative flavonoid substrate, and the relative activity was quantitatively analyzed by HPLC and LC/ESI-MS. Contrary to the flavanone-specific prenyltransferases SfN8DTs that accept only DMAPP as the prenyl donor (14), SfG6DT recognized DMAPP (100%), GPP (4.5%), and FPP (trace) as the prenyl substrates, indicating that the SfG6DT protein had broad substrate specificity for prenyl diphosphate when compared with other flavonoid-specific prenyltransferases isolated from plant species thus far (Fig. 4D).

Divalent cations were definitely required for the enzyme activity of SfG6DT, Mg²⁺ being most effective (100%) followed by Ni²⁺ (39%), Mn²⁺ (16%), and Ca²⁺ (7.9%) (supplemental Fig. S3A). The optimum temperature was about 30 °C, and the optimum pH was in a broad range between 8.0 and 10.0 (supplemental Fig. S3, B and C). The apparent Kₘ values for DMAPP and genistein were calculated to be 99 and 55 μM, respectively. These values are in a similar range as those of recombinant SfN8DT, GmG4DT protein, and native flavonoid prenyltransferases in other plant species (26, 27).

Analyses on Substrate Specificity with Chimeric Enzymes between SfN8DT-1 and SfG6DT—To clarify the determinant region responsible for the flavonoid substrate recognition in the G6DT polypeptide, we prepared 10 serial chimeric enzymes between SfG6DT and SfN8DT-1 in which amino acid sequences were recombined within the loop domains (Fig. 5A).

Utilizing these chimeric enzymes, the activities of both naringenin 8-dimethylallyltransferase (N8DT) and genistein 6-dimethylallyltransferase (G6DT) were measured with a standard protocol. Due to the unsuccessful protein expression and complete loss of enzyme activities, which often occur in membrane proteins having multiple transmembrane β-helices, only two clones, SfG2N and SfG3N, showed appreciable enzyme activities (Fig. 5, B and C). SfG2N possessed purely N8DT activity (0.99 ± 0.1 nmol h⁻¹ mg of protein⁻¹) despite the absence of the N-terminal sequence derived from N8DT (243 amino acids). Contrary to this, SfG3N possessed only G6DT activity (0.21 ± 0.02 nmol h⁻¹ mg of protein⁻¹), although its 33% of the C terminus is from N8DT polypeptide (Fig. 5B). Neither chimeric enzyme could catalyze the prenylation reaction for other types of flavonoid (kaempferol, apigenin, naringenin chalcone). These results strongly suggest that the determinant region for the flavonoid substrate specificity, either flavanone or isoflavone, is located between junctions 2 and 3, i.e. around the fifth transmembrane β-helix.

Expression of SfG6DT Genes in S. flavescens—In intact S. flavescens plants, a large number of prenylated flavonoids such as kuranorine, kushenol I, and sophoraflavanone G are exclusively accumulated in root tissues, in particular, in the root bark to cork layer (14, 28). In accordance with the accumulation pattern of prenylated flavonoids, SfN8DT-1 was exclusively expressed in the root bark tissue. In fact, SfG6DT mRNA was also solely detected in root tissues, and no detectable expression was seen in aerial tissues (supplemental Fig. 4A). However, SfG6DT mRNA was not detectable in the root bark tissue, but specifically accumulated in the peeled root, which is in clear contrast to that of SfN8DT-1 expression (supplemental Fig. 4B).

The prenyltransferase activity in cultured S. flavescens cells was inducible by the application of methyl jasmonate and yeast extract, which mimics defense reactions against insect and fungal attacks. When monitored by RNA gel blot analysis, SfG6DT expression in cultured cells was also strongly induced by only salicylic acid, but not by methyl jasmonate and yeast extract (supplemental Fig. 4C). This induction pattern was also apparently different from that of SfN8DT-1 expression, which was induced by any of these elicitors, methyl jasmonate, yeast extract, or salicylic acid. This result suggests that SfG6DT is associated with biotrophic resistance via a salicylic acid signaling pathway.

Subcellular Localization of SfG6DT—Two dimethylallyl groups of lupalbigenin (6,3’-di-dimethylallylated genistein) in S. flavescens cultured cells were biosynthesized via 2-C-methyl-d-erythritol 4-phosphate supplemental experimental procedures, pathway, which was shown by incorporating a study using 13C-labeled glucose (29). In the computer analysis, however, only WoLF PSORT gave high scores for a transit peptide at the N terminus when the full polypeptide sequence of SfG6DT was submitted to various prediction programs (Target P, ChloroP, and WoLF PSORT). To confirm the subcellular localization of SfG6DT, its N-terminal sequence (52 amino acids) was fused to green fluorescent protein (GFP), which was expressed under the control of the CaMV35S promoter in onion peels via a transient expression experiment with particle bombardment. As a positive control of plastid protein targeting, Waxy fused to DsRed (WxTP-DsRed) was used (21). In the transient expression experiment, the green fluorescence derived from SfG6DT-GFP was localized to dotted organelles in the epidermal peel cells of the onion, and the fluorescence pattern completely matched the red fluorescence derived from WxTP-DsRed (Fig. 6, A and C). Control GFP showed a fluorescence pattern typical of cytosol localization (Fig. 6B). This result indicates that the subcellular localization of SfG6DT in plant cells is plastid as the N-terminal sequence of SfG6DT functions as a transit peptide for the plastid sorting of the polypeptide.

DISCUSSION

Prenylation reaction of aromatic compounds is an important biosynthetic reaction step in plant secondary metabolism as it largely contributes to the structural diversity and biological activities of aromatic natural products (30, 31). Many prenylated aromatic compounds, such as flavonoids, coumarins, xanthones, and chlorogluconin, have been identified in various medicinal plants, and their biological and pharmacological activities have been studied. In particular, prenylated isoflavonoids found mainly in the Leguminosae family have drawn...
much attention as phytoalexins (32), in addition to some beneficial activities for humans (1, 8). Studies on plant prenyltransferase genes for the prenylation of native polyphenolic compounds has begun from the identification of SfN8DT-1, the first flavonoid-specific plant prenyltransferase. The sequence information of SfN8DTs and the GmG4DT potentially enables us to discover new prenyltransferase genes for other types of flavonoids, coumarins, xanthones, and so on.
In this study, isoflavone- and chalcone-specific prenyltransferase genes, *SfG6DT* and *SfiLDT*, respectively, have been isolated from *S. flavescens* by utilizing their genetic sequence information. These new membrane-bound prenyltransferases are grouped in a branch where legume flavonoid prenyltransferases such as *SfN8DT* (64% amino acid identity) and *GmG4DT* (48% identity) are clustered. The phylogenetic tree (Fig. 2) suggests that prenyltransferases for flavonoids, at least in legume plants, evolved from homogenitise prenyltransferases responsible for the biosynthesis of vitamin E. Further discoveries of flavonoid prenyltransferases from non-legume plant families will help to argue for the molecular evolution of this membrane-bound enzyme family in more detail. Moreover, identification of other new orthologues having different aromatic substrate preferences for e.g. phenylpropanes, xanthones, coumarins, and phloroglucinols is also highly expected to comprehend a thorough overview of the phylogenetic relationship of these prenyltransferases. One of those candidates is the prenyltransferase gene, *HIPT1*, isolated from hop (*Humulus lupulus*) (33), whose amino acid sequence is classified as a unique member (Fig. 2). This divergence may reflect the taxonomical distance of hop (Cannabinaceae) from Leguminosae or the difference in enzymatic functions such as different substrate preference. Partial sequence of prenyltransferase putatively involved in cannabinoid biosynthesis has also been found in *Cannabis sativa* (Cannabinaceae) EST data (34), and this partial sequence is similar to *HIPT1*. However, the possibility cannot be eliminated that other membrane-bound prenyltransferases, from the *p*-hydroxybenzoate prenyltransferase family involved in the biosynthesis of ubiquinone (35, 36) and naphthoquinone (19), serve another evolutionary origin for those divergent prenyltransferases from other aromatic substrates.

Because of the unique specificity for flavonoid substrates of plant-derived flavonoid prenyltransferases, *i.e.* flavanone-specific *SfN8DT*-1 and isoflavone-specific *SfG6DT* that share 64% amino acid identity with each other, we have narrowed down the dominant region for substrate specificity using chimeric enzymes between them. Because both *SfN8DT*-1 and *SfG6DT* possess almost an identical hydrophobicity profile, chimeric enzymes were generated by recombination at the loop domain (Fig. 5A). *SfG2N*, which possessed the N-terminal 240 amino acids of *SfG6DT* and the C-terminal 167 amino acids of *SfN8DT*-1 by connecting between transmembrane α-helices 4 and 5 (Fig. 5B), showed
the transferase activity of dimethylallyl moiety to narigenin, but not to genistein (Fig. 5C). Although SFG3N, which is a chimera of both enzymes between transmembrane α-helices 5 and 6, catalyzed the dimethylallylation of genistein at 6-position, there was no prenylation of naringenin. These results suggest that the sequence around transmembrane α-helix 5 is important in determining substrate specificity. However, in general, proteins having multiple transmembrane α-helices are very difficult to functionally express in heterologous organisms (37), and thus, other chimeric enzymes prepared in this study including SFIGN (the relevant domain of SFN8DT-1 at the transmembrane α-helix 5 was inserted into SFG6DT) did not show any activity.

For prenyl donor substrate specificity, SFG6DT accepted GPP and FPP adding to DMAPP as a prenyl donor. This broad substrate specificity for prenyl diphosphate is a unique property of SFG6DT and not observed in other flavonoid prenyltransferases reported thus far. In nature, several geranylated isoflavones occur, and SFG6DT is the first aromatic substrate prenyltransferase that accepts GPP as substrate in plants. In prokaryotes, some polyphenol prenyltransferases accepting GPP as prenyl donor have been cloned from Streptomyces (38, 39), but these are soluble proteins, and there is no significant sequence similarity with plant prenyltransferases. In addition, Streptomyces prenyltransferases as well as fungal prenyltransferases, reveal, in general, broad substrate specificity for aromatic compounds, which is another large difference from plant-derived prenyltransferases (40).

Similar to other flavonoid-specific prenyltransferases in plants, SFG6DT is also localized to plastids. It is noteworthy that the final prenylated products are mostly accumulated in apoplastic spaces, e.g. the cell wall, to the best of our knowledge (41). This suggests that after prenylation, the prenylated flavonoids mobilize from plastids to apoplast by transporting across both plastidial and plasma membranes.

In addition to SFG6DT, we have identified a cDNA coding for isoliquiritigenin (chalcone structure) dimethylallyltransferase (SfILDT) in this study. Thus, the isolation of serial genes coding for prenyltransferases, which specifically recognize either group of flavonoid (flavanone, isoflavone, chalcone, or pterocarpan) as prenyl acceptor, has been achieved. Although the native substrate of SfILDT may be different from isoliquiritigenin because prenylated isoliquiritigenin derivatives have not been identified in S. flavescens yet, production of various prenylated flavonoids became feasible by biotransformation using a yeast transformant expressing this prenyltransferase, as we previously showed with SFN8DT-1 (42). In application to plants, the isoflavone-specific prenyltransferase SFG6DT is expected to be a useful tool for metabolic engineering toward the molecular breeding of pathogen-resistant crops such as the pea, alfalfa, and lico-rice. Our recent study on the metabolic engineering using prenyltransferase genes in tomato fruits is an example (43). By choosing a combination of introduced prenyltransferase genes and the host plant, various prenylated polyphenols will be produced, for production of valuable compounds in transgenic plants as well as for the sake of breeding pathogen-resistant crops.

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**REFERENCES**

1. Tahara, S., and Ibrahim, R. K. (1995) *Phytochemistry* **38**, 1073–1094
2. Barron, D., and Ibrahim, R. K. (1996) *Phytochemistry* **43**, 921–982
3. Ahmed-Belkacem, A., Pozza, A., Muñoz-Martínez, F., Bates, S. E., Castaños, S., Gamarrro, F., Di Pietro, A., and Pérez-Victoria, J. M. (2005) *Cancer Res.* **65**, 4852–4860
4. Han, A. R., Kang, Y. J., Windonno, T., Lee, S. K., and Seo, E. K. (2006) *J. Nat. Prod.* **69**, 719–721
5. Wang, B. H., Ternai, B., and Polya, G. (1997) *Phytochemistry* **44**, 787–796
6. Maitrejean, M., Conte, G., Barron, D., El Kirat, K., Conseil, G., and Di Pietro, A. (2000) *Bioorg. Med. Chem. Lett.* **10**, 157–160
7. Murakami, A., Gao, G., Omura, M., Yano, M., Ito, C., Furukawa, H., Takashashi, D., Koshimizu, K., and Ohigashi, H. (2000) *Bioorg. Med. Chem. Lett.* **10**, 59–62
8. Bottla, M., Menendez, P., Zappia, G., de Lima, R. A., Torge, R., and Monachea, G. D. (2009) *Curr. Med. Chem.* **16**, 3414–3468
9. Wang, X., Li, R. T., Mao, Y. L., Zhang, H. J., Li, S. H., Song, Q. S., and Sun, H. D. (2005) *J. Agric. Food Chem.* **53**, 267–271
10. Turbek, C. S., Smith, D. A., and Schardl, C. L. (1992) *FEMS Microbiol. Lett.* **94**, 187–190
11. Zahringer, U., Ebel, J., Kreuzaler, F., and Grisebach, H. (1977) *H-S Z Physiol. Chem.* **358**, 1303–1304
12. Laflamme, P., Khouri, H., Gulick, P., and Ibrahim, R. (1993) *Phytochemistry* **34**, 147–151
13. Ahn, B. Z., Baik, K. U., Kweon, G. R., Lim, K., and Hwang, B. D. (1995) *J. Med. Chem.* **38**, 1044–1047
14. Sasaki, K., Mitto, K., Ohara, K., Yamamoto, H., and Yazaki, K. (2008) *Plant Physiol.* **146**, 1075–1084
15. Akashi, T., Sasaki, K., Aoki, T., Ayabe, S., and Yazaki, K. (2009) *Plant Physiol.* **149**, 683–693
16. Yamamoto, H., Kawai, S., Mayumi, J., Tanaka, T., Inuma, M., and Mizuno, M. (1991) *Z. Naturforsch. C.* **46**, 172–176
17. Cornforth, R. H., and Popjak, G. (1969) *Methods Enzymol.* **15**, 359–390
18. Rentsch, D., Laloi, M., Rouhara, I., Schmelzer, E., Delrot, S., and Frommer, W. B. (1995) *FEMS Lett.* **370**, 264–268
19. Yazaki, K., Kunihisa, M., Fujisaki, T., and Sato, F. (2002) *J. Biol. Chem.* **277**, 6240–6246
20. Sasaki, K., Ohara, K., and Yazaki, K. (2005) *FEBS Lett.* **579**, 2514–2518
21. Kitajima, A., Asatsuma, S., Okada, H., Hamada, Y., Kaneko, K., Nanjo, Y., Kawagoe, Y., Toyooka, K., Matsuoka, K., Takeuchi, M., Nakano, A., and Mitsu, T. (2009) *Plant Cell* **21**, 2844–2858
22. Collakova, E., and DellaPenna, D. (2001) *Plant Physiol.* **127**, 1113–1124
23. Schledz, M., Seidler, A., Beyer, P., and Neuhaus, G. (2001) *FEBS Lett.* **499**, 15–20
24. Savidge, B., Weiss, J. D., Wong, Y. H., Lasser, M. W., Mitsu, T. A., Shewmaker, C. K., Post-Beittenmiller, D., and Valentín, H. E. (2002) *Plant Physiol.* **129**, 321–332
25. Cahoon, E. B., Hall, S. E., Ripp, K. G., Ferrari, M., Hitz, W. D., and Coughlan, S. J. (2003) *Nat. Biotechnol.* **21**, 1082–1087
26. Dhillon, D. S., and Brown, B. A. (1976) *Arch. Biochem. Biophys.* **177**, 74–83
27. Vitali, A., Giardina, B., Delle Monache, G., Rocca, F., Silvestrini, A., Tafi, A., and Botta, B. (2004) *FEBS Lett.* **557**, 33–38
28. Yamamoto, H., Ichimura, M., Ishikawa, N., Tanaka, T., Inuma, M., and
Isoflavone-specific Prenyltransferase

Mizuno, M. (1992) Z. Naturforsch. C 47, 535–539
29. Yamamoto, H., Zhao, P., and Inoue, K. (2002) Phytochemistry 60, 263–267
30. Heide, L. (2009) Curr. Opin. Chem. Biol. 13, 171–179
31. Yazaki, K., Sasaki, K., and Tsurumaru, Y. (2009) Phytochemistry 70, 1739–1745
32. Daniel, M., and Purkayastha, R. P. (eds) (1995) Handbook of Phytoalexin Metabolism and Action, pp. 333–373, CRC Press, Inc., Boca Raton, FL
33. Tsurumaru, Y., Sasaki, K., Miyawaki, T., Mommma, T., Umemoto, N., and Yazaki, K. (2010) Plant Biotechnol. 27, 199–204
34. Marks, M. D., Tian, L., Wenger, J. P., Omburo, S. N., Soto-Fuentes, W., He, J., Gang, D. R., Weiblen, G. D., and Dixon, R. A. (2009) J. Exp. Bot. 60, 3715–3726
35. Okada, K., Ohara, K., Yazaki, K., Nozaki, K., Uchida, N., Kawamukai, M., Nojiri, H., and Yamane, H. (2004) Plant Mol. Biol. 55, 567–577
36. Ohara, K., Yamamoto, K., Hamamoto, M., Sasaki, K., and Yazaki, K. (2006) Plant Cell Physiol. 47, 581–590
37. Ohara, K., Muroya, A., Fukushima, N., and Yazaki, K. (2009) Biochem. J. 421, 231–241
38. Kuzuyama, T., Noel, J. P., and Richard, S. B. (2005) Nature 435, 983–987
39. Haagen, Y., Unsöld, I., Westrich, L., Gust, B., Richard, S. B., Noel, J. P., and Heide, L. (2007) FEBS Lett. 581, 2889–2893
40. Li, S. M. (2009) Appl. Microbiol. Biotechnol. 84, 631–639
41. Yamamoto, H., Yamaguchi, M., and Inoue, K. (1996) Phytochemistry 43, 603–608
42. Sasaki, K., Tsurumaru, Y., and Yazaki, K. (2009) Biosci. Biotechnol. Biochem. 73, 759–761
43. Koeduka, T., Shitan, N., Kumano, T., Sasaki, K., Sugiyama, A., Linley, P., Kawasaki, T., Ezura, H., Kuzuyama, T., and Yazaki, K. (2011) Plant Biol. 13, 411–415