Molecular Cloning and Characterization of a Geranyl Diphosphate-Specific Aromatic Prenyltransferase from Lemon[1][W]

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Prenyl residues confer divergent biological activities such as antipathogenic and antiherbivorous activities on phenolic compounds, including flavonoids, coumarins, and xanthones. To date, about 1,000 prenylated phenolics have been isolated, with these compounds containing various prenyl residues. However, all currently described plant prenyltransferases (PTs) have been shown specific for dimethylallyl diphosphate as the prenyl donor, while most of the complementary DNAs encoding these genes have been isolated from the Leguminosae. In this study, we describe the identification of a novel PT gene from lemon (*Citrus limon*), *ClPT1*, belonging to the homogentisate PT family. This gene encodes a PT that differs from other known PTs, including flavonoid-specific PTs, in polypeptide sequence. This membrane-bound enzyme was specific for geranyl diphosphate as the prenyl donor and coumarin as the prenyl acceptor. Moreover, the gene product was targeted to plastid in plant cells. To our knowledge, this is the novel aromatic PT specific to geranyl diphosphate from citrus species.

Prenylation is an important derivatization of plant aromatics, contributing to the chemical diversification of phenolic secondary metabolites in plants due to differences in prenylation positions, prenyl chain lengths, and further modifications of prenyl chains. To date, about 1,000 prenylated aromatic compounds have been isolated as biologically active substances from various plant species, including many medicinal plants.

Coumarins (α-benzopyrones) are a large group of plant secondary metabolites. Many biologically active coumarins are prenylated, with the prenyl residue enhancing the biological activities of the aromatic core compound. For example, imperatorin (dimethylallylated xanthotoxol), a strong inhibitor of a *Manduca sexta* midgut cytochrome P450, has 100-fold greater activity than the nonprenylated coumarin compound, suggesting that prenylation is involved in chemoprevention against biotic stress in plants (Neal and Wu, 1994). Prenylated compounds are also beneficial for human health. For example, geranylation of umbelliferone at the OH position to form auraptene results in a 25-fold enhancement of the inhibition of Epstein Barr virus activity, a test used to screen antitumor compounds (Murakami et al., 1997). Moreover, in tuberculosis, 8-geranyloxypsoralen was reported to decrease the growth rate of *Mycobacterium smegmatis* (Adams et al., 2006).

There are many reports on the detection of prenyltransferase (PT) activities for coumarins in various plant species. For example, umbelliferone-dimethylallyltransferase activities were reported in cultured parsley (*Petroselinum crispum*) cells, *Ruta graveolens*, and *Ammi majus*, and plastidial localization of the enzyme activity is also reported (Ellis and Brown, 1974; Dhillon and Brown, 1976; Tietjen and Matern, 1983; Hamerski and Matern, 1988; Hamerski et al., 1990). In addition, bergaptol 5-*O*-geranyltransferase activity, which yields bergamottin, a

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major coumarin derivative, was characterized using the microsomal fraction of lemon (*Citrus limon*) peel flavedo, the outer part of the lemon fruit (Frérot and Decorzant, 2004; Munakata et al., 2012). In the lemon flavedo, 8-geranyltransferase activity for umbelliferone was also detected (Munakata et al., 2012). To date, only one gene encoding these enzymes has been described; this gene, which encodes a parsley PT (PcPT), was very recently isolated (Karamat et al., 2014).

The first flavonoid-specific PT identified was naringenin 8-dimethylallyltransferase (SfN8DT1) from a leguminous medicinal plant, *Sophora flavescens* (Sasaki et al., 2008). Since then, genes encoding various flavonoid PTs have been identified in Leguminosae (Akashi et al., 2009; Sasaki et al., 2011; Shen et al., 2012). Although other prenylated aromatic compounds, including coumarins, xanthons, phenylpropanoids, and phloroglucinols, have been isolated from many plant species, no gene encoding a PT for those aromatics has been isolated, except for the gene encoding a phloroglucinol-specific enzyme (HlPT1) from hops (*Humulus lupulus*) and a the recently isolated coumarin dimethylallyltransferase from parsley (Tsurumaru et al., 2010, 2012; Karamat et al., 2014). These isolated plant aromatic PTs show strong preference for dimethylallyl diphosphate (DMAPP) as the prenyl donor substrate, although in nature, many geranylated phenolics and less farnesylated phenolics have been described. This raises questions about the enzymes and reaction mechanisms involved in the synthesis of these phenolic compounds, such as substrate specificity and prenylation sites. Better understanding of these reactions requires the identification of PTs with other enzymatic activities. It is also necessary to identify PTs producing prenylated phenolics in nonleguminosaeous plants. Four different tracks should be explored to identify enzymes that (1) recognize nonflavonoid substrates, e.g. coumarins, phenylpropanoids, and xanthons, (2) are specific for longer chain prenyl diphosphates such as geranyl diphosphate (GPP) and farnesyl diphosphate (FPP), (3) are from nonlegume origins, and (4) catalyze *O*-prenylation.

Citrus species, including lemons, contain large quantities of geranylated coumarins. We therefore isolated a complementary DNA (cDNA) encoding a PT from lemon peel, identifying the novel PT-encoding gene *ClPT1*. Phylogenetic analysis showed that this enzyme shares homologies with homogentisate PTs involved in vitamin E and plastoquinone biosynthesis but is located in a new clade. We provide evidence showing that this unique enzyme is highly specific for GPP as a prenyl donor and coumarin as a prenyl acceptor. We also show that the gene product is targeted to plastid in plant cells.

RESULTS

Identification of a Candidate cDNA Encoding Coumarin-Specific PT from Lemon

To isolate cDNA candidates encoding PTs from lemon, we designed degenerated primers based on the conserved amino acid sequences in the PT family (Supplemental Table S1) and used to amplify cDNA prepared from the flavedo of a commercially available lemon. Of the resulting cDNAs, one encoded an amino acid sequence similar to that of homogentisate PT. The full-length coding sequence was obtained by 5′- and 3′-RACE. This cDNA, which encodes a protein of 407 amino acid residues, was tentatively designated as ClPT1. Because the lemon cultivar used to isolate this cDNA could not be formally identified later, we reisolated the gene from the RNA of a Lisbon lemon. This cDNA was designated *ClPT1a*, whereas the former was designated *ClPT1b*.

The polypeptide sequences encoded by *ClPT1a* and *ClPT1b* were of the same length and 99% identical, with a mismatch of four amino acids in their C-terminal regions (Supplemental Fig. S1A). Further analysis was mainly performed using ClPT1a, hereafter designated ClPT1. In silico translation of the coding sequence showed two Asp-rich motifs, NQxxDxxID and KD(I/L)PDx(E/D)GD, the former conserved among plant-derived PTs for aromatics and the latter only found among homogentisate PT family members (Supplemental Fig. S1B). TMHMM Server v. 2.0 predicted that the ClPT1 polypeptide has multiple transmembrane α-helices. Various computer programs (iPSORT, PSORT, WoLF PSORT, and ChloroP) were used for in silico analysis of the subcellular localization of ClPT1. iPSORT predicted that ClPT1 has a transit peptide at its N terminus, and PSORT found a putative cleavage site between G36 and G37 (Supplemental Fig. S1A).

Phylogenetic analysis showed that the polypeptide sequence of ClPT1 shares moderate identity with PTs involved in vitamin E biosynthesis (Arabidopsis [*Arabidopsis thaliana*] homogentisate phytyltransferase2-1 [AtVTE2-1], 39%; *Hordeum vulgare* homogentisate geranylgeranyltransferase [HvHGGT], 37%), enzymes involved in the biosynthesis of furanocoumarins (PcPT, 36%), prenylated flavonoid (SfN8DT1, 32%), plastoquinone (*Chlamydomonas reinhardtii* homogentisate solanesyltransferase [CrHST], 26%), bitter acid (HlPT1, 21%), shikonin (*Lithospermum erythrorhizon p*-hydroxybenzoate geranyltransferase1 [LePGT1], 11%), and ubiquinone (*Oryza sativa p*-hydroxybenzoate polyprenyltransferase1 [OsPPT1], 10%; Collakova and DellaPenna, 2001; Savidge et al., 2002; Yazaki et al., 2002; Ohara et al., 2006; Sadre et al., 2006; Sasaki et al., 2008; Tsurumaru et al., 2010; Yang et al., 2011; Karamat et al., 2014). ClPT1 could not be grouped into any previously identified clade, including membrane-bound PTs (Fig. 1; Supplemental Fig. S1B).

Functional Expression of ΔTP-ClPT1 in Yeast and Its Enzymatic Characterization

Because the presence of an N-terminal transit peptide may decrease the efficiency of expression in yeast (*Saccharomyces cerevisiae*; e.g. soybean [*Glycine max*; Gm] glycinol 4-dimethylallyltransferase [GmG4DT] and lupine [*Lupinus albus*] LaPT1; Akashi et al., 2009; Shen et al., 2012), we generated a deletion mutant lacking the nucleotide sequence corresponding to the 36 N-terminal

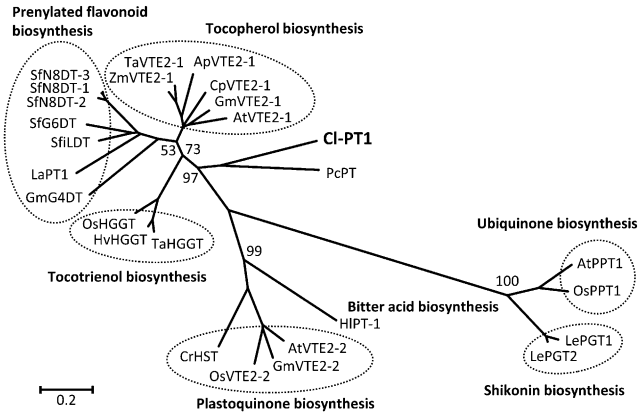

Figure 1. Phylogenetic tree showing the association of ClPT1 and other plant-derived PTs for aromatic substrates. A neighbor-joining phylogram for amino acid sequences of ClPT1 and related PTs accepting aromatic substrates was drawn using a ClustalW alignment, followed by 1,000 bootstrap tests using MEGA5 software. Bootstrap values (maximum 100) are shown only at nodes generating clades. The scale bar represents 0.2 of amino acid substitution per site. Abbreviations for species are: Ap, *Allium porrum*; At, *Arabidopsis thaliana*; Cp, *Cuphea pulcherrima*; Cr, *Chlamydomonas reinhardtii*; Gm, soybean; Hl, hops; Hv, *Hordeum vulgare*; La, lupine; Le, *Lithospermum erythrorhizon*; Os, *Oryza sativa*; Pc, parsley; Sf, *S. flavescens*; Ta, *Triticum aestivum*; and Zm, *Zea mays*. Homogentisate phytyltransferases (VTE2-1s) and homogentisate geranylgeranyltransferases (HGGTs), homogentisate solanesyltransferases (HST and VTE2-2s), and *p*-hydroxybenzoate polyprenyltransferases (PPTs) are involved in the biosynthesis of vitamin E, plastoquinone, and ubiquinone, respectively. Accession numbers are: ApVTE2-1, DQ231057; AtPPT1, AB052533; AtVTE2-1, AY089963; AtVTE2-2, DQ231060; ClPT1, AB813876; CpVTE2-1, DQ231058; CrHST, AM285678; GmG4DT, AB434690; GmVTE2-1, DQ231059; GmVTE2-2, DQ231061; HlPT1, AB543053; HvHGGT, AY222860; LaPT1, JN228254; LePGT1, AB055078; LePGT2, AB055079; OsHGGT, AY222862; OsPPT1, AB263291; PcPT, AB825956; *S. flavescens* isoliquiritigenin dimethylallyltransferase (SfiLDT), AB604223; *S. flavescens* genistein 6-dimethylallyltransferase (SfG6DT), AB604224; SfN8DT1, AB325579; SfN8DT2, AB370330; SfN8DT3, AB604222; TaHGGT, AY222861; TaVTE2-1, DQ231056; ZmVTE2-1, DQ231055; and OsVTE2-2, gi|50938601.

amino acids. This *delta transit peptide* (*ΔTP*)-*ClPT1* fragment was inserted into the pDR196 shuttle vector, and the corresponding protein was expressed in yeast. The microsomal fraction prepared from the transgenic yeast was incubated with various substrate combinations in the presence of $MgCl_2$ at 30°C for 60 min. Incubation with umbelliferone and GPP as substrates resulted in the generation of 8-geranylumbelliferone (8GU; 13 ± 1 nmol h^{-1} mg^{-1} of protein). The reaction product was identified by direct comparison with a standard specimen of 8GU (Fig. 2, A–C). A second monogeranylated umbelliferone was also detected but at a much lower quantity (0.9 ± 1 nmol h^{-1} mg^{-1} of protein; Fig. 2, B and C). This product may be 6-geranylumbelliferone (6GU) but could not be identified due to the unavailability of a standard specimen. These products were not observed in the absence of GPP, umbelliferone, or enzyme, with heat-denatured enzyme (95°C for 20 min), or in presence of 10 mM EDTA instead of $MgCl_2$ (Supplemental Fig. S2). No products resulted when the substrates were incubated with the microsomal fraction of the yeast transformed with the pDR196 empty vector (Fig. 2B). The effect of the deleted N-terminal 36 amino acids was evaluated by expressing the full-length ClPT1 in yeast; the same enzymatic reaction products were observed, but the activity was much lower than that of ΔTP-ClPT1 (Supplemental Fig. S3). We also assessed the catalytic activity of ClPT1b, which was isolated from an unidentified lemon cultivar and had a four-amino acid difference. Truncated ΔTP-ClPT1b expressed in yeast had the same enzymatic activity, including substrate and product specificities, as truncated ΔTP-ClPT1 (data not shown).

In silico analysis of ClPT1 using the TMHMM program predicted that this protein contains multiple transmembrane domains. Ultracentrifugation of the cell-free extract of yeast ΔTP-ClPT1 at 100,000*g* for 30 min resulted in the complete recovery of umbelliferone 8-C-geranyltransferase (U8GT) activity in the microsomal fraction (Fig. 2D), consistent with the native U8GT activity detected in lemon flavedo (Supplemental Fig. S4). The yeast recombinant protein of ΔTP-ClPT1 had apparent mean ± SE K_m values for umbelliferone and GPP of 6.1 ± 0.3 and 4.8 ± 0.6 μM, respectively. These values for both the prenyl acceptor and donor were much lower than those reported for aromatic PTs of flavonoids and phloroglucinols (Sasaki et al., 2008, 2011; Akashi et al., 2009; Shen et al., 2012; Tsurumaru et al., 2012).

We also analyzed the substrate specificity of recombinant ΔTP-ClPT1 for prenyl acceptors by using several aromatic compounds (Fig. 3A), with GPP used as the prenyl donor. Recombinant ΔTP-ClPT1 catalyzed the geranylation of umbelliferone, esculetin, 5,7-hydroxycoumarin, and 5-methoxy-7-hydroxycoumarin, yielding a major and a minor reaction product for each coumarin substrate (Fig. 3B; Supplemental Fig. S5). Tandem mass spectrometry (MS^2) analyses in the positive ion mode showed that all of these products were *C*-geranylated compounds because they had lost a 124 mass unit due to the cutoff of C_9H_{16} in the geranyl chains, a finding specific to geranyl groups bound to an aromatic ring by C-C bonds (Pannala and Rice-Evans, 2001; Supplemental Fig. S5). The specificity for prenyl acceptors of the enzyme was also assessed by testing other coumarin derivatives, phenylpropanoids with hydroxylation patterns corresponding to umbelliferone, esculetin, and 5,7-dihydroxycoumarin, as well as flavonoids and homogentisate (Fig. 3A), but none was accepted by ΔTP-ClPT1 as a prenyl acceptor (Fig. 3B). Prenyl donor specificity was assessed by using DMAPP, with umbelliferone and esculetin as acceptors, but no reaction products could be detected (Fig. 3C). Longer prenyl donors, FPP and geranylgeranyl diphosphate (GGPP), were also tested but they were not accepted as prenyl substrates for this enzyme (Supplemental Fig. S6).

Metabolite Analysis of Transgenic *R. graveolens* Plants Overexpressing *ClPT1*

The in vivo function of ClPT1 was investigated by comparing metabolites of transgenic *R. graveolens* plants

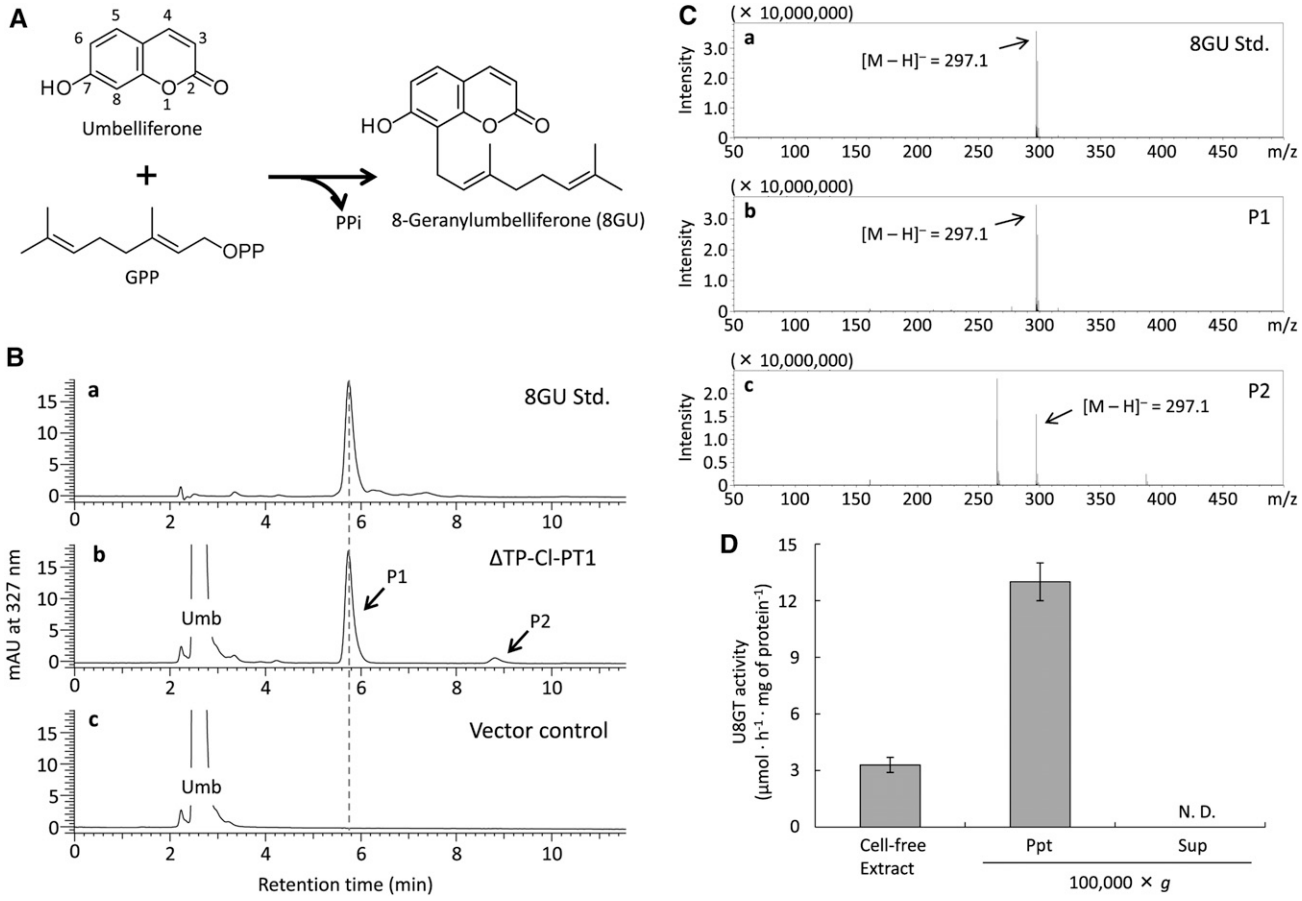

Figure 2. Identification of enzymatic reaction products of recombinant ΔTP-ClPT1. A, Full assay of the U8GT reaction. B, UV chromatograms of the reaction products detected at 327 nm. a, A standard specimen of 8GU. b, Reaction mixtures with the crude enzymes from yeast harboring pDR196-ΔTP-ClPT1. c, pDR196 empty vector as a negative control. The dotted line indicates the retention time of standard 8GU (5.7 min). P1 (retention time, 5.7 min) and P2 (retention time, 8.8 min) are the enzymatic reaction products formed with ΔTP-ClPT1. C, Mass spectra of enzyme reaction products in LC-IT-TOF/MS analysis; standard specimen of 8GU (a), P1 (b), and P2 (c). The negative ion mode was used to identify enzymatic reaction products. D, Membrane localization of ΔTP-ClPT1. Cell-free extract, microsome (Ppt), and soluble fraction (Sup) following ultracentrifugation at 100,000*g* were assayed. Values are means ± SD of triplicate experiments. N. D., Not detected; Umb, umbelliferone; Std., standard; PPi, diphosphate; mAU, m absorbance unit.

overexpressing *ClPT1* and plants transformed with the empty binary vector pBIN as a negative control. The presence of the heterologous DNA insertion *Cauliflower mosaic virus 35S promoter* (*CaMV35Spro*)-*ClPT1* and empty vector was confirmed by genomic PCR, and the expression of *ClPT1* was monitored by real-time PCR as described in "Materials and Methods." These analyses found that *ClPT1* mRNA was highly expressed in five independent transgenic plants, which were subjected to further detailed metabolite analysis. Coumarin derivatives were extracted from whole plantlets of these transgenic lines and quantitatively analyzed by liquid chromatography (LC)/mass spectrometry (MS). In addition to 8GU, we focused on five representative molecules of the furanocoumarin pathway: umbelliferone, psoralen, bergapten, xanthotoxin, and isopimpinellin. Quantitative comparison of those metabolites showed that the content of umbelliferone, an in vivo substrate, was significantly lower in transgenic plants overexpressing *ClPT1* (0.4 ± 0.3 ng g^{-1} fresh weight [FW]) than in control plants (1.7 ± 1.5 ng g^{-1} FW). This decrease is correlated with the appearance of 8GU, which was undetectable in the transgenic plants carrying the empty vector (Fig. 4, A and B). No significant change was, however, observed for the four other furanocoumarin derivatives (Fig. 4C) in transgenic *R. graveolens*, which is possibly related to the endogenous regulation of the coumarin intermediate (umbelliferone) and the final products. Furanocoumarins shown in Figure 4C are major coumarin compounds accumulated in *R. graveolens* as final products, and their levels are more than 10 ng g^{-1} FW, whereas umbelliferone is an intermediate to be converted to furanocoumarins and 8GU in transgenic *R. graveolens*, and the endogenous level is 10 times lower than the final products. Due to the additional utilization of umbelliferone for 8GU by ClPT1, its level decreased significantly, but the plant is probably

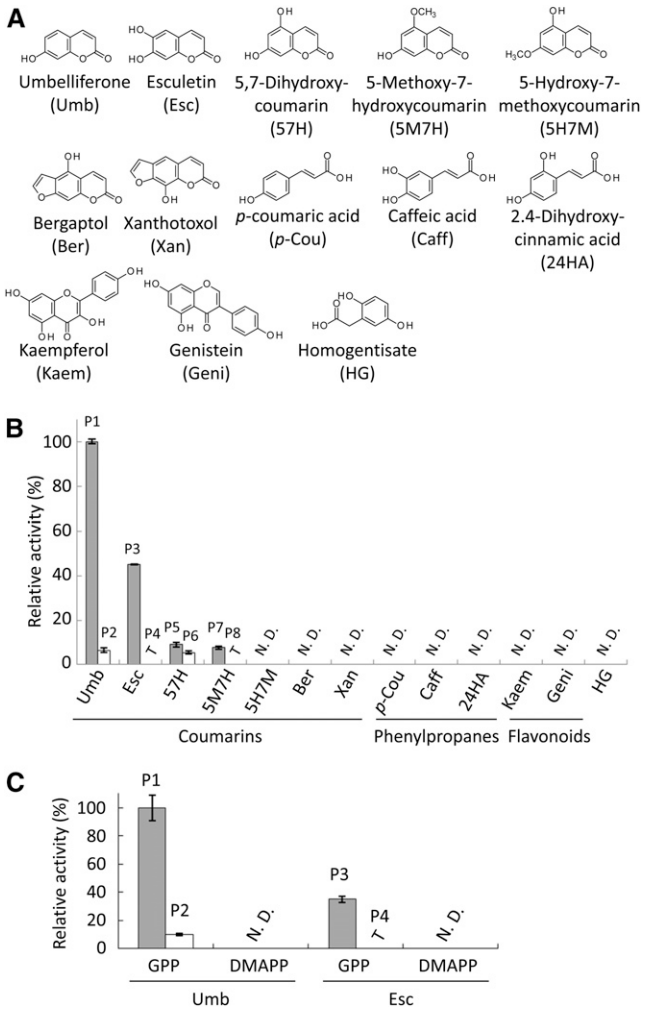

Figure 3. Substrate specificity of recombinant ΔTP-ClPT1. A, Chemical structures of aromatic substrates used in the experiments. B, Geranyltransferase activities with various prenyl acceptors. C, Substrate preference for prenyl donors using umbelliferone and esculetin as representative donors. The quantities of geranylated esculetin, 5,7-dihydroxycoumarin, and 5-hydroxy-7-methoxycoumarin were equivalent to those of the corresponding coumarin substrates, and the quantities of by-products derived from geranyltransferase activity for umbelliferone were equivalent to those of 8GU. Relative activities compared with U8GT activity (100%) are shown as percentages. P1 to P8 represent reaction products. Values are means ± SD of triplicate experiments. T, Trace; N. D., not detected; Umb, umbelliferone; Esc, esculetin; 57H, 5,7-dihydroxycoumarin; 5M7H, 5-hydroxy-7-methoxycoumarin; 5H7M, 5-hydroxy-7-methoxycoumarin; Ber, bergaptol; Xan, xanthotoxol; *p*-Cou, *p*-coumaric acid; Caff, caffeic acid; 24HA, 2,4-dihydroxycinnamic acid; Kaem, kaempferol; Geni, genistein; HG, homogentisate.

able to maintain the normal level of final products. A similar result was also observed in our earlier study using PcPT (Karamat et al., 2014).

Detection of 8GU in Lemon

To date, the enzymatic reaction product of ClPT1, 8GU, has not been detected in citrus so far, although this compound has been isolated from other genera of Rutaceae, including from the aerial parts of *Eriostemon tomentellus* and *Boronia lanceolata*, the leaves of *Boronia inornata* and *Luvunga sarmentosa*, and the root bark of *Severinia buxifolia* and *Pamburus missionis* (Rashid et al., 1992; Ahsan et al., 1994, 1995; Kumar et al., 1994; Chen et al., 2001; Lien et al., 2002). To confirm the presence of 8GU in lemon, the freeze-dried flavedo was extracted with methanol and analyzed by HPLC. Little 8GU was detectable by UV monitoring, whereas large amounts of 8-geranyloxypsoralen, bergamottin, and 5-geranyloxy-7-methoxycoumarin were detected as primary coumarin derivatives (8-geranyloxypsoralen, 490 ± 10 μg g^{-1} dry weight [DW]; bergamottin, 701 ± 7; and 5-geranyloxy-7-methoxycoumarin, 337 ± 5). More sensitive LC-ion trap (IT)-time-of-flight (TOF)/MS analysis was therefore performed, with a peak corresponding to 8GU detected in mass chromatograms (Fig. 5). Similar results were obtained using methanol extracts from freeze-dried leaves of lemon plants, i.e. high amounts of *O*-geranylated coumarins were observed by UV (8-geranyloxypsoralen, 1,110 ± 30 μg g^{-1} DW; bergamottin, 222 ± 7; and 5-geranyloxy-7-methoxycoumarin, 4.2 ± 0.2 in mature

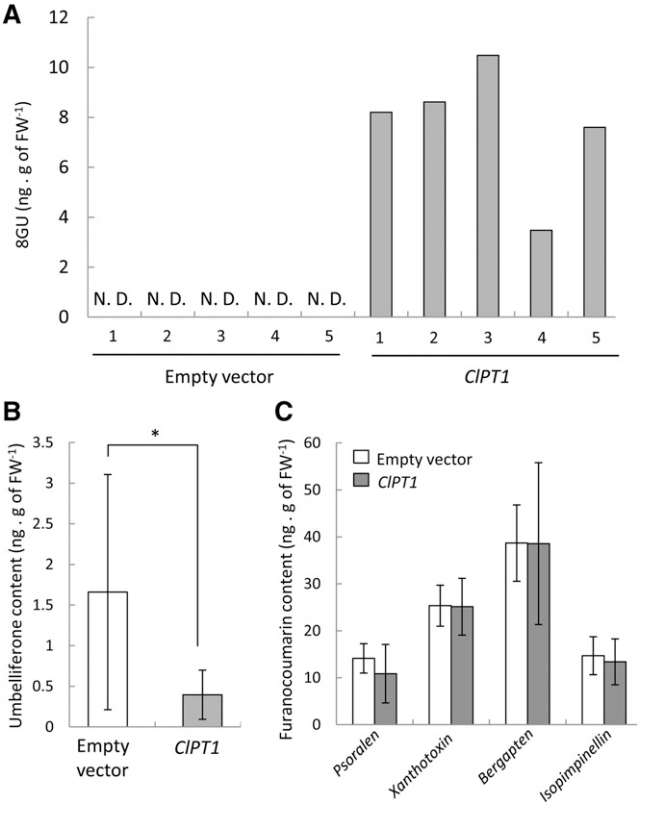

Figure 4. Quantification of coumarin derivatives in transgenic *R. graveolens*. Contents of 8GU (A), umbelliferone (B), and furanocoumarins (C) in transgenic lines of *R. graveolens* expressing *ClPT1*, as quantified by LC/MS, compared with the contents of control lines. Values are means ± SD of five biological replicates for B and C. The asterisk indicates a statistically significant difference by Student's *t* test ($P < 0.05$). N. D., Not detected.

leaves), with 8GU detectable only by LC-IT-TOF/MS analysis (Supplemental Fig. S7). To determine whether 8GU is present in other citrus species, we analyzed its presence in 56 different citrus peels (Supplemental Table S2), finding that 8GU was present in the peel of six of these species, at amounts ranging from 1 to 11 $\mu g\ g^{-1}$ FW.

Subcellular Localization of ClPT1

The iPSORT program predicted that ClPT1 contains a transit peptide-like sequence at its N terminus, whereas the other common prediction programs did not. To verify its plastid localization experimentally, a protein fusing the N-terminal region, including the predicted cleavage site, and GFP was constructed (ClPT1-TP-GFP) and transiently expressed in onion (*Allium cepa*) epidermal cells. As a positive control for plastid localization, the transit peptide region of waxy gene fused to red fluorescent protein (WxTP-DsRed) was coexpressed (Kitajima et al., 2009). Microscopic examination showed that fluorescence related to ClPT1-TP-GFP was localized to dotted organelles. This pattern was identical to that of WxTP-DsRed, with predominant yellow dots in the merged picture (Fig. 6A). By contrast, control GFP without a transit peptide localized in the cytosol and nucleus (Fig. 6B). Similar dotted organelles with green fluorescence were observed when ClPT1-TP-GFP was expressed in *Nicotiana benthamiana* leaves by agroinfiltration (Supplemental Fig. S8A), a pattern identical to that of chloroplast-sorted protein fused to GFP (Arsova et al., 2010). These results strongly suggest that the N-terminal sequence of ClPT1 functions as a transit peptide and that this GPP-specific coumarin PT localizes to plastids.

Organ-Specific Expression of *ClPT1* in Lemon

Quantitative reverse transcription-PCR analysis of total RNA prepared from various organs of lemon plants, including albedo, flavedo, buds, young leaves, and mature leaves was performed using primers specific to *ClPT1*, with lemon 26S ribosomal RNA (rRNA) used as an internal standard (De Felice and Wilson, 2010). High levels of *ClPT1* mRNA were detected in the flavedo of lemon peels, but it was almost undetectable in albedo (Fig. 7), a finding consistent with the localization of endogenous U8GT activity in lemon fruits (Supplemental Fig. S4). Unexpectedly, the levels of expression of *ClPT1* transcripts were much (>10-fold) higher in young and mature leaves than in flavedo, whereas the 8GU content in leaves was also trace as in the flavedo (Supplemental Fig. S7).

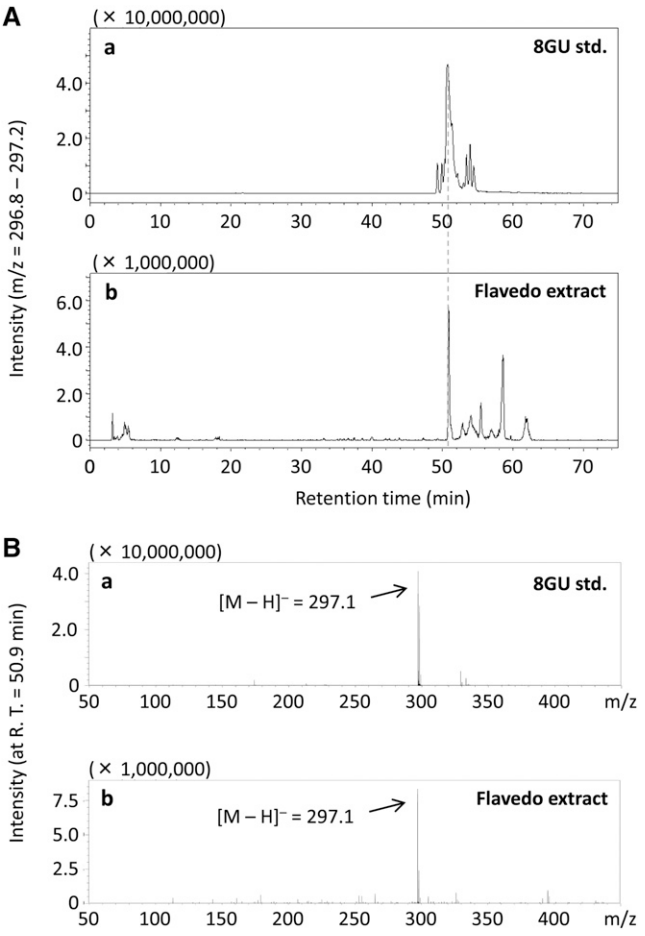

Figure 5. LC-IT-TOF/MS analysis of a methanol extract of lemon flavedo. A, Mass chromatograms scanned at mass unit (*m/z*) 296.8 to 297.2 in the negative ion mode. The dotted bar indicates a retention time of 8GU (50.9 min). B, Mass spectra of the peak at 50.9 min monitored at *m/z* 90 to 510. In A and B, section a is a standard (std.) specimen of 8GU, and section b is the methanol extract of lemon flavedo.

DISCUSSION

Coumarins are a unique group of plant phenols, with more than 1,000 derivatives isolated to date from more than 800 plant species (Croteau et al., 2000). Coumarins are ubiquitously found in higher plants (Bourgaud et al., 2006) but are more chemically diversified in Rutaceae and Apiaceae, including many citrus fruits, herbs, and vegetables, e.g. orange (*Citrus sinensis*), lemon, parsley, celery (*Apium graveolens*), and carrot (*Daucus carota*; Lacy and O'Kennedy, 2004). In particular, the flavedo of citrus species contains high amounts of various prenylated coumarins (Frérot and Decorzant, 2004).

Prenylation has particular importance in coumarin metabolism, both regarding chemical diversification and in the furanocoumarin biosynthetic pathway. This enzymatic reaction is essential in determining whether the end products will be linear- or angular-type furanocoumarins (Brown and Steck, 1973). In addition, the high abundance of *O*-prenylated compounds, in which prenyl chains are bound to aromatic rings by a C-O bond, is unique to citrus species. Furthermore, the chemical diversity due to prenylation largely contributes to the various bioactivities of coumarin derivatives, e.g. antibacterial

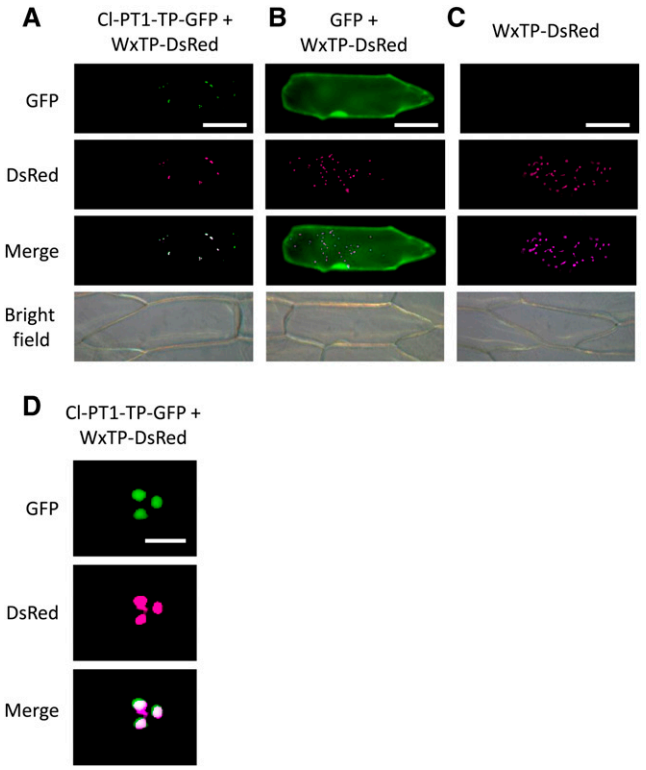

Figure 6. Microscopic analysis of subcellular localization of ClPT1 transit peptide (TP) fused with GFP in onion epidermal cells. A, B, and D, Plasmids containing *ClPT1-TP-GFP* and *WxTP-DsRed* (A and D), *CaMV35Spro-GFP* (pHKN29) and *WxTP-DsRed* (B), or *WxTP-DsRed* alone (C) were mixed and introduced into onion epidermal cells by particle bombardment. Bars = 100 μm (A–C) and 10 μm (D).

and antiherbivorous activities. Prenylation was thought to elevate bioactivity by altering hydrophobicity, thus enhancing cellular uptake. Recently, however, bioactivity was thought to be enhanced by possible interactions between prenylated polyphenols and target proteins (Neal and Wu, 1994; Adams et al., 2006; Mukai et al., 2012).

This study described the isolation from lemon flavedo of *ClPT1* cDNA, which encodes a GPP-specific PT, U8GT. It is to be noted that ClPT1 is a novel PT specifically recognizing GPP as prenyl donor and a non-flavonoid prenyl acceptor substrate, and from citrus species. Phylogenetic analysis showed that although ClPT1 was a member of the membrane-bound PT family involved in vitamin E and plastoquinone biosynthesis, in which homogentisate is the prenyl acceptor substrate, it possessed a unique polypeptide sequence when compared with other members of this protein family (Collakova and DellaPenna, 2001; Yazaki et al., 2002; Ohara et al., 2006; Sadre et al., 2006; Yang et al., 2011). Recently identified PcPT that is involved in furanocoumarin biosynthesis showing strict specificity to dimethylallyl diphosphate (DMAPP) as prenyl substrate shares moderate sequence similarity (36%) with ClPT1, while it accepts coumarin as the aromatic substrate (Fig. 1). It is still unclear whether the sequence similarity reflects to the substrate compounds or taxonomical distance. Further isolation of plant PTs having different enzymatic properties from the same plant species, and those with the same properties from different plant origins, is necessary to assess the molecular evolution of this membrane-bound enzyme family.

Analysis of the substrate specificity of ClPT1 enzyme showed that it had the strongest preference for umbelliferone but also accepted esculetin, 5,7-dihydroxycoumarin, and 5-methoxy-7-hydroxycoumarin as substrates, with each compound yielding two products. This specific property of ClPT1 differs from other aromatic substrate PTs, which generally yield only one product per prenyl donor (Sasaki et al., 2008, 2011; Akashi et al., 2009; Shen et al., 2012; Tsurumaru et al., 2012). Methylation of the hydroxy group at position 7 of 5,7-dihydroxycoumarin, yielding 5-hydroxy-7-methoxycoumarin, abolished the geranylation activity of ClPT1, suggesting that this hydroxy group is critical for the recognition of coumarin derivatives as substrates and/or to transfer the geranyl moiety from GPP. Testing of other prenyl acceptors, including furanocoumarins, phenylpropanoids, flavonoids, and homogentisate involved in plastoquinone and vitamin E biosyntheses, yielded no reaction products, indicating that ClPT1 has a narrow specificity for prenyl acceptors, similar to other plant-derived PTs (Sasaki et al., 2008, 2011; Akashi et al., 2009; Shen et al., 2012; Tsurumaru et al., 2012). Because the major coumarin derivatives in lemon peel are furanocoumarins, it was not surprising if ClPT1 would show 6-PT activity with a substrate preference for DMAPP, resulting in demethylsuberosin, a key intermediate of furanocoumarins (Brown and Steck, 1973). This enzyme, however, did not accept DMAPP as a prenyl donor, suggesting that the protein encoded by this gene is not involved in major furanocoumarin biosynthesis. Further substrate analysis with longer prenyl chains, i.e. FPP and GGPP, indicates that ClPT1 recognizes GPP as a sole prenyl donor.

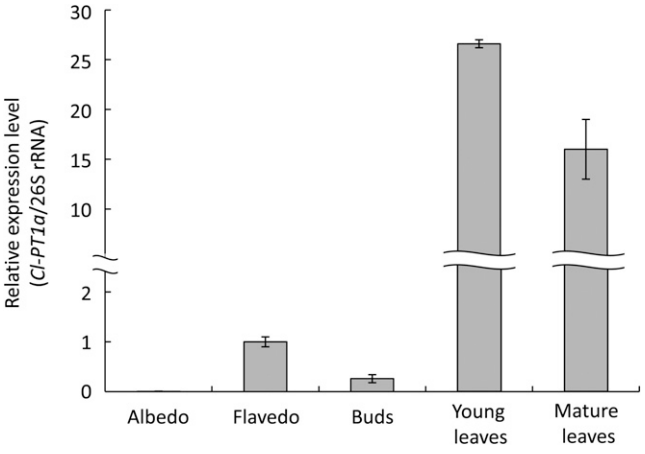

Figure 7. Real-time PCR analysis of *ClPT1* expression in different organs of lemon trees. Relative expression levels of *ClPT1* in albedo, flower buds, young leaves, and mature leaves relative to expression in flavedo (1.0) and normalized relative to the level of expression of 26S rRNA. Values represent means ± SD (young leaves, $n = 3$; others, $n = 4$).

During the secondary metabolism of most *C*-prenylated phenolics in plants, prenyl chains are transferred to the benzene ring at a position *ortho* to the hydroxy group, suggesting that geranylation takes place at the 6 and 8 positions of benzene rings in coumarin molecules (Yazaki et al., 2009). In addition, because of the preference of recombinant ClPT1 for the 8-*C*-geranylation of umbelliferone and the shorter retention time of 8GU than that of P2 in HPLC analysis, the major enzyme products (P5 and P7) are likely 8-*C*-geranylated, whereas the minor products (P2, P6, and P8) are likely 6-*C*-geranylated compounds. The hydroxylation pattern in its benzene ring suggests that only esculetin was likely geranylated by ClPT1 at the 5 and 8 positions.

To confirm the enzymatic activity of ClPT1 in plant cells, we selected *R. graveolens* as the expression host, because this rutaceaeous plant produces both coumarin derivatives and monoterpenoids, indicating in vivo supplies of GPP and umbelliferone. Metabolite analysis of *R. graveolens* expressing *ClPT1* showed the production of 8GU and a significant decrease in the endogenous content of in vivo umbelliferone, strongly suggesting that ClPT1 functioned as a U8GT in plant cells. U8GT activity may alter furanocoumarin contents in transgenic *R. graveolens* due to their lower levels of umbelliferone, as the latter is the common precursor of all furanocoumarins. However, the major furanocoumarin contents of *ClPT1* and empty vector transformants of *R. graveolens* were similar, suggesting that a regulatory system may maintain certain endogenous levels of major end products to resist against artificial turbulence of metabolic flow, as described above.

Phytochemical analysis showed the presence of 8GU in the extracts of lemon flavedo and leaves, as well as in several other citrus species, including yuzu (*Citrus junos*) and shekwasha mandarin (*Citrus depressa*; Supplemental Table S2). We found that the native U8GT activity of lemon flavedo was 12% of the activity of bergaptol 5-*O*-geranyltransferase activity, which yields bergamottin, the main coumarin derivative in the lemon flavedo (Munakata et al., 2012). In contrast to its high native enzymatic activity in lemon flavedo, the endogenous level of 8GU in these lemon organs was very low, suggesting that 8GU serves as an intermediate that is converted to other unidentified metabolites, which accumulate, along with other coumarin components, in the oil glands of citrus flavedo (Frérot and Decorzant, 2004).

Similar to flavonoid- and homogentisate-specific PTs, ClPT1 protein localizes to plastids, a finding consistent with the subcellular localization of GPP biosynthesis via the methylerythritol phosphate pathway. Large amounts of monoterpenes also accumulate in lemon flavedo, with GPP being the key biosynthetic precursor of these molecules. This suggests that monoterpene synthases and PTs compete for GPP molecules or that GPP is regulated to control the proportions of final products in flavedo. It has not yet been determined whether, following their biosynthesis, prenylated coumarins, like monoterpenes, are transported from plastids to secretory glands by passing across multiple membranes.

The highest expression of *ClPT1* mRNA in lemon was observed in the leaves, with expression being about 20-fold higher than in the flavedo. This result was unexpected, because, to our knowledge, lemon leaves have not been reported to accumulate large amounts of coumarin derivatives. In quantifying major coumarin derivatives in young and mature lemon leaves, we found that these leaves accumulate comparable levels of prenylated coumarins. In particular, the content of 8-geranyloxypsoralen, a geranylated furanocoumarin, was higher in mature leaves than in flavedo.

We also analyzed 56 citrus species for the presence of the enzymatic product 8GU. Although the contents of this compound were not very high, citrus species from Asian countries tended to contain 8GU, while it is unclear whether the productivity of 8GU is regulated on a genetic level according to citrus species. Recently, genome sequences are available for a couple of citrus species, i.e. orange and clementine (*Citrus clementina*). In homology search, we could detect a single copy of gene sharing high sequence identity (95%) with *ClPT1* for each species. Coumarin derivatives are, however, barely detectable in these species, suggesting that the pathway to supply coumarin is not active despite the existence of a homolog of *ClPT1* in their genomes.

Although 8GU has not been shown to possess biological activity, its isomers, auraptene (7-*O*-geranylumbelliferone) and ostruthin (6-geranylumbelliferone), were found to inhibit cutaneous cancer and infectious bacteria, respectively (Murakami et al., 1997; Schinkovitz et al., 2003). Our findings suggest that metabolic engineering of a heterologous plant overexpressing *ClPT1* can produce 8GU. Screening of appropriate host organisms and optimizing metabolic engineering may produce sufficient 8GU for pharmacological studies. Moreover, recombinant ΔTP-ClPT1 showed lower apparent K_m values for both the prenyl donor and acceptor than other membrane-bound PTs. These properties favor its biotechnological application in agriculture, the food industry, and pharmaceutical sciences.

MATERIALS AND METHODS

Plant Materials and Reagents

Fruits and other organs of lemon (*Citrus limon* 'Lisbon') trees used for gene sources and 8GU detection were kindly provided by the Institute of Fruit Tree Science, National Agriculture and Food Research Organization (Japan). Lemon fruits (cv Lisbon) for biochemical experiments were purchased at a local market (Kyoto, Japan). Different citrus species for 8GU survey were harvested at the Institut National de la Recherche Agronomique research center of San Giuliano (France). *Ruta graveolens* seeds were provided by Conrad Appel, Samen, und Pflanzen GmbH. 8GU (Uto et al., 2002) and DMAPP (Cornforth and Popják, 1969) were synthesized as described. GPP was kindly provided by Dr. Tomohisa Kuzuyama (The University of Tokyo) and Dr. Takashi Kawasaki (Ritsumeikan University). FPP and GGPP were kindly provided by Dr. Seiji Takahashi (Tohoku University).

Isolation of ClPT1 cDNA

Total RNA was isolated from the flavedo tissue of a lemon fruit obtained from a local market using the RNeasy Plant Mini Kit (Qiagen). The RNA was

treated with DNase in the DNA-free kit (Life Technologies), and first-strand cDNA was synthesized using Superscript III reverse transcriptase (Life Technologies) with oligo(dT) primers. To isolate a partial *PT* gene, the first-strand cDNAs were utilized as a template for PCR using the degenerate primers DGP-F1 (forward) and DGP-R1 (reverse; Supplemental Table S1). The resulting PCR products were used as a template for the second PCR with the primers DGP-F2 (forward) and DGP-R1 (reverse). Amplification with these primers was performed as described (Koeduka et al., 2009). The 300-bp-long amplified PCR fragments were isolated into the vector pGEM-T easy (Promega) and sequenced. The complete coding sequence of *ClPT1* was obtained by 5′- and 3′-RACE with internal gene-specific primers (Supplemental Table S1) using the SMARTer RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instructions. The sequenced isolates of 5′- and 3′-RACE covered the initiation and termination codons, respectively.

To determine the origin of *ClPT1* transcription, we reisolated *ClPT1* cDNA from the total RNA of the flavedo of a lemon (cv Lisbon) by PCR using DNA polymerase KOD-plus (Toyobo) and the gene-specific primer pair 5′UTR-F and 3′UTR-R (Supplemental Table S1) designed from the 5′ and 3′ untranslated regions of the above-described *ClPT1* sequence. The cDNA obtained from the Lisbon cultivar was used for the enzymatic characterization of ClPT1. To distinguish the cDNAs from the Lisbon cultivar and the fruit purchased in a market, they are designated ClPT1a (accession no. AB813876) and ClPT1b (accession no. AB813877), respectively, in Supplemental Table S1. The two isolates differ by only four amino acids at the C terminus (Supplemental Fig. S1A) but encode proteins with similar enzymatic properties. The transmembrane domain of the gene product was analyzed by TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Subcellular localization of ClPT1 was analyzed by iPSORT (http://ipsort.hgc.jp/), PSORT (http://psort.hgc.jp/), WoLF PSORT (http://www.genscript.com/psort/wolf_psort.html), and ChloroP (http://www.cbs.dtu.dk/services/ChloroP/). Phylogenetic analysis was performed using MEGA5 software (http://www.megasoftware.net/).

Heterologous Expression of ClPT1 in Yeast

A truncated open reading frame of *ClPT1*, lacking the nucleotide sequence encoding 36 amino acids at the N terminus, *ΔTP-ClPT1*, was amplified by PCR with Phusion High-Fidelity DNA Polymerase (Finnzymes) and the primer set ΔTP-ClPT1a-F and ΔTP-ClPT1a-R (Supplemental Table S1). After double digestion with *Spe*I and *Xho*I, *ΔTP-ClPT1* was inserted into the pDR196 vector, which is driven by a strong constitutive promoter, yeast (*Saccharomyces cerevisiae*) plasma membrane ATPase promoter, to yield the plasmid pDR196-ΔTP-ClPT1 (Rentsch et al., 1995). This vector was introduced into the yeast strain W303-1A-Δ*coq2*, in which an endogenous *p*-hydroxybenzoate prenyltransferase gene involved in coenzyme Q biosynthesis (*COQ2*) was disrupted (Yazaki et al., 2002). The microsome fraction of W303-1A-Δ*coq2* expressing pDR196-ΔTP-ClPT1 was prepared as described (Yazaki et al., 2002), resuspended in 100 mM Tris-HCl (pH 7.5) containing 125 μM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol, and used for enzymatic assays. Protein content was measured by Bradford's method with bovine serum albumin as a standard (Bradford 1976).

Enzymatic Assays

A standard mixture (200 μL), containing 1.0 mM prenyl acceptor, 1.0 mM prenyl donor, 10 mM $MgCl_2$, and the microsome fraction, was incubated at 30°C for 60 min. The reaction was stopped by adding 100 μL of 3 N HCl, and the mixture was extracted with 300 μL of ethyl acetate. When determining apparent K_m values, 10 μL of 3 mM testosterone propionate was added to the reaction mixture as an internal standard just before the extraction with ethyl acetate. This extract was evaporated to dryness and dissolved in 50 μL of methanol. After centrifugation at 20,400*g* for 5 min, the supernatant was used for HPLC and LC-IT-TOF/MS analyses.

HPLC and LC-IT-TOF/MS Analyses of Enzyme Reaction Products

Reaction products were quantified by HPLC (D-2000 Elite HPLC System with L-2455 photodiode array detector, Hitachi) using a LiChrosphere RP-18 column (4 × 250 mm, Merck). A methanol:water:acetic acid (80:20:0.3) solvent was used for routine analyses of geranyltransferase activities of umbelliferone. To assess substrate specificity, reaction mixtures with other aromatic compounds were analyzed with the isocratic program described above or with a gradient program of 20% (v/v) to 80% (v/v) solvent B (methanol with 0.3% [v/v] acetic acid) in solvent A (0.3% [v/v] acetic acid) over 60 min at 40°C and a flow rate of 1 mL min^{-1}. Products were detected spectrophotometrically at 254 to 330 nm.

Reaction products were identified by LC-IT-TOF/MS (Shimazu) using a TSK gel ODS-80Ts column (2 × 250 mm, Tosoh). After separation by a gradient program with solvent A (0.3% [v/v] formic acid) and solvent B (acetonitrile) where solvent B was eluted from 20% (v/v) to 80% (v/v) for 60 min at 40°C with a flow rate of 0.2 mL min^{-1}, reaction products were ionized in the negative ion mode. The detection range was from *m/z* 90 to 510.

Construction of Recombinant Plasmids for Transformation of *R. graveolens*

The PT encoding sequence was amplified by PCR (*Taq* HIFI polymerase, Life Technologies), using the primers ClPT1b-F2 and ClPT1b-R2 (Supplemental Table S1). The resulting PCR product was isolated using the pCR8/GW/TOPO TA Cloning Kit (Life Technologies), according to the manufacturer's instructions. The gene was subsequently transferred into pBIN-GW plasmid by LR recombination (LR Clonase, Life Technologies) as described (Lievre et al., 2005).

Stable Transformation of *R. graveolens* and Molecular Characterization

Transgenic *R. graveolens* lines were obtained as described (Lievre et al., 2005; Vialart et al., 2012). The presence of the transgene was assessed using the PHIRE plant direct PCR (Finnzymes) approach with a leaf disc as the template. PCR amplification was performed using the forward primer CaMV35Spro-F, which anneals to the *Cauliflower mosaic virus* 35S promoter, and the reverse primer ClPT1b-R2 (Supplemental Table S1). The amplification protocol consisted of an initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 15 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The expression of *ClPT1b* was monitored by quantitative PCR using a Step One device (Applied Biosystem, http://www.appliedbiosystems.com) and the SYBRGREEN detection/quantification method (Fast SYBRGREEN Master Mix, Applied Biosystem). Total RNAs were extracted with RNeasy plant mini kit (Qiagen), and cDNA was synthesized using a High-Capacity RNA-to-cDNA Kit (Applied Biosystems) and the primer pair ClPT1b-qPCR-F and ClPT1b-qPCR-R (Supplemental Table S1). The amplification protocol consisted of an initial denaturation at 98°C for 5 min, 40 cycles of denaturation at 98°C for 5 s, annealing at 59°C for 5 s, extension at 72°C for 30 s, and a final extension at 72°C for 1 min.

LC/MS Metabolic Quantification of Transformed *R. graveolens* Plants

Total coumarins/furanocoumarins were extracted as described (Dugrand et al., 2013). Coumarin was added to the samples as a control molecule, and samples were diluted 10- and 30-fold to quantify molecules present at very high or low concentrations.

The coumarins and furanocoumarins were quantified using an HPLC/MS system (ThermoFisher Scientific), equipped with an Linear Trap Quadripole ion trap as a mass analyzer. Data were processed using Xcalibur software (version 2.1). Chromatographic separation was performed on a C18 Alltima reverse phase column (150 × 2.1 mm, 5 μm porosity; Grace/Alltech) at 25°C, with the mobile phases consisting of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). The mobile phase consisted of gradients of A:B (v/v), 90:10 at 0 min, 80:20 at 5 min, 53:47 between 15 and 30 min, 0:100 from 35 to 40 min, and 90:10 from 40.10 to 46 min, at a flow rate of 0.2 mL min^{-1}. The eluent was monitored by a photodiode array and MS throughout. For MS, electrospray ionization was used in a positive ionization mode. Sheath, auxiliary, and sweep gases were set at 40, 10, and 10 arbitrary units min^{-1}, respectively, capillary temperature at 300°C, and capillary voltage at 36 V, and the voltages of the tube, split, and front lenses were set at 80, –44, and –3.25 V, respectively. Ion optics parameters were optimized by automatic tuning using a standard solution of isopimpinellin at 0.1 g L^{-1} infused in mobile phase (A:B [1:1]) at a flow rate of 5 μL min^{-1}. Compounds were detected sensitively and specifically by monitoring daughter ions obtained from MS^2 fragmentation of pseudomolecular $[M + H]^+$ ions.

HPLC and LC-IT-TOF/MS Analysis of the Lemon Flavedo Extract

The lemon flavedo was freeze dried overnight and ground with a mortar and pestle. After addition of methanol (10 mL g^{-1} DW), coumarins were extracted by shaking at 200 rpm and 37°C for 16 h (for HPLC analysis) or 2 d (for LC-IT-TOF/MS analysis). The supernatant was filtered through Minisart RC4 (0.2-μm pore, Sartorius Stedim Biotech) and used for HPLC or LC-IT-TOF/MS analysis. HPLC analysis was performed essentially as described previously (Sugiyama et al., 2011). LC-IT-TOF/MS analysis was performed under the same conditions as those used for the enzyme assay mentioned above.

Construction of a Plasmid Expressing GFP-Fused Protein

A nucleotide sequence corresponding to the N-terminal region (81 amino acids) of ClPT1, designated *ClPT1-TP*, was amplified by PCR with KOD-plus (Toyobo) and the primer pair ClPT1a-TP-F and ClPT1a-TP-R (Supplemental Table S1). The amplified DNA fragment was double digested and subcloned into pENTR2B vector (Life Technologies), followed by LR recombination with pGWB505 vector to yield the construct *CaMV35Spro-ClPT1-TP-GFP* (Nakagawa et al., 2007). Particle bombardment and microscopic analysis were performed as described (Sasaki et al., 2011). The plasmid pHKN29 containing *CaMV35Spro-GFP* and pWxTP-DsRed were used as controls for GFP and plastid localization, respectively (Kumagai and Kouchi, 2003; Kitajima et al., 2009).

Real-Time PCR for Tissue-Specific Expression of *ClPT1* in Lemon

Total RNA was extracted from the albedo and flavedo of lemon fruits, young leaves, and mature leaves with hexadecyltrimethylammonium bromide solution (2% cetyl-trimethyl-ammonium bromide [CTAB], 0.1 M Tris-HCl, 20 mM EDTA, 1.4 M NaCl, and 1% 2-mercaptoethanol, pH 8.0). After extraction with chloroform and precipitation with 2 M LiCl, total RNA was purified with the RNeasy Plant Mini kit (Qiagen). Total RNA of flower buds was extracted using an RNeasy Plant Mini kit (Qiagen). RNAs were reverse transcribed using ReterTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo). Levels of expression in tissue samples were compared by quantitative reverse transcription-PCR with THUNDERBIRD SYBR qPCR Mix (Toyobo) and the *ClPT1* primer pairs ClPT1a-qPCR-F and ClPT1a-qPCR-R. The amplification protocol consisted of an initial denaturation at 95°C for 1 min and 40 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. As an internal control, 26S rRNA was amplified using the primers 26S rRNA-qPCR-F and 26S rRNA-qPCR-R (De Felice and Wilson, 2010). Emitted fluorescence was measured with Roter-Gene 3000A (Corbett Research).

Sequence data from this article can be found in the DNA Data Bank of Japan/GenBank/EMBL data libraries under accession numbers AB813876, Cl-PT1a and AB813877, Cl-PT1b.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. In silico analysis of ClPT1 polypeptide.

Supplemental Figure S2. U8GT activity in full assay and various negative control assays.

Supplemental Figure S3. Negative effect of the ClPT1 N-terminal sequence on U8GT activity.

Supplemental Figure S4. Membrane-bound property of native U8GT activity in lemon fruits.

Supplemental Figure S5. Identification of enzymatic reaction products of ΔTP-ClPT1 by HPLC and LC-IT/MS^2 analyses.

Supplemental Figure S6. Substrate specificity of recombinant ΔTP-ClPT1 for FPP and GGPP.

Supplemental Figure S7. Detection of 8GU in lemon leaves.

Supplemental Figure S8. Transient expression of ClPT1 transit peptide fused with GFP (ClPT1-TP-GFP) in *N. benthamiana* leaves.

Supplemental Table S1. PCR primers used in this study.

Supplemental Table S2. Quantitative analysis of 8GU in peel of various citrus species.

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